# THE 7TH INTERNATIONAL CONFERENCE ON FOOD AND APPLIED BIOSCIENCE 2024

February 8 - 9, 2024

# PROCEEDING

# FAB 2024

"FOOD AND BIOTECHNOLOGY FOR A SUSTAINABLE & BIO-CIRCULAR-GREEN ECONOMY"

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# International Conference on Food and Applied Bioscience

## February 8-9, 2024

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# PROCEEDING

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***Oral Presentation***

# Enhancing the Functional Properties of Okara through Fermentation with *Rhizopus Oligosporus* TISTR 3138: A Study on Total Phenolic Compounds and Antioxidant Activity

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**Abstract**

Okara, or soybean pulp, is a by-product from the soymilk production process. To enhance its value, it should be developed as a functional food. The purpose of this research was to study the changes in total phenolic compounds and antioxidant activity of fermented okara by Rhizopus oligosporus TISTR 3138. The fermented okara was used to enhance the functional properties in porridge products. Okara was fermented with R. oligosporus TISTR 3138 for 72 h at 35°C. The fermented okara was then processed into porridge products using the foam-mat drying method. The experiments consisted of four conditions: 1) rice porridge, 2) fermented okara porridge, 3) porridge from rice mixed with fermented okara 75% (w/w), and 4) porridge from rice mixed with fermented okara 50% (w/w). Analysis of the samples included determination of total phenolic content (TPC), 2,2-diphenyl -1-picrylhydrazyl (DPPH) scavenging activity. The results revealed that the fermented okara had a total phenolic content of 331.48 mg GAE/g extract and antioxidant activity against DPPH of 46.27 ± 3.14%, which were higher than those in unfermented okara, which were 2.68 mg GAE/g extract and 2.95 ± 0.16%, respectively. The porridge made from fermented okara exhibited the highest total phenolic content at 458.68 mg GAE/g extract, with an antioxidant activity of 66.82 ± 1.33%. These values were notably higher compared to rice porridge, by 30 times for total phenolic content and 6 times for antioxidant activity. These results indicate that incorporating fermented okara can improve the functional properties of porridge products. Consequently, the utilization of fermented okara as a functional ingredient in food products presents an intriguing prospect.

***Keywords**:* Okara; Fermentation; Functional food; Porridge; *Rhizopus oligosporus*

## 1. Introduction

Okara, also referred to as soy pulp or tofu dregs, constitutes a nutrient-rich by-product originating from the production processes of soymilk and tofu [(1)]. The fibrous residue produced during the coagulation and pressing stages of soymilk extraction, traditionally deemed as waste, has recently garnered attention for its nutritional benefits, leading to increased interest in its diverse applications [(2)].

Possessing a comprehensive nutritional profile, okara emerges as a valuable addition to diverse diets. Abundant in proteins, it furnishes essential amino acids crucial for bodily functions [(3)] Furthermore, its elevated fiber content contributes to digestive health and potential weight management [(4)]. With a substantial carbohydrate content, okara enhances energy profiles, while the presence of B vitamins, including folate, supports metabolism and

overall health. Additionally, okara serves as a mineral source, offering calcium and iron for bone health and oxygen transport [5].

Researchers and food scientists have explored multiple applications of okara, unveiling its potential in different realms. Investigations into functional foods indicate that okara enhances the nutritional value of products like bread, cookies, and snacks [6]. Furthermore, through fermentation processes, okara can yield bioactive compounds and improved functional properties, demonstrating antioxidant and antimicrobial potential [7].

Various microorganisms, including bacteria (e.g., *Bacillus subtilis*) and fungi (e.g., *Aspergillus oryzae*), have been employed in fermenting okara to enhance its nutritional quality [8]. Okara fermentation can increase total phenolic content and antioxidant activity, providing potential health benefits. Fermented okara can be incorporated into functional food products, contributing to improved nutritional profiles [9]. Okara fermentation aligns with sustainable practices by transforming a by-product into a valuable resource [10].

This study's focal point lies in processing fermented okara into ready-made porridge, with the primary objective of examining its total phenolic content and antioxidant activity.

## 2. Materials and Methods

### 2.1 Materials and Microorganisms

Fresh okara was generously supplied by Lactasoy Co., Ltd. (Bangkok, Thailand) and stored at -20ºC prior to use. *Rhizopus oligosporus* TISTR3138, obtained from the Thailand Institute of Scientific and Technological Research (TISTR), was employed. *R. oligosporus* was activated on Potato Dextrose Agar (PDA agar) and incubated at 37°C for 48 h. The culture was preserved in 15% glycerol at -20ºC for further use. To prepare the inoculum for the starter culture in solid-state fermentation, the culture was inoculated into Potato Dextrose Agar and incubated at 37°C for 48 h. A suspension of *R. oligosporus* containing 3 Log CFU/mL was prepared using sterile water.

### 2.2 Okara Fermentation

Okara (1 kg) was placed in a container and sterilized at 12°C for 30 min. After cooling to room temperature, *R. oligosporus* TISTR3138 was inoculated at a concentration of 3 Log CFU/mL, then mixed well. One hundred g of inoculated okara were placed into perforated plastic bags (12 × 17 cm). Solid-state fermentations were conducted at 35ºC for 72 h. Samples were taken every 24 h and kept at -20ºC before analysis for cell concentration. Unfermented okara and fermented okara at 72 h were analyzed for total phenolic compounds, and antioxidant activity.

### 2.3 Preparation of instant porridge using Foam-mat drying method.

Instant porridge was prepared according to the method of Khuenpent and Polpued [11]. The experiments were divided into 4 conditions as shown in Table 1.

**Table 1.** Instant porridge formulars.

| Experimental conditions | Broken jasmine rice (%w/w) | Fermented okara (%w/w) |
|---|---|---|
| Femented okara porridge | - | 100 |
| Broken jasmine rice porridge + Femented okara 75% | 25 | 75 |
| Broken jasmine rice porridge + Femented okara 50% | 50 | 50 |
| Broken jasmine rice porridge | 100 | - |

Instant porridge ingredient included broken jasmine rice. The rice was washed with water and boiled until cooked before being mixed in with the fermented okara. In conditions without broken jasmine rice as an ingredient, fermented okara was cultivated until it formed a homogeneous mass. One percent maltodextrin was added, and the boiling temperature was maintained at 80-85ºC. The mixture was then allowed to cool at 30-32ºC and weighed. Prior to preparing the foam-forming solution, 1% carboxymethyl cellulose (w/w) was added by dissolving the foam-forming substance in water at a temperature of 90ºC, and then it was incorporated into the prepared porridge. The mixture was blended with a blender at a low speed for 1 min, followed by an electric mixer at the highest speed for 15 min. The resulting mixture was poured into a tray lined with wax paper, baked in a hot air oven at a temperature of 60ºC for 7 h. After drying, the mixture was processed in a blender to produce powder.

**2.4 Extraction of sample**

Sample extraction was modified following the methods of Dajanta *et al.* [12] and Samruan *et al.* [13]. Samples as shown in Table 2, were extracted with 80% methanol in a ratio of 1:3, using an ultrasonic bath at a temperature of 40ºC for 1 h. Subsequently, they were centrifuged at 4ºC at 4,000 rpm for 30 min. The clear portion of the sample was stored at 4ºC, awaiting further analysis.

**Table 2.** Sample codes.

| Sample | Code |
|---|---|
| Unfermented okara | UO |
| fermented okara (72 h) | FO |
| Fermented okara porridge | PFO100 |
| Broken jasmine rice mixed with 75% fermented okara porriidge | PFO75 |
| Broken jasmine rice mixed with 50% fermented okara porriidge | PFO50 |
| Broken jasmine rice porridge | R100 |

**2.5 Determination of total phenolic compound**

The total soluble phenolic content (TPC) of fermented okara extracts was determined using the Folin-Ciocalteu reagent with gallic acid as a standard phenolic compound [14]. Twenty µL of each diluted extract was added to 100 µL of Folin-Ciocalteu's reagent and allowed to stand for 3 min. Then, 80 µL of a 10% (w/v) sodium carbonate solution was added to the mixture. The solution was incubated at 35ºC for 1 h in dark conditions, and the absorbance of the samples was measured at 765 nm. TPC was calculated as gallic acid equivalents (GAE) from the calibration curve obtained from the gallic acid standard solution and expressed as µg GAE/g okara.

**2.6 Evaluation of antioxidant activities**

The 2,2-Diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity was determined using a modification from Chatatikun and Chiabchalard [15]. Briefly, 60 µL of sample extracts or standard solutions of Trolox in absolute methanol were added to 140 µL of 0.16 mM DPPH reagent in a 96-well plate. Absolute methanol was used for the reagent blank. The reaction mixtures were mixed well and incubated for 30 min at 30ºC in the dark. The absorbance was measured at 517 nm with a Microplate Reader (PerkinElmer, United States). Experiments were all conducted in triplicate. The percentages of DPPH free radical scavenging activity were calculated as follows:

$$\%\text{Scavenging activity} = \frac{Ab_{control} - Ab_{sample}}{Ab_{control}}$$

### 2.7 Statistical analysis

The data from this study were presented as mean ± standard deviation. Statistical analysis was conducted using One-way Analysis of Variance (ANOVA) with SPSS Statistics 27.0 software (SPSS Inc., Chicago, IL). Group differences were assessed using Duncan's post-hoc test, with a significance level set at P>0.05. [16-17]

## 3. Results & Discussion

In this research, okara underwent a 72 h fermentation process with the fungus *Rhizopus oligosporus* TISTR 3138. The primary objectives of the study were to analyze fungal growth throughout the fermentation process. Subsequently, the fermented okara underwent processing to create instant porridge. Following this processing step, the instant porridge was analyzed to determine the quantity of total phenolic compounds and assess its antioxidant activity.

### 3.1 Growth of *Rhizopus oligosporus* TISTR 3138 during okara fermentation

The study on the growth of *Rhizopus oligosporus* TISTR 3138 during the fermentation of okara at 0, 24, 48 and 72 h revealed optimal growth at 48 h, with a growth value of 6.56 Log CFU/g, decreasing at 72 h, as depicted in Figure 1.

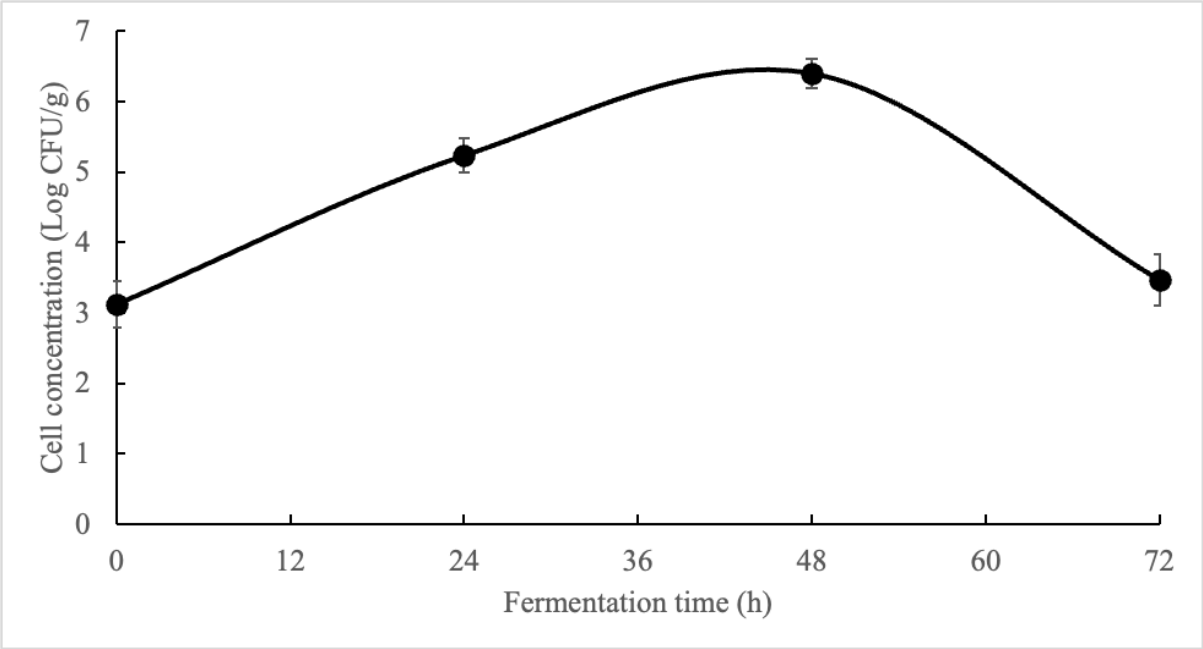


**Figure 1**. Growth of *Rhizopus oligosporus* TISTR 3138 during okara fermentation.

Figure 1 illustrates continuous fungal growth from hour 0, reaching its peak at 48 h before gradually decreasing until hour 72. This pattern aligns with the microbial growth theory proposed by Swinnen *et al.* [18], which outlines four stages of microbial growth: 1) Lag phase, where microorganisms adapt to the environment; 2) Log phase, characterized by rapid division after adaptation; 3) Stationary phase, indicating equilibrium in growth and decrease rates; and 4) Death phase, where the death rate exceeds the growth rate due to unfavorable conditions, leading to a decline in cell division rate. This finding is consistent with previous study [19] that have shown a similar pattern of growth for *Rhizopus oligosporus*

during fermentation of various substrates. Fungal growth is influenced by several factors, including substrate composition, pH, temperature, and moisture content. In the case of okara fermentation, the substrate provides a nutrient-rich environment for fungal growth, leading to an increase in fungal biomass over time. The peak growth observed at 48 h suggests that this is the optimal time point for harvesting the fermented okara to maximize fungal biomass production.

After reaching its peak at 48 h, fungal growth gradually decreases until 72 h. This decline in growth could be attributed to nutrient depletion or the accumulation of inhibitory compounds in the substrate.

### 3.2 Total phenolic content (TPC)

From the analysis of the total phenolic compounds in the samples of fermented okara and instant porridge, the results are presented in Figure 2.

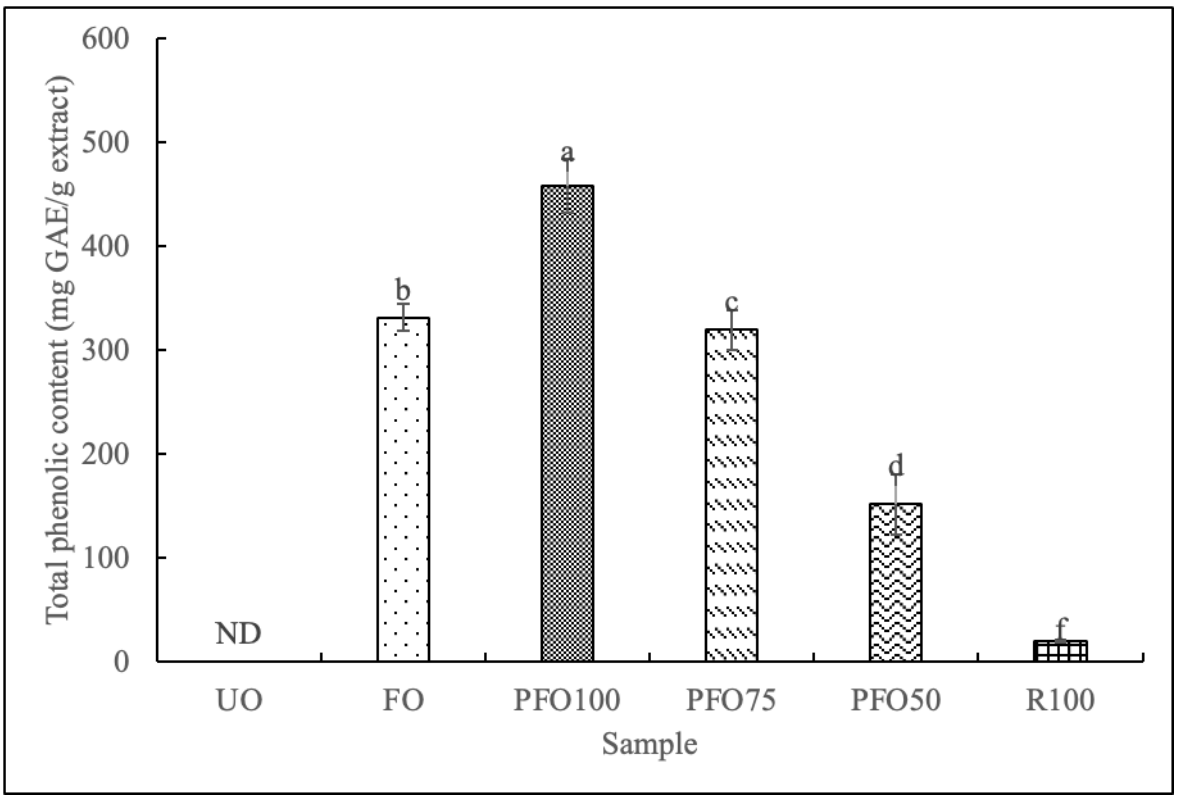


**Figure 2.** The amount of total phenolic compounds in the samples
Results were expressed as mean ±standard deviation; Sample (n=3). Different lowercase letters are significantly different (P<0.05) by Duncan's post-hoc test. *ND = Non detected

The analysis of total phenolic compounds revealed that fermented okara for 72 h (FO) had an increased quantity of total phenolic compounds after fermentation, measuring 331.48 mg GAE/g extract, according to the study by Nout and Kiers [20]. The study suggested that *Rhizopus oligosporus* can produce enzymes, including α and β–glucosidase, during fermentation. These enzymes can break down cell walls, releasing phenolic compounds in the form of aglycone isoflavones, leading to an increase in total phenolic compounds in the fermented okara. Additionally, McCue *et al.* [21] demonstrated that *Rhizopus oligosporus* is an effective microorganism for enhancing phenolic content in defatted soybean powder. They also highlighted the importance of β-glucosidase, the primary carbohydrate-cleaving enzyme, for efficient mobilization of phenolics from soybean powder.

Furthermore, the instant porridge from fermented okara (PFO100) significantly exhibited the highest amount of total phenolic compounds at 458 mg GAE/g extract, surpassing the content in okara fermented for 72 h. These values were notably higher compared to rice porridge, by 30 times for total phenolic content. This is attributed to the heat during the processing into instant porridge, which destroys the cell walls of okara, leading to the increased release of cell wall phenolic compounds. Our results align with Hong *et al.* [21] who studied the effect of boiling, steaming, microwave baking, far-infrared baking, steam explosion, and deep frying on bioactive compound, and phenolic compounds of Qingke (highland hull-less barley). The finding presented that the thermal methods including steam explosion and deep frying had a higher content of tocopherol, phenolic, and flavonoid. In contrast, the instant porridge from Broken jasmine rice showed the lowest amount of total phenolic compounds, measuring 20 mg GAE/g extract. These findings highlight the potential of fermented okara as a rich source of phenolic compounds, especially when processed into instant porridge, which enhances the bioavailability and functionality of these compounds.

**3.3 Antioxidant activity of fermented okara instant porridge**

The study on the antioxidant activity of fermented okara and instant porridge from fermented okara, assessed through the DPPH method, revealed that fermented okara (FO) significantly exhibited a higher antioxidant activity (46.269% ± 3.14) compared to unfermented okara. Furthermore, when the fermented okara was processed into instant porridge, the sample (PFO100) demonstrated the highest antioxidant activity, measuring 66.82% ± 1.33. In contrast, instant porridge from Broken jasmine rice (R100) showed the lowest antioxidant activity at 11.21% ± 3.72, as indicated in Table 3. The values for antioxidant activity were notably higher compared to rice porridge, being 6 times greater. These findings correspond to the total phenolic compound quantities mentioned earlier in Section 3.2.

**Table 3**. The percentage of antioxidant activity using the DPPH method for samples at a concentration of 100 mg/mL

| Sample | % Scavenging activity |
|---|---|
| UO | $2.95 \pm 0.163^{f}$ |
| FO | $46.269 \pm 3.14^{b}$ |
| PFO100 | $66.82 \pm 1.33^{a}$ |
| PFO75 | $41.37 \pm 2.95^{c}$ |
| PFO50 | $32.32 \pm 1.47^{d}$ |
| R100 | $11.21 \pm 3.72^{e}$ |

Results were expressed as mean ± standard deviation; Sample (n=3). Different lowercase letters are significantly different (P<0.05) by Duncan's post-hoc test.

When considering the concentration of substances capable of reducing the DPPH concentration by 50% ($IC_{50}$), it was found that instant porridge from fermented okara retained the greatest ability to inhibit free radicals, with an $IC_{50}$ value of 60.96 mg/mL. The study did not find the concentration of the substance that could reduce the DPPH concentration by 50% for unfermented okara and Broken jasmine rice porridge, as illustrated in Figure 3.

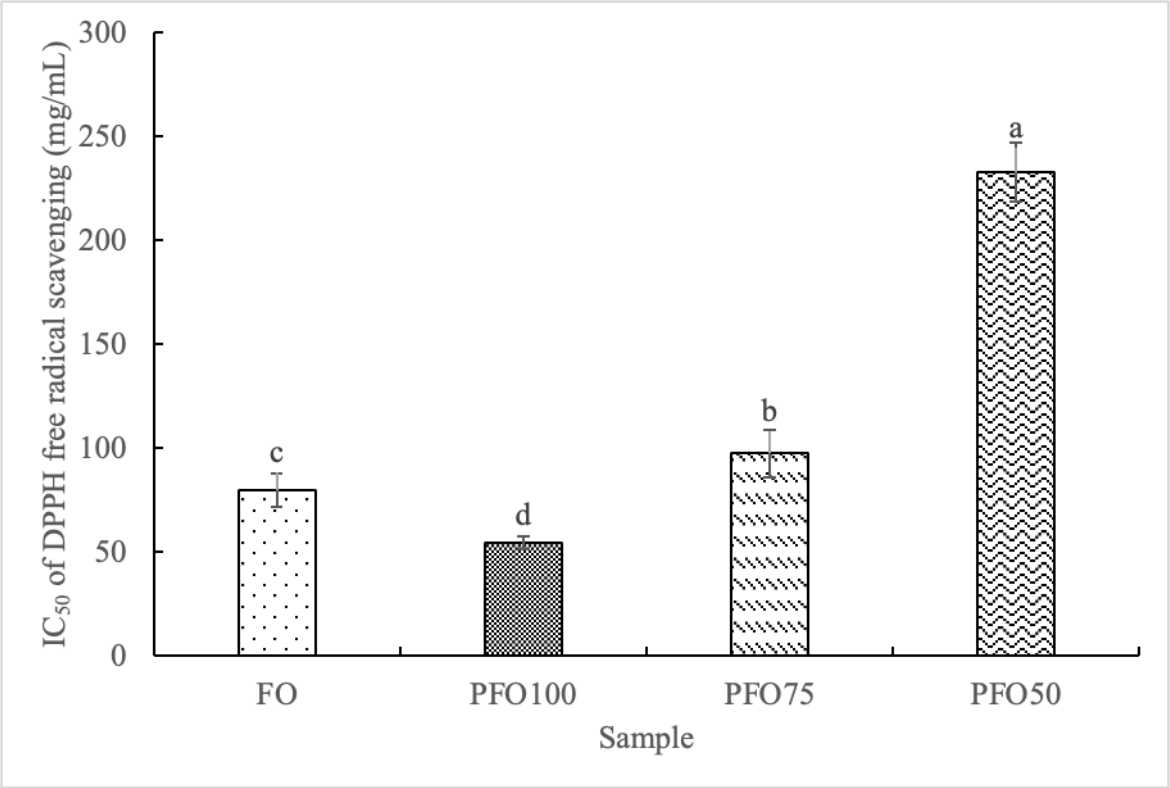


**Figure 3.** The concentration of the substance required to reduce the concentration of DPPH by 50% ($IC_{50}$).
Results were expressed as mean ± standard deviation; Sample (n=3). Different lowercase letters are significantly different ($P<0.05$) by Duncan's post-hoc test.

In accordance with Hong *et al.* [22], thermal process can increase antioxidant activities. The study revealed that steam explosion-processed Qingke (highland hull-less barley) showed the highest antioxidant activities including $Fe^{2+}$ chelating abilities and ABTS radical scavenging activity. Additionally, Mayachiew *et al.* [23] investigated the impact of incorporating soybean on the functional properties of rice porridge powder. The soybean mixed with rice porridge at a ratio of 3:7 exhibited higher TPC and DPPH radical scavenging activity, with concentrations of 4.37 mg GAE/g sample and 3.07 µmoL Trolox/g dry sample, respectively, compared to rice porridge powder, which had TPC and DPPH radical scavenging activity at concentrations of 1.09 mg GAE/g sample and 0.21 µmol Trolox/g dry sample, respectively.

## 4. Conclusion

This research demonstrated the significant potential of enhancing the functional properties of okara, a by-product of soymilk production, through fermentation with *Rhizopus oligosporus* TISTR 3138. The fermented okara exhibited a substantial increase in total phenolic content. Furthermore, the utilization of fermented okara in porridge products, particularly in conditions where it replaced a portion of rice, resulted in products with elevated total phenolic content and enhanced antioxidant activity. These findings underscore the potential of fermented okara as a functional ingredient in food products, offering improved nutritional and antioxidant qualities. The utilization of fermentation techniques opens avenues for enhancing the value and applications of soybean by-products in the food industry.

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# Evaluation of Antioxidant Activity, Total Phenolic Content, and Total Flavonoid Content of Betong Watercress

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## Abstract

Antioxidants play an important role in mitigating various diseases. Watercress (*Nasturtium officinale* R.Br.), a member of the cruciferous vegetable family, contains various nutrients and phytochemicals. However, the antioxidant information of the watercress cultivated in Thailand, Betong watercress, is insufficient. The purpose of the current study was to determine the levels of phenolic and flavonoid compounds and evaluate the antioxidant properties of Betong watercress. Aqueous and ethanolic extracts of Betong watercress were prepared for the in vitro experiments. The total phenolic content and total flavonoid content of watercress extracts were evaluated. The antioxidant activities of the extracts were measured using the ferric reducing antioxidant power (FRAP) assay, the 2,2-diphenyl-1-picrylhydrazyl hydrate (DPPH) radical scavenging assay, and the 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) radical scavenging assay. The results demonstrated that the amounts of total phenolic and total flavonoid in the ethanolic extract, 41.14 ± 0.66 mg gallic acid equivalent/g extract and 13.94 ± 0.44 mg quercetin equivalent/g extract, respectively, were significantly higher than those in the aqueous extract, 33.98±1.56 mg gallic acid equivalent/g extract and 7.87 ± 0.59 mg quercetin equivalent/g extract, respectively. The antioxidant activities of the aqueous extract, as analyzed by FRAP assay, DPPH radical scavenging assay, and ABTS radical scavenging assays, were 52.60 ± 0.64, 43.73 ± 3.53, and 95.36 ± 4.16 mg trolox equivalent/g extract, respectively. In comparison, those of the ethanolic extract were 62.55 ± 3.18, 66.48 ± 3.81, and 119.09 ± 1.31 mg trolox equivalent/g extract, respectively. Similarly to the results on total phenolic content and total flavonoid content, the ethanolic extract of Betong watercress showed significantly higher antioxidant properties than the aqueous extract. Moreover, the antioxidant activities of watercress extract had strong correlations with the total phenolic content and the total flavonoid content. In conclusion, the antioxidant potential of Betong watercress might be due to its polyphenol compounds. Nevertheless, further studies are required to confirm the health benefits of Betong watercress consumption.

***Keywords****:* Betong watercress; Antioxidant activity; Total phenolic content; Total flavonoid content

## 1. Introduction

Cellular metabolism generally produces the harmful byproduct known as free radicals. Environmental factors, in addition to cellular metabolism, can exacerbate the production of cellular free radicals. The environmental factors include ultraviolet (UV) light exposure, cigarette smoke, air pollutants, ozone, heavy metal ions, pesticides and insecticides, and toxin[1]. Free radicals are oxygen-containing molecules that have one or more unpaired electrons. Free radicals easily bind to other molecules due to their unstable condition [2]. Free radicals can impair cellular structure and components, including proteins, lipids, and DNA. The body naturally has an antioxidant system to counteract free radicals [1]. However, if there is an imbalance between free radical and antioxidant levels for a long time, it leads to oxidative stress [3]. Oxidative stress is the cause of various health conditions such as the aging process, neurodegenerative disease, asthma, diabetes, cancer, and cardiovascular disease [1-3].

Antioxidant is a compound that can neutralize free radicals, such as oxygen, nitrogen, and lipidic radicals, and protect a biological system. Antioxidants neutralize free radicals by donating their electrons to free radical molecules [4]. Natural antioxidants are divided into two main categories. The enzymatic antioxidant, or endogenous antioxidant, is the antioxidant generated in our body against free radicals. The well-known enzymatic antioxidants include glutathione peroxidase, catalase, and superoxide dismutase. Whereas the non-enzymatic antioxidant, or exogenous antioxidant, can be found in natural sources. The non-enzymatic antioxidants include minerals (iodine, zinc, copper, and selenium), vitamins (vitamin A, vitamin E, and vitamin C), polyphenols (flavonoids, gingerol, polyphenolic acid, and curcumin), carotenoids, and more. The consumption of non-enzymatic antioxidants is expected to support our enzymatic antioxidant system [5].

Watercress, scientifically known as *Nasturtium officinale* R.Br., belongs to the Brassicaceae family. It is a leafy, semi-aquatic vegetable [6]. The main groups of bioactive phytochemicals found in watercress include glucosinolates (gluconasturtiin and glucobrassicin), isothiocyanates, phenolic compounds (caffeic acid, gallic acid, coumaric acid, and chlorogenic acid), and flavonoids (rutin and quercetin-3-O-rutinoside) [7-8]. The consumption or supplementation of watercress has been associated with numerous health benefits. Many biological health benefits of watercress have been studied, such as effects on the antioxidant system, DNA damage, hepatotoxicity, anticancer potential, and anti-diabetic effects [9].

The native lands of this plant are western Asia, India, Europe, and Africa [6-7]. However, the growth environment can impact plant morphology, nutritional quality, and the nutraceutical benefit of plants. Qian *et al*. [10] conducted a study comparing watercress cultivated in warm and dry conditions in California with that cultivated in cool and wet conditions in the United Kingdom. The results revealed the significant differences in morphological characteristics, biochemical content, and antioxidant capacity.

In Thailand, watercress is abundantly cultivated in Betong district, Yala province. The Thai local name of watercress is “Betong watercress” or “Phak Nam Betong” [11]. The studies on Betong watercress remains limited, particularly concerning its antioxidant profile. Therefore, this study aimed to determine the levels of phenolic and flavonoid compounds and evaluate the antioxidant properties of Betong watercress.

## 2. Materials and Methods

### 2.1 Plant material

Betong watercress was purchased from the Zhi Wu watercress farm in Betong district, Yala province, Thailand. A voucher specimen of Betong watercress (No. 0023371) was identified and deposited in the Department of Pharmaceutical Sciences, Faculty of Pharmacy, Chiang Mai University. The plants were air-dried before being ground into powder. The powder was stored away from sunlight in desiccator cabinet until usage.

### 2.2 Preparation of the Betong watercress extracts

For the preparation of the aqueous extract, 10 g of ground watercress were boiled in 100 mL of water for 20 min. The aqueous extract was filtered through Whatman No. 1 filter paper using the vacuum filtration technique. The filtered solution was then lyophilized and stored at -20°C. For the ethanolic extract, 10 g of ground watercress were soaked in 100 mL of 80% ethanol at room temperature for 24 h. The solution was filtered and then evaporated by a rotary evaporator at 40°C. Afterward, the ethanolic extract was lyophilized and stored at -20°C.

### 2.3 Total phenolic content

The total phenolic content determination of watercress extracts was performed using the Folin-Ciocalteu (FC) colorimetric method modified from Kulprachakarn *et al.* [12]. In a 96-well plate, 20 µL of watercress extracts were mixed with 100 µL of FC reagent, which was diluted in deionized water with a ratio of 1:10. After 3 min of incubation, 80 µL of sodium carbonate solution ($Na_2CO_3$, 1 M) were added. Absorbance at 765 nm was measured using a spectrophotometer. The total phenolic content was calculated from the standard calibration curve of gallic acid at the following concentrations (0-0.5 mg/mL). The total phenolic content was expressed as mg gallic acid equivalent (GAE)/g extract.

### 2.4 Total flavonoid content

The determination of total flavonoid content in watercress extracts was performed using an aluminum chloride colorimetric method modified from Faizy *et al.* [13]. In a 96-well plate, 50 µL of watercress extracts were mixed with 10 µL of aluminium chloride ($AlCl_3$, 10% w/v), 150 µL of 50% methanol, and 10 µL of sodium acetate ($CH_3COONa$, 1 M). The mixture was equilibrated at room temperature for 30 minutes. Absorbance at 415 nm was measured using a spectrophotometer. The total flavonoid content was calculated from the standard calibration curve of quercetin at the following concentrations (0-0.5 mg/mL). The total flavonoid content was expressed as mg quercetin equivalent (QE)/g extract.

### 2.5 Ferric reducing antioxidant power (FRAP) assay

The FRAP assay was conducted following the method from Xiao *et al.* [14]. This antioxidant assay can directly measure the antioxidants in the sample. The FRAP working solution was made from acetate buffer (300 mM, pH 3.6), ferric chloride hexahydrate ($FeCl_3 \cdot 6H_2O$, 20 mM), and TPTZ (10 mM) in a ratio of 10:1:1. In a 96-well plate, 5 µL of watercress extracts were incubated with 180 µL of FRAP working solution at 37°C for 5 min. Then, the absorbance measurement was performed at 593 nm using a spectrophotometer. Trolox was used as the standard at a concentration of 0-1 mg/mL. The antioxidant activity, FRAP value, was calculated from the standard calibration curve and expressed as mg trolox equivalent (TE)/g extract.

### 2.6 DPPH radical scavenging assay

The 2,2-diphenyl-1-picrylhydrazyl hydrate (DPPH) radical scavenging assay was performed using the method from Xiao *et al.* [14] with slight modifications. In a 96-well plate, 10 µL of watercress extracts were mixed with 195 µL of DPPH solution. The mixture was equilibrated in the dark at room temperature for 30 min. Then, the absorbance measurement was performed at 517 nm using a spectrophotometer. Trolox was used as the standard at a concentration of 0-0.2 mg/mL. The antioxidant activity, DPPH radical scavenging value,

was calculated from the standard calibration curve and expressed as mg TE/g extract. The DPPH radical percentage of inhibition was also calculated using the equation below:

$$\% \text{ inhibition} = [(A_0-A_1)/A_0] \times 100$$

Where $A_0$ is the absorbance of the blank and $A_1$ is the absorbance of the sample. The DPPH radical percentage of inhibition was then used to estimate the DPPH radical scavenging activity, $IC_{50}$ value, which is the concentration of sample required to scavenge 50% of the DPPH radicals.

**2.7 ABTS radical scavenging assay**

The 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) radical scavenging assay was performed using the method from Xiao *et al.* [14] with slight modifications. The ABTS working solution was made from ABTS (7 mM) and potassium persulfate ($K_2S_2O_8$, 2.45 mM) in a ratio of 1:1. The ABTS working solution was equilibrated in the dark at room temperature for 12 h. The absorbance of the ABTS working solution should be 0.7 ± 0.02 at 743 nm before use. In a 96-well plate, 10 µL of watercress extracts were mixed with 195 µL of ABTS working solution. The mixture was equilibrated in the dark at room temperature for 30 min. Then, the absorbance measurement was performed at 734 nm using a spectrophotometer. Trolox was used as the standard at a concentration of 0-0.2 mg/mL. The antioxidant activity, ABTS radical scavenging value, was calculated from the standard calibration curve and expressed as mg TE/g extract. The ABTS radical percentage of inhibition and the ABTS radical scavenging activity, $IC_{50}$ value, were also calculated using the same method as described earlier.

**2.8 Statistical analysis**

All statistical analysis was performed by GraphPad Prism Software version 10.0 for Windows. The experiments were performed in triplicate, and the results were expressed as the mean ± standard deviation (SD). The independent t-test and one-way analysis of variance (ANOVA) with a Tukey post hoc test were used to compare means between groups. The relationship between two variables was determined using the Pearson correlation coefficient. A *P*-value of less than 0.05 is considered significant.

**3. Results & Discussion**

**3.1 Polyphenol content of Betong watercress extracts**

The polyphenol content of Betong watercress extracts, including total phenolic and total flavonoid content, is summarized in Table 1. The total phenolic content of ethanolic extract (41.14 ± 0.66 mg GAE/g extract) was significantly higher than that of aqueous extracts (33.98 ± 1.56 mg GAE/g extract). Similarly, the total flavonoid content of the ethanolic extract (13.94 ± 0.44 mg QE/g extract) was significantly higher than that of aqueous extracts (7.87 ± 0.59 mg QE/g extract). From the results, ethanol could draw more phenolic and flavonoid content than water. The results of the current study align with previous studies. Zeb [6] mentioned the higher total phenolic content in the alcoholic extract than in the aqueous extract of wild watercress collected in Pakistan, 3.21 and 2.31 mg GAE/g dried watercress, respectively. Fenton-Navarro *et al.* [15] demonstrated that the alcoholic extract of watercress purchased from Maxico showed significantly higher total phenolic and total flavonoid content, 552.5 ± 39.12 mg GAE/g extract and 5,067 ± 116.83 mg QE/g extract, respectively, when compared to the aqueous extract, 61.47 ± 8.47 mg GAE/g extract and 773 ± 64.38 mg QE/g extract, respectively.

**Table 1.** Total phenolic content and total flavonoid content of aqueous extract and ethanolic extract of watercress.

| Sample | Total phenolic content [a] | Total flavonoid content[b] |
|---|---|---|
| Aqueous extract | 33.98 ± 1.56 | 7.87 ± 0.59 |
| Ethanolic extract | 41.14 ± 0.66** | 13.94 ± 0.44*** |

Data are mean±SD of triplicate determinations.
[a]Total phenolic content was expressed as mg gallic acid equivalent/g extract.
[b]Total flavonoid content was expressed as mg quercetin equivalent/g extract.
*Significantly different from aqueous extract (P<0.05).
**Significantly different from aqueous extract (P<0.01).
***Significantly different from aqueous extract (P<0.001).

The presence of a higher level of phenolics and flavonoids in ethanolic extract is due to its ability to form hydrogen bonds. Hydrogen bonds play an essential role in the extraction reaction. During the extraction process, the hydroxyl group (-OH) of the ethanol molecule can form a hydrogen bond with the oxygen atom of phenolic and flavonoid. This aligns with previous reports asserting that ethanol is more effective than water in extracting phenolic compounds (16). However, water molecules also have a hydroxyl group and can form hydrogen bonds. Water can be added to ethanol to enhance the ability to form hydrogen bonds and draw polyphenol compounds (17). The amount of water added to ethanol is important. It has been verified that the higher the amount of water, the weaker the intermolecular hydrogen bond formation (18). The appropriate amount of water added to ethanol for polyphenol extraction was around 20-30% (18, 19).

### 3.2 Antioxidant properties of Betong watercress extracts

The FRAP assay, the DPPH radical scavenging assay, and the ABTS radical scavenging assay were used to determine the antioxidant properties of Betong watercress extracts, as shown in Figures 1-3. The reducing power of Betong watercress extract can be assumed by the FRAP assay. In this method, the antioxidants reduce the ferric-tripyridyltriazine ($Fe^{3+}$-TPTZ) complex to its ferrous form ($Fe^{2+}$-TPTZ). The amount of $Fe^{2+}$-TPTZ is correlated with the amount of antioxidant in Betong watercress extract (17). The FRAP value was calculated from the standard curve of Trolox and expressed as mg TE/g extract. Based on the results, the aqueous extract and ethanolic extract of Betong watercress had the ability to reduce $Fe^{3+}$-TPTZ. The FRAP value of ethanolic extract (62.55 ± 3.18 mg TE/g extract) was significantly higher than that of aqueous extract (52.60 ± 0.64 mg TE/g extract) (Figure 1A).

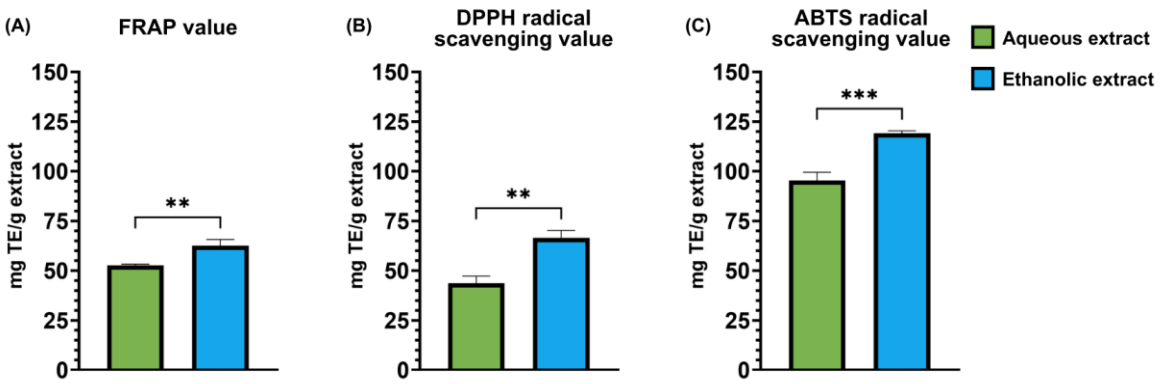


**Figure 1.** Antioxidant activities of aqueous extract and ethanolic extract of watercress.
(A) FRAP value, (B) DPPH radical scavenging value, (C) ABTS radical scavenging value were expressed as mg TE/g extract
Data are mean ± SD of triplicate determinations.
*Significantly different (P<0.05)
**Significantly different (P<0.01)
***Significantly different (P<0.001)
FRAP, ferric reducing antioxidant power
DPPH, 2,2-diphenyl-1-picrylhydrazyl hydrate
ABTS, 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) radical scavenging
TE, trolox equivalent; SD, standard deviation

The DPPH radical scavenging assay was used to assess the scavenging activity of Betong watercress extracts on DPPH radicals. In this method, the antioxidant stabilizes DPPH radical molecules by donating their hydrogen to the DPPH radicals [18]. The antioxidant activity from the DPPH radical scavenging assay can be expressed as the DPPH radical scavenging value (mg TE/g extract), DPPH radical percentage of inhibition (%), or DPPH $IC_{50}$ value. The DPPH radical scavenging value of Betong watercress extracts is presented in Figure 1B. Ethanolic extract exhibited a significantly higher DPPH radical scavenging value (66.48 ± 3.81 mg TE/g extract) compared to the aqueous extract (43.73 ± 3.53 mg TE/g extract). The DPPH radical inhibition percentages at different concentrations of Betong watercress extract are shown in Figure 2A. Based on the results, at the same concentration, ethanolic extract had a higher ability to inhibit DPPH radicals than aqueous extract. The $IC_{50}$ values represent the concentration required to scavenge 50% of DPPH radicals. The DPPH $IC_{50}$ value of Betong watercress extracts was compared with Trolox, which is the standard antioxidant compound. The results demonstrated that Trolox was significantly less used than Betong watercress extracts to scavenge 50% of DPPH radicals. Moreover, the DPPH $IC_{50}$ value of the ethanolic extract was significantly lower than that of the aqueous extract (Figure 3A).

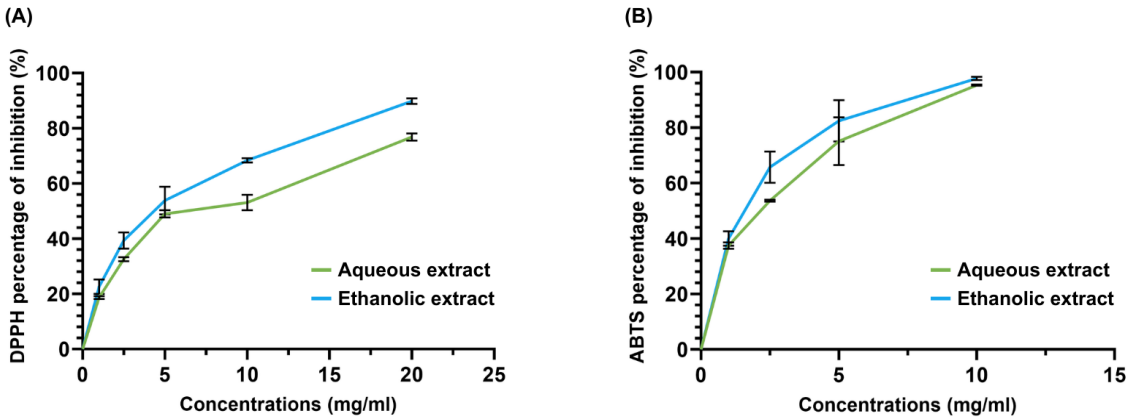


**Figure 2.** Free radical percentage of inhibition of watercress aqueous and ethanolic extracts at different concentrations. (A) DPPH radical percentage of inhibition and (B) ABTS radical percentage of inhibition.
Data are mean ± SD of triplicate determinations.
DPPH, 2,2-diphenyl-1-picrylhydrazyl hydrate
ABTS, 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid)
SD: standard deviation.

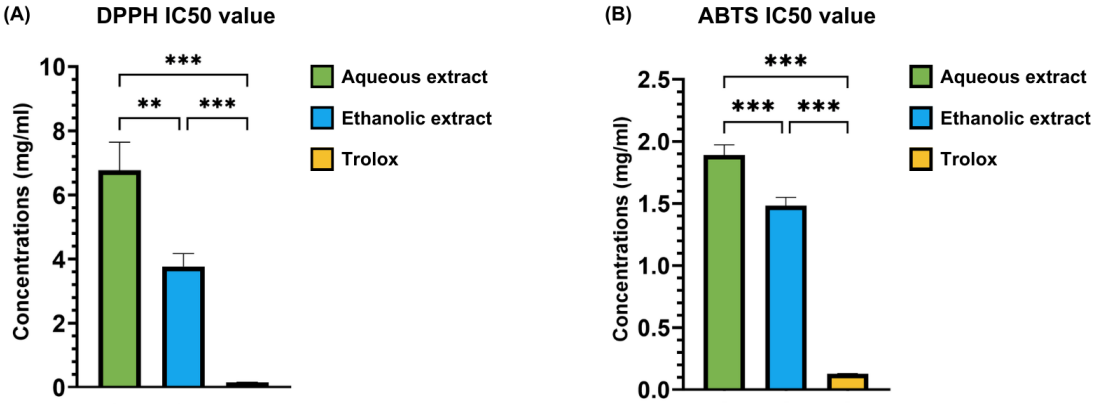


**Figure 3.** Free radical scavenging activities ($IC_{50}$) of trolox, aqueous extract, and ethanolic extract of watercress. (A) DPPH radical scavenging activity (DPPH $IC_{50}$ value) and (B) ABTS radical scavenging activity (ABTS $IC_{50}$ value) were expressed as mg/mL.
Data are mean ± SD of triplicate determinations.
*Significantly different ($P < 0.05$).
**Significantly different ($P < 0.01$).
***Significantly different ($P < 0.001$).
DPPH, 2,2-diphenyl-1-picrylhydrazyl hydrate
ABTS, 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid)
SD: standard deviation.

The ABTS radical scavenging assay principle is quite similar to the DPPH radical scavenging assays. The antioxidants donate their hydrogen to the nitrogen atom of ABTS radicals [14]. The antioxidant activity measured by the ABTS radical scavenging assay can be represented as the ABTS radical scavenging value (mg TE/g extract), ABTS radical percentage of inhibition (%), or ABTS $IC_{50}$ value. Figure 1C shows the ABTS radical scavenging value of the extracts of Betong watercress. The ethanolic extract demonstrated a significantly higher ABTS radical scavenging value (119.09 ± 1.31 mg TE/g extract) compared to the aqueous extract (95.36 ± 4.16 mg TE/g extract). Figure 2B displays the percentage of inhibitions of ABTS radicals at various concentrations of Betong watercress extract. According to the findings, at an equal concentration, ethanolic extract could scavenge more ABTS radicals than aqueous extract. The ABTS $IC_{50}$ values of Betong watercress extracts and Trolox were compared. The findings revealed that Trolox had a lower $IC_{50}$ value than ethanolic extract and aqueous extract, respectively (Figure 3B).

The previous study also demonstrated the antioxidant properties of other variants of watercress. Boligon *et al.* [19] analyzed the antioxidant activity of watercress planted in a hydroponic system. The DPPH $IC_{50}$ value of the crude extract of watercress was 30.76 ± 0.19 µg/mL. Burçin Uyumlu *et al.* [20] demonstrated the antioxidant capacity of watercress from Turkey. The 500 µg/mL of watercress methanolic extract had 290.7 ± 2.18 µg/mL of DPPH $IC_{50}$ value. Yazdanparast *et al.* [17] reported the FRAP value and DPPH $IC_{50}$ value of the ethanolic extract of watercress cultivated in Iran. The watercress extract showed 680.2 ± 30.5 µM/mg of FRAP value and 114.7 µg/mL of DPPH $IC_{50}$ value. Aires *et al.* [21] analyzed the antioxidant capacity of baby-leaf watercress purchased from an organic farm in Protugal. The FRAP value of watercress methanol extract was 14.3 ± 2.2 µM equivalent $FeSO_4$/mg extract.

**3.3 Correlation between polyphenol content and antioxidant activity**

The relationship between polyphenol content and antioxidant activity of Betong watercress extract was analyzed using the Pearson correlation coefficient. The variables can be assumed to have a strong relationship when the correlation coefficient is 0.8-1.0. Based on the results displayed in Table 2, the Pearson correlation demonstrated a strong positive relationship between total phenolic content and FRAP value, DPPH radical scavenging value, and ABTS radical scavenging value, with correlation coefficients of 0.88, 0.91, and 0.99, respectively. Total flavonoid also had a strong positive relationship with FRAP value, DPPH radical scavenging value, and ABTS radical scavenging value, with correlation coefficients of 0.91, 0.98, and 0.95, respectively. We can assume from these findings that the antioxidant capacity, including FRAP value, DPPH radical scavenging value, and ABTS radical scavenging value, will increase as the levels of total phenolic and total flavonoid of watercress increase. On the other hand, total phenolic content and total flavonoid content had strong negative relationship with $IC_{50}$ values of both DPPH and ABTS radicals. The correlation coefficients between total phenolic content and DPPH $IC_{50}$ value and ABTS $IC_{50}$ value were -0.82 and -0.89, respectively. The correlation coefficients between total flavonoid content and DPPH $IC_{50}$ value and ABTS $IC_{50}$ value were -0.95 and -0.98, respectively. Which means the higher the amount of total phenolic and total flavonoid, the lower the concentrations used to scavenge 50% of free radicals. According to the findings, the higher the polyphenol content, the higher the antioxidant properties.

Polyphenols are the major group of non-enzymatic antioxidants. The polyphenol has been reported to have significant antioxidant potential [5]. Polyphenol compounds can enhance the activity and production of enzymatic antioxidants. Polyphenol can inhibit mechanisms related to free radical production, such as the reactions of NADPH oxidase (NOX), xanthine oxidase (XO), lipoxygenase (LOX), monoamine oxidase (MAO), and inducible nitric oxide synthase (iNOS). Polyphenol itself can donate electrons to free

radicals.Polyphenol can also bond with free metal ions, which are the catalysts of free radical synthetic reactions [22, 23].

**Table 2.** Correlation coefficients between polyphenol contents, including total phenolic content and total flavonoid content, and antioxidant activity measured with FRAP value, DPPH radical scavenging value, ABTS radical scavenging value, DPPH $IC_{50}$ value, and ABTS $IC_{50}$ value.

| | Total phenolic content | Total flavonoid content |
|---|---|---|
| FRAP value | 0.88 | 0.91 |
| DPPH radical scavenging value | 0.91 | 0.98 |
| ABTS radical scavenging value | 0.99 | 0.95 |
| DPPH $IC_{50}$ value | -0.82 | -0.95 |
| ABTS $IC_{50}$ value | -0.89 | -0.98 |

TPC, total phenolic content; TFC, total flavonoid content; FRAP, ferric reducing antioxidant power; DPPH, 2,2-diphenyl-1-picrylhydrazyl hydrate; ABTS, 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid).

## 4. Conclusion

We have shown the antioxidant properties of Betong watercress. Despite the environmental factor, the extraction method also affects the polyphenol and antioxidant properties of watercress. Specifically, our results showed that the ethanolic extract of Betong watercress contains higher polyphenol content and antioxidant capacity compared to the aqueous extract. Moreover, the polyphenol content of Betong watercress had a strong relationship with its antioxidant capacity. These findings align with previous research emphasizing the antioxidant properties of watercress. In conclusion, Betong watercress represents a promising source of antioxidants, and this might be due to its polyphenol compounds. Adding Betong watercress in daily diets may provide the antioxidant benefits. Nevertheless, further studies are required to confirm the precise quantity and other associated health benefits of Betong watercress consumption.

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# Effect of High Pressure Processing on the Quality of Kum Doi Saket Variety Grass Juice

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**Abstract**

Kum Doi Saket (KDS) is a black purple glutinous rice cultivar (*Oryza sativa* L.) that is widely grown in the north and north-east of Thailand. Juice squeezed from KDS rice grass harvested at the jointing stage exhibited the considerable levels of phenolic compounds, antioxidants, and anthocyanin. However, the limitation of rice grass juice is that it is perishable and has a short shelf-life. Therefore, it is necessary to develop appropriate preservation strategies to enhance the shelf life of rice grass juice while maintaining its phytochemical properties and guaranteeing product safety. The objectives of this research were to find out the conditions of high pressure processing (HPP) and heat treatment (HT) which can inactivate pathogenic bacteria up to the target of 5 log reduction and to evaluate the influence of HPP and HT on phytochemical properties of KDS rice grass juice. In this study, KDS rice grass harvested at day 9 after planting was used to make the juice. The juices with the inoculation of *Escherichia coli* K12 ATCC 10798 and *Listeria innocua* JCM 32814 were treated by HPP (400-600MPa/25ºC/5-15 min) and HT (80ºC/2-8 min), respectively. The results of HPP treatment showed that the elevation of pressure from 400MPa to 600MPa remarked the significant increase in microbial reduction. At the pressure of 600MPa, the bacterial inactivation increased significantly as the holding treatment time was extended from 5min to 10min. Overall, HPP at 600 MPa for 10 min and HT at 80°C for 8 min could significantly inactivate both strains of bacteria for more than 5 log reduction. However, compared to control sample, the sample treated by HT (80°C, 8 min) resulted in the significant decrease in total phenolic content (45.96% reduction), antioxidant activity (46.34% reduction), and anthocyanin content (48.46% reduction) while the HPP (600 MPa, 10min) did not significantly change the properties of rice grass juice. In conclusion, HPP can be considered as a potential innovative technique to eliminate the targeted bacteria in KDS juice while maintaining the bioactivities similar to those of the fresh juice.

***Keywords:*** High pressure processing; Kum Doi Saket rice grass juice; Log reduction; Phytochemical properties

## 1. Introduction

Since the last century, cereal grass has been considered as a functional food with "blood-building" function [(1)]. Wheatgrass juice is a concentrated source of nutrients, containing considerable amounts of vitamins, minerals, amino acids, active enzymes (over 80 have been identified), antioxidants and chlorophylls [(2)]. However, due to the high price, history, and cultivation area of wheat, the products from wheatgrass are not commonly launched and consumed in Asian countries. Belonging to the *Poaceae* family similar to wheat, rice (*Oryza sativa*) is the most important staple food crop in Asia where is also the leading continent in rice production, accounting for nearly 90% of the market share all over the world [(3)]. Although there are just a few studies related to rice grass, most of them showed

that rice grass juice had similar or even superior phytochemical characteristics compared to wheatgrass juice [4]. Khanthapoka [5] revealed that among 15 juice samples from 7 cultivars of white grass, 7 cultivar of color grass, and 1 cultivar of wheat grass, rice-grass juice from Kum Doi Saket cultivar exhibited the highest content of total phenolic content (4.3 mg GAE/g DE) and the greatest radical scavenging activity with a half maximal effective concentration ($EC_{50}$) of 0.11 mg dried extract/mL [5]. A recent study of Wongpoomchai *et al.* [6] indicated the cancer chemopreventive potential of Kum Doi Saket rice grass. Rice grass extract from black rice Kum Doi Saket is also a potential source of chlorophyll (2.16 mg/g), anthocyanin (7.37 mg/g), carotenoid (1.05 mg/g), and flavonoid (84.55 mg/g). This research also determined numerous phenolic compounds in Kum Doi Saket extract by HPLC such as protocatechuic acid, chlorogenic acid, vanillic acid, syringic acid, p-coumaric acid, rutin, isorhamnetin-3-glucoside, luteolin, apigenin, quercetin-3-glucoside, and quercetin (6). On the other hand, the study of Phimphilai *et al.* [7] suggested that Sukhothai-1 rice grass was safe for consumption with no observation of toxicity in hepatocyte culture, acute toxicity in mice, sub-chronic toxicity in rats [7]. Overall, due to the following reasons: i) rice is the main staple food in Asia; ii) reasonable price; and iii) black rice grass is a great source of polyphenols and antioxidants, rice grass juice can be developed competitively to wheatgrass juice as an alternative functional beverage.

However, quality and safety preservation strategies of a beverage product are complicated. Rice grass juice, similar to wheatgrass juice, can be categorized as low-acid vegetable juice with pH > 4.6. The United States Food and Drug Administration (USFDA) defined “vegetable juice" as “the liquid unfermented but fermentable product or lactic acid fermented product intended for direct consumption obtained from the edible part of one or more sound vegetables and preserved exclusively by physical means”. The products must be free from microorganisms capable of development under normal conditions of storage [8]. Today, global fruit and vegetable juice market is expanding. Consumers not only concern about the sensorial characteristics of food products but also pay more attention to their safety, nutritional value, and additive residues. Although thermal preservation technologies effectively reduce levels of food microorganisms, such preservation methods can alter natural taste and flavor and degrade nutritional quality of the product. Therefore, alternative or novel food-processing technologies are being explored and implemented to provide safe, fresh-tasting, and nutritive foods without using heat or chemical preservatives [9]. Among them, high pressure processing (HPP), a non-thermal technique or cold pasteurization, can satisfy two targets of reducing the numbers of microorganisms up to 5-log reduction and retaining the sensorial quality as well as bioactive compounds of the product. There are four principles which describes the action of HPP: (1) Le Chatelier’s principle: if a process in equilibrium is disturbed, the process responds in such a way as to reduce the disturbance, so any that leads to a decrease in volume during HPP is favored [10]; (2) Isostatic principle: the pressure effects are instantaneously and homogenously distributed within the food, regardless of geometry and size [11]; (3) Microscopic ordering principle: a constant temperature, an increase in pressure increases the degree of ordering of molecules of a given substance [12]; and (4) Transition state theory: the velocity of a given reaction can increase or decrease by changing pressure, according to whether the intermediate state is less or more voluminous. [13].

For fruit and vegetable juices and smoothies, HPP is usually applied as pasteurization method with three main characteristics: i) pressures in the range of 400-600 MPa; ii) treatment times of 1-15 min and iii) prolonging the shelf-life of food products up to 4-6 weeks at refrigerated temperature (4$^{o}$C) [11]. So far, many research have shown that HPP could preserve the safety and organoleptic quality of fruit and vegetable beverage such as orange juice [14-15], apple juice [16-17], strawberry juice [18-19], carrot juice [20], raspberry juice [21], and so on.

The aim of this work was to investigate the effect of HPP (400 – 600 MPa/5-15 min) on lethality of *Escherichia coli* K12 ATCC 10798 and *Listeria innocua* JCM 32814 inoculated in Kum Doi Saket rice grass juice and the phytochemical properties of the juice after treatment.

## 2. Materials and Methods

### 2.1 Preparation of Rice Grass Juice (RGJ)

Kum Doi Saket rice grass was provided by Lanna Rice Research Center, Chiang Mai University, Chiang Mai, Thailand. Fresh grass was harvested by cutting above the ground at day 9 of post-planting when the plant was about 22-23 cm long and before the appearance of the third young leaf. All fresh grass was washed with tap water and pretreated for dirt removal and a microbial reduction. For preliminary treatment experiments, the grass was weighed and divided into equal amount then treated by (1) soaking in detergent (sodium bicarbonate 0.1%, 15 min), (2) blanching (85ºC, 1 min), and (3) soaking in detergent following by blanching, respectively. RGJ was extracted by Oscar DA1200 juicer (Thailand) and filter through cheese cloth for three times. The juice was then transferred into 100 mL PET bottles and stored at -20ºC until and thawed in a water bath at room temperature prior to use. The influences of preliminary treatment methods on RGJ quality were investigated by examining the changes in total phenolic content (TPC), antioxidant activity (AOA), and anthocyanin content (AC) of RGJ after treatment.

### 2.2 Bacterial strains and growth conditions

Bacterial strains used in this study were *Escherichia coli* K12 ATCC 10798 (Gram negative bacteria) and *Listeria innocua* JCM 32814 (Gram positive bacteria) obtained from The American Type Culture Collection and Japan Collection of Microorganisms, respectively.

These are surrogate strains for pathogenic *E. coli* O157:H7 and *L. monocytogenes*, respectively. The bacteria were maintained in stock cultures at –20ºC in Tryptic Soy Broth (TSB) supplemented with 50% glycerol. Prior to experiments, cell cultures were activated by streaking on Tryptic Soy Agar (TSA) and incubated at 37ºC for 24 h. After that, a single bacterial colony from TSA was subculture into 10 ml of TSB at 37ºC until the strains reached early stationary phase (approximately $10^9$ CFU/mL). Then, cells were washed twice by sterilized sodium chloride solution (NaCl 0.85% w/v) and harvested by centrifugation (6000xg/10 min/4ºC). The bacterial pellets were resuspended in saline solution (0.85% w/v NaCl). The cell concentration was adjusted to approximately $10^9$ CFU/mL for further experiments.

### 2.3 Bacterial inoculation and Enumeration of viable and sublethal injured cells

Cell cultures were inoculated into juice sample with approximate final concentration of $10^6$ CFU/mL. The inoculated samples were kept at 4°C not exceeding 1 h prior to HT or HPP. Immediately after treatment, the experiments for bacterial enumeration were conducted. Appropriate decimal dilutions of cell suspensions were drop-plated onto TSA. All plates were incubated at 37°C for 24 - 36 h. Then, typical colonies were counted and reported as log reduction which was calculated as the difference between the logarithmic counts of colonies in untreated ($N_0$) and treated (N) samples using Eq.1:

$$Log\ reduction = log10\ (N_0) – log10\ (N)$$

The experiments of enumeration of sublethal injured cells were follow the method of Zhao *et al.* [22] with some modification. Treated samples were plated on selective medium which was TSA supplemented with 2% (w/w) sodium chloride (TSA – NaCl 2%) for both *E. coli* ad *L.innocua*. All plates were incubated at 37°C for 24-36 h. The difference between

selective and nonselective media was expressed as the injured survivors. The percentage of injured cells was calculated according Eq.2 [22]:

$$\textit{The percentage of sublethally injured cell (\%)} = \frac{(1 - CFU/mL_{TSA\ NaCl\ 2\%})}{CFU/mL} \times 100$$

where $CFU/mL_{TSA\ –\ NaCl\ 2\%}$ was the counts on selective medium; and $CFU/mL_{TSA}$ was the counts in nonselective medium.

**2.4 Heat treatment**

Ten-milliliter of the inoculated RGJ was heated in a water bath at 80°C. The holding treatment time was ranging from 2 to 10 min. Immediately after treatment, samples were transferred to cold water bath to cool down. Subsequently, the inoculated RGJ samples were examined for the survival and sublethal injury of bacteria cells (Section 2.3).

**2.5 HPP treatment**

The inoculated RGJ samples in 30 mL polyethylene terephthalate (PET) bottle was subjected to HPP system (HHP-650, Baotou Kefa Co., Ltd., Inner Mongolia, China) with a capacity of 7.0 L. This unit pressurized at 2 MPa/s with the decompression time of 7 to 8 s. Pure water was used as the pressure transmitting fluid. The samples were treated at pressure levels ranging from 400 to 600 MP, for 5 to 15 min at room temperature. After treatment, inoculated RGJ samples were investigated for viability and sublethal ratio of bacterial cell (Section 2.3).

**2.6 Total phenolic content (TPC)**

TPC of uninoculated HT and HPP-treated RGJ was determined using the Folin-Ciocalteu method according to Khanthapoka *et al.* [5] with some modifications. 2.5 mL of Folin-Ciocalteu reagent 10% (v/v) was mixed with 0.5 mL of diluted RGJ (1:25). The mixture was incubated in the dark for 5 min. Then 2mL $Na_2CO_3$ 7.5% (w/v) was added to the reaction mixture followed by incubating in dark for 60 min. The absorbance of the solution was measured at 765 nm using a UV-VIS Spectrophotometer. The gallic acid (0-18 mg/L) standard curve was used to determine TPC which was expressed as milligrams of gallic acid equivalents per liter of juice (mg GAE/L) [5]

**2.7 ABTS (2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) assay**

Radical scavenging activity was determined using the method of Re *et al.* [23] with some modifications. In short, the $ABTS^{\cdot+}$ radical cation was generated by mixing ABTS 7 mM and $K_2S_2O_8$ 2.45 mM with the ratio of 1:1. The mixture was incubated in dark at room temperature for 12-16 h. The dark blue solution was diluted with water until reach the absorbance of 0.7 ± 0.02 at 734 nm using a UV-VIS Spectrophotometer. For measurement, 3 mL of $ABTS^{\cdot+}$ solution was mixed with 50 µl diluted RGJ (1:25) and incubated in dark at room temperature for 30 min. The absorbance was measured at 734 nm. Trolox solution (0-4µM) was used to make a standard curve. Antioxidant activity was reported as millimoles of Trolox equivalent per liter of juice (mmol TE/L) [23].

**2.8 Ferric reducing ability power (FRAP) assay**

The ferric reducing property of the RGJ was determined using the method of Benzie and Strain with some modifications. FRAP working solution (300 mM acetate buffer pH 3.6, 10 mM TPTZ, and 20 mM $FeCl_3$) was reacted with 0.1 mL diluted juice (1:25) and then incubated for 30 min in the dark. The absorbance of the reaction mixtures was measured at 593 nm using a UV-VIS Spectrophotometer. A standard curve for $FeSO_4$ (0-200 µmol $Fe^{2+}$/L) was plotted. The FRAP value will be calculated from the standard curve and is expressed in micromoles $Fe^{2+}$ per liter of juice (µmol $Fe^{2+}$/L) (Benzie and Strain, 1996).

### 2.9 Total anthocyanin content (TAC)

TAC of RGJ was investigated according to the differential method according to the method of Lee *et al.* [24] with some modification. Briefly, two buffer solution including pH 1.0 buffer (potassium chloride, 0.025M) and pH 4.5 buffer (sodium acetate, 0.4M) were prepared. Then, the undiluted juice was added was added into each buffer solution respectively with the ratio of 1:4 (1 part test portion, 4 parts buffer) so as not to exceed the buffer capacity of the reagents and incubated for 20 min. The absorbance of sample diluted with pH 1.0 buffer and pH 4.5 buffer was measured at both 520 and 700 nm using a UV-VIS Spectrophotometer. The TAC was expressed as milligrams of cyanidin-3-glucoside equivalents per liter of juice (mg/L) and determined by using Eq.3 [24]

$$\text{TAC (mg/L)} = \frac{A \times MW \times DF \times 10^3}{\varepsilon \times 1}$$

where A (absorbance) = $(A_{520} - A_{700})_{pH\ 1.0}$ - $(A_{520} - A_{700})_{pH\ 4.5}$; MW (molecular weight) = 449.2 g/mol for cyanidin-3-glucoside; DF = dilution factor; 1 = pathlength in cm; ε = 26900 molar extinction coefficient, in $L.mol^{-1}.cm^{-1}$; and $10^3$ = factor for conversion from g to mg.

### 2.10 Statistical analyses

Each experiment was conducted in triplicate. Data were analyzed by using JMP 12 software (SAS Institute, Cary, NC). To evaluate the effects of pressure, treatment time, and their interaction on log reduction of bacteria, a full factorial design was used with three levels for treatment pressure and three levels for treatment time. Two-way analyses of variance (ANOVA) were performed and means were compared using Tukey's HSD test. Significance was assigned to comparisons with a P-value of less than 0.05 ($P<0.05$).

## 3. Results & Discussion

### 3.1 Effect of preliminary treatment on the quality of RGJ

Before making the juice, RG was preliminarily treated by (1) detergent, (2) blanching (85°C, 1 min), and (3) detergent + blanching in order to remove dirt and reduce microbial load. The results of the quality of RGJ after each treatment were shown in Table 1. Obviously, compared to control sample, the sample treated by heat resulted in the significant decrease in TPC, AOA, and TAC.

**Table 1**. Effects of preliminary treatment on the quality of RGJ

| Sample | Wj (g) | TPC (mg GAE/L) | FRAP (mmol $Fe^{2+}$/L) | ABTS (mmol TE/L) | TAC (mg/L) |
|---|---|---|---|---|---|
| $S_c$ | 45.10 ± 0.01$^d$ | 4080.70 ± 49.14$^a$ | 32.58 ± 0.77$^a$ | 45.11 ± 2.14$^a$ | 370.88 ± 8.42$^a$ |
| $S_{det}$ | 53.71 ± 0.35$^c$ | 3994.10 ± 133.56$^a$ | 31.87 ± 0.43$^a$ | 36.59 ± 4.84$^b$ | 355.35 ± 1.26$^a$ |
| $S_{bla}$ | 55.03 ± 0.78$^b$ | 1455.20 ± 15.06$^b$ | 9.26 ± 0.10$^b$ | 4.91 ± 1.97$^c$ | 212.91 ± 7.71$^b$ |
| $S_{det\&bla}$ | 58.82 ± 0.18$^a$ | 1243.10 ± 46.77$^c$ | 7.86 ± 0.49$^c$ | 4.15 ± 1.21$^c$ | 182.85 ± 15.93$^c$ |

Wj: the weight of juice per 100g of grass
Sc: control sample
Sdet: grass treated by detergent
Sbla: grass treated by blanching
Sdet&bla: grass treated by both detergent and blanching
Different letters (a, b, c, d) represented a significant difference ($P<0.05$). N = 36.

Blanching also caused the change of juice color from purple black to brown due to the degradation of TAC (Figure 1). Thermal blanching damages cytoplasmic and other membranes, which become permeable and result in loss of cell turgor [18]. Consequently, water and solutes pass into and out of cells, resulting in nutrient losses as well as the increase in the obtained weight of juice per 100g of grass (Table 1). The treatment by detergent followed by heat caused the excessive damage of cell tissue resulting in the highest loss of anthocyanins, antioxidants, and phenolic compounds (Table 1). Only the sample treated by detergent (sodium bicarbonate 0.1%, 15 min) can keep the properties similar to fresh juice. Therefore, soaking in detergent (sodium bicarbonate 0.1%) for 15 min was chosen as the preliminary treatment for RG.

**3.2 Effects of thermal treatment on log reduction of bacteria**

The microbial inactivation in RGJ treated with thermal processing at 80°C for 2-8 min was exhibited in Figure 2. The results showed that the increase in holding time caused the significant increase in lethality of bacteria ($P<0.05$). In addition, Thermal treatment at 80°C for 8 min decreased the counts of both *E. coli* and *L. innocua* up to 5-log CFU/mL. The main events that occur during the vegetative bacterial cell exposed to heat include the loss of membrane-associated functions, the leakage of intracellular components, the loss of specific protein functions and DNA alterations which cause the death of bacterial cells [26].

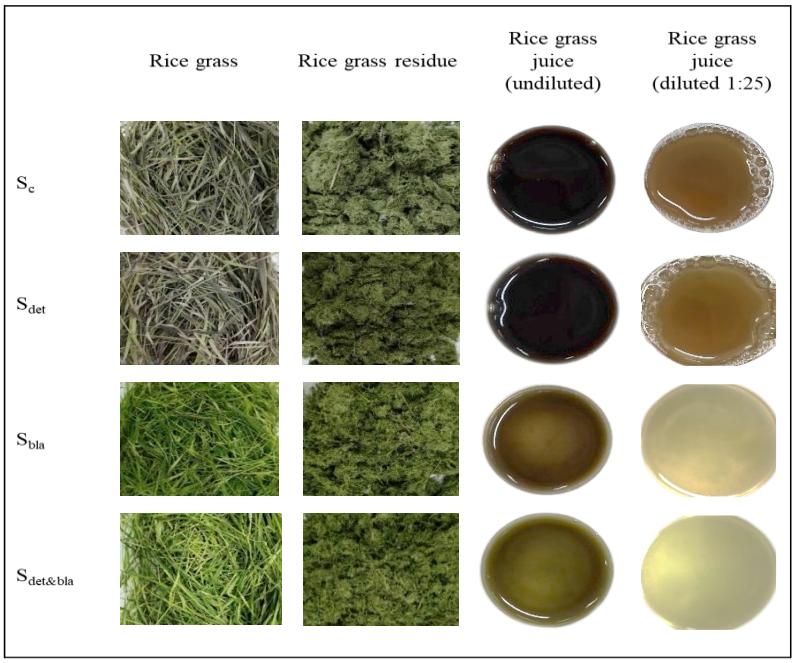


**Figure 1.** Effects of preliminary treatment on the color of RG

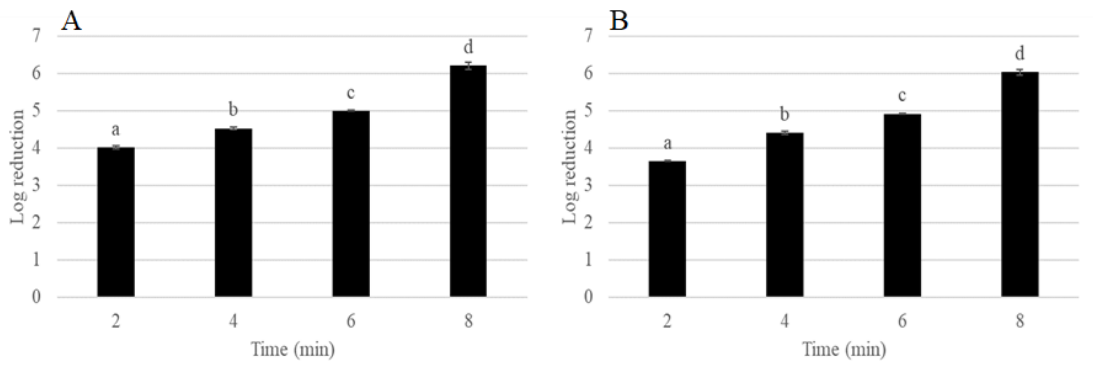


**Figure 2**. Effects of thermal treatment on log reduction of E.coli (A) and L.innocua (B). Different letters (a, b, c, d) represented a significant difference (P<0.05).

### 3.3 Effects of HPP treatment on on log reduction of bateria

For non-thermal treatment, RGJ was subjected to HPP system with the pressure of 400, 500 and 600 MPa during 5, 10 and 15 min. The initial count of bacteria in the juice prior to treatment was approximately $10^6$ CFU/mL. The log reduction results were illustrated in Figure 3. The statistical analysis showed that two main factors (pressure and time) and their interaction significantly affected on the lethality of both strains of bacteria (P<0.05). With respect to attaining a 5 log CFU reduction, the treatment at 400 MPa and 600 MPa for all treatment time failed to attain the desirable level of inactivation for both *E. coli* and *L. innocua*. On the other hand, over 5 log reduction was achieved by applying the pressure of 600 MPa with the treatment time of 10 min. Previous studies on low-acidity juice such as carrot juice [27;14;28], coconut juice [29], and pitaya juice [30] also revealed that only the pressure up to 600 MPa was sufficient to support a 5-log CFU reduction of the target bacteria.

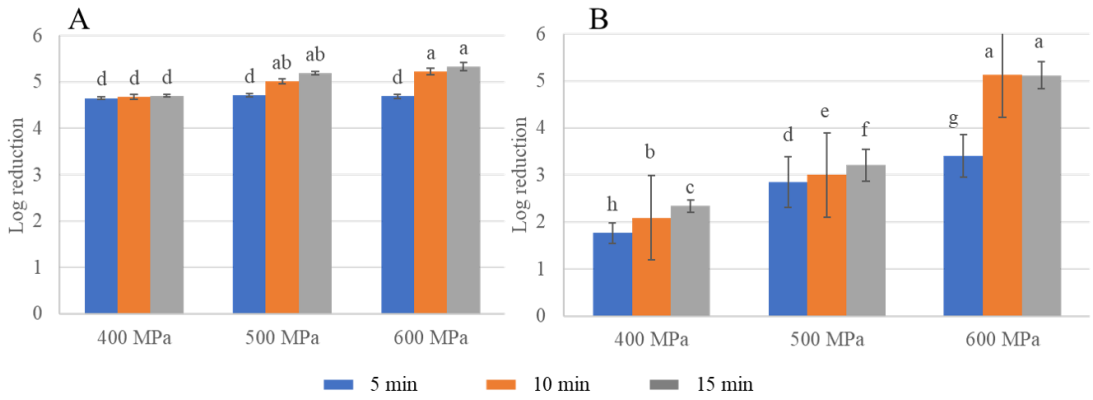


**Figure 1.** The effect of HPP on log reduction of E.coli (A) and L.innocua (B). Different letters represented a significant difference (P<0.05).

Furthermore, resistance responses to high pressure and holding treatment time of *E. coli* and *L. innocua* seemed to be different. It could be observed that at the pressure of 400 MPa, the increase in holding time could not significantly increased the log reduction of *L. innocua* (P>0.05). On the other hand, the extension of treatment time significantly decreased the viable counts of *E. coli* at any level of pressure. In general, gram-positive bacteria are more resistant to high pressure compared to gram-negative bacteria due to the presence of rigid teichoic acid in its cell wall [31]. Moreover, the cell membrane is generally recognized as the main site of pressure damage in microorganisms [32]. The mechanisms of inactivation of microorganisms under pressure can be explained by altering noncovalent

bonds in proteins that are responsible for replication, cellular integrity and metabolism (e.g. enzymes involved in DNA replication and transcription and a variety of biochemical changes). The irreversible denaturation of one or more of these critical proteins results in cell injury or death. At high pressure, cellular morphology is altered. There are reductions in cell volume, collapse of intracellular vacuoles, and membrane disruption or an increase in membrane permeability, leading to the loss of intracellular constituents. Overall, there are four main factors could be attributed to the inactivation of microorganisms by HPP: i) unfolding of proteins and enzymes, including partial or complete denaturation; ii) phase transition and fluidity changes in the cell membranes; iii) disintegration of ribosomes in their subunits; iv) pH changes in the intracellular compartments, which are related to the inactivation of enzymes and membrane damage [25, 33-36].

**3.4 Determination of injured cells after treated by HT and HPP**

The effects of HT and HPP on *E. coli and L. innocua* cells were determined by comparing the survival levels of the samples plated onto nonselective and selective media (supplementing with 2% NaCl) after exposure to high temperature (80°C/2-8 min) or high pressure (400-600 MPa/10 min) (Table 2). On the other word, the sublethal ratio (%) expressed the percentage of injured cells which can grow on non-selective medium (TSA) but cannot recovered on selective one (TSA-NaCl 2%). At some treatment condition of HPP like 600 MPa/10min, there was no colony detected on selective medium, so it was assumed that 100% of cells are injured after treatment and unable to recovered on TSA-NaCl 2%. The results revealed that there was a significant difference ($P<0.05$) in microbiological enumeration between nonselective and selective media after all treatment conditions. For thermal treatment, it can be observed that the injured population of *E. coli* and *L. innocua* increased from 32.89% to 72.22% and from 35.13% to 76.11%, respectively, after exposure to heat (80°C) as the holding time ranging from 2 to 8 min. On the other hand, an increase in injury rate of *E. coli* (89.55-100%) and that of *L. innocua* (78.93-100%) treated with HPP was observed when the pressure raised from 400 MPa to 600 MPa. Although both HT and HPP were capable of achieving the lethality up to 5 log reduction, HPP treatment resulted in higher sublethal injury ratio (%) compare to HT. Kimura *et al.* [37] observed colony formation of *E. coli* in 400 MPa-treated tomato juice, while no colonies were detected in 500 or 600 MPa-treated samples [37]. The study of Nasiłowska *et al.* (38) found that the sublethal injury ratio of *L. innocua* CIP 80.11 T in McIlvain buffer (pH 7.0) rose with increasing pressure (200-400 MPa) and exposure time (1, 5 and 10 min) [38]. Generally, the pressure higher than 400 MPa could lead to the denaturation of proteins [39]. HPP treatment could cause damage to bacterial cell membrane, membrane proteins, enzymes and ribosomes [40].

**Table 2** Sublethal ratio (%) of E. coli and L. innocua in HT-treated and HPP-treated RGJ

| | Treatment condition | Sublethal ratio (%) of *E. coli* | Sublethal ratio (%) of *L. innocua* |
|---|---|---|---|
| HT | 80°C/2min | $32.89 \pm 0.77^{a}$ | $35.13 \pm 5.22^{a}$ |
| | 80°C/4min | $33.23 \pm 3.18^{a}$ | $54.47 \pm 2.32^{b}$ |
| | 80°C/6min | $67.22 \pm 2.45^{b}$ | $61.62 \pm 0.94^{c}$ |
| | 80°C/8min | $72.22 \pm 4.81^{b}$ | $76.11 \pm 3.47^{d}$ |
| HPP | 400 MPa/10min | $89.55 \pm 0.096^{A}$ | $78.93 \pm 0.07^{A}$ |
| | 500 MPa/10min | $100.00 \pm 0.00^{B}$ | $91.28 \pm 2.22^{B}$ |
| | 600 MPa/10min | $100.00 \pm 0.00^{B}$ | $100.00 \pm 0.00^{C}$ |

Different letters represented a significant difference ($P<0.05$). N = 63

### 3.5 Effects of HT and HPP on TPC, AOA, and TAC of RGJ

Effect of HT and HPP on phytochemical properties of RGJ was showed in Table 3. Obviously, compared to control sample, the sample treated by HT (80°C, 8 min) resulted in the significant decrease in total phenolic content (45.96% reduction); antioxidant activity (46.34% reduction), and anthocyanin content (48.46% reduction) while the HPP-treated samples still retain the properties of as fresh RGJ. Generally, when pressure is applied to foods, bioactive and sensorial compounds, such as vitamins, flavor compounds, and color pigments, however, are minimally affected [41].

**Table 3.** Effects of HT and HPP on chemical properties of RGJ

| Sample | TPC (mg GAE/L) | FRAP (mmol $Fe^{2+}$/L) | ABTS (mmol TE/L) | TAC (mg/L) |
|---|---|---|---|---|
| Control | 2790.54 ± 67.44$^{a}$ | 31.87 ± 0.35$^{a}$ | 57.28 ± 0.17$^{a}$ | 375.47 ± 2.76$^{a}$ |
| 400 MPa/10min | 2757.91 ± 60.48$^{a}$ | 24.43 ± 0.21$^{b}$ | 44.77 ± 2.12$^{b}$ | 355.35 ± 1.03$^{a}$ |
| 500 MPa/10min | 2676.33 ± 00.28$^{a}$ | 24.11 ± 0.03$^{b}$ | 49.27 ± 0.32$^{b}$ | 304.25 ± 1.11$^{a}$ |
| 600 MPa/10min | 2602.21 ± 20.31$^{a}$ | 23.78 ± 0.02$^{b}$ | 45.58 ± 1.23$^{b}$ | 349.25 ± 0.09$^{a}$ |
| 80°C/8min | 1507.91 ± 85.41$^{b}$ | 13.13 ± 0.77$^{c}$ | 30.68 ± 1.18$^{c}$ | 209.65 ± 5.26$^{b}$ |

Different letters (a, b, c) represented a significant difference ($P<0.05$). N = 45

The positive preservation of HPP on bioactive compounds in fruit and vegetable juice can be explained by two reasons: i) high pressure affects only non-covalent bonds (hydrogen, ionic, and hydrophobic bonds). This effect on non-covalent bonds causes macro-molecules such as protein chains to unfold under high pressure but has little effect on chemical constituents associated with desirable food quality components such as flavor, color, and nutritional compounds and ii) as compared to thermal processing at atmospheric pressure, HPP has a great advantage of instantaneously transmitting uniform pressure from all sides on the food material, irrespective of the size and shape of the product [11;28]. The research of Ali *et al.* [42] on wheat grass juice reported that HPP process would be preferentially selected over thermal treatment on the basis of preserving antioxidants [42-43].

## 4. Conclusion

The changes occurring in RGJ during HPP and thermal treatment were assessed in this study. It was clear that HPP at 600 MPa for 10 min and HT at 80°C for 8 min could significantly inactivate both *E. coli* K 12 and *L. innocua* strains of bacteria for more than 5 log reduction meeting the regulation of FDA for fruit and vegetable juice (FDA-2002-D-0298). However, HPP treatment resulted in higher sublethal injury ratio (%) compare to HT. Moreover, it was notable with the thermally treated juice with the significant decrease in TPC, AOA, and TAC whereas HPP-treated samples still retained the phytochemical properties as fresh juice. Overall, HPP is the potential treatment method for RGJ pasteurization based on the retention of nutritive composition while delivering the required microbial reduction.

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# Valorization of Asian Seabass By-Products from Ko Yo Island, Songkhla, Thailand

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**Abstract**

Asian seabass (*Lates calcarifer*) is cultured mainly by residents of Ko Yo Island located in the Songkhla Lake, Songkla, Thailand. Commercialization of Asian seabass is under the operation of Rak Ko Yo Community Enterprise. The fish is mainly sold in fresh whole fish or smoked fillets. The secondary products, i.e., head, tail, and fish bones with flesh, are cheaply sold in a frozen form. One strategy to increase residents' income was to add value to the fish's undervalued parts by turning them into concentrated broth. Bones with flesh and tails were simmered with some vegetables and spices. The filtered, concentrated broth was filled in a 300 mL. pouch and sterilized. A sterility test proved that there were no *Clostridium botulinum*, mesophilic and thermophilic flat-sour. The concentrated broth pH was 5.49 ± 0.01. The pH slightly increased after onefold and two-fold dilution. The water activity was 0.99 ± 0.00 at 25℃ and did not change with dilution. A test for heavy metals showed that mercury was detected with less than 0.01 ppm; meanwhile, there was no detection of cadmium and lead. Accelerated shelf-life testing showed that at 35℃, the product would last 12 months, while at 45℃, it would last 6 months. A product acceptance test using the Just About Right scale revealed that six product attributes, i.e., color, turbidity, aroma, flavor, saltiness, and sweetness, were in the range of 70-90 with net effects of turbidity, aroma, and flavor that were less than 20. Twenty-nine out of 30 consumers (96.67%) accepted the product. The concentrated broth developed from Asian seabass cheaper cuts has the potential to be commercialized.

***Keywords***: Valorization; Asian seabass; Undervalued fish parts; Concentrated broth; Food product characterization

## 1. Introduction

Aquaculture is the principal way for residents of Ko Yo Island, Songkhla Province, Thailand, to earn a living. Asian seabass (*Lates calcarifer*) is one of the major cultured species. Commercialization of Asian seabass is under the operation of Rak Ko Yo Community Enterprise. The fish is mainly sold in fresh whole fish or smoked fillets. The secondary products, i.e., head, tail, and fish bones with flesh, are cheaply sold in a frozen form. One strategy to increase residents' income was to add value to the fish's undervalued parts. Although fisheries by-products are sometimes less interesting when considering their selling price, they still contain nutritional values such as protein, bioactive peptides, omega-3 oils, collagen, and cartilage calcium. These bioactive components can be used as functional ingredients that improve food products' nutritional and sensorial qualities and help create business benefits [(1-2)]. Exploiting secondary products as raw materials for new fish-based food products not only adds value to those parts but also increases food choices to meet the

increasing consumer demand for food [3]. The objectives of this study thus are to valorize Asian seabass's undervalued parts, such as bones with flesh and tails, by using them to develop a concentrated broth and characterize the product properties with the aim of making a marketable food product.

## 2. Materials and Methods

### 2.1 Preparation of the Asian seabass concentrated broth

Asian seabass bones with flesh or tails were mixed with salt (3% by wt.) and commercially dried Chinese tea leaves (1% by wt.) for 15 min before thoroughly rinsing with boiled water (100℃) to remove the fishy odor. Fishbone stock was then prepared by simmering it with vegetables such as carrots, Chinese radishes, and herbs such as spring onion, garlic, and coriander roots for 60 min. The mixture was filtered with a clean white cloth to keep only the liquid. Consequently, fishbone stock was seasoned with salt, fermented soybean, and white pepper, then boiled with ginger and galanga until the temperature reached 100℃. After filtering, the fishbone broth was obtained. Three hundred milliliters of the broth were filled in an aluminum foil pouch and heat-sealed at 180℃. The product was sterilized at 117℃, 1.5 bar for 20 min.

### 2.2 Product acceptance test

The level of appropriateness for concentrated broth attributes was determined using the Just About Right scales. Thirty consumer panels were asked to taste product samples and give their opinions by rating six product attributes: color, turbidity, aroma, flavor, saltiness, and sweetness. Demographic information of each respondent was collected.

### 2.3 Characterization of Asian seabass concentrated broth

According to the Bacteriological Analytical Manual, the concentrated broth product has undergone sterility testing using the direct inoculation method [4]. The water activity and pH were also determined. The product was tested for possible contamination of heavy metals, i.e., cadmium, lead, and mercury, by an ISO/IEC 17025 laboratory using an in-house method based on AOAC (2019) 999.10, 986.15, and 974.14, respectively. Accelerated shelf-life testing was carried out where the products were stored at 35℃ and 45℃ for 60 days. During the experiments, product appearance, color, aroma, water activity, pH, total plate count, yeast & mold, and the number of *Escherichia coli* were monitored periodically

## 3. Results and Discussion

### 3.1 Product acceptance test

30 consumer panels tasted the concentrated broth to evaluate the intensity of 6 sensory attributes, which are color, turbidity, aroma, flavor, saltiness, and sweetness, by using the Just-about-right scale (Table 1). The majority of consumer panels chose 5 attributes, i.e., color (76.67%), turbidity (86.67%), aroma (66.67%), flavor (76.67%), and sweetness (66.67%) at the just-about-right level meanwhile 50% of consumers found the product was a little too salty. The net effects of turbidity (13.33), aroma (6.67), and flavor (16.67) were less than 20, whereas the net effects of color (23.33) and sweetness (26.67) were slightly over 20. The highest value of the net effect was from saltiness (56.67). The results showed that the saltiness and the sweetness of the broth needed to be adjusted.

**Table 1.** The just-about-right (JAR) scale percentages of responses for concentrated fishbone broth and the percentage of responses are shown in parentheses.

| Product Attributes | Respondents (%) for just-about-right (JAR) test | | | | | Net effect |
|---|---|---|---|---|---|---|
| | Very weak | Slightly weak | Just about right | Slightly strong | Very strong | |
| Color | 1 (3.33) | 6 (20.00) | 23 (76.67) | 0 (0.00) | 0 (0.00) | 23.33 |
| Turbidity | 0 (0.00) | 4 (13.33) | 26 (86.67) | 0 (0.00) | 0 (0.00) | 13.33 |
| Aroma | 0 (0.00) | 4 (13.33) | 20 (66.67) | 6 (20.00) | 0 (0.00) | 6.67 |
| Flavor | 0 (0.00) | 1 (3.33) | 23 (76.67) | 5 (16.67) | 1 (3.33) | 16.67 |
| Saltiness | 1 (3.33) | 0 (0.00) | 11 (36.67) | 15 (50.00) | 3 (10.00) | 56.67 |
| Sweetness | 1 (3.33) | 8 (26.67) | 20 (66.67) | 0 (0.00) | 1 (3.33) | 26.67 |

Net effect = |Total percentage of strong effect -Total percentage of weak effect |

Demographic information of consumer panels was grouped by age, gender, income level, and educational level according to Figure 1. Most consumers were females aged between 21 and 30 years, who had obtained a bachelor's degree and earned between 10,001 and 15,000 THB/month. In general, twenty-nine out of thirty consumers (96.67%) accepted the product.

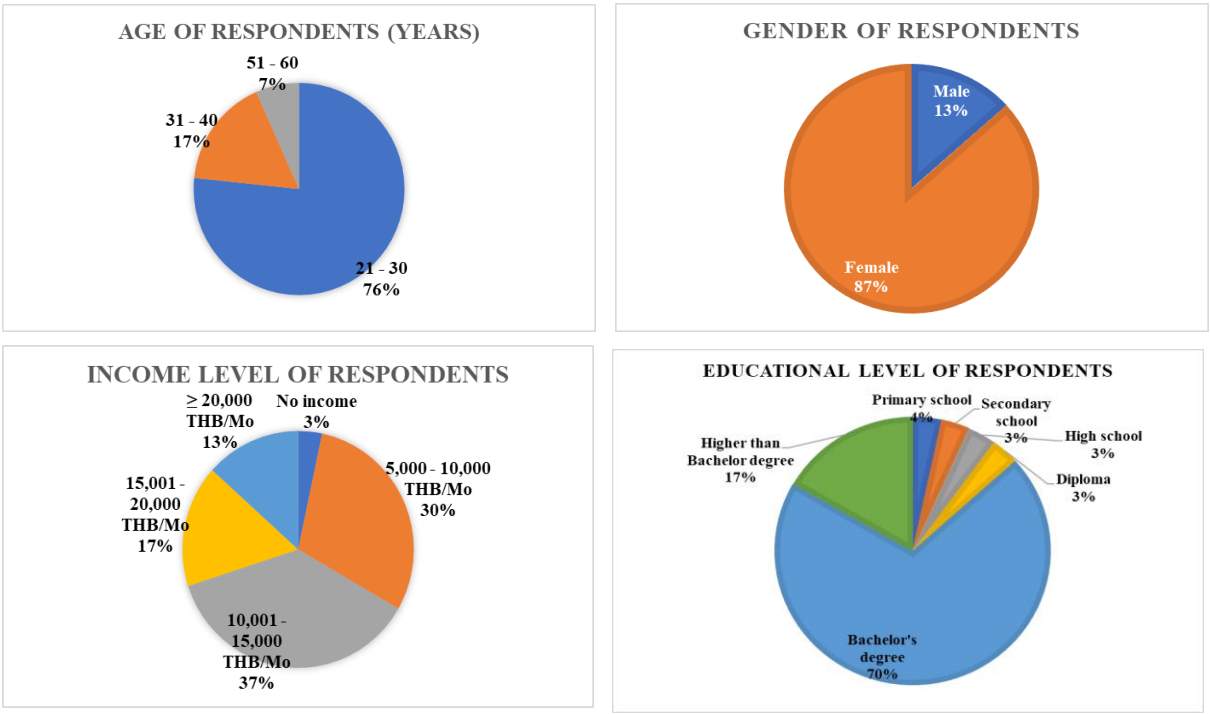


**Figure 1.** Demographic information of respondents grouped by age, gender, income level, and educational level

### 3.2 Characterization of Asian seabass concentrated broth

Water activity (Aw) and pH of the concentrated broth were determined without dilution and with onefold and twofold dilutions, as shown in Table 2. The Aw values did not change by dilution. The pH values showed a slight increase after onefold and twofold dilutions. The mean comparison confirmed a significant difference among the three dilution groups at 0.05 level. A slight increase in pH after water dilution of the concentrated broth could be explained by the result of brine dilution [5].

**Table 2.** Water activity (Aw) and pH of concentrated broth from Asian seabass bones with flesh with onefold and twofold dilution

| Product | Dilution | Water activity (Aw) | pH |
|---|---|---|---|
| Concentrated broth | 1 | 0.99 ± 0.00 | 5.49 ± 0.01 |
| | onefold | 0.99 ± 0.00 | 5.62 ± 0.02 |
| | twofold | 0.99 ± 0.00 | 5.71 ± 0.00 |

The product in a 300 mL. aluminum pouch underwent a sterility test in a hermetically sealed container to verify the presence or absence of viable microorganisms that cause food spoilage (Table 3). The result showed no detection of mesophilic and thermophilic flat sour as well as *Clostridium botulinum*. This proved that the sterilization process was efficient.

**Table 3.** Sterility test of concentrated broth from Asian seabass bones with flesh

| Product | Incubation Test | Mesophilic flat sour | Thermophilic flat sour | *Clostridium botulinum* |
|---|---|---|---|---|
| Concentrated broth | Acceptable | Not Detected | Not Detected | Not Detected |

Contaminated heavy metals in water resources can accumulate in the tissues of aquatic animals through gills, skin, and oral ingestion of food and water [6]. The bioaccumulated quantity does not diminish even after cooking [7]. In Table 4, the concentrated broth product was tested for possible contamination of cadmium (Cd), mercury (Hg), and lead (Pb) from fish raw materials [8]. The result showed no detection of Cd and Pb. The mercury concentration found in the sample was less than 0.01 ppm, which was far lower than the maximum limit allowed by the Thai Food Drug Administration (1 ppm. for predatory fish).

**Table 4.** Heavy metals analysis for concentrated broth from Asian seabass bones with flesh

| Product | Cadmium | | | Lead | | | Mercury | | |
|---|---|---|---|---|---|---|---|---|---|
| Concentrated broth | Result Mg/kg | LOD. | LOQ. | Result Mg/kg | LOD. | LOQ. | Result Mg/kg | LOD. | LOQ. |
| | Not detected | 0.007 | 0.010 | Not detected | 0.010 | 0.020 | <0.010 | 0.005 | 0.010 |

The shelf life of the sterilized product was estimated by accelerated shelf-life testing conducted at 35℃ and 45℃. Product appearance, Aw, and pH were periodically monitored at 0, 15, 30, 45, and 60 days (Table 5). Total Plate Count and yeast & mold were tested at 0, 30, and 60 days (Table 6). The product appeared as a light brown liquid with a savory smell from day 0 to day 60. The appearance remained the same at both tested temperatures. Water activity and pH were almost the same at both tested temperatures and for the whole test period as well. The microbial enumeration tests revealed that at the beginning of the test, after 30 days, and after 60 days of storage, there was no detection of aerobic, mesophilic

microorganisms as well as yeast & mold at both 35℃ and 45℃. *Escherichia coli* was not found in the product, reassuring the efficiency of the sterilization process. From the test results, it can be predicted that if the storage temperature were 35℃, the product would last 12 months. If the storage temperature were 45℃, the product would last six months. Considering the normal storage temperature, between 25℃ and 30℃, the product would last approximately 18 months.

**Table 5.** Accelerated shelf-life testing for concentrated broth from Asian seabass bones with flesh

| Storage (days) | Product Appearance | | Water activity | | pH | |
|---|---|---|---|---|---|---|
| | 35°C | 45°C | 35°C | 45°C | 35°C | 45°C |
| **0** | Light brown liquid, savory smell | Light brown liquid, savory smell | 0.95 | 0.95 | 5.12 | 5.12 |
| **15** | Light brown liquid, savory smell | Light brown liquid, savory smell | 0.95 | 0.94 | 5.16 | 5.10 |
| **30** | Light brown liquid, savory smell | Light brown liquid, savory smell | 0.95 | 0.95 | 5.14 | 5.16 |
| **45** | Light brown liquid, savory smell | Light brown liquid, savory smell | 0.95 | 0.95 | 5.14 | 5.14 |
| **60** | Light brown liquid, savory smell | Light brown liquid, savory smell | 0.95 | 0.95 | 5.14 | 5.14 |

**Table 6.** Microbiological test of concentrated broth from Asian seabass bones with flesh under accelerated shelf-life testing

| Microbiological test | Storage temperature (°C) | Storage (days) | | |
|---|---|---|---|---|
| | | **0** | **30** | **60** |
| Total Plate Count (CFU/mL) | 35 | <1 | <1 | <1 |
| | 45 | | <1 | <1 |
| Yeast & Mold (CFU/mL) | 35 | <1 | <1 | <1 |
| | 45 | | <1 | <1 |
| *E.coli* (MPN/mL) | RT | <3[a] | n/a | n/a |

[a] *E.coli* was enumerated at room temperature only when receiving the sample.

## 4. Conclusion

Developing a new product line using by-products from the existing business helps add value to those cheaper parts of raw materials and eventually increases business revenue. In this study, bones with flesh and tails of Asian seabass were used as raw materials in making a sterilized concentrated broth. Sensory evaluation indicated that 96.67% of the sampled consumers accepted the product; the saltiness and the sweetness of the broth needed to be further adjusted. Sterility tests confirmed the efficiency of the sterilization process. There were no observations of cadmium and lead in the product. The quantity of mercury was much lower than the allowed limit. The accelerated shelf-life testing revealed an estimated shelf life of 18 months at normal storage temperature. In summary, the developed product has the potential to be commercialized.

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# Water Activity Optimization of Sliced Garlic Using Vacuum Drying by Response Surface Methodology

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**Abstract**

Optimizing conditions for vacuum drying of sliced garlic were investigated to control the water activity and study the properties of vacuum dried garlic such as moisture content, color, texture, and the amount of allicin. The experimental design was determined using Box-Behnken with the Response Surface Methodology (RSM). The factors include temperature (50-70°C), pressure (5-25 inHg), and time (2-6 h). It was found that the optimum conditions for vacuum drying of sliced garlic were 69°C, 20 inHg, and 3.5 h. The predicted water activity value and the observed value of the vacuum-dried garlic at the optimum conditions were 0.3003 and 0.3547, respectively, which are in the range that can inhibit microbial growth and reduce the rate of lipid oxidation. The properties of vacuum dried garlic were studied including moisture content at 5.23%, color value *L*a*b** at 86.67, 11.81, and 40.91, respectively, hardness at 25056 (g) force and allicin concentration at 3.722 mg/g.

***Keywords**:* Garlic; Water Activity; Response Surface Methodology; Vacuum Drying

## 1. Introduction

Garlic is a medicinal plant in Thailand that can be used as one of the main ingredients in cooking. It has a unique taste and many nutritional compounds, including protein, fat, and organosulfur substances such as allicin and ajoene [1, 2], which can reduce cholesterol and blood pressure in the body [3], prevent the risk of heart disease [4] and can also stimulate the death of cancer cells. Therefore, it is one of the options for healthy consumption. However, fresh garlic contains more than 60% moisture content, leading to its short shelf life. To extend the shelf life of garlic, most garlic was stored in the dried form; it was processed into various products such as garlic powder, pickled garlic, fried garlic, etc. [5, 6]. The commonly used method of drying garlic is hot air drying [7], but it also has limitations. Hot air drying will reduce important substances in garlic that are beneficial to consumers, making the dried garlic using this method have the lowest quality and lowest nutritional benefits compared to other drying processes [6].

Vacuum drying is a drying process that works under conditions where the pressure is lower than atmospheric pressure. It is a method of preserving food by reducing the amount of free water by evaporating, which can better maintain the nutritional value of the garlic [8]. Free water in garlic is an essential factor that can control its quality and prevent unwanted changes in garlic [9], such as the growth of microorganisms and oxidation of fats, which affect its shelf life [10, 11]. At water activity (aw) below 0.6, garlic will contain free water at a safe level that can inhibit the growth of microorganisms and the formation of mycotoxins [12], which causes spoilage and danger in food. The water activity of garlic at 0.2-0.5 is an appropriate range that slows down lipid oxidation [13].

This research aims to study the optimum conditions for vacuum drying sliced garlic using response surface methodology (RSM) to control the amount of free water at a safe level that can inhibit the growth of microorganisms, make the dried garlic have a long storage shelf life, and safe for consumers at the commercial level.

## Materials and methods

### 2.1 Materials

Garlic was purchased from a local department store in Bangkok (Thailand). Ethyl alcohol (AR grade, Qrec, New Zealand), L-cysteine (Bioplus Laboratory, Thailand), DTNB (Ellman's Reagent, Bioplus Laboratory, Thailand), and HEPES buffer (Loba, India).

### 2.2 Preparation of sliced garlic

Garlic heads were separated into cloves, and the cloves were peeled and stored at 4°C. The cloves were put at room temperature to dry the garlic and then sliced into a 2 mm thickness. The sliced garlic was placed in an aluminum tray and dried in a vacuum oven.

### 2.3 Statistic design of experiments

The experiment design used the Box-Behnken experimental design by MINITAB software version 19.0 (Pennsylvania, USA). RSM analyzed the optimum conditions of sliced garlic. The three studied factors were temperature (°C), pressure (inHg), and time (h). The level of parameters was as shown in Table 1, with a total of 30 experimental runs. The response was the amount of free water contained in dried garlic, measured as aw value, determined by the water activity meter (4TE, METER Group, Inc., USA).

**Table 1.** Experimental design of vacuum drying on sliced garlic.

| Factor | Level | | |
|---|---|---|---|
| | **-1** | **0** | **1** |
| $X_1$: Temperature (°C) | 50 | 60 | 70 |
| $X_2$: Pressure (inHg) | 5 | 15 | 25 |
| $X_3$: Time (h) | 2 | 4 | 6 |

### 2.4 Characterization of vacuum-dried garlic

The moisture content of dried sliced garlic was analyzed using a moisture balance IR (HB43 Moisture analyzer, Mettler Toledo, Switzerland). Color measurement of dried sliced garlic was analyzed using a chroma meter (CR-400, Konika Minolta, Japan).

The hardness of dried sliced garlic was analyzed using a texture analyzer (TA.XT Plus, Stable Micro Systems, Godalming, UK) with a P/25 probe at a constant speed of 2 mm/s. Each sample was compressed at 1 cm.

The concentrations of allicin were measured according to Sun and Wang [14]. The dried sliced garlic was ground into a powder of approximately 2 g and mixed with 8 ml of 95 % ethyl alcohol. The samples were placed in an ultrasonic bath for 20 min and centrifuged at 4000 rpm. The samples were then left at room temperature for 15 minutes. A volume of 0.25 mL cysteine solution (1 mmol/L) was mixed with a volume of 1 mL DTNB (2 mmol/L), then mixed with 0.25 mL of sample or HEPES buffer (blank), and adjusted the volume with HEPES buffer to 6 ml. Left the solutions at room temperature before measuring with a visible spectrophotometer (SP-880, Metertech, Taiwan) at a wavelength of 412 nm and calculated as shown in Eq.1:

$$C_{Allicin} = \frac{(A_0 - A) \times d \times 162.26}{(2 \times 14150)} \times 0.7 \quad (1)$$

While $A_0$ is the absorbance of the blank at 412 nm, A is the absorbance of the sample at 412 nm, and d is dilution times.

## 3. Results and discussions

### 3.1 Evaluation of experimental

The optimization of sliced garlic using vacuum drying was determined by measuring the water activity in dried garlic. The result of the observed aw value varied from 0.1545 to 0.8728, as shown in Table 2. From the experimental design, the relationship of difference factor level between temperature (*X*1), pressure (*X*2), and time (*X*3) was used to model the equation predicted the water activity of dried garlic (*Y*1) as shown in Equation 2:

$$Y_1 = 3.225 - 0.0620\ X_1 + 0.0505X_2 - 0.4285\ X_3 + 0.000325\ X_1^2 + 0.000134\ X_2^2 + 0.01916\ X_3^2 - 0.000509X_1X_2 + 0.003820X_1X_3 - 0.00315X_2X_3 \quad (2)$$

Figure 1 shows residual plots for the water activity of sliced garlic, indicating the data's accuracy and reliability. A standard probability plot related to the discrepancies in the data is shown in Figure 1(a). The results show that the data spreads straight, indicating that the predicted water activity value is accurate and reliable. In Figure 1(b), versus fits showed the stability of the data variance. It was found that the data has low variance. Figure 1(c) of the histogram showed a standard distribution, which looks like an inverted bell, and Figure 1(d) of versus order showed that most of the data has values close to the zero line, indicating a normal distribution.

**Table 2.** Process parameters and response for vacuum drying of sliced garlic.

| Run | Temperature (°C) | Pressure (inHg) | Time (h) | Observed $a_w$ | Predicted $a_w$ |
|---|---|---|---|---|---|
| 1 | 50 | 25 | 4 | 0.6670 | 0.6774 |
| 2 | 60 | 15 | 4 | 0.4166 | 0.3255 |
| 3 | 60 | 15 | 4 | 0.2147 | 0.3255 |
| 4 | 70 | 5 | 4 | 0.1876 | 0.1673 |
| 5 | 60 | 25 | 6 | 0.2355 | 0.2802 |
| 6 | 50 | 25 | 4 | 0.6760 | 0.6774 |
| 7 | 50 | 5 | 4 | 0.3911 | 0.3726 |
| 8 | 60 | 15 | 4 | 0.3378 | 0.3255 |
| 9 | 60 | 5 | 2 | 0.5024 | 0.4251 |
| 10 | 70 | 15 | 6 | 0.1787 | 0.1835 |
| 11 | 50 | 15 | 6 | 0.2821 | 0.3378 |
| 12 | 70 | 15 | 2 | 0.3596 | 0.3786 |
| 13 | 50 | 5 | 4 | 0.3253 | 0.3726 |
| 14 | 70 | 15 | 2 | 0.3921 | 0.3786 |
| 15 | 70 | 25 | 4 | 0.2902 | 0.2685 |
| 16 | 60 | 5 | 2 | 0.3437 | 0.4251 |
| 17 | 60 | 5 | 6 | 0.1556 | 0.1530 |
| 18 | 50 | 15 | 2 | 0.8381 | 0.8385 |
| 19 | 70 | 15 | 6 | 0.1545 | 0.1835 |
| 20 | 60 | 25 | 2 | 0.6916 | 0.7039 |
| 21 | 70 | 25 | 4 | 0.2751 | 0.2685 |
| 22 | 60 | 5 | 6 | 0.1744 | 0.1530 |
| 23 | 60 | 15 | 4 | 0.3524 | 0.3255 |
| 24 | 50 | 15 | 2 | 0.8728 | 0.8385 |
| 25 | 60 | 15 | 4 | 0.2588 | 0.3255 |
| 26 | 60 | 25 | 6 | 0.3288 | 0.2802 |
| 27 | 60 | 25 | 2 | 0.6921 | 0.7039 |
| 28 | 60 | 15 | 4 | 0.3726 | 0.3255 |
| 29 | 70 | 5 | 4 | 0.1587 | 0.1673 |
| 30 | 50 | 15 | 6 | 0.3991 | 0.3378 |

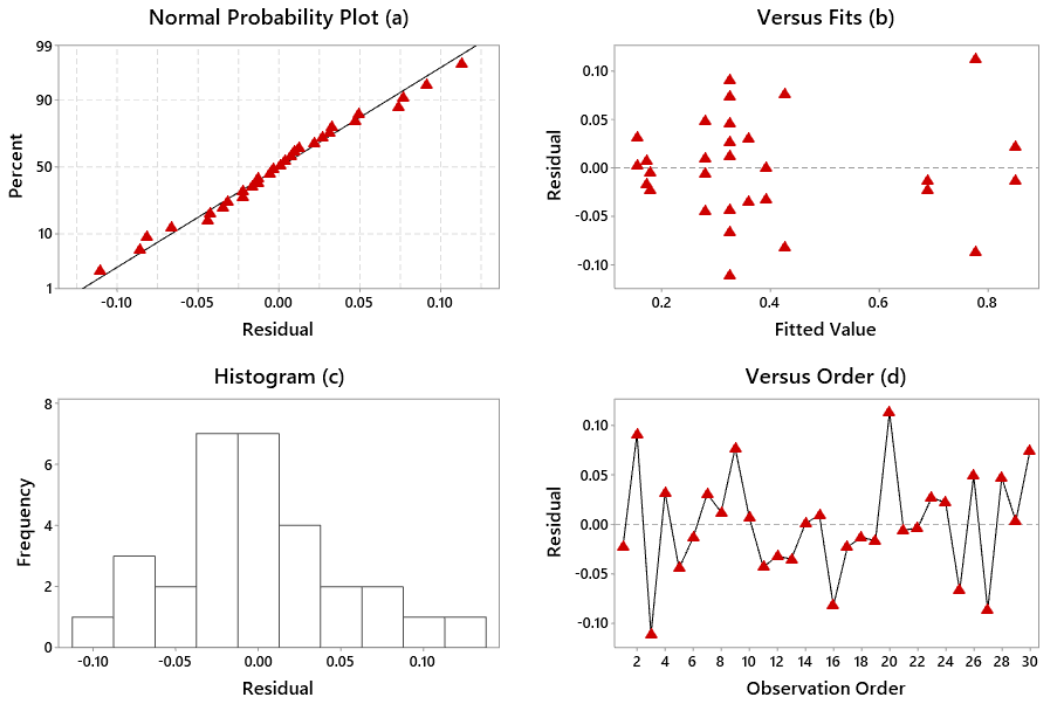


**Figure 1** Residual plots for water activity of vacuum drying of sliced garlic.

### 3.2 Effects of factors on water activity of the dried garlic

The analysis of variance (ANOVA) for the water activity of vacuum-dried garlic is shown in Table 3. The three main variables, temperature, pressure, and time, have a statistically significant effect (P<0.05) on the water activity in vacuum-dried garlic at an R2 value of 94.21%, which is within the acceptable range.

**Table 3.** Analysis of variance for water activity of vacuum dried garlic.[a]

| Term | Water Activity | | |
|---|---|---|---|
| | coefficient | T-value | P-value |
| Constant | 0.3255 | 12.67 | 0.000 |
| $X_1$ | -0.1534 | -9.75 | 0.000 |
| $X_2$ | 0.1136 | 7.22 | 0.000 |
| $X_3$ | -0.1865 | -11.86 | 0.000 |
| $X_1X_1$ | 0.0325 | 1.40 | 0.176 |
| $X_2X_2$ | 0.0134 | 0.58 | 0.570 |
| $X_3X_3$ | 0.0766 | 3.31 | 0.003 |
| $X_1X_2$ | -0.0509 | -2.29 | 0.033 |
| $X_1X_3$ | 0.0764 | 3.43 | 0.003 |
| $X_2X_3$ | -0.0629 | -2.83 | 0.010 |

[a] R-squared = 94.21%

Main factors affecting the water activity in vacuum-dried garlic are shown in Figure 2. The results showed that the water activity was decreased as the temperature increased, due to an increase in temperature will increases the heat transfer rate which results in a decrease in the moisture content and free water of vacuum dried garlic [15, 16]. The same trendline was also shown in the time factor, the water activity was decreased as the time trendline increased. However, the pressure factor has a trend opposite to the temperature and time

factors. It was found that as the pressure rises, the water activity of vacuum-dried garlic also increases. Because the boiling point of water can be reduced by lowering the pressure with a vacuum [17], as the pressure in vacuum condition increased, the boiling point of water was higher and lead to the lower evaporation rate of free water content, which resulted in higher water activity value in vacuum dried garlic.

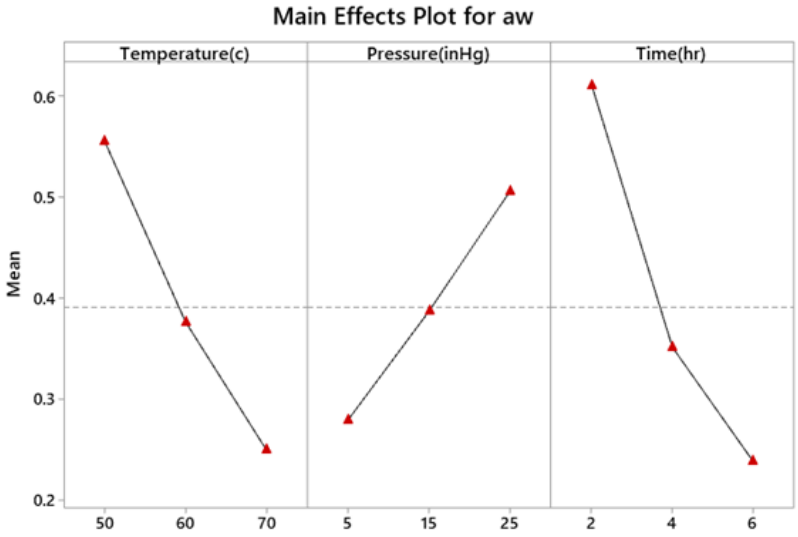


**Figure 2.** Main factors affecting the water activity in vacuum drying of sliced garlic.

The results of the interacting factors affecting the water activity are shown in Figure 3. In the response surface plot, pressure and temperature caused the amount of free water to be reduced and resulted in a lower water activity value. When the pressure is reduced in vacuum conditions, the drying temperature can be lower compared to drying at standard conditions. The relationship between the time and pressure factors showed that the water activity of vacuum-dried garlic reduced when pressure decreased and time increased. At low pressure, the drying time was reduced. The water activity was lower at the decreasing pressure and the increasing time. In the vacuum drying process of sliced garlic, the interaction of temperature, pressure, and time affects the reduction in the amount of free water remaining.

The experimental results showed that the optimum conditions for vacuum drying of sliced garlic were temperature of 69°C, pressure of 20 inHg, and time of 3.5 h, respectively. The predicted water activity value of vacuum-dried garlic was 0.3003. Therefore, the three repetitive tests were conducted to confirm the values under optimum conditions. It was found that the observed water activity value was equal to 0.3547, which is close to the predicted value obtained by the calculation of Eq.2, indicating that the prediction equation is accurate in finding the optimum conditions for determining the water activity value of vacuum-dried garlic.

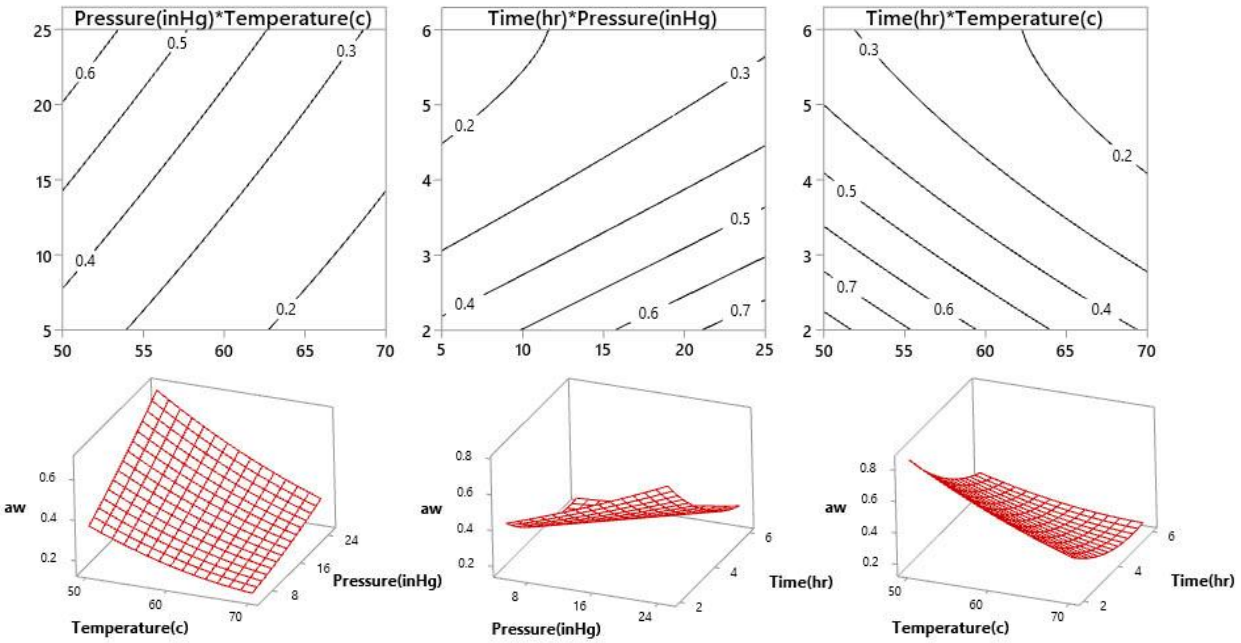


**Figure 3.** Response surface of water activity in vacuum drying of sliced garlic

### 3.3 Characterization of vacuum dried garlic

The physical characterization of dried sliced garlic obtained using vacuum drying showed that the dried sliced garlic contained moisture content and aw at 5.23 % and 0.3547, respectively. The color measurement showed that the *L*a*b** values were 66.67, 11.81, and 40.91, respectively. The hardness of dried sliced garlic was 25056.52 (g) force. The chemical characterization included allicin detected in sliced garlic before and after vacuum drying. The concentration of allicin were 5.011 and 3.722 mg/g, respectively, with loss of allicin potential during drying around 25.72 %, which was less than the research results from Rahman et al. [18], which had an allicin loss of about 35.3 %. Allicin is an unstable and volatile secondary substance [19]. The effect of temperature can cause an evaporation of the allicin content. The efficiency of allicin decreases when temperatures exceed 42°C [20]; the higher the temperature, the higher the evaporation rate of water. Moreover, lowered pressures induce faster water evaporation from the food at a relatively low temperature [7], and a shorter time affects the amount of allicin loss due to the reduced time of heat exposure.

## 4. Conclusions

The vacuum drying conditions of sliced garlic were optimized to study the water activity level of dried sliced garlic products. The aw value of dried garlic should be in the range of 0.2-0.5, suitable for dried sliced garlic products. The optimum conditions for vacuum were at a temperature of 69°C, a pressure of 20 inHg, and a time of 3.5 h, with the response of the observed water activity value at 0.3547 and the predicted water activity value at 0.3003, respectively. An increase in temperature and time will cause the water activity to decrease, but on the other hand, an increase in pressure will cause the water activity to increase. The dried sliced garlic had a moisture content of 5.23% and a hardness of 25056 (g) force. The allicin concentration of dried sliced garlic was 3.722 mg/g, and the allicin loss from the vacuum drying process was 25.72%.

## Acknowledgements

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# Development of Non-Alcoholic Beer from Malted Barley Added with Kum Jao Morchor 107 Rice Bran

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## Abstract

The development of non-alcoholic beer is becoming popular due to the increasing health awareness of people nowadays. This research aimed to investigate the effects of Kum Jao Morchor 107 rice bran substituted with malted barley on the qualities of non-alcoholic beer. Malted barley added with 5, 10 and 15% (w/w) Kum Jao Morchor 107 rice bran was fermented with *Saccharomyces cerevisiae* var. *chevalieri*, a maltose negative yeast strain. The %alcohol by volume (%ABV), pH, total soluble solids (TSS) and color were measured during 6 days of fermentation at 20°C. The samples at day 3 of fermentation were chosen since the %ABV did not exceed 0.5% (requirement of non-alcoholic beer), to further analysis in ethanol concentration by high performance liquid chromatography, reducing sugar, total phenolic content (TPC), total flavonoid content (TFC), total anthocyanin content (TAC), and antioxidant capacities (DPPH and FRAP assay). The results showed that increasing the amount of Kum Jao Morchor 107 rice bran increased TPC, TFC and TAC of the products. The pH value and TSS were decreased with increasing fermentation time, meanwhile TPC and TFC were decreased. Besides, the antioxidant activities assessed by DPPH and FRAP assays were enhanced with the increasing amount of Kum Jao Morchor 107 rice bran and tended to decrease at day 3 of fermentation. For sensory evaluation of the product fermented for 3 days using 9-point hedonic scale, the acceptance scores showed no significant difference in all attributes except the mouthfeel. Non-alcoholic beer products with 5% and 10% (w/w) Kum Jao Morchor 107 rice bran gained the highest acceptance for mouthfeel with no significant difference. Therefore, the addition of Kum Jao Morchor 107 rice bran substituted to malted barley could improve the TPC, TFC, TAC and antioxidant properties of non-alcoholic beer while retaining the sensorial acceptability.

***Keywords**:* Non-alcoholic beer; *Sacharomyces crevisiae* var. *chevalieri*; Kum Jao Morchor 107 rice bran; Total anthocyanin content; Antioxidant activity

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## 1. Introduction

Beer is considered among the earliest fermented beverages and is one of the most globally consumed alcoholic drinks, especially at parties and various celebratory occasions. However, with the rising global trend of increased consumption of health drinks, many businesses have responded to changing consumer lifestyles by developing non-alcoholic beverages. These beverages offer alternative drink options suitable for consumption during daily activities or in specific conditions [(1)]. The non-alcoholic beer market is expected to have a compound annual growth rate of 4.954 % from 2023-2028 [(2)]. In terms of non- alcoholic beverages, products like non-alcoholic beer or beer-like beverages are designed to mimic the taste of beer while containing no more than 0.5% alcohol by volume (ABV) [(5)].

The general methods for producing non-alcoholic beer involve limiting ethanol formation and removing ethanol through various processes such as physical, chemical, or biological methods. The use of special yeasts was distinguished by their characteristics that differ from conventional yeasts in alcohol production. Special yeasts are typically maltose-negative, resulting in minimal to zero alcohol production.

Nowadays, non-alcoholic beer beverages are crafted from a variety of raw materials to deliver health-promoting benefits while contributing to the distinctive taste of beer. Many researchers studied the addition of other plant-base ingredients in wort boiling. For instance, the addition of pulp and dried okra into the cloudy wheat beer enhanced the foam texture and stability in beer and also improved the aroma characteristics of beer [(4)]. Another example involves enhancing the antioxidant capacity of beer by adding dried goji berries during the wort boiling process [(5)]. So, adding nutritious ingredients will increase the functional benefits of beer.

Rice bran, the outer layer of rice grain, is an industrial by-product of the rice milling process [(6)]. It contains many bioactive phytochemicals such as phenolic compounds, flavonoids, and saponins which have anti-carcinogenic, anti-inflammatory and antioxidant properties and are beneficial to health [(7-8)]. Kum Jao Morchor 107 rice bran is rice bran from Thai variety rice with black or purple membranes rich in anthocyanin [(9)].

Thus, this study aimed to evaluate the appropriate ratio of Kum Jao Morchor 107 rice bran substituted to malted barley on the physicochemical, organoleptic properties, and the antioxidant activities of non-alcoholic beer fermented with *Saccharomyces cerevisiae* var. *chevalieri*.

## 2. Materials and methods

### 2.1 Non-alcoholic beer from malted barley added with Kum Jao Morchor 107 rice bran production

*2.1.1 Wort production*

Commercial pale malt (Homebrewthai, Thailand) and Kum Jao Morchor 107 rice bran (Lanna rice research center, Chiang Mai university, Thailand) were mixed at 4 levels of varying amounts of Kum Jao Morchor 107 rice bran replacing malt at 0% (control), 5%, 10% and 15% (w/w). The mixtures were added with water at 70°C. Then, the temperature was controlled at 66-67°C for 1 h (mashing). After that, the wort was filtered with a nylon filter cloth and the malt residue was washed with 76-77°C of hot water (sparging). The collected wort was boiled then added with 0.55 g/L of cascade hop pellets (Homebrewthai, Thailand). At the last 5 min of boiling, 0.55 g/L of the cascade hop pellets was further added. Finally, the wort was rapidly cooled until the temperature reached around 37°C.

*2.1.2 Fermentation of non-alcoholic beer*

After being filtered with a nylon filter cloth to remove the hop sediment, the obtained wort was added with 0.5 g/L of *Saccharomyces cerevisiae* var. *chevalieri* (Fermentis, France) and incubated at 20°C for 6 days.

### 2.2 Analysis of non-alcoholic beer from malted barley added with Kum Jao Morchor 107 rice bran

*2.2.1 Physicochemical properties*

The following analysis was performed daily during 6 days of fermentation. The alcohol content was measured with a hydrometer (home winery shop, Thailand) and displayed in the unit of %alcohol by volume (%ABV). The total soluble solids (TSS) was determined using a hand refractometer (ATAGO, Japan). The pH was analyzed with a pH meter (FiveGo F2, Mettler Toledo, Switzerland). The color values ($L^*$, $a^*$ and $b^*$) were measured with a ColorQuest XE (Hunter lab, USA). The optimal fermentation period was

assesed by a %ABV value of no more than 0.5. The sample from the selected fermentation period was further analyzed and compared to the sample in day 0.

*2.2.2 Reducing sugar analysis*

Reducing sugar contents were measured by the Miller method with DNS reagent [10]. One milliliter of DNS solution was added to 1 mL of sample then mixed and heated at approximately 100°C for 10 min. Then, the mixture was added with 5 mL of distilled water and mixed well. Maltose was used as a standard and the absorbance was measured at the wavelength of 540 nm using a UV-vis spectrophotometer (Genesys 180, Thermo Fisher Scientific, Waltham, MA, USA).

*2.2.3 Total phenolic contents (TPC)*

TPC analysis was determined using the method described by Phongthai and Rawdkuen [11]. Fifty microliter of the sample was mixed with 1.25 mL of Folin-Ciocalteu reagent (1:9 dilution) followed by addition of 1 mL of 7.5%w/v $Na_2CO_3$. Next, the mixture was incubated at 50°C for 15 min. After that, the absorbance was measured at 760 nm using a UV spectrophotometer. Gallic acid was used as a standard. The values were displayed as mg gallic acid equivalent per L (mg GAE/L) of sample.

*2.2.4 Total flavonoid contents (TFC)*

TFC was analyzed by aluminum chloride colorimeter assay, which was adapted from the method of Pukklay and Chuesaard [12]. The 0.5 mL of sample was mixed well with 1.5 mL of 95%(v/v) ethanol, 0.1 mL of 10% $AlCl_3$, 0.1 mL of 1 M $CH_3COOK$ and 2.8 mL of distilled water. The mixture was incubated in the dark at room temperature for 30 min. Then, the absorbance of the solution was measured at the wavelength of 405 nm using a UV spectrophotometer. The quantification of flavonoids in the sample was expressed in units of mg quercetin equivalent per 100 mL (mg QE/100mL) of sample.

*2.2.5 Total anthocyanin contents (TAC)*

TAC was examined by pH-differential method described by Spessoto *et al.*[13]. The 0.2 mL of sample was added with 1.8 mL of KCl buffer (pH 1.0). Another 0.2 ml of sample was added with 1.8 mL of $CH_3COONa$ buffer (pH 4.5). The absorbance of each mixture solution was measured at the wavelength of 510 nm and 700 nm, respectively. The TAC was calculated using the following equation and expressed as mg cyanidin-3-glucoside equivalent per L (mg/L) of sample.

$$\text{TAC (mg/L)} = \frac{\text{Abs} \times \text{MW} \times \text{D} \times 1{,}000}{\varepsilon \times 1}$$

Where: Abs is absorbance calculated from $(Abs_{510nm}\text{-}Abs_{700nm})_{pH\ 1.0} - (Abs_{510nm}\text{-}Abs_{700nm})_{pH\ 4.5}$;

Mw is the molecular weight of cyanidin-3-glucoside (449.2 g/moL); D is the dilution factor; $\varepsilon$ is a molar absorptivity of cyanidin-3-glucoside (26,900 L/moL/cm)

*2.2.6 Antioxidant activities*

*2.2.6.1 DPPH radical scavenging activity assay*

The method described by Phongthai *et al.* [14] was used to measure the DPPH free radical scavenging activity of the sample. The 1 mL of 0.1 mM DPPH in 95% ethanol was mixed with 0.25 mL of the sample. The mixture was incubated in the dark at room temperature for 30 min. The absorbance value was determined at 517 nm using a UV spectrophotometer. The values were displayed as mmol Trolox per 100 mL (mmoL Trolox/100mL) of sample.

*2.2.6.2 Ferric reducing antioxidant power (FRAP) assay*

FRAP assay was measured according to the method of Phongthai *et al.* [14]. The FRAP reagent was prepared by mixing 300 mM pH 3.6 acetate buffer, 10 mM TPTZ (2,4,6-Tris(2-pyridyl)-s-triazine) in 40 mM HCl and 20 mM $FeCl_3$ at a ratio of 10:1:1, respectively. Next, the 60 µL of sample was mixed with 180 µl of distilled water and 1.8 mL of FRAP reagent. The mixture solution was placed in the dark for 10 min. Then, the absorbance was measured at 593 nm using a UV spectrophotometer. The FRAP values were expressed as units of mM $FeSO_4$ per 100 mL (mM $FeSO_4$/100 mL) of sample.

*2.2.7 Ethanol concentration*

The ethanol concentration in the selected samples was measured by a high performance liquid chromatography (HPLC; Hitachi Chromaster), coupled with a refractive index detector (RID, VWR Hitachi Chromaster 5450) using Aminex HPX-85H (300 mm × 7.8 mm; Bio-Rad, Hercules, CA, USA) column. The samples and standards were eluted with 5 mM $H_2SO_4$ (RCI Labscan), as a mobile phase at 0.75 mL/min. The column temperature was 55°C and the detector temperature was 40°C. Samples were filtered using a 0.22 µm nylon filter before analysis. The values were displayed as %ethanol concentration.

*2.2.8 Sensory analysis*

The sample from the suitable fermentation duration was carbonated and evaluated for the sensory properties in terms of color, aroma, taste, bitterness, mouthfeel and overall acceptability using 9-point hedonic scale (1 – dislike extremely, 2 – dislike very much, 3 – dislike moderately, 4 – dislike slightly, 5 – neither like nor dislike, 6 – like slightly, 7 – like moderately, 8 – like very much and 9 – like extremely) by 30 untrained panelists with no sample sequence.

*2.2.9 Statistical analysis*

The results were statistically analyzed using SPSS V.17.0 software for windows (SPSS Inc., Chicago, IL, USA) and were reported as mean ± S.D. Analysis of variance (ANOVA) and Duncan's multiple range test (DMRT) was performed to test the difference between the sample means at a significant level of $P<0.05$.

## 3. Results

### 3.1 Physicochemical properties, reducing sugar and ethanol concentration during fermentation

The physicochemical properties of samples from the fermentation of malted barley added with Kum Jao Morchor 107 rice bran with *S. cerevisiae* var. *chevalieri* were shown in Figures 1 to 3. The results in Figure 1 showed that starting on day 3 of fermentation, increasing Kum Jao Morchor 107 rice bran decreased the %ABV while significantly increased the TSS from day 5 of fermentation ($P<0.05$). This might be because barley and rice bran had distinct structures of starch which allowed the releasing of fermentable sugars in the wort during mashing [13]. Fabian *et al.* [16] reported that starch existing in rice bran had gelatinization properties similar to those in rice endosperm. The addition of rice in beer has been reported to increase the total amount of starch while decrease the amount of starch hydrolytic enzymes, resulting in more soluble starch and less fermentable sugar remaining in the wort after mashing that caused difficulty for yeast metabolism during fermentation [15,16]. Consequently, the addition of Kum Jao Morchor 107 rice bran resulted in lower alcohol contents in the product compared with the control. On the other hand, prolonging the fermentation period increased the %ABV of all samples. The %ABV was dramatically increased after 4 days of fermentation (Figure 1) due to yeast activity. The wort was fermented by yeasts in an anaerobic environment by converting sugar into carbon dioxide and ethanol [17], which was consistent with the decreasing TSS value during fermentation.

As mentioned above, this study focuses on the production of non-alcoholic beer with an alcohol content of not more than 0.5% ABV [3]. Although the alcohol content of all samples during 6 days of fermentation ranged from 0 to 1.25 %ABV, the %ABV exceeding 0.5 was only demonstrated from day 4 in 5% rice bran sample (Figure 1). Therefore, in this study, the samples fermented for 3 days was selected for further analysis.

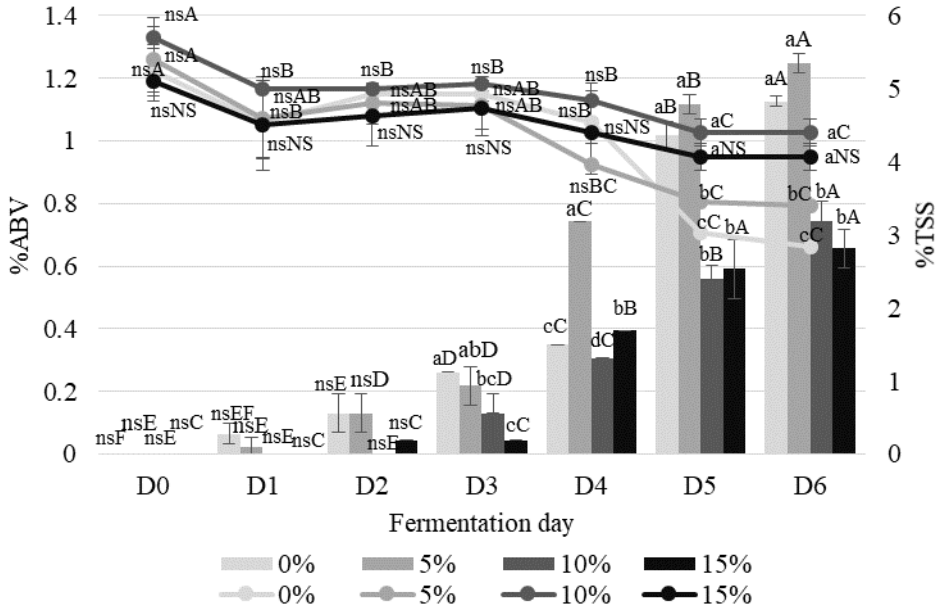



**Figure 1.** %ABV and %TSS of samples from malted barley added with 0-15% Kum Jao Morchor 107 rice bran fermented with *S. cerevisiae* var. *chevalieri* during 6 days
Different lowercase letters indicate a significant difference in %rice bran (P<0.05)
Different uppercase letters indicate a significant difference in fermentation duration (P<0.05)

It was shown that the addition of rice bran did not affect the pH value except on day 1 of fermentation, in which the pH was rising with increasing rice bran content (Figure 2). This might be due to the relatively higher pH value of the rice bran demonstrating 6 to 6.9 [19]. During the fermentation period, it was found that pH did not decrease significantly (P<0.05) in the first 3 days. However, the pH sharply dropped from a range of 4.94 ± 0.01 to 5.22 ± 0.05 on day 3 to a range of 4.7 ± 0.01 to 4.9 ± 0.12 on day 4 of fermentation and further dropped with no significant difference to a range of 4.5 to 4.9 at day 6 of fermentation. The lowest pH was found in control sample (4.53 ± 0.15) similar to the result of Simões *et al.*[20], using *Sacharomyces crevisiae* var. *chevalieri* providing the final pH on day 6 at about 4.4. The reduction of pH was caused by the carbon dioxide produced from yeast metabolism [21], which corresponded to the reduction of TSS and increase in %ABV of the products.

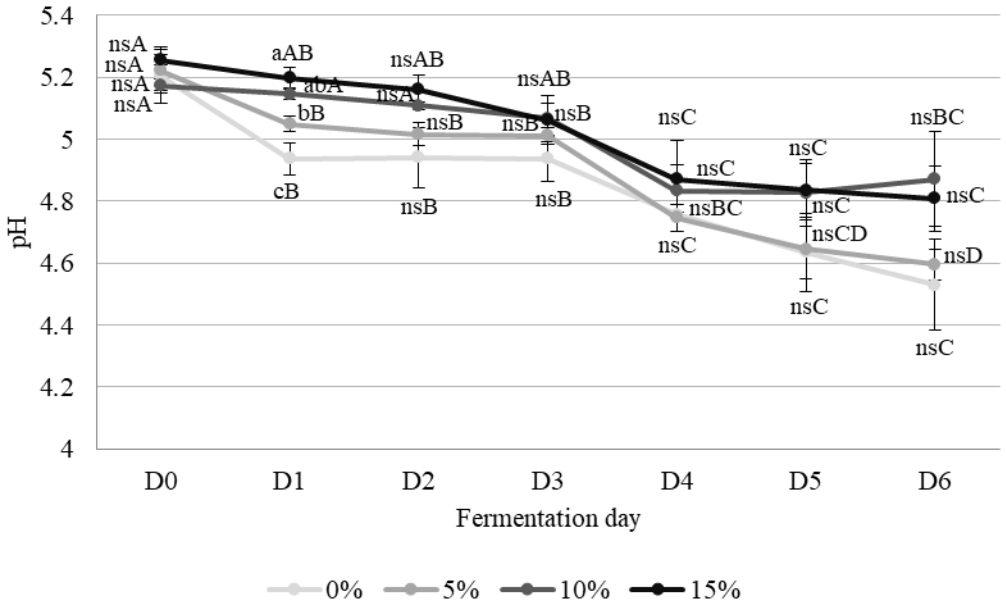
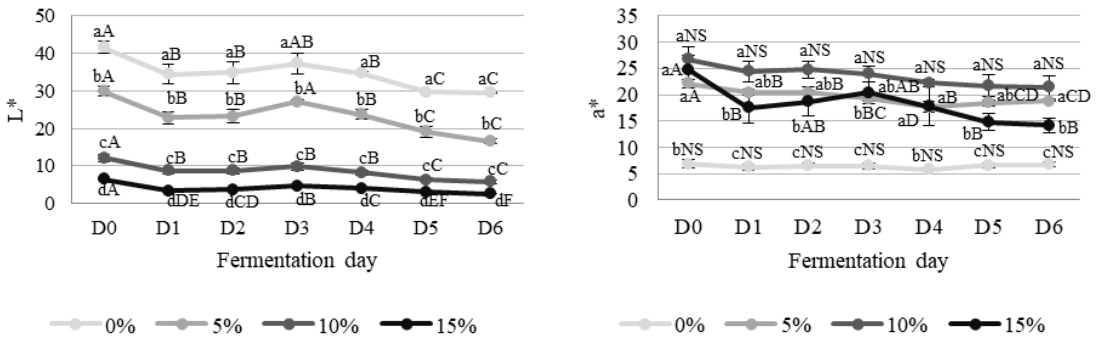
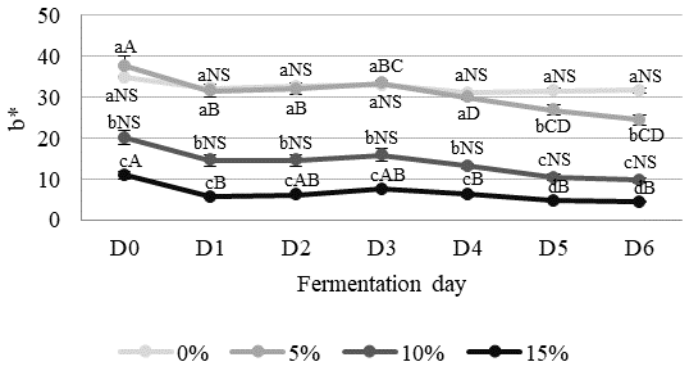



**Figure 2.** The pH values of samples from malted barley added with 0-15% Kum Jao Morchor 107 rice bran fermented with *S. cerevisiae* var. *chevalieri* during 6 days
Different lowercase letters indicate a significant difference in %rice bran (P<0.05)
Different uppercase letters indicate a significant difference in fermentation duration (P<0.05)

The color parameters of the samples are illustrated in Figure 3. It was found that the brightness (*L**) value significantly decreased with the addition of Kum Jao Morchor 107 rice bran (P<0.05) and tended to decrease with longer fermentation (P<0.05). For the redness (*a**) value, the control had the lowest redness compared to samples substituted by Kum Jao Morchor 107 rice bran (P<0.05). However, the *a** value tended to increase with no significant difference (P>0.05) among the rice bran-added samples. As the fermentation time increased, the 5% and 15% rice bran added samples inclined to decrease in *a** value. The changes in *L** and *a** values in samples containing rice bran might be due to the presence of anthocyanins [22] in Kum Jao Morchor 107 rice bran exhibiting dark purple color. In terms of yellowness (*b**) value, it was found that increasing amounts of Kum Jao Morchor 107 rice bran significantly decreased *b** value of samples (P<0.05).




(A) (B)




(C)

**Figure 3.** Color in terms of *L** (A), *a** (B) and *b** (C) of samples from malted barley added with 0-15% Kum Jao Morchor 107 rice bran fermented with *S. cerevisiae* var. *chevalieri* during 6 days
Different lowercase letters indicate a significant difference in %rice bran (P<0.05)
Different uppercase letters indicate a significant difference in fermentation duration (P<0.05)

As previously mentioned, samples fermented for 3 days were selected for further analysis. Figure 3 demonstrated that rice bran-added samples contained significantly lower reducing sugar than control (P<0.05) on day 0. Furthermore, there was a significant decrease in reducing sugar content in samples containing 10% and 15% rice bran compared to the control group. However, there was no significant decrease in reducing sugar contents between day 0 and day 3 of fermentation (P>0.05). This might be because the yeast used in this study, *Sacharomyces crevisiae* var. *chevalieri*, is a commercial maltose-negative yeast strain. Maltose and maltotriose are a primary fermentable sugar for yeast consumption [(23)]. However, this yeast strain cannot utilize maltose and maltotriose, and only ferment the usable residual sugars in trace amounts due to a lack of maltose transporter and maltase [(20)]. Therefore, reducing sugar contents representing maltose remained at day 3 of fermentation. These results corresponded to the results of TSS, in which no significant decrease in the first 3-4 days of fermentation.

The %ethanol concentration determined by HPLC was shown in Table 1. The %ethanol content of the control was the highest (0.82 ± 0.02), exceeding 0.5% (v/v) which could not be labeled as a non-alcoholic beer. Nonetheless, it could be labeled as low-alcoholic beer, containing alcohol content ranging from 0.5 to 1.2% v/v [(20)]. The value obtained was still lower than that of Simões *et al*. [(20)], studying the production of beer using the same yeast strain demonstrating the %ethanol content (v/v) of 1.2% in day 3 of fermentation. In this study, the sample added with 15% rice bran provided the lowest %ethanol content at 0.26 ± 0.02. These results corresponded to the results of %ABV as previously described.

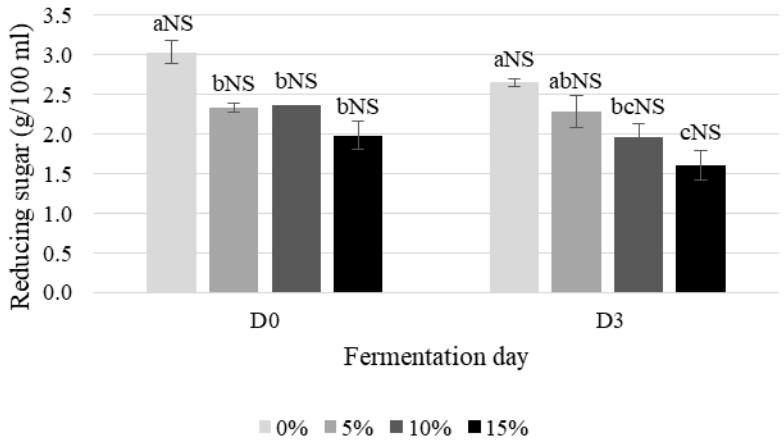



**Figure 4.** Reducing sugar contents of samples from malted barley added with 0-15% Kum Jao Morchor 107 rice bran fermented with *S. cerevisiae* var. *chevalieri* on day 3 compared to day 0 of fermentation from maltose standard curve
Different lowercase letters indicate a significant difference in %rice bran (P<0.05)
Different uppercase letters indicate a significant difference in fermentation duration (P<0.05)

**Table 1.** Ethanol concentration using HPLC of samples from malted barley added with 0-15% Kum Jao Morchor 107 rice bran fermented with *S. cerevisiae* var. *chevalieri* on day 3 of fermentation

| %Rice bran | Ethanol concentration (%) |
|---|---|
| 0 | $0.82 \pm 0.02^{a}$ |
| 5 | $0.33 \pm 0.01^{b}$ |
| 10 | $0.34 \pm 0.01^{b}$ |
| 15 | $0.26 \pm 0.02^{c}$ |

Means ± standard deviation (S.D.) with different lowercase letters (a, b, c) represent significant difference (P<0.05)

### 3.2 Bioactive compounds of non-alcoholic beer from malted barley added with Kum Jao Morchor 107 rice bran

The results of TPC, TAC and TFC are demonstrated in Table 2. Polyphenol contents are one of the crucial parameters that affect beer quality, related to flavor, color, and organoleptic properties [24-25]. It was found that the increasing amounts of Kum Jao Morchor 107 rice bran in samples increased TAC, TPC and TFC values, except on day 3 of fermentation, in which TAC and TFC of 5 and 10% rice bran added samples were not significantly different (P>0.05). Kum Jao Morchor 107 rice bran is a source of various types of phenolic compounds majoring ferulic acid, p-coumaric acid and diferulate [26]. Additionally, anthocyanin, a class of flavonoids that includes cyanidin-3-O-glucoside, is abundant in Kum Jao Morchor 107 rice bran [27]. For these reasons, TPC, TAC, and TFC values increased as the amount of rice bran increased. The TPC values of all samples on day 3 ranged from 105.56 ± 3.93 to 211.11 ± 3.93 mg GAE/L sample which were lower than the value of non-alcoholic beer revealed by Mitić *et al.* [25] exhibiting 340.40 mg GAE/L. However, the values obtained are within the range of TPC in the research of Martinez-Gomez *et al.* [27] (75 to 366 mg GAE/L). The difference in values probably varied from the different amounts of malts, the main source of phenolics in beer [28], the concentration of yeast, and the final fermentation time [29].

As the fermentation day increased, the TPC and TFC values significantly decreased ($P<0.05$) except the TPC value of the sample with 5% rice bran, which had no significant difference during fermentation ($P>0.05$). Nevertheless, the TAC value had no statistical differences among all samples on day 3 compared to day 0 of fermentation. The reduction of TPC during fermentation can be described as an activity of the yeast. In addition to consuming sugar, this yeast strain also utilizes phenolic compounds, such as ferulic acid and coumaric acid, using enzymes to decarboxylate them to 4-vinylguaiacol and 4-vinylphenol, contributing to spicy and clove-like flavor in beer [30-31]. In terms of flavonoids, decreasing values during fermentation might be due to reactive oxygen species attack causing the degradation of flavonoids during fermentation [33].

**Table 2.** TPC, TAC and TFC of samples from malted barley added with 0-15% Kum Jao Morchor 107 rice bran fermented with *S. cerevisiae* var. *chevalieri* on day 3 compared to day 0 of fermentation

| Bioactive compound | %Rice bran | Fermentation day | |
|---|---|---|---|
| | | 0 | 3 |
| TPC (mg GAE/L) | 0 | $158.33 \pm 15.71^{cA}$ | $105.56 \pm 3.93^{cB}$ |
| | 5 | $177.78 \pm 19.64^{cNS}$ | $125.00 \pm 7.86^{cNS}$ |
| | 10 | $261.11 \pm 11.79^{bA}$ | $169.44 \pm 23.57^{bB}$ |
| | 15 | $319.44 \pm 15.71^{aA}$ | $211.11 \pm 3.93^{aB}$ |
| TAC (mg/L) | 0 | $1.20 \pm 0.43^{cNS}$ | $0.53 \pm 0.11^{cNS}$ |
| | 5 | $3.01 \pm 0.43^{bcNS}$ | $1.95 \pm 0.00^{bcNS}$ |
| | 10 | $4.43 \pm 1.17^{bNS}$ | $3.38 \pm 0.74^{bNS}$ |
| | 15 | $6.91 \pm 0.43^{aNS}$ | $6.46 \pm 1.70^{aNS}$ |
| TFC (mg QE/100ml) | 0 | $6.29 \pm 0.13^{dA}$ | $3.55 \pm 0.3^{cB}$ |
| | 5 | $10.77 \pm 0.27^{cA}$ | $4.86 \pm 0.4^{bcB}$ |
| | 10 | $15.16 \pm 0.72^{bA}$ | $5.96 \pm 0.74^{bcB}$ |
| | 15 | $20.55 \pm 0.10^{aA}$ | $10.17 \pm 0.3^{aB}$ |

Means ± standard deviation (S.D.) with different lowercase letters (a, b, c) represent significant difference ($P<0.05$)
Different lowercase letters indicate a significant difference in %rice bran ($P<0.05$)
Different uppercase letters indicate a significant difference in fermentation duration ($P<0.05$)

### 3.3 Antioxidant activity of non-alcoholic beer from malted barley added with Kum Jao Morchor 107 rice bran

The results of antioxidant activity through DPPH and FRAP assays as shown in Table 3 demonstrated that the increase of Kum Jao Morchor 107 rice bran increased the DPPH and FRAP values. Moreover, as fermentation day increased, the DPPH values increased significantly in control and 5% rice bran added sample ($P<0.05$), however, remained not significantly different in samples with 10 and 15% rice bran ($P>0.05$). In FRAP values, it was significantly lower only in samples with 5% rice bran on day 3 compared to day 0 of fermentation ($P<0.05$). The disagreement between DPPH and FRAP values during fermentation can be explained by the different mechanisms during antioxidant measurement of each technique. The increase of antioxidant activity in DPPH values might be due to synergistic effect of the metabolite changes from yeast [33]. According to the results of bioactive compounds at day 3 of fermentation, it could be said that antioxidant activity probably increased from other substances besides phenolics groups such as B vitamins. It has been reported that B vitamins especially vitamins B3, B6 and B12 can be synthesized from yeast [34]. The B vitamins groups can donate hydrogen atoms to stabilize the antioxidant products which affect the DPPH but had no effect on the FRAP values [35]. Moreover, the

reduction of antioxidant activity during fermentation may be due to a decrease in activity of yeast or degradation of phenolics and flavonoids as shown in reduced TPC and TFC values[32]. The DPPH values of samples ranged from 0.032 ± 0.00 to 0.079 ± 0.00 mmoL Trolox/100ml, which were higher than that observed in alcohol-free beer from the study of Mitić *et al.* [25], which averaged to 0.031 mmol Trolox/100mL. The increment of DPPH and FRAP values in the addition of Kum Jao Morchor 107 rice bran might be due to the higher values of TPC, and TFC, as previously discussed. Kum Jao Morchor 107 rice bran contains many polyphenols in high amounts, such as tocopherols and γ-oryzanol providing antioxidant activity [8].

**Table 3.** TPC, TAC and TFC of samples from malted barley added with 0-15% Kum Jao Morchor 107 rice bran fermented with *S. cerevisiae* var. *chevalieri* on day 3 compared to day 0 of fermentation

| Antioxidant activity | %Rice bran | Fermentation day | |
|---|---|---|---|
| | | 0 | 3 |
| DPPH (mmol Trolox/100mL) | 0 | $0.012 \pm 0.00^{dB}$ | $0.032 \pm 0.00^{dA}$ |
| | 5 | $0.024 \pm 0.00^{cB}$ | $0.053 \pm 0.00^{cA}$ |
| | 10 | $0.065 \pm 0.00^{bNS}$ | $0.065 \pm 0.00^{bNS}$ |
| | 15 | $0.072 \pm 0.00^{aNS}$ | $0.079 \pm 0.00^{aNS}$ |
| FRAP (mmol $FeSO_4$/100mL) | 0 | $0.28 \pm 0.00^{cNS}$ | $0.29 \pm 0.01^{dNS}$ |
| | 5 | $0.42 \pm 0.00^{bA}$ | $0.37 \pm 0.00^{cB}$ |
| | 10 | $0.51 \pm 0.03^{aNS}$ | $0.52 \pm 0.01^{bNS}$ |
| | 15 | $0.52 \pm 0.03^{aNS}$ | $0.57 \pm 0.00^{aNS}$ |

Means ± standard deviation (S.D.) with different lowercase letters (a, b, c) represent significant difference (P<0.05)
Different lowercase letters indicate a significant difference in %rice bran (P<0.05)
Different uppercase letters indicate a significant difference in fermentation duration (P<0.05)

Table 4 shows the sensorial properties of samples on day 3 of fermentation by 30 untrained panelists. The results showed that the addition of Kum Jao Morchor 107 rice bran did not significantly affect color, aroma, taste, bitterness and overall acceptability (P>0.05). However, the sample with 15% rice bran had the lowest score of mouthfeel attribute. *S. crevisiae* var. *chevalieri* has been reported as being capable of producing low-alcoholic beer with high levels of sensory acceptance and optimum amounts of volatile compounds [20]. According to the findings of the sensory testing, it is clear that the panelists accepted it quite positively. It might be said that this non-alcoholic beer drink tastes similar to beer, which led the panelists to accept it as one of the beer products. In non-alcoholic or low-alcoholic beer, non-fermentable maltose, maltotriose, and dextrin which cannot be utilized by yeast, are left unfermented after fermentation and therefore remain in the produced beer. They add body, mouthfeel, and palate fullness to the beer, and contribute to its flavor [18].

**Table 4.** Sensorial properties of samples from malted barley added with 0-15% Kum Jao Morchor 107 rice bran fermented with *S. cerevisiae* var. *chevalieri* on day 3 of fermentation

| %Rice bran | Color ns | Aroma ns | Taste ns | Bitterness ns | Mouthfeel | Overall acceptability ns |
|---|---|---|---|---|---|---|
| 0 | 6.9 ± 1.66 | 6.4 ± 1.19 | 6.2 ± 1.53 | 5.7 ± 1.74 | 6.3 ± 1.20[a] | 6.2 ± 1.04 |
| 5 | 7.3 ± 1.44 | 6.5 ± 1.28 | 6.0 ± 1.88 | 6.1 ± 1.83 | 6.4 ± 1.87[a] | 6.1 ± 1.53 |
| 10 | 6.8 ± 2.06 | 6.3 ± 1.46 | 5.7 ± 1.37 | 5.8 ± 1.95 | 6.5 ± 1.46[a] | 6.1 ± 1.16 |
| 15 | 6.8 ± 1.99 | 6.5 ± 1.36 | 5.7 ± 1.67 | 5.7 ± 1.82 | 5.6 ± 1.47[b] | 5.9 ± 1.49 |

Means with different lowercase letters (a, b, c) represent significant difference (P<0.05)
Ns indicate non-significant differences (P>0.05)

## 4. Conclusion

In this study, it was found that adding Kum Jao Morchor 107 rice bran from 5 to 15% replacing malted barley and incubated with *S. crevisiae* var. *chevalieri*, a maltose-negative yeast strain for 3 days at 20°C enabled the production of non-alcoholic beer with ethanol contents in the range of 0.26 to 0.34% (v/v). In addition, it was found that by increasing the amount of rice bran in beer, the TPC, TAC, TFC, and antioxidant values tended to increase. In sensory analysis, the addition of bran at 5 and 10% obtained the highest acceptance scores in mouthfeel properties similar to the control, with no significant differences in color, aroma, taste, bitterness, and overall acceptability scores among all samples.

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# Consumption Pattern on PET-bottled Beverages and Intention to Recycle PET Bottles

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**Abstract**

Polyethylene terephthalate (PET) packaging has been shaped by an increase in the consumption of PET bottles for both beverage and non-beverage. Because of the lack of a recycling collection system for PET and the rapid growth of PET bottle consumption, PET-bottled beverages were the majority of plastic pollution. Moving toward the Bio-Circular-Green economy (BCG economy) in Thailand, the recycling of PET bottles has become an important segment in the circular economy goals; however, the intention to recycle PET bottles varies among generations. The objective of this study is to analyze the relationship between age and the consumption of PET-bottled beverages by using descriptive statistics, Pearson's chi-square correlation, and Pearson's correlation coefficient. The data were collected from 207 participants by using the questionnaires on consumption of five types of PET-bottled beverages including drinking water, soft drinks, vegetable and fruit juices, ready-to-drink tea, and functional drinks. Findings showed that the average consumption of PET-bottled beverages was estimated at 442.9 g per week or 35 PET bottles (600 mL) per person per week. Drinking water (55.8%) carried the highest consumption of PET bottles followed by soft drinks (19.9%), vegetable and fruit juices (10.6%), ready-to-drink tea (9.6%), and functional drinks (4.1%). The young group (aged 18-35 years old) consumed PET-bottled beverages higher than the adult group (aged greater than 35 years old), accounting for 61 percent higher than the average of PET-bottled beverage consumption by the adult. The results showed a significant relationship between age and the intention to recycle PET bottles (P-value < 0.05). However, both groups had insignificant associations with the separation of waste. Thus, the promotion of the benefits of recycling PET bottles and waste collection systems should be encouraged to both groups by related stakeholders of the beverage industry.

***Keywords**:* PET bottles; Beverages; Consumption pattern; Recycle PET; BCG economy

## 1. Introduction

PET bottles are widely used in various industries and applications, leading to a significant amount of waste in PET bottles. However, the lack of management policies in Thailand has resulted in these waste bottles entering the informal collection and recycling sectors, causing environmental pollution [(1)]. Thai people have increasingly depended on the use of plastic for any application as it is convenient and available without any charges. Thailand gained recognition as one of the leading contributors to ocean plastic waste in Asia, ranking among the top five [(2)]. However, it lagged behind many other ASEAN countries in efforts to address the plastic waste issue. In 2017, more than 185,000 tonnes of plastic were generated in Thailand each year, but that only less than half of the waste from plastic bottles is effectively recycled. The remaining 100,000 tonnes of plastic bottles are thrown away in landfills, and the rest become marine waste and pollute the ecosystem worldwide [(3)]. Following these situations, the government has identified strategies for lowering plastic trash

production. Through the release of a roadmap for the management of plastic waste from 2017 to 2030 [4], as well as the adoption of a resolution at the Cabinet meeting to "stop using" three different types of plastic by the year 2019, including Oxo plastic bags, bottle caps seal, and microbeads and four different types of plastic by the year 2021, including Foam food containers, plastic cups thickness less than 100 microns, plastic bags thickness less than 36 microns, and plastic straws.

However, the situation of COVID-19 pandemic has driven the rapid growth of the food delivery industry, which has increased the amount of single-use plastic waste [6,10]. According to the Thai Environment Institute, the COVID-19 situation has caused an increase in plastic waste of 15% from an average of 5,500 tons per day to 6,300 tons per day by the year 2020. As a result, in 2020, Thailand was ranked as the fifth largest source of plastic waste, especially in the sea and landfill, in the world. The environment, ecosystem, and climate change are all significantly impacted by plastic waste. One of the main reasons is single-use plastic such as plastic cups, plastic bottles, straws, plastic bags, and food packaging, which has a short period of use and takes more than 450 years to decompose [8, 10]. Furthermore, there is no separation and proper management of plastic trash in the country that drives the effective reuse of single-use plastics [5,10]. To achieve the carbon neutrality goal in 2030, the government has set a goal to use 100% recycled plastic by 2027, which both the public and private sectors must work together [4,5]. In 2022, Thailand published new legislation relating to food contact plastics. Thai Food and Drug Administration has unlocked its regulation allowing the use of recycled plastic. However, the government's initiatives may not be sufficient to achieve its objective of using 100% recycled plastic by 2027. Even though there have been several campaigns offered by public and private sectors to encourage people to separate garbage after use, lack of awareness of plastic waste is still a major challenge for the recycling process [4,5].

This study aims to study the consumption patterns of beverages in PET bottles and analyze the relationship between age and intention to reuse plastic bottles. Moreover, the relationship between age and the level of waste separation from plastic bottles is investigated to understand the after-use of plastic bottles.

## 2. Materials and Methods

This study tackles current PET-bottle beverage consumption patterns, the intention of reusing plastic bottles, and the level of separation of plastic bottle waste within Thailand. The survey on PET bottle consumption was carried on by an online questionnaire on a sample of people who live in Thailand [9]. The respondents aged 18 years or older were selected for the survey. To investigate the differences between the age groups, the respondents were segmented into two age groups: the young group (who were born after 1988) and the adult group (who were born before 1988).

The questionnaire was divided into 3 parts: (i) sociodemographic characteristics of respondents (gender, age, income) (ii) Data on the behavior of PET-bottle beverage consumption and management of PET bottles after use which PET-bottled beverages divided into 5 categories according to drinking water, carbonated drink, ready-to-drink tea, vegetable and fruit juice and functional drink [7], and (iii) intention for reusing PET bottles and separation of recycled waste. The intention to reuse PET bottles is categorized into different percentages: 0%, 20%, 40%, 60%, 80%, and 100%. The level of separate recycling waste is categorized into different percentages: 0%, 20%, 40%, 60%, 80%, and 100%.

Data on demographic characteristics and the consumption of PET-bottle beverages in different types of beverages were analyzed with descriptive statistics. The relationship between the consumption of PET bottles and age was analyzed using Pearson's correlation test. The relationship between age and intention to reuse plastic bottles and the relationship between age and the level of waste separation from plastic bottles were analyzed using Pearson's chi-square.

## 3. Results & Discussion

### 3.1 Sample demographic

The characteristics of 207 respondents involved in the survey (gender, age, income, education level) were reported in Table 1. The majority of respondents in the study were female, accounting for 78%, while males and others accounted for 19% and 3% respectively. Most respondents were in the young group (less than or equal to 35 years), representing 84%. Respondents' educational level appears quite high since the main part of respondents have a bachelor's degree (64%), and 28% of the total respondents have a master's degree. Lastly, most of the respondents had income between 5,001 and 15,000 THB (29%).

**Table 1.** Demographic of the sample

| | | Frequency | Percentage |
|---|---|---|---|
| Gender | | | |
| | Male | 40 | 19% |
| | Female | 161 | 78% |
| | Other | 6 | 3% |
| Age | | | |
| | Less than or equal to 35 years old | 174 | 84% |
| | More than 35 years old | 33 | 16% |
| Income | | | |
| | Lower 5,000 THB | 11 | 5% |
| | Between 5,001-15,000 THB | 60 | 29% |
| | Between 15,001-25,000 THB | 55 | 27% |
| | Between 25,001-35,000 THB | 40 | 19% |
| | Between 35,001-45,000 THB | 19 | 9% |
| | Between 45,001-55,000 THB | 7 | 3% |
| | Between 55,001-65,000 THB | 5 | 2% |
| | More than 65,001 THB | 10 | 5% |
| Education level | | | |
| | Secondary school | 6 | 3% |
| | Diploma | 3 | 1% |
| | Bachelor's degree | 133 | 64% |
| | Master's degree | 58 | 28% |
| | Doctoral degree | 7 | 3% |
| Total | | 207 | 100% |

### 3.2 Beverage consumption

The average consumption of PET bottles per week per person for different types of beverages is presented in Table 2. The results show that the average consumption of PET-bottle beverages drinking water was the highest at 35.7 bottles per person per week followed by carbonated drinks (11.2 bottles per person per week), vegetable and fruit juice (6.2 bottles per person per week), ready-to-drink tea (5.6 bottles per person per week), and functional drinks (2.7 bottles per person per week). The respondents who were in the young group had an average of 38.4 bottles per person per week, which accounts for 62%, which was a higher beverage consumption than those over 35 years old who consumed an average of 23.1 bottles per person per week (38%). This is supported by the study of the consumption of PET bottles in China [(11)]. This provides a better understanding of the consumption patterns of different age groups and their impact on PET bottle consumption.

**Table 2.** The consumption of PET-bottle beverages in different types of beverages

| Age group | Average consumption of PET bottles (bottle/person /week) | | | | | |
|---|---|---|---|---|---|---|
| | **Drinking water** | **Carbonated drink** | **Ready-to-drink tea** | **Vegetable and fruit juice** | **Functional drink** | **PET bottle consumption** |
| Young group | 21.1 | 7.9 | 3.7 | 4.1 | 1.5 | 38.4 (62%) |
| Adult group | 14.6 | 3.3 | 1.9 | 2.1 | 1.2 | 23.1 (38%) |
| Total | 35.7 | 11.2 | 5.6 | 6.2 | 2.7 | 61.5 |

Pearson's correlation test was used for statistical analysis to explore the relationship between the consumption of five types of PET-bottle beverages and age group (Table 3). Overall, the correlation coefficient of the relationship between age and the total PET bottle consumption values at -0.119 (*P-value*<0.1) giving the higher PET bottle consumption by the young group. The significant relationship between the consumption of types of PET-bottle beverages includes drinking water (-0.067) and vegetable and fruit juice (-0.117), indicating an inverse correlation. This reveals that carbonated drinks and vegetable and fruit juice had more consumption in the young group compared to the adult group. However, there is no relationship between age and other types of PET-bottle beverages including drinking water, ready-to-drink tea, and functional drinks.

**Table 3.** The correlation between age and the consumption of PET-bottle beverages

| | | **Drinking water** | **Carbonated drink** | **Ready-to-drink tea** | **Vegetable and fruit juice** | **Functional drink** | **PET bottle consumption** |
|---|---|---|---|---|---|---|---|
| ge | Pearson Correlation | -0.067 | -0.123* | -0.080 | -0.117* | 0.020 | -0.119* |
| | Sig. (2-tailed) | 0.335 | 0.078 | 0.253 | 0.092 | 0.772 | 0.087 |

* Correlation is significant at the 0.1 level (2-tailed).

Table 4 shows the relationship between age groups and intention to reuse and the relationship between age groups and the level of separate recycled bottles using Pearson's chi-square. The correlation values and statistical significance for the relationship between age and the intention for reuse (*P-value*<0.05), indicate a significant association between age and intention to reuse PET bottles. The young group has a higher intention to reuse PET bottles than the adult group. However, there is no significant difference between age and the level of separate recycling waste (*P-value*>0.05), indicating that the level of separate recycling waste was not strongly influenced by age.

**Table 4.** Correlation between age, intention to reuse, and level of separate recycled bottles.

| | | **Intention to reuse PET bottles** | | | | | | **Pearson's chi-square (*P-value*)** |
|---|---|---|---|---|---|---|---|---|
| | | **0%** | **20%** | **40%** | **60%** | **80%** | **100%** | |
| Age | Young group | 6 | 32 | 43 | 33 | 38 | 22 | 11.494** (0.042) |
| | Adult group | 4 | 7 | 6 | 11 | 2 | 3 | |
| | | **Level of separation recycle waste** | | | | | | **Pearson's chi-square (*P-value*)** |
| | | **0%** | **20%** | **40%** | **60%** | **80%** | **100%** | |
| Age | Young group | 58 | 5 | 21 | 42 | 32 | 16 | 2.621 (0.758) |
| | Adult group | 8 | 2 | 6 | 9 | 5 | 3 | |

**Correlation is significant at the 0.05 level (2-tailed).

## 4. Conclusion

The consumption of PET-bottle beverages, particularly drinking water, carbonated drinks, vegetable and fruit juice, ready-to-drink tea, and functional drinks has significantly contributed to plastic pollution. The young age group (less than or equal to 35 years old) consumes more PET-bottle beverages compared to the adult group at the difference of 15 bottles per person per week, indicating a higher demand for these products among younger individuals. However, the intention to recycle PET bottles is higher among the young group compared to adults. On the other hand, there is no difference in the level of separation of recycled waste and age groups. One possible solution to address the issue of low waste separation among respondents is to implement educational campaigns and awareness programs. By educating consumers about the importance of waste separation and recycling, they can develop a better understanding of the impact of their actions on the environment. Additionally, providing instructions and accessible recycling facilities can make it easier for consumers to properly dispose of their plastic waste. These initiatives can help increase waste separation rates and contribute to a more sustainable waste management system.

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# Plant-Based Seasoning as Umami Ingredient: Process, Free Amino Acid Profile and Application in Traditional Thai Fermented Fish Sauce

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**Abstract**

*Phong nua*, or traditional umami ingredients originated by *Isan* ethnic wisdom for home use, is made by mixing various vegetable and herb paste, subsequently sun-drying to powder. The cost-availably hot air drying (HA) process at drying temperature of 50, 60, and 70°C was proposed compared with the current time-consuming solar drying (SD) (3 days). The drying time was obtained for 10, 9, and 7 h at drying temperature of 50, 60, and 70°C, respectively**.** The HA sample at 60°C (HA60) gave the highest overall acceptance score (7.06) and flavor liking (7.02) when sensorially tested in soup medium ($P \leq 0.05$). As a result, monosodium glutamate (MSG)-like free amino acids (FAA) (glutamic acid and aspartic acid) was found in the greatest amount in the HA60 sample (82.44 μg/g DW). On the other hand, the sample at lower temperature (50°C) released the highest FFA group linked to sweet taste (351.66 μg/g DW). The physicochemical properties of the selected sample (HA60) showed low moisture (5.81% DW) and low $a_w$ (0.238), acceptable dissolvability (63.24%) and dispersibility (0.325), with pH (4.59) and color value ($L^*$ 59.48, $a^*$ -4.10, and $b^*$ 23.98). When applying the HA60 sample to *nam-pla-ra* products, it generated the higher liking score than regular recipe ($P \leq 0.05$), with well-accepted in *som-tum* menu. Due to the presence of umami compounds in local plant-based seasoning, it can be a potential alternative as a newly natural flavor enhancer and HA process was high-throughput and cost-effective for better production.

***Keywords****:* Flavor enhancer; Free amino acids (FFA); Hot air drying; Monosodium glutamate; Plant-based seasoning; Umami taste

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## 1. Introduction

Improving food quality is crucial to meet the demands of global consumers impacted by socioeconomic development, diseases, and changing lifestyles. Ready prepacked food is a convenient option for modern lifestyle; however, the use of artificial additives, like monosodium glutamate, have become a villain to consumers' perception [1-2]. Therefore, future trends in enhancing the taste of food are anticipated to be lively, driven by a movement towards reducing levels of salt, sugar, and fat in food products. Consumers favor natural products, emphasizing the need for cost-effective ingredient management and a shift towards environmentally-friendly processes [3].

The umami taste is mainly attributed to the presence of L- glutamic acid (L -Glu) and its salt, monosodium glutamate (MSG). However, the excessive consumption may cause Chinese restaurant symptoms or an MSG allergy, which subsequently contributes the degeneration of the brain and nervous system. Consequently, it seems MSG become unhealthy ingredient from consumers' perspective and they rather search for natural and herbal alternatives. Subsequently, food manufacturers engage in the formulation of several natural seasoning products devoid of monosodium glutamate (MSG). The reduction of sodium in food is a highly discussed subject currently, given that excessive sodium

consumption increases the risk of chronic non-communicable diseases such as hypertension and cardiovascular problems [4].

Monosodium glutamate (MSG) is one of several forms of glutamic acid found in foods, in large part because glutamic acid (an amino acid) is pervasive in nature. MSG is used in the food industry as a flavor enhancer with an umami taste that intensifies the meaty, savory flavor of food, as naturally occurring glutamate does in foods such as stews and meat soups. Though the umami featured monosodium γ-glutamate (MSG) and 5′-ribonucleotides are naturally present in many foodstuffs [5], their largely industry-scale production of these compounds as food additives has resulted in a negative consumer image. In 2017, the European Food Safety Authority set safe levels for glutamate food additives in response to consumer concerns [6]. Umami ingredients are very important for food seasoning and are widely used in food production. They also show many health benefits. Umami compounds can play an important role in the process of reducing sodium in foods, since they enhance the perception of salty taste [7-8], avoiding loss in sensory quality and consequent re- jection of food products. MSG is the most widely used umami compound to enhance food flavor. However, consumers consider food without added MSG to be safer and healthier. [9]

Glutamic acid can be isolated from numerous vegetable sources, such as wheat gluten, soybean meal, casein and residue from “Steffen waste”. Another widely used technique for the production of glutamic acid is microbial fermentation [10].Vegetables contribute a variety of flavors to food, including umami, sour, sweet, bitter, and spicy. And have significant nutritional and health benefits and are rich in bioactive compounds [11]. Amino acids are abundant in vegetables, and their concentrations are important for nutrition. Determination of amino acid content in vegetable several techniques based on liquid chromatography coupled with mass spectrometry (LC-MS) offer versatile tools for addressing the identification and quantification of a wide number of compounds in plant samples. Reversed-phase high-performance liquid chromatography/ultra- performance liquid chromatography (HPLC/UPLC) [12-13] is the commonly used separation techniques in the study of natural plant active substances.

*Phong nua*, or seasoned vegetable powder, is a traditional condiment originated by Isan local wisdom. It provides tasty flavor by blending 16 different kinds of vegetables and herbs with their own sour, sweet, spicy, bitter, and aromatic properties. Community Enterprise Group of Safe Agricultural Herbs in the Sila Sub-district of Khon Kaen Province developed *Phong nua* formula based on this concept. However, too many varieties of vegetables in the recent recipe might be not appropriate for quality controlling, and future scale-up production. Additionally, some herbs have not registered yet. The current solar drying is time-consuming up to 72 h with unpredictable weather. Therefore, the purpose of this study is to propose cost-availably hot-drying processes and investigate the key components involving seasoned vegetable powder’s taste and flavor attributes in order to develop the new formula containing few vegetable types for practical production. Furthermore, the trial in ethnic *nam-pla-ra* (fermented fish sauce) were evaluated the performance as alternative to MSG.

## 2. Materials and Methods

### 2.1 Chemicals and reagents

L-aspartic acid 5960 (≥98%), L- glutamic acid 33032 (≥99%), L-asparagine 6267 (≥98%), L-histidine 6274 (≥98%), L-serine 5951 (≥99%), L-glutamine 5961 (≥99%), L-cysteine 5862 (≥99%), L- arginine 6322 (≥99%), glycine 750 (≥99%), L-threonine 6288 (≥98%), L-alanine 5950 (≥ 99%), L-tyrosine 6057 (≥98%), L-tryptophan 6305 (≥98%), L-valine 6287(≥98%), L-methionine 6137 (≥98%), L-phenylalanine 6140 (≥98%),

L-isoleucine 6306 (≥98%), L-leucine 6106 (≥98%), and L-lysine 5962 (≥98%) were obtained from Sigma Aldrich (St. Louis, MO, USA). Hydrochloric acid, 9-fluorenylmethyl chloroformate (FMOC reagent), o-phthalaldehyde and 3-mercaptopropionic acid (OPA), Borate Buffer were purchased from Agilent technology. HPLC grade methanol and acetonitrile were purchased from Duksan (Korea).

**2.2 Fresh vegetable sample preparation**

The vegetable used in this study were obtained from local market in Nai Muang District, Khon Kaen Province area. Seasoning vegetable powders are made from 11 different varieties of vegetables, including: wild sweet vegetables, mulberry leaves, young moringa leaves, chinese chives, chamuang leaves, coriander leaves, spinach, pandan leaves, shallots, garlic and pumpkin. All vegetables should be washed and selected them that have the complete leaves. Afterwards 1 minute of streaming, the sample were dropped into cold water, drained, and mixed thoroughly. All vegetables samples weighed 1 kg each. Except for wild sweet vegetables was weighed 2 kg. A sample of vegetables should be combined with 200 g. of salt and sugar each in stainless steel mixing bowl. After the vegetables have been dried, all veggies were mashed for 1 min or until finely milled in a high-powered blender, then filtered through a 100-sieve size. The samples were sealed in aluminum foil pouches and stored at -18°C until needed.

**2.3 Effect of drying temperatures on drying rate of hot- air dried vegetable powder**

Samples of seasoned vegetable powder were dried in a hot air dryer using temperatures of 50, 60 and 70°C by weighing every 5 min for 1 h, every 15 min for 1 h and 30 min for 1 h, and 60 min throughout the drying period until the weight is stable. For each temperature, repeat three times. After that, the drying rate was calculated. Create a drying curve for seasoned vegetable powder at each temperature using the result. After the drying process is complete, were using a fine grinder to blend the seasoned vegetable powder and filtered using a sieve with a mesh size of 100. The seasoned vegetable powder samples were collected in aluminum foil bags (*Aluminum laminate polyethylene*) and stored at -18°C for further analysis.

**2.4 Physicochemical characteristics and sensorial acceptance of vegetable powder**

*2.4.1 Color, solubility, dispersibility*

Color was determined using a colorimeter (Hunter lab Ultra scan XE reflected color spectrophotometer) by reading the *L**(lightness), *a*(*redness), *b**(yellowness) parameters, with xenon lamp. The solubility was determined as described by Cano-Chauca Stringheta, Rumos, and Cal-Vidal [(14)]. In blender (Philip, R12160, 550 W, Barueri, Brazil), weighing 1.5 g (Dry weight-DW) of the sample were dissolved in 150 mL of distilled water and stirred for 4 mins by magnetic stir until the solution was dissolved. Afterwards the solution was centrifuged at 3000 rpm for 5 min. Aliquots of 25 mL of the supernatant transferred to previously weighed Petri dishes and dried at 105°C in hot-air oven for 5 h or until constant weight. Solubility (%) was calculated as the weight of dry solids in the supernatant represented as a percentage of the initial sample weight. The dispersibility was measured by weighing 0.5 g of the sample were dissolved in 25 mL of water and centrifuged at 2000 rpm for 5 min. The absorbance meter (ABS) at 520 nm was used to measure the dispersibility of sample. These analyses were performed on three sample of each treatment, with three readings for each sample.

*2.4.2 Moisture content, $a_w$, pH*

The moisture content was determined by drying inside an oven at 105 ºC till constant weight (15 %MC) accomplished. Water activity ($a_w$) was determined in triplicate for vegetable powder samples using an Aqualab apparatus (Aqua Lab). The pH value was measured using a pH meter (Eutech-Mettler, Model pH 510) with a glass penetration

electrode. These analyses were performed on three sample of each treatment, with three readings for each sample.

*2.4.3 Sensorial acceptance*

Sensory testing of seasoned vegetable powders dried at 50, 60 and 70ºC was carried out by dissolving 10 g of each sample in 1,000 mL of pork stock and thoroughly mixing it. Then, to prepare the test subject, pour it into a 20 mL plastic cup. Each time the panelist tastes a sample, they must gargle with plain water before tasting the next one. Using panelists, a 9-point Hedonic scale was employed in this test, with 1 being the most disliked and 9 being the most liked. In terms of appearance, color, aroma, overall taste, and overall liking, 50 untrained panelists scored their preferences.

**2.5 Determination of amino acid profile**

*2.5.1 High Performance liquid chromatography (HPLC) analysis*

Each vegetable powder sample at 50, 60 and 70ºC (10 g) was measured and mixed with 35 mL of 50% methanol. Then it was centrifugated at 3,000 rpm for 5 min (Hermle LaborTechnik GmbH - Z 200 A Universal Compact Centrifuge). After that, prior to HPLC injection, the supernatant was filtered from each sample with a nylon syringe filter (pore diameter of 0.22 µm) into a HPLC vial for analysis (United States Department of Agriculture, 2011 with some modifications). Samples were store at 4°C until analysis. Mobile phase A was prepared 1,000 mL, weighing out 1.4 g anhydrous $Na_2HPO_4$ and 3.8 g $Na_2B_4O_7$ .10 $H_2O$ in 1,000 mL of distilled water. Adjust to approximately pH 8.4 with 1.2 mL concentrated HCl and then modify until the pH reached 8.2. Prior to adjusting pH, allow the borate crystals to dissolve completely while stirring. Filter through regenerate cellulose membrane using 0.22 µm. Acetonitrile, Methanol (Duksan,Korea) and distilled water were combined in a 500 ml that ratio 45:45:10 for preparing mobile phase B. Apply for prepared 100 mL of mobile phase A and 0.4 mL concentrated $H_3PO_4$ provide the injection dilution. This solution was prepared in a 100 mL bottle that should be stored at 4°C. A 18 min separation of the amino acids was achieved by the following gradient program, 2% Mobile phase B for 0- 0.35 min, gradually increase from 2% to 57% B, 0.35- 13.4 min, gradient increase to 100% of elute B, 13.4 - 13.5 min then linearly increase up to 100% B kept 100 % B, 13.5-15.7 min, then return to initial condition 2% elute B, 15.8-18 min and remain %B until end. The flow rate was 1.5 mL/min and the injection volume was 1 ml. For the derivatization of amino acids, an OPA-Borate buffer solution + FMOC (OPA + Borate buffer + FMOC, filterd in 0.22 µm) was added to HPLC vials containing vegetable powder samples or amino acid standards solution.Amino acids were separated using a HPLC system was carried out out by reverse phase liquid chromatography (Pump model 600, injector model 486, and autosampler model 717, Waters, Milford, MA). Detection was carried out by a 2475 fluorescence detector. The detector was determined the transition point needed with fluorescence detector (FLD), it is necessary to perform two separate runs. The first using excitation 340 nm, Emission 450 nm to detect the OPA derivatized amino acids and the second using excitation 260 nm, Emission 325 nm to detect the FMOC derivatized amino acids. The column HPLC was Column Agillent Poroshell 120 EC-C18, 3.0 × 100 mm, 2.7µm and the column temperature was 40°C. Total running time was 18 min for each sample.

*2.5.2 Validation*

To obtain accurate validation results, Stock solutions of 20 amino acid standards were prepared with 0.1 N HCl and sonicate until dissolved. Finally fill to mark with distilled water for final concentration of 2.5 µg/mL. The final concentrations were than serially diluted 0.08, 0.16, 0.32, 0.64, 1.28 µg/mL for the calibration curve. The linearity of the calibration curves of different amino acids were evaluated by injecting 5 µL of the serially diluted standard solutions. The calibration graph was obtained by plotting the HPLC peak area against their concentrations. The limit of detection (LOD) and limit of quantifications (LOQ) were

determined with signal to noise ratios (S/N) of 3 and 10, respectively. Analyzed in three replicates of the same standard solution. After that, each solution was filtrated by a syringe filter 0.22 µm to obtain clearer solutions before HPLC injection and which were kept in vials at 4°C.

**2.6 Sensorial acceptance of fermented fish sauce**

The preference of vegetable powder in fermented fish sauce was investigated. To test the preference and acceptance of fermented fish sauce with vegetable powder instead of MSG, the temperature that produces the best physicochemical research results and acceptance will be selected. Consumer preferences and acceptance of fermented fish sauce with MSG were compared. Sensory testing of seasoned vegetable powders in fermented fish sauce was carried out by dissolving 10 g of vegetable powder in 1,000 ml of fermented fish sauce and thoroughly mixing it. Then, to prepare the test subject, pour it into a 20 mL plastic cup. Each time the panelists test a sample, they must rinse with plain water before tasting the next one. Using panelists, a 9-point Hedonic scale was employed in this test, with score range 1 (extremely dislike) to 9 (extremely like). In terms of appearance, color, aroma, overall taste, and overall preference, 50 untrained panelists scored their preferences.

**2.7 Statistical Analysis**

The results are expressed in mean ± standard error and analyzed by the statistical program of IBM SPSS version 25 (SPSS Inc., State) were package and processed by one-way analysis of variance (ANOVA) to evaluate significant difference ($P \leq 0.05$) and was used to evaluate the temperature on vegetable powder, Physiochemical characteristics and sensorial acceptance. The mean values were separated using Duncan's multiple-range test was conducted to evaluate the effect of temperature on amino acid separation.

## 3. Results & Discussion

**3.1 Effect of drying temperatures on drying rate of hot- air dried vegetable powder**

In this experiment, 11 various types of freshly ground vegetables were combined with sugar and salt. These vegetables included wild sweet greens, mulberry leaves, young moringa leaves, chives, chamuang leaves, coriander, spinach, pandanus leaves, shallots, garlic, and pumpkin. Drying condition by hot air oven of steamed-treated vegetable mixture was investigated for replacing the conventional method (sun drying). The drying time was obtained for 10, 9, and 7 h at drying temperature of 50, 60, and 70°C, respectively, to achieve moisture content at equilibrium <15% db (according to dried food standard). It was shorter time-consuming than sun drying (72 h or 3 days). The results showed drying pattern of powdered mixed vegetable only a short period of falling rate (or decreasing drying rate). The water content in the samples decreased with longer drying times. There was a pattern of decreasing curves with longer drying times at all temperatures. However, there was not a significant variance in the drying rate at either temperature until the moisture balance started to stabilize. Vegetable weights tended to decrease until they stabilized after a period of time. This occurs as a result of the vegetables' water evaporating and producing steam. The sample's moisture content consistently decreases as the drying temperature increases. This can be attributed to a significant increase in the rate of evaporation [15]. The sample's moisture content consistently decreases as the drying temperature increases. It is obvious that the drying time required to accomplish moisture balance and maintain to preservation food standards tends to decrease with increasing drying temperature.

### 3.2. Physicochemical characterisitics and sensorial acceptance of vegetable powder

*3.2.1 Color, solubility, Dispersibility*

The seasoning powder prepared using solar energy was found to have lower L* and a* color values at all three temperatures than the seasoning vegetable powder product heated with hot air drier. The products with the lowest *L** and *a** values are characterized by a darker and less green tint. This is due to the reason that the reference sample did not dry the vegetables after steaming them to eliminate the enzymes. Enzymatic reactions that could result in color changes are inhibited by blanching. raw materials' flavor and aroma during processing and storage [16]. The effect of intermolecular adhesion is reduced by blanching. resulting in the formation of a thin layer on the raw material's surface. Water vapor evaporation is slowed down as a result. When comparing the hot air oven's and heating temperature's results. It is apparent that while *b** did not significantly change, the values of L* and a* decreased ($P \leq 0.05$) as the temperature increased. This is caused by the reason that high temperatures result product drying to brown more quickly [17]. Moisture absorption during drying at low temperatures is minimal. and reduce the process that amide components and sugar interact with drug usage. Less than high temperature drying, browning results from non-enzymatic reactions [18]. Due to an increased amount of chlorophyll, In the Table 1. samples at 50 and 60ºC had higher *a** values than samples at 70°C. With increasing temperatures, dried vegetable products lost more chlorophyll. The seasoned vegetable powder product that was dried using solar energy was found to have a higher *b** value 29.05 than the seasoned vegetable powder product. that had a *b** value between 23.21-24.39 which had been heated at all temperatures in a hot air oven, indicating that the sample dried in a hot air oven was less yellow-colored than the reference sample. The seasoned vegetable powder products that are solar-dried will react with a browner color as a result. Including products made from seasoned vegetable powder that are dried at a higher temperature in a hot air dryer. Temperature causes the color's intensity to increase. In the food company, drying is a common procedure, and hot air dryers are still widely used to dry products. This is due to the reason that it can decrease storage costs and increase product shelf life. The chemical composition and nutritional content of the finished dried vegetables are also significantly influenced by the drying process. The Maillard reaction and vitamin deterioration are two chemical changes brought due to drying. taste loss, discoloration, and oxidation of fat. To preserve the nutritional value of vegetable products with the least amount of loss, these chemical reactions must be avoided or minimized. Ingredients made from fresh vegetables are frequently treated to maintain their quality. In recent years, research has become increasingly concentrated on the problems surrounding the preparation of vegetable raw materials prior to drying in order avoid and reduce the deterioration of vegetable quality during the drying process. These vegetables were all made of chemically derived plant materials. Phytochemicals include minerals, vitamins, pigments, antioxidants, and other biological materials. Nevertheless, drying usually results in the degradation of these phytochemicals. due to its sensitivity to changes in temperature, light, and oxygen [19], particularly color. This has an immediate effect on how pigments including betalains, phenols, carotenoids, and chlorophyll are retained. Thus, prior to drying in this study, vegetable samples were prepared using blanching pretreatment method. High-temperature water or steam is typically used infrequently and only when certain conditions are satisfied. The sample's color, flavor, texture, and nutritional value may all be affected by blanching. and the general dried vegetable consumer approval score increased [20].

**Table 1.** The color values (*L**, *a**, *b**) of seasoned vegetable powders dried at 50, 60, and 70ºC (HA50, HA60, and HA70, respectively) in a hot air oven with reference sample (Solar dried sample)

| ***Phong nua* powder samples** | **color** | | | **% Solubility** | **Dispersibility** |
|---|---|---|---|---|---|
| | ***L**** | ***a**** | ***b**** | | |
| Reference Sample | $52.49^{d}$±0.005 | $-1.28^{d}$±0.005 | $29.05^{a}$±0.005 | $64.55^{a}$±0.26 | $0.861^{a}$±0.001 |
| HA50 | $59.38^{b}$±0.004 | $-5.32^{a}$±0.007 | $23.21^{c}$±0.004 | $50.84^{b}$±2.64 | $0.183^{d}$±0.001 |
| HA60 | $59.48^{a}$±0.004 | $-4.10^{b}$±0.011 | $23.98^{b}$±0.009 | $63.24^{a}$±1.55 | $0.325^{c}$±0.001 |
| HA70 | $56.95^{c}$±0.005 | $-1.43^{c}$±0.008 | $23.21^{c}$±0.005 | $63.17^{a}$±1.98 | $0.444^{b}$±0.001 |

*Means with different letters in the same columns were significantly different (P≤0.05).
**HA 50 = Hot air drying at 50°C, HA 60 = Hot air drying at 60°C, HA 70 = Hot air drying at 70°C

For the solubility value, (Table 1) it was discovered that the reference sample had results that were same at both 60 and 70°C. and higher than the sample that was dried at 50°C. Regarding the seasoned vegetable powder's dispersion ability, it was discovered that the reference formula's seasoning vegetable powder had the highest value (0.861), and that seasoning vegetable powder at 70, 60, and 50°C had the following dispersibility values (P<0.05): 0.444, 0.325, and 0.183, respectively. Because the reference sample's dispersibility was higher than the sample that was dried using a hot air dryer in that it took the reference sample 72 h to dry. The product would distribute more effectively at higher temperatures.

*3.2.2 $a_w$, moisture content, pH*

At 50, 60 and 70°C, the percentage of moisture was 5.82, 5.81 and 5.66% db, respectively, however it differed significantly from the reference sample. The reference sample's moisture content was determined to be as high as 20.14%, exceeding the requirements for product standards, according to that the moisture percentage of a product must be less than 18%. Nonetheless, samples dried in all three conditions had moisture contents that reached the required ranges. However, when the drying curve's moisture content is determined, the result is a little less than the result obtained from the drying curve in Section 3.1. Nevertheless, the results of this topic's moisture calculation will determine the amount that water weighs in comparison to the sample of vegetable powder's dry weight following hot air drying. Products based on powder that have a moisture level of less than 3% frequently have higher flowing properties. Better microbiological safety is indicated by powder products with a moisture content of less than 10% [(21)]. Considering the water activity level Essential characteristics of dry food items. The water activity value of dried vegetables considerably decreased in the range of 0.22-0.27 (P≤0.05) when the drying temperature was increased. This was due to the food's free water quickly evaporating. Due to their low free water activity, dried vegetables are less likely to encourage the growth of microbes with longer storage time (P<0.05). All the seasoning vegetable powder samples exhibited acidic pH values, although they varied when compared to the reference samples. Considerably, the sample dried at 70°C had the highest value with a pH of 4.72. Samples dried at 50 and 60°C had pH values of 4.68 and 4.59, respectively. The reference sample had the lowest value of pH 4.27. It is apparent that the value was impacted by the drying process.

**Table 2.** % Moisture content, $a_w$ and pH of seasoned vegetable powders dried at 50, 60 and 70°C (HA50, HA60, and HA70, respectively) in a hot air oven with reference sample (Solar dried sample).

| ***Phong nua* powder samples** | **% Moisture Content** | **$a_w$** | **pH** |
|---|---|---|---|
| Reference Sample | $20.14^{b} \pm 1.22$ | $0.27^{a} \pm 0.022$ | $4.27^{c} \pm 0.38$ |
| HA50 | $5.82^{a} \pm 0.11$ | $0.24^{b} \pm 0.001$ | $4.68^{a} \pm 0.03$ |
| HA60 | $5.81^{a} \pm 0.17$ | $0.24^{bc} \pm 0.002$ | $4.59^{b} \pm 0.01$ |
| HA70 | $5.66^{a} \pm 0.14$ | $0.22^{c} \pm 0.001$ | $4.72^{a} \pm 0.01$ |

*Means with different letters in the same columns were significantly different (P≤0.05).
**HA 50 = Hot air drying at 50°C, HA 60 = Hot air drying at 60°C, HA 70 = Hot air drying at 70°C

*3.2.3 Sensorial acceptance*

The responsibility of assessing the preferences of their products' appearance was assigned to fifty panelists. The results showed that the seasoning vegetable powder products that were dried using solar energy and hot air dryers at 50, 60, and 70°C scored in the neutral to slightly like range. Their sensory rating scores, which were 5.28, 6.02, 6.36 and 6.30 scores, respectively, were statistically significant (P≤0.05).

**Table 3.** The sensory evaluation of seasoned vegetable powder at different temperature (HA50, HA60, and HA70, respectively) and the reference sample (Solar dried sample).

| ***Phong nua* powder samples** | **Appearance** | **Aroma** | **Color** | **Overall Taste** | **Overall liking** |
|---|---|---|---|---|---|
| Reference product | $5.28^{b} \pm 1.21$ | $5.38^{b} \pm 0.85$ | $5.32^{c} \pm 1.04$ | $5.62^{c} \pm 0.70$ | $5.58^{c} \pm 0.81$ |
| HA50 | $6.02^{a} \pm 1.23$ | $6.30^{a} \pm 0.95$ | $6.26^{b} \pm 1.16$ | $6.66^{b} \pm 0.80$ | $6.58^{b} \pm 0.95$ |
| HA60 | $6.36^{a} \pm 1.06$ | $6.58^{a} \pm 0.84$ | $6.82^{a} \pm 0.98$ | $7.02^{a} \pm 0.91$ | $7.06^{a} \pm 1.00$ |
| HA70 | $6.30^{a} \pm 0.95$ | $6.40^{a} \pm 0.70$ | $6.62^{ab} \pm 0.92$ | $6.76^{ab} \pm 0.74$ | $6.78^{b} \pm 0.79$ |

*Means of hedonic scores with different letters in the same columns were significantly different (P≤0.05).
**HA 50 = Hot air drying at 50°C, HA 60 = Hot air drying at 60°C, HA 70 = Hot air drying at 70°C

Panelists preferences were scored at a slight like level about the aroma of the seasoned vegetable powder products that were dried using solar energy and dried in a hot air drier at temperatures of 50, 60, and 70°C. and, with scores of 5.38, 6.30, 6.58, and 6.40 points, respectively, were significantly different (P<0.05). Panelists preferences ranged from neutral to slightly satisfactory when it related to the color of the seasoned vegetable powder products that were dried using solar energy and a hot air drier set at temperatures of 50, 60, and 70°C. and had scores of 5.32, 6.26, 6.82, and 6.62 points, respectively, for the overall taste of seasoning vegetable powder products dried using solar energy and dried using a dryer. These differences were statistically significant (P<0.05). Hot air dried with 50, 60, and 70°C temperatures. The level of panelists preferences is moderate. The scores for vegetable powder products that were dried using a hot air dryer were 6.66, 7.02, and 6.76, in that order. The scores for solar-dried vegetable powder flavoring. The overall taste liking score was significantly different (P≤0.05) and at the neutral level of 5.62 points. Panelists' overall liking ranged into the moderate range for the seasoned vegetable powder products that were dried using solar energy and heated air dryers at 50, 60, and 70 degrees Celsius. The scores for vegetable powder products that were dried using a hot air dryer were 6.58, 7.06, and 6.78, in that order. The overall liking score was significantly different (P≤0.05) and at the neutral level of 5.58 points.

### 3.3 Amino acid profile

Products with vegetable powder seasoning are used for combining regional herbs and vegetables with flavors variety from sweet and sour to bitter, oily and spicy to aromatic and spicy to provide food a pleasant taste. Thus, analysis occurs out to determine the product's flavor. The amino acid types and contents in seasoning vegetable powder dried using a hot air drier (HA50, HA60, and HA70) and those in dried with solar drying (reference sample) was shown in Table 4. Each of these 20 amino acids has a different flavor. All three oven-dried vegetable powder samples had the highest amount of overall sweet amino acids (Alanine, Glycine, Threonine, Asparagine, Glutamine, Serine, and Proline), but individually the highest content was arginine (100.47 µg/g), as bitter taste, followed by aspartic acid (74.50 µg/g) (umami), and glutamine (61.89 µg/g) (sweet). The findings showed that the quantity of amino acids also decreased as temperatures.

**Table 4.** The total amount of all 20 amino acids in seasoning vegetable powder dried at 50, 60 and 70°C in a hot air oven was compared to reference samples (Solar dried sample).

| **Amino Acid (µg/g)** | ***Phong nua* powder samples** | | | |
|---|---|---|---|---|
| | **Reference (Solar dried sample)** | **Example of seasoned vegetable powder in the hot air oven** | | |
| | | **50°C** | **60°C** | **70°C** |
| Umami Flavor | | | | |
| Aspartic acid | 0.00 | 27.84 | 74.50 | 21.30 |
| Glutamic acid | 0.00 | 7.40 | 7.94 | 6.82 |
| **Total amino acid umami flavor** | **0.00** | **35.24** | **82.44** | **28.12** |
| Sweet Flavor | | | | |
| Alanine | 5.92 | 182.91 | 67.93 | 3.33 |
| Glycine | 2.68 | 11.75 | 3.60 | 3.33 |
| Serine | 8.21 | 50.30 | 17.80 | 17.42 |
| Asparagine | 0.00 | 57.19 | 9.38 | 16.58 |
| Glutamine | 61.89 | 25.75 | 8.61 | 11.17 |
| Threonine | 9.96 | 22.75 | 9.85 | 10.43 |
| Proline | 1.41 | 1.01 | 0.89 | 0.86 |
| **Total amino acid sweet flavor** | **90.07** | **351.66** | **118.06** | **63.12** |
| Bitter Flavor | | | | |
| Histidine | 16.75 | 21.03 | 34.95 | 32.33 |
| Arginine | 0.00 | 272.33 | 14.00 | 11.31 |
| Tyrosine | 3.28 | 7.57 | 6.99 | 8.72 |
| Tryptophan | 2.06 | 2.48 | 1.27 | 2.74 |
| Cysteine | 2.65 | 1.43 | 1.33 | 0.69 |
| Lysine | 0.87 | 48.40 | 20.69 | 10.24 |
| Phenylalanine | 2.20 | 1.03 | 0.83 | 0.58 |
| Methionine | 0.36 | 4.03 | 0.04 | 1.24 |
| Valine | 0.57 | 10.05 | 6.66 | 4.05 |
| Isoleucine | 6.74 | 8.61 | 0.60 | 2.81 |
| Leucine | 6.07 | 6.03 | 2.08 | 0.30 |
| **Total amino acid bitter flavor** | **41.55** | **382.99** | **89.44** | **75.01** |

Aspartic acid and glutamic acid are included in the group of umami flavors. Alanine, glycine, proline, serine, threonine, asparagine, and glutamine are varieties of sweet tastes. These consist of tryptophan, valine, tyrosine, cysteine, isoleucine, leucine, lysine, methionine, phenylalanine, and histidine [22]. According to the study, seasoned vegetable powder products that were dried with a hot air dryer showed the highest amounts of amino acids in the sweet flavor group. Amino acids, which impart a bitter and umami flavor, follow next. Research has indicated that the quantity of each variety of amino acid extracted from products that contain seasoning vegetable powder and are dried in a hot air dryer at 50, 60, and 70°C was different from the amounts of amino acids found in products that are sun-dried. Products for flavoring vegetable powder are dried with solar energy, as Table 3 shows. Alanine, which relates to within the sweet taste group, and arginine, which relates to the bitter flavor group, are the most usually found amino acid contents. The amounts of amino acids in seasoned vegetable powder products that were dried with a hot air dryer is shown in Table 3. The quantity of amino acids decreases as the drying temperature increases. According to a Hidatada report, temperature variations that impact different pK values cause changes in amino acid composition [23]. Following the study, the amounts of amino acids provided the different flavors It was discovered that the seasoned vegetable powder products dried with solar energy had a lower amino acid content than the products that were dried with hot air dryers.

**3.4 Sensorial acceptance of fermented fish sauce**

*3.4.1 Sensorial acceptance of commercial fermented fish sauce and the formula containing seasoned vegetable powder.*

The researcher selected vegetable powder that was dried in a hot air dryer set to 60°C based on the results of the previous study (part 3.2 and 3.3 result). When considering the results to continue developing new products. Panelists scored the vegetable seasoning powder that had been dried in a hot air dryer at 60°C the highest score based on the results of the preference test. To test the preference for the seasoned fermented fish sauce product, the researcher used seasoned vegetable powder that had previously been dried in a hot air dryer at 60°C. To compare with samples obtained from producers that employ commercial *pla-ra* formulations. Samples of fermented fish sauce and somtam samples containing the commercial formula seasoned fermented fish sauce product and the formula containing seasoned vegetable powder were sensory applying the 9 Hedonic scale test on a total of 50 panelists. The samples were dried in a hot air dryer at 60°C

**Table 5.** The sensory evaluation of fermented fish sauce with seasoned vegetable powders dried at 60 degrees Celsius in a hot air oven compare with commercial fermented fish sauce.

| attributes | Commercial fermented fish sauce | Fermented fish sauce with seasoned vegetable powder | t | sig |
|---|---|---|---|---|
| Appearance | 5.54 ± 1.61 | 5.78 ± 1.47 | 0.778 | 0.438 |
| Aroma | 4.46 ± 2.07 | 4.82 ± 2.18 | 0.846 | 0.400 |
| Color | 5.28 ± 1.36 | 5.38 ± 1.50 | 0.350 | 0.727 |
| Salty | 4.90 ± 1.22 | 5.16 ± 1.35 | 1.014 | 0.313 |
| Sweet | 4.28 ± 1.31 | 4.50 ± 1.34 | 0.829 | 0.409 |
| Umami | 5.10 ± 1.23 | 5.60 ± 1.36 | 1.930 | 0.057 |
| Bitter | 2.80 ± 1.46 | 3.16 ± 1.31 | 1.297 | 0.198 |
| Overall Taste | 5.12 ± 1.32 | 5.78 ± 1.49 | 2.346 | 0.021* |
| Overall liking | 5.14 ± 1.29 | 5.82 ± 1.52 | 2.408 | 0.018* |

50 panelists were used to gather consumer preferences on appearance, aroma, color, salty, sweet, umami and bitter each tester was conditioned to view the product in regular lighting (natural light). the vegetable powder samples received by oven drying at 60°C for 9 h, gave the highest overall liking scores (7.06) and overall taste scores (7.02) when the panelists tested in soup medium (P≤0.05). Correspondingly, among amino acids in their profile revealed that the highest content of aspartic acid content (one of umami taste) compared to other samples.

*3.4.2 Sensorial acceptance of commercial fermented fish sauce and the formula containing seasoned vegetable powder in somtum.*

When applying derive vegetable powder to *nam-pla-ra* products, it could be substituted for 0.5% monosodium glutamate in its original recipe. Also, *nam-pla-ra* products with seasoned vegetable powder instead of MSG provided the higher liking score as regular recipe (added MSG) (P≤0.05), with the same trend in *somtum* menu (Table 6).

**Table 6.** The sensory evaluation of *somtum* with fermented fish sauce with seasoned vegetable powders dried at 60°C in a hot air oven compare with commercial fermented fish sauce.

| Attributes | ***somtum* with commercial fermented fish sauce** | ***somtum* with seasoned vegetable powder fermented fish sauce** | t | sig |
|---|---|---|---|---|
| Appearance | 6.64 ± 1.05 | 6.72 ± 1.03 | 0.385 | 0.701 |
| Aroma | 6.16 ± 0.93 | 6.56 ± 0.93 | 2.147 | 0.034* |
| Color | 6.18 ± 0.87 | 6.22 ± 0.91 | 0.224 | 0.823 |
| Overall Taste | 6.36 ± 0.88 | 6.76 ± 0.85 | 2.323 | 0.022* |
| Overall liking | 6.36 ± 0.85 | 6.84 ± 0.87 | 2.688 | 0.008* |

## 4. Conclusion

*Phong nua*, or seasoned vegetable powder, is a traditional condiment with its origins in Isan local knowledge. It achieves a delicious taste by blending 16 various vegetables and herbs, each possessing its own sour, sweet, spicy, bitter, and aromatic characteristics. The current solar drying is time-consuming up to 72 h (3 days) with unpredictable weather. This research was able to determine optimal hot air drying (10, 9, and 7 h at drying temperature of 50, 60, and 70 °C, respectively). The vegetable powder samples received by oven drying at 60°C for 9 h, gave the highest overall acceptance score (7.06) and flavor liking (7.02) when sensorially tested in soup medium (P≤0.05). Correspondingly, among amino acids in their profile revealed that the highest content of aspartic acid content (one of umami taste) compared to other samples. When applying derive vegetable powder to *nam-pla-ra* products, it could be substituted for 0.5% monosodium glutamate in its original recipe. Also, *nam-pla-ra* products with seasoned vegetable powder instead of MSG generated the higher liking score as regular recipe (added MSG) (P≤0.05), with the same trend in *som-tum* menu. In summary, the proposed drying method delivered high-throughput drying management, potential quality control, and cost-efficiency for manufacturing in small and medium scale such as Community Enterprise, SMEs.

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# Exopolysaccharide Production from *Halomonas* sp. SS3: Cultural Optimization and *In Vitro* Antioxidant Activity

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**Abstract**

Microbial exopolysaccharides (EPS) have gained increasing attention due to the growing demand for natural polymers. Halophilic bacteria, particularly those from solar saltern environments, are a promising source of novel EPS with unique functional properties. This study investigated the production, optimization, and antioxidant activity of EPS from halophilic bacteria isolated from solar saltern soil in Phetchaburi province, Thailand. Out of the eight EPS-producing halophilic isolates obtained, *Halomonas* sp. SS3 demonstrated the highest EPS yield (0.51 $g \cdot L^{-1}$). The optimization of EPS production was performed using the one-factor-at-a-time (OFAT) approach in a malt-yeast extract (MY) medium cultivation system. Results indicated that sucrose at a concentration of 100 $g \cdot L^{-1}$ served as a suitable carbon source for EPS production. Moreover, the most effective cultural condition was under 15% (w/v) NaCl, medium pH of 8.0, an incubation temperature of 30°C, an agitation speed of 200 rpm, an inoculum size of 20% (v/v), an incubation time of 7 days, and an extraction ratio of 1:2 (supernatant:EtOH). Under this optimized condition, the maximum EPS yield was 1.28 $g \cdot L^{-1}$, representing a remarkable 151% or 2.5-fold increase (from 0.51 $g \cdot L^{-1}$ to 1.28 $g \cdot L^{-1}$) compared to the initial EPS yield from a screening step mentioned above. Furthermore, the EPS from *Halomonas* sp. SS3 exhibited noteworthy antioxidant activity. The EPS demonstrated antioxidant activity, with DPPH and ABTS scavenging activities of 79.50% and 43.94%, respectively, at a concentration of 5 $mg \cdot mL^{-1}$. The FRAP assay indicated a $Fe^{2+}$ equivalent of 41.40 µM $FeSO_4$ at the same concentration. Total antioxidant activity revealed that 5 $mg \cdot mL^{-1}$ EPS was equivalent to 432.25 µM ascorbic acid. The EPS produced by *Halomonas* sp. SS3 exhibited notable antioxidant activity, suggesting its potential as a promising antioxidant agent in foods, nutraceutical, and pharmaceutical applications. Further exploration of the EPS's structure and detailed analysis of its antioxidant mechanisms will be investigated.

***Keywords***: Exopolysaccharide; Halophilic bacteria; Fermentation optimization; Antioxidant activity; *Halomonas* sp.

## 1. Introduction

The utilization of natural polymers has significantly increased across various industrial sectors, particularly polysaccharides in food and pharmaceuticals. Microorganisms offer exceptional sources for polysaccharide production under controlled cultivation [(1)]. Notably, certain microbes possess the ability to synthesize polysaccharides that are subsequently secreted to coat their cells, forming structures known as exopolysaccharides (EPS) [(2)]. EPS can be found in variety groups of microbes such as bacteria, archaea, filamentous fungi, and microalgae [(3)]. Microbes produce EPS for various purposes including cell adhesion, protection against environmental stress, and biofilm formation [(4)]. Microbial EPS have increased significant attention due to their diverse biological properties, exhibiting antioxidant, anti-inflammatory, immunomodulatory, and antitumor activities. Interestingly, the specific types of EPS produced and their associated bioactivities vary among different microbial species [(3)].

Over the past few decades, research interest in extremophilic microorganisms and their unique metabolites has intensified. Extremophiles, encompassing acidophiles, halophiles, alkaliphiles, psychrophiles, and thermophiles, thrive in extreme environments, producing specialized enzymes (extremozymes) and protective biomolecules (extremolytes) for survival [(5)]. As part of their adaptation and survival strategies, extremophiles synthesize various substances, notably EPS [(6)]. The study of extremophilic EPS has seen an increasing trend, especially EPS produced by halophilic bacteria and marine bacteria. Halophilic microbes, which inhabit saline environments, can be classified based on their salt requirements: slight halophiles (2-5% NaCl), moderate halophiles (5-15% NaCl), and extremely halophiles (20-30% NaCl). Halophiles produce a variety of biomolecules, including enzymes, carotenoid pigments, biopolymers, bacteriorhodopsins, and halocins, showcasing significant biotechnological advantages [(7)]. Halophilic bacteria are not commonly associated with human infections. There are few reports of virulence factors of halophiles. On the other hand, they can produce metabolites with antimicrobial efficiency, and they have been considered as an emerging source of antibiotics [(8)]. Halophilic bacteria produce various types of EPS with unique properties. Several studies have reported that halophilic EPS demonstrate diverse biological activities, such as antioxidant, antitumor, and immune-enhancing properties [(9-12)].

*Halomonas* spp. are Gram-negative halophilic bacteria and commonly found in hypersaline habitats. They require salt for growth, which can be categorized into two groups: moderate and extreme *Halomonas*, depending on their salt requirement. The increasing interest in *Halomonas* spp. lies in their ability to synthesize diverse high-value-added products, such as EPS, PHAs, ectoine, hydroxyectoine, and biosurfactants [(12)]. EPS from *Halomonas* spp. have been garnering considerable interest due to their properties and biological activities. Various strains of *Halomonas* demonstrate the capability to produce a variety of bioactive EPS. For instance, *H. maura* produced a sulfated EPS named Maura, which displayed an antioxidant activity and could be applied as biomaterials, nanoparticles, and nanofibers [(13-15)]. Another EPS produced by *H. desertis* G11 exhibited antioxidant activity and inhibited the growth of cancer cell lines [(16).] *Halomonas* sp. 2E1 produced a bioactive EPS a bioactive EPS with immunomodulatory activity, stimulating the production of nitric oxide (NO), COX-2, TNF-α, IL-1β, and IL-6 in RAW264.7 macrophage cell lines (11). Additionally, Mata *et al*, [(17)] reported that EPS from *H. ventosae* and *H. anticariensis* showed an ability to absorb heavy-metal cations ($Cu^{2+}$, $Pb^{2+}$, and $Co^{2+}$).

Due to the increasing interest of EPS from marine microbes and halophiles, we would like to explore new EPS with unique properties produced by halophilic microbes isolated from Thailand. This study focuses on *Halomonas* sp. SS3 isolated from solar saltern soils in Phetchaburi province, Thailand. We aim to optimize EPS production conditions for this strain and evaluate its potential antioxidant activity.

## 2. Materials and Methods

### 2.1 Isolation of EPS-producing halophilic bacteria

EPS-producing halophiles was isolated using modified MY medium supplemented with NaCl (consisting of: 3 $g \cdot L^{-1}$ malt extract; 3 $g \cdot L^{-1}$ yeast extract; 5 $g \cdot L^{-1}$; peptone; 20 $g \cdot L^{-1}$ sucrose; 10-20% (w/v) NaCl; pH 7.0). Solar saltern soil samples were collected from salt ponds located in Phetchaburi province, Thailand. To achieve enrichment of halophiles, 10 g of soil sample was introduced into 50 mL of MY medium with varying salt concentrations and incubated at 30°C, 100 rpm for 7 days. Subsequently, pure cultures of EPS-producing bacteria were isolated on MY agar medium. Selection was based on identifying mucoid and slime colonies present on the agar medium. The molecular identification of the selected strains was performed by rRNA gene sequence determination (MU-OU: CRC, Mahidol University, Thailand). The sequences were compared with BLAST database online.

### 2.2 EPS production and Isolation

EPS production by halophilic bacteria was conducted using the modified MY medium containing 10% (w/v) NaCl. The 10%(v/v) of freshly prepared inoculum ($OD_{600}$ of 0.8) was transferred to a 250-mL flask containing 50-mL of the MY medium. The cultural flasks were incubated at 30°C with orbital shaking at 100 rpm for 7 days (18). After the fermentation, the culture broth was centrifuged at 9000 rpm for 10 min for eliminating bacterial cells. For the protein precipitation, trichloroacetic acid (TCA) was added to the cell-free supernatants at the final concentration of 4% (w/v). The mixture was then placed at 4°C for 30 min, followed by another centrifugation step (9000 rpm, 10 min) to collect the resulting supernatant. EPS isolation was achieved using the ethanol precipitation method. Cold ethanol (90% v/v) was added into the supernatants at the ratio of 1:3 (supernatant:EtOH) and stored at -20°C for overnight. After that, the mixtures were centrifuged (9000 rpm, 10 min) and collected the crude EPS. Finally, the EPS pallets were dried by lyophilization [19, 20].

### 2.3 Optimization for EPS production

To optimize the cultural conditions, the one-factor-at-a-time method was used in this experiment. Various factors were varied to estimate the optimal values for the EPS production. The variables consisted of carbon source (glucose, sucrose, lactose, and fructose), sugar concentration (10, 20 50, 100, 150, and 200 $g \cdot L^{-1}$), NaCl concentration (5, 7.5, 10,12.5, and 15% w/v), medium pH (5.0, 6.0, 7.0, 8.0, and 9.0), temperature (25, 30, 32, 35, and 40°C), agitation speed (0, 100, and 200 rpm), inoculum size (5, 10, and 20% v/v), incubation time (5, 7, and 9 days), and extraction ratio (1:1, 1:2, and 1:3 (supernatant:EtOH)).

*2.3.1 DPPH Scavenging Assay*

The DPPH assay was proceeded following the method of Jan *et al.* [21]. A 0.1 mM DPPH solution was prepared in 100% methanol and kept in the dark for 2 h. Different concentrations of EPS (ranging from 0 to 5 $mg \cdot mL^{-1}$) were prepared and combined with the DPPH solution in the ratio of 1:1. The mixture was incubated at room temperature for 30 min in darkness. After incubation, the absorbance was measured at 517 nm (methanol was used as a blank). Ascorbic acid (Vc) was used as a positive control. The DPPH scavenging activity of EPS was obtained from the following equation:

$$\text{Scavenging activity (\%)} = \frac{A_{Control} - A_{Sample}}{A_{Control}} \times 100 \quad (1)$$

where $A_{control}$ represents the absorbance of the solution with DPPH and distilled water and $A_{sample}$ is the absorbance of testing sample solution.

*2.3.2 ABTS Scavenging Assay*

The ABTS scavenging assay was done following the method of Bomfim *et al.* [22] with minor adaptations. The ABTS working solution was prepared by mixing a 7 mM of ABTS solution (7 mM) and a 2.45 mM of potassium persulfate ($K_2S_2O_8$) solution in a ratio of 1:1. This mixture was then kept in the dark at room temperature for 12–16 hr. Then, the absorbance of an ABTS working solution was adjusted to 0.7 ± 0.02 using deionize water at 734 nm. The reaction was done by adding 150 $\mu$L of EPS solutions (0-5 mg·mL$^{-1}$) to 750 $\mu$L of the ABTS working solution. The mixture was incubated at room temperature for 7 min. After incubation, the absorbance was measured at 734 nm (n = 3). Ascorbic acid (Vc) was used as a positive control. The ABTS scavenging activity was calculated from the following equation:

$$\text{Scavenging activity (\%)} = \frac{A_{Control} - A_{Sample}}{A_{Control}} \times 100$$

where $A_{control}$ represents the absorbance of the control group (distilled water rather than the sample solution), and $A_{sample}$ represents the absorbance of the experimental group [23].

*2.3.3 FRAP Assay*

The FRAP assay was performed following the method of Bomfim *et al.* [22] with slight modifications. The FRAP working solution was prepared by combining acetate buffer (300 mM), TPTZ solution (10 mM), and $FeCl_3$ solution (20 mM) in a ratio of 10:1:1 and kept at 37°C in the dark. The reaction was proceeded by mixing 900 $\mu$L of FRAP working solution and 100 $\mu$L of EPS solutions (0-5 mg·mL$^{-1}$). Then, the reaction mixture was incubated at 37°C for 15 min in darkness. The absorbance was determined at a wavelength of 593 nm (n = 3). Moreover, $FeSO_4 \cdot 7H_2O$ (200-

1000 µmol·L$^{-1}$) was used to perform the calibration curve. The results were expressed as $Fe^{2+}$ equivalents ($\mu$M).

*2.3.4 Total Antioxidant Capacity (Phosphomolybdate Assay)*

The total antioxidant capacity, assessed through the phosphomolybdate assay, relies on the conversion of Mo (VI) to Mo (V) by antioxidant agents, resulting in the formation of a green phosphate Mo (V) complex under acidic pH conditions. A 0.1 mL of sample solutions (0-5 mg·mL$^{-1}$) was combined with 1 mL of the reagent containing 0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate. Then, the mixture was incubated at 95°C for 90 min in a water bath. After that, the mixture was cooled to room temperature and the absorbance was measured at 695 nm (n = 3). Ascorbic acid was used as a standard. The results were expressed ascorbic acid equivalents (µg·mL$^{-1}$) [9,24].

## 3. Results & Discussion

### 3.1 Screening and Identification of EPS-producing halophiles

Eight halophilic strains were isolated from soil samples using the modified MY medium supplemented with different concentrations of NaCl. The eight strains were named SS1, SS2, SS3, SS5, SS7, SS9, SS10, and SS12. They were found to produce slimy mucoid colonies on solid MY medium. To identify their genera, partial rRNA gene sequences were performed, and the results of % similarity were showed in Table 1. The results showed that five isolates belonged to *Salibacterium halochares* and three isolates were identified as *Halomonas* sp. (Table 1). According to the screening of EPS production, all strains were cultivated on the modified MY medium with 10% (w/v) NaCl. The highest EPS yield of 0.51 ± 0.10 g·L$^{-1}$ was observed in *Halomonas* sp. SS3, while *Salibacterium halochares* SS7 produced the least EPS at 0.13 ± 0.02 g·L$^{-1}$ (Table 1). Therefore, *Halomonas* sp. SS3 was chosen for further experiments. It could be found that the morphology of *Halomonas* sp.

colonies were mucoid, glistening, circular, smooth, and convex shape and appeared coral-pink color on the modified MY medium (Figure 1).

**Table 1.** Identification of EPS-producing halophiles and EPS yields.

| Strain | %Similarity | Identified Bacteria | EPS yield ($g \cdot L^{-1}$) | Biomass ($g \cdot L^{-1}$) |
|---|---|---|---|---|
| SS1 | 100.00% | *Salibacterium halochares* | $0.32 \pm 0.02^{b}$ | $0.76 \pm 0.53$ |
| SS2 | 100.00% | *Salibacterium halochares* | $0.37 \pm 0.02^{b}$ | $0.80 \pm 0.66$ |
| SS3 | 99.71% | *Halomonas* sp. | $0.51 \pm 0.10^{a}$ | $1.74 \pm 0.36$ |
| SS5 | 100.00% | *Salibacterium halochares* | $0.31 \pm 0.03^{b}$ | $0.51 \pm 0.13$ |
| SS7 | 100.00% | *Salibacterium halochares* | $0.13 \pm 0.02^{c}$ | $0.43 \pm 0.10$ |
| SS9 | 99.72% | *Halomonas* sp. | $0.37 \pm 0.05^{b}$ | $8.11 \pm 0.11$ |
| SS10 | 100% | *Halomonas* sp. | $0.33 \pm 0.01^{b}$ | $1.15 \pm 0.35$ |
| SS12 | 100% | *Salibacterium halochares* | $0.29 \pm 0.02^{b}$ | $1.32 \pm 0.05$ |

The different lower-case letters in the column indicate a significant difference ($P<0.05$).

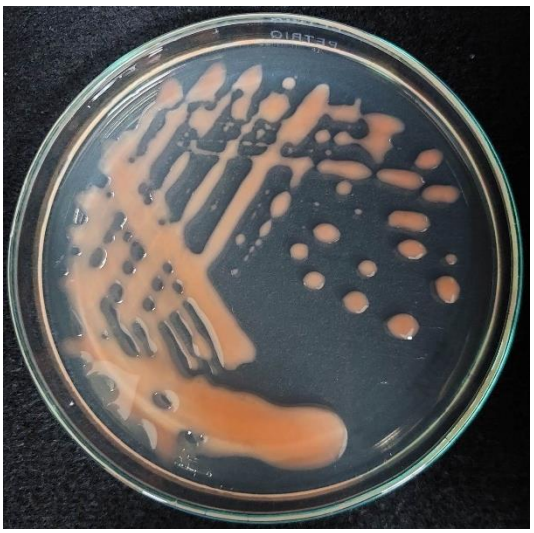

**Figure 1.** Colony morphology of Halomonas sp. SS3 on MY medium with 10% (w/v) NaCl.

### 3.2 Optimization of Cultural Conditions for EPS production

*3.2.1 Effect of Carbon Sources*

Different types of sugar were added to MY medium at a concentration of 20 $g \cdot L^{-1}$. The effect of carbon sources was determined using glucose, sucrose, lactose, and fructose. While the EPS yields from different sugar types showed no significant differences, the highest EPS yield was obtained from sucrose (Figure 2A), reaching a maximum of $0.51 \pm 0.11$ $g \cdot L^{-1}$. When a carbon source was glucose, lactose, and fructose, the ESP yields were $0.40 \pm 0.04$, $0.42 \pm 0.15$, and $0.42 \pm 0.01$ $g \cdot L^{-1}$, respectively.

*3.2.2 Effect of Sugar Concentration*

In this experiment, sucrose was used as a sole carbon source in MY medium at different concentrations (10, 20, 50, 100, 150, and 200 $g \cdot L^{-1}$). At a sucrose concentration of 100 $g \cdot L^{-1}$, EPS yield was higher than other concentrations and significantly different compared to the others. The maximum EPS yield from *Halomonas* sp. SS3 was $0.87 \pm 0.04$ $g \cdot L^{-1}$ (Figure 2B). This study highlighted the influence of sugar concentrations on EPS production, indicating that an excess of sugar might impede bacterial growth and subsequently affected EPS production.

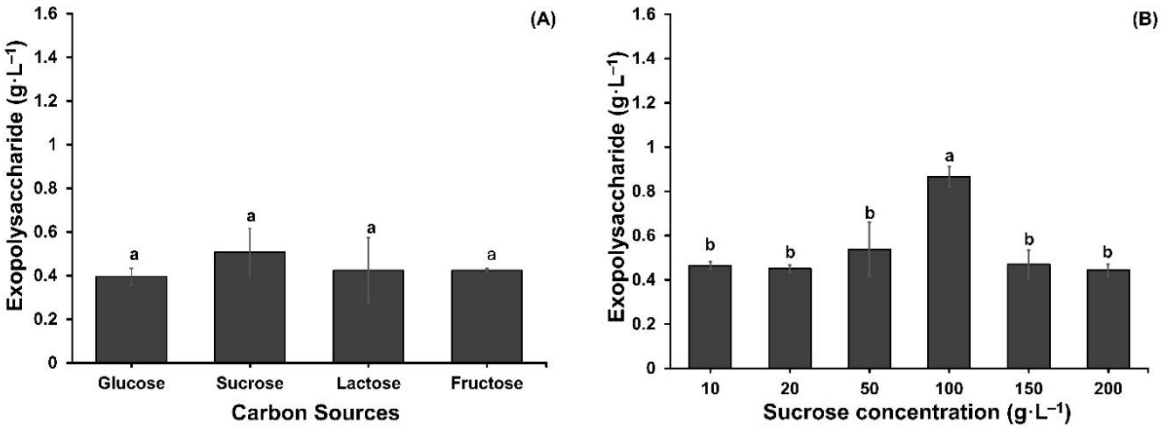


**Figure 2.** Influence of types of carbon sources (A) and sugar concentrations (B) on EPS production by *Halomonas* sp. SS3.

*3.2.3 Effect of NaCl Concentration*

Salt is essential for the growth of halophilic bacteria. *Halomonas* sp. SS3 could grow on a wide range of NaCl concentrations (salt tolerance). Different NaCl concentrations were added to the MY medium. The highest EPS yield was 0.99 ± 0.12 g·L$^{-1}$, observed at a NaCl concentration of 15% (w/v) (Figure 3A). Conversely, the lowest EPS yield of 0.54 ± 0.10 g·L$^{-1}$ was observed at 5% (w/v) NaCl.

*3.2.4 Effect of Initial Medium pH*

The initial medium pH is an important factor that influences bacterial growth. For EPS production from *Halomonas* sp. SS3, MY medium with different initial pH (pH of 5.0, 6.0, 7.0, 8.0, and 9.0) was prepared. At an initial pH of 8.0, the EPS yield was the highest amount reaching 1.11 ± 0.02 g·L$^{-1}$. The lowest EPS yield (0.56 ± 0.14 g·L$^{-1}$) was obtained from the medium with pH of 5.0 (Figure 3B). These findings suggested that *Halomonas* sp. SS3 exhibits a preference for an alkaline environment.

*3.2.5 Effect of Temperature*

A wide range of temperatures (25, 30, 32, 35, and 40°C) was tested for EPS production by *Halomonas* sp. SS3. Incubation at 30°C gave the highest EPS yield, which was significantly different from the others. The maximum EPS yield was 0.99 ± 0.04 g·L$^{-1}$ (Figure 3C). Conversely, the least EPS yield was 0.81 ± 0.04 g·L$^{-1}$ when incubation was conducted at 40°C.

3.*2.6 Effect of Agitation Speed*

Agitation speed plays a crucial role in promoting bacterial cell growth. It was observed that the agitation speed significantly impacted both the growth of *Halomonas* sp. SS3 and its EPS production. With increased agitation speed, there was a notable rise in EPS yield. Hence, the agitation speed of 200 rpm gave the highest EPS yield. The maximum EPS yield rose to 1.20 ± 0.05 g·L$^{-1}$ when the cultivation was done at the agitation speed of 200 rpm (Figure 3D).

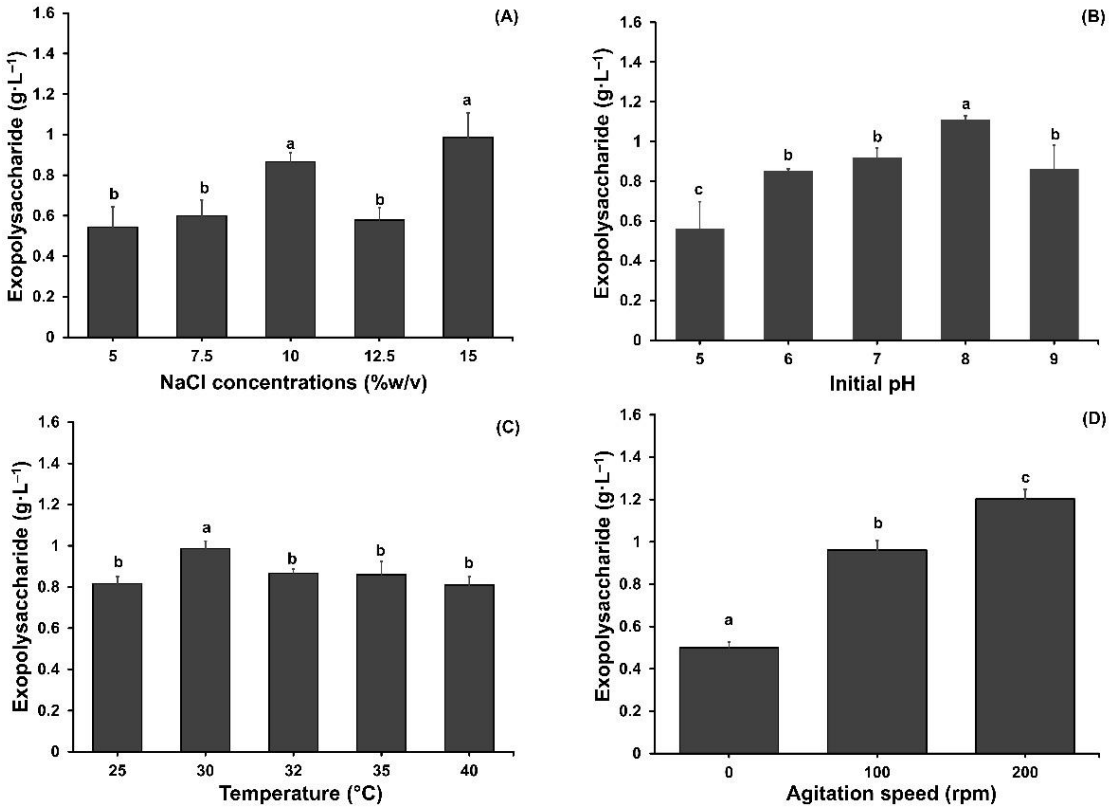


**Figure 3.** The effects of NaCl concentrations (A), initial pH (B), temperature (C), and agitation speed (D) on the EPS production by Halomonas sp. SS3.

*3.2.7 Effect of Incubation Time*

In this study, incubation times of 5, 7, and 9 days were tested for EPS production by *Halomonas* sp. SS3, and other factors were fixed at the optimized values mentioned above (3.2.1-3.2.6). The results indicated that the highest EPS yield occurred after 7 days of incubation, reaching 1.07 ± 0.05 $g \cdot L^{-1}$. With prolonged incubation (9 days), there was a slight reduction in EPS yield (Figure 4A).

*3.2.8 Effect of Inoculum Size*

The initial inoculum dose exerted a significant influence on EPS production in the MY medium. A freshly prepared inoculum ($OD_{600\ nm}$ = 0.8) was added to the cultural medium at varying doses (5, 10, and 20% v/v), and the fermentation was performed under the optimized conditions from previous experiments (3.2.1-3.2.7). Figure 4B showed that the EPS yields increased proportionally with the escalating inoculum doses. The optimum inoculum size was 20% (v/v), resulting in the highest EPS yield of 1.13 ± 0.05 $g \cdot L^{-1}$.

*3.2.9 Effect of Extraction Ratio*

The EPS production was conducted under the optimized conditions from previous experiments (3.2.1-3.2.8). The EPS isolation was performed using the ethanol precipitation method, where cold ethanol (EtOH, 90% v/v) was added to the supernatant. The different ratios of supernatant:EtOH were varied as 1:1, 1:2 and 1:3. The results showed that the ratio of 1:2 gave the highest EPS yields (1.28 ± 0.32 $g \cdot L^{-1}$). According to statistical analyses, there was no significant difference between EPS yields derived from all extraction ratios (Figure 4C). The ratio of 1:1 should be chosen due to the lesser use of ethanol and cost savings.

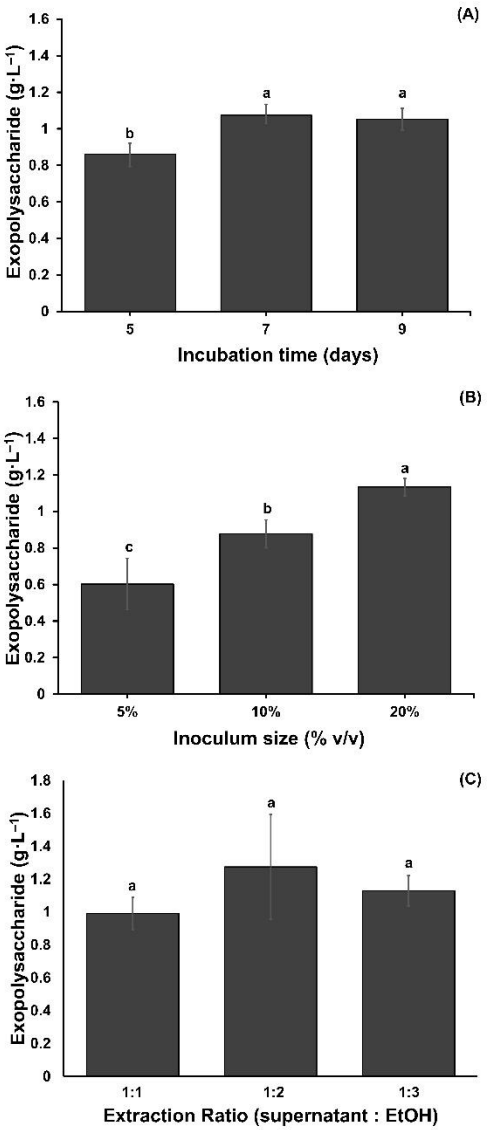


**Figure 4.** Influence of incubation time (A), inoculum size (B), and extraction ratio (C) on the EPS production by Halomonas sp. SS3

Under the optimal conditions, *Halomonas* sp. SS3 exhibited a maximum EPS yield of 1.28 g·L$^{-1}$. Comparison to other reports, *Halomonas* sp. SS3 produced EPS higher than *H. smyrnensis* K2, *Halomonas marina* HMA103, and *H. nitroreducens*, which produced EPS yields of 0.074, 0.69, and 1.25 g·L$^{-1}$, respectively [25-27]. Nevertheless, our study's EPS yield was lower compared to several reports (Table 2). For instance, *H. almeriensis* M8T, *H. stenophila*, and *H. xianhensis* SUR308 gave the large amount of EPS yields of 1.70, 3.89 and 2.56 g·L$^{-1}$, respectively [28-30]. The future work should prioritize strategies to enhance EPS yield from *Halomonas* sp. SS3 for pilot scale production.

**Table 2.** The EPS production from different *Halomonas* species.

| Microbe | Medium | Carbon source | EPS yield ($g \cdot L^{-1}$) | Bioactivities | References |
|---|---|---|---|---|---|
| *Halomonas* sp. SS3 | MY medium with 15% (w/v) NaCl | Sucrose | 1.28 | Antioxidant | **This study** |
| *Halomonas smyrnensis* K2 | Semi complex medium (SCM) with 15% (w/v) NaCl | Glucose | 0.074 | Antibiofilm; Antioxidant | Joulak *et al.* [25] |
| *Halomonas marina HMA 103* | Modified basal synthetic medium with 10% (w/v) NaCl | Glucose | 0.69 | N/A | Pal *et al.* [26] |
| *Halomonas nitroreducens* | MY medium with 5% (w/v) NaCl | Glucose | 1.25 | Antioxidant | Chikkanna *et al.* [27] |
| *Halomonas almeriensis* M8T | MY medium with 7.5% (w/v) NaCl | Glucose | 1.70 | N/A | Llamas *et al.* [28] |
| *Halomonas stenophila* | MY medium with 5% (w/v) NaCl | Glucose | 3.89 | N/A | Amjres *et al.* [29] |
| *Halomonas xianhensis* SUR308 | MY medium with 2.5% NaCl, 0.5% casein hydrolysate | Glucose | 2.56 | Antioxidant | Biswas *et al* [30] |

N/A, not available

### 3.3 Antioxidant Activities

This study has proved that the EPS extracted from *Halomonas* sp. SS3 exhibited a biological activity, especially the antioxidant activity. The DPPH assay revealed that the EPS exhibited increasing DPPH scavenging activity in a dose-dependent manner (1-5 $mg \cdot mL^{-1}$). At a concentration of 5 $mg \cdot mL^{-1}$, the DPPH scavenging activity was 79.50 ± 3.36%. Figure 5A illustrates the DPPH scavenging activity of the EPS compared to an ascorbic acid (a positive control). The DPPH assay is a common method for assessing antioxidant activity. Table 3 presented that EPS from other microbes exhibited high values of DPPH scavenging activity (51.52-84%) at different EPS concentrations. The EPS from *Halomonas sp.* SS3 showed higher DPPH scavenging activity using a low amount of EPS (5 $mg \cdot mL^{-1}$) compared to several studies (Table 3).

The ABTS radical scavenging activity of the EPS compared to an ascorbic acid was shown in Figure 5B. The EPS exhibited positive responses to the reaction. The ABTS scavenging activity increased in a dose-dependent manner of the EPS. The activity reached 43.94 ± 2.49 % at the EPS concentration of 5 $mg \cdot mL^{-1}$. However, the activity of the EPS was lower than ascorbic acid at the same concentrations.

In the FRAP assay (Figure 5C), the EPS derived from *Halomonas* sp. SS3 exhibited the capability to reduce $Fe^{3+}$-TPTZ, generating $Fe^{2+}$ -TPTZ complexes. $FeSO_4$ was used as a positive control and the results were expressed as the equivalents of $FeSO_4$. The $FeSO_4$ equivalence of the EPS showed an incremental pattern corresponding to the rise in EPS concentrations. At the EPS concentration of 5 $mg \cdot mL^{-1}$, the equivalent was 41.40 ± 3.04 µM $FeSO_4$.

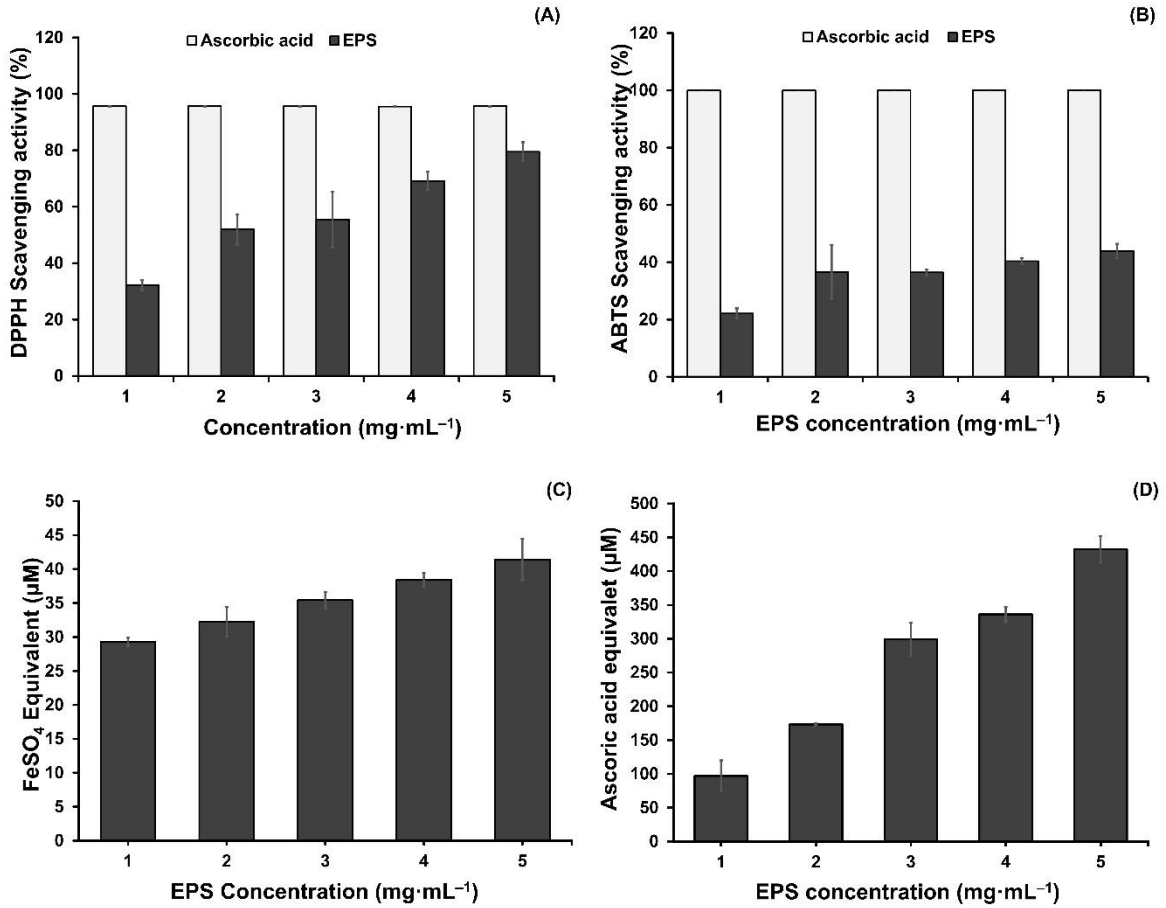


**Figure 5.** Antioxidant activity of EPS produced by *Halomonas* sp. SS3 evaluated in vitro by different methodologies. DPPH assay (A); ABTS assay (B); FRAP assay (C); total antioxidant capacity (D).

Total antioxidant capacity relies on the reduction of Mo (VI) to Mo (V), which forms a green phosphate Mo (V) complex at acidic pH. The EPS also showed an ability to reduce Mo (VI) to Mo (V). The results of this assay were expressed ascorbic acid equivalents (µM). Figure 5D showed that the ascorbic acid equivalents increased in a dose-dependent manner of the EPS. The EPS concentration of 5 mg·mL$^{-1}$ exhibited the highest activity, which the maximum ascorbic acid equivalent was 432.25 ± 19.65 µM of ascorbic acid.

The antioxidant activity of EPS is primarily influenced by its structural characteristics, for example, functional groups, monosaccharide compositions, glycosidic linkages, structural configuration, and molecular weight. Functional groups like hydroxyl (-OH), carboxyl (COOH), acetyl ($C_2H_3O$), and sulfate groups ($SO_4^{2-}$) exhibit the capacity to donate hydrogen or electrons when interacting with free radicals. Additionally, some functional groups (COOH, $SO_4^{2-}$, OH, sulfhydryl (SH) and amide groups ($CONH_2$)) show metal binding ability by donating electrons or losing protons. Beyond functional groups, the presence of reducing sugars also influences the antioxidant capacity. Furthermore, molecular weight of EPS plays a role in its bioactivities; the low-molecular weight EPS are able to liberate more protons [30]. According to our results, we expected that the EPS synthesized by *Halomonas* sp. SS3 might contain some functional groups or possess distinct structural configurations. Future studies should focus on characterizing the structure of EPS from *Halomonas* sp. SS3 to comprehend its mode of action in exhibiting antioxidant activity. This study demonstrated that *Halomonas*

sp. SS3 could produce bioactive EPS. The EPS exhibited an antioxidant activity. Thus, the EPS might be served as antioxidant agent and could be applied in various fields.

**Table 3.** DPPH scavenging activity of EPS produced by *Halomonas* sp. SS3 compared to other microbes.

| Microbes | EPS concentration ($mg \cdot mL^{-1}$) | DPPH Scavenging activity (%) | References |
|---|---|---|---|
| *Halomonas* sp. SS3 | 5 | 79.50 | This study |
| *Halolactibacillus miurensis* | 10 | 84 | (9) |
| *H. nitroreducens* WB1 | 5 | 83.3 | (27) |
| *Marinobacter* sp. N4 | 2.5 | 72.75 | (18) |
| *Halomonas elongata* S6 | 10 | 60.59 | (31) |
| *Lactobacillus plantarum* CNPC003 | 10 | 51.52 | (22) |
| *Haloterrigena turkmenica* | 10 | 68.2 | (32) |

## 4. Conclusion

*Halomonas* sp. SS3, a novel isolate from Thai solar saltern soil, exhibits significant potential for exopolysaccharide (EPS) production and antioxidant activity. This study optimized EPS production on MY medium, achieving a maximum yield of 1.28 $g \cdot L^{-1}$ under the following conditions: 100 $g \cdot L^{-1}$ of sucrose, 15% (w/v) of NaCl, initial pH of 8.0, agitation speed of 200 rpm, inoculum dose of 20% (v/v), incubation at 30°C for 7 days, and extraction ratio of 1:2 (supernatant:EtOH). According to the absence of significant difference between extraction ratios, the EtOH ratio of 1:1 should be used for cost savings. This represents a remarkable 151% increase compared to the initial yield of 0.51 $g \cdot L^{-1}$. Furthermore, the EPS from *Halomonas* sp. SS3 exhibited moderate antioxidant activities *in vitro* assays such as DPPH, ABTS, FRAP, and total antioxidant capacity. The antioxidant activities increased in a dose-dependent manner of the EPS concentrations.

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# Comparison of Three Different Plant Proteins in Oil-in-Water Emulsion System

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## Abstract

This study investigated the emulsifying properties of three plant-based proteins—soy protein isolate, mung bean protein isolate, and cassava leaf protein concentrate—across varying concentrations in oil-in-water emulsion system. The emulsions were prepared using a sodium phosphate buffer at pH 7, incorporating 3.5% corn oil. The emulsification process involved a high-speed mixture (10,000 rpm for 1 min) followed by a high-pressure homogenizer (20 MPa for 3 cycles). Model emulsion systems were established with soy protein isolate and mung bean protein isolate at concentrations of 1.5%, 2.5%, and 3.5% w/v, and cassava leaf protein concentrate at concentrations of 2.5%, 3.5%, and 4.5% w/v. The droplet characteristics were examined, including the Creaming Index (CI) during a 14-day storage period at 20°C and the mean particle diameters (d32). Results showed stable soy protein isolate emulsions, maintaining mean particle diameters between 0.490-0.570 μm without cream layer formation. In contrast, mung bean protein isolate emulsions at concentrations of 1.5% and 2.5% exhibited cream layer formation after 3 and 9 days, respectively. Emulsion containing 3.5 % mung bean protein isolate showed no cream layer formation and mean particle diameters were 0.494±0.021 μm. Cassava leaf protein concentrate emulsions at 2.5% and 3.5 % exhibited cream layer formation after 7 and 11 days, respectively. Emulsions containing 4.5 % cassava leaf protein showed no cream layer formation with mean particle diameters of 0.732 ± 0.127 μm. The data revealed that increasing mean particle diameter of unstable mung bean protein isolate and cassava leaf protein concentrate emulsions were found with increasing storage time. Our results showed that stable emulsion could be achieved with soy protein isolate at a concentration of 1.5%. Whilst cassava leaf protein concentration exhibited good emulsifying properties at higher concentration (4.5%) compared to soy protein isolate and mung bean protein isolate (1.5% and 3.5%, respectively). These findings contribute valuable insights into the formulation of stable emulsion systems using plant-based proteins.

***Keywords**:* Oil-in-water emulsion; Soy protein isolate; Mung bean protein isolate; Cassava leaf protein concentrate; Emulsion stability.

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## 1. Introduction

In the past ten years, plant proteins-like those found in legumes-have drawn a lot of interest as more ecologically friendly alternatives to animal-derived proteins, including soy protein and mung bean protein. This is because they are rich in essential amino acids and have a higher lysine content than other bean protein sources, with a level comparable to that of eggs [(1)]. Furthermore, cassava leaves represent a type of plant protein rich in protein, typically containing 20-40% crude protein on a dry matter basis, with an essential amino acid content comparable to that of eggs [(2)]. However, the applications of cassava protein in foods are limited. Investigation of using cassava protein in an emulsion system should extend its applications in foods and feeds.

In the context of food, emulsions typically exhibit rheological behaviors. Food is a system made up of water and oil, forming a mixture with varying surface tensions. Consequently, mixing the two liquids is not possible. Emulsion stability can be achieved, in part, by adding an emulsifier. Emulsifiers are amphiphilic substances with both hydrophilic and hydrophobic properties. They are crucial in lowering the surface tension that separates water from oil, allowing the oil to permeate into the water molecules. Plant proteins have a crucial functional characteristic called emulsifying ability. Research on the emulsifying qualities of plant proteins, particularly soy protein, has been done extensively because proteins can function as surfactants [(3)]. It was discovered that 90% of soy protein is made up of glycinin and beta-conglycinin, which are good amphiphilic compounds with emulsifying qualities. The several studies could formulate the stable emulsion with 1 to 5% soy protein isolate emulsion (O/W) with $d_{32}$ at 3.5 to 8 μm [(4)] and 1 to 4% soy protein isolate emulsion (W/O/W) with $d_{32}$ at d at 14 and 33 μm [(5)].

As known, there are substantial studies on the emulsifier application of soy protein but not of mung bean and/or leaf protein. In order to better understand the emulsifying qualities of these three plant-based proteins, this study examined their effects at different concentrations in an oil-in-water emulsion system: soy protein isolate, mung bean protein isolate, and cassava leaf protein concentrate.

## 2. Materials and Methods

### 2.1 Materials

Soy protein isolate (90% protein content) was sourced from Sinoglory Soypro Co., Ltd, Shandong, China. Mung bean protein (80% protein content) was obtained from Yantai Shuangta Food Co., Ltd, Shandong, China. Cassava leaf protein concentrate (32% protein content) from KU 50 variety, was prepared using alkali extraction with isoelectric point precipitation, followed by freeze-drying.

### 2.2 Emulsion Preparation Using Three Different Plant Proteins

Three different plant proteins-soy protein isolate (SPI), mung bean isolate (MBI), and cassava leaf protein concentrate (CLPC) at different concentrations (1.5%, 2.5%, 3.5% for SPI and MBI, and 2.5%, 3.5%, 4.5% for CLPC) were utilized to develop the emulsion model system. To prepare the emulsion, the aqueous phase was created by dispersing the required amount of plant protein in sodium phosphate buffer (pH 7), and the suspension was stirred for 1 h. Subsequently, 3.5% corn oil (dispersed phase) was added to the continuous phase. The system was then homogenized using a dispersing tool (IKA T25 digital ULTRA-TURRAX®, China) at 10,000 rpm for 1 min, followed by three cycles through a high-pressure homogenizer (Izumi Food Co., Ltd. Model HV-0A3-2.25, Japan) at 20 MPa. $NaN_3$ (0.01 w/v) was added to the emulsion to prevent microbial growth during storage. The emulsion was collected and stored in an incubator at 20 °C for analysis.

### 2.3 Emulsion Stability in Emulsion Model System

Creaming stability was investigated to estimate emulsion stability immediately after the preparation of the emulsion. Emulsion samples were transferred into a set of glass tubes and sealed to prevent evaporation. These test tubes were then placed in an incubator at 20°C, and creaming was visually monitored for 14 days. The observed separation consisted of the top layer (Cream) and the bottom layer (Serum). The total emulsion height (HT) and the height of the serum layer (HS) were determined [(6)]. The Creaming Index (CI) was then calculated using equation (1) as follows:

$$CI\ (\%) = HS\ /HT \times 100 \quad (1)$$

### 2.4 Emulsion Characterization in Emulsion Model System

*2.4.1 Optical Microscopy*

Emulsions formed on day 1 were analyzed under optical microscopy using a Fluorescence Microscope (Olympus BX51, Japan & Canon EOS 75DD, Japan) with a magnification of 40X [(7)][7].

*2.4.2 Particle Size Measurement*

Malvern MasterSizer 3000 (Malvern Instruments Ltd, Worcestershire, UK), utilizing laser diffraction to measure the size distribution of particles in a sample, was employed for determining the oil droplet size ($d_{32}$) on days 1, 5, and 10 [(7)].

### 2.5 Statistical Analysis

All experiments were conducted in triplicates. The statistical analysis was performed using IBM SPSS Statistics software version 22 (SPSS Inc., Chicago, IL).

## 3. Results & Discussion

### 3.1 Impact of Protein Type and Concentration on Emulsion Stability

The condition of 14-day storage at 20°C represented a model of a milk-based product. The findings revealed the absence of cream layer formation in soy protein isolate emulsions at concentrations of 1.5-3.5% SPI. In contrast, mung bean protein isolate emulsions at concentrations of 1.5% displayed cream layer formation after 3 days of storage. There was a separation of the cream layer on top and a clear layer below. Mung bean protein isolate emulsions at concentrations of 2.5% displayed cream layer formation after 9 days of storage. There was a slight precipitate at the bottom of the test tube, forming a clear layer in the middle of the test tube. and a cream layer forms on top. Notably, emulsions containing 3.5% mung bean protein isolate exhibited no cream layer formation, as illustrated in Figure 2. Krstonosic *et al.* [(4)] reported that the emulsion of soy protein isolate concentration at 3 % and 5 % with sunflower oil at 30 % (w/w) showed that the creaming index was not noticed for entire 14 days of storage at 25°C [(4)]. The study of Wang *et al.* [(1)] reported that emulsion containing canola oil and 8% mung bean protein isolate solution at ratio of 1:9 (v/v), pH 7, displayed cream layer formation after 3 days of storage at 25°C [(1)]. For cassava leaf protein concentrate emulsions, cream layer formation was observed at 2.5% and 3.5% concentrations after 7 and 11 days, respectively. Protein precipitate was found at the bottom of the test tube. However, emulsions containing 4.5% cassava leaf protein exhibited no cream layer formation, as depicted in Figure 3.

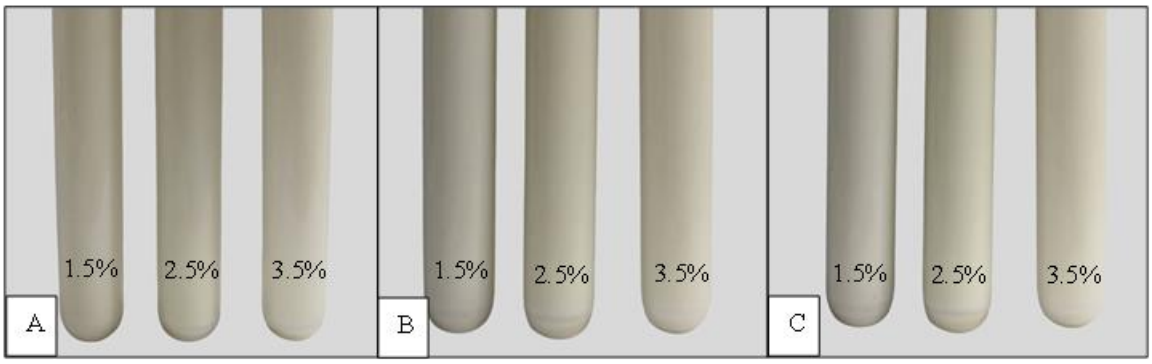


**Figure 1.** Impact of protein concentration on the emulsion stability of soy protein isolate emulsions. (A) Day 1; (B) Day 7; (C) Day 14.

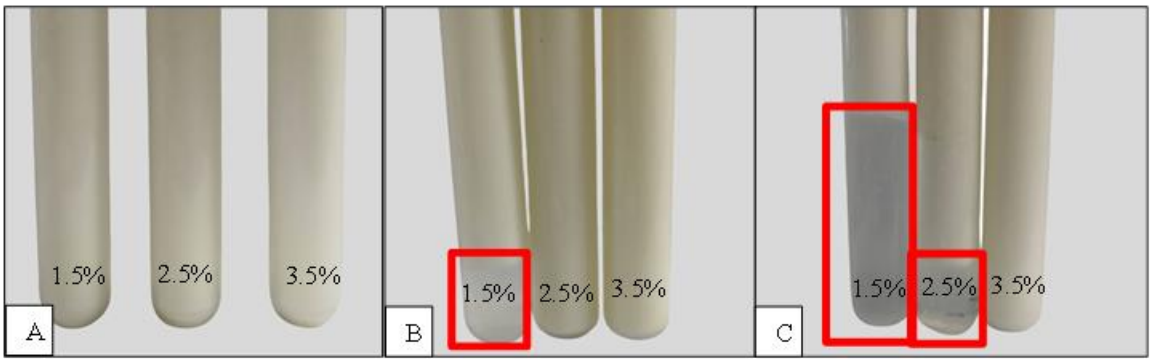


**Figure 2.** Impact of protein concentration on the emulsion stability of mung bean protein isolate emulsions. (A) Day 1; (B) Day 7; (C) Day 14.

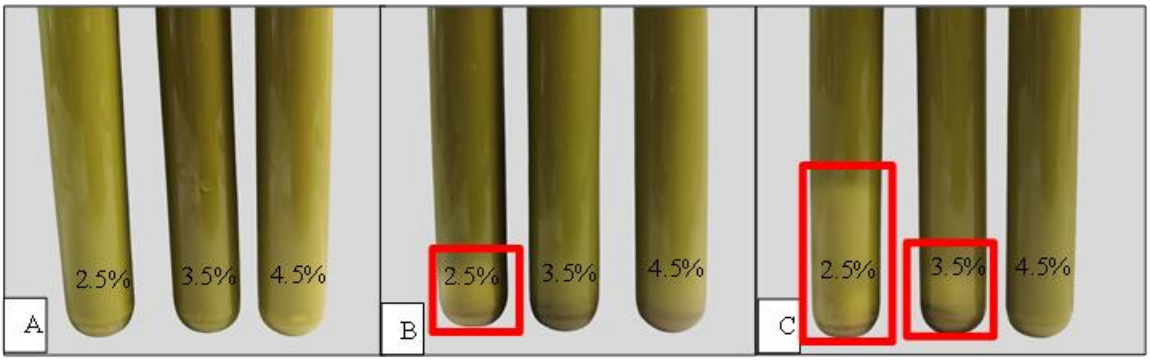


**Figure 3.** Impact of protein concentration on the emulsion stability of cassava leaf protein concentrate emulsions. (A) Day 1; (B) Day 7; (C) Day 14.

### 3.2 Impact of Protein Type and Concentration on Emulsion Characterization

*3.2.1 Impact of Protein Type and Amount on Microstructure of Emulsions*

The investigation into the impact of protein type and concentration on emulsion formation included a detailed examination of their microstructure using optical microscopy. Soy protein isolate emulsion displayed small oil droplets with a well-distributed pattern on day 1, day 5, and day 10 of storage, as illustrated in Figure 4. No agglomeration of oil droplets was observed across all concentrations of SPI used. The droplet distribution of soy protein isolate emulsion emulsions was more uniform than mung bean protein isolate in the study of Zhang *et al.* [8]. This favorable outcome can be attributed to the emulsifying properties of beta-conglycinin and glycinin in soy proteins [9], acting as effective amphiphilic molecules. These proteins reduced the surface tension between water and oil, facilitating the distribution of oil within water molecules and contributing to emulsion stability.

In contrast, mung bean protein isolate emulsion required a higher protein concentration, specifically 3.5%, to achieve a well-distributed emulsion with small oil droplets. For cassava leaf protein concentrate emulsions, the minimum concentration needed for optimal performance was 4.5%. At concentrations of 2.5% and 3.5% for cassava leaf protein, oil droplet aggregations were evident, as depicted in Figures 6A and 6B.

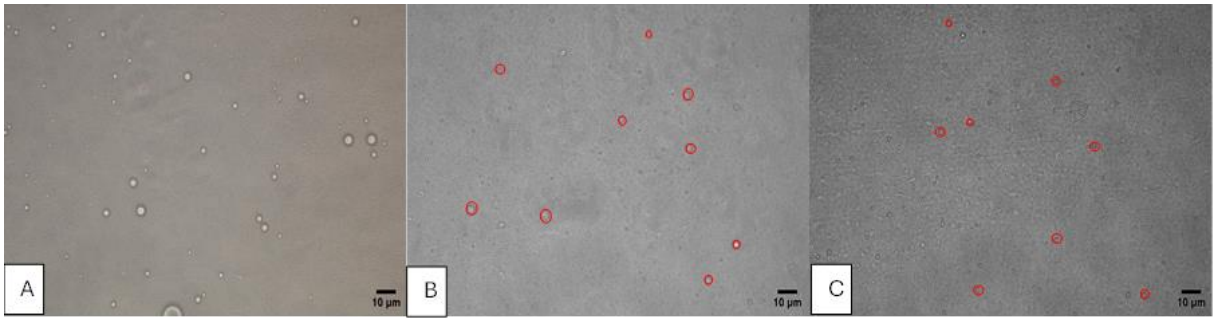


**Figure 4.** Microstructures of soy protein isolate emulsions observed by optical microscopy using a 40 × objective lens. (A) 1.5%; (B) 2.5%; (C) 3.5%

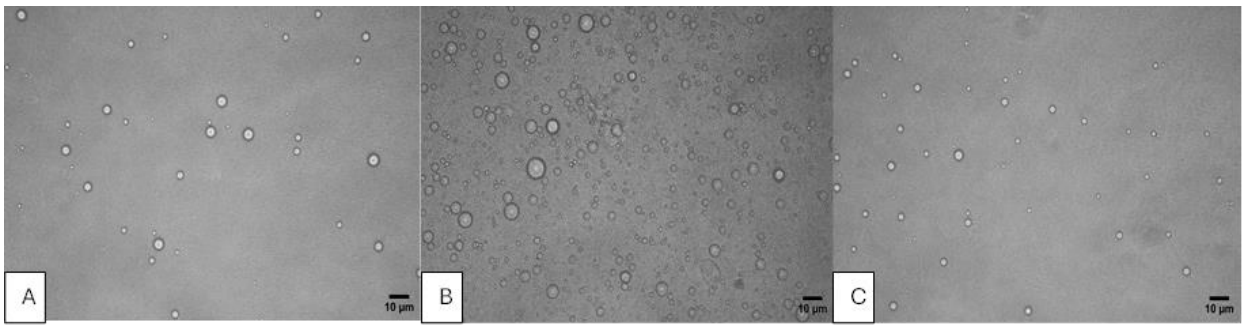


**Figure 5.** Microstructures of mung bean protein isolate emulsions observed by optical microscopy using a 40 × objective lens. (A) 1.5%; (B) 2.5%; (C) 3.5%.

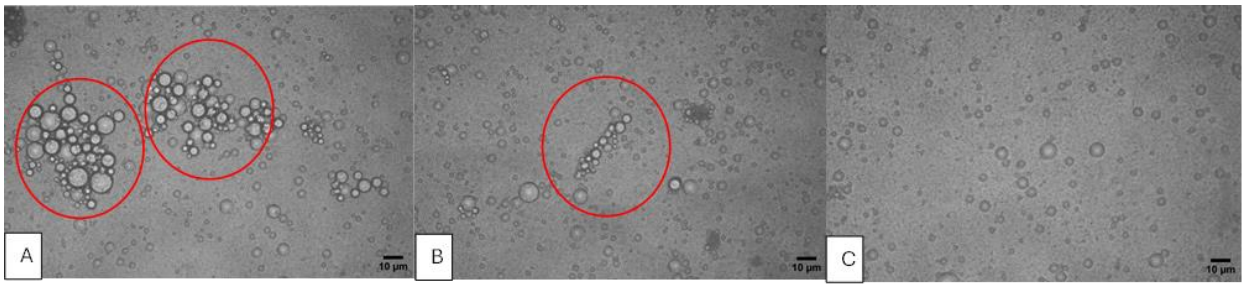


**Figure 6.** Microstructures of cassava leaf protein concentrate emulsions observed by optical microscopy using a 40 × objective lens. (A) 2.5%; (B) 3.5%; (C) 4.5%.

*3.2.2 Impact of Protein Type and Concentration on Mean Particle Diameters ($d_{32}$) and Emulsion Size Distribution*

The investigation delved into the influence of protein type and concentration on the particle size characteristics of the emulsions. Throughout the storage period on days 1, 5, and 10, the results revealed a consistent particle size distribution in soy protein isolate emulsions, with mean particle diameters ranging between 0.490-0.570 µm. Correspond to the study of Keerati-u-rai and Corredig [4] which reported that the emulsion of soy protein isolate concentration at 1 % (w/v) with soybean oil at 10 % (w/w) showed a monomodal distribution of particle sizes with an average diameter of about 1 µm [10]. As the concentration of mung bean isolate or cassava leaf protein increased, the emulsion exhibited a monomodal size distribution of oil droplets with smaller diameters. This reduction in particle size was attributed to the adequate amount of protein surrounding the oil droplet in homogenization process. Mung bean protein could form the emulsion of 3.5% corn oil at 3.5% with similar

$d_{32}$ to soy protein. While cassava leaf protein at 4.5% w/v could form the emulsion with monomodal size distribution but much bigger $d_{32}$ to soy protein. However, with an extended storage period, the emulsifying properties of mung bean protein isolate and cassava leaf protein concentrate diminished due to the protein's reduced ability to absorb the interface. The data indicated that emulsions at lower protein concentrations exhibited poor dispersion, resulting in larger particle sizes and particle agglomeration, ultimately causing cream layer formation faster. Li *et al.* [11] found that the mean particle size of the emulsions of soy protein isolate at 0.5% (w/v) increased with the storage time from 2 to 8 µm after storage for 12 days [11]. The results demonstrated the distinguish emulsifier abilities among these three plant protein resulting from the different protein types and composition in each source. The diverse proteins in mung bean and cassava leaf and their application modification are needed to be studied further.

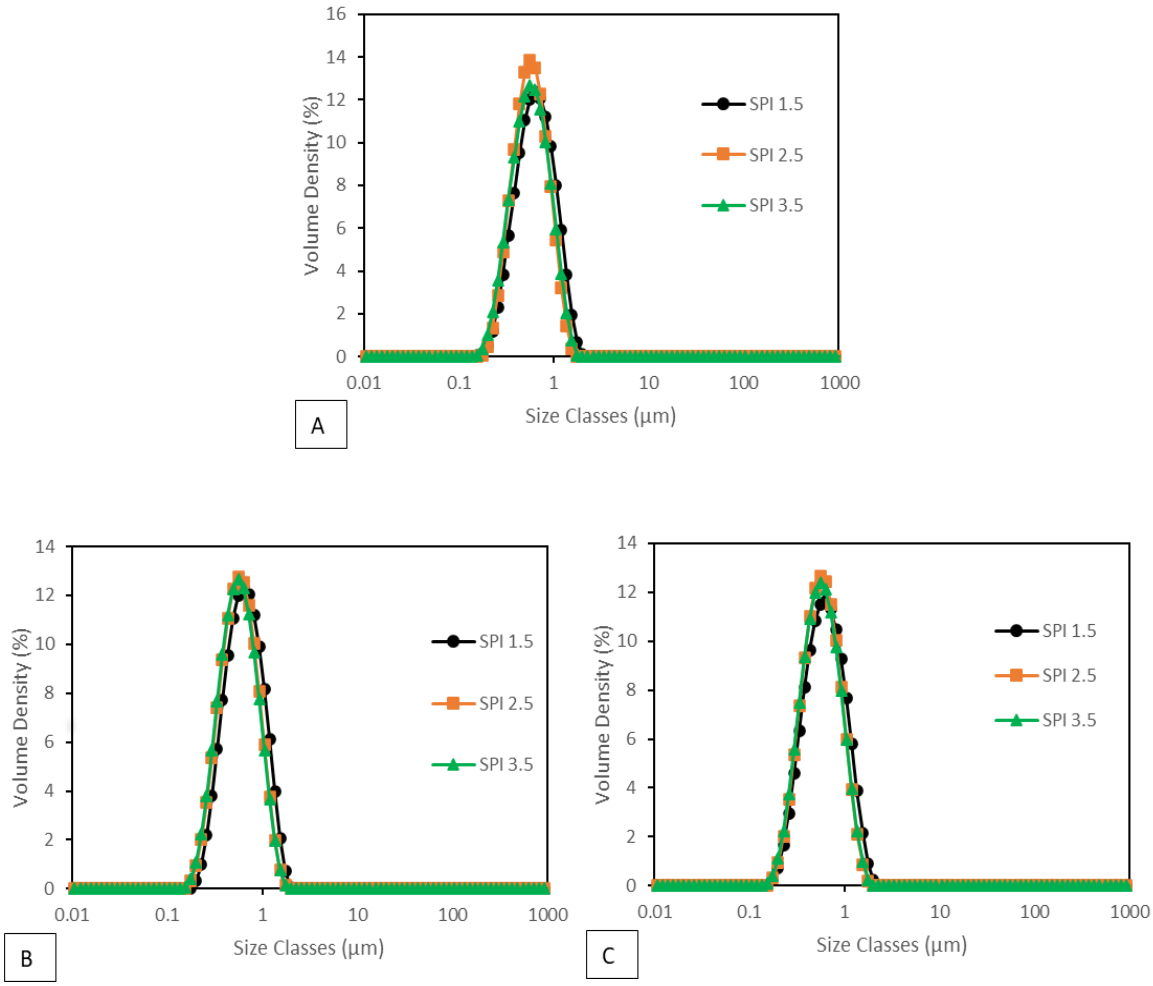


**Figure 7.** Impact of protein concentration on the particle size distribution of soy protein isolate emulsions. (A) Day 1; (B) Day 5; (C) Day 10.

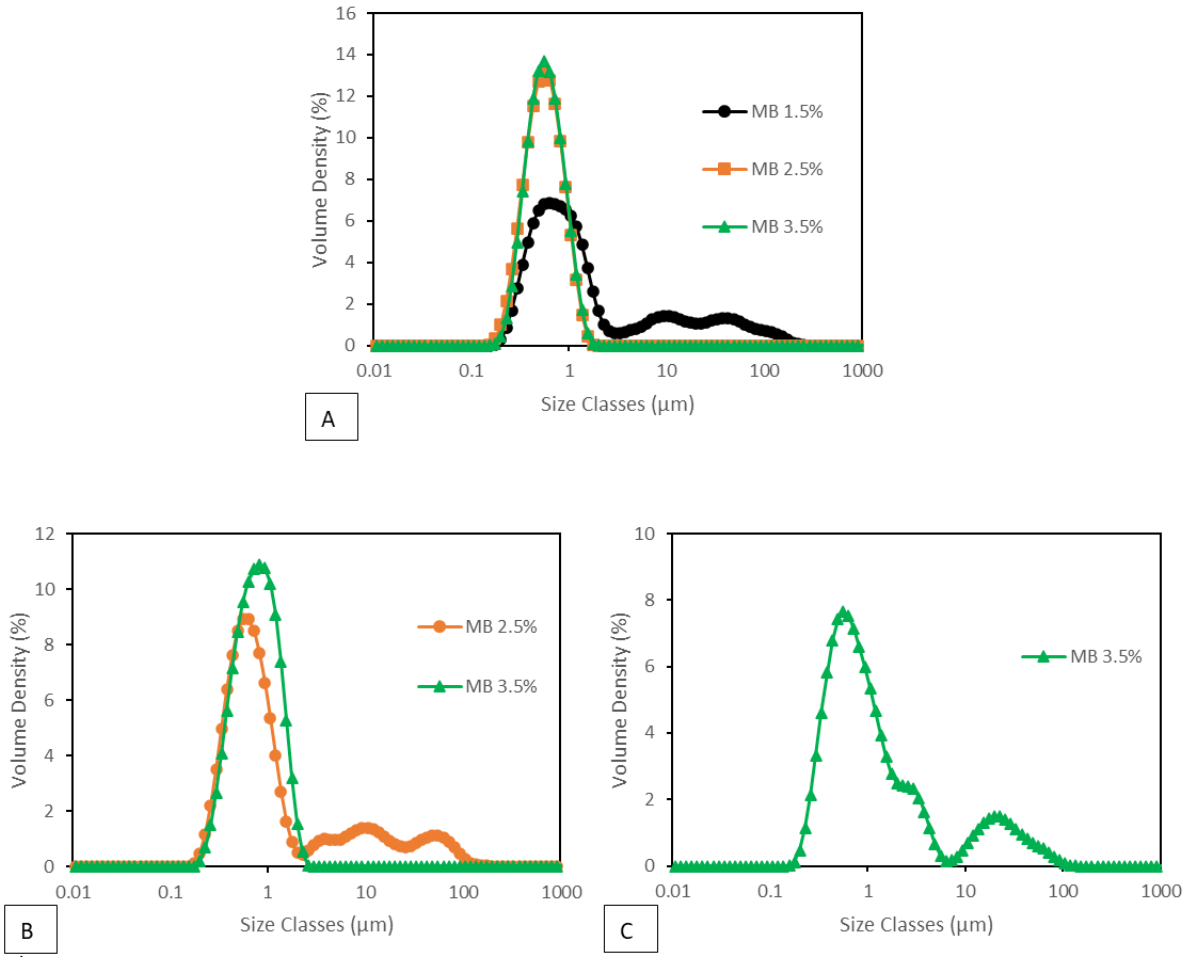


**Figure 8.** Impact of protein concentration on the particle size distribution of mung bean protein isolate emulsions. (A) Day 1; (B) Day 5; (C) Day 10.

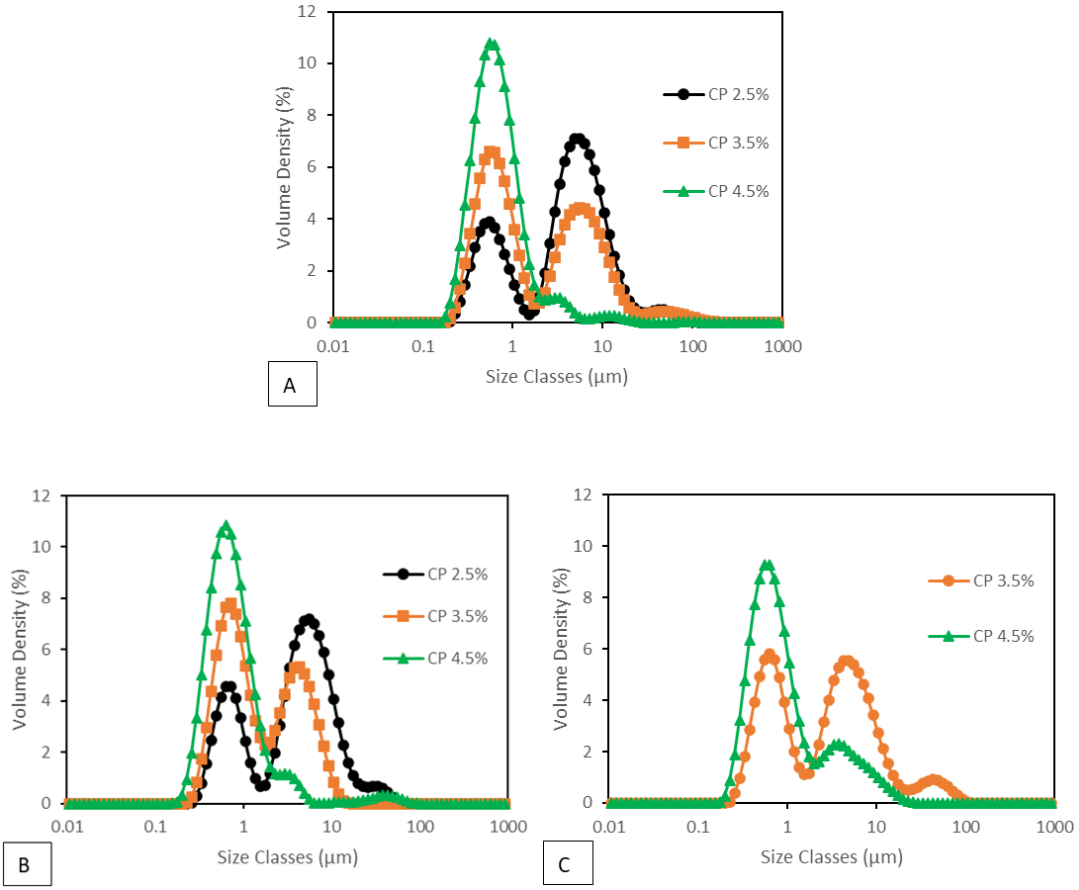


**Figure 9.** Impact of protein concentration on the particle size distribution of cassava leaf protein concentrate emulsions. (A) Day 1; (B) Day 5; (C) Day 10.

## 4. Conclusion

In conclusion, this study delved into the intricate dynamics of protein type and concentration, elucidating their significant impact on the stability and size distribution of plant-based emulsions. Notably, soy protein isolate emerged as a superior emulsifier, outperforming mung bean protein isolate and cassava leaf protein concentrate. The findings underscore that stable emulsions could be achieved with a soy protein isolate concentration as low as 1.5%. Moreover, elevating the protein concentration, particularly with mung bean protein isolate and cassava leaf protein concentrate, resulted in improved size distribution characterized by smaller oil droplet sizes, ensuring stable emulsions over a 14-day storage period. This emphasizes the nuanced relationship between protein characteristics and the formulation of stable plant-based emulsion systems, paving the way for informed applications in the food industry.

## Acknowledgements

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# Effects of Pre-Treatment and Temperature of Screw Press Extraction on Physical and Thermal Properties of Tiger Peanut (*Arachis Hypogaea*) Crude Oil

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**Abstract**

The effects of pre-treatment and extraction temperature on physical and thermal properties of crude oil were investigated on Tiger peanut (Kalasin 2) variety. The highest yield was obtained from pre-heated kernels at extraction temperature of 160°C with a yield of 38.25%, the viscosity and density of 0.0562 Pa.s and 0.869 g/ml respectively and the color parameter of $L^*$ value of 89.25, $a^*$ value of -6.91 and $b^*$ value of 28.32. The lowest yield was obtained from unheated kernels extracted at 80°C with yield of 26.89%. Pretreatment and extraction temperature had a significant effect on the extraction yield and physical properties of oil ($P \leq 0.05$). Increase in the extraction temperature had an impact on thermal behaviour of oil. The interaction of pre-treatment and extraction temperature significantly affected the extraction yield and properties of oil ($P > 0.05$). Differential scanning colorimeter (DSC) analysis showed that oil extracted at 80°C had the lowest freezing temperature of -3.06°C and melting temperature of 0.93°C. Oil extracted at 200°C had various endotherms from peak temperature of -37.36°C, -13.44°C and 0.93°C. As the extraction temperature increased additional peaks were identified at 117.13°C, 159.68°C and 170.77°C. The oil extracted at 120°C with heat pretreatment at 80°C for 15 min, exhibited rheological properties suitable for alternative use for Tiger peanut oil in food industry such as salad dressing formulation and frying process.

***Keywords**:* Oil extraction; Temperature; Physical and rheological properties

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## 1. Introduction

Peanut oil is regarded as quality oil due to its high smoking point, oxidation and thermal stability [(1)], its constitution ratio of saturated fatty acids, mono-unsaturated fatty acids and poly-unsaturated fatty acids, and the essential fatty acid ratio [(2)]. Different varieties and extraction methods have shown variation in physical and chemical properties of oil [(3)]. Therefore, efficient and economic extraction methods are essential for oil recovery without compromising the quality and efficacy of the extracted oil for desired application.

Conventional techniques of oil extraction using solvents such as hexane, ethanol and ethyl acetate have been studied and found to have high extraction yields [(4)], although poses health, safety and environmental concerns [(5)] and oil refining is needed for human consumption. Novel extraction techniques have been explored which includes microwave assisted extraction, ultrasound assisted extraction [(6)], supercritical fluid and pulse electric field assisted extraction. This techniques have high extraction yields [(7)], however they uses expensive and complex equipment which are not economically feasible for local producers and farmers.

Oil extraction by mechanical presses is commonly used to reduce potential adverse effects of solvent extraction and these involves use of hydraulic and screw presses [8]. Cold pressed peanut oil is one of the traditional edible oil in Asia and is more preferred than refined oil because they are regarded as virgin oils and offers health benefits due to preserved physical and chemical properties [9]. Screw pressing offers an efficient and economical method of extracting peanut oil with preserved natural nutrients, flavour and physical properties resulting in high quality oil [10]. Screw pressing extraction has high extraction yields than hydraulic pressing extraction [11], which contributed to the development of oil extraction screw expellers even at household level. However, the efficiency of this expellers can be influenced by factors such as extraction temperature, pre-treatment methods and screw speed [12]. Extreme extraction parameters were found to alter the physical and chemical properties that have functional properties and stability of the oil [13]. The extraction temperature plays a pivotal role in the quality and characteristics of extracted oil which affects its application in culinary and industrial purposes. Different extraction temperature leads to variation in oil composition and overall yield. Elevated temperature causes oil degradation such as formation of free fatty acid [14], affecting its quality and stability. Pre-treatment operations such as heat-treatment, size reduction and moisture content alteration usually precedes the process of oil extraction with the intention to achieve maximum yield and extraction efficiency with oil of acceptable quality.

Different authors reported different optimum extraction conditions, [15] found the optimum extraction temperature to be 50°C, [12] found it be 66°C and [16] when evaluating the performance of continuous screw pressing found out it was effective at 90°C. [17] obtained optimum yield on pre-heated kernels at 81.93°C for 7 min using hydraulic pressing and [18] obtained the highest yield on gourd seeds at heating temperature of 100°C. Although some existing automatic screw expellers has been developed for oil extraction with the highest temperature of 200°C, most research used extraction temperature ranging from 50°C to 110°C not reaching the maximum temperature of the screw expeller. In this study, Tiger peanut (Kalasin 2) was used, which is mainly cultivated by local farmers and consumed as raw snack in Thailand due to its richer flavour and unique buttery texture for alternative application. The extraction factors and their interaction for screw press extraction were investigated using full factorial design with two factors of pretreatment and extraction temperature. Extraction yield, physical properties and thermal stability of extracted crude oil were investigated.

## 2. Materials and Methods

### 2.1 Sample preparation

Tiger peanuts (KAC431) were purchased from the local market in Mae Hong Son Province. The kernels were be sorted to remove damaged seeds, that is cracked or broken seeds and were stored in cool dry place until extracted. For pretreatment, using the method described by Gao *et al.* [17], the kernels were pre-heated at 80°C in a hot air oven for 15 min before extraction, and raw Tiger peanuts were used as untreated sample for comparison.

### 2.2 Oil Pressing extraction

Samples of 100 g of unheated and pre-heated peanut kernels was carried out using automatic screw pressing expeller (GagalU, 300-800W automatic screw press) at a different temperatures as per experimental treatment. A full factorial design was used to determine the interaction between two factors being; Factor A – pretreatment and factor B – extraction temperature. Factor A had two levels (unheated (A1) and pre-heated (A2)). For factor B as independent variable had four levels (80°C (B1), 120°C (B2), 160°C (B3) and 200°C (B4)). The treatment were replicated three times. After extraction, oil samples were centrifuged at

4000 rpm for 10 min to eliminate impurities and crude oil were transferred into amber bottles and stored in darkness, and dry place at ambient for one week for further analysis.

**2.3 Physical property**

*Viscosity*

Oil viscosity was measured using Brookfield LVDV-II+ Viscometer (Brookfield engineering laboratories Inc, Germany) according to [19]. The spindle number 18 at the shear speed of 40 rpm, giving the shear rate of 52.8 1/s and the small sample holding set was used. The heating mantle was set at 25°C. After recording the viscosity the heating mantle was adjusted to different temperature range until the maximum temperature of 90°C. The viscometer reading were recorded at 25. 30, 50, 70 and 90°C from the triplicated treatments.

*Colour*

The colour of oils was measured in terms of $L^*$, $a^*$ and $b^*$ [20] and using the CIE Hunter Lab system Color Quest XE, (Color Global co. ltd, Thailand). The measurement was taken for oil from different extraction treatments. The measurements were taken from the triplicated treatments.

*Density*

The density of oil was determined by measuring the volume and the mass to calculated the density of each sample. The exact amount of oil was pipetted into the cylinder and the mass was measured to determine the density of each sample.

*Thermal properties using DSC*

Thermal properties of oil samples were measured with a differential scanning calorimeter (Perkin_Elmer DSC 8000, PerkinElmer Inc. Germany) equipped with a thermal analysis data station. The samples of 5mg were weighed into aluminum pans. The pans were sealed and equilibrated at room temperature for 10 min before DSC analysis. The samples were heated at scan temperature of -30°C to 180°C and isothermal temperature of 30°C at a heating rate of 10°C/ min, using an empty aluminum pan as the reference. The thermal transition parameters (onset (To), peak (Tp) and end (Te) temperatures and enthalpy change ($\Delta H$) were determined from the data recording software.

**2.4 Statistical analysis**

Means of triplicated treatment were determined from a complete factorial design of two independent variables. Statistical differences were estimated from MANOVA testing at a 5% level of significance and the Duncan multiple comparison test ($P \leq 0.05$). Pretreatment differences were subjected to paired independent t-test at 5% confidence level. The statistical analyses were carried out using SPSS program, release 17.0 and the optimum conditions were determined using Design – Expert version 6.0.2 based on desirability criteria.

**3. Results & Discussion**

The experimental results as shown in Table 1 shows the effects of pretreatment and extraction temperature on extraction yield, viscosity, density and color of Tiger peanut oil extracted using screw pressing. The statistical analysis showed that pretreatment and extraction temperature had significant ($P<0.05$) effects on the dependent variables and their interaction was significant. An independent sample t-test was conducted to compare the dependent variables for pretreatment. There was interaction between pre-treatment levels and extraction temperature levels in determining the yield, viscosity and density of peanut oil (Figure 1).

**Table 1.** Effect of pretreatment and extraction temperature on physical properties

| Oil Properties | Extraction temperature | | | |
|---|---|---|---|---|
| | **80**°C | **120**°C | **160**°C | **200**°C |
| *Yield (%)* | | | | |
| Unheated | 26.89 ± 4.01$^{b}$ | 30.20 ± 1.63$^{ab}$ | 30.50 ± 1.03$^{ab}$ | 35.70 ± 1.67$^{a}$ |
| Preheated | 32.32 ± 0.98$^{b}$ | 33.49 ±2.37$^{ab}$ | 38.25 ± 0.65$^{a*}$ | 37.30 ± 2.20$^{a}$ |
| *Viscosity (Pa.s)* | | | | |
| Unheated | 0.0564±0.0001$^{a}$ | 0.0542±0.0042$^{b}$ | 0.0542±0.0000$^{b}$ | 0.0542±0.0014$^{b}$ |
| Preheated | 0.0564±0.0028$^{ab}$ | 0.0568±0.0000$^{a*}$ | 0.0562±0.0014$^{b*}$ | 0.0561±0.0014$^{b*}$ |
| *Density (g/ml)* | | | | |
| Unheated | 0.834 ± 0.010$^{b}$ | 0.858 ± 0.006$^{a}$ | 0.857 ± 0.001$^{a}$ | 0.868 ± 0.004$^{a}$ |
| Preheated | 0.855 ± 0.002$^{b}$ | 0.865 ± 0.005$^{a}$ | 0.869 ± 0.001$^{a*}$ | 0.870 ± 0.007$^{a}$ |
| *L** | | | | |
| Unheated | 87.84 ± 0.00$^{c*}$ | 89.66 ± 0.01$^{b*}$ | 91.99 ± 0.01$^{b*}$ | 92.07 ± 0.01$^{a*}$ |
| Preheated | 86.02 ± 0.01$^{d}$ | 86.65 ± 0.04$^{c}$ | 89.25 ± 0.01$^{b}$ | 90.94 ± 0.00$^{a}$ |
| *a** | | | | |
| Unheated | -5.22 ± 0.01$^{a}$ | -7.05 ± 0.21$^{b}$ | -7.17 ± 0.01$^{c}$ | -7.28 ± 0.01$^{d}$ |
| Preheated | -5.02 ± 0.01$^{a*}$ | -6.09 ± 0.03$^{b*}$ | -6.91 ± 0.01$^{c*}$ | -7.01 ± 0.01$^{d*}$ |
| *b** | | | | |
| Unheated | 27.12 ± 0.01$^{d*}$ | 29.94 ± 0.03$^{a*}$ | 29.36 ± 0.03$^{b*}$ | 29.69 ± 0.01$^{c*}$ |
| Preheated | 26.33 ± 0.04$^{d}$ | 28.54 ± 0.03$^{b}$ | 28.32 ± 0.02$^{c}$ | 28.72 ± 0.01$^{a}$ |

Data are means ± SD of triplicates (n=3)
The * shows significant difference based on the independent t-test at P≤0.05
Means ± SD with followed by different letter in a row are significantly different using DMCT at P≤0.05

### 3.1 Yield

Table 1 shows that oil yield varied with treatment and the highest yield was found in extraction of pre-heated kernels and extraction temperature of 160°C at the yield of 38.25%, the lowest yield was obtained from unheated kernels and extracted at 80°C at yield of 26.89%. The highest extraction temperature of 200°C yielded 35.70% and 37.30% in unheated and preheated kernels respectively. The statistical comparison between the pre-heated and unheated samples shows that a significant difference was found at extraction temperature of 160°C. Extraction temperature had an impact on the extraction yield as yield increased with the increase of extraction temperature. The main effects interaction was found between the extraction temperature of 160°C and 200°C on preheated kernels (Figure 1) which resulted in high yields. This scenario agrees with the research conducted on tobacco seed oil yield as influenced by temperature and pretreatment at the optimum yield of 73.38% and optimized condition for mechanical oil expeller [15]. The temperature affects oil extraction as it causes the cell wall to rapture and expel the oil content therefore during screw pressing more oil will be recovered. Figure 1 (a) shows the interaction of pretreatment and extraction temperature, the treated peanut had relatively high extraction yield than untreated peanut and increasing with increase in extraction temperature even though the difference was not significant.

### 3.2 Color

Color measurement in terms of *L**, *a** and *b**, the highest L-value was 92.07 of oil unheated and extracted at 200°C and the lowest L-value was 86.02 for oil extracted at 80°C (Table 1). A significant difference (P<0.05) was found between oil samples extracted at 80°C and 120°C while the results obtained from 160°C and 200°C were not significantly different from each other and paired comparison showed that pretreatment significantly affected the

colour of oil at all extraction temperature. According to Figure 1D, the L-value and b-value of unheated samples were higher than the pre-heated samples and increasing with the increase in extraction temperature, while the a-value was lower and decreasing with increase in extraction temperature. This means that as the extraction temperature increases the oil lightness also increases. Darker colours indicate the presence of colour pigment and phenolic compounds affected by pre-heating treatment and extracted with oil during crushing process [21]. High temperatures were found to play a significant role in dissolving phosphorus in the oil [22] making them to appear pale yellow or amber and this relates to increasing in L-value and b-value. Oil color plays important role in consumer visual acceptance and product colour, darker colored oils are regarded as unrefined oils with pigments such as chlorophyll and other impurities [23].

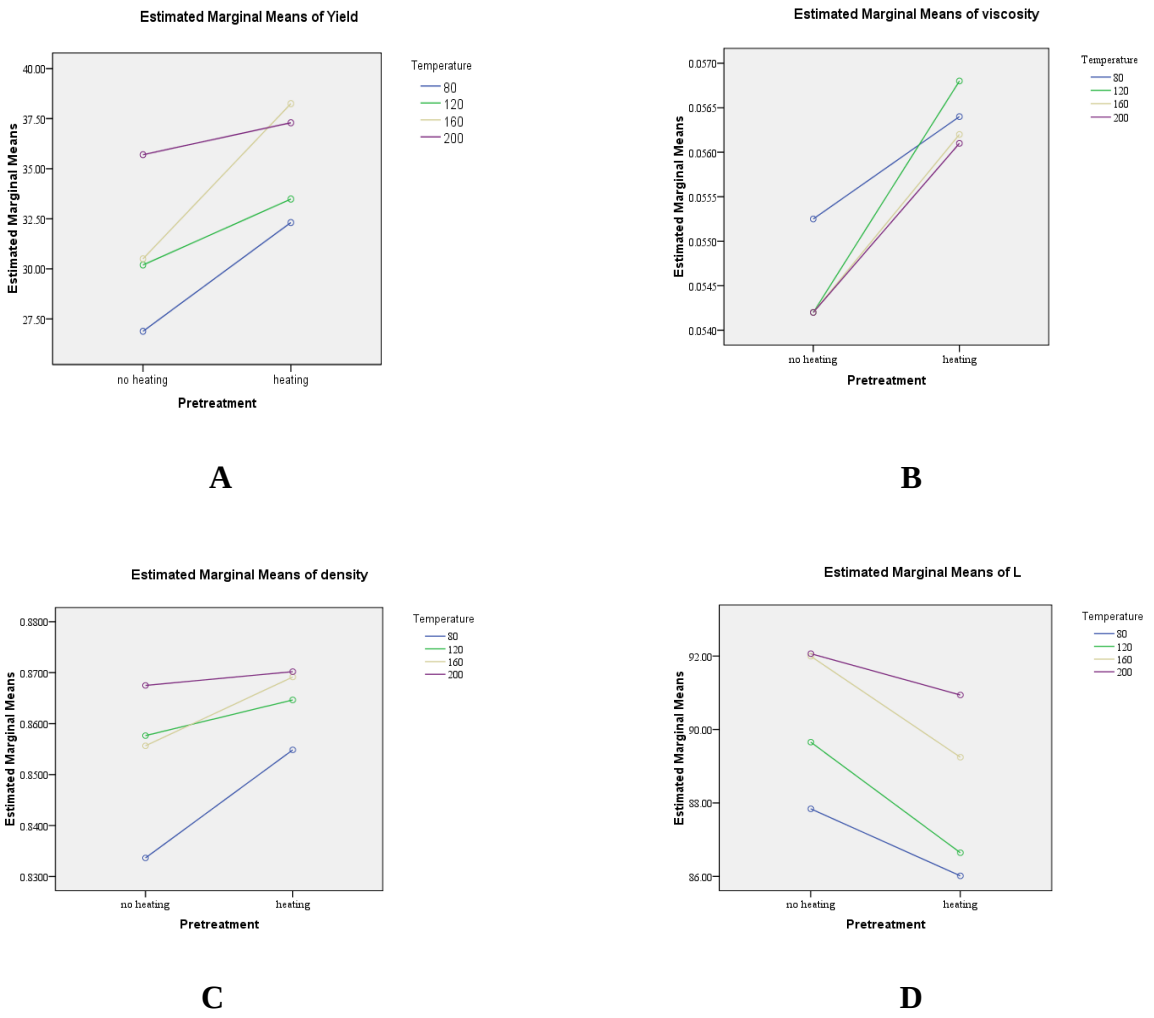


**Figure 1** Interaction of pretreatment and extraction temperature for A – yield, B – Viscosity, C – density and D – *L** - value.

### 3.3 Viscosity

Vegetable oil exhibits a characteristic viscosity that can vary depending on factors such as temperature and the presence of impurities or additives. The pre-treatment and viscometer temperature had significant effect ($P<0.05$) on the viscosity of oil, the significant effect of pretreatment was found in oil extracted at 120, 160 and 200°C (Table 1). At 25°C the oil viscosity was 0.0545 Pa.s and 0.0554 Pa.s for unheated and preheated samples respectively. The difference between the viscosity was detected by interaction between pre-treatment and

extraction temperature of 80°C and 120°C (Figure 1B). Pre-heated peanut oil had high viscosity and decreased with the increase in extraction temperature. As the extracted oil was exposed to different temperatures using viscometer resulted in decrease of the viscosity, the results shows that as temperature was increased to 90°C the viscosity decreased to 0.313 Pa.s and 0.0314 Pa.s for unheated and pre-heated samples respectively (Table 2). The linear relationship of viscosity to temperature agrees with [24] when determining the model for viscosity used in food oils at high temperatures. The reduction in viscosity as temperature increases is the results of degradation by breaking down of long chains of fatty acids that constitutes to triacylglycerol [25]. In food application such as salad dressing formulation, viscosity plays important role in emulsion stability as high viscosity in oils was found to increase the droplet size of the emulsion and increase in the increase in droplet size affect the rate of destabilization [26], lower viscous oils produces small droplets which are relatively stable [27]. Generally, vegetable oils have a low viscosity ranging from 0.040 to 0.063 Pa.s at ambient temperature [28], allowing them to flow easily due to the internal friction between the number of molecules that makes it up, which is affected by the degree of unsaturation. Tiger peanut oil was consistent with the published data of viscosity of different vegetable oil at different temperatures [29].

**Table 2**. Viscosity variation for screw pressed oil at different temperature range

| Temperature (ºC) | Unheated viscosity (Pa.s) | Pre-heated viscosity (Pa.s) |
|---|---|---|
| 25 | $0.0545 \pm 0.0051^{a}$ | $0.0564 \pm 0.0032^{a}$ |
| 30 | $0.0482 \pm 0.0026^{b}$ | $0.0488 \pm 0.0035^{b}$ |
| 50 | $0.0268 \pm 0.0041^{c}$ | $0.0266 \pm 0.0025^{c}$ |
| 70 | $0.0163 \pm 0.0034^{d}$ | $0.0157 \pm 0.0024^{d}$ |
| 90 | $0.0108 \pm 0.0050^{e}$ | $0.0106 \pm 0.0024^{e}$ |

Data are means ±SD of triplicates (n=3)
Means ± SD with followed by different letter in a row are significantly different using DMCT at P≤0.05

### 3.4 Density

According to Table 1, the significant effect from pretreatment was from the extraction temperature of 160°C with the density of 0.857 g/mL and 0.869 g/mL for untreated and treated peanut oil respectively. The treated peanut had high density (Figure 1C) and was increasing with increase in extraction temperatures which showed interaction at extraction temperature of 120°C and 160°C. Density of edible oils are correlated to its purity, exposing oils to high pretreatment and extraction temperatures results in polymerization [28] and as extractions temperature increases it causes the cell wall to rapture and expel the oil content and other impurities [30]. Less dense oils are used in salad dressing formulation and shortenings. The results obtained with Tiger peanut are within the recommended density of edible oils ranging from 0.84 to 0.93 g/mL [31] and can be suitable in formation of stable emulsion in salad dressing formulation due to the size of the droplets [27].

### 3.5 Thermal stability

The thermogram results of oil extracted at different temperatures at heating rate of 10°C /min in range of -40°C to 180°C are shown in Table 2 and 3. Extraction temperature exhibits different thermograms of the cooling and heating curve. The DSC shows the freezing, melting and degradation temperature of edible oils.

**Table 3**. DSC parameters of the peak and enthalpy from the cooling curve

| Extraction Temperature | $T_{onset}$ (ºC) | $T_{peak}$ (ºC) | $T_{end}$ (ºC) | ΔH (J/g) |
|---|---|---|---|---|
| 80 | -3.06 | -4.66 | -7.1 | 1.9710 |
| 120 | -2.75 | -4.08 | -7.19 | 3.4407 |
| 160 | -2.42 | -4.01 | -6.83 | 2.9313 |
| 200 | -2.37 | -3.61 | -5.69 | 0.7270 |

**Table 4**. DSC parameters of the peak and enthalpy from the heating curve

| Extraction temperature | Peak | $T_{onset}$ (ºC) | $T_{peak}$ (ºC) | $T_{end}$ (ºC) | ΔH (J/g) |
|---|---|---|---|---|---|
| 80 | 1 | -2.76 | 0.93 | 3.36 | 0.9191 |
| 120 | 1 | -6.38 | 0.14 | 4.87 | 3.5481 |
| | 2 | 127.61 | 127.90 | 128.42 | 0.0266 |
| | 3 | 154.50 | 155.05 | 155.29 | 0.0171 |
| 160 | 1 | -35.48 | -33.22 | -34.71 | 0.5935 |
| | 2 | 3.41 | 5.20 | 8.00 | 0.7232 |
| | 3 | 109.64 | 110.56 | 111.97 | 0.0221 |
| 200 | 1 | -38.26 | -37.36 | -36.16 | 0.1346 |
| | 2 | -15.31 | -13.44 | -11.83 | 0.2206 |
| | 3 | -1.79 | 0.93 | 3.11 | 2.1196 |
| | 4 | 80.93 | 81.4 | 82.1 | 0.1013 |
| | 5 | 116.67 | 117.13 | 118.14 | 0.076 |
| | 6 | 158.79 | 159.68 | 160.1 | 0.5791 |
| | 7 | 170.52 | 170.77 | 171.18 | 0.0274 |

The cooling curve obtained from oil extracted at 80°C to 200°C exhibited the freezing temperature of -4.66°C, -4.08°C, -4.01°C and -3.61°C respectively, with oil extracted at 80°C having lower onset temperature of -3.06°C (Table 3). All the samples shown only one freezing exotherm and almost at the same region and this corresponds to [32] about the relationship between the exothermal crystallization curve and triacylglycerol composition.

Extracted oil at 80°C had a melting point of 0.93°C and when heated to high temperature the oil sample was stable, this could be due to oil purity and oil major Tag groups not affected by extraction temperature [33] high melting temperature also correlates with the melting of TAGs containing myristic and palmitic chains [34]. The endothermal peaks of oil extracted at 160°C were identified at -33.22°C and 5.20°C, oil extracted at 200°C had various endotherms from peak temperature of -37.36ºC, -13.44ºC and 0.93ºC (Table 4). Different transition at lower temperature shows transition of one form of fat crystals to another and this correspond to the melting point of α – crystals followed by β – crystals (32). The broad melting peaks of oil extracted at 120°C and 200°C could be the results of two or more TAG structures melting simultaneously [25]

As the scanning temperature increased, various peaks were identified for oil extracted at high temperatures (Table 3). Oil extracted at 200°C had peak temperature at 117.13°C, 159.68°C and 170.77°C. This shows that high temperature extraction had effect on the thermal stability of oil. High temperatures are associated with the decomposition of TAG groups especially polyunsaturated fatty acids with the formation of compounds such as dimers and polymers [32]. The various peaks at high temperature scanning could be due to impurities of the oil as high temperature degrades other components of the oil bodies and being extracted with the oil during screw pressing.

## 4. Conclusion

Extraction temperature had a significant effects on physical and thermal properties of oil, and the pre-treatment had a significant effect on the colour and viscosity of the extracted oil. The pre-heated peanut had higher yield than unheated peanut at all extraction temperatures. According to desirability of quality parameters, the recommended condition was from pre-heated kernels at extraction temperature of 120°C complied to the standard values for edible oils for viscosity, density and desirable color. However the highest yield was obtained from extraction of pre-heated kernels and extraction temperature of 160°C at the yield of 38.25%. The linear relationship of viscosity to temperature was identified that as temperature increases the viscosity reduces. The DSC results shows that oil extracted at lower temperatures are more thermal stable than those extracted at higher temperatures. This phenomenon provides the correct selection of application of extracted oil such as in salad dressing and cooking. Formulation of salad dressing from Tiger peanut oil and its nutritional values will be further investigated.

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# Conjugation Factors Screening for Conjugated Compound of Rice Bran Peptides and Resveratrol Using Plackett and Burman Design

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**Abstract**

The purpose of this study was to investigate factors affecting the conjugation of rice bran peptides (RBPs) with resveratrol (RSV), and the biological activities of conjugated compounds. Using mild-subcritical alkaline water extraction, peptides were isolated from rice bran and then hydrolyzed by proteases G6 and GN. Peptides were fractionated to obtain three fractions with molecular weight < 3, 3-10, and > 10 kDa. The conjugate is prepared from RBPs MW < 3 kDa and RSV using Plackett and Burman design under a variety of conditions, including pH (3-9), oxygen flushing (0-10 $cm^3/s$), temperature (25-45°C), RSV concentration (0.10-1.00 mg/mL), and conjugation time (30-120 min). A total of 15 experiments were conducted to determine the significance of five factors on conjugation. According to the results of the regression analysis: pH, RSV concentration, and conjugation time were significant. The alkaline condition of pH increases DPPH radical scavenging activity and the conjugation of RBPs and RSV. Increasing RSV concentration directly affects the biological activity of the conjugated compound. Extended conjugation time increases conjugation between RBPs and RSV. For further optimization by the central composite design (CCD), the following factors were chosen: pH, RSV concentration, and conjugation time; these were set at 25°C and 10 $cm^3/s$, respectively, for the temperature and oxygen flushing.

***Keywords***: Rice Bran Peptides; Resveratrol; Conjugation; Plackett and Burman design

## 1. Introduction

Rice is a staple food for the Asian population. The production of rice in the world and Thailand in 2020 was 502.5 and 24.1 million tons, respectively [(1)]. For rice milling, there are 4 products, including white rice (70%), rice husk (20%), rice bran (8%), and rice germ (2%). But rice bran is the most nutritious part of rice, which contains lipids 15.0-19.7%, carbohydrates 34-62%, total dietary fiber 19-29%, ash 6.6-9.9%, water 8-12%, and protein 11.3-14.9% [(2)].

Currently, rice bran is being converted into rice bran protein and developed as an ingredient for food. This is due to the protein's hypoallergenic properties, ease of digestion, and high content of the essential amino acids in rice bran. The major essential amino acids found in rice bran protein were leucine, lysine, phenylalanine, threonine, and valine [(3)]. The antihypertensive properties of rice bran protein are its most notable biological benefit to the body. However, rice bran protein also has additional biological properties, including cholesterol-lowering, anti-cancer, anti-diabetic, and antioxidant activities [(4)].

Resveratrol is a natural polyphenol that has been identified in over 70 plant species, mostly in the skin and seeds of grapes. It has also been found in trace amounts in red wines and a variety of foods consumed by humans [5]. Research indicates that the primary source of resveratrol in Thailand is berry fruits, particularly mulberries. Resveratrol can be considered a nutraceutical because of its benefits, such as anticancer activity, anti-inflammatory activity, antimicrobial activity, antioxidant activity, and cardiovascular protection. The French paradox refers to the inverse relationship between red wine consumption and the prevalence of cardiovascular diseases [6]. Nevertheless, because of its low solubility and bioavailability, resveratrol utilization remains a major concern.

Foods typically contain two ingredients: protein and polyphenol. They bind each other by noncovalent and covalent interactions. Under oxidative conditions, polyphenols convert to o-quinones and then form covalent conjugates by nucleophilic reactions with active residues in proteins [7]. Conjugation may modify the chemical structures of phenolics and associated proteins, changing their biological [8-12], and functional properties [13-16]. According to a study by Fu *et al.* [17] Chlorogenic acid-gelatin (CGA-Gel) conjugates had a higher free radical scavenging than free chlorogenic acid, even though CGA-Gel has a lower amount of chlorogenic acid less than 9 %. In vitro, conjugated compounds derived from rapeseed peptides and phenolic compounds showed reduced antioxidant activity, however, cellular studies demonstrated increased antioxidant activity [18]. Wu *et al.* [19] found that the ß-lactoglobulin (ßLG) - polyphenol conjugated was effective in reducing immunoglobulin E binding capacity and improved thermal stability with high antioxidant activity. Moreover, de Morais *et al.* [20] reported that the complexation can protect phenolic compounds under the in vitro digestion condition, and the digestibility of whey protein isolated and complexes showed no difference.

There is a lot of research reported on the conjugation of rice bran protein with polyphenols, such as ferulic acid [21-22], catechin [23-24], chlorogenic acid [25], and epigallocatechin-3-gallate [26]. These research mainly used crude rice bran protein hydrolysate for the conjugation to improve its emulsifying or functional properties and use as a food additive. However, there were a few data reported recently on purified or partially purified rice bran proteins or peptides conjugated with phenolic acids, especially those that focus on biological activities improvement. Besides, there was no data reported on the conjugation of RBPs and resveratrol.

Protein-phenolic interactions are influenced by a wide range of factors, including pH, temperature, protein/phenolic ratio, oxidation, and time. Therefore, the objective of this study was to investigate factors affecting the conjugation of RBPs with RSV, and the biological activities of conjugated compounds.

## 2. Materials and Methods

### 2.1 Materials

Khao Dawk Mali 105 (*Oryza sativa* L.) Thai jasmine rice bran is obtained from rice bran oil processing using cold-pressed extraction. Resveratrol (≥ 98% purity) was purchased from Shaanxi Jintai Biological Engineering Co., Ltd. (Shaanxi, China).

### 2.2 Extraction of Rice Bran Protein Hydrolysate

According to Senaphan *et al.* [27], Khao Dawk Mali 105 rice bran was separated using a 50-mesh screen and then suspended in water at a 1:9 ratio, protein was extracted under a mild-subcritical alkaline water condition at 120°C under 15 psi for 120 min with a pH of 9.5. Then, the mixture was adjusted to pH 8.0 and 55°C before hydrolysis. The protease G6 with 580,000 DU/mL was added at an enzyme-to-RB ratio of 20 mL/kg and the protein was hydrolyzed for 4 h. After that, the protease GN with 580,000 DU/ml was added at the same ratio and further the hydrolysis process for 2 h. The protease activity was inhibited by heating

at 90°C for 15 min. After the mixture had reached room temperature, it was centrifuged for five minutes at 4,800 rpm. The supernatant was collected and the pH was adjusted to 7.5. The total soluble solid content was adjusted to 25°Brix by adding maltodextrin before spray drying at the inlet and outlet temperatures of 190°C and 90°C, respectively. The Kjeldahl technique was used to determine the protein content of the RBPH powder [28], after which it was sealed in an aluminum foil bag and stored at 5°C before use for 12 months.

**2.3 Rice Bran Peptides Fractionation**

Five grams of RBPH was dissolved in 100 mL distilled water and stirred for 10 min at room temperature and the concentration of the protein was adjusted with distilled water to obtain the final protein concentration of 10 mg/mL. Then, the RBPH solution was centrifuged for ten minutes at 4,800 rpm. The supernatant was separated into three fractions of MW < 3, 3-10, and > 10 kDa using Amicon ultra centrifugal filters with molecular weight cutoffs of 10 and 3 kDa. This was done using the method of Uraipong and Zhao *et al.*[29]. The free amino group content and biological activities of the three rice bran peptides (RBPs) fractions were examined. The fraction with the highest free amino group content and biological activities was selected for further conjugation.

**2.4 Conjugation of RBPs and RSV**

Alkali-induced covalent conjugation was used to produce RBPs–RSV conjugates, with slight modifications made following Zhou *et al.* [7]. RBPs were diluted in distilled water (2.0 mg/mL). Bovine serum albumin (BSA) was used to create a standard curve for the Biuret assay [30] which was used to calculate the protein content. Resveratrol was diluted in 25% ethanol to attain the desired RSV concentrations of 0.2, 1.1, and 2.0 mg/mL. The RBPs and RSV solutions at each concentration were mixed in a ratio of 1:1.

The effect of the various factors on the conjugation method was studied using the Plackett & Burman design using Minitab 16 for experimental design. The factors assessed during conjugation are listed in Table 1 with their corresponding values marked as -1, 0, and +1. According to Plackett and Burman design, 12 combinations of factors with three center points were organized (Table 3). Minitab 16 Statistical Software was used to generate the results of the statistical analysis.

**Table 1.** The Plackett & Burman design factor levels and their corresponding actual values.

| Factors | Code | -1 | 0 | +1 |
|---|---|---|---|---|
| pH | $X_1$ | 3 | 6 | 9 |
| Oxygen flushing ($cm^3/s$) | $X_2$ | 0 | 5 | 10 |
| Temperature (°C) | $X_3$ | 25 | 35 | 45 |
| RSV concentration (mg/ml) | $X_4$ | 0.10 | 0.55 | 1.00 |
| Conjugation time (min) | $X_5$ | 30 | 75 | 120 |

**2.5 Analyses**

*2.5.1 Determination of Free Amino Group Content*

RBPs free amino group content was measured by TNBS (2,4,6-trinitrobenzene sulfonic acid) assay [31] with some modification. 100 µl of the sample was mixed with 200 µL of 0.01% (w/v) TNBS in 0.1 M sodium bicarbonate, pH 8. After two hours of incubation at 37°C, the mixture reacted with 200 µl of 10% (w/v) SDS and 100 µL of 1 N HCl. Following, the absorbance at 335 nm was measured by a UV-Vis spectrophotometer, and the free amino group content was computed using a standard curve generated with L-leucine and expressed as nmol/mg protein.

*2.5.2 Determination of Total Phenolic Content*

The Folin-Ciocalteu technique was utilized to calculate the total phenolic contents with a few minor adjustments [32]. 1 mg of RSV was dissolved in methanol, bringing the total volume up to 10 mL; the standard solutions were obtained from dilution to various concentrations. 200 µl of the 0.2 N Folin-Ciocalteu reagent was well mixed with 50 µL of the sample or standard solution. After that, the mixture was left for ten minutes at room temperature in the dark. Following the addition of 500 µl of a 7.5% $Na_2CO_3$ (w/v) solution, which was thoroughly mixed and monitored, the reaction proceeded for 30 minutes at 25°C in the dark. A UV-Vis spectrophotometer was utilized to measure the absorbance at 765 nm, with distilled water provided as a blank control. The outcome is displayed as mg of RSVE/mg of protein.

*2.5.3 Determination of DPPH Radical Scavenging Activity*

According to Phongthai *et al*. [33], the DPPH free radical scavenging operations were carried out. One milliliter of 0.1 mM DPPH in 80% methanol was mixed with fifty microliters of the sample. The mixture was incubated in the dark at 25°C. The absorbance was measured at 517 nm using a UV-Vis spectrophotometer. RSV was utilized to make a standard curve, while distilled water served as a blank control.

*2.5.4 Determination of Ferric Reducing Antioxidant Power*

The ferric-reducing activity power (FRAP) assay was conducted by the protocol outlined by Thaipong *et al.* [34]. The FRAP reagent was prepared by combining 300 mM of pH 3.6 acetate buffer, 10 mM TPTZ in 40 mM HCl, and 20 mM $FeCl_3$ in distilled water in a 10:1:1 ratio. Fifty microliters of the sample or standard solutions were combined with 950 µL of the FRAP reagent. The mixture was well combined, then left for 30 min to sit in the dark at 25°C. RSV was utilized to generate a standard curve, distilled water served as a blank control, and a UV-Vis spectrophotometer was used to measure the absorbance at 593 nm.

*2.5.5 Determination of Angiotensin-I-Converting Enzyme (ACE) Inhibitory Activity*

The ACE activity assay kit (CS0002, Sigma-Aldrich) was modified slightly to evaluate the ACE inhibitory activity [35]. 25 µL of the sample was incubated with 25 µL ACE positive control in 96-well flat bottom plates at 37°C for 5 min. 50 µL of the substrate should be added to the sample wells to start the reaction. As soon as possible, read the fluorescence in kinetic mode in 5 cycles for 5 min. At 37°C, the fluorescence emission was measured at 405 nm (with excitation at 320 nm) using a microplate reader (Spark, Tecan Group Ltd., Mannedorf, Switzerland). ACE positive control was used as a control by mixing 25 µL of ACE positive control with 25 µl assay buffer and reacting with 50 µL of the substrate. The following equation was employed to calculate the inhibitory activity of ACE.

$$\text{ACE inhibitory activity (\%)} = [(\text{control-sample})/\text{control}] \times 100$$

Where control is ACE positive control activity (mU), and the sample is sample activity (mU).

**2.6 Statistical analysis**

Minitab 16 statistical software was used for the design and analysis of the Plackett and Burman design studies. ANOVA was used to analyze the experimental results of RBPs fractionation.

## 3. Results & Discussion

### 3.1 RBPs Fractionation

After fractionation, free amino group content and biological activities of three fractions of RBPs were analyzed and the results are shown in Table 2. The findings exhibited that RBPs with MW < 3 kDa had more free amino groups than peptides with MW 3–10 and > 10 kDa, respectively. Zaky et al. (36) reported that peptides with MW < 3 kDa had the most free amino group and higher levels of the amino acids Phe, Met, Val, Ala, and Tyr than peptides with MW 3–10 and > 10 kDa when the rice bran was hydrolyzed by Flavorzyme. In addition, RBPs with MW < 3 kDa also have higher antioxidant activities than other fractions. This might be due to the aromatic ring structure of the amino acids tyrosine and phenylalanine which were able to donate protons to scavenge the free radicals [37, 38]. Protein with longer chain lengths relocate their amino acids with nonpolar and hydrophobic R groups, such as Tyr and Phe, clustering together on the inside of the protein [39]. Therefore, DPPH and FRAP activity was decreased with the increasing molecular weight of RBPs. In addition, ferulic acid was reported to be a major phenolic acid found in rice bran. The extraction of ferulic acid from the crude Khao Dok Mali 105 rice bran was found to increase from 22.79 ± 0.40 µg/g to 222.89 ± 0.31 µg/g when mild-subcritical alkaline water extraction was applied [40]. Therefore, in this study, phenolic acids might be extracted to a high amount in the RBPs. After fractionation and elution with water several times, almost of phenolic acids might be eluted with peptides of MW < 3 kDa because of their lesser molecular. Therefore, the peptide fraction with lower molecular weight showed the highest TPC and antioxidant activities. Moreover, RBPs with MW < 3 kDa showed the highest ACE inhibitory activity. This could be because peptides with two or three amino acid residues from the rice bran might bind to ACE's catalytic sites due to the similar structure or chain length of the peptides and angiotensin I (ACE substrate). Therefore, the ACE activity was inhibited when the peptide fraction with MW < 3 kDa was used in the analysis [29]. Wang *et al.* [41] reported RBPs MW < 4 kDa were found to be the most effective in inhibiting ACE when compared to other RBPs fractions. Tyr-Ser-Lys tripeptide from RBPH has been shown to block ACE more effectively than peptides from other plant proteins, such as Lys-Phe-Tyr-Gly from the hydrolyzed wakame seaweed protein or Cys-Lys-Glu-Pro from the hydrolyzed pistachio protein. This suggests that one of the factors influencing the peptide's action is the kind of amino acid contained in its sequence. Based on the aforementioned findings, it may be inferred that RBPs MW < 3 kDa possess greater levels of biological activities and free amino groups, which could be highly probable to conjugate. Therefore, RBPs MW < 3 kDa were selected for further experiments by conjugation with RSV.

**Table 2**. Free amino group content and biological activities of each RBPs fraction.

| Fractions | RBPs < 3 kDa | RBPs 3-10 kDa | RBPs > 10 kDa |
|---|---|---|---|
| Free amino group (mmol/mg protein) | 1612.09 ± 42.91[a] | 367.77 ± 10.33[b] | 253.79 ± 3.36[c] |
| Total phenolic content (µg RSVE/mg protein) | 168.62 ± 2.91[a] | 90.79 ± 3.06[b] | 35.44 ± 1.83[c] |
| DPPH radical scavenging activity (µg RSVE/mg protein) | 226.19 ± 2.15[a] | 93.97 ± 2.60[b] | 43.01 ± 1.31[c] |
| Ferric reducing antioxidant power (µg RSVE/mg protein) | 222.00 ± 2.81[a] | 80.32 ± 2.54[b] | 41.75 ± 0.99[c] |
| ACE inhibitory activity (%) | 78.66 ± 1.22[a] | 19.25 ± 0.68[b] | 9.49 ± 1.29[c] |

[a] Different letter within the same row indicates a statistically significant difference in the mean value ± SD (P≤0.05).

### 3.2 RBPs and RSV Conjugation

In this study, screening of factors affecting the conjugation of RBPs with RSV was conducted. From the results in Table 3, the highest TPC (792.77 µg RSVE/mg protein), FRAP activity (579.13 µg RSVE/mg protein), and DPPH radical scavenging activity (2172.92 µg RSVE/mg protein) were noticed in treatment 4 and 5, respectively. TPC, DPPH radical scavenging activity, and FRAP activity enhanced at high concentrations of RSV. At low levels of all factors, especially pH, Treatment 12 has the strongest ACE inhibitory activity. Furthermore, the lowest amount of free amino group is found in treatment 7. This is most likely caused by the oxygen flushing effect, which is seen in treatments 2, 3, 5, 6, and 11.

Table 4-5 displays the findings of the regression analysis about the main effect and P-value of each factor. RBPs-RSV conjugation, the factors were significant if the P-value was less than 0.2. If the main effect was negative (-1), the amount used in the optimization stage has to be less than what is considered a low level. despite this, if the main effects were positive, then a larger value than the high level (+1) recommended should be used in further optimization [(42)].

From Table 4, the P-value of 2 factors namely, pH, and RSV concentration were less than 0.2 indicating that those 2 factors were significant. RSV concentration is a factor affecting the TPC, DPPH radical scavenging activity and FRAP activity were statistically significant (P<0.2). This is due to the fact that RSV is an antioxidant by nature; when concentration was raised, so was the quantity of TPC and the antioxidant activity. These results show that RBPs antioxidant activity can be enhanced by covalent interaction with RSV. Thus, the results demonstrated that RSV might increase RBPs antioxidant activity through the addition of hydroxyl groups [(21)]. Moreover, another significant (P<0.2) factor that influences the DPPH antioxidant activity is pH. Because changes in the pKa value correlate with changes in the ionized hydroxyl group or other functional groups of phenolic compounds, raising the pH causes an increase in the DPPH radical scavenging activity [(43)].

**Table 3.** Plackett and Burman design experiment for factors screening of RBPs-RSV conjugation.

| Treatments | Factors [a] | | | | | Responses [b] | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | $X_1$ | $X_2$ | $X_3$ | $X_4$ | $X_5$ | TPC | DPPH | FRAP | ACE | Free amino group |
| 1 | 1 | -1 | 1 | -1 | -1 | 243.23 | 297.22 | 271.13 | 77.97 | 1225.73 |
| 2 | 1 | 1 | -1 | 1 | -1 | 597.88 | 1947.01 | 446.78 | 83.51 | 1377.87 |
| 3 | -1 | 1 | 1 | -1 | 1 | 275.71 | 299.67 | 307.24 | 96.00 | 1297.05 |
| 4 | 1 | -1 | 1 | 1 | -1 | 792.77 | 1763.13 | 579.13 | 88.76 | 1325.57 |
| 5 | 1 | 1 | -1 | 1 | 1 | 720.85 | 2172.92 | 492.74 | 89.94 | 1458.70 |
| 6 | 1 | 1 | 1 | -1 | 1 | 236.73 | 225.77 | 292.34 | 76.46 | 1401.65 |
| 7 | -1 | 1 | 1 | 1 | -1 | 429.29 | 1411.13 | 423.54 | 93.43 | 1054.56 |
| 8 | -1 | -1 | 1 | 1 | 1 | 697.65 | 1920.74 | 468.50 | 92.30 | 1686.92 |
| 9 | -1 | -1 | -1 | 1 | 1 | 597.88 | 1579.25 | 414.95 | 93.40 | 1453.95 |
| 10 | 1 | -1 | -1 | -1 | 1 | 251.66 | 339.95 | 297.90 | 82.97 | 1392.14 |
| 11 | -1 | 1 | -1 | -1 | -1 | 248.95 | 291.96 | 303.46 | 91.02 | 1278.03 |
| 12 | -1 | -1 | -1 | -1 | -1 | 268.36 | 310.88 | 319.37 | 99.40 | 1653.64 |
| 13 | 0 | 0 | 0 | 0 | 0 | 156.26 | 466.88 | 384.13 | 82.20 | 1886.61 |
| 14 | 0 | 0 | 0 | 0 | 0 | 119.52 | 396.83 | 361.90 | 79.15 | 2076.80 |
| 15 | 0 | 0 | 0 | 0 | 0 | 160.51 | 592.96 | 393.23 | 80.57 | 2167.13 |

[a] $X_1$: pH; $X_2$: Oxygen flushing ($cm^3/s$); $X_3$: Temperature (°C); $X_4$: RSV concentration (mg/mL); $X_5$: Conjugation time (min) / [b] TPC: (µg RSVE/mg protein); DPPH: (µg RSVE/mg protein); FRAP: (µg RSVE/mg protein); ACE: (%); Free amino group: (mmol/mg protein)

**Table 4.** Regression analysis of Total phenolic content (TPC), DPPH radical scavenging activity (DPPH), and Ferric reducing antioxidant power (FRAP) of the Plackett and Burman design for RBPs-RSV conjugates.

| Factors | Code | TPC | | DPPH | | FRAP | |
|---|---|---|---|---|---|---|---|
| | | Main effect | P-value | Main effect | P-value | Main effect | P-value |
| pH | $X_1$ | 18.1 | 0.315 | 51.8 | 0.176[a] | 7.9 | 0.396 |
| Oxygen flushing ($cm^3/s$) | $X_2$ | -11.4 | 0.292 | 4.6 | 0.833 | -2.8 | 0.609 |
| Temperature (°C) | $X_3$ | -0.2 | 0.974 | -12.1 | 0.282 | 1.1 | 0.687 |
| RSV concentration (mg/ml) | $X_4$ | 856.2 | 0.000[a] | 3344.0 | 0.000[a] | 383.0 | 0.000[a] |
| Conjugation time (min) | $X_5$ | 0.7 | 0.528 | 1.9 | 0.435 | -0.3 | 0.673 |

[a] Variables showed significant effects for RBPs-RSV conjugates when P-value ≤ 0.2

As indicated in Table 5, pH is the only variable that influences the RBPs-RSV conjugates percentage of ACE inhibition. Since RSV is persistent in acidic conditions, the amount of ACE inhibition decreases as pH rises, and when the pH is higher than 6.8 starts to decompose [(44)]. In addition, conjugation modifies the net charge of protein molecules, influencing the solubility of the protein. This results in decreased antioxidant activity in vitro while increasing antioxidant activity in cellular antioxidant activity [(18)]. Therefore, RBPs-RSV can't bind to ACE's catalytic sites, which reduces its ACE inhibitory activity. Factors that affect the free amino group are the oxygen flushing and the conjugation time. This is because there's a greater possibility that phenolic compounds will oxidize to o-quinone and form interactions with free amino groups in proteins when there's more oxygen gas present in the system [(45)]. As a result, the amount of free amino group is decreased. Zhou *et al.* [(7)] investigated the interaction between soy protein isolate and (-)-epigallocatechin gallate (EGCG). It was discovered that the quantity of free amino groups decreased as EGCG concentration increased. That demonstrates the covalent binding of phenolic compounds with some free amino groups in proteins. Increasing the conjugation time is associated with higher RBPs and RSV bonding, as evidenced by the fact that it decreases the free amino group. It can be concluded that longer times result in binds between RSV and some free amino groups in RBPs.

**Table 5.** Regression analysis of ACE inhibitory activity (ACE), and Free amino group content of the Plackett and Burman design for RBPs-RSV conjugates.

| Factors | Code | ACE | | Free amino group | |
|---|---|---|---|---|---|
| | | Main effect | P-value | Main effect | P-value |
| pH | $X_1$ | -3.66 | 0.002[a] | -13.47 | 0.685 |
| Oxygen flushing ($cm^3/s$) | $X_2$ | -0.15 | 0.776 | -29.00 | 0.169[a] |
| Temperature (°C) | $X_3$ | -0.26 | 0.339 | -10.38 | 0.311 |
| RSV concentration (mg/ml) | $X_4$ | 6.49 | 0.279 | 40.50 | 0.854 |
| Conjugation time (min) | $X_5$ | -0.01 | 0.846 | -2.87 | 0.195[a] |

[a] Variables showed significant effects for RBPs-RSV conjugates when P-value ≤ 0.2

**Table 6.** Summary of factors affecting response values of RBPs-RSV conjugates.

| Responses | Factors affecting response value |
|---|---|
| TPC | RSV concentration |
| DPPH | pH, RSV concentration |
| FRAP | RSV concentration |
| ACE | pH |
| Free amino group | Oxygen flushing, Conjugation time |

The data from Table 6 revealed that the overall TPC, DPPH radical scavenging and FRAP activity of the RBPs-RSV conjugates antioxidant activities are influenced by the RSV concentration. For the effect of pH, the results showed that pH impacts the ACE inhibitory activity of the RBPs-RSV conjugated compounds as well as the antioxidant activity of DPPH. The percentage of inhibition of ACE activity was reduced while DPPH radical scavenging activity was increased with the increasing pH in the alkaline pH range. In addition, RSV was found to be more sensitive to oxidation to produce o-quinone and formed bonds with free amino groups in RBPs in alkaline conditions [(45)].

The oxygen flushing and conjugation time resulted in a reduction in the amount of free amino group, which means the bonding between RBPs and RSV. In the treatments with and without oxygen flushing to study the effect of the presence of oxygen in the reaction system, it was found that the presence of oxygen gas in the reaction mixture pronounced the conjugation of RBPs and RSV. However, the oxygen flushing rate showed no significant difference in the conjugation of the RBPs and RSV.

## 4. Conclusion

The studied parameters including pH, resveratrol concentration, and time were found to affect the conjugation of RBPs and RSV as indicated by the central composite design (CCD) studied. Temperature and oxygen flushing rate in the studied range showed no significant difference effect on the conjugation of the two substances but the presence of oxygen in the reaction system was necessary. Conjugation of RBPs-RSV causes a significant increase in antioxidant activities and ACE inhibitory activity. These findings suggested that peptides with lower molecular weight exhibited higher biological activities regarding the exposure of their reactive amino acid groups. This also enhanced the conjugation of the peptides with the reactive group of resveratrol and improved their biological activities. Therefore, the conjugation technique could be a promising tool for adding value to rice bran protein or peptides for nutraceutical product purposes.

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# Effect of Hydrocolloid Types and Concentration on the Properties of Three-Dimensional Food Printing Ink

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**Abstract**

Effects of types and concentrations of hydrocolloids on physico-chemical properties, apparent viscosity, and textural properties of soy protein isolate (SPI)-gelatin-based three-dimensional (3D) food ink were investigated. The proximate compositions and colors of the food ink were slightly affected by the types and concentrations of the hydrocolloid used. The apparent viscosity of the 3D-food ink was continuously decreased when the temperature was increased from 15 up to 30°C, but apparent viscosity was found to increase when the temperature was cooled back to lower than 25°C. The apparent viscosity change characteristic of the food ink with different types and concentrations of the hydrocolloid as a function of temperature was considered to support the printing ability and structure forming of the 3D-food ink. The SPI-gelatin-based with 0.5% and 1.0% sodium alginate showed the highest structure-holding ability among all types and concentrations of hydrocolloids used. SPI-gelatin-based in combination with sodium alginate, was a promising formula for the development of 3D-food ink

***Keywords**:* 3D-food Ink; Hydrocolloid; Galatin; Sodium alginate; Food printing

## 1. Introduction

One of the innovative technologies to help design bite-sized portions, obtaining adequate energy and protein, is 3D-food printing. It is a new technology to design food for the elderly or patients with swallowing problems [(1)]**.** By producing food based on the needs of consumers considering the nutritional value, calorie content, and quality is the core. Nowadays, 3D-printing technology is widely applied in food structure forming instead of molding owing to its technology in shape creation and design. The reported food ingredients for 3D-food printing were mainly carbohydrates and fats for printing cookie dough and chocolate [(2, 3)]. One of the advantages of 3D-printing is a variety of food ingredients can be mixed together to form food. However, the material must be able to easily flow out of the nozzle tip and at the same time support the weight of the next print layer without deformation [(4)].

Hydrocolloids are hydrophilic polymers obtained from plants, animals, and microorganisms, including natural or synthetically modified polymers. Normally, hydrocolloids are derived from various parts of plants such as seeds, rubber such as locust bean gum, gum arabic, roots, and stems such as starch, or from seaweed such as carrageenan, or animals such as chitin, or fermentation by microorganisms such as xanthan gum [(5)]. Typically, these hydrocolloids are a large molecular size and consist of numbers of hydroxy

(-OH) groups making it able to soluble in water. These hydrocolloids display important functions in foods such as thickeners, gelling agents, emulsifiers, and stabilizer, etc [6].

Protein composed of amino acids both essential and non-essential amino acids depending on the protein sources. These amino acids plays an important role in chemical, physical and functional properties of the proteins [7]. However, proteins are not able to perform the printing properties regarding to flowability and stability properties in the formability of the food structure itself. Therefore, additives are needed to help improve the chemical, physical, and printability of food. For example, gelatin and sodium alginate were hydrocolloids that could improve the printability, mechanical properties, and structural stability of food printing materials [8]. In addition, blending of protein and sodium alginate in the presence of water to sufficient amount could help protein to form thermoreversible gel [9] which helps facilitate loading of the ink into the printer syringe [10] and thereafter printing through the nozzle of the 3D-food printer.

This study aimed at investigation of formulation of 3D-food inks containing soy protein isolate (SPI) as a main ingredient, 3 types of hydrocolloids (sodium alginate, cappa-carrageenan, and xanthan gum) with different concentrations. The rheological, textural and physical properties as well as printability of the food inks were also examined.

## 2. Materials and Methods

### 2.1 Materials

Soy protein isolate (SPI) with 90.6% protein (dry basis) was purchased from Specialty Natural Products Co., LTD (Chonburi, Thailand). Gelatin and Sodium alginate were purchased from Union Science (Union Science, Chiang Mai, Thailand) Carrageenan and Xanthan gum were purchased from Wendt Chemie Company GmbH (Hamburg, Germany).

### 2.2 Methods

*2.2.1 Preparation of 3D Food Inks*

The 3D food inks were prepared according to the method of Chen *et al.,* 2019 [11] with some modifications. The mixtures of SPI, gelatin and 3 types of hydrocolloids including sodium alginate (S), cappa-carrageenan (C), and xanthan gum (X) at various concentrations were prepared. The concentration of the hydrocolloids were varied at the level of 0.25 to 1.00% (w/w). Firstly, all hydrocolloids were dissolved into water and stirred for 2 h. Then, 10.0% (w/w) of gelatin was added into each prepared hydrocolloid mixture and incubated at 65°C for 1 h in water bath. After that, 20% (w/w) of SPI powder was added into mixture and stirred at 800 rpm for 10 min. this mixture was referred to as “3D-food ink”. The 3D-food ink was transferred into the syringe and stored at 4°C for 24 h and warmed in the water bath at 45°C prior to use.

*2.2 2 3D-printing process*

The 3D printing process was performed using an extrusion 3D printer (Food Bot D2, Shiyin Technology Co. Ltd, China), the machine was designed and modified for food materials printing. The maximum volume of the syringe was 50 ml and the 0.84 mm diameter nozzle tip was equipped with the 3D printer. In addition, the printing parameters was fixed at 100% flow rate and printing temperature at 37°C to established the cylinder shape sample with 100 % geometry fill density with the width of 2 cm and height of 2.5 cm. The 3D-food ink sample with various types and concentration of hydrocolloids in the syringe was loaded into the 3D-food printer. After that, the syringe plunger was pushed until the food ink was extruded through the nozzle tip and deposited on the 3D-printer plate to form the first layer and then was continuously deposited layer by layer to form the designed cylindrical shape structure. All the 3D printing samples were collected in plastic box and the temperature was controlled at 25°C until analysis within 12 h.

**2.3 Analyses**

*2.3.1 Physico-chemical properties*

Physico-chemical properties of 3D-printed food sample were analyzed as follows: moisture, protein, fat and ash content [12]. Energy value was determined by Bomb calorimeter (6200, Parr instrument, USA). The color values of the sample were determined using a Hunter Lab (CR-410, Konica Minolta, Japan) and expressed as *L**, *a**, *b** values.

*2.3.2 Apparent viscosity*

The apparent viscosity of 3D-food ink was investigated using a rheometer (AR 2000, TA Instruments, USA) with modifications [11], using cone and plate with diameter of 50 mm and 0 degree geometry with the gap of 1 mm. The temperature sweep was carried out at 1 Hz of frequency with constant shear rate of $1s^{-1}$ over a temperature in a range of 50 to 15°C with a cooling rate at 2°C/min in order to measure the apparent viscosity of sample.

*2.3.3. Texture profile analysis (TPA)*

The mechanical properties of 3D-printed food samples were investigated using a texture analyzer (TA.XTplus, Stable Micro Systems, UK) with an acrylic cylindrical plate of 35 mm diameter (P/35). 3D printed food sample was placed on the texture analyzer plate.The TPA was determined according to Chen *et al.*, 2019 [11] with modifications. The test operation speed was 1.0 mm/s with 25% compression of sample deformation. The compression-decompression cycle was repeated after a time interval of 5 sec. All measurements were done in 5 replicates and the results were reported as hardness, adhesiveness, springiness, cohesiveness, gumminess, and chewiness. Each sample was tested, and the results are reported as the mean ± standard deviation.

**2.4. Statistical analysis**

All experiments were carried out in triplicate. Results were expressed as mean ± standard deviations, and the statistical significance of the data was determined by ANOVA and Duncan's multiple range tests ($P<0.05$) using SPSS software for Windows (Version 17.0, SPSS Inc.)

**3. Results & Discussion**

**3.1 Physico-chemical properties**

The Physicochemical composition analysis of 3D food ink formulas was presented in Table 1. In general, 3D-food inks formulated with different types and concentrations of hydrocolloids exhibited different physico-chemical properties ($P<0.05$). The proximate composition of 3D-food inks including moisture, protein, fat, and ash of all sample were the range of 66.81-67.68%, 16.92-17.60%, 0.04-0.11% and 4.16-4.62% respectively. Regardless of hydrocolloid type, increasing of hydrocolloid concentration resulted in the decrease of moisture content. Among all hydrocolloids used, the lowest moisture content was observed from when S and X ($P<0.05$) were used at the concentration of 1.0% wt. The highest protein content was obtained from the 3D-food ink with 0.25% C and the lowest value of fat content was found in 0.5% C 3D-food ink sample. For the energy values, the 3D food ink showed the energy value in the range of 3.29 to 3.43 kcal/g sample. Using of 0.5% S gave the highest energy value. The color parameter *L* a** and *b** were presented in Table 2. Increasing the concentrations of hydrocolloids resulted in a slight changed in lightness (*L**), redness (*a**) and yellowness (*b**) values of the food inks.

**Table 1.** Proximate composition and energy value of 3D-food ink from different types and concentrations of hydrocolloid

| Formulas | Concentration (%wt) | Moisture (%) | Protein (%) | Fat (%) | Ash (%) | Energy (kCal/g) |
|---|---|---|---|---|---|---|
| SPI-S | 0.25 | 67.15 ± 0.27$^{abc}$ | 17.59 ± 0.13$^{ab}$ | 0.04 ± 0.01$^{ab}$ | 4.39 ± 0.04$^{ab}$ | 3.41±0.01$^{ab}$ |
| | 0.50 | 66.81 ± 0.08$^{c}$ | 17.60 ± 0.40$^{ab}$ | 0.05 ± 0.01$^{ab}$ | 4.20 ± 0.12$^{ab}$ | 3.43±0.03$^{a}$ |
| | 1.00 | 67.48 ± 0.60$^{a}$ | 17.16 ± 0.18$^{c}$ | 0.04 ± 0.01$^{ab}$ | 4.53 ± 0.14$^{ab}$ | 3.39±0.35$^{bc}$ |
| SPI-C | 0.25 | 67.37 ± 0.46$^{ab}$ | 17.74 ± 0.06$^{a}$ | 0.05 ± 0.01$^{ab}$ | 4.62 ± 0.07$^{a}$ | 3.32±0.01$^{ed}$ |
| | 0.50 | 67.41 ± 0.49$^{ab}$ | 16.92 ± 0.03$^{ad}$ | 0.04 ± 0.01$^{ab}$ | 4.31 ± 0.06$^{bc}$ | 3.41±0.15$^{ab}$ |
| | 1.00 | 66.93 ± 0.09$^{bc}$ | 17.42 ± 0.05$^{b}$ | 0.05 ± 0.02$^{ab}$ | 4.48 ± 0.05$^{ab}$ | 3.3 ± 0.03$^{cd}$ |
| SPI-X | 0.25 | 66.70 ± 0.16$^{c}$ | 17.08 ± 0.15$^{cd}$ | 0.04 ± 0.01$^{ab}$ | 4.44 ± 0.06$^{ab}$ | 3.29±0.20$^{e}$ |
| | 0.50 | 66.93 ± 0.39$^{bc}$ | 17.51 ± 0.08$^{ab}$ | 0.06 ± 0.01$^{b}$ | 4.43 ± 0.06$^{ab}$ | 3.33±0.20d$^{e}$ |
| | 1.00 | 67.68 ± 0.11$^{a}$ | 17.12 ± 0.24$^{cd}$ | 0.11 ± 0.02$^{a}$ | 4.16 ± 0.15$^{c}$ | 3.32±0.15$^{ed}$ |

**Note:** * % wet basis; physico-chemical properties
Data were expressed by means ± standard deviation (SD), values with different letters in a column are significantly different (P<0.05), according to Duncan's multiple range tests.

**Table 2.** Color values of 3D-food ink from different types and concentrations of hydrocolloid

| Formulas | Concentration (%wt) | *L** | *a** | *b** |
|---|---|---|---|---|
| SPI-S | 0.25 | 60.79 ± 0.32$^{c}$ | 0.57 ± 0.03a | 16.740.18$^{ab}$ |
| | 0.50 | 60.75 ± 0.21$^{ab}$ | 0.54 ± 0.04$^{ab}$ | 16.65 ± 0.33$^{ab}$ |
| | 1.00 | 61.02 ± 0.35$^{b}$ | 0.48 ± 0.02$^{cd}$ | 16.68 ± 0.34$^{ab}$ |
| SPI-C | 0.25 | 60.36 ± 0.12$^{ab}$ | 0.48 ± 0.01$^{cd}$ | 16.36 ± 0.11$^{b}$ |
| | 0.50 | 60.56 ± 0.33$^{ab}$ | 0.49 ± 0.02$^{cd}$ | 17.02 ± 0.43$^{a}$ |
| | 1.00 | 60.57 ± 0.53$^{ab}$ | 0.47 ± 0.02$^{d}$ | 17.06 ± 0.20$^{a}$ |
| SPI-X | 0.25 | 60.69 ± 0.34$^{ab}$ | 0.49 ± 0.02$^{cd}$ | 16.65 ± 0.12$^{ab}$ |
| | 0.50 | 61.06 ± 0.17$^{b}$ | 0.58 ± 0.02$^{a}$ | 16.74 ± 0.31$^{ab}$ |
| | 1.00 | 62.07 ± 0.45$^{a}$ | 0.52 ± 0.03$^{bc}$ | 16.59 ± 0.14$^{ab}$ |

**Note:** Data were expressed by means ± standard deviation (SD), values with different letters in a column are significantly different (P<0.05), according to Duncan's multiple range tests.

### 3.2 Apparent viscosity of 3D-food ink

The apparent viscosity generally affected the printability of the 3D-food ink [(4)], Alterations in 3D food inks viscosity were also interfered their flow characteristics. This assessment was conducted at the temperature in the range of 50 to 15°C on the temperature sweep curve which presented in Figure. 1.

The results showed that an increase in hydrocolloid concentration in 3D-food ink resulted in the increased of viscosity of all types of hydrocolloids, especially when the temperature was decreased from 50 to 15°C. These viscosity-temperature changing patterns of each hydrocolloid used in the food ink revealed the unique behavior of hydrocolloid type. The concentration was also determined the viscosity behavior. The hydrocolloid used should exhibit an apparent viscosity that was sufficiently low to facilitate smooth extrusion through a narrow diameter nozzle. The viscosity profiles of this investigation suggested that using of printing temperature at higher than 30°C would cause the viscosity of food inks with all type and concentration of hydrocolloids to be low enough to extrude through the printer nozzle. The similar characteristics were also reported [(14)]. However, after the printing the viscosity should be high enough to support the deposited structures layer by layer. Moreover, the combination of hydrocolloids with gelatin showed the liquid-like behavior at temperatures above 30°C and became gel-like at temperatures below 30°C, which was similar to that of the flow behavior of gelatin [(15)]. From this studies, apparent viscosity of 3D-food ink could be

modified using several types and concentration of hydrocolloids, however the amount used should also comply with food safety standards [3,16].

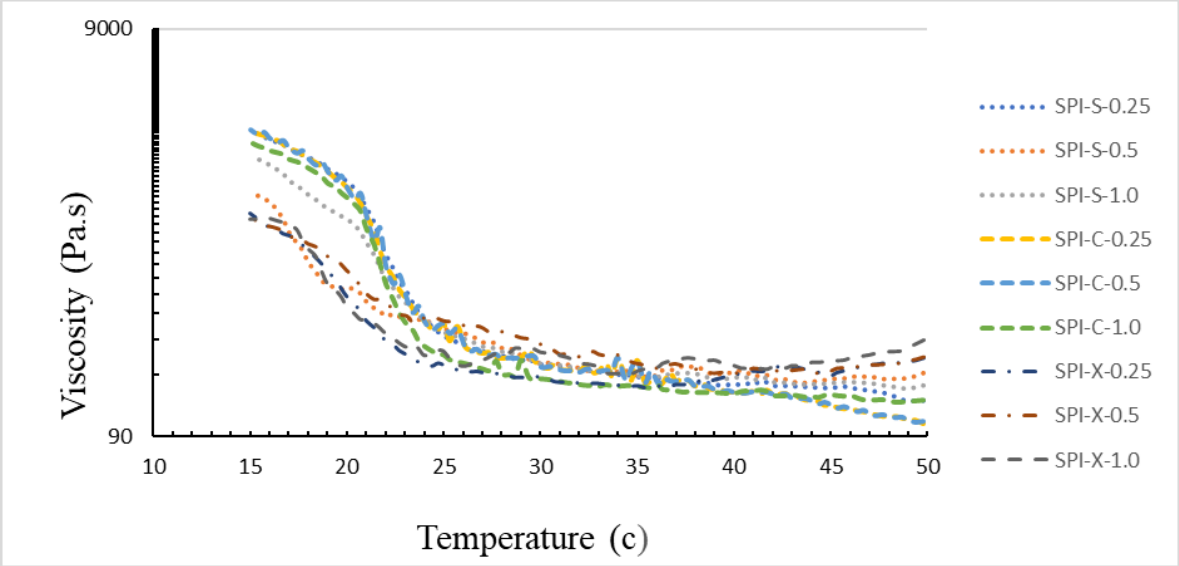


**Figure 1.** Temperature sweep curves of 3D-food Inks at temperature of 50°C to 15°C with a cooling rate 2°C/min with a constant shear rate of $1s^{-1}$

### 3.3. Texture profile analysis

The effect of types and concentrations of hydrocolloid on the texture profiles of the 3D printed food were shown in Table 4. The hardness was referred to the maximum force of the first compression. Adhesiveness was measured by the negative work between two cycles. Cohesiveness indicated adhesion within the sample. Springiness was described by the product that was able to spring back after deformation. Chewiness and gumminess were the force required to chew a product until it was ready to be swallowed [17]. From the results, hardness and adhesiveness increased with increasing hydrocolloid concentration from 0.25% to 0.5% but decreased with further increasing of concentration to 1.0%. Cohesiveness, springiness, gumminess and chewiness were increased with increasing concentration. Furthermore, an increase in the concentration of hydrocolloid was also resulted in the increasing stability of the 3D-printed food as shown in Figure 2. The increasing of hydrocolloid concentrations from the left-hand-side to the right-hand-side in Figure 2 showed that the printed food could hold its structure to a higher extent when the concentration of the hydrocolloids was increased. Among all type of hydrocolloids used, 3D-printed food with sodium alginate exhibited a higher hardness and chewiness values at the same concentration.

**Table 3.** Texture profile analysis (TPA) of 3D-printed food from different types and concentrations of hydrocolloid

| Formulas | Concentration %wt | Hardness (g) | Adhesiveness (g.s) | Cohesivenes | Springiness (%) | Gumminess | Chewiness |
|---|---|---|---|---|---|---|---|
| SPI-S | 0.25 | 475.92±23.28$^{ab}$ | -120.32±3153$^{ab}$ | 0.35±0.04$^{e}$ | 35.38±2.01$^{b}$ | 168.42±29.91$^{b}$ | 59.94±13.99$^{c}$ |
| | 0.50 | 465.18±10.73$^{ab}$ | -161.32±3475$^{bc}$ | 0.42±0.04$^{de}$ | 34.95±5.42$^{b}$ | 206.83±26.94$^{ab}$ | 90.35±10.93$^{ab}$ |
| | 1.00 | 454.26±29.19$^{ab}$ | -166.07±3026$^{c}$ | 0.46±0.12$^{cde}$ | 43.45±2.49$^{b}$ | 205.89±30.64$^{ab}$ | 91.2±13.81$^{ab}$ |
| SPI-C | 0.25 | 467.16±5.52$^{ab}$ | -118.13±11.34$^{at}$ | 0.36±0.03$^{e}$ | 33.29±0.33$^{b}$ | 169.98±9.21$^{b}$ | 56.68±3.96$^{c}$ |
| | 0.50 | 389.91±6.78$^{bc}$ | -129.73±12.62$^{ab}$ | 0.58 ± 0.13$^{abc}$ | 42.74±0.56$^{b}$ | 221.47±8.16$^{ab}$ | 88.29±7.83$^{ab}$ |
| | 1.00 | 312.13±5.46$^{cd}$ | -90.90±6.16$^{a}$ | 0.60±0.02$^{ab}$ | 59.90±1.02$^{a}$ | 187.07±2.14$^{b}$ | 106.44±1.22$^{ab}$ |
| SPI-X | 0.25 | 441.68±27.02$^{ab}$ | -156.90±9.88$^{bc}$ | 0.51±0.03$^{bcd}$ | 38.34±5.83$^{b}$ | 225.97±28.53$^{ab}$ | 85.09±1.23$^{ab}$ |
| | 0.50 | 543.97±28.46$^{a}$ | -215.54±15.24$^{d}$ | 0.18±0.03$^{bcde}$ | 36.96±3.64$^{b}$ | 259.53±18.55$^{a}$ | 96.13±4.20$^{ab}$ |
| | 1.00 | 284.99±10.85$^{e}$ | -93.19±9.18$^{a}$ | 0.68±0.05$^{a}$ | 61.30±1.57$^{a}$ | 195.22±24.49$^{b}$ | 120.30±5.83$^{a}$ |

**Note:** Data were expressed by means ± standard deviation (SD), values with different letters in a column are significantly different (P<0.05), according to Duncan's multiple range tests.

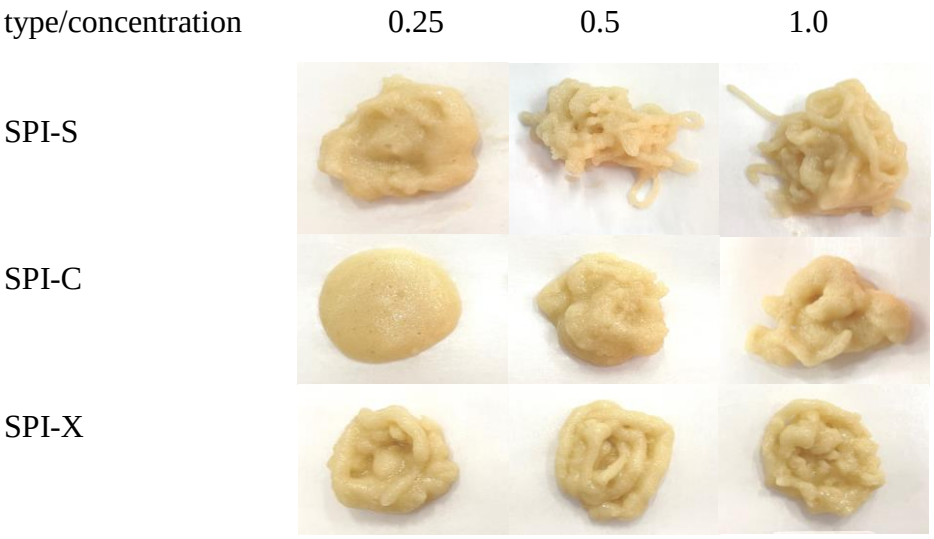


**Figure 2.** Geometry shape of 3D-printed food from different types and concentrations of hydrocolloid

## 4. Conclusion

Type and concentration of hydrocolloid were found to influence the physico-chemical properties, apparent viscosity and textural properties of 3D-food ink in which imply the printing ability of the food ink. Soy protein isolate-gelatin based 3D-food ink in combination with sodium alginate could performed a proper printability among all type of hydrocolloids in this investigation. The printability of the 3D-food ink was observed at the temperature above 30°C. However, the optimum printing and cooling condition of this food ink need to be more studied. The data obtained from this investigation could be a benefited for further research on formulation of 3D-food ink and also the application for commercialization.

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***Poster Presentation***

# The Effects of Dried Oyster Mushroom on the Property of Cake and Its Quality Changes during Storage

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## Abstract

The cultivation of edible mushrooms such as oyster mushrooms in Thailand has been gaining attention. It can grow on several lignocellulosic residues such as agro-forestry wastes and agro-industrial wastes. Mushrooms are the fruiting bodies of certain types of fungi. It is well appreciated for its exquisite taste and flavor and rich in fiber. The study aimed to observe the effect of different levels of dried oyster mushroom on physicochemical properties and sensory characteristics of cakes. Wheat flour was replaced with dried oyster mushrooms at 0% (as a control), 10%, 20% and 30 % by weight. Results demonstrated that the increase in dried oyster mushroom tended to decrease water activity of cakes but increase 1.54 to 2.41 folds in fiber content compared to control. Increasing the level of dried oyster mushrooms significantly increased specific volume and redness ($b^*$) of cakes meanwhile decreasing in lightness ($L^*$) of crumb. The cake containing 20% dried mushroom was found to be similarly acceptable to control. The quality change during storage 0, 2, 4, 6 days was evaluated. The moisture content of mushroom cake decreased during storage. Meanwhile, water activity and sensory score values of dried oyster mushroom cake presented similar qualities during 6 days. The presence of total plate count and yeast and mold were lower than the Thai Community Product Standard (459/2549). Additionally, consumer acceptance and factors affecting consumer's purchase decisions were also investigated. The overall acceptance of the final product was 4.16 from a total score of 5 indicating like slightly. The results revealed that the most important factor for a purchase decision was the taste of the product. It can be concluded that the cake replacement wheat flour with 20% dried oyster mushroom could be used for cake preparation to achieve higher fiber content and acceptable cake quality to produce an alternative product for consumers.

***Keywords****:* Dried mushroom; Cake; Physicochemical properties; Consumer acceptance

## 1. Introduction

Thailand is an agricultural country which is one of the world leaders in agricultural products and exports [(1)]. After harvesting there will be a large amount of agricultural residues; rice straw, and other crops. In common practice, these residues often end up by sending to landfills [(2)] or in some cases are not properly managed without taking care of the surrounding environment. Meanwhile these residues can be applied to many utilizations such as the source of biomass feedstocks for supplementary fuel [(3, 4)] production of cellulose for application in biocomposites material [(2, 5)] and cultivation of mushrooms. The conversion of lignocellulosic wastes for cultivation of mushrooms using biotechnological techniques is a friendly environmental method with provides rich nutrient products [(1)]. Mushrooms are incredibly popular in different parts of the world. They are well appreciated for their delicate taste and

unique flavor [6]. Edible mushrooms have a great nutrient since they are rich in protein with essential amino acids, fiber, vitamins (B1, B2, B12, C, D and E). Moreover, they are low in calories, sodium, fat, and free-cholesterol [7-10]. Among different varieties, oyster mushroom (*Pleurotus sajorcaju*) is very famous. It is the third most edible mushroom cultivated in the temperate and subtropical regions of the world [1]. The production of *Pleurotus* mushrooms is approximately 25% of that total cultivated mushrooms globally [11]. The saccharidic complex of oyster mushrooms is characterized by a low content of digestible carbohydrates and by a relatively high content of polysaccharides that take part in the formation of edible fiber [6]. Moreover, previous studies indicated that specific mushrooms can be considered as a source of natural antioxidant, antimicrobial, and anticancer compounds [6, 12, 13]. In recent years, dietary fiber has received increasing attention from researchers and industry due to its beneficial effects on the reduction of coronary heart-related diseases, cholesterol levels, diabetes incidence and risk of colon cancer [14, 15]. Although the utilization of mushrooms in various products such as sauces, pickles, soups, etc. are favorite globally, however, the use of mushroom powder in cereal and bakery products such as cakes, breads, and biscuits is very scanty. Therefore, the study of incorporation of mushrooms in food products like bakeries has gained more attention by partially substituting the wheat flour to add up the protein and fiber content, and other nutrients as well in the functional product such as baked goods [6, 16-19]. Some previous studies reported that the volume of cake increases with increasing mushroom content and then the cake becomes softer [17]. However, the addition of fibers sometimes causes a negative impact on the quality of bakery products, and leads to decrease of sensory acceptance [20, 21]. The lightness of cake decreases with increasing levels of mushroom [16, 17]. The study of consumer behavior is concerned with how individuals decide to spend their available resources such as money, time, and effort to get a product or service [22]. Understanding buyer behavior is complicated because several factors can influence consumer behavior before making a purchase decision such as social media, store atmosphere (23), perceived value and sale promotion [24] and brand [22] so the marketing team has to create an effective marketing plan that motivates consumers to purchase. Therefore, this research aimed to observe the effect of different levels of dried mushroom on physico-chemical properties and sensory characteristics. And the further assessments were to investigate factors affecting consumer's purchase decisions and consumer acceptance and evaluate the change of quality during storage.

## 2. Materials and Methods

### 2.1 Material

The oyster mushroom was collected from the mushroom farm located in Ban Wanghong, Phrae province, Thailand. Wheat flour (Red Lotus brand, UFM Food Center Co., Ltd, Thailand), butter (Allowri brand), white sugar (Mitr Phol Co., Ltd.), baking powder (Imperial brand), sterilized milk (Carnation Extra brand), Vanilla flavoring agent (Winner brand) and fresh whole eggs (~50 g/piece) were used in this study.

### 2.2 Preparation of dried mushroom

Mushrooms were prepared according to the modified method of Al-zamani *et al.*[18]. In brief, mushrooms were washed with tap water to remove dust, sand and other undesirable material before use. The clean mushrooms were then teared into small pieces of 3 mm width and dried in a tray dryer at 70°C for 3 h until moisture content of 10%. The dried mushrooms were cooled at room temperature, ground with a grinder into small pieces as shown in Figure 1. The ground mushroom was sieved to pass through a 40-mesh sieve and packed in an airtight box and stored in ambient condition.

**Figure 1.** Dried oyster mushroom

### 2.3 Preparation of mushroom cakes

The cakes were prepared by partially replacing the wheat flour at four different levels of dried mushroom (0%, 10%, 20%, and 30%) along with other basic formulation as shown in Table 1. All ingredients were weighed accurately. The cake was manufactured using the following steps. The wheat flour and baking powder were sieved through a 150-mesh sifter. Next, the white sugar and butter were mixed in a mixing machine for 15 min to produce cream. The fresh egg, sterilized milk, vanilla flavoring agent, wheat flour and dried mushroom were mixed in a mixer at lower speed for 3 min to ensure distribution of the components. The batter was scaled into a pre-greased cake pan. All cakes were baked in an electric oven (Sharp EO-70K) for 30 min at 170°C. After baking, it was removed from the baking pan and left to cool for 1 h at room temperature. Physical properties and sensory evaluation were carried out on the cakes. The rest of cooled cakes were kept in an airtight box and put in a freezer at 8°C until used for further chemical analysis. The selected formulation was used for further study on the change of quality during storage and consumer evaluation.

**Table 1.** The formulation of cakes.

| Ingredients (g) | Ratios of wheat flour to dried mushroom | | | |
|---|---|---|---|---|
| | **100:0 (Control)** | **90:10** | **80:20** | **70:30** |
| Cake flour | 380 | 342 | 304 | 266 |
| Dried mushroom | 0 | 38 | 76 | 144 |
| Fresh egg | 48 | 48 | 48 | 48 |
| White sugar | 350 | 350 | 350 | 350 |
| Sterilized milk | 220 | 220 | 220 | 220 |
| Butter | 335 | 335 | 335 | 335 |
| Baking powder | 12 | 12 | 12 | 12 |
| Vanilla flavoring agent | 14 | 14 | 14 | 14 |

**2.4 Method of analysis**

*2.4.1 Chemical properties analysis*

The chemical properties of cakes include moisture content, water activity and fiber content. Moisture content (oven at 105°C) and fiber were evaluated according to the official methods of the AOAC [25]. Water activity was measured using a water activity meter (Novasina, Model NV260 0179, Switzerland). All analyses were performed in triplicates.

*2.4.2 Physical properties analysis*

The physical characteristics of cakes include specific volume and color of crumb. The specific volume (mL.g-1) of the cakes was obtained by the ratio between the apparent volume (mL) and the mass (g) after baking. The mass was obtained by weighing the cakes on a precision scale (Sartorius, Entris 224, Germany). The apparent volume of the cakes was determined by the method of displacement of sesame seeds [26]. The color of crumb was measured using a colorimeter (Colorimeter, NH300, China). Results were expressed in CIE color values; $L^*$ = lightness (0 = black, 100 = white), $a^*$ ($-a^*$ = greenness, $+a^*$ = redness) and $b^*$ ($-b^*$ = blueness, $+b^*$ = yellowness). All the analyses were performed in triplicates.

*2.4.3 Sensory evaluation*

Sensory characteristics were evaluated by 30 untrained panelists who are staff and students of Maejo University-Phrae Campus, Thailand. The level of preference for mushroom cakes was scored using a 9-points hedonic scale test. The panelists obtained samples and were requested to rate them based on level of preference on a 9-point hedonic scale (1 = dislike extremely, 2 = dislike very much, 3 = dislike moderately, 4 = dislike slightly, 5 = neither like nor dislike, 6 = like slightly, 7 = like moderately, 8 = like very much, and 9 = like extremely) to evaluate the product attributes (appearance, color, flavor, taste, texture, and overall acceptance). Panelists were instructed to rinse their mouths with water between samples to minimize any residual effect following Penjumras *et al.* [27]. The randomized complete block design (RCBD) was used for sensory evaluation due to untrained panelists. The analysis of variance, and comparing the difference between the average by Duncan's New Multiple Range Test method at the 95% confidence level was performed.

*2.4.4 The measurement of quality during storage*

The quality of selected formulation was observed during storage for 6 days. After baking, cakes were removed from pans and cooled at room temperature then packed in polyethylene bag and stored at room temperature for 6 days. Moisture content, water activity, sensory preference using 9-point hedonic scale were determined. The microbial growth of both total plate count (TPC) [28] and yeast and mold [28] were evaluated during storage for 6 days.

*2.4.5 Consumer testing*

The selected formulation was studied on consumer testing. Purchase decision was measured using a five-item scale. Evaluation of factors affecting consumer's purchase decision on cake was collected using a survey method from 100 consumers (scale: 1 = not important, 2 = of little importance, 3 = moderately important, 4 = important, 5 = very important). Furthermore, the measurement of consumer acceptance in the final product by scoring was also investigated (1 = dislike very much, 2 = dislike slightly, 3 = neither like nor dislike, 4 = like slightly, 5 = like very much).

*2.4.6 Statistical analysis*

Data were subjected to Analysis of Variance (ANOVA) using SPSS for Window version 24. In case of any differences in mean, multiple comparisons were performed using Duncan's Multiple Range Test (DMRT) at 5% level of significance ($P \leq 0.05$).

## 3. Results & Discussion

### 3.1 Effects of different levels of dried oyster mushroom on the properties of the cake

The cakes were prepared by incorporation of dried mushroom at different ratios of wheat flour to dried mushroom for 100:0 (control), 90:10, 80:20, and 70:30. Chemical, physical properties and sensory characteristics of cakes were investigated. The results are presented in Tables 2, 3 and 4, respectively.

**Table 2.** The effect of ratios of wheat flour to dried mushroom on chemical properties of cake.

| Chemical Properties | Ratios of wheat flour to dried mushroom | | | |
|---|---|---|---|---|
| | 100:0 (Control) | 90:10 | 80:20 | 70:30 |
| Moisture content (%) | $24.09 \pm 1.33^{a}$ | $24.31 \pm 0.48^{ab}$ | $25.18 \pm 0.29^{ab}$ | $25.62 \pm 0.26^{b}$ |
| Water activity | $0.88 \pm 0.02^{d}$ | $0.82 \pm 0.10^{c}$ | $0.77 \pm 0.09^{b}$ | $0.76 \pm 0.03^{a}$ |
| Fiber content (%) | $0.22 \pm 0.01^{a}$ | $0.34 \pm 0.00^{b}$ | $0.42 \pm 0.00^{c}$ | $0.53 \pm 0.01^{d}$ |

Mean ± standard deviation values followed by a different letter within the same row are significantly different (P≤0.05) by Duncan's multiple range test

**Table 3.** The effect of ratios of wheat flour to dried mushroom on physical properties of cake.

| Physical Properties | Ratios of wheat flour to dried mushroom | | | |
|---|---|---|---|---|
| | 100:0 (Control) | 90:10 | 80:20 | 70:30 |
| Specific volume ($cm^3/g$) | $1.51 \pm 0.00^{a}$ | $1.71 \pm 0.02^{b}$ | $1.80 \pm 0.23^{c}$ | $1.82 \pm 0.00^{c}$ |
| Color values | | | | |
| - *L** | $63.26 \pm 0.63^{c}$ | $52.66 \pm 0.44^{b}$ | $52.47 \pm 0.41^{b}$ | $37.54 \pm 0.10^{a}$ |
| - *a** | $6.40 \pm 0.34^{a}$ | $7.77 \pm 0.19^{b}$ | $9.89 \pm 1.41^{c}$ | $12.58 \pm 2.80^{c}$ |
| - *b** | $38.73 \pm 0.93^{a}$ | $40.28 \pm 1.68^{a}$ | $39.63 \pm 1.58^{a}$ | $37.28 \pm 1.53^{a}$ |

Mean ± standard deviation values followed by a different letter within the same row are significantly different (P≤0.05) by Duncan's multiple range test

From Table 2, ANOVA showed significant differences (P≤0.05) in chemical properties of cakes. The moisture content showed significantly different results when replacing dried mushrooms at 30% meanwhile other levels provided similar results to control but the decrease in water activity of cake samples was observed with raising the replacement level. The increase in dried mushroom tends to increase 1.54 to 2.41 folds in fiber content. This result is in agreement with Sheikh et al. and Al-zamani *et al.* [16, 18] who found that moisture content of cakes increases with the increase of mushroom powder. The increase in fiber content of cakes could be related to their high quantities in dried mushrooms which are rich in fiber content [6-10]. However, the increasing level of mushroom led to reduction of water activity of the cakes. This could be related to free water being entrapped by fiber then led to decreased water activity of the cakes.

Table 3. shows significant differences (P≤0.05) of specific volume and color values of cake's crumb (*L**, *a**, *b**). The specific volume of baked cake indicates the amount of air that remains in the final product [29]. The specific volume of cakes was found to increase with the

increase of dried mushrooms. In general, a high concentration of soluble dietary fibers such as hemicellulose and pectin can induce a high water binding capacity [30] then the structure setting must be expanded by water vapor during oven-baking [31]. Thus, the resulting cake is highly aerated and gives higher specific volume. This study found a similar result with previous study of Jahanbakhshi and Ansari [30] who reported that the specific volume of cake caused an increase in the olive stone powder which is rich in dietary fiber. Lebesi and Tzia [32] also reported that the volume of cake increased significantly parallel to increasing level of dietary fiber from wheat, oat, barley and maize. In addition, the similar result was also found by Al-Sayed and Ahmed [33]. The specific volume of cake increases by increase of dietary fiber from watermelon rinds and sharlyn melon peels [33]. However, the result found in this study contrasts with the previous study of Aydogdu *et al.* [15] who found that cakes enriched with lemon fiber had the lowest specific volume and the highest hardness value. Among fibers, lemon fiber had the highest water binding capacity. When the fiber with high water binding capacity was used, wheat flour could not absorb enough water to develop of gluten-protein network and rigid structure occurred [34]. Similar result with Aydogdu *et al.* [15] was observed by Das *et al.* [6], the cake enriched with freeze dried mushroom powder had higher in moisture content but lower in specific volume. Concerning crumb color, in general, as the dried mushroom level increased $L^*$ value decreased but the $a^*$ value increased, while there was no significant difference in $b^*$ value. This indicated that the crumb color became darker and more redness. However, the temperature of the cake crumb during baking might not be sufficient for the Maillard reaction and for caramelization. Therefore, the crumb color is largely defined by ingredients [30, 32]. This could be related to the dark color of dried mushroom compared to wheat flour. Thus the positive $a^*$ values or redness were higher for the cake made from a higher ratio of dried mushroom. This result is in agreement with Salehi *et al.* [17]. Al-Sayed and Ahmed [33] also reported that the addition of water rinds powder affected the crumb color and caused $L^*$ and $b^*$ values to decrease. The difference of chemical and physical properties due to the incorporation of dried mushroom found to influence on sensory characteristics as shown in Table 4.

**Table 4.** The effect of ratios of wheat flour to dried mushroom on sensory characteristics of cake

| Characteristics | Ratios of wheat flour to dried mushroom | | | |
|---|---|---|---|---|
| | **100:0 (Control)** | **90:10** | **80:20** | **70:30** |
| Appearance | $7.13 \pm 1.41^{c}$ | $7.03 \pm 1.07^{b}$ | $6.60 \pm 1.40^{b}$ | $5.76 \pm 1.43^{a}$ |
| Color | $7.50 \pm 0.83^{b}$ | $7.10 \pm 0.76^{b}$ | $5.82 \pm 0.26^{a}$ | $5.36 \pm 1.03^{a}$ |
| Flavor | $7.33 \pm 1.03^{b}$ | $6.63 \pm 1.19^{ab}$ | $5.13 \pm 0.51^{a}$ | $4.66 \pm 0.12^{a}$ |
| Taste | $7.06 \pm 0.58^{c}$ | $6.66 \pm 1.35^{bc}$ | $5.86 \pm 0.26^{b}$ | $4.46 \pm 1.03^{a}$ |
| Texture | $6.66 \pm 0.28^{b}$ | $6.80 \pm 1.10^{b}$ | $5.53 \pm 1.02^{ab}$ | $4.96 \pm 0.48^{a}$ |
| Overall acceptance | $6.93 \pm 0.98^{b}$ | $6.85 \pm 1.10^{ab}$ | $5.70 \pm 1.02^{ab}$ | $4.76 \pm 0.48^{a}$ |

Mean ± standard deviation values followed by a different letter within the same row are significantly different ($P \leq 0.05$) by Duncan's multiple range test

The organoleptic properties of cakes at various levels of dried mushroom were investigated and presented in Table 4. The received score values significantly decreased in all quality attributed with increasing levels of dried mushroom. Jahanbakhshi and Ansani [30] stated that fiber contained in the product can reduce chewing ability and make coarse particles in the mouth. The amount of insoluble compounds causes the gluten-starch matrix unable to situate these fiber particles between the matrix then affecting the texture of products. This

may result in a loss of sensory properties [15]. It was observed that the cake made from a ratio of wheat flour to dried mushroom of 90:10 provides a very similar preference to control. Observing the overall acceptance, it can be stated that there was no significant ($P>0.05$) difference of score compared to the control for incorporation of dried mushroom reached 20%. All sensory characteristics had scores of replacement of dried mushroom of 10% and 20% higher than 5, this means that panels accepted cake made from dried mushroom. Meanwhile, the cake incorporated with 30% dried mushroom showed a sensory score of flavor, taste, texture and overall acceptance less than 5, indicating unacceptable sensory quality from panelists. The similar level of mushroom incorporated cake was found by Thakur and Singh [20]. They found that mushroom powder at 15 % addition level significantly improved color, flavor and texture of cake. The texture and overall acceptability of the cakes with mushroom powder was equally acceptable to control cake without mushroom. Moreover, the similar trend is also reported by Talib *et al.* [21]. The cake with 15% incorporated mushroom was reported to be sensory acceptable and palatable. Therefore, the cake made from incorporation of 20% of dried mushroom should be selected for further steps.

**3.2 The change of quality during storage**

The selected formulation of 20% replacement wheat flour by dried mushroom cake was evaluated at 2-day intervals for 6 days according to our preliminary study of butter cake with similar formulation of control, it was found that the cake was rejected by panelists due to rancidity. The quality of cakes during storage of 6 days demonstrates in Tables 5, 6 and 7.

**Table 5.** The change of moisture content and water activity during storage

| Properties | Storage time (Day) | | | |
|---|---|---|---|---|
| | **0** | **2** | **4** | **6** |
| Moisture content (%) | $24.71 \pm 0.68^{d}$ | $20.31 \pm 0.28^{c}$ | $19.25 \pm 0.19^{b}$ | $17.66 \pm 0.57^{a}$ |
| Water activity | $0.83 \pm 0.01^{a}$ | $0.82 \pm 0.00^{a}$ | $0.82 \pm 0.00^{a}$ | $0.81 \pm 0.00^{a}$ |

Mean ± standard deviation values followed by a different letter within the same row are significantly different ($P \leq 0.05$) by Duncan's multiple range test

Table 5 presents the change of moisture content and water activity of cakes during storage. It was found that moisture content of cakes gradually decreased meanwhile there was no change of water activity during storage. This could be related to loss of free water to the atmosphere while bound water was absorbed by fiber in cake which has high water absorption capacity [33]. From a previous study of Al-Sayed and Ahmed [33], they recommended that although moisture content of cake decreases during storage, the substituting of wheat flour with dietary fiber can decrease loss of water content compared to wheat flour batch without dietary fiber. The loss of water from cake normally affects sensory characteristics as shown in Table 6.

**Table 6.** The change of sensory preferences during storage.

| Characteristics | Storage time (Day) | | | |
|---|---|---|---|---|
| | 0 | 2 | 4 | 6 |
| Appearance | 7.06 ± 1.26[a] | 6.03 ± 0.76[a] | 5.76 ± 0.73[a] | 5.63 ± 0.67[a] |
| Color | 7.10 ± 1.23[a] | 6.26 ± 1.19[a] | 5.20 ± 0.87[a] | 5.16 ± 1.21[a] |
| Flavor | 6.73 ± 0.83[a] | 5.16 ± 0.74[a] | 5.23 ± 0.77[a] | 4.03 ± 0.96[a] |
| Texture | 6.50 ± 1.04[a] | 6.33 ± 0.55[a] | 5.16 ± 0.76[a] | 4.30 ± 1.56[a] |
| Overall acceptance | 6.73 ± 0.69[a] | 5.63 ± 0.89[a] | 5.63 ± 0.76[a] | 5.13 ± 1.24[a] |

Mean ± standard deviation values followed by a different letter within the same row are significantly different (P≤0.05) by Duncan's multiple range test

From Table 6 there was no significant difference in sensory scores during storage but the sensory scores were found to decrease especially flavor and texture. At 6 days, the result demonstrated that flavor and texture score values were lower than 5. It means that storage of the cake longer than 6 days tends to be unacceptable for the panelist. This trend could be attributed to the staling of the gluten-starch network in cake samples and makes cake samples harder during storage [30,33]. The oxidation reaction is generally generated in bakery products due to fatty matter in the formulation. This reaction leads to compounds linked not only to flavor but also sometimes to safety issues [35]. Lipid oxidation requires low energy along with oxygen so this reaction can continuously appear during storage which influences the flavor of baked products. Therefore, the score value of flavor could be affected by lipid oxidation.

Al-Sayed and Ahmed [33] reported that peroxide value and acid value of the cake increase during storage. These values are normally used for monitoring of lipid oxidation.

**Table 7.** The change of microbial populations during storage.

| Microbial populations | Storage time (Day) | | | |
|---|---|---|---|---|
| | 0 | 2 | 4 | 6 |
| Total Plate Count (TPC) (CFU/g) | ND | ND | $9\times10^{2}$ | $1.6\times10^{5}$ |
| Yeast and Mold (CFU/g) | ND | <30 | <30 | <30 |

**Note:** ND means not detected
Thai Community Product Standard (459/2549): TPC < 1×106 and Yeast and Mold <100 CFU/g

Table 7 presents microbial growth of both total plate count (TPC) and yeast and mold in cake was evaluated during 6 days of storage at ambient conditions. It was found that microbial populations of both TPC and yeast and mold were lower than the Thai Community Product Standard (459/2549). It indicated that this mushroom cake maintains its quality for 6 days and is safe for consumption by consumers.

### 3.3 Consumer evaluation

Data were collected from 100 persons consisting 74% of female and 26% of male. Their age ranged from 20 to above 50 years. The highest range was 20-29 years with 58%. The most favorite place for purchase was convenience stores at 66.00%. The consumer testing consists of study of factors affecting consumer purchase and consumer acceptance of the final product as shown in Tables 8 and 9, respectively.

**Table 8.** The factors affecting consumer's purchase decision on cake.

| Factors | Scale | Level of scale |
|---|---|---|
| Taste | 4.58 ± 0.57 | Important |
| Packaging | 3.94 ± 0.65 | Important |
| Serving size | 4.14 ± 0.73 | Important |
| Novelty | 3.98 ± 1.04 | Important |
| Convenience of purchase | 3.94 ± 0.71 | Important |
| Price | 3.68 ± 0.79 | Important |
| Promotion | 3.22 ± 1.13 | Moderately Important |
| Brand | 3.14 ± 1.03 | Moderately Important |

Based on the result, it has been identified that the first concern of consumer participants in purchasing and consuming a final product was a taste of the cake with a scale of 4.58 indicating an important factor. Serving size got the second highest with 4.14 following novelty with the scale of 3.98. The packaging and convenience of purchase got the same scale of 3.94 following price, promotion and brand with the scale of 3.68, 3.22, and 3.14 respectively. Taste always plays an important role in food choice [36]. Serving size was the second factor for consumer decisions that could be related to the quality of cake changing rapidly within a short time as result in Tables 5 and 6. Due to the staling and oxidation usually occurs in bakery products [30, 33, 35]. Third factor impacted on the decision was novelty. Curutchet *et al.* [37] said that in recent years, consumers' concern about nutrition facts, thus, novelty of products related to healthy trends has gained attention then becomes the important factor. Therefore, the product development of cake should focus on taste, serving size and novelty. The acceptance of the final product from consumers as shown in Table 9.

**Table 9.** The acceptance of consumers on mushroom cake.

| Characteristics | Score | Level of score |
|---|---|---|
| Appearance | 4.00 ± 0.70 | Like slightly |
| Color | 4.02 ± 0.59 | Like slightly |
| Flavor | 4.12 ± 0.66 | Like slightly |
| Taste | 4.34 ± 0.63 | like slightly |
| Texture | 3.70 ± 0.86 | like slightly |
| Overall acceptance | 4.16 ± 0.74 | like slightly |

Table 9 shows the score of the acceptance level of the cake. The results obtained showed that consumers accepted all of the attributes of cake, especially taste with a score of 4.34 indicating like slightly.

## 4. Conclusion

Based on this study, it can be concluded that the increase of dried mushroom significantly increased fiber content, specific volume and redness ($a^*$) of cake but decreased water activity and lightness ($L^*$). The sensory characteristics were found to decrease significantly ($P \leq 0.05$) for all characteristics with an increasing ratio of dried mushrooms. The selected level was 20% dried mushroom which was similar to the control. The moisture content of 20% dried mushroom cake decreased during storage and sensory score values decreased. The microbial population of TPC and yeast and mold were lower than the Thai Community Product Standard (459/2549). It indicated that this mushroom cake maintained the quality for 6 days at ambient temperature and was safe for consumption. The most

important factor affecting consumer's purchase was taste following by serving size and novelty. The overall acceptance score was 4.16 indicating like slightly. It can be concluded that the cake replacement wheat flour with 20% dried mushroom could be applied for cake compositions to achieve higher fiber content and acceptable cake quality to produce an alternative product for consumers.

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# Boiled Corn Wastewater Wine Production by *Saccharomyces Cerevisiae* TISTR 5019

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**Abstract**

Sweet corn is widely cultivated and consumed for its notable sweetness and low-fat content. Thailand is a significant area for corn cultivation, where boiling corn is a common cooking, resulting in substantial amounts of wastewater. The objective of this research was to reuse boiled corn wastewater to valuable by alcoholic beverage fermentation. Comparison of substrates of alcoholic beverage production such as boiled corn wastewater, corn juice and corn juice with pomace. Along with study of the effect of adding sugar on alcohol production in alcoholic beverage fermentation, comparing no sugar-added samples and sugar-added samples, fermented with *Saccharomyces cerevisiae* TISTR 5019 for 12 days. The results showed that the sugar-added samples were produced more alcohol than the no sugar-added samples (boiled corn wastewater with added sugar produced ethanol concentration of 84.34±1.70 g/L, yielding 0.32 g/g) and the sensory test results showed that the samples with the corn pomace adding was favorite by testers because it produced a good aroma. The results of suitable initial sugar concentration in alcoholic beverage fermentation showed that the boiled wastewater with pomace and added 22 brix sugar concentration was the best sample by ethanol concentration 95.63±0.85 g/L, yield 0.32 g/g and satisfaction of 64% of the testers. It can be concluded that boiled corn wastewater contains a small amount of sugar. It needed to add sugar if there wanted a good taste, and the corn pomace addition made the aroma beverage better.

***Keywords**:* Wine Production; Sweet Corn; Wastewater; Boiled Corn; Pomace

## 1. Introduction

Sweet corn is an economic crop that is very popularly grown around the world. and Thailand is one of the important cultivation areas. Sweet corn can be cultivated in all regions of Thailand and can be grown all year round. The production volume of Thailand is 450,358 tons [(1)]. It is the most popular type of corn for consumption. Because it has high sweetness and low fat. It can help reduce heart disease and cancer. It contains an antioxidant called ferulic acid, which helps in boosting the body's immune system. Sweet corn can be used for cooking and can be processed in many ways. Before being put through the process, corn is often boiled first. This causes a lot of wastewater from boiling corn. The wastewater from boiling corn has a unique aroma and a mild sweet taste. Therefore, some researchers have begun to use wastewater from boiling corn to add value. An agricultural company takes baby corn blanching water and processes it into vinegar. It was successful and could later be produced industrially [(2)].

Wine is a type of alcoholic beverage produced by fermenting grapes or grape juice with the yeast strain *Saccharomyces cerevisiae*. Yeast consumes the sugar in the fruit for growth and converts it into alcohol and carbon dioxide. Nowadays, fruits are used as raw materials for making wine called fruit wine. Fruit wine has an alcohol content of 8-10 degrees [3]. In wine, some components are beneficial to the body, including vitamins minerals, and antioxidants such as the phenolic compound flavonoid carotenoid, these substances prevent cardiovascular disease, delay aging, and help reduce the occurrence of irritable bowel syndrome [4, 5]. If wine is consumed in the right amount, there will be benefits to the body. Corn wine is a popular wine as well. Because it has a unique smell and taste. and high nutritional value [6].

The objective of this research is to reuse wastewater from boiling corn as a raw material to produce alcoholic beverages to add value to waste from industry and households. Studied factors affecting the quality of beverages, including the type of starting material and the appropriate amount of starting sugar. To get quality drinks and tastes that are acceptable to consumers.

## 2. Materials and Methods

### 2.1 Sample preparation

Sweet corn materials were obtained from Huatakeh market in Ladkrabang District, Bangkok, Thailand. Peel and soak the corn for 10 min. Separate the corn pulp from the cobs, and boil at 100°C (corn and water ratio 1:9). After 20 min, wait for the temperature to cool down and filter to separate the pulp from wastewater.

### 2.2 Yeast strain and inoculum preparation

A commercial *Saccharomyces cerevisiae* TISTR 5019 was used in the experiments. The inoculum was prepared by cultivation of the yeast in 250 mL Erlenmeyer flasks containing 100 mL of PDB medium ( Potato dextrose broth composed of 200 g/L Potato and 20 g/L Dextrose in 1 L water, pH 5.1) Cells were cultivated under shaking conditions (150 rpm), at 30°C for 24 h, being subsequently recovered by centrifugation (5500 × g, 20 min), washed with distilled water and resuspended in the fermentation medium to obtain an initial concentration of $1 \times 10^7$ cells ($OD_{660}$ = 0.5) at the beginning of fermentations.

### 2.3. Fermentation medium for corn wastewater wine

Fermentation experiments were carried out in 6 samples as W-, W+, J-, J+, JP- and JM+ as described in Table 1. 2000-mL of Erlenmeyer flasks containing 1000 mL of culture medium and glucose (22°Brix). Sterilized by pasteurization for 10 min. Inoculate 5 % of *S. cerevisiae* TISTR 5019 into the medium. The fermentations were performed at 25°C for 12 days, under static conditions. Samples were taken periodically (at 2, 4, 6, 8, 10 and 12 days, respectively).

**Table 1.** Describe the preparation of 6 medium types for fermentation.

| Process for preparing fermented food | Sugar addition | Sample name |
|---|---|---|
| Boiled, separated pulp, filter only liquid. (Wastewater) | - | W- |
| | + | W+ |
| Boiled, blended until it becomes juice, filter only liquid. (Juice) | - | J- |
| | + | J+ |
| Boiled, blended until it becomes juice, filter only liquid, add pomace. (Juice with pomace) | - | JP- |
| | + | JP+ |

[a] Triplicates for each medium samples

**2.4 Effect of sugar concentration for wine fermentation.**

A sample of fermentation medium was added to various sucrose concentration variables 20, 22 and 24°Brix. Sterilized by pasteurization for 10 min. Inoculate 5% of *S. cerevisiae* TISTR 5019 into the medium. The fermentations were performed at 25°C for 12 days, under static conditions. Samples were taken periodically (at 2, 4, 6, 8, 10 and 12 days, respectively).

**2.5 Analytical methods**

The parameters measured in this experiment were total sugar and bioethanol content. The total sugar content was determined using the phenol-sulfuric method described by Dubois *et al.* [7]. Glucose was utilized as a standard. A total of 1 mL of the sample was combined with 1 mL of 5% (w/v) phenol and 5 mL of 98% sulfuric acid. At 490 nm, the combined solution was measured using a Spectrophotometer Shimadzu UV-Vis 1800. Ethanol content was measured with a Gas Chromatography machine (GC-2014) using a DB-1 column, setting the temperature at 60 degrees Celsius, using a Flame Ionization Detector (FID), setting the temperature at 180 degrees Celsius, and using helium gas as mobile phase.

**2.6. Fermentation parameters and statistical analysis**

The ethanol yield ($Y_{P/S}$, g/g) was calculated by the ratio between ethanol produced (g/L) and glucose consumed (g/L). The ethanol productivity (QP, g/L h) was calculated by the ratio between the ethanol produced (g/L) and the fermentation time (h).

All the fermentation experiments were operated in Completely Randomized Design (CRD) . The results were presented as mean ± SD of three independent experiments and significantly different values of each extract (ANOVA followed by Tukey's HSD tests, ($P<0.05$)

**3. Results & Discussion**

**3.1 Comparison of different substrates for wine fermentation**

After inoculating *Saccharomyces cerevisiae* TISTR 5019 to 6 samples. All experiments had a similar trend. The ethanol concentration increased continuously inversely proportional to the initial sugar concentration that decreased continuously, as shown in Figure 1. This is due to the rapid growth and reproduction of microorganisms in the early stage of fermentation. After 6 days of fermentation, the number of yeast cells was relatively stable. This is because yeast reduces the rate of cell multiplication. and enters the alcohol fermentation phase [8]. Yeast converts sugar into ethanol and flavor substances [9]. A similar trend was observed by Chen *et al.* [10] studied in the fermentation of Corn wine, the initial sugar rapidly decreased during D0 - D6 and the percentage of Alcohol rapidly increased too.

Comparing all types of samples, it was found that samples with added sugar (W+, J+, and JP+ produced alcohol concentrations 84.34 ± 2.8, 97.36 ± 0.2 and 98.65 ± 0.5 g/L, respectively) that produced significantly more ethanol than samples without added sugar (W-, J-, and JP- was produced alcohol concentration 12.62 ± 0.56, 11.84 ± 1.6 and 14.51 ± 2.4 g/L, respectively), as shown in Figure 1. because there is more sugar available for the yeast's

consumption. However, the initial carbohydrate content of the three samples (W+, J+, and JP+) was not the same because J+ and JP+ were composed of a part of the starch and sugar than W+. But comparing the alcohol yield values, it was found that all samples had not slightly different alcohol yield values. Sugar concentration and ethanol concentration relationship were directly proportional [11].

**Table 2.** The concentration of alcohol and sugar from the fermentation of different substrates

| Samples | Ethanol Concentration(g/L) | Sugar concentration(g/L) | Productivity ($gl^{-1}h^{-1}$) | Yield $_{P/S}$ (g/g) |
|---|---|---|---|---|
| W- | $12.62 \pm 0.58^{c}$ | $34.52 \pm 1.5^{d}$ | 0.05 | $0.37^{a}$ |
| W+ | $84.34 \pm 1.70^{b}$ | $262.50 \pm 1.0^{c}$ | 0.35 | $0.32^{c}$ |
| J- | $11.84 \pm 0.46^{c}$ | $32.50 \pm 1.5^{d}$ | 0.04 | $0.36^{b}$ |
| J+ | $97.36 \pm 0.37^{a}$ | $349.04 \pm 0.4^{a}$ | 0.34 | $0.28^{c}$ |
| JP- | $14.52 \pm 0.87^{c}$ | $28.75 \pm 1.0^{d}$ | 0.35 | $0.32^{b}$ |
| JP+ | $98.63 \pm 1.29^{a}$ | $288.46 \pm 0.9^{b}$ | 0.34 | $0.34^{c}$ |

$^{a,b,c,d}$ The data of different superscript letters revealed significantly different values for each extract (ANOVA followed by Tukey's HSD tests, $P < 0.05$)

The results of the wine aroma test. Wine with the addition of corn pomace is the favorite test for testers. Figure 1. The volatile flavor substances such as esters and ethanol were produced during the production of alcohol which had a great aroma on wine [12]. This is because the addition of pomace helps to add more good aromas to the wine. From the results of this experiment. Therefore, a sample of wastewater with added sugar was selected for the next experiment to be consistent with the main objectives of the research and added corn pomace to help with the aroma.

### 3.2 Influence of the initial concentration of sugar on the fermentation of wine.

After fermenting the wine in the wastewater medium containing different sugar concentrations. It was found that the samples added with 20, 22 and 24°Brix of sucrose produced alcohol concentrations of 92.39 ± 1.14, 95.63 ± 0.85, and 106.67 ± 0. 7 8 g/L, respectively. The samples added with 24°Brix gave the highest alcohol concentration on 12 days. The amount of alcohol increased corresponding to the amount of sugar decreased, as shown in Figure 1. However, the yield of product and productivity were compared, and it was found that the values were not significantly different. According to Suwanposri *et al.* [13] studied the effect of initial sugar concentration in corn wine fermentation. It found that the corn wine added 18, 20 and 22°Brix of sugar. But in statistics, there was no significant difference. Initial substrate concentration has direct effects on the rate of fermentation as well as the growth of yeast cells. Because the fermentation rate increases with the increase in sugar concentration up to a certain level [14]. High sugar concentration has a direct effect on the fermentation rate and growth of microbial cells during fermentation due to high osmotic stress. However, excessively high sugar concentration exceeds the cells' sugar uptake capacity, which results in a steady state in the fermentation [15]. Thus, a high initial sugar concentration does not necessarily mean a high ethanol concentration.

The results of the sensory test from 25 random people found that 64% of the testers liked the sample with 22°Brix of sugar added because of its moderate taste. Because of the mellow taste and not too sweet.

**Table 3**. The concentration of alcohol and sugar from the fermentation of various initial concentrations of sugar

| °Brix | Ethanol concentration(g/L) | Sugar concentration(g/L) | Productivity ($gl^{-1}h^{-1}$) | Yield $_{P/S}$ (g/g) |
|---|---|---|---|---|
| 20 | $92.39 \pm 1.14^{c}$ | $285.86 \pm 1.91^{c}$ | 0.38 | $0.32^{a}$ |
| 22 | $95.63 \pm 0.85^{b}$ | $297.12 \pm 0.30^{b}$ | 0.40 | $0.32^{a}$ |
| 24 | $106.67 \pm 0.78^{a}$ | $361.54 \pm 1.42^{a}$ | 0.44 | $0.30^{b}$ |

$^{a,b,c,d}$ The data of different superscript letters revealed significantly different values for each extract (ANOVA followed by Tukey's HSD tests, $p < 0.05$)

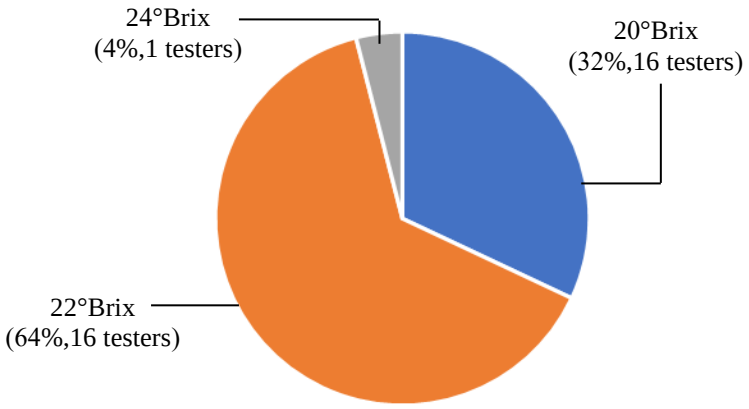


**Figure 1.** Percentage of satisfaction of random testers with wines that fermented of various initial concentrations of sugar.

## 4. Conclusion

Based on all the results of this study it can be concluded that the boiled corn wastewater can be the substrate of wine fermentation by *Saccharomyces cerevisiae* TISTR 5019 and must add sugar for increase the rate of alcohol concentration enough for good taste beverage. Adding pomace is another option for improving the flavor of wine. Alcoholic beverages are an interesting alternative for reusing boiled corn wastewater to add value. Thus, the results of this laboratory-scale experiment must be further developed to develop at the industrial level.

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# Antioxidant Capacity of Duckweed Protein Extracts Produced via Ultrasound-Assisted Alkaline Extraction

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**Abstract**

This study aimed to investigate the antioxidant capacity of proteins extracted from *Wolffia arrhiza* using ultrasound-assisted alkaline extraction (UAAE). The duckweed protein extract (DPE) was obtained through a process of mixing rehydrated duckweed powder with a pH 8.5 aqueous solution at a weight ratio of 1:6. Ultrasound energy at an intensity of 107 W/cm$^2$ was applied to disrupt cells, followed by 2-h stirring and protein precipitation at pH 4.2. The resulting DPE was analyzed for its chemical composition, total contents of amino acids (TAAC), phenolics (TPC), and flavonoids (TFC). The antioxidant activities of the DPE were evaluated using two different extracting solvents: pH 9.0 aqueous buffer and 95% (v/v) ethanol. The results showed that the DPE contained 64.2 ± 0.2% protein and 506.5 mg/g$_{\text{dry sample}}$ total amino acids (TAA), with the essential amino acids (EAA) comprising 46.3% of the TAA. The top three EAAs in the DPE were Leu, Phe, and Lys. Furthermore, the DPE was proved to contain EAAs with an amount comparable to the FAO/WHO recommended protein intake for preschool-aged children. The TFC in the DPE increased from 47.5 ± 1.0 in native duckweed to 58.4 ± 2.8 mg.RE/g.sample, while the TPC decreased from 9.5 ± 0.5 to 8.0 ± 0.1 mg.GAE/g.sample ($P<0.05$). The antioxidant capacity of the DPE's aqueous extract was 54.3 ± 0.3, in terms of ABTS, while that of the DPE's ethanol extract was 15.1 ± 0.3 μM.TE/g.sample. In terms of FRAP, the activity of the aqueous extract was 85.8 ± 1.5, while that of the ethanol extract was 53.9 ± 1.9 μM.TE/g.sample. The results indicated that the DPE's antioxidant capacity was associated with both water-soluble and insoluble compounds, in which the antioxidant activities determined in aqueous extract were higher than those obtained from ethanol extract. According to the analysis, DPE's aqueous extract was mainly composed of 70.0 ± 2.3% proteins (dry weight basis), it might be possible that the DPE antioxidant capacity in aqueous extract was primarily associated with the protein component. The DPE produced may have significant implications in the future development of functional foods and feed.

***Keywords**:* Alternative proteins; Antioxidant; Duckweed; Plant proteins; Ultrasound-assisted extraction

## 1. Introduction

Protein is an essential nutrient that plays a vital role in the growth, maintenance, and repair of the body's tissues. Proteins also serve as messengers to transport critical molecules, maintain homeostasis, and function as building blocks for molecules such as hormones, antibodies, blood, and other related fluids. Peptides and amino acids derived from gastrointestinal digestion are crucial for promoting health and preventing diseases, including lowering blood pressure, modulating the immune system, controlling stress, and providing antimicrobial benefits. The deficiency of these physiological functions leads to serious non-communicable chronic diseases (NCDs), such as atherosclerosis, hypertension, type-2 diabetes, Alzheimer's disease, and cancer, which are directly associated with oxidative stress.(1) Proteins and peptides that have excellent antioxidant capacity can be beneficial for the promotion of health and oxidative stress-related disease therapy.(2,3) However, traditional sources of protein such as meat, dairy, and soy raise concerns over sustainability, environmental impacts, and health effects. As a result, there is growing interest in finding alternative sources of protein, particularly plant-based proteins. Duckweed, a fast-growing aquatic plant, is a promising source of plant proteins (4). *Wolffia*, the predominant genus in Asia, with *W. arrhiza* being one of the most abundant species in Thailand.

Currently, we have produced duckweed protein extracts (DPE) from *W. arrhiza* via ultrasound-assisted alkaline extraction or UAAE (5). The DPE contains a high protein content and has been shown to have good oil-holding capacity and emulsifying properties. The UAE method has been noted as a potent technique for protein extraction from various raw materials.(5-7) As previously mentioned, proteins and peptides have antioxidant activity, while phenolic and flavonoid compounds are noted to be presented in *Wolffia*. The two phytochemicals are widely known for their potent antioxidant capacity.

This study therefore aimed to investigate the amino acid profiles and antioxidant capacity of DPE, as well as to compare it with native duckweed to evaluate the effect of UAAE process on the antioxidant activity of the DPE. The antioxidant capacity of DPE based on ABTS radical scavenging and ferric-reducing antioxidant power (FRAP) assays was evaluated in both aqueous buffer pH 9.0 and 95% ethanol. Using the two different polarity solvents can help elucidate the influence of polar and non-polar compounds in contributing to the DPE's antioxidant capacity. This study provides insight into the nutritional and antioxidant properties of DPE, which could have implications for the development of new functional food products and supplements.

## 2. Materials and Methods

### 2.1 Materials

Fresh duckweed (*W. arrhiza*) was purchased from Areeya Duckweed Farm (Kalasin Province, Thailand). The duckweed's chemical composition in dry basis (d.b.) measured via proximate analysis was 30.07 ± 0.19% protein, 28.55 ± 0.34% total dietary fiber, 18.78 ± 0.02% ash, 16.28 ± 0.58% carbohydrate, and 6.32 ± 0.03% lipid. The fresh duckweed was dried using a hot-air rotary oven at a controlled temperature of 53 ± 1°C to obtain a final moisture content of 5.5 ± 0.7 % (d.b.). The dried duckweed was ground to duckweed powder (DPwd) with an average particle size of ~250 ± 10 μm using an ultra-centrifugal mill (Retsch, ZM200, Haan, Germany).

**2.2 Duckweed protein extract (DPE) preparation**

Thirteen grams (d.b.) of DPwd were soaked in 430 g of distilled water for 12 h. The suspension was ultrasonicated (Sonics & Materials, VCX-750 Vibra-cell, Oxon, UK) with using a 25-mm diameter titanium alloy tip at an ultrasonication of 20 kHz with energy intensity of 107 W/cm$^2$. The sample temperature was maintained at 10 ± 1°C throughout the UAE using an ice bath for cooling. After ultrasonication, pH of the suspension was adjusted to 8.5 and subjected to alkaline extraction at 25 ± 1°C for 2 h. The resulting mixture was centrifuged, and the supernatant was collected. The pH of the supernatant was then adjusted to 4.2 using 1 and 0.5 N HCl. The resulting protein pellet was pre-frozen at -20°C then freeze-dried at -35°C under a vacuum pressure of 20 Pa for 30 h (Christ, Alpha 1-4 LSCPlus, Germany) to obtain dried DPE with a moisture content of 5.8 ± 0.4% (d.b.).

**2.3 Chemical composition analysis**

The protein content of the sample was evaluated using Dumas nitrogen analysis method as described in the studies by Grossmann *et al.* [8] and Nieuwland *et al.* [9]. Calibration curves were prepared using EDTA, with a known nitrogen content of 9.58%. The crude protein content was calculated using a nitrogen-to-protein conversion factor of 6.25, as mentioned in the works of Appenroth *et al.* [10], Duangjarus *et al.* [11], and EFSA [12]. The other compositions, including lipids, total dietary fibers, ash, and carbohydrates, were determined according to the methods described by Grossmann *et al.* [8] and Nieuwland *et al.* [9]. The carbohydrate content was calculated as the remaining percentage.

**2.4 Determination of total amino acid**

The amino acid profile of DPwd and DPE was determined using a modified microwave extraction method based on the Moldoveanu [10] protocol. Firstly, 19 ± 1 mg of DPE was dissolved in 10 mL of 6N HCl containing 0.1% (v/v) phenol. The mixture was then flushed with nitrogen and subjected to microwave digestion at 130°C for 30 min using a MultiWave3000 (Anton Paar GmbH, Graz, Austria). Once the mixture was cooled to ambient temperature, it was filtered through a 0.45-µm PTFE membrane. Lastly, the amino acid content of the DPE sample was analyzed using an amino acid analyzer (Biochrome 30+, Biochrom, Cambridge, UK) equipped with a PEEK-Oxidized Feedstuff Column (u-3327), high resolution (200 × 4.6 mm) (Biochrom Ltd., Cambridge, UK). Mobile phases included 0.2 M sodium citrate buffer (pH 2.65, 3.35, 4.25), 0.5 M sodium citrate/sodium borate buffer (pH 8.60), and 0.4 M sodium hydroxide buffer (pH 14.0). Analysis of the system was performed using a post column derivatization by ninhydrin. The eluent flow rate was operated at 25 mL/h, while that of reagent or ninhydrin was 35 mL/h. Amino acid contents were reported as amino acid residues/1000 residues.

**2.5 Determination of total phenolic and flavonoid contents (TPC, TFC)**

*2.5.1 Sample extract in ethanol and aqueous buffer preparation*

In this study, the antioxidant of the samples was determined in 95% ethanol extract and aqueous glycine buffer pH 9.0 extract. For the former, a sample solution was prepared by mixing the sample with 95% ethanol at a ratio of 1:100 (w/v) and shaking for 30 min at 50°C. For aqueous extract preparation, the sample was extracted in 1% (w/v) glycine buffer pH 9.0 at a weight ratio of 1/100 at 25°C for 30 min. Sample suspension was centrifuged at 5,000× for 20 min, and the supernatant was then collected for further analysis.

*2.5.2 TPC determination*

The TPC in the DPwd and DPE samples was determined using Folin–Ciocalteu reagent. The experiment was conducted using the method of Hu *et al.* [14] with some modifications. In brief, 25 µL of DPE solution was added to 25 µL of Folin-Ciocalteu reagent aqueous solution at a ratio of 1/1. After incubation for 5 min, 200 µL of distilled water was added and mixed with 25 µL of 10% (w/v) sodium carbonate ($Na_2CO_3$) in an aqueous solution. After that, the mixed reagent was vigorously shaken and allowed to stand for 1 h at 25°C before measuring

the sample at a wavelength of 765 nm using a spectrophotometer. The TPC value was calculated based on a standard solution of gallic acid and reported as mg of gallic acid equivalents per gram of DPE dry weight (mg GAE/g dry sample).

*2.5.3 TFC determination*

The TFC was determined according to the method of Hu *et al.* [14]. with some modifications. Briefly, 25 µL of DPE dissolved in 60% ethanol (100 µL) was reacted with 7.5 µL of 5% $NaNO_2$. After incubation for 6 min in ambient, 7.5 µL of 10% $AlCl_3.6H_2O$ was added. After further incubation in the dark for 6 min, the reaction mixture was treated with 100 µL of 1 M NaOH, and the volume was added up to 1 mL with 95% ethanol. After 15 min, the absorbance of the solution was measured at 510 nm using a microplate reader. Rutin hydrate was used as a standard, and the results of total flavonoid content were expressed as mg of rutin hydrate equivalent/g dry sample.

**2.6 *In vitro* Antioxidant Activity Determination**

The assessment of the in vitro antioxidant activity of the sample was carried out based on their ability to scavenge the 2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonate) (ABTS) radical and chelate ferrous ions (Ferric reducing antioxidant power or FRAP). Sample in ethanol extract and in aqueous extract prepared as described in 2.5.1 was used for the antioxidant activity evaluation. Both ABTS and FRAP assays were performed according to the methods of Hu *et al.* [14] with some modifications as described below.

*2.6.1 ABTS radical scavenging assay*

The ABTS radical scavenging activity assay was performed by preparing ABTS and potassium persulfate solutions at concentrations of 7 mM and 2.45 mM, respectively. The two solutions were then mixed vigorously at a volume ratio of ABTS-to-potassium persulfate of 2/1 and kept incubated in the dark for 16 h before use. After incubation, the ABTS solution was diluted with methanol (1:20) to a spectrophotometric absorbance value of 0.70 ± 0.02 at a wavelength of 734 nm using a UV spectrophotometer (Shimadzu, Kyoto, Japan). Next, a 30-µL sample solution (from 2.5.1) was mixed vigorously with 250-µL of ATBS, incubated for 6 min, and then the sample absorbance at 734 nm was measured by using a microplate reader. A standard curve was prepared using Trolox in the range of 0-250 (µM/L). To determine the Trolox equivalent antioxidant capacity (TEAC) value, the gradient of the plot of the %absorbance reduction versus sample concentration was divided by the gradient of the Trolox plot. All experiments were done in triplicate. The ABTS scavenging activity of the samples was expressed as a TEAC (µM/L Trolox equivalents per gram of sample).

*2.6.2 Ferric reducing antioxidant power (FRAP)*

For the FRAP assay, the FRAP reagent was prepared from a mixture of acetate buffer (300 mM, pH 3.6), TPTZ (10 mM), and $FeCl_3.6H_2O$ (20 mM) at a vol. ratio of 10:1:1. The FRAP reagent was then warmed to 37°C. A 20-µL sample was mixed with 200-µL FRAP reagent and incubated at 37°C for 30 min. After incubation, the absorbance was measured at 593 nm using a microplate reader. A standard curve was prepared using Trolox in the range of 0-1000 (µM/L). The FRAP value of the samples was expressed as a TE (µM/L Trolox equivalents per gram of sample). All experiments were done in triplicate.

**2.7 Statistical analysis**

All analyses of the DPE properties were conducted at least in duplicate. The experimental data is presented as X ± SD. To assess the differences among the data, an analysis of variance (ANOVA) was conducted, and Tukey's range test was performed at a confidence level of 95% ($P<0.05$) using the SPSS package software.

## 3. Results & Discussion

### 3.1 Chemical composition and amino acid profiles of DPE

The chemical composition of DPwd and DPE via proximate analysis is presented in Table 1. When comparing DPE to DPwd, the protein content showed an increase of approximately 2.6 times, from 25.21% to 64.19%, and the lipid content also increased from 6.66% to 12.35%. UAE is noted as a highly efficient method for the extraction of lipids and their derivatives from plant cells [15]. In this study, the higher percentage lipid composition in DPE could be due to both non-polar and polar lipids comprising duckweed cell organelles and cuticle wax components that were hydrolyzed through ultrasonication and co-extracted with proteins. According to a study by Zhang *et al.* [7] ultrasounds could induce the liberated molecules to form protein-lipid complexes which were difficult to separate from each other. As a result, the lipid content in the DPE was increased. The results suggested that the UAAE technique is an efficient method for duckweed protein extraction, with a production yield and protein recovery of about 16% and 35%, respectively.

Compared to the study of Duangjarus *et al.* [11] that prepared DPE *W. globosa* with UAE applied, the protein content of the resulting extracts obtained in our study was slightly higher with a significantly higher protein recovery. This could be because the alkaline extraction used in our study helps maximize protein liberation. The protein recovery was rather high compared to other leaf protein extracts reported in the study of Santamaría-Fernández and Lübeck [16]. The results suggested that *Wolffia* is an excellent plant source of plant-based protein production, and UAAE is a promising approach for *Wolffia* protein production.

**Table 1.** Chemical composition of $DP_{wd}$ and DPE produced using UAAE

| Composition (g/100 gSample, dry weight basis) | $DP_{wd}$ | DPE |
|---|---|---|
| Protein | 25.21 ± 0.05 | 64.19 ± 0.20 |
| Lipid | 6.66 ± 0.24 | 12.35 ± 0.59 |
| Carbohydrate | 28.41 ± 0.28 | 13.59 ± 0.65 |
| Dietary fiber | 22.14 ± 0.01 | 8.93 ± 0.16 |
| Ash | 17.58 ± 0.11 | 0.94 ± 0.02 |

Table 2 presents the amino acid (AA) profiles and total amount of essential amino acids (EAA) of DPE, as well as the EAA recommended protein intake for preschool-aged children by WHO/FAO/UNU. The findings reveal that the total AA content of DPE was 506.49 mg/g sample equivalent to 789.09 mg/g protein, with all EAAs included. However, tryptophan (Trp) was not measured in this study. The top three predominant AAs in DPE were glutamic acid (Glu), aspartic acid (Asp), and leucine (Leu), while the major EAAs were Leu, phenylalanine (Phe) and lysine (Lys). The results also indicated that the EAA ratio accounted for approximately 46% of the TAA. The EAA ratio of DPE in comparison to the recommended values for histidine (His), isoleucine (Ile), leucine (Leu), lysine (Lys), aromatic amino acids (AAA) including phenylalanine (Phe), tyrosine (Tyr) and tryptophan (Trp), as well as threonine (Thr), and valine (Val) were 1.8, 1.0, 1.2, 0.9, 2.0, 1.6, and 1.0, respectively. However, the content of sulfur-containing amino acids (SAAs) in DPE was approximately 0.8-fold of the recommended value. The result indicated that the EAAs found in DPE are likely to align with the recommended values. Methionine (Met) and cysteine (Cys) are two essential amino acids often very limited in crop and vegetable proteins. Furthermore, leucine (Leu), isoleucine (Ile), lysine (Lys), and sulfur-containing amino acids (SAAs) are frequently deficient in most legumes and nut proteins, such as quinoa, beans, lentils, chickpeas, and

wheat. The DPE could therefore serve as a beneficial nutritional protein ingredient when combined with other plant proteins to enhance the nutritional quality of plant-based foods.

**Table 2.** Amino acid profiles of DPE produced using UAAE and WHO/FAO recommended EAAs in protein intake for pre-school-aged children

| Amino acids | DPE (mg/gSample) | DPE (mg/gProtein) | WHO/FAO/UNU recommended values* (mg/gProtein) | EAA ratio of DPE/recommended values |
|---|---|---|---|---|
| Alanine | 32.79 | 51.08 | | |
| Arginine | 39.76 | 61.94 | | |
| Aspartic acid | 49.00 | 76.34 | | |
| Glutamic acid | 66.60 | 103.75 | | |
| Glycine | 29.96 | 46.67 | | |
| Proline | 28.50 | 44.40 | | |
| Serine | 25.56 | 39.82 | | |
| Histidine | 18.78 | 29.26 | 16 | 1.8 |
| Isoleucine | 19.77 | 30.80 | 30 | 1.0 |
| Leucine | 46.61 | 72.61 | 61 | 1.2 |
| Lysine | 27.76 | 43.25 | 46 | 0.9 |
| SAA | 11.42 | 17.81 | 23 | 0.6 |
| Cysteine | (2.77) | | | |
| Methionine | (8.66) | | | |
| AAA | 58.89 | 91.74 | 47 | 1.1 |
| Phenylalanine | (37.55) | | | |
| Tyrosine | (21.34) | | | |
| Tryptophan | (N/A) | | | |
| Threonine | 25.71 | 40.05 | 25 | 1.7 |
| Valine | 25.38 | 39.54 | 40 | 1.0 |
| Total AA | 506.50 | 789.06 | | |
| Total EAA | 234.33 | 365.06 | 248.00 | 1.5 |
| % EAA (gEAA/100gTAA) | 46.26 | 46.26 | | |

*****EAA values of protein diet recommended by WHO/FAO/UNU [17] for preschool-aged children
N/A means "Not available"

### 3.2 TPC, TFC of DPE

Table 3 presents the TPC (total phenolic content) and TFC (total flavonoid content) of DPwd (duckweed powder) and DPE (duckweed protein extract). The TPC of DPwd and DPE were 9.50 ± 0.48 and 8.04 ± 0.09 mg/g dry sample, respectively, while the TFC of the two samples were 45.47 ± 1.01 and 58.37 ± 2.79 mg/g dry sample. The TPC and TFC values of DPwd were similar to those reported by Hu *et al.* [14]. On the other hand, DPE had a lower TPC value but a higher TFC value than DPwd. This suggests that the protein production process of duckweed caused the changes in these two compounds. In this work,

DPE was extracted from duckweed cells under alkaline conditions with ultrasonication applied to facilitate the extraction. According to the study of Aksoylu and Karakaya [18], the flavonoid content in cocoa significantly diminished during the alkalization process. In this study, it therefore was possible that the phenolic and flavonoid contents were reduced during the alkaline extraction. On the other hand, Toydemir *et al.* [19] found that ultrasonication significantly helped increase the phenolics and flavonoids liberated from cells. In addition, the high energy intensity generated by ultrasonication can break down the conjugation of phenolic and flavonoid molecules from complex molecules, thereby increasing the extent of phenolic and flavonoid compounds in duckweed undergoing ultrasonication. Accordingly, the UAAE process used for duckweed protein extraction had both positive and negative effects on the phenolic and flavonoid contents of the resulting DPE. However, as above reported *Wolffia* naturally has a high flavonoid content with a much lower amount of phenolics. Therefore, the TFC remained in the DPE was higher than the TPC.

**Table 3**. TPC, TFC of DP and DPE produced using UAAE

| 3 | TPC | TFC | ABTS (TE) | | FRAP (TE) | |
|---|---|---|---|---|---|---|
| | | | Buffer (aq.) | 95%EtOH | Buffer (aq.) | 95%EtOH |
| $DP_{wd}$ | 9.50 ± 0.48[a] | 47.45 ± 1.01[b] | 55.28 ± 0.20[a] | 5.19 ± 0.85[b] | 74.82 ± 0.94[b] | 65.75 ± 1.85[a] |
| DPE | 8.04 ± 0.09[b] | 58.37 ± 2.79[a] | 54.39 ± 0.33[a] | 15.10 ± 0.26[a] | 85.79 ± 1.47[a] | 53.95 ± 1.86[b] |

Value are X ± SD
Values in the same column with the different superscripts are significantly different at $P<0.05$.
TPC: total phenolics content (mg of gallic acid equivalent/g sample, dry weight basis).
TFC: total flavonoids content (mg of rutin equivalent/g sample, dry weight basis).
TE: Trolox equivalent (μM TE/g sample, dry weight basis).

### 3.3 Antioxidant capacity of DPE

The high levels of phenolic and flavonoid compounds found in duckweed have been recognized for their potent antioxidant activity. Additionally, peptides have been scientifically reported to possess exceptional antioxidative properties. With this in mind, a study was conducted to investigate the antioxidant capacity of duckweed protein extract via ABTS and FRAP assays in both aqueous buffer pH 9.0 and 95% ethanol.

The antioxidant capacity of duckweed powder (DPwd) and duckweed protein extracts (DPE) were analyzed and shown in Table 3. The values of ABTS and FRAP of DPwd in 95% ethanol extracts were found to be 55.28 ± 0.20 and 74.82 ± 0.94, respectively, while those of DPE were 54.34 ± 0.33 and 85.79 ± 1.47. The results showed that the TPC and TFC values, as well as the ABTS and FRAP values of duckweed, were similar to the values reported by Hu *et al.* [14]. This confirmed the antioxidant capacity in 95% ethanol contributed by the TPC and TFC in *W. arrhiza*. Interestingly, the study also revealed that the antioxidant activities of both DPwd and DPE in the aqueous buffer extract were considerably higher than that determined in the 95% ethanol extract. This outcome suggests that both polar and non-polar compounds present in DPE contributed to its antioxidant activity, with polar compounds exhibiting a more significant effect compared to non-polar compounds. Furthermore, protein was identified to be the main component comprised the aqueous extract of up to 70.0 ± 2.3%. The result might suggest that protein could be the significant component associated with the antioxidant of the DPE aqueous extract.

This study, for the first time, shows a new finding regarding the antioxidant capacity of *Wolffia arrhiza* and its protein extracts. The study found that the antioxidant capacity of *Wolffia* and *Wolffia* protein extract is primarily influenced by the constitutive polar compounds rather than the nonpolar compounds. These findings provide valuable insights into the mechanisms underlying antioxidant activity in duckweed and may have significant implications for the development of future antioxidant-based therapies and nutritional supplements. Further studies are underway to identify these polar compounds and explore their potential uses in food, feed, and pharmaceutical applications.

## 4. Conclusion

In conclusion, this study has demonstrated the application of the UAAE technique for duckweed (*W. arrhiza*) protein production, resulting in a protein-rich extract with EAAs content comparable to the values of EAAs in the protein diet recommended by FAO/WHO/UNU for preschool-aged children. Additionally, the study has shown that the DPE contained a high level of flavonoids, contributing to its antioxidant activity. For the first time, the study has revealed that the antioxidant activity of the DPE was primarily influenced by the constitutive polar compounds rather than nonpolar compounds. Overall, the findings suggested that the DPE fabricated via UAAE was rich in EAAs and having antioxidant capacity that could be applied in the development of functional food/feed and nutraceuticals.

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# Effect of Foaming Agent Concentration on Properties of Foam-mat Dried Shrimp (*Litopenaeus Vannamei*) Powder

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**Abstract**

Shrimp is one of the most nutritious seafoods, which is a rich source of amino acids, peptides, protein, vitamins, and minerals, as well as various extractable and valuable compounds. Due to shrimp presents the highly perishable nature, several researchers have been focused on new methods to extend its shelf life such as chilling, freezing, and drying. Among such methods, a liquid or semi-liquid is whipped with or without foaming agent to produce stable foam that a rapid drying rate at lower temperature. However, ternary blend of xanthan gum (XG), carboxymethyl cellulose (CMC) and guar gum (GG) as foaming agents to form stable foam of shrimp powder production for application as ingredients in various foods, especially dysphagia diet were not investigated. Therefore, this research was aimed to determine a suitable ternary blend of those foaming agents for shrimp powder production by foam-mat drying for use as an alternative food with good nutrients and safe to swallow for dysphagia patients. The effect of combined foaming agents namely, XG, CMC, and GG at different ratio on properties of shrimp powder was studied. Foaming agent solutions were prepared under five different conditions (S1, S2, S3, S4 and S5) and were mixed with shrimp puree in the ratio of 7:1 (w/w). Foam density and drainage volume of foam-mat samples were investigated. After drying at 70°C for 4 h, moisture content, water activity ($a_w$), bulk and tapped densities, solubility, sedimentation ratio, and viscosity were evaluated. The results showed that drainage volume of foam, moisture content, $a_w$, bulk and tapped densities, solubility and sedimentation ratio were not significantly different ($P>0.05$). However, the suitable ratio of three foaming agents (XG: CMC: GG) was 1.0: 1.0: 4.0 (S2), which showed the lowest foam density and sedimentation ratio, while the highest solubility. Additionally, sample S2 at concentration of 20% (w/v) exhibited shear-thinning behavior with viscosity of 17.21 Pa·s. This result indicated that foam-mat dried shrimp powder can improve the thin liquid foods into pudding-like consistency correspond thickened liquid foods need for dysphagic patients.

***Keywords***: Carboxymethyl cellulose; Guar gum; Solubility; Viscosity; Xanthan gum

## 1. Introduction

Shrimp is a rich source of protein, amino acids, calcium, peptides, vitamins, and various extractable compounds. However, shrimp presents the highly perishable nature, several researchers have been focused on new methods to produce new products, reduce production costs, and extend its shelf life such as chilling, freezing, and drying [1-2].

Foam is colloidal system in which tiny air bubbles are dispersed in a continuous phase (liquid or solid). The addition of surface active agents such as proteins, surfactants, polymers, or particles [2-3] lead to form stable foam. Foam-mat drying has recently achieved considerable attention as a new effective technique of food preservation. Generally, foam-mat drying is carried out by conversion of liquid or semi-liquid materials are whipped to form a stable foam

by adding stabilizing agent and gas [4]. Foaming ability and foam stability are two main characteristics of foams [1]. Subsequently, stable foam was dehydrated using several techniques such as hot-air and freeze drying [1]. The larger surface area caused by the more porous the foam structure can enhance drying rate, which suitable for drying viscous materials and can improve product quality compared with the non-foamed material dried [3-4]. In addition, foam mat drying is ideal for products that require retention of high nutrition, high reconstitution characteristics, and cost-effectiveness [5]. Foam-mat drying has been applied for the several materials such as shrimp [1-4], cantaloupe [5], mango [6], and beetroot (*Beta vulgaris*) [7].

Hydrocolloid such as xanthan gum (XG), carboxymethyl cellulose (CMC) and guar gum (GG) are widely used in the food industry as stabilizers, emulsifiers, and thickeners [2]. The interaction of XG and whey protein isolate resulted in decrease protein-protein interaction and increase the film viscosity-flexibility of foam [8]. However, the solubility of whey protein isolate is reduced by the XG addition [8]. Thuy *et al.* [9] reported that CMC can increase the viscosity of the surfactant solution and can produce a smaller microbubble structure of stable foam. However, the use of CMC alone led to lack of its ability to give a gel-like structure with small yield stress value [10]. According to Simiqueli *et al.* [11], the addition of GG increased the viscosity of the continuous phase, leading to reduce the coalescence of bubbles and the drainage rate of foam. Nevertheless, the higher the viscosity of the aqueous medium resulted in the smaller volume of foam [11].

Several researchers have studied the effect of drying conditions, type of foaming agents, protein additives, and their concentrations on foam or foam-mat dried powder properties. Previous studies reported that the foam density was significantly increased caused by XG, guar, tragacanth, and methylcellulose, while Arabic gum had negative effect on the foam density [2]. Additionally, Hayati *et al.* [10] reported ternary synergism among XG, GG and CMC exhibit the formation of smaller droplets, and high viscosity, result in the stability of O/W emulsion. However, ternary blend of XG, CMC and GG as foaming agents to form stable foam of shrimp powder production for application as ingredients in various foods, especially dysphagia diet were not investigated. Therefore, this research was aimed to determine a suitable ternary blend of those foaming agents for shrimp powder production by foam-mat drying for use as an alternative food with good nutrients and safe to swallow for dysphagia patients.

## 2. Materials and Methods

### 2.1 Materials

Pacific white shrimp (*Litopenaeus vannamei*) was purchased from Makro food service Ladkrabang (Bangkok, Thailand). Salt (Aro, Thai Refined Salt Co., Ltd., Nakhon Ratchasima, Thailand). Food-grade xanthan gum (XG), carboxymethyl cellulose (CMC), and guar gum (GG), were purchased from Krungthepchemi Co., Ltd. (Bangkok, Thailand).

### 2.2 Preparation of shrimp puree

The shrimps were peeled, cleaned, and then frozen at -18°C. After thawing at 4°C for 24 h, shrimps were washed and boiled in salt solution at concentration of 2% (w/v) for 3 min to deactivate enzymes and kill harmful microorganisms [2]. Boiled shrimps were mashed using a kitchen blender (700 W, HR2221/00, Philips, China) at a maximum speed to achieve a uniform and homogeneous puree. Then, shrimp puree was calculated the moisture content and solid content.

### 2.3 Preparation of foam-mat shrimp puree

Foaming agent (0.5 g) at different ratio (Table 1) was dissolved in 139.6 g distilled water. After that shrimp puree was mixed with foaming agent solution in the ratio of 1:7 (w/w) to achieve the total mass of 160 g. Foam-mat shrimp puree was prepared using hand mixer (Sokany, SK-6621, China) at a maximum speed for 5 min. Control sample (S5) was conducted without adding foaming agent. All foam-mat samples were measured in terms of foam density and drainage volume.

**Table 1.** Formulation of foam-mat shrimp puree

| Sample | Water (g) | Shrimp (g dry solid) | Ratio of foaming agent | | |
|---|---|---|---|---|---|
| | | | XG | CMC | GG |
| S1 | 139.6 | 19.9 | 1.0 | 1.0 | 1.0 |
| S2 | 139.6 | 19.9 | 1.0 | 1.0 | 4.0 |
| S3 | 139.6 | 19.9 | 4.0 | 1.0 | 1.0 |
| S4 | 139.6 | 19.9 | 1.0 | 4.0 | 1.0 |
| S5 | 140.1 | 19.9 | 0.0 | 0.0 | 0.0 |

### 2.4 Production of foam-mat dried shrimp powder

The foam-mat samples were spread uniformly on an aluminum plate (15 cm diameter) at thicknesses of 3 mm, and then was dried at 70°C using tray dryer (Progress, Thailand) for 4 h. During drying, samples were determined the moisture content for drying kinetics. The foam-mat dried shrimp sample was milled by pin mill (Retsch, ZM1000, Singapore) with 10,000 rpm for 30 min and sieved (particle size is approximately 250 µm).

### 2.5 Characterization of foam properties

*2.5.1 Foam density determination*

Foam density of sample was conducted based on the method of Azizpour *et al.* [3] with some modification. The foam (50 mL) was poured very carefully to a 50-mL graduated cylinder. The foam density was calculated as:

$$\text{Foam density} = \frac{\text{Weight of foam (g)}}{\text{Volume of foam (mL)}} \quad (1)$$

*2.5.2 Drainage volume*

Drainage volume was measured following the methods of Azizpour *et al.* [2] with some modification. The foam (50 g) was poured into a graduated cylinder (50 mL). The volume (mL) of liquid drained after 1 h was reported as drainage volume.

### 2.6 Characterization of dried shrimp powder

*2.6.1 Moisture content*

Moisture content of foam-mat dried shrimp powder was performed using the halogen moisture analyzer (Mettler toledo, model HX204) at 105°C.

*2.6.2 Water activity ($a_w$)*

Water activity ($a_w$) of foam-mat dried shrimp powder was measured based on the methods of Hamzeh *et al.* [4] with some modification. Dried sample (2 g) was placed in the instrument cup, and then $a_w$ was conducted using a water activity meter (Aqualab, 4TE, USA) at the temperature of 24.5 ± 0.5 °C.

*2.6.3 Bulk and tapped densities*

Bulk and tapped densities were conducted as described by Kamali *et al.* [12] with some modification. Foam-mat dried shrimp powder (3 g) was freely poured into a 10-mL graduated cylinder. Bulk density was calculated without tapping cylinder. After that, the cylinder was tapped from a height of 5 cm (30 times). Bulk and tapped densities were calculated by using Equation (2) and (3).

$$\text{Bulk density} = \frac{\text{Mass of Power (g)}}{\text{Volume of power (mL)}} \quad (2)$$

$$\text{Tapped density} = \frac{\text{Mass of Power (g)}}{\text{Final tapped volume (mL)}} \quad (3)$$

*2.6.4 Solubility*

Solubility of dried shrimp powder was measured as described by Tran *et al.*[13] with a slight modification. Foam-mat dried shrimp powder (1 g) was suspended in 15 mL of distilled water (85°C) [14]. The suspension was stirred continuously for 10 min and centrifuged using refrigerated centrifuge (Eppendorf, 5804R, US) at 5,000 rpm (15 min). The supernatant was poured into an aluminum foil cup and dried at 105°C until a constant weight was reached. The solubility was calculated as following formula:

$$\text{Solubility} = \frac{\text{Mass after drying} \times 100}{\text{Initial sample volume}} \quad (4)$$

*2.6.5 Sedimentation ratio*

The stability of suspension was evaluated by determining the sedimentation ratio according to the methods described by Yilmaza & Yilmaz [15] with some modification. Foam-mat dried shrimp powder was dissolved in warm distilled water (85°C) [14] at the concentration of 10 and 20% (w/v). The suspension was stirred for 24 h at room temperature. The sedimentation ratio was calculated as following formula:

$$\text{Sedimentation ratio} = \frac{b}{a + b} \quad (5)$$

where *a* is the height of the aqueous layer (cm) and *b* is the height of the sediment layer (cm)

*2.6.6 Shear viscosity*

The apparent shear viscosity of all samples was prepared by foam-mat dried shrimp powder (4 g) and 20 mL of distilled water (85°C) [14] compared to distilled water without sample, then was analyzed using a rheometer (Anton Paar, MCR 302, Austria). Parallel plates (50-mm) were used for the measurements and the gap between the plates was 1 mm. The apparent viscosity of the samples was carried out as a function of shear rate from 0.1-1000 $s^{-1}$ at constant temperature of 25°C [16]. The flow behavior of the samples was described by Power-law model [16]. The apparent viscosity at the shear rate of 50 $s^{-1}$ was then calculated as per Eq. (6):

$$\eta_a = K\dot{\gamma}^{n-1} \quad (6)$$

where $\eta_a$ is the apparent viscosity (Pa·s), *K* is the consistency index (Pa·$s^n$), $\dot{\gamma}$ is the shear rate ($s^{-1}$) and *n* is the flow behavior index (dimensionless).

### 2.7 Statistical analysis

All results were performed in two replicates using SPSS® software, version 22 (SPSS Inc., Chicago, IL, USA). The data was carried out with one-way analysis of variances (ANOVA) with Tukey's test for mean comparison at 5% significance.

## 3. Results and Discussion

### 3.1 Foam density and drainage volume

Table 2 shows the foam density and drainage volume of foam-mat shrimp puree. Foam density is usually used to investigate the whipping properties [5]. The lower foam density suggests more air was trapped in the foam[2]. The foam density of foam-mat shrimp puree was in the range of 0.63-0.74 g/mL. Although, there were no significant differences in foam density of all samples, S2 exhibited the lowest foam density. This may be due to the surface activity of XG and CMC, as well as non-surface activity of GG at the ratios of 1.0, 1.0, 4.0, respectively, by their emulsifying property which placed in the air-liquid interface can help to increase foam-formation by reducing the surface tension, resulting in a reduction of foam density [2,10]. Particularly, GG had higher ratio than XG and CMC may reduce the surface tension of aqueous systems with its hydrophilic/hydrophobic structure are adsorbed at the air-liquid interface more than other conditions [21].

Foam-mat shrimp puree without foaming agent (S5) exhibited the highest drainage volume (3.85 mL) (Table 2). Foam drains excessively may be due to decrease the viscosity of foam, which resulting in the liquid around the bubbles migrates from the cell wall into the intersection of the bubbles, the coalescence of air bubbles is occurs, and the drainage of liquid is increase[1]. Similar results for the highest degree of juice separation of shrimp foam without gum addition was reported by Azizpour *et al.* [2]. However, this result found no significant change in the drainage volume in the case of foam-mat shrimp puree with foaming agents (S1-S4). This is due to the addition of foaming agents leading to an increase in the water holding capacity of foam and reduces the rate of the liquid drains from foam structure compared to control sample (S5).

**Table 2.** Foam density and drainage volume of foam-mat shrimp puree

| Sample | Foam density (g/mL) $^{ns}$ | Drainage volume (mL) |
|---|---|---|
| S1 | 0.73 ± 0.04 | ND |
| S2 | 0.63 ± 0.01 | ND |
| S3 | 0.74 ± 0.01 | ND |
| S4 | 0.72 ± 0.07 | ND |
| S5 | 0.73 ± 0.02 | 3.85 ± 0.21 |

ns indicates that values in the same column are not significantly different ($p > 0.05$). ND: No drained.

### 3.2 Drying kinetics of foam-mat shrimp puree

The drying curve of foam-mat shrimp puree is shown in Figure 1. The moisture content decreased with an increased in drying time. However, in the case of S5 (without foaming agent) showed a higher drying rate compared to other samples. This is probably because the lower viscosity caused by without addition of foaming agent, led to more open foam structure and water removing increased[2].

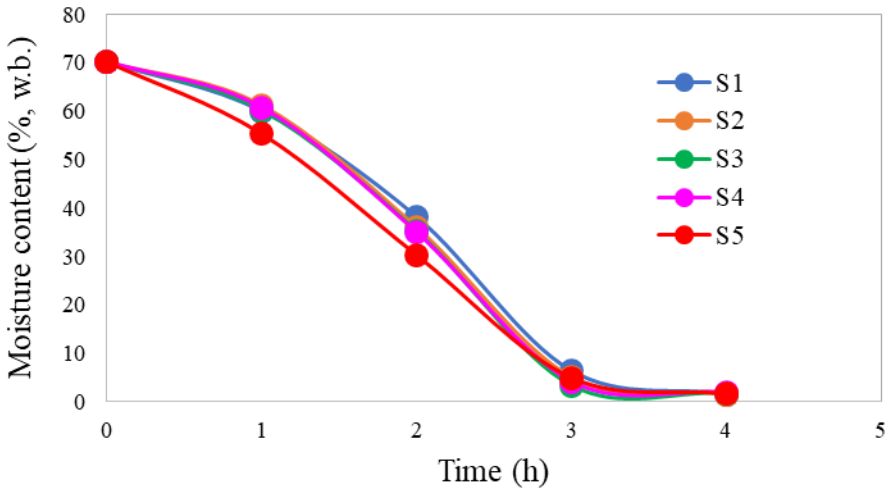


**Figure 1.** Drying curves of foam-mat shrimp puree

### 3.3 Moisture content and water activity ($a_w$)

Moisture content and water activity as shown in Table 3. The moisture contents of foam-mat dried shrimp powder were in the range of 1.57-1.97%. This is because the more porous the foam structure may accelerate the moisture loss during drying[4]. $a_w$ values of foam-mat dried shrimp powder were in the range of 0.17-0.20, which indicates the powders could be regarded microbiologically stable [(4)]. The loss of water and protein denaturation may reduce their water binding ability, which resulting in reduction of water activity of the powder[4]. However, both moisture content and $a_w$ of all foam-mat dried shrimp powder were not significantly different.

**Table 3.** Moisture content and $a_w$ of foam-mat dried shrimp powder.

| Sample | Moisture content (%) $^{ns}$ | $a_w$ $^{ns}$ |
|---|---|---|
| S1 | 1.64 ± 0.42 | 0.20 ± 0.13 |
| S2 | 1.57 ± 0.42 | 0.20 ± 0.14 |
| S3 | 1.88 ± 0.42 | 0.18 ± 0.09 |
| S4 | 1.97 ± 0.45 | 0.18 ± 0.11 |
| S5 | 1.78 ± 0.16 | 0.17 ± 0.02 |

ns indicates that values in the same column are not significantly different ($p > 0.05$).

### 3.4 Bulk and tapped densities

Table 4 shows bulk and tapped densities of foam-mat dried shrimp powder. The bulk density of all foam-mat dried shrimp powder was 0.49 g/mL, while tapped density was in the range of 0.54-0.57 g/mL. However, bulk and tapped densities of all foam-mat dried shrimp powder were not significantly different.

**Table 4.** Bulk and tapped densities of foam-mat dried shrimp powder.

| Sample | Bulk density (g/mL) $^{ns}$ | Tapped density (g/mL) $^{ns}$ |
|---|---|---|
| **S1** | 0.49 ± 0.07 | 0.57 ± 0.18 |
| **S2** | 0.49 ± 0.03 | 0.55 ± 0.11 |
| **S3** | 0.49 ± 0.07 | 0.54 ± 0.13 |
| **S4** | 0.49 ± 0.03 | 0.54 ± 0.13 |
| **S5** | 0.49 ± 0.01 | 0.55 ± 0.09 |

ns indicates that values in the same column are not significantly different ($p > 0.05$).

### 3.5 Solubility

Solubility is a property that affects sensory attributes such as the taste perception in a final product, which relates to the consumption characteristics of foam-mat dried shrimp powder [3]. The solubility of foam-mat dried shrimp powder was in the range of 12.28-12.80% as shown in table 5. It was observed that all foam-mat dried shrimp powder with foaming agent exhibited lower solubility than the control sample S5 (without foaming agent). Similar result was observed by Susanti *et al*. [8] for the solubility of whey protein isolate is reduced by the XG addition. An important functional property of shrimp proteins is affected by the protein-water and protein-protein interactions, which also correlates with the hydrophobicity and the potential conformational rearrangement to create an elastic film [8,22]. However, there is no significant difference (P>0.05) between foam-mat dried shrimp with or without foaming agent. This is due to exposing the hydrophobic groups within the shrimp protein molecules caused by the protein unfolding during higher temperatures in boiling process, resulting in aggregation of large protein and reduction of solubility of foam-mat dried shrimp powder [17].

**Table 5. S**olubility and sedimentation ratio of foam-mat dried shrimp powder.

| Sample | Solubility (%) $^{ns}$ | Sedimentation ratio | |
|---|---|---|---|
| | | 10 % (w/v) | 20% (w/v) |
| S1 | 12.56 ± 0.43 | 61.11 ± 2.62$^{a}$ | NS |
| S2 | 12.61 ± 0.51 | 50.03 ± 2.57$^{bc}$ | NS |
| S3 | 12.35 ± 0.32 | 65.74 ± 1.31$^{a}$ | NS |
| S4 | 12.28 ± 0.10 | 57.41 ± 2.62$^{ab}$ | NS |
| S5 | 12.80 ± 0.33 | 46.30 ± 2.62$^{c}$ | 62.85 ± 0.49 |

ns indicates that values in the same column are not significantly different (P>0.05). NS: No sedimented.

The stability of foam-mat dried shrimp powder when redispersed in water was evaluated by determining the sedimentation ratio (Table 5). The sedimentation ratio of redispersed foam-mat dried shrimp powder at the concentration of 10 and 20% (w/v) was in the of 46.30-65.75% and 0.00-62.85%, respectively. There is a significant difference (P<0.05) in the sedimentation ratio of redispersed foam-mat dried shrimp powder with and without foaming agent (control sample S5). In the case of 10% (w/v) concentration, S5 showed the lowest sedimentation ratio, which corresponds to the highest solubility compared to other samples. Interestingly, among all redispersed foam-mat dried shrimp powder with foaming agent at 10% (w/v) concentration, S2 exhibited the lowest sedimentation ratio. On the other hand, S5 exhibited a significantly higher sedimentation ratio than other samples. However, in the case of 20% (w/v) concentration of foam-mat shrimp puree with foaming agents (S1-S4) observed no significant change in the sedimentation ratio (Table 5). This is probably because the effect of foaming agent in sample S1-S4 helped to increase viscosity of aqueous phase[2] and helped inhibit free movement of insoluble particles, some protein fractions that insolubilized during shrimp thermal processing [23], resulting then in enhanced stability.

### 3.7 Shear Viscosity

As the result above, sample S2 was selected for evaluation as dysphagia diet by determining the viscosity when redisperse in water. This is because sample S2 exhibits the foam density, drainage volume, moisture content, bulk and tapped densities when compared with other samples. Additionally, this sample also showed the highest solubility and sedimentation ratio compared with other foaming agents. The viscosity of thickened water at a shear rate of 50 $s^{-1}$ (25°C) can be classified into four categories as described by the National Dysphagia Diet Task Force [18], namely, thin liquid (0.001-0.05 Pa·s), nectar (0.051-0.35 Pa·s), honey (0.0351-1.75 Pa·s) and pudding (> 1.75 Pa·s) [16]. Constant the apparent viscosity

under shear rate was observed in water (Figure 2), corresponding to Newtonian behavior [19]. Water exhibited the apparent viscosity was 0.001 ± 0.00 Pa·s, which corresponds to thin liquid-like consistency. However, the apparent viscosity of S2 decreased with increased shear rate, indicating that the sample S2 exhibited shear-thinning behavior[16]. This is probable due to the entangled protein structure and polysaccharide molecule network is immediately disrupted under high shear rate [20]. The experimental apparent viscosity of sample S2 was well fitted to the power-law model, with $R^2$ of 0.999. The values of *K*, *n* was 792.62 ± 15.02 Pa·s$^n$ and 0.03 ± 0.00, respectively. The viscosity of sample S2 at shear rate of 50 s$^{-1}$ ($\eta_{a,50}$) was 17.21 ± 0.48 Pa·s, corresponding to the pudding-like consistency. The result indicated that redispersion of sample S2 in water provides higher viscosity, which can be used as dysphagia diet for improvement the thin liquid foods into pudding-like consistency.

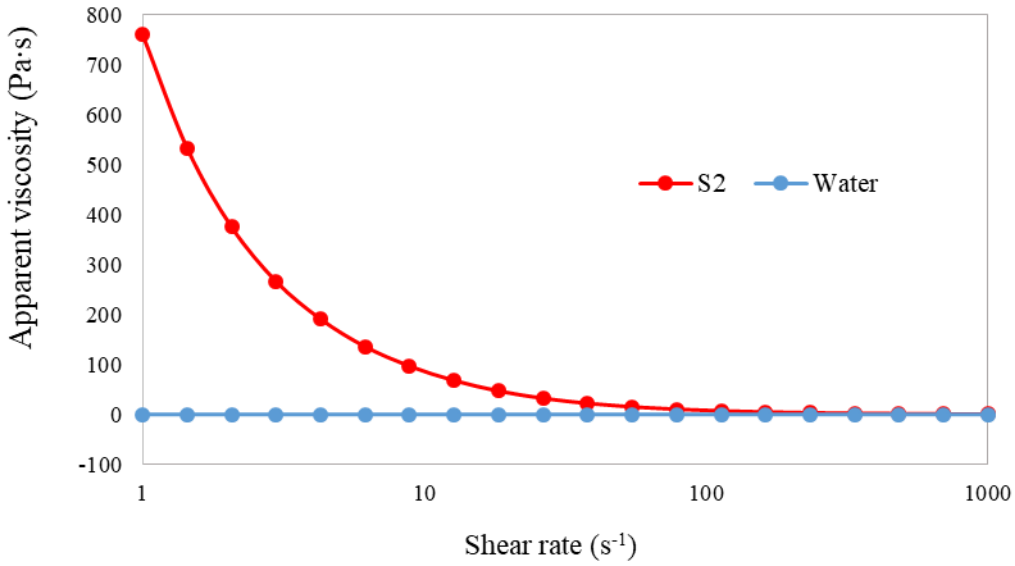


**Figure 2.** Viscosity curves of water and water thickened by foam-mat dried shrimp powder

## 4. Conclusion

Production of foam-mat dried shrimp powder with or without foaming agent was investigated. This study demonstrated that drainage volume of foam, moisture content, aw, bulk and tapped densities, solubility, and sedimentation ratio of foam-mat shrimp sample with or without foaming agent were not significantly different (P>0.05). However, the lowest foam density and sedimentation ratio, while the highest solubility was observed in sample S2. Moreover, sample S2 at concentration of 20% (w/v) exhibited shear-thinning behavior with viscosity of 17.12 Pa·s with pudding-like consistency. Therefore, foam-mat dried shrimp powder with foaming agent namely, XG, CMC, and GG at the ratios 1.0: 1.0: 4.0, respectively can be used as an alternative food with good nutrients and safe to swallow for dysphagia patients.

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# Influence of Protein Concentrations and Protein to Alginate Ratios on Characteristics and Qualities of Spirulina Protein-Spherification

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**Abstract**

A leftover product called spirulina residue is a by-product generated from phycocyanin extraction. This by-product is rich in protein, containing approximately 30-40% of protein, which offers the potential opportunity for value-added production and effective utilization. A nutritious spirulina protein was successfully prepared from the by-product in our previous work. In this work, the potential use of spirulina protein extracts for non-meat-based food applications was explored in the form of spirulina protein nutritious beads. The amino acid profile of the resulting spirulina protein was also examined. The major aim of this study was to investigate the effects of the concentration of spirulina protein and spirulina protein-to-alginate ratio on the characteristics and qualities of spirulina protein spherification. Two concentrations of spirulina protein, namely 5% and 10% (w/w), were employed, while the concentration of alginate solution was fixed at 2% (w/w). The spirulina protein-to-alginate volume ratio was tested at 1/1, 3/1, and 9/1. The spherification process was carried out in a 2.5% calcium chloride solution. The results showed that the spirulina protein extracts contained all the essential amino acids (EAA) with a high EAA ratio, accounting for 36.5% of total amino acids. Furthermore, the study exhibited that the sphericity of spirulina protein beads, prepared using 10% protein concentration at the spirulina protein-to-alginate ratios of 1/1 and 3/1, were not significantly different and were in a range of 1.02-1.05. The results of the texture analysis showed that the spirulina protein beads prepared at the ratio of 1/1 were harder than those at the 3/1 ratio. Sensorial results indicated that the spirulina protein beads prepared at the ratio of 1/1 received the highest acceptance scores of 6.95 ± 1.43 which meant that it was liked slightly to moderately. The findings suggest that spirulina protein extracts could be a promising alternative protein ingredient for nutritious protein diet applications.

***Keywords**:* Alternative proteins; *Arthrospira platensis*; Plant-based foods; Spherification; Spirulina

## 1. Introduction

Spirulina is a blue-green algae which is a potent source of nutrients. It is a unicellular alga with a high protein content of 60-70% of dry matter [(1)]. It is an important source of vitamins B1, 2, 3, and 12, vitamin C, vitamin E and beta-carotene. It also contains gamma-linolenic acid (γ-linolenic acid, GLA), a source of omega-3 fatty acids [(2)], which are considered essential fatty acids for the body. Generally, spirulina is extracted to produce phycocyanin, a pigment-protein complex in the alga. This pigment shows potential health

benefits and is used commercially in medicine and food applications [3]. Although it has a high protein content, spirulina's by-products are only used as animal feed or fertilizer which are of low value. Great attention has been paid to the reuse of the byproduct. The residual biomass contains about 30-40% protein [4]. Recently, there has been a great interest in alternative proteins. Conversion of the residual biomass into value-added protein materials: plant-based protein spherification not only follows green, sustainable, and environmentally friendly principles but also achieves great economic benefits in industrial applications.

Spherification technique is commonly used to achieve a flavors and textural sensation in modernist cooking. It is the jellification process that transforms any liquid into spheres. There are two main techniques of spherification, direct and reverse spherification [5]. In the former, the gelling agent such as sodium alginate is mixed with the sample before dropping into a calcium solution. Sodium ions in alginate liquid are exchanged with calcium ions resulting in cross-linking and gel-forming structure. The bead from aforementioned process has a very thin membrane which results in a delicate mouth feel and a rounder sphere. In the latter, the substance containing the calcium solution is dropped into an alginate bath [6]. The bead membrane is thicker and more difficult to obtain a perfectly spherical bead. Spherification technique is commonly used to achieve a flavorous and textural sensation in modernist cooking. Yuasa *et al.* [7] employed spherification technique to produce mentsuyu caviar (MC) from a liquid and seasoned Japanese noodle soup base (mentsuyu). He found that the texture of MC depended on the length of time the sample was exposed to calcium lactate and the most appropriate time was 1.5 min. Spherification can mask the off flavor like a burnt aftertaste caused by algae. Rajmohan and Bellmer [8] encapsulated spirulina powder using ionic to camouflage bitterness or astringency in food products. They reported that the hydrogels prepared by external gelation had better qualities as compared with those prepared by internal method. Though there has been some research involving spirulina, no work has been done on spirulina protein encapsulation. In this study, spherification of spirulina protein by internal gelation was prepared. The study aimed to investigate factors, i.e., spirulina protein concentrations and spirulina protein to alginate ratios on characteristics and qualities of spirulina protein beads.

## 2. Materials and methods

### 2.1 Materials

Dried spirulina (*Arthrospira platensis*) residue was procured from Pilot Plant Development and Training Institute (PDTI), Thailand. Sodium alginate ($C_6H_7O_6Na$, Mol. Wt. 85000) and calcium chloride dehydrate AR grade were supplied by Krungthepchemi Co. Ltd, Thailand.

### 2.2 Preparation of spirulina protein concentrate

Protein extraction method is referred to the study of Yucetepe *et al.* [9]. Dried spirulina residue was extracted in alkaline water at pH 9 and stirred at 700 rpm at 25±2°C for 60 min and further extracted by ultrasonic (VGT-1730QT, Gtsonic, China) at a frequency of 45 kHz for 120 min. The dispersion was centrifuged (BECKMAN COULTER, Avanti J-26 XPI, USA) at 10,000 rpm for 15 min. The supernatant was collected, and its pH was adjusted to pH 4 or pI with 1 M HCl. The insoluble protein was separated by centrifuging at 10,000 rpm for 15 min. The protein was resuspended in distilled water and neutralized to pH 7. Thereafter, it was freeze dried (Christ, Alpha 1-4 LSCPlus, Germany) and kept in aluminum foil bag. The proximate composition of protein concentrate was performed according to the AOAC International methods [10]. The amino acid composition was analyzed using a high-speed amino acid analyzer (AminoSAAYA, LA8080, Japan)

**2.3 Determination of suitable spirulina protein concentration and protein-to-alginate ratios for spirulina protein beads**

The protein extracted from spirulina residue was used to determine the suitable ratio of protein and alginate in spherification technique. Two concentrations of spirulina protein concentrate (5 and 10%) were incorporated in 2% (w/w) of sodium alginate solutions with different volume ratios of 1:1, 3:1, and 9:1. The liquid mixture was dropped slowly with help of 50 mL syringe into 2.5% (w/v) aqueous calcium chloride solution followed by stirring. After stirring (incubation) for 1 h, the formed spirulina protein balls were separated by filtration, washed with distilled water, and dried at ambient temperature. The characteristics of prepared beads, i.e., protein content (Combustion, LECO, CN828, USA) protein payload [(11)] and encapsulation efficiency [(12)] of spirulina protein base beads were determined as the following equations.

$$\%\text{Protein} = \frac{\text{Protein in the extract} \times 100}{\text{Weight of sample}}$$

$$\%\text{Protein payload} = \frac{\%\text{Protein in bead} \times 100}{\text{Weight of capsules}}$$

$$\%\text{Encapsulation efficiency} = \frac{\text{Protein in bead}}{\text{Total protein in extract to prepare the bead}} \times 100$$

**2.4 Determination of incubation periods on physical and sensorial properties of spirulina protein beads**

The spirulina protein beads were prepared using the suitable protein extract concentration from the previous section. Three incubation periods (10, 30, and 60 min) were carried out. The prepared balls were preserved in a zip lock bag and stored at $4\pm1^{o}$C for further evaluation of sphericity, hardness, and sensorial properties. All sample evaluation was completed within one week after sample collection.

**2.5 Physical analysis**

*Sphericity*

The photo images of 100 sample beads were taken by LENOVO (LENOVO, G40, Chinese). The length and the width of each sample were measured from the captured image via Photoshop program. Then, the ratio between length and width was calculated. An ideal spherical shape has the ratio between length and width of 1.0.

*Hardness*

The texture of spirulina protein beads was analyzed using a texture analyzer (TA-XT 2i) equipped with 50 kg of load cell. A cylindrical probe (P/50) was used to examine samples at a test speed of 1.0 mm/s. The maximum force or hardness of the sample was recorded. The results were performed with 3 replicates.

**2.6 Sensory evaluation**

Sensory characteristics of spirulina protein beads were estimated by scoring and hedonic tests using a semi-trained panel of 20 members, 10 men and 10 women. All panelists are normally able to discriminate and effectively communicate the differences. They were asked to rate the intensity of their perceptions of appearance (5 = spherical with smooth and uniform surface and 1 = not very spherical with rough surface), flavor (5 = no seaweed or fishy odor and 1 = the strongest seaweed or fishy odor), texture (5 = the firmest and the most crunchy and 1 = the most mushiness, and overall acceptance (5 = like very much and 1 = dislike very much).

### 2.7 Statistical analysis

Statistical analysis was performed using SPSS Statistics (Version 25.0). All data are expressed as mean ± standard deviation. Analysis of variance was conducted and if there were any statistical differences among the means, Duncan's new multiple was used for mean comparison. The difference was considered statistically significant when $P \leq 0.05$.

## 3. Results and discussions

### 3.1 Proximate compositions

Proximate compositions of the spirulina protein concentrate were as presented in Table 1. The extracted protein had 64.90 ± 0.04% protein, 2.50 ± 0.16% lipid, 1.11 ± 0.07% total dietary fiber, 1.56 ± 0.01% ash and 29.93 ± 0.14% carbohydrate. The high protein content indicated that spirulina residue should be exploited as an alternative protein source for food products. However, the protein concentrate value in this study was less than reported by Parimi *et al* (80.6 %) [13] and Bleakley and Hayes (85.50%) [14]. The differences between these results and other studies could be attributed to variations in cultivation conditions, harvesting time of seeds, and the extraction methods used.

**Table 1.** Proximate compositions of spirulina protein concentrate (d.b.)

| Component | Amount (g/ 100g) |
|---|---|
| Protein | 64.90 ± 0.04 |
| Lipid | 2.50 ± 0.16 |
| Total dietary fiber | 1.11 ± 0.07 |
| Ash | 1.56 ± 0.01 |
| Carbohydrate | 29.93 ± 0.14 |

### 3.2 Amino acid composition of spirulina protein concentrate

The amino acid composition of spirulina protein concentrate was analyzed using an amino acid analyzer and the results are shown in Table 2. The spirulina protein extracts contained all the essential amino acids (EAA) with a high EAA ratio, accounting for 36.5% of total amino acids.

The most dominant amino acid was glutamic acid (129.9 mg/g protein) followed by aromatic amino acid (90.4 mg/g protein), aspartic acid (90.0 mg/g protein), and leucine (78.5 mg/g protein), respectively. A similar result was reported by Voltarelli *et al* [15] that amino acids in spirulina were dominated by glutamic acid, followed by aspartic acid and leucine. Due to the presence of hydrophobic amino acids such as lysine, leucine, and valine, this protein concentrate may demonstrate antioxidant properties [16]. Table 2 indicates that spirulina protein contains all essential amino acids for preschool children [17] and these amino acids are also mentioned in WHO standards [18]. Based on the findings, spirulina protein concentrate could be a promising alternative protei ingredient for nutritious protein diet applications.

### 3.3 Suitable spirulina protein concentrate concentration and protein-to-alginate ratios for spirulina protein beads

The protein content, protein payload, and encapsulation efficiency of spirulina protein bead were calculated to investigate the potential to be developed on a large-scale production. Table 3 shows the effect of protein concentrate concentration and protein-to-alginate ratios on the properties of spirulina protein beads. Irrespective of the protein-to-alginate ratios, the samples prepared from 5% protein concentrate had protein content, protein payload, and encapsulation efficiency ranging from 2.78-8.63%, 14-41%, and 19-43%, respectively while those prepared from 10% protein concentrations ranging from 10.09-13.30%, 45-59 and 53-60%, respectively. The observations indicated that protein concentrations had a significant

impact on protein content, protein payload, and encapsulation efficiency (P≤0.05). Moreover, the increase in those parameters also depended on the increased ratios between protein concentrate and alginate. For example, at 10% protein concentrate, the samples prepared from protein-to-alginate ratios of 1/1, 3/1, and 9/1 had protein contents of 10.09, 11.73 and 13.30%: protein payloads of 45, 50 and 59% and encapsulation efficiencies of 55, 53, and 60%, respectively. The results implied that the amount of alginate used in this study was sufficient to encapsulate the protein. Since the protein concentration had a pronounced effect on the properties of spirulina protein beads, 10% of protein concentrate and all 3 protein-to-alginate ratios were exploited in the further study.

**Table 2.** Amino acid composition of spirulina protein concentrate.

| Amino acid | spirulina protein (mg/g Protein) | Pre-school child [12] (mg/g protein) | WHO recommended (18) (mg/g protein) |
|---|---|---|---|
| Alanine | 66.6 | | |
| Arginine | 65.7 | | |
| Aspartic acid | 90.0 | | |
| Glutamic acid | 129.9 | | |
| Glycine | 61.0 | | |
| Proline | 34.3 | | |
| Serine | 49.8 | | |
| Histidine | 23.1 | 15 | 18 |
| Isoleucine | 39.4 | 30 | 31 |
| Leucine | 78.5 | 59 | 63 |
| Lysine | 34.0 | 45 | 52 |
| Sulfur amino acid (Cysteine and Methionine) | 20.0 | 22 | |
| Methionine | | | 26 |
| Aromatic amino acid (Phenylalanine, tryptophan, and tyrosine) | 90.4 | 47 | 46 |
| Threonine | 39.3 | 23 | 27 |
| Valine | 47.0 | 39 | 42 |

**Table 3.** Effect of protein concentrate and protein-to-alginate ratios on properties of spirulina protein beads.

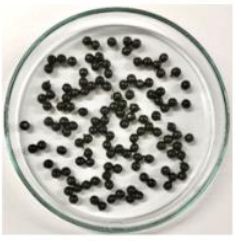
| Protein concentrate (w/w) | Volume of protein-to-alginate ratio | Protein content* (%w.b) | Protein Payload* (%) | Encapsulation efficiency* (%) |
|---|---|---|---|---|
| 5% | 1/1 | $2.78^{f} \pm 0.11$ | $13.92^{f} \pm 0.77$ | $19.49^{f} \pm 1.08$ |
| | 3/1 | $6.95^{e} \pm 0.14$ | $34.73^{e} \pm 0.26$ | $39.37^{e} \pm 0.29$ |
| | 9/1 | $8.63^{d} \pm 0.26$ | $43.14^{d} \pm 0.85$ | $45.06^{d} \pm 0.89$ |
| 10% | 1/1 | $10.09^{c} \pm 0.55$ | $50.47^{c} \pm 0.50$ | $60.56^{c} \pm 0.59$ |
| | 3/1 | $11.73^{b} \pm 0.59$ | $58.63^{b} \pm 0.13$ | $62.54^{b} \pm 0.14$ |
| | 9/1 | $13.30^{a} \pm 0.82$ | $66.51^{a} \pm 1.36$ | $67.99^{a} \pm 1.39$ |

*Different letters in the same column indicated that the mean values ± SD are significantly different (P≤0.05)

### 3.4 Effect of incubation periods on physical properties of spirulina protein beads

Spirulina protein beads were prepared at different incubation periods. The appropriate incubation period was evaluated by physical and sensory evaluation. Table 4 demonstrates that the incubation period did not affect the appearance of the beads which meant that the beads prepared at the same ratio had similar sizes, regardless of the incubation time. Unlike the incubation period, the bead size increased as the protein-to-alginate ratios increased. The sample beads at all ratios were almost spherical, except for the 9/1 ratio which was round and flat. This could be described as the 9/1 ratio beads having the thinnest and most delicate surface due to the highest proportion of protein. As a result, it is less able to withstand internal forces and is deformed.

**Table 4.** Appearance of the spirulina protein beads prepared from 10% protein concentrate at different protein-to-alginate ratios and incubation periods.

| Ratio | Time (min) | | |
|---|---|---|---|
| | 10 | 30 | 60 |
| 1/1 |  |  |  |
| 3/1 |  |  |  |
| 9/1 |  |  |  |

The results agreed with the diameter and sphericity values in Table 5. At protein-to-alginate ratios of 1/1 and 3/1, the beads had diameter values of 4.17-4.76 mm and sphericity close to 1 (1.02-1.05), regardless of the incubation time. The beads prepared from the ratio 9/1 had a much larger diameter in the range of 5 .33-6 .02 mm and a sphericity value of 3.26-3.69, indicating that they were far from spherical. This phenomenon was due to the alginate content. Generally, the carboxyl group of alginate binds to calcium ions to form a strong, protein-encapsulated structure [19]. However, the beads prepared from a protein-to-alginate ratio of 9/1 had a small proportion of alginate compared to spirulina protein, resulting in insufficient alginate to create the encapsulation. Hence, the beads provided a less-strength encapsulation structure than those prepared from other ratios.

**Table 5.** Diameter and Sphericity of spirulina protein beads prepared from 10% protein concentrate at different protein-to-alginate ratios and incubation periods.

| Protein-to-alginate ratio | Incubation time (min) | Diameter* (mm.) | Sphericity* |
|---|---|---|---|
| 1/1 | 10 | $4.17^{a} \pm 0.22$ | $1.02^{a} \pm 0.04$ |
| | 30 | $4.20^{b} \pm 0.11$ | $1.03^{b} \pm 0.03$ |
| | 60 | $4.20^{b} \pm 0.24$ | $1.03^{b} \pm 0.03$ |
| 3/1 | 10 | $4.53^{a} \pm 0.17$ | $1.05^{b} \pm 0.04$ |
| | 30 | $4.56^{a} \pm 0.17$ | $1.04^{a} \pm 0.03$ |
| | 60 | $4.76^{b} \pm 0.17$ | $1.04^{ab} \pm 0.06$ |
| 9/1 | 10 | $5.33^{a} \pm 0.49$ | $3.26^{a} \pm 0.26$ |
| | 30 | $5.73^{b} \pm 0.52$ | $3.49^{b} \pm 0.27$ |
| | 60 | $6.02^{c} \pm 0.42$ | $3.69^{c} \pm 0.24$ |

*Different letters in the same column indicated that the mean values ± SD are significantly different ($P \leq 0.05$)

The hardness of spirulina protein beads is presented in Figure 1. The results indicated that the protein-to-alginate ratio had a significant influence on the hardness of the spirulina protein beads. The hardness of the bead prepared at a protein-to-alginate ratio of 1/1 was the significantly highest followed by the beads at ratios of 3/1 and 9/1, respectively. During spherical process, the mixture of alginate and protein solution could be separated from the syringe tip only when it gained enough weight to overcome the surface tension and dropped into the encapsulated solution. The alginate reacted with calcium chloride and generated gelatinous (jello-like) material. The bead prepared at spirulina protein-to-alginate of 1/1 which had the highest proportion of alginate than those at other ratios was completely encapsulated with a thicker wall than the beads at other ratios which had a lower alginate-to-protein ratio and had thinner bead walls. In this study, it was noted that the hardness of the spirulina protein beads was unchanged after incubating for 10 min for all protein-to-alginate ratios.

### 3.5 Sensory evaluation of spirulina protein beads prepared from 10% protein concentrate at different protein-to-alginate ratios

The spirulina protein beads prepared from 10% protein concentrate at different protein-to-alginate ratios were served on a salad for evaluation of sensory properties using a 5-level scoring test for appearance, flavor, taste, and texture. The results in Table 6 show that there were significant differences ($P<0.05$) among those three ratios. The appearance, flavor, taste, and texture of spirulina protein beads at the ratio of 1/1 showed the highest score which meant that the beads had a smooth and uniform spherical shape, slightly seaweed flavor, and taste. Its texture was firm but not hard and did not stick to hands. For the preference test, the overall acceptability using a 9-level hedonic test was used. The results agreed with the scoring results that spirulina protein beads at the ratio of 1/1 showed the highest score which was in the range of like slightly and like moderately. Compared to the other samples, the sample with a ratio of 9/1 was the least acceptable of all properties. This may be due to the high ratio of protein-to-alginate which made the bead quite poor appearance and texture quality. High spirulina protein in proportion made the beads strong seaweed flavor and taste as well.

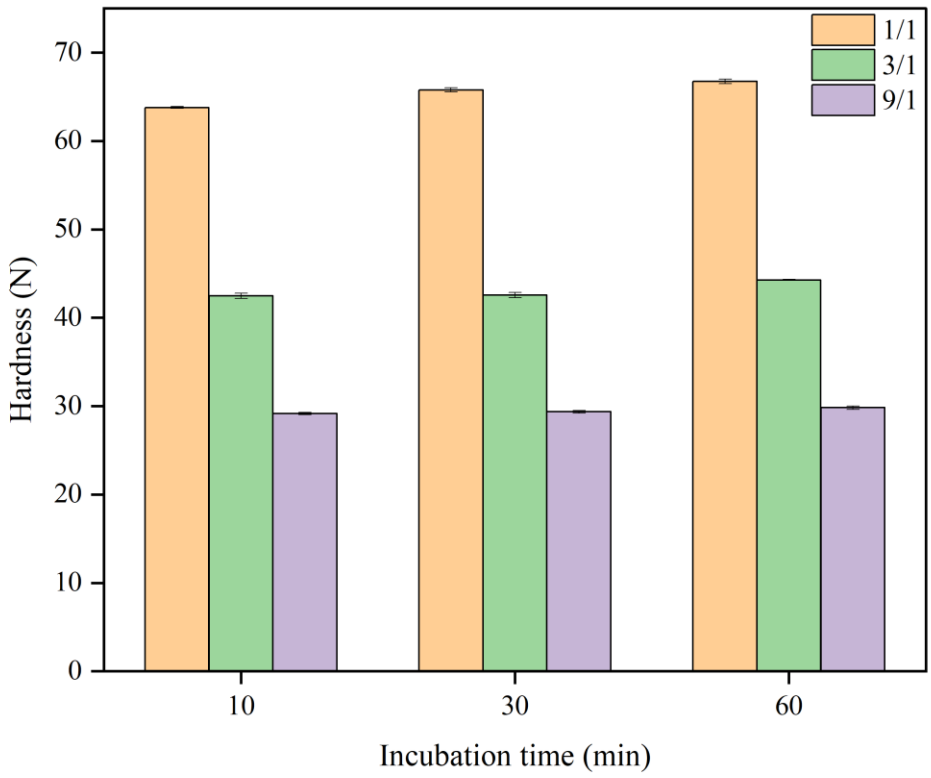



**Figure 1.** Hardness (N) of spirulina protein beads prepared from 10% protein concentrate at different protein-to-alginate ratios (1/1, 3/1 and 9/1) and incubation periods (10, 30 and 60 min).

**Table 6.** Sensory evaluation of spirulina protein beads prepared from 10% protein concentrate at different protein-to-alginate ratios and incubated for 10 min.

| Ratio | Appearance* | Flavor* | Taste* | Texture* | Overall Acceptability* |
|---|---|---|---|---|---|
| 1/1 | $4.60^{a} \pm 0.50$ | $3.65^{a} \pm 0.60$ | $3.60^{ab} \pm 0.59$ | $3.85^{a} \pm 0.81$ | $6.95^{a} \pm 1.43$ |
| 3/1 | $4.10^{a} \pm 1.16$ | $2.80^{bc} \pm 1.36$ | $3.10^{b} \pm 0.85$ | $2.80^{b} \pm 0.83$ | $5.65^{b} \pm 1.76$ |
| 9/1 | $1.90^{b} \pm 1.21$ | $1.75^{c} \pm 0.72$ | $1.4^{c} \pm 0.51$ | $1.25^{c} \pm 0.44$ | $1.80^{c} \pm 1.00$ |

*Different letters in the same column indicated that the mean values ± SD are significantly different ($P \leq 0.05$)

## 4. Conclusions

Spirulina protein contains all essential amino acids. Some of them are hydrophobic amino acids, indicating that the protein may exhibit antioxidant activity. In the spirulina protein spherification process, the protein concentration had a pronounced effect on the properties of spirulina protein beads. The spirulina beads prepared from 10% protein concentrate provided higher protein content, protein payload, and protein efficiency as compared to those prepared from 5% protein concentrate. Incubation time did not affect the size or shape of the beads. An increase in protein-to-alginate ratio increased bead diameter and made the bead less spherical. Spirulina beads prepared at a protein-to-alginate ratio of 1/1 demonstrated the highest hardness because they contained the highest alginate proportion. The hardness of the spirulina protein beads was unchanged after incubating for 10 min for all

protein-to-alginate ratios. Spirulina protein beads at the ratio of 1/1 obtained the highest scores, on both the scoring test and the hedonic test. the beads had a smooth and uniform spherical shape, slightly seaweed flavor, and taste. Its texture was firm but not hard and did not stick to hands. Based on its satisfying characteristics, it was accepted by the panelists at a slightly to moderately like level.

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# Efficiency of Cannabis Flower Oil Extract in β-Cyclodextrin Encapsulation by Freeze-Drying Method

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**Abstract**

Several countries have legalized the utilization of *Cannabis sativa L.* for both medicinal and recreational purposes. Low solubility affects the usage of cannabinoids including cannabidiol (CBD) and tetrahydrocannabinol (THC) in food. THC affects the central nerve and circulatory systems and is psychotropic. Rapid release and difficult to control release of THC and CBD in products have been reported to be linked with adverse health effects. To reduce substance abuse and overdose, encapsulation can be applied to regulate key component release and improve cannabis solubility. A pilot study of encapsulated CBD in β-cyclodextrin by freeze-drying demonstrated its promise as a coating material. This study aimed to investigate the physical properties and encapsulation efficiency of the cannabis flower oil extract encapsulated in the polysaccharide which was β-cyclodextrin as the coating material by freeze-drying method. Cannabis oil extract formed inclusion complex with β-cyclodextrin with ratios of core to wall material at 1:1 and 1:2 (w/w), and different emulsifiers such as Tween 20 and 40% ethanol were employed for the development process by freeze-drying method. Encapsulation efficiency ranges from 23.93% to 85.90%.

***Keywords**:* Cannabis; Cannabinoid; Encapsulation; β-cyclodextrin; Freeze drying

## 1. Introduction

*Cannabis sativa* L. can be widely utilized for medicinal and recreational purposes in many countries around the world. It is currently legal in various countries [(1)]. Some countries, such as Thailand and Italy, have legalized those plants. Low solubility affects the usage of cannabis cannabinoids including cannabidiol (CBD) and tetrahydrocannabinol (THC) in cosmetics and pharmaceuticals [(2)]. THC is also a psychoactive substance. Cannabis has two THC isomers, delta-8-tetrahydrocannabinol (Δ8-THC) and delta-9-tetrahydrocannabinol (Δ9-THC) [(3)], which impact various physiological systems, including the central nervous system and circulatory system [(4)]. THC and CBD are used in a variety of food products, including cakes, brownies, and candies, which have been linked to negative health impacts [(5)]. In order to mitigate the unsuitable utilization and overdose of substances. Encapsulation represents a potential strategy for regulating the controlled release of key compounds upon their entry into the human body. The previous study applied a freeze-drying method to encapsulate cannabidiol (CBD) in β-cyclodextrin and its derivative and investigated their physicochemical properties, water solubility, and dissolution rate. The study indicated that the encapsulation efficiency was more than 90% and the in vitro dissolution rate of CBD was also promoted after complexation [(2)]. However, the effect of cannabis flower oil extract encapsulated in β-cyclodextrin coating materials by freeze-drying techniques on physical properties and efficiency has not been studied.

The process of encapsulating food ingredients and nutraceuticals through freeze-drying involves dissolving, dispersing, or emulsifying these key components into coating material matrix systems, followed by co-lyophilization [6]. Surfactants, also known as emulsifiers, are frequently used to create precise and stable emulsions during the freeze-drying process. A widely used nonionic surfactant that is suitable for the preparation of oil-in-water emulsions are polysorbate (Tween) and ethanol [7]. Encapsulates prepared using different methods and/or under different conditions exhibit different microstructures, which affect encapsulation efficiency and retention of encapsulated core materials in different ways. The microstructure of the obtained complexes can be irregular or sphere-shaped, depending on the applied coating material used [8]. Therefore, it was hypothesized that the coating material may affect the character, physical properties, and encapsulation efficiency of the cannabis flower oil extract encapsulated. Thus, it is possible to apply polysaccharides as a coating material to protect the bioactive compounds in cosmetics and pharmaceutical applications and improve the solubility of cannabis oil extract for a water-based product.

## 2. Materials and methods

### 2.1 Plant material

Cannabis (*Cannabis sativa* L.) Hang Kra Rog Phu Phan cultivar were harvested from Sala, Ko Kha District, Lampang Province, Thailand in 2022. The map location is at 18.419074566415787, 99.35854145303016.

### 2.2 Materials and Chemicals

Materials and chemicals used in the study n-hexane AR (RCI Labscan), methanol HPLC (Thomas Baker), polysorbate 20 (Tween® 20) (Venus) from Chemico Inter Corporation Co., Ltd., ethanol, yeast β-glucan powder purchased from AP operations Co., Ltd., β-cyclodextrin powder (98-102%), Δ9-THC (CRM) standard, Δ8-THC (CRM) standard, Cannabidiol (CRM) standard, THCA-A (CRM) standard. All other chemicals were chemical grade. Deionized water was used throughout the study.

### 2.3 Proximate analysis and Fatty acid profile analysis

Proximate analysis and fatty acid profile analysis of fresh leaves and flowers of Cannabis (*Cannabis sativa* L.) Hang Kra Rog Phu Phan cultivar was performed by the Analytical service unit of Institute of Nutrition, Mahidol University.

### 2.4 Cannabis flower oil extraction

The solvent extraction technique adapted from Namdar *et al.* [9]. In brief, dried cannabis flower was extracted in hexane and methanol mixture at 7:3 (v/v) and shaken for 45 min at 250 rpm in a shaker at room temperature then centrifuged at 4600 rpm for 10 min and concentrated in a rotary evaporator at 35°C.

### 2.5 Preparation of the different emulsion mixture prior encapsulation

*2.5.1 Tween 20*

The formulation adopted here was modified from a prior study conducted. β-cyclodextrin was dissolved in deionized water and magnetic stirred continuously for an hour at a temperature of 30 ± 1°C to formulate solutions. After that, 1 g of emulsifier Tween 20 and cannabis flower oil extract were combined by magnetically stirring for 6 h, The oil loading and coating materials ratios were 1:1 and 1:2 (w/w).

*2.5.2 40% Ethanol*

The formulation adopted here was modified from a prior study conducted by Li *et al.*[2]. The coating materials and cannabis flower oil extract were weighed at 1:1 and 1:2 (w/w) ratios. β-cyclodextrin was solubilized using 20 mL of 40% ethanol following an hour of continuous magnetic stirring. After dissolving cannabis flower oil extract in 500 µL of ethanol, it was gradually added to the coating materials solution. After that, the solution was magnetic stirring for 6 h at 30 ± 1°C.

**2.6 Freeze drying of the prepared emulsion**

The prepared emulsions were dried by using a vacuum freeze dryer, (Kinetic, Model LD-0.5). Condition at -30ºC for 24 to 48 h, all encapsulated were kept in a desiccator at room temperature until analysis.

**2.7 LC-MS/MS assay for CBD and THC content**

The measurement of THC and CBD content was performed by LC-MS/MS (Thermo Scientific, Ultimate 3000 series UHPLC, TSQ Quantis Triple quadrupole mass spectrometer) according to a previous study [10-11].

*2.7.1 Cannabinoid extraction from cannabis flower oil extract*

Weight 10 mg of sample to a conical centrifuge tube, add 1 mL of 80% methanol and shake at 330 rpm for 30 min after that sonicated for 30 min and centrifuged at 4600 rpm for 15 min, collect supernatant and filtrated the supernatant by using PTFE syringe filter 0.22 µm before analysis.

*2.7.2 HPLC condition*

HPLC condition: column used an Accucore RP-MS 100 × 2.1 mm particle size 2.6 µm. The mobile phases consisted of (A) 0.1% formic acid in acetonitrile and (B) 0.1% formic acid in water at a flow rate of 0.3 mL/min. The separation was achieved by using a gradient for 23 min of total run time (Table 1).

**Table 1.** Gradient elution ratio. [10-11]

| Time (min) | %A 0.1% formic acid in acetonitrile | %B 0.1% formic acid in water |
|---|---|---|
| 0 | 50 | 50 |
| 5 | 70 | 50 |
| 7 | 75 | 25 |
| 10 | 80 | 20 |
| 15 | 95 | 5 |
| 16 | 95 | 5 |
| 18 | 50 | 50 |
| 23 | 50 | 50 |

*2.7.3 ESI-MS/MS condition*

ESI-MS/MS condition: cannabis extract was individually infused into the mobile phase and directed to the ESI source of the mass spectrometer via an infusion pump to determine and optimize MS/MS parameters. Two different ion transitions were used for Δ8-THC, Δ9-THC, and CBD content. The ion spray voltage was set to 4000 V, Sheath gas to 30, Aux gas to 10, and Sweep gas to 1 arbitrary unit. The ion transfer tube was set to 325°C and the vaporizer temperature was set to 300°C. Argon was used as collision gas at 0.5 mTorr and Q1 resolution was set to 0.7 and Q3 resolution to 1.2 FWHM.

**2.8 Encapsulation efficiency (EE)**

The encapsulation efficiency of each sample will be calculated by measuring the difference between the CBD content added to the solution before encapsulation and the CBD content of the non-encapsulated CBD remaining in the aqueous supernatant. In this method adapted from Li *et al.* [2], 10 mg of dry encapsulated particles of β-cyclodextrin were added to 1 ml of 80% methanol, then the solution was treated with ultrasonic processing at room temperature for 30 min. After that, the solution was centrifuged at 4600 rpm for 15 min. The supernatant will be collected and filtrated the supernatant by using PTFE syringe filter

0.22 µm before analysis. LC-MS/MS will be used to measure it. The following equation will be used to calculate encapsulation efficiency.

$$\text{Encapsulation efficiency (\%)} = M_E/M_T \times 100 \quad (1)$$

$M_E$ = content of CBD released from particles
$M_T$ = content of CBD added initially

### 2.9 Data analysis

Statistical analysis was calculated using the SPSS program version PASW statistic 18. All experiments were performed in triplicate (n=3). A comparison of the mean of the result of each experiment between 2 different emulsifiers will be analyzed by using a one-way analysis of variance (ANOVA). P-value < 0.05 will be considered statistically significant.

## 3. Results

### 3.1 Proximate analysis of *Cannabis sativa* L. (Hang Kra Rog Phu Phan)

The proximate analysis of *Cannabis sativa* L. (Hang Kra Rog Phu Phan) is presented in Table 2, displaying the energy, moisture, protein, fat, carbohydrate, and ash content of the two different sections of cannabis. The energy levels observed in the study ranged from 92.26 kcal to 172 kcal, with the fresh leaves exhibiting the lowest energy content. The moisture level of the samples varied between 59.57 and 72.15 g/100 g sample, with the greatest observed in the fresh leaves. The protein content exhibited a range of 6.20 to 8.27 g/100 g sample, with the maximum concentration observed in fresh flowers. In comparison, fresh flowers have higher levels of total fat and total carbohydrate than fresh leaves. In contrast, the ash content exhibited a range of 4.73 to 5.77 g/100 g sample, with higher levels observed in fresh leaves as compared to fresh flowers (Table 2).

**Table 2.** Proximate analysis of *Cannabis sativa* L. (Hang Kra Rog Phu Phan)**.**

| Constituents | Fresh leaves | Fresh flowers |
|---|---|---|
| Energy (kcal) | 92.26 ± 0.81 | 172.60 ± 2.38 |
| Moisture (g) | 72.15 ± 0.20 | 59.57 ± 0.42 |
| Protein (Nx6.25) (g) | 6.20 ± 0.04 | 8.27 ± 0.03 |
| Total fat (g) | 0.78 ± 0.01 | 5.96 ± 0.00 |
| Total carbohydrate (g) | 15.12 ± 0.23 | 21.47 ± 0.45 |
| Ash (g) | 5.77 ± 0.00 | 4.73 ± 0.00 |

All data are expressed as mean ± standard deviation (SD) of triplicate experiments (n=3). Reported as g/100 g sample.

The proximate analysis of *Cannabis sativa* L. (Hang Kra Rog Phu Phan) revealed variations compared to the previous work conducted by Audu *et al.* [12] In 2014, which examined the proximate analysis of the leaves, stem and seeds of *Cannabis sativa* L. obtained from Jos, Nigeria [12]. In their study shown protein content and ash in cannabis leave was 23.78% and 11.18% respectively, in the other hand in this study found protein content and ash in cannabis leave was 6.20% and 5.77% respectively. In addition, it also gives results that are different from the study by Waris *et al.* [13] in 2018, which examined the proximate analysis of whole plant of *Cannabis sativa* L. obtained from Pakistan. The climate could be the cause of the variation in ingredient composition. According to the type of soil, the climate, and the portion of the plant utilized, plants are dependent on these factors [13].

### 3.2 Fatty acid profile analysis

The fatty acid profile analysis of *Cannabis sativa* L. (Hang Kra Rog Phu Phan) is presented in Table 3. In *Cannabis sativa* L. (Hang Kra Rog Phu Phan), a total of 9 fatty acids have been identified. These are capric acid, lauric acid, myristic acid, palmitic acid, palmitoleic acid, stearic acid, oleic acid, linoleic acid, and gamma linoleic acid. The fresh cannabis flower demonstrated a significant presence of linoleic acid, accounting for 50.59% of the total fatty acid content.

**Table 3.** Fatty acid profile of *Cannabis sativa* L. (Hang Kra Rog Phu Phan)**.**

| Fatty acid | | % of total fatty acid | |
|---|---|---|---|
| | | **Fresh leaves** | **Fresh flowers** |
| Caproic acid | C6:0 | 0.00 | 0.00 |
| Caprylic acid | C8:0 | 0.00 | 0.00 |
| Capric acid | C10:0 | 0.09 | 0.00 |
| Lauric acid | C12:0 | 0.36 | 0.03 |
| Myristic acid | C14:0 | 1.54 | 0.34 |
| Myristoleic acid | C14:1 | 0.00 | 0.00 |
| Palmitic acid | C16:0 | 35.75 | 9.95 |
| Palmitoleic acid | C16:1 | 1.56 | 0.13 |
| Stearic acid | C18:0 | 5.58 | 3.23 |
| Oleic acid | C18:1 | 7.28 | 14.14 |
| Linoleic acid | C18:2, n-6 | 12.82 | 50.59 |
| Gamma linoleic acid | C18:3, n-6 | 35.01 | 0.23 |
| Linoleic acid | C18:3, n-3 | 0.00 | 21.35 |
| Arachidic acid | C20:0 | 0.00 | 0.00 |
| Eicosaenoic acid | C20:1 | 0.00 | 0.00 |
| Eicosadienoic acid | C20:2 | 0.00 | 0.00 |
| Eicosatrienoic acid | C20:3, n-6 | 0.00 | 0.00 |
| Eicosatrienoic acid | C20:3, n-3 | 0.00 | 0.00 |
| Arachidonic acid | C20:4, n-6 | 0.00 | 0.00 |
| Eicosapentaenoic acid | C20:5, n-3 | 0.00 | 0.00 |
| Behenic acid | C22:0 | 0.00 | 0.00 |
| Erucic acid | C22:1 | 0.00 | 0.00 |
| Docosadienoic acid | C22:2, n-6 | 0.00 | 0.00 |
| Docosahexaenoic acid | C22:6, n-3 | 0.00 | 0.00 |
| Lognoceric acid | C24:0 | 0.00 | 0.00 |
| Nervonic acid | C24:1 | 0.00 | 0.00 |

Table 3 presents the fatty acid composition of fresh cannabis leaves and flowers, revealing that linoleic acid exhibited the highest concentration (50.59%). The findings of the fatty acid composition align with the previous investigation conducted by Sundar *et al.* [(14)] on the oil derived from hemp (*Cannabis sativa* L.) seeds [(14)]. It has been reported that hemp seed oil contains a significant proportion of linoleic acid, constituting around 54.61% of the total unsaturated fatty acids present.

### 3.3 Cannabis flower oil extract

The extraction of dried cannabis flower powder was conducted using a mixture of hexane and methanol in a volumetric ratio of 7:3. This extraction process resulted in a product yield of 19.7%.

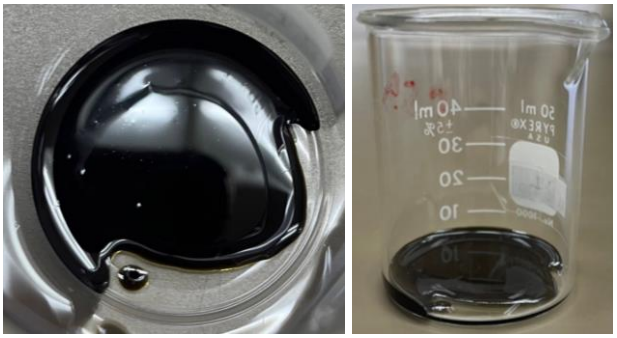
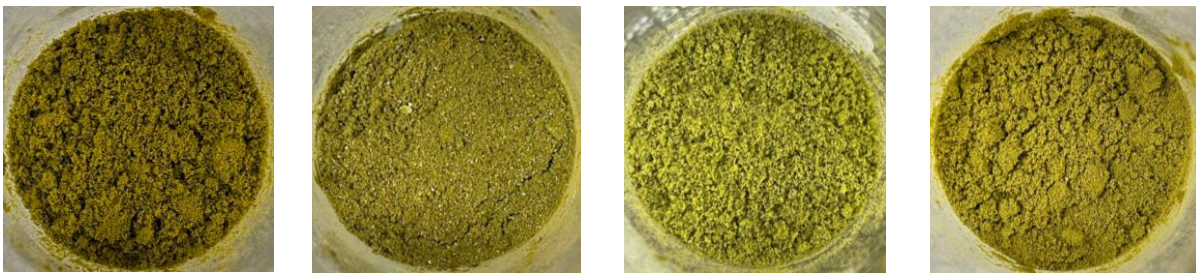


**Figure 1.** Cannabis flower oil extract , Close up of extracted cannabis flower oil (Left) The liquid and stick texture of cannabis oil extracted (right)

This study selected the cannabis flower for the extraction method based on previous research that demonstrated the flower has the highest concentration of cannabinoids compared to the leaf [15]. The extraction of cannabis in this research was performed by utilizing a combination of hexane and methanol in a volumetric ratio of 7:3. The resulting product yield was determined to be 19.7%. Prior research has established that the extraction processes employed have a significant impact on the yield and bioactivity of phytochemicals. Various parameters, including the composition of plant materials, extraction methods and duration, choice of solvents, pH levels, temperature, pressure, and the ratio of material to solvent, might impact the efficacy of extracting bioactive chemicals from plant matrices [16].

### 3.4 Cannabis flower oil extract encapsulation

The study involved the encapsulation of cannabis flower oil extract using β-cyclodextrin as wall material. The volume ratio of core to wall material was varied at 1:1 and 1:2, and different emulsifiers such as Tween 20 and ethanol were employed for the development process. The encapsulating products are depicted in Figure 2. The utilization of wall materials and various emulsifiers has been observed to result in distinct variations in particle characteristics. There is no noticeable difference observed in the resulting products derived from various emulsifiers.



(a) Cannabis flower oil extract/β-cyclodextrin-Tween20 (1:1)

(b) Cannabis flower oil extract/β-cyclodextrin-40% EtOH (1:1)

(c) Cannabis flower oil extract/β-cyclodextrin-Tween20 (1:2)

(d) Cannabis flower oil extract/β-cyclodextrin-40% EtOH (1:2)

**Figure 2.** Cannabis flower oil extract encapsulated products.

### 3.5 Quantitative analysis of THC and CBD by LC/MS-MS

The quantitative analysis of *Cannabis sativa* L. (Hang Kra Rog Phu Phan) extract and their encapsulation product were presented in Table 4. The cannabis flower oil extract, when encapsulated in β-cyclodextrin with Tween 20 at a ratio of 1:2, exhibited the highest CBD and Δ8-THC content in comparison to other encapsulated products. However, when compared to other encapsulated products, the cannabis flower oil extract encapsulated in β-cyclodextrin at a 1:1 ratio with 40% ethanol showed the greatest THCA-A level. Furthermore, our investigation revealed no significant variations in the Δ9-THC level across groups. The findings can be stipulated that Tween 20 could offer better surfactant property compared to ethanol for effectively encapsulating both CBD and THC from cannabis flower oil extract. The data indicates that Tween 20's potentially dissolve and formed micelles which enhanced the accessibility of these hydrophobic cannabinoids micelles. On the other hand, ethanol's ability to solubilize these substances may be limited, and it may compete with cannabinoid content for binding sites on the β-cyclodextrin [17]. Furthermore, this pattern is likely to occur when the oil-to-coating material ratio is high. However, to obtain a more conclusive study, further statistical tests and investigating a broader range of oil-to-coating material ratios are needed. In summary, the surfactant, concentration, and oil-to-coating material ratio are critical factors that can affect the levels of THC and CBD in the encapsulated product. Gaining insight into the impact of these parameters on solubility, partitioning, and encapsulation efficiency is necessary for optimizing formulations and attaining the optimal cannabinoid content in the encapsulated product [8].

**Table 4.** CBD and THC content of cannabis oil extract from flowers encapsulated in different coating materials.

| Sample | | Ratio oil : coating material | Content (mg/g oil) | | | |
|---|---|---|---|---|---|---|
| | | | CBD | Δ9-THC | Δ8-THC | THCA-A |
| Cannabis flower oil extract | | | $0.26^{e}$±0.00 | 0.04±0.00 | $0.68^{c}$±0.01 | ND to 0.15± 0.00 |
| β-cyclodextrin | Tween 20 | 1:1 | $0.06^{a}$±0.00 | ND | $0.38^{a}$±0.01 | $1.61^{c}$±0.03 |
| β-cyclodextrin | 40% EtOH | | $0.18^{c}$±0.00 | 0.46±0.00 | $0.63^{b}$±0.00 | $2.13^{d}$ ±0.07 |
| β-cyclodextrin | Tween 20 | 1:2 | $0.22^{d}$±0.02 | 0.02±0.00 | $0.67^{c}$±0.04 | $0.61^{a}$ ±0.05 |
| β-cyclodextrin | 40% EtOH | | $0.08^{b}$±0.00 | 0.24±0.00 | ND | $1.11^{b}$ ± 0.00 |

All data are expressed as mean ± standard deviation (SD) of triplicate experiments (n=3). Data with different superscript letters in the same column indicate significantly different at P<0.05 calculated by one-way analysis of variance (ANOVA).
ND = Not detect

### 3.6 Encapsulation efficiency (EE)

Encapsulation efficiency is a critical statistic that is widely used to calculate the amount of guest molecules contained in the cavity of coating materials. The encapsulation efficiency of *Cannabis sativa* L. (Hang Kra Rog Phu Phan) flower oil extract encapsulated in β-cyclodextrin were presented in Table 5. The maximum encapsulation efficiency, 85.90%, was shown by the cannabis flower oil extract encapsulated in β-cyclodextrin with Tween 20 at a 1:2 ratio.

**Table 5.** Encapsulation efficiency of Cannabis flower oil extract encapsulation.

| Sample | | Ratio oil : coating material | Encapsulation efficiency (%) |
|---|---|---|---|
| β-cyclodextrin | Tween 20 | 1:1 | $23.93^{a} \pm 0.10$ |
| β-cyclodextrin | 40% EtOH | | $69.22^{c} \pm 0.81$ |
| β-cyclodextrin | Tween 20 | 1:2 | $85.90^{d} \pm 5.88$ |
| β-cyclodextrin | 40% EtOH | | $31.51^{b} \pm 0.08$ |

All data are expressed as mean ± standard deviation (SD) of triplicate experiments (n=3). Data with different superscript letters in the same column indicate significantly different at $P<0.05$ calculated by one-way analysis of variance (ANOVA).

Microencapsulation is a technology used to preserve sensitive compounds and create materials with enhanced qualities. Microencapsulation is the act of encapsulating particles that are on a micron scale within a shell made of polymers. Various methodologies exist for the encapsulation of pharmaceutical compounds. The encapsulation efficiency of microparticles, microspheres, or microcapsules is contingent upon various aspects, including the polymer concentration, polymer solubility in the solvent, rate of solvent removal, and the solubility of the organic solvent in water. Then the encapsulation of THC and CBD by using β-cyclodextrin. β-cyclodextrin can sparingly dissolve in water therefore encapsulation efficiency was quite low as 23-85%. These results are consistent with the findings of Ahmad *et. al.* [18], which studied microencapsulation of saffron anthocyanins using β-cyclodextrin and β-glucan. In their study reported the encapsulation efficiency of β-cyclodextrin and β-glucan was 63.25% and 45% respectively [18]. The larger internal cavity and their structure of β-cyclodextrin may be responsible for its higher encapsulation efficiency than β-glucan. In addition, during freeze drying the process, sublimation from solid to gas removes ice crystals from samples in freeze drying. This process may result in a porous microstructure, altering their physical form. Hollow or porous microstructure may affect particle surface area and porosity, which may decrease encapsulation efficiency as well as poorer core protection by affecting substance accessibility to the environment [19]. Tween 20 is a non-ionic surfactant that has the ability to dissolve both polar and non-polar substances. Additionally, it creates micelles, which are spherical clusters capable of capturing certain molecules such as THC and CBD (key components found in cannabis flower oil) and improving their delivery to the oil phase. The enhanced solubility and formation of micelles are likely to contribute to the observable improvement in encapsulation efficiency when using higher oil to wall ratio for Tween 20. In contrast, ethanol, a polar solvent, primarily targets polar compounds. While it might solubilize some cannabinoids, it has a weaker effect on micellization compared to Tween 20. Additionally, ethanol might compete with some target molecules for binding sites on the β-cyclodextrin, hindering their extraction and potentially reducing encapsulation efficiency with a higher oil-to-coating material ratio. Furthermore, the concentration of β-cyclodextrin, which serves as the coating material, can also affects the percentage of encapsulation efficiency (%EE). At a lower oil-to-coating material ratio, such as 1:1, there may be a lower concentration of surfactant in comparison with the number of binding sites on the β-cyclodextrin. This might results in saturation, a condition when all accessible sites are filled, and more binding is restricted. By increasing the ratio of oil-to-coating material, such as 1:2, a greater number of surfactant molecules are introduced. This has the potential to overcome saturation and enable more effective extraction, particularly for substance that have a weaker affinity for β-cyclodextrin. To improve the encapsulation efficiency, it is imperative to consider additional aspects such as operating factors, maintaining a similar quantity of total solid content in the feed for both samples and ensuring an appropriate ratio of wall material to

core material [20]. Previous studies showed that the combination of wall materials can increase encapsulation efficiency [8].

## 4. Conclusion

The finding of this study showed that *Cannabis sativa* L. Hang Kra Rog Phu Phan cultivar. Proximate analysis revealed that these fresh leaves contain energy 92.26 kcal, moisture 72.15 g/100 g sample, protein 6.20 g/100 g sample, total fat 0.78 g/100 g sample, total carbohydrate 15.12 g/100 g sample, and ash 5.77 g/100 g sample. The proximate analysis of fresh flower contains energy 172.60 kcal, moisture 59.57 g/100 g sample, protein 8.27 g/100 g sample, total fat 5.96 g/100 g sample, total carbohydrate 21.47 g/100 g sample, and ash 4.73 g/100 g sample. Fatty acid profile analysis reported this plant was a great source of linoleic acid. The combination of hexane and methanol in a volumetric ratio of 7:3 was applied to extract cannabis flowers. The resulting product yield was determined to be 19%. Cannabis flower oil extract formed an inclusion complex with β-cyclodextrin with a volume ratio of core to wall material was varied at 1:1 and 1:2, and different emulsifiers such as Tween 20 and ethanol were employed for the development process by freeze-drying technique. Encapsulation efficiency ranges from 23.93% to 85.90%. This study offers experimental evidence to support the increasing scope of application of cannabis flower oil extract or oil-based components in the fields of pharmaceuticals, cosmetics, and the chemical industry.

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# Impact of Bio-Calcium from Nile Tilapia Bone as Supporting Material for Probiotic Immobilization

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## Abstract

Nile tilapia bone is considered as low value by product, which affects the environment by difficulty to decompose and provides unpleasant smell. Increase value of this waste by processing as bio-calcium is the remarkable strategy. The physical and chemical properties of bio-calcium are interesting to develop as supporting material for probiotic immobilization, which can future apply on various food products as functional foods. This study evaluated the impact of bio-calcium from Nile Tilapia bone, as several formulations, on survivability of probiotic (*Lactobacillus acidophilus* TISTR 1338) in various harsh conditions (freeze-drying and temperature levels). Bio-calcium at three concentrations (5%, 10%, and 15%; w/v) mixed with 10% (v/v) of probiotic suspension, supplemented with 10% (w/v) of sucrose were studied at 30, 60, and 90 min of agitation (200 rpm). The result showed that bio-calcium at 15%, agitated for 90 min presented the optimal formulation by providing the highest probiotic survivability after freeze-drying (99.4 ± 0.2%). Whereas the formulation of sucrose alone (without bio-calcium) showed 80.8 ± 0.7% of survivability. Water activity of developed immobilized probiotic formulations as dry powder showed up to 0.19. Stability of probiotic immobilized by optimal formulation was evaluated in various temperature levels (6 ± 2°C, 25 ± 2°C, and 50 ± 2°C). After 3 h of incubation at 6±2°C and 25 ± 2°C, optimal formulation showed probiotic survivability at 95.7 ± 4.6% and 100.0 ± 0.5%, respectively. Whereas probiotic as free cell (the control) showed 92.2 ± 1.3% and 97.8 ± 1.5%, respectively. In addition, no probiotic could survive at 50 ± 2°C for both optimal formulation and probiotic as free cell. Scanning electron microscope (SEM) revealed the images presenting physical structure of bio-calcium combined with sucrose as supporting material for probiotic immobilization. The result showed that numerous cells of probiotic were immobilized inside the porous structure of bio-calcium, with no damaged cell was observed. Overall results indicated that bio-calcium from Nile Tilapia bone presented as remarkable supporting material for probiotic immobilization. However, the formulation should be improved in the future study to increase the efficiency of immobilized probiotic in high temperature and other conditions.

***Keywords**:* Nile Tilapia Bone; Bio-Calcium; Probiotics; Supporting material; Immobilization

## 1. Introduction

Nile Tilapia bone is the by product from food industry, which generally considered as low value material [1]. The structure of this fish bone is difficult to decompose, results in unpleasant smell which affect the environments. From our previous study, bio-calcium from Nile tilapia bone has been successively produced with good nutritional value that is rich in calcium and phosphorus. Bio-calcium prepared from animal bone has been reported as the source of good nutritional value that can stimulate calcium absorption through animal intestines [2]. The remarkable properties of bio-calcium have been found from our previous study on the production of bio-calcium from Nile tilapia bone [3]. Calcium crystals in the fish bone have been reported as the form of hydroxyapatite crystals, which are the same form found in human bones [4]. Moreover, bio-calcium also contained with protein and collagen that influenced the absorption of calcium after digestion [3]. On the other hand, probiotics is generally known as the provider of health benefits and increasingly recommended to use as metabolic syndrome disorder treatment [5]. Nevertheless, the stability and survivability of those microorganism under product processes, storage as well as gastrointestinal tract, were highly concerned. Thus, many techniques have been studied to protect and improve the survivability of the probiotics for food application [6]. Cell immobilization on a solid carrier was based on the physical adsorption due to electrostatic forces or by covalent binding between the cell membrane and the carrier [6]. One of the most interesting with practicable process is fixing or immobilization of microorganism with adsorption method, which has no chemical use, low energy consumption and less adverse effect on microorganism [7]. In addition, with porosity structure of supporting material the cell could be trapped in that matrix, which was also another physical mechanism to fix the cell. Therefore, the bio-calcium power from fish bone that's rich of biomaterial as protein with the strong solid porous structure [8], which might be a good choice as support material for fixing microorganism by both mechanism, adsorption and entrapment. The objective of this study was aimed to develop food supplement as functional food with high stabilized probiotic content and good source of calcium and phosphorous by fixing the probiotic with bio-calcium power. The current study developed several formulations of immobilized probiotic as dry powder by using bio-calcium from Nile tilapia bone supplemented as supporting materials. The optimal formulation, which provided the highest survivability of probiotic after freeze-drying, was selected to evaluate the stability in various temperature levels. Physical, chemical and microstructure of immobilized probiotic with bio-calcium were also investigated.

## 2. Materials and Methods

### 2.1 Chemical and reagents

Chemicals and reagents for bio-calcium preparation was purchased from Lab-Scan (Bangkok, Thailand). Probiotic strain (*L. acidophilus* TISTR1338) was purchased from the TISTR Culture Collection, Thailand. All culture media were purchased from HiMedia Laboratories Pvt. Ltd., India.

### 2.2 Bio-calcium preparation

Raw bone of organic Nile tilapia was obtained from Faculty of Fisheries Technology and Aquatic Resources, Maejo University, Thailand, which is the by-product from fish fillet process line. Bone samples was subjected to boil in boiling water (100ºC) for 2 min before cleaning using a high-pressure water jet cleaner (Zinsano Andaman, Bangkok, Thailand) at a pressure of 120 bar with a flow rate of 360 L/h for 1-2 min. The non-collagenous protein was removed as described by Sriket *et al.* [3] using 2.0 M KOH solution in a ratio of bone and KOH solution of 10:1 (v/w) at 100°C for 30 min. The obtained bones were dried using a cabinet tray dryer (Binder FED115, Tuttlingen, Germany) at 50°C for 2 h., then reduce the sample size with a grinder to three-four millimetres. The lipid content was then removed by

soaking in 95% ethanol with ratio of 1:10 (w/v) at 25 °C and stirred for 60 min [3]. Lipid removed bone was bleached by soaking in 1 % (v/v) hydrogen peroxide ($H_2O_2$) solution with a ratio of bone and $H_2O_2$ solution of 1:10 (w/v) at ambient temperature for 30 min followed by continuous stirring. Treated bone sample was then washed with ten volumes in running water, dried in a cabinet tray dryer at 50°C for 5 h. After that, the sample was ground into a powder using a pin mill. The resulting bone powder (60 mesh) will be defined as bio-calcium powder.

**2.3 Bacterial growth condition**

The cultivation of probiotic strain was described by Petsong *et al*. [9]. A single colony of *L. acidophilus* TISTR1338 was cultivated De Man, Rogosa and Sharpe (MRS) (HiMedia Laboratories Pvt. Ltd., India) and incubated at 37°C for 36-60 h, under anaerobic conditions.

**2.4 Probiotic immobilization**

The bio-calcium powder was used as support material. All materials used for immobilization was autoclaved (121°C, 15 min) to eliminate the initial contaminating microorganisms. Cell immobilization was carried out as described previously [10]. Different concentrations of bio-calcium powder (5, 10, and 15 % (w/v)) were mixed with probiotic suspension (10 mL; $10^9$ CFU/mL) in 25 mL flasks. The mixture was agitated at 200 rpm at different fixing times (30, 60, and 90 min) at room temperature, then mixed with 10% (w/v) of sucrose as cryoprotectant. Similarly, the free cells mixed with 10% (w/v) of sucrose was studied as the control. Both immobilized and free cell suspensions in sucrose solutions was frozen at -60°C for 24 h before freeze-dried using a laboratory-scale freeze-dryer (Gamma2-16LSC, Christ, Germany) for 48-72 h. The dry powders from each immobilization conditions were prepared in three separate batches.

The method to enumerate the number of immobilized probiotics after freeze-drying was carried out according to a previous study [9] with minor modifications. Immobilized probiotic powder was mixed with 50 mL of 1 M sodium phosphate buffer (pH 7.5). The mixture was agitated at 200 rpm for 60 min at room temperature. A spread plate technique was performed on MRS agar, and the samples was incubated at 37°C under anaerobic conditions for 48-72 h. The survivability of the immobilized probiotics after freeze-drying was calculated as follows. The number of probiotics recovered from the powder (RP) was divided by the initial number of probiotics added for immobilization (IP) and then multiplied by 100:

$$\text{Survivability (\%)} = (RP \times 100)/IP$$

**2.5 Color of immobilized probiotic formulations**

All samples from section 2.4 were evaluated color quality by the CIE $L^*$ $a^*$ $b^*$ system ($L^*$ indicates lightness, $a^*$ indicates redness/greenness, and $b^*$ indicates yellowness/blueness). Hunter lab UltraScan XE colorimeter (HunterLab Reston, VA, USA) was used to evaluate all samples.

**2.6 Chemical characteristic of immobilized probiotic formulations**

Water activity ($a_w$) of immobilized probiotic formulations as dry powder was evaluated to determine the quality of food products. Water activity of immobilized probiotic was measured by Pre-Aqua Lab Water Activity Analizer (Decagon Devices, Inc., Pullman, WA) at 25°C. The experiment was performed in three independent studies.

**2.7 Stability of immobilized probiotic in various temperature levels**

Among ten formulations of probiotic immobilization in section 2.3, the optimal formulation was selected by the highest survivability. To demonstrate the stability of selected immobilized probiotic at temperature 6 ± 2°C, 25 ± 2°C, and 50 ± 2°C to perform refrigeration temperature, ambient temperature, and high temperature, respectively. Immobilized probiotic as dry powder (1 g) or probiotic as free-cell form (1 mL) was

suspended in phosphate buffer saline (PBS) solution (pH 7.4) at ratio 1:10. The mixtures were incubated at 6 ± 2°C, 25 ± 2°C, and 50 ± 2°C individually. The samples were collected every hour for 3 h enumerate the number of living probiotic cells.

**2.8 Microstructure of immobilized probiotic formulations**

To study physical relationship of probiotic cells and supporting materials, the images revealed by SEM were used to demonstrate. Physical structure of immobilized probiotic formulations (optimal- and control- formulation) was examined using a Field-Emission Scanning Electron Microscope (FE-SEM) (TESCAN: MIRA, Czech Republic). The samples were fixed on aluminum stubs with double adhesive tape and vacuum coated with a layer of gold before being visualizing under magnification of 5 kx and 20 kx. Observations were carried out at an acceleration voltage acceleration of 5 kV.

**2.9 Statistical analysis**

All experiments were performed in three replications. Data were analyzed using IBM SPSS Statistics 26. Analysis of Variance (ANOVA) was used to compare survivability and $a_w$ of immobilized probiotic from various developed formulations. Comparison of means was declared by Duncan's multiple range tests ($P<0.05$). Mean comparison between survivability of probiotic as optimal formulation and free-cell form at various temperature levels were analyzed using Student's t-test.

## 3. Results & Discussion

**3.1 Survivability of immobilized probiotic after freeze-drying**

This study evaluated the effect of bio-calcium concentration (5, 10 and 15 %) and agitation time (30, 60, and 90 min) of mixing supporting material and probiotic. A total of 10 formulations of immobilized probiotics developed in this study, including the control (sucrose alone), were evaluated the efficiency to protect probiotic cells through the conditions of freeze-drying. Survivability of immobilized probiotic formulations presented from 60.9 ± 2.2 to 99.4 ± 0.2% (Table 1). It was noted that increase of bio-calcium concentration could effectively increase the survivability of probiotic comparing in the same agitation time. On the other hand, the longest agitation time (90 min) would be required to reach the significant increase of survivability, excepted those from 10% bio-calcium ($P>0.05$). The organic material as bio-calcium powder with the porous structure could be potentially attached by probiotic and protect them from the harsh condition during product processes [(7)]. Where the higher concentration of bio-calcium powder used, the higher supporting surface for probiotic could be provided for probiotic attachment. The result showed that formulation of bio-calcium at 15% (w/v) provided the highest survivability of probiotic. Whereas formulation of bio-calcium at 5% (w/v) supplemented provided the lower survivability, compared with that of the control ($P<0.05$). This might indicate that the lowest level of bio-calcium powder used could not provide enough space or contact surface for probiotic cell to adsorb, while the major amount of probiotic was suspended as free cell. In addition, the sucrose in the formulation that functions as anti-freezing agent and protects the cell by coating, was diluted. Some of sucrose might be divided to cover the bio-calcium powder particle and the less amount of sucrose remained for coating the free cell resulting the less amount of cell survivability. The impact of jackfruit inner skin fiber and whey protein isolate as supporting materials for *L. acidophilus* TISTR 1338 immobilization was reported with 83.1 ± 6.1% of survivability after freeze-drying [(9)]. Indicating the higher efficiency of bio-calcium in this study to protect probiotic cells from freeze-drying conditions compared to those materials. In this study, the formulation which provided the highest survivability of probiotic was selected as optimal formulation.

**Table 1.** Survivability of immobilized probiotic from various formulations after freeze-drying.

| Formulation | Bio-calcium concentration (%; w/v) | Agitation time (min) | Survivability (%) |
|---|---|---|---|
| 1 | 5 | 30 | $60.9 \pm 2.2^{f}$ |
| 2 | | 60 | $61.5 \pm 4.0^{f}$ |
| 3 | | 90 | $72.3 \pm 1.1^{e}$ |
| 4 | 10 | 30 | $82.4 \pm 2.7^{cd}$ |
| 5 | | 60 | $86.9 \pm 2.5^{c}$ |
| 6 | | 90 | $86.4 \pm 1.5^{c}$ |
| 7 | 15 | 30 | $87.3 \pm 1.5^{bc}$ |
| 8 | | 60 | $91.7 \pm 1.5^{b}$ |
| 9 | | 90 | $99.4 \pm 0.2^{a}$ |
| 10* | without bio-calcium | 10 | $80.8 \pm 0.7^{d}$ |

*Indicates formulation of sucrose alone (10 g) (without bio-calcium). Lowercase letters as superscript indicate significant difference (P<0.05) of survivability (%) among various formulations.

### 3.2 Color and water activity of immobilized probiotic formulations

The color and water activity ($a_w$) of various immobilized probiotic formulations in this study. It was found that there was no significant difference on color for all samples tested (P>0.05) for $L^*$ (71.81–75.10), $a^*$ ((-1.34) – (-1.08)) and $b^*$ (5.64-6.46) values (data not shown). These suggested no impact of bio-calcium powder at different concentrations used on appearance, particularly color, of final product. Table 2 showed the water activity of all tested formulations. The similar water activity was observed for all samples with bio-calcium (0.04 ± 0.0) (P>0.05), which was lower than that of the control (0.19 ± 0.0) (P<0.05). The $a_w$ is generally regarded as one of the most important indicators of food quality and food safety [(11)]. Thus, $a_w$ less than 0.6, indicates the safety of this developed immobilized probiotic formulations from microbial proliferation including bacteria, yeast, and mold during storage time [(12)].

**Table 2.** Water activity of immobilized probiotic from various formulations after freeze-drying.

| Formulation | Bio-calcium concentration (%; w/v) | Agitation time (min) | Water activity |
|---|---|---|---|
| 1 | 5 | 30 | $0.11 \pm 0.01^{b}$ |
| 2 | | 60 | $0.04 \pm 0.00^{c}$ |
| 3 | | 90 | $0.04 \pm 0.00^{c}$ |
| 4 | 10 | 30 | $0.04 \pm 0.00^{c}$ |
| 5 | | 60 | $0.04 \pm 0.00^{c}$ |
| 6 | | 90 | $0.04 \pm 0.00^{c}$ |
| 7 | 15 | 30 | $0.04 \pm 0.00^{c}$ |
| 8 | | 60 | $0.04 \pm 0.00^{c}$ |
| 9 | | 90 | $0.04 \pm 0.00^{c}$ |
| 10* | without bio-calcium | 10 | $0.19 \pm 0.00^{a}$ |

*Indicates formulation of sucrose alone (10 g) (without bio-calcium). Lowercase letters as superscript indicate significant difference (P<0.05) of water activity among various formulations.

### 3.3 Stability of immobilized probiotic in various temperature levels

In this study, the stability of optimal formulation against various temperature levels (6 ± 2°C, 25 ± 2°C, and 50 ± 2°C represented low temperature, room temperature, and high temperature) was compared to *L. acidophilus* as free cell (Table 3). At 25 ± 2°C, the result showed significant difference ($P<0.05$) between survivability of probiotics as optimal formulation and free cell at 100.0% and 97.8%, respectively. Nevertheless, there was no significant difference at low temperature condition between optimal formulation and the control ($P>0.05$). Unfortunately, no survival cells of probiotic were detected at 50 ± 2°C. These results suggested that *L. acidophilus* could be stable at cold condition but higher temperature. Bio-calcium powder used as supporting material for fixing this probiotic could improve its stability at room temperature in some degree. However, at the high temperature up to 50ºC, the present formulation could not support the probiotic survivability. The result was in agreement with the previous report which explained that probiotic microorganisms belong to the group of products with low thermal stability. Since, high temperatures can denature intracellular proteins and destabilize cell membranes, which in turn leads to cell death [13]. In 2024, Ardsiri *et al.* [14] and co-workers developed supporting material formulation (pea protein isolate supplemented with inulin and glycerol) to encapsulate *L. acidophilus* TISTR 1338. They reported that the developed supporting material could incredibly protect probiotic cells from various temperatures (refrigeration temperature; 6 ± 2°C, ambient temperature; 26 ± 2°C, and high temperature; 55 ± 2°C) by 100% of survivability. Suggesting that protein structure could play the major role to protect probiotic cells from the adverse temperatures. Therefore, the stability of immobilized probiotic of the present study at the high temperature could possibly be improved by incorporating of protein-based materials and bio-calcium for the future study.

**Table 3**. Stability of immobilized probiotic at optimal formulation and free-cell form at low temperature (6 ± 2°C), room temperature (25 ± 2°C), and high temperature (50 ± 2°C) after 3 h of incubation.

| Temperature levels | Survivability (%)[a] |
|---|---|
| Low temperature | |
| Optimal formulation | $95.7 \pm 4.6^{NS}$ |
| Control | $92.2 \pm 1.3^{NS}$ |
| Room temperature | |
| Optimal formulation | $100.0 \pm 0.5^{A}$ |
| Control | $97.8 \pm 1.5^{B}$ |
| High temperature | |
| Optimal formulation | ND |
| Control | ND |

[*]Survivability (%) reported as mean ± standard deviation (n=3). Uppercase letters as superscript indicate significant difference ($P<0.05$) between optimal formulation and control (free cells). ND indicates that living probiotic cells were not detected. NS indicates non-significant difference.

### 3.4 Microstructure of immobilized probiotic formulation

The microstructure of immobilized probiotic formulation containing 15% bio-calcium with 90 min of agitation time is shown in Figure 1. The application of bio-calcium powder as supporting material showed the cell that attached on the surface of bio-calcium particle. The functional group of organic components in bio-calcium could facilitate the interaction of cell membrane and supporting material [6]. Moreover, the rough surface and porous structure could also facilitate the attachment and entrapment of probiotic in bio-calcium particle (Figure 1A and 1C). In addition, sucrose in the formulation could function as coating and

antifreezing reagent that could protect the cell from freezing damage and environment condition. On the other hand, without bio-calcium powder (Figure 1B and 1D), there is only sucrose with smooth surface that covered the probiotic cell. This result related with the higher survivability of immobilized probiotic with bio-calcium power as supported material, compared with the control (Table 1). Whole cell immobilization was the physical confinement or localization of cells that intact on surface or the region of supporting material space, which preserved the cell survivability or some desired catalytic activity [15,6]. The bio-calcium powder could give the physical protective to the probiotic as supporting material with absorption and entrapment mechanism, resulting the improvement of probiotic survivability and stability during preparation, storage and further application.

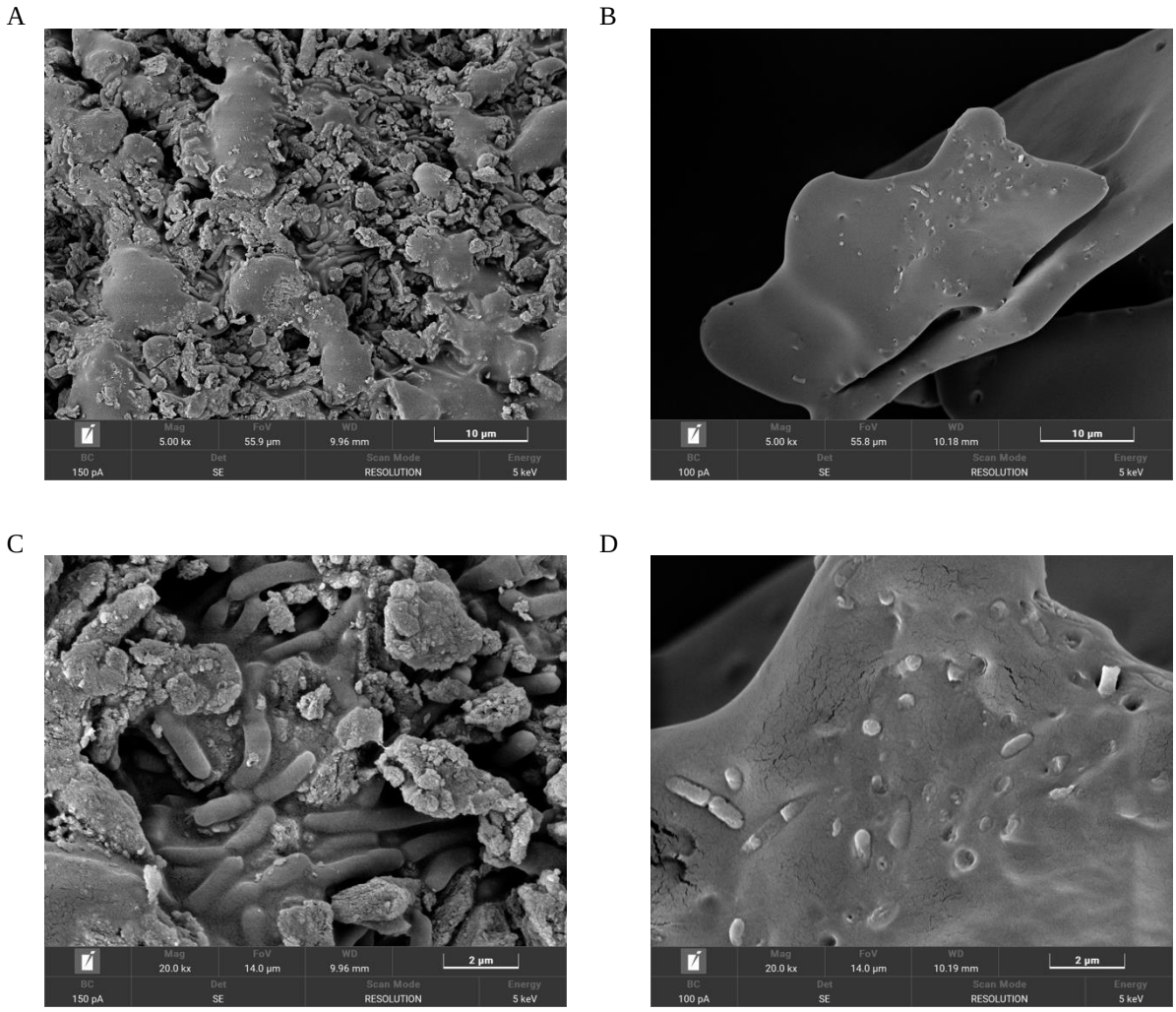


**Figure 1.** Images of immobilized probiotic at optimal formulation (A; 5 kx and C; 20 kx) and sucrose alone (B; 5 kx and D; 20 kx) under scanning electron microscope.

## 4. Conclusion

This study highlighted the high potential of bio-calcium from Nile tilapia bone as supporting material for probiotic immobilization. Concentration of the bio-calcium at 15% (w/v) with 90 min of agitation was suggested to obtain high survivability of probiotic after transformation to be dry powder via freeze-drying. The optimal formulation developed in this study showed the perfect immobilized probiotic cells with no cell damages was observed. Moreover, the developed product showed chemical characteristic as dry powder with low $a_w$ (<0.6), which indicated the desired property to meet food quality and food safety regulations.

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# Application of X-Rays for Eliminating Microorganisms In *Biancaea Sappan* Powder (Fang)

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## Abstract

*Biancaea sappan* (Fang) is a commonly known herb that has many medicinal properties such as nourishing the blood, curing diarrhea, treating athlete's foot, etc. Fang powder is normally found to be contaminated with microorganisms. The aim of this study was to assay the effects of X-rays radiation on the microorganism contamination, bioactive substances content, and antioxidant activities on Fang powder. Fang powder samples were exposed to X-rays at doses of 5, 10, 15, and 20 kGy for 2.5, 5, 7.5 and 10 hours, respectively. The study revealed high number of yeasts and molds in non-irradiated sample. The study showed that the X-rays irradiation at dose 5 kGy was able to eradicate the number of total plate count, yeasts and molds, and pathogenic bacteria, *Bacillus cereus* content to below 10 CFU/g. In the evaluation of the bioactive substance content in Fang, which is brazilin, it was found that irradiation of X-rays resulted in a significant decrease on the brazilin content. Irradiation at doses of 15 and 20 kGy caused the greatest reduction brazilin content. In terms of the antioxidant activities, the irradiation at doses 10-20 kGy significantly decreased the total phenolic content from 94.21 mgGAE/g of non-irradiated sample to 84.73 mgGAE/g of 20 kGy irradiated sample but had no effect on DPPH (68.67-71.11 mgAAE/g) and FRAP (905.54-940.13). In this study, it was concluded that X-rays irradiation of 5 kGy on Fang powder was sufficient to improve the microbial quality and only has lightly changed the content of bioactive substances and antioxidant properties. The successful application of X-rays irradiation to improve the microbial quality of Fang powder opens up possibilities for its use in other food products such as spices, herbs, and other powdered ingredients.

***Keywords***: *Biancaea sappan*; brazilin; antioxidant activity; microbial quality; X-ray irradiation

## 1. Introduction

Fang is a local plant in the tropical regions of Asia. They can be found in different countries. Those are in the tropics include India, myanmar, Thailand, Laos, Cambodia, Sri Lanka, Bangladesh, Vietnam, and southern China. In Thailand, they can be found in dry evergreen forests, mixed deciduous forests, deciduous forests and limestone mountains in the northern, northeastern and some parts of the central region, etc. [(1)] Fang has many medicinal properties including treating diarrhea, blood tonic, expectorant, menstrual and fever. It also has anti-acne [(2)], anti-inflammatory [(3)], antibacterial, antioxidant and antifungal properties [(4)].

An important issue that should be considered regarding the safety of herbal use is microbial contamination. Microorganisms may be found in leaves, stems, flowers, seeds, and roots, which are components of herbal products. [(5)] Spices and herbs are generally processed using a gentle drying method to preserve as much bioactive compounds and volatile components as possible. Additionally, herbs and spices are often produced in open

environments such as sun drying. This process results in high number of microorganism. [6] If these products are exposed to moisture, this can result in the growth of pathogenic microorganisms.[7] Irradiation is one method that can reduce the amount of microbial contamination in food or herbs to be within the standard criteria as announced by the Ministry of Public Health. There are 3 types of sources of radiation that are allowed to be used in irradiating food and herbs according to the announcement of the Ministry of Public Health regarding irradiated food issue, B.E. 2010. Gamma rays from cobalt 60 or cesium 137, X-rays from X-ray generators operating at or above 5 Mev, and electrons from electron accelerators operating at or above 10 Mev. However, most of the research published in the past has involved the use of radioactive gamma rays. The obtaining permission for the possession and application of radioactive substances is burdensome, so in the future the use of radiation from electron accelerators may replace the use of radioactive substances such as electron beams or X-rays [8]. Therefore, the objective of this research is to study the effect of X-rays on Fang powder in terms of microbiological and chemical properties. This will lead to increase confidence in entrepreneurs who irradiate food and herbal products with X-rays in order to gain maximum benefit from using this technology

## 2. Materials and Methods

### 2.1 Samples and X-ray irradiation

Sample of Fang powder was purchased from herbal stores in Bangkok, Thailand. Samples were placed in sealed aluminum foil envelopes and then subjected to X-rays at the irradiation center of Thailand Institute of Nuclear Technology (Public Organization). An X-ray generator with an energy level of approximately 5 Mev from a 50 KW electron accelerator which was installed by Mevex Corporation, Canada. X-ray irradiation was performed at doses of 5, 10, 15, and 20 kGy which employed 2.5, 5, 7.5 and 10 h, respectively. The number of samples used per dose were 6 bags, each containing 200 g.

### 2.2 Microbial load, antioxidant properties, and brazilin content determination

Three bags of each non-irradiated and irradiated samples were used for microbial determination and another three bags were used for chemical assay. The total plate count [9], yeast and mold [10] and *Bacillus cereus* [11] were examined. The study of antioxidant properties can be proceeded by total phenolic content evaluation [12], DPPH radical scavenging activity assay (DPPH assay) [13], and ferric ion reducing antioxidant power assay (FRAP assay) [14]. The brazilin content in Fang powder was determined by using high performance liquid chromatography (HPLC) [15]. The extraction of brazilin in samples can be performed by using water extraction at a temperature of 95° C for 30 min. The experiment conditions are as follows.

Column: C18 (4.6 × 150 mm), Column temperature: 25° C, Injection volume: 10 µL, Run time: 38 min, PDA detector: 280 nm, Mobile phase: 0.3% acetic acid and 100% acetonitrile (Ratio = 85:15), Standard substance: brazilin

### 2.3 Statistical analysis

Data of antioxidant qualities and brazilin evaluation were reported as the means ± standard deviation (SD) of three replicate determinations. Where appropriate, the data were tested by one-way ANOVA followed by Duncan post hoc test using IBM SPSS v.21 with $P<0.05$ was considered significant.

## 3. Results & Discussion

### 3.1 Microbial evaluation

From this study, it was found that X-ray irradiation resulted to decrease the number of microorganisms in Fang powder as shown Table 1. It revealed that the number of total plate count, yeast and mold and *Bacillus cereus* in the non-irradiated sample were $8.5 \times 10^3$, $2.0 \times 10^2$ and 30 CFU/g, respectively. Meanwhile, irradiated samples with 5-20 kGy of X-rays contained total plate count, yeast and mold and *Bacillus cereus* < 10 CFU/g. In general, the sources of microbial contamination of herbs are soil, water, air, anthropogenic, invalid harvest, storage and processing [16]. The production of Fang powder can be done by grinding Fang wood into powder. Therefore, in this process it may cause a high risk of microbial contamination into the product. Reducing microbial contamination of herbal powders can be achieved with and without heat treatment. For heat treatment, the steam is used for many herbal raw materials. The steam temperature is usually between 100 and 200°C then the plant material is dried with hot air and subjected to rapid cooling [16]. Food irradiation by gamma, electron beam or X-ray are known as a 'Cold Process' as the temperature of the processed product does not significantly increase. It is not dependent on humidity, temperature, vacuum or pressure. Thus, the packaging remains intact, as the seals are not stressed. The only variables are source strength and exposure time. The X-ray irradiation is a process that can reduce microbial contamination without generating heat. In general, X-rays are widely used in medical system. There are lightly studies on this application for use in food and herbal. This study revealed x-ray irradiation has the effect of reducing the number of microorganisms in Fang powder. The decontamination of microorganisms is due to X-rays destroying the DNA of microorganisms, causing them to be unable to repair cells damaged by radiation as well as causing the molecular dynamics of the cells to become weak, resulting in the microorganisms being unable to grow [16]. Similarly, the report of Frink *et al*. [16] showed that X-ray irradiation at dose of 2.5 kGy is a feasible method for *Aspergillus* decontamination of cannabis flower. In the same as fresh dairy cheese product, *Pseudomonas* spp. and *Enterobacteriaceae* were decreased by X-ray irradiation. The irradiated samples at doses 2 and 3 kGy showed a significant shelf-life increase, compared to the untreated samplesp [17].

**Table 1.** Effects of X-ray irradiation at various doses on the number of microorganisms in Fang powder.

| Dose (kGy) | Total plate count (CFU/g) | Yeast and mold (CFU/g) | *Bacillus cereus* (CFU/g) |
|---|---|---|---|
| 0 | $8.5 \times 10^3$ | $2.0 \times 10^2$ | 30 |
| 5 | <10 | <10 | <10 |
| 10 | <10 | <10 | <10 |
| 15 | <10 | <10 | <10 |
| 20 | <10 | <10 | <10 |

### 3.2 Antioxidant properties and bioactive substance content determination

*3.2.1 Total phenolic content*

The content of phenolic compounds is shown in Table 2. The irradiated Fang powder tended to have a lower total phenolic content than the non-irradiated sample. This study revealed that X-ray irradiation at a dose of more than 5 kGy resulted in a significant decrease on the total phenolic content ($P \leq 0.05$). The non-irradiated and irradiated samples at 5 kGy showed higher a total phenolic content than the irradiated Fang powder samples at doses of 10, 15, and 20 kGy. The phenolic content of non-irradiated and irradiated at dose 5 kGy samples were 94.21 and 96.64 mgGAE/g, respectively whereas the phenolic content of irradiated samples at doses 10 to 20 kGy were between 86.16 and 84.73 mgGAE/g.

The reduction in a total phenolic content due to product deterioration by radiation [18]. This study showed that higher doses of irradiation resulted in a decrease on total phenolic content, which was according to the experiment of Gumus *et al*. [19] They studied on three spices that are most widely used in Turkey. Their research exhibited that the total phenolic content on these spices were reduced at higher dose of irradiation. On the other hand, there are many reports showing that higher doses of irradiation cause a total phenolic content to increase. Khawory *et al* [20]. studied on effects of radiation treatment in three different medicinal plants. The report revealed that the content of gallic acid in irradiated samples with doses 6-13 kGy increased significantly from before irradiation around 50%. There was report of gamma irradiation affects the total phenol in three different Persian pistachio nuts that was conducted by Akbari *et al* [21]. They showed that irradiation at the dose of 1 and 2 kGy increased total phenolic content in these nuts, whereas it was decreased at a radiation dose of 4 kGy. The effects of irradiation on antioxidant activities vary depending on the type of plant or product may be due to different chemical compositions and other characteristics such as crosslinking, fragmentation and polymerization of phenolic constituents [22].

*3.2.2 Antioxidant properties*

There are several methods for the antioxidant activities determination in food or herbal samples. Evaluating antioxidant activities by DPPH radical scavenging activity assay and ferric ion reducing antioxidant power assay used in this experiment are two favorite methods. The DPPH assay method is based on the principle that antioxidants react with the radical of 1, diphenyl-2-picyldrazyl radical (DPPH). As for the FRAP assay, the principle is that antioxidants can transfer electrons to a complex compound $(Fe_3TPTZ2)^{3+}$. This study illustrated that the irradiation did not significantly alter DPPH and FRAP assay values in Fang powder which were shown in Table 2. Similar to these finding, radiation at doses of 10 and 25 kGy have not changed DPPH value in caraway seeds which reported by Fatemi *et al*. [23]. Lee *et al.* [24] indicated that in fresh ready-to-use tamarind juice a non-significant increase in DPPH radical scavenging activity was observed as the irradiation dose increased.

*3.2.3 Brazilin content*

*Biancaea sappan* or Fang in Thai name is a herbal plant with medicinal properties that grow throughout Southeast Asia [25], called Brazil wood or Sappon wood. Bioactive substance in Fang, brazilin, is commonly found in heartwood. Brazilin is a bioactive substance that provides many benefits such as anti-allergic, antioxidant, antibacterial and anti-acne [26]. Therefore, the present study aims to evaluate the brazilin content in X-ray irradiated Fang powder by HPLC method. This study indicated that irradiation significantly affected to amount of brazilin in Fang powder at $P \leq 0.05$. It showed that irradiated samples contained less brazilin than non-irradiated sample. The irradiated sample at 20 kGy had significantly the least of brazilin content. The effects of irradiation on the bioactive compounds of herbal plants depends on several factors, the species and the irradiation doses are the main factor [27]. The reports with results are similar to the current study that irradiation respond in the reduction of bioactive substances include: decrease on carotenoids in sage, oregano, cinnamon parsley and bird pepper at dose of 10 kGy, [28] decrease on tannic acid in Espinheirasanta at dose 100 Gy [29], decrease the content of synapic acid, ferulic acid and p-coumaric acid in clove at 10 kGy [30] and decrease the amount of curcuminoids in turmeric at dose 100 Gy [31].

**Table 2.** Effects of X-ray irradiation at various doses on antioxidant properties and brazilin content in Fang powder

| Dose (kGy) | Total phenolic content (mgGAE/g) | DPPH$^{ns}$ (mgAAE/g) | FRAP$^{ns}$ (µmol $FeSO_4$/g) | Brazilin content (mg/g) |
|---|---|---|---|---|
| 0 | 94.21 ± 2.48$^{b}$ | 68.67 ± 2.40 | 936.54 ± 31.44 | 11.93± 0.30$^{d}$ |
| 5 | 96.64 ± 4.03$^{b}$ | 69.56 ± 2.14 | 940.13 ± 7.34 | 10.67 ± 0.11$^{c}$ |
| 10 | 86.16 ± 6.39$^{a}$ | 69.56 ± 3.36 | 905.54 ± 15.24 | 9.80 ± 0.20$^{b}$ |
| 15 | 82.76 ± 4.12$^{a}$ | 71.11 ± 1.02 | 922.82 ± 7.71 | 10.00 ± 0.34$^{b}$ |
| 20 | 84.73 ± 5.07$^{a}$ | 68.89 ± 2.69 | 920.40 ± 58.86 | 9.17 ± 0.25$^{a}$ |

Different superscript letters in the same column mean statistically significant difference (P≤0.05).
ns = not significant

## 4. Conclusion

X-ray irradiation has the effect of reducing microbial contamination in Fang powder, resulting in better product quality. In this study, it was concluded that 5 kGy of X-ray irradiation was sufficient to reduce microbial contamination and resulted in a slight decrease in the quality of antioxidant and the content of bioactive substance.

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# That is My Cup of Tea: Emotion and Sensory Characteristics of Tisane Infusions

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**Abstract**

Tisanes is a healthy herbal tea considered a functional food rich in antioxidants. This study aimed to survey consumers' behavior and sensory emotions toward tea infusions blended with herbs for relaxation and calmness. The tea was infused with chamomile, lavender, mimosa, rose, and stevia. The survey involved 126 participants, comprising 31.7% males and 68.3% females. Consumer acceptance of Tisane was assessed using 9-point hedonic scale. The results indicated the majority of respondents were company employees with an average income of 10,000-20,000 baht per month. Overall liking was 7.76 ± 1.15 and was unrelated to gender ($P>0.05$). The hedonic scale for various attributes was assessed, including color, clearness, chamomile aroma, sweet aroma, spices aroma, sweetness, chamomile flavor, aftertaste, and astringency. The mean scores for these attributes were 7.52 ± 1.43, 7.72 ± 1.24, 7.72 ± 1.23, 7.52 ± 1.42, 6.79 ± 1.77, 7.02 ± 1.84, 7.52 ± 1.29, 7.07 ± 1.84, and 7.60 ± 1.35, respectively. Age significantly impacts hedonic rating on astringent attributes ($P\leq0.05$). In terms of 'Just About Right' evaluations, participants indicated that color, clearness, chamomile flavor, and aftertaste were perceived as just about right. However, other attributes were reported to be either too low or too high, with spice aroma and astringency specifically leaning towards a too-low level. The sensory emotions, measured through 5 scores, indicated strong feelings of relaxation (3.71 ± 0.83) and calmness (3.36 ± 0.90) toward the tea formulation. In conclusion, consumers most desired the tea that promotes relaxation, promotes sleep, and decreases stress. The findings will benefit developing the formulation of tisanes in the future. It provides more knowledge of herbal tea infusions with medicinal properties for relaxation and calmness.

***Keywords***: Consumers acceptance; Functional food; Tisanes; Sensory Emotional; Lifestyle Medicine

## 1. Introduction

Non-communicable diseases (NCDs) are chronic diseases resulting from a combination of genetic, environmental and behavioral factors. NCDs kill 41 million people, accounting for 74% of death worldwide every year [(1)]. In Thailand, NCDs are the main cause of death, accounting for 76% of all deaths reported in 2023 [(2)]. Because most death occurs earlier than 70 years of age, NCDs severely hinder economic and social development [(1)].

Lifestyle and dietary behaviors are major modifiable factors for NCDs. Healthy diet, exercise, and sleep are parts of Lifestyle Medicine (LM) that have been integrated into public health recommendations to prevent and manage several NCDs [(1, 3-6)]. LM is a growing and well-known medical field that is focused on holistic lifestyle behaviors for the prevention and treatment of chronic diseases [(7-8)].

Herbs are an important component of LM medicine that has been around for at least 5,000 years [11-13]. Adaptogenic herbs promote feelings of calm and relaxation [14] with purported benefits of reducing fatigue, improving mental performance, and relieving depression and anxiety [15-19]. Chamomile is one of the most popular adaptogens that has been used as a natural sleep aid. Another type of herbs that promote relaxation and sleep is called nervines. Nervines specifically inhibit the nervous system to promote calmness, possibly by γ-aminobutyric acid modulation [21-21]. Examples of nervines include lemon balm, lavender, chamomile, and valerian [13,20-21]. In traditional Thai, Chinese, and Indian medicine, often blended with various infusions [22-25]. Due to these benefits, adaptogens and nervines are often blended and consumed as tea in the LM practice.

Herbal tea or tisane is an uncaffeinated beverage made from soaking or boiling dried herbs and spices in hot water to extract flavor and aromatic compounds, as well as phytochemicals. Because tisanes were often a blend of various plant parts (stems, roots, leaves, flowers, bark, rhizomes, and/or shrubs), this type of tea is rich in phytochemicals and antioxidants, considered one of the functional foods and an important health beverage [26-28].

This makes it an enticing product in the growing functional food market. The objectives of this study were 1) to survey the attitude, emotional feeling, and behavior of Thai consumers toward herbal tea (tisanes) and 2) to measure the antioxidant properties of and sensory emotion of the consumers toward an herbal tea formula used in Thai traditional medicine practice.

## 2. Materials and Methods

### 2.1 Tea sample and preparation

Flowers, fruits, leaves, barks, and other plant parts were used to brew tea by following the traditional formulation of the Phrommin Thai Traditional Medicine Clinic, Chiang Mai, Thailand [46] [in Thai]. An herbal tea blend was created using five different herbs, including chamomile (*Chamaemelum nobile* L.), lavender (*Lavandula angustifolia Mill*), mimosa (*Mimosa pudica* Linn*)*, rose (*Rosa damascena*), and stevia (*Stevia rebaudiana Bertoni*). The tea powder quality was assessed using standard methods. Specifically, moisture content was 6.94%. Water activity ($a_w$) was 0.49. Color parameters were $L^*$ = 96.90 ± 0.18, $a^*$ = -3.77 ± 0.91, and $b^*$ = 17.93 ± 0.45.

Additionally, total phenolic content and antioxidant capacity were measured. The total phenolic content was 39.39 ± 1.70 mg GAE/g tea powder. DPPH activity was 46.90 ± 3.36 mg TE/g tea powder. FRAP activity was 114.94 ± 6.98 mM $Fe^{2+}$ equivalent /g tea powder. ABTS activity was 89.03 ± 2.66 mg TE/ g tea powder.

The herbal tea preparation involved steeping each tea sachet (1-1.5 g) in 200 mL of water at 96°C ± 1°C for 5 min. This method was adapted from the study by McAlpine and Ward [29].

### 2.2 Participants and Study Protocol

Quota sampling was used to select participants equally from three age groups: 18-40, 41-60, and over 60 years old. A total of 126 participants enrolled in the study. The survey was conducted in Chiang Mai province during the 15 October to November 2023. The inclusion criteria included those with experience in tea drinking and no history of allergy to herbal or tea formulations. Eligible participants were asked to fill out a questionnaire, which had three parts. The first part gathered demographic data, including gender, age group, professional career, allergic history, education level, occupation, and monthly income. The second part was focused on general opinions about herbal tea consumption and emotions toward the herbs used in this study, along with a preference ranking score. The third part was dedicated to sensory testing of tisanes products and their acceptance, utilizing a 9-point hedonic scale and a 5-point Just About Right score. For parts 2 and 3, participants were served 20 mL of tea

formulation in clear plastic cups, with the temperature meticulously maintained between 60-65°C.

**2.3 Emotion and sensory evaluation**

This study delved into consumer attitudes, emotional feelings towards herbs, behavior related to tea consumption, and acceptance of tisanes. The survey, which included 126 participants, consisted of three parts. The first part gathered demographic data, including gender, age group, professional career, allergic history, education level, occupation, and monthly income. The second part focused on general opinions about herbal tea consumption and emotional feelings toward the herbs used in this study, along with a preference ranking score. The third part was dedicated to sensory testing of tisanes products and their acceptance, utilizing a 9-point hedonic scale and a 5-point Just About Right score. The questionnaire consisted of the evaluation of sensory tests and emotional feelings. Sensory evaluation of the tea formulation was conducted using 9-point hedonic scale. Which of the nine levels of sensory hedonic scales. Scale 1.) Dislike extremely 2.) Dislike very much 3.) Dislike moderately 4.) Dislike slightly 5.) Neither like nor dislike 6.) Like slightly 7.) Like moderately 8.) Like very much 9.) Like extremely. Which, 5 scores of emotional feeling scaling were 1.) feeling weak 2.) mild feeling 3.) moderately feeling 8.) feeling very much 9.) extremely feeling. The attributes consisting of color, clearness, chamomile aroma, sweet aroma, spices aroma, sweet flavors, chamomile flavor, astringent, aftertaste, and overall liking modified from Mutumon [47] [in Thai].

**2.4 Statistical analysis**

Data was inspected prior to analysis. Differences in sensory scores by participant characteristics were analyzed by using t-test or ANOVA. Chi-square tests were used for emotion questionnaires. Logistic regression analysis was used to analyze acceptance and intention of purchasing.

## 3. Results & Discussion

**3.1 General demographic and behavior of tea consumption data**

The study involved 126 participants, with a gender distribution of 31.7% male and 68.3% female. Additionally, 36.5% of participants aged between 18-40 years, 31.0% between 41-60 years, and 32.5% over 60 years. Educational backgrounds varied, with 35.7% having a bachelor's degree, 27% having completed high school, and 20.6% holding a master's degree. The predominant professions among participants were general employees (21.4%), students (20.6%), and housewives/husbands (13.5%). Most people reported an average monthly income of 10,000-20,000 baht (Table 1).

**Table 1.** General participant characteristics (N = 126)

| Socio-demographic data | Number (N=126) | Percent (%) |
|---|---|---|
| **Sex** | | |
| Male | 40 | 31.7 |
| Female | 86 | 68.3 |
| **Age** | | |
| 18-40 years | 46 | 36.5 |
| 41-60 years | 39 | 31.0 |
| > 60 years | 41 | 32.5 |
| **Education** | | |
| High school | 34 | 27.0 |
| Diploma | 6 | 4.8 |
| Bachelor degree | 45 | 35.7 |
| Master degree | 26 | 20.6 |
| Philosophy of doctoral | 4 | 3.2 |
| other | 11 | 8.7 |
| **Occupation** | | |
| Student | 26 | 20.6 |
| General employee | 27 | 21.4 |
| Civil servant | 14 | 11.1 |
| Retirement | 11 | 8.7 |
| State enterprise employees | 3 | 2.4 |
| Private company employee | 8 | 6.3 |
| Businessperson | 2 | 1.6 |
| Housewife/household (male, female) | 17 | 13.5 |
| Business owner/ personal shop | 10 | 7.9 |
| Othe | 8 | 6.3 |
| **Salary (Baht)** | | |
| Less than 10,000 | 44 | 34.9 |
| 10,001-20,000 | 40 | 31.7 |
| 20,001-30,000 | 16 | 12.7 |
| 30,001-40,000 | 6 | 4.8 |
| > 40,000 | 20 | 15.9 |
| **Allergy** | | |
| Yes | 2 | 1.6 |
| No | 124 | 98.4 |

In this survey examining tea consumption behaviors, most participants (62.7%) preferred ready-to-drink beverages and only 5.6% disliked tea. A notable 94.4% were inclined towards healthy beverage diets. Regarding consumption volume, 34.1% reported drinking around 150 mL of tea daily, and 76.9% consumed tea either daily or 2-3 times a month. Shopping malls, convenience stores, and healthy beverage shops were the most favored locations for purchasing herbal tea, preferred by 55.6%, 53.2%, and 45.2% of participants, respectively (Table 2).

**Table 2**. Consumer behavior and opinion (N = 126)

| Behavioral | Number | Percent (%) |
|---|---|---|
| **Interest in Tisane/ tea healthy drink** | | |
| Interested | 119 | 94.4 |
| Not interested | 7 | 5.6 |
| **Frequency of tea Consumption** | | |
| everyday | 9 | 7.1 |
| 2-6 times/week | 27 | 21.4 |
| 1 time /wk | 29 | 23.0 |
| 2-3 times/month | 32 | 25.4 |
| Less than 1 time/month | 22 | 17.5 |
| other | 7 | 5.6 |
| **Opinions on the price of herb infusion in the market** | | |
| Cheap | 8 | 6.3 |
| Costly/ expensive | 11 | 8.7 |
| Depending on the Tea type | 53 | 42.1 |
| Reasonable | 54 | 42.9 |
| **Form of tea drink** | | |
| Ready to drink | 79 | 62.7 |
| Tea powder | 28 | 22.2 |
| Other | 19 | 15.1 |
| **Volume of consuming** | | |
| 100 milliliters | 30 | 23.8 |
| 150 milliliters | 43 | 34.1 |
| 200 milliliters | 25 | 19.8 |
| 250 milliliters | 23 | 18.3 |
| Other | 5 | 4.0 |
| **Place of purchasing** | | |
| General grocery store | 37 | 29.4 |
| Shopping mall | 70 | 55.6 |
| Healthy shop | 57 | 45.2 |
| Market | 13 | 10.3 |
| Food truck | 18 | 14.3 |
| Restaurant with freezer | 13 | 10.3 |
| Coffee shop | 37 | 29.4 |
| Convenient store | 67 | 53.2 |
| Coin-operated freezer | 2 | 1.6 |
| Other | 14 | 11.1 |

The top motives for consuming tisanes were health promotion, good taste, and quality of ingredients, receiving preference scores of 179, 147, and 120, respectively. Relaxation, help with sleeping, and decreased stress were the top three motivating factors for consumers to drink herbal tea (Table 3). Regarding the reason for purchasing, the participants showed a strong preference for tea that was developed to enhance health, offer a pleasing taste, and contain high-quality ingredients, as reflected in the respective sum scores of 258, 212, and 96.

**Table 3**. Consumers' rating on consumption motivation and reason for purchasing (N = 126)

| Consumption motivation | Sum score | Reason for purchasing | Sum score |
|---|---|---|---|
| Promote Relaxation | 179 [1] | Promote Health | 258 [1] |
| Promote Sleep | 147 | Taste | 212 |
| Release stress | 120 | Ingredient | 96 |
| Health- Fresh | 90 | Cost | 84 |
| Nervous system tonic | 88 | Cultivar | 51 |
| Blood tonic | 65 | Package | 22 |
| GI Tonic | 25 | Color | 14 |
| Pandan- YaNang | 19 | Brand | 12 |
| Chamomile | 16 | Promotion | 2 |
| Other | 7 | Other | 0 |

[1]The gradient in color from red to green shown the high frequency to the lowest

### 3.2 Emotions toward Herbs and Tisanes Infusions tea

Relaxation and calming emotions toward the study herbs and tea formulation were unrelated to sex or age (Chi square = 0.83) with a moderately strong feeling. The relaxation feeling was highest for chamomile, lavender, and rose, having 3.83 ± 1.07, 3.46 ± 0.92, and 3.44 ± 0.97 rating scores respectively, while *Mimosa Pudica* Linn and stevia received the lowest relaxation feeling scores (2.06 ± 1.15 for relaxation and 2.35 ± 1.27 for calmness) including stevia (2.83 ± 1.13). (Figure 1a). Similar results were observed for calmness emotion, in which chamomile, lavender, and rose showed the highest scores (Figure 1b). When all five herbs were blended and brewed into tea, participants reported moderate to strong feelings of relaxation (3.71 ± 0.83) and calmness (3.36 ± 0.90) (Figure 1c). Results of hedonic assessments suggested that these healthy tisanes beverages were likely to be accepted and purchased by consumers.

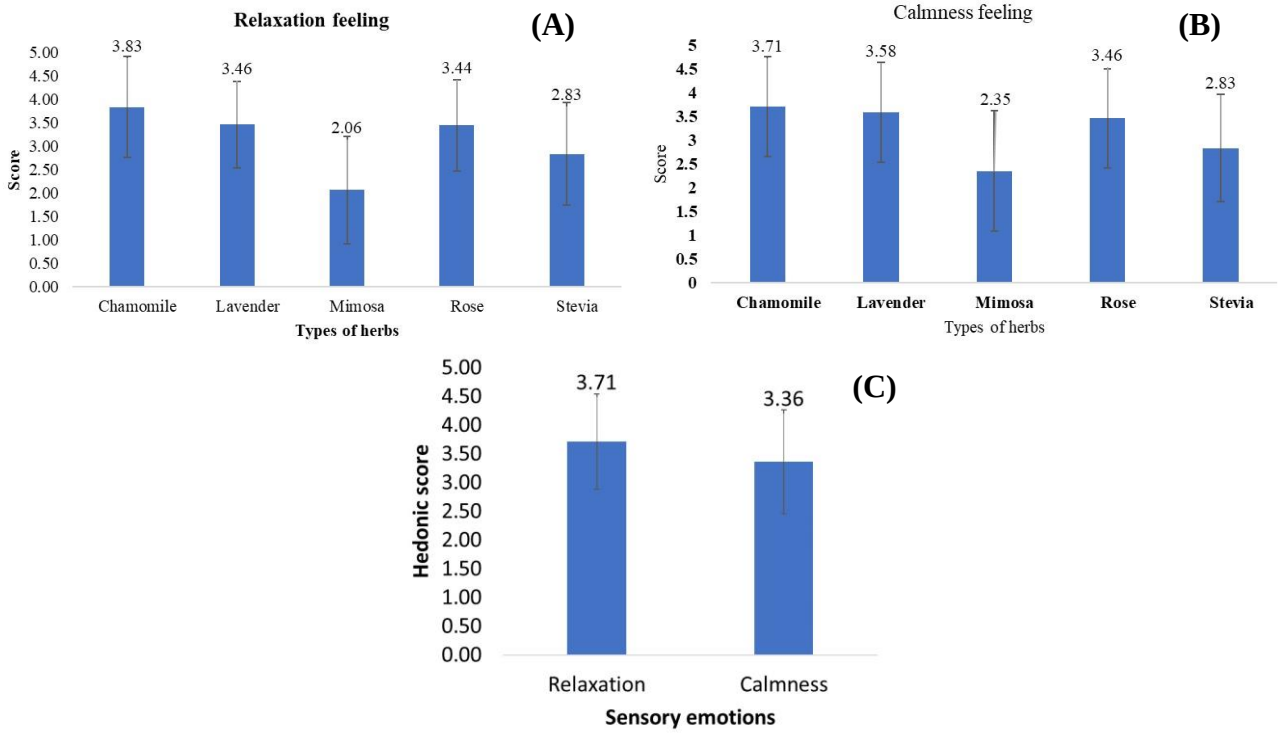


**Figure 1.** Relaxation (A) and calmness emotions (B) toward five herbs, and strength of relaxation and calmness emotions toward tisanes made from these herbs (C)

### 3.3 Consumer acceptance of herbal tea (tisane infusion)

Consumer acceptance was evaluated using 9-point hedonic ratings. Mean overall liking of the tisane infusion was 7.76 ± 1.15 with no significant differences observed between genders (P>0.05;Figure 2). On average, participants liked the following attributes moderately: color (7.52 ± 1.43), clearness (7.72 ± 1.24), chamomile aroma (7.72 ± 1.23), sweet aroma (7.52 ± 1.42), sweetness (7.02 ± 1.84), chamomile flavor (7.52 ± 1.29), aftertaste (7.07 ± 1.84), and astringency (7.60 ± 1.35). Comparison between age groups showed that participants aged 41-60 years had a lower mean astringent score than the others (P< 0.001; Table 4).

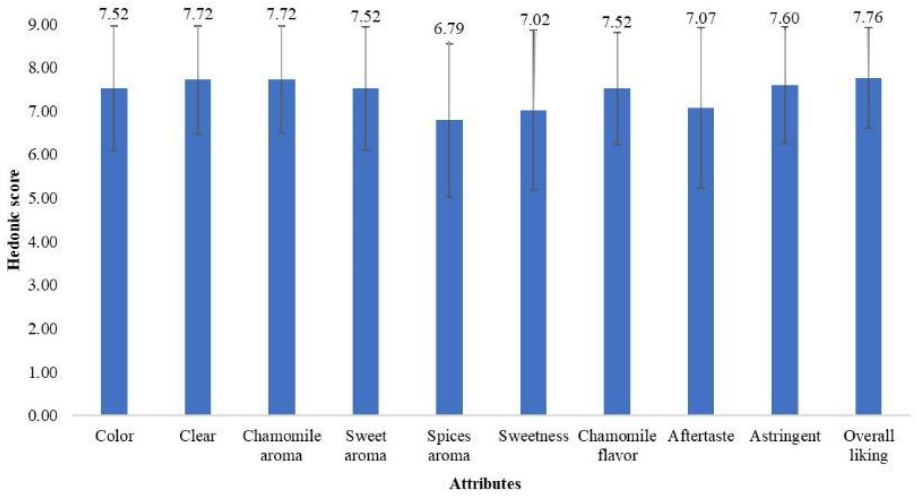


**Figure 2.** Nine-point hedonic scores of tisanes infusion

To understand the direction of consumer preference, the Just About Right scale was used. Figure 3 showed that ≥70% of participants found the color, clarity, chamomile flavor, and aftertaste of the study tea formulation to be just about right. However, <70% were satisfied with the sweetness, sweet aroma, and chamomile aroma. Based on this result, the tea formulation should be adjusted to increase the aroma of spices and sweetness while reducing its astringency.

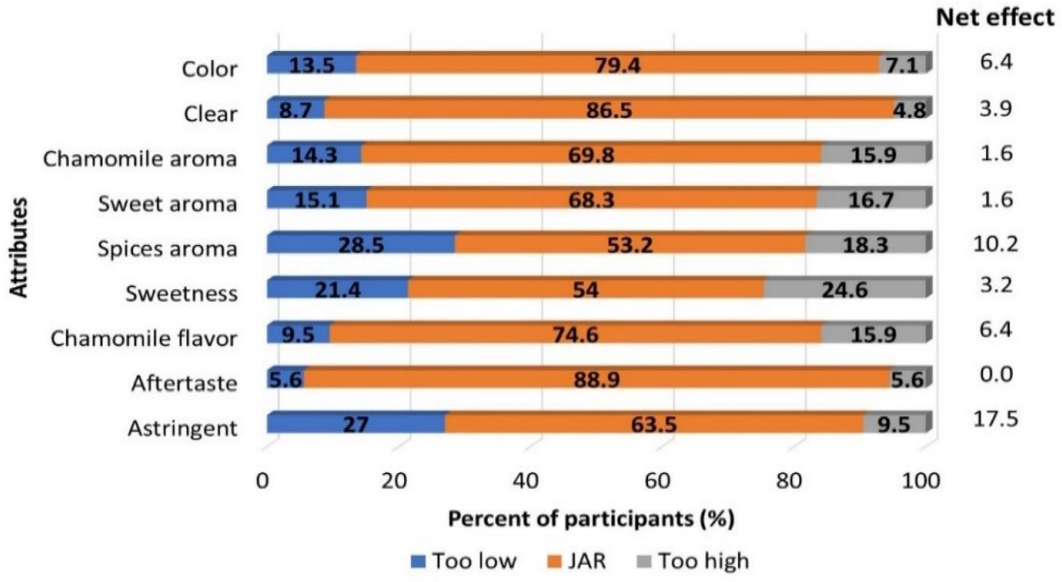


**Figure 3**. Just About Right (JAR) scale of the tisane attributes. ≥70% JAR was considered satisfactory.

**Table 4**. Nine-point hedonic ratings of tisanes by sex and age[2]

| Parameter | Attribute | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Aroma | | | Flavor | | | | |
| | Color | Clearness | Chamomile | Sweet | Spices | Sweetness | Chamomile | Astringent | Aftertaste | Overall liking |
| **Sex** | | | | | | | | | | |
| **Male** | 7.52$^{ns}$±1.43 | 7.50$^{ns}$±1.41 | 7.53$^{ns}$±1.45 | 7.40$^{ns}$±1.37 | 6.70$^{ns}$±1.96 | 6.77$^{ns}$±1.75 | 7.33$^{ns}$±1.31 | 6.95$^{ns}$±1.80 | 7.45$^{ns}$±1.26 | 7.52$^{ns}$±1.24 |
| **Female** | 7.51$^{ns}$±1.44 | 7.83$^{ns}$±1.15 | 7.81$^{ns}$±1.11 | 7.57$^{ns}$±1.44 | 6.83$^{ns}$±1.68 | 7.14$^{ns}$±1.88 | 7.62$^{ns}$±1.28 | 7.13$^{ns}$±1.87 | 7.67$^{ns}$±1.38 | 7.87$^{ns}$±1.10 |
| **Age** | | | | | | | | | | |
| **18-40** | 7.30$^{ns}$±1.33 | 7.76$^{ns}$±1.23 | 7.72$^{ns}$±1.11 | 7.48$^{ns}$±1.09 | 6.74$^{ns}$±1.51 | 7.15$^{ns}$±1.49 | 7.59$^{ns}$±1.20 | 7.20$^{a}$±1.76 | 7.50$^{ns}$±1.31 | 7.63$^{ns}$±1.02 |
| **41-60** | 7.38$^{ns}$±1.73 | 7.41$^{ns}$±1.53 | 7.77$^{ns}$ ±1.39 | 7.54$^{ns}$±1.89 | 6.46$^{ns}$±1.85 | 6.64$^{ns}$±2.06 | 7.28$^{ns}$±1.60 | 6.26$^{b}$±2.23 | 7.41$^{ns}$±1.59 | 7.69$^{ns}$±1.32 |
| **>60** | 7.88$^{ns}$±1.17 | 7.98$^{ns}$±0.85 | 7.68$^{ns}$±1.23 | 7.54$^{ns}$±1.25 | 7.15$^{ns}$±1.92 | 7.24$^{ns}$±1.96 | 7.68$^{ns}$±1.01 | 7.71$^{a}$±1.15 | 7.90$^{ns}$±1.01 | 7.98$^{ns}$±1.13 |

[2] Data value was mean ± standard deviation. Nine levels of sensory hedonic scales were 1 = strongly dislike, 2 = dislike very much, 3 = moderately dislike, 4 = dislike a little, 5 = neutral, 6 = little like 7 = moderately like, 8 = strong like, 9 = very strong like. Letters a and b show a statistically significant difference ($p<0.05$) between sex or age groups; ns= not significant

### 3.4 Factors associated with the purchase intention of tisanes

Logistic regression analysis, based on responses from 126 participants, generated an equation for predicting purchase intention. This equation, presented in Table 5, explained 95.2% of variation ($R^2$) in purchase intention. The probability of purchasing was significantly influenced by sweetness and aftertaste. Specifically, for each 1-point increase in the hedonic score for sweet flavor, the chance of purchasing increased by 88%. Similarly, a 1-point increase in the aftertaste was associated with an increased chance of purchasing by ~150%.

**Table 5.** Logistic regression analysis showing factors affecting the purchase intention of tisane products. Model $R^2$ = 95.2%

| Attributes | Beta | Significant (P≤0.05) | Odd Ratio [Exp(B)] | 95% C.I.for EXP(B) | |
|---|---|---|---|---|---|
| | | | | Lower | Upper |
| Color | -.310 | .439 | .734 | .335 | 1.608 |
| Clearness | -.468 | .359 | .626 | .231 | 1.700 |
| Chamomile aroma | .828 | .073 | 2.288 | .925 | 5.659 |
| Sweet aroma | -.506 | .152 | .603 | .302 | 1.204 |
| Spices aroma | .279 | .332 | 1.321 | .753 | 2.318 |
| **Sweetness** | **.633** | **.007** | **1.884** | **1.193** | **2.974** |
| Chamomile flavor | -.550 | .311 | .577 | .199 | 1.673 |
| Astringent flavor | -.191 | .523 | .826 | .460 | 1.484 |
| **Aftertaste** | **.910** | **.017** | **2.485** | **1.175** | **5.256** |
| Constant | -1.196 | .630 | .303 | | |

Purchase intention (Y) =-1.196-0.310 Color-0.468 clearness + 0.828 Chamomile aroma -0.506 sweet aroma+0.279 Spices aroma + **0.633Sweetness***-0.550 Chamomile flavor-0.191 Astringent + **0.910Aftertastes***

## 4. Discussions

*Mimosa pudica*, despite being recognized in Traditional and Ayurvedic medicine for its medicinal properties that promote sleep and soothe the nervous system [22-26], receives the lowest score on relaxation and calmness in the sensory emotion assessment of tisanes as well as stevia. The emotions that general consumers feel towards chamomile, lavender, and rose range from moderate to high, aligning with the widespread knowledge of these herbs being recognized for their relaxing and calming properties [30]. This discrepancy could indicate a lack of general consumer knowledge about this plant. Stevia is commonly used as a sugar substitute or to enhance taste, and it was frequently studied concerning the acceptance of the development of energy-free snack products [31-32]. The survey indicated a consumer preference for purchasing blended tisanes, largely due to their high health-promoting properties, appealing taste, and quality ingredients. In terms of specific interests, tisanes that promote relaxation were most popular, followed by those aiding in sleep and relieving stress. The study also uncovered those sensory attributes, particularly taste and flavor, are crucial in determining consumer liking and their readiness to try these healthier beverage options. Furthermore, it was noted that texture significantly influences consumer preferences and their willingness to make a purchase [34-35]. In terms of 'Just About Right' evaluations, participants indicated that color, clearness, chamomile flavor, and aftertaste were perceived as just about right. However, other attributes were reported to be either too low or too high, with spice

aroma and astringency specifically leaning towards a too-low level. The limitations of astringent or spices sensory surveys in general consumers compared to panelists can be attributed to several factors. General consumers may have limited knowledge and experience with astringent sensations, making it challenging for them to accurately identify and describe these attributes [36]. The results of the taste function were affected by age, the ability to identify sweet and salty tastes was not affected by age, but bitter was affected by age [37]. Gender was not affected by the ability to perceive astringency corresponding to Michon *et al.* [38].

From the logistic regression in each variable, the possibility of purchasing was shown significantly of the sweetness hedonic score of aftertastes more likely to buy respectively corresponding to the works by Deliza [39] and Novela *et al.* [40]. Based on research conducted in the field of consumer behavior, it has been observed that a 1-score level increase in the sweet flavor hedonic score significantly enhances the likelihood of purchasing a product. Additionally, when the aftertaste is more favorable, it tends to generate a stronger intention to buy more of the product This influence on sensory perception, suggests that a positive aftertaste significantly contributes to enhanced hedonic ratings [39-40]. A favorable aftertaste was known to create a positive perception, influencing consumers' intention to buy the product and attitude plays a significant role in the relationship between motivation and purchase intention. Certainly, consumer behavior, including choices in healthy food and herbal teas, was influenced by various factors. Here are aspects of consumer behavior along with relevant ones. Most people prefer good health and wellness, in this recent study, consumers desired the tisanes tea that promotes healthy, good sleep and decreases stress which was relevant to the study from Grunert [41]. In conclusion, the chance of purchasing a product was likely to increase when there was a higher sweetness flavor hedonic score and a more favorable aftertaste [42]. Customers were more inclined to purchase a product perceived to offer high quality and a pleasurable taste, aligning with the findings of Kotler *et al.* [43]. Consumers were increasingly seeking out health-promoting products. They were often chosen for their potential therapeutic properties and natural ingredients, appealing to those who prefer a more holistic approach to health while contributing to the desired health promotion of tisanes [44]. Health Benefits, consumers are motivated to purchase teas for their perceived health benefits [45].

## 5. Conclusions

The sensory and emotional survey on tisanes/herbal tea revealed significant insights into how its ingredients impact consumers' senses and emotions, providing compelling evidence of their positive effects. This study highlights the potential of these ingredients to not only enhance sensory experiences but also promote emotional well-being. The ability of the tea to induce a sense of calmness and emotional balance particularly underscores its potential as a natural remedy for stress relief and relaxation. These insights can be invaluable for refining existing tea blends or developing new ones that align more closely with consumer expectations. The appeal of these teas in terms of taste, aroma, and overall sensory experience was crucial, as consumers are more likely to purchase and regularly consume products, they find appealing.

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# Size Reduction of Rice Starch Granule by Acid Hydrolysis Combining with Ball Milling

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**Abstract**

The aim of this study was to devise a technique for reducing the size of rice starch granules by the process of acid hydrolysis in conjunction with ball milling. Rice starch was hydrolyzed with 3.16 M $H_2SO_4$ for every 4 hours up to 24 h at 40ºC. The hydrolysis time that resulted in the maximum hydrolysis of the amorphous lamellae in the rice starch granule was measured by observing the changes in the weight yield and the height ratios of the FTIR bands at 1047 and 1022 $cm^{-1}$ of the hydrolyzed starch. The findings indicated a notable decline in the yield weight within the initial 16 h, followed by a gradual fall thereafter. The ratios of the FTIR bands at 1047 and 1022 $cm^{-1}$, which are indicative of the level of crystallinity in starch granules, exhibited a gradual increase until the 16-h mark, followed by a subsequent reduction. Subsequently, the rice starch that had undergone a 16-hours acid hydrolysis process was subjected to wet milling using a ball mill in 96% ethanol, with a weight ratio of 1:3, for a duration of 3 h. The morphologies of rice starches were examined using SEM, while the particle size distribution was analyzed using a particle size analyzer. The acid-hydrolyzed starch granules exhibited a more uneven surface compared to the native starch granules, however their granule size remained comparable to that of the native starch. The starch granules that underwent ball milling exhibited fractured granules. The acid-hydrolyzed starch, when combined with ball-milled starch, showed the formation of small, irregular-shaped particles that have a tendency to congregate and form agglomerates. The volume average diameter (D[4,3]), for native, acid-hydrolyzed, ball-milled, and acid-hydrolyzed combined with ball-milled starches were 9.54, 8.92, 6.07, and 5.16 μm, respectively. The study demonstrated the combined impact of acid hydrolysis and ball-milling on reducing the size of rice starch granules, resulting in a synergistic effect.

***Keywords**:* Rice starch; Acid hydrolysis; Ball milling; Starch small particle

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## 1. Introduction

Due to their distinctive characteristics, small particles made from starch have attracted considerable attention in a range of fields, especially in biomedical and food industry applications. These small particles, which have dimensions in the nanometer range, possess unique qualities when compared to larger particles. These qualities include increased stability, solubility, and improved capacity to penetrate biological barriers. Recently, there has been an increasing amount of study dedicated to creating and using tiny particles made from starch in several fields. This includes using them as carriers for delivering drugs [(1)] and as stabilizers in emulsions [(2)]. These particles are made from starch, which is a plentiful, inexpensive, safe, naturally renewable, and biodegradable substance obtained from many plants.

Ball-milling is a mechanical technique employed to alter different materials, such as starch. Ball milling has been extensively employed in various domains due to its simplicity, cost-efficiency, and eco-friendliness. Starch modification can be achieved by utilizing ball milling to generate fine starch particles. Prior research indicated that starch particles with a number-average diameter of 260 nm were produced during the process of ball milling. Additionally, there were also particles smaller than 100 nm present [3]. Consequently, the extended duration of ball milling resulted in a reduction in the crystalline structure of starch [4].

The utilization of acid hydrolysis has been extensively employed in the production of fine particles from starch granules. Starch is a complex carbohydrate consisting of two constituents: amylose and amylopectin. Starch granules possess both crystalline and amorphous areas. Upon acid exposure, the initial phase occurs swiftly as a result of the hydrolysis of the non-crystalline regions, while the subsequent phase is longer and linked to the hydrolysis of the crystalline regions [5]. The non-crystalline areas of the starch granules are selectively broken down through hydrolysis, resulting in the disintegration of the granule structure. This method produces fragile residues that are highly prone to mechanical degradation. There have been publications indicating that the utilization of ball milling, following acid pretreatment, has demonstrated significant efficacy in the disintegration of maize starch granules [6]. However, there is currently no documented report on the process of producing small starch particles by combining acid hydrolysis with a ball mill utilizing rice starch.

The aim of this study was to devise a technique for decreasing the particle size of rice starch granule by the process of acid hydrolysis in conjunction with ball milling.

## 2. Materials and Methods

### 2.1 Materials

Rice starch with approximately 25.2% amylose was purchased from Thai Flour Industry Co., Ltd. (Thailand). All other chemicals and reagents were purchased from NT chemical Co., Ltd. (Thailand).

### 2.2 Preparation of acid-hydrolyzed starches

The acid hydrolysis procedure was carried out based on a prior study [7] with certain alterations. The experiment involved the combination of 20 grams of unmodified rice starch with 250 milliliters of an aqueous solution containing sulfuric acid at a concentration of 3.16 M. The combination underwent continuous stirring at a rate of 150 rpm and was heated at a temperature of 40ºC for a duration of 4 h, repeated every 4 h for a total of 24 h. Following the completion of the hydrolysis process, the starch suspension was subjected to centrifugation (using a Kubota centrifuge from Tokyo, Japan) at a speed of 10,000 rpm for a duration of 15 min. Distilled water was used for washing until the pH value approached neutrality. The obtained precipitate was subsequently freeze-dried using a freeze drier manufactured by LABCONCO in Kansas, USA. The acid-hydrolyzed starch yield was determined by dividing the weight of starch after acid hydrolysis by the weight of starch before hydrolysis.

### 2.3 Ball milling

The ball milling process was conducted using a Pulverisette 6 ball mill manufactured by FRITSCH Inc. in Germany. A quantity of ten grams of rice starch, which had undergone a 16-h acid hydrolysis process, was placed in a stainless-steel mill pot with a volume of 250 mL. Subsequently, 30 mL of ethanol with a concentration of 96% was added to evenly distribute the starch. Using ethanol as a humectant instead of water reduces gelatinization, a process that occurs during milling in a liquid environment [8]. The starch samples were ground using stainless steel balls, specifically 50 balls of 10 mm in diameter. The operation was

performed at a rolling speed at number 2 for a duration of 3 h. The starch that underwent ball milling was freeze-dried for a duration of 24 h.

**2.4 FT-IR spectroscopic analysis**

The starch samples were analyzed using FTIR spectroscopy. The analysis was conducted using a Perkin-Elmer Spectrum 3 spectrophotometer equipped with a diamond ATR attachment. The resolution was set at 4 $cm^{-1}$ and 64 scans were performed. The spectral range analyzed was from 1200 $cm^{-1}$ to 800 $cm^{-1}$. The spectra were converted to absorbance and underwent automatic baseline correction for the regions between 1200 $cm^{-1}$ and 800 $cm^{-1}$ before being analyzed [9]. Prior to analysis, the starches were subjected to a drying process at a temperature of 105°C for a duration of 12 h to prevent any potential interference caused by moisture. The ratio between the height at 1045 $cm^{-1}$ and the height at 1022 $cm^{-1}$ was computed to quantify the short-range orderly arrangement of starch.

**2.5 Morphology by scanning electron microscopy**

The morphology of starch samples was examined using a JSM-7610F scanning electron microscope (JEOL Ltd., Tokyo, Japan). The specimens were covered with a layer of gold. The scanning electron microscope (SEM) was operated at a low accelerating voltage of 2 kilovolts (kV) at magnifications of 1,000x, 5,000x, and 50,000x. The main objective of this SEM examination was to examine the surface characteristics and visual aspects of the starch granules.

**2.6 Particle size analysis**

The particle size distribution of starch granules was determined using Malvern Mastersizer 3000 (Malvern Instruments Ltd., UK). The samples were suspended in ethanol and stirred at 2,500 rpm. Ultrasound (20 kHz) energy was applied for 30 s to break the clumps. The general model with irregular shape was used with starch refractive and absorption indices of 1.52 and 0.1, respectively. The refractive index of water used was 1.33.

**2.7 Statistical analysis**

The statistical methods employed to identify significant differences ($P \leq 0.05$) among treatments were one-way analysis of variance (ANOVA) and Tukey's honestly significant difference test (HSD). The analyses were conducted using the statistical software IBM® SPSS® Statistics version 28 for Windows.

## 3. Results & Discussion

**3.1 Acid hydrolysis of rice starch**

Figure 1 illustrates the amount of acid-hydrolyzed starch produced at different periods of hydrolysis. The starch yield had a rapid decrease within the initial 16 h, followed by a subsequent gradual decline. Acid hydrolysis proceeds in two distinct stages. The initial stage mostly entails the disintegration of the amorphous regions. The fast hydrolysis observed in this stage is attributed to the degradation of the non-crystalline regions present in starch granules. Acidic molecules can readily access and engage with the amorphous regions, characterized by their less structured and loosely arranged compositions. The subsequent deceleration phase of hydrolysis involves the concurrent destruction of the crystalline regions within the starch granules. In contrast to the amorphous portions, the crystalline sections have a highly ordered and densely packed structure, rendering it more challenging for the acid to access and disrupt the chemical bonds. As a result, the hydrolysis of these regions shows a lower rate compared to the amorphous regions [5].

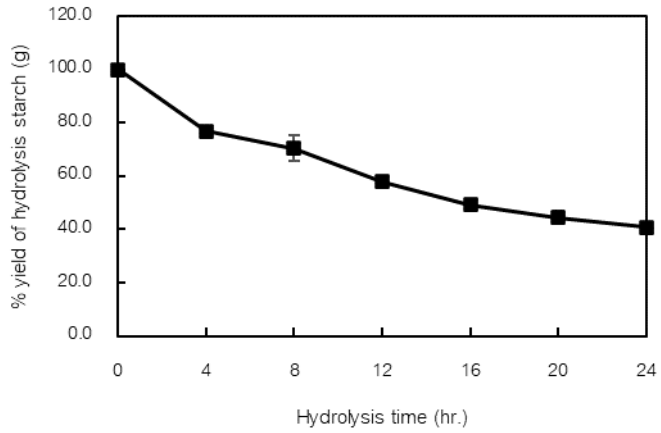



**Figure 1.** The yield of acid hydrolysis of rice starch at different hydrolysis times (Data are averages of three replications).

### 3.2 Fourier-transform infrared spectroscopy

The FTIR spectroscopy could provide information about the glycosidic linkages in starch molecules. The FTIR band observed at a wavenumber of 1047 $cm^{-1}$ is responsive to alterations in the crystalline lamellae, whereas the band at 1022 $cm^{-1}$ is responsive to changes in the amorphous lamellae [10]. Hence, the relative heights of the FTIR bands at 1047 and 1022 $cm^{-1}$ could serve as an indicator of crystallinity in starch granule. The findings show that the ratio of the heights of the FTIR bands at 1047 and 1022 $cm^{-1}$ increased progressively during acid hydrolysis, reaching its highest point at 16 hours, and then started to decrease (Figure 2). The increase in the FTIR height ratio indicates that the acid largely breaks down the non-crystalline components of the starches during the earliest stages of hydrolysis. The decrease in band height at 1047 $cm^{-1}$ seen after 16 h of hydrolysis can be attributed to the dissolution of the crystalline lamellae. Like the previous work [11], the influence of acid hydrolysis on the 1022 $cm^{-1}$ band is apparent, as it is significantly affected and disappears in the initial stages of the treatment. The slowdown of the process at a later stage can be attributed to the breakdown of the crystalline components. These results were consistent with the previously mentioned drop in the yield of hydrolysis.

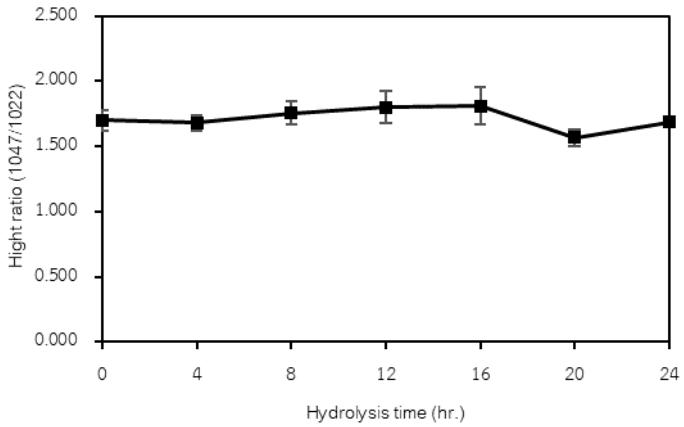
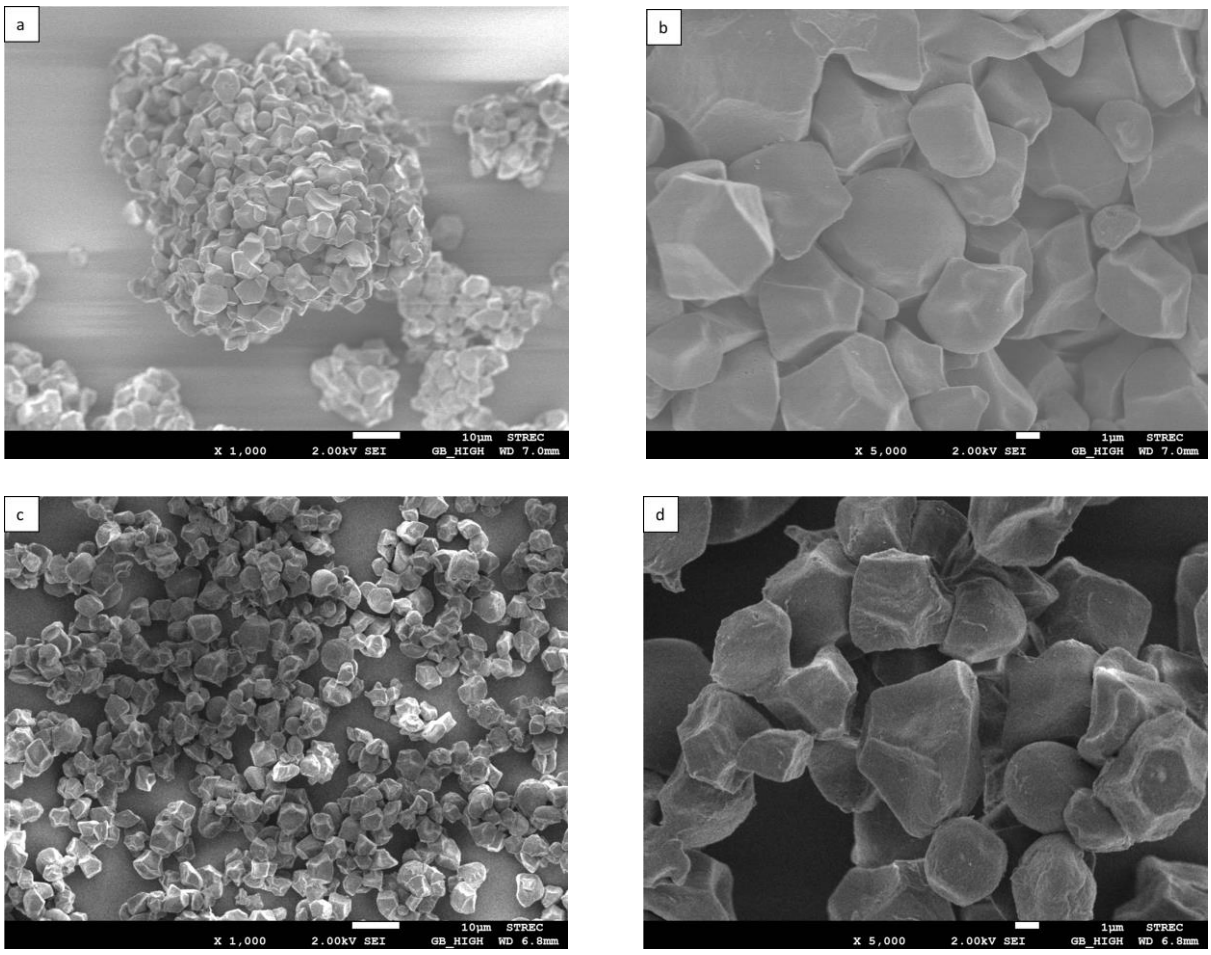



**Figure 2.** The height ratios of the FTIR bands at 1047 and 1022 $cm^{-1}$ of acid hydrolyzed rice starch with different hydrolysis times (Data are averages of three replications).

Starch granules become brittle and easily breakable during the milling process once the amorphous lamellae are eliminated [6]. Based on the alterations in the yield and height ratios of the FTIR bands at 1047 and 1022 $cm^{-1}$ of rice starch during acid hydrolysis, it can be concluded that the rice starch granules subjected to 16-h acid hydrolysis would exhibit the lowest quantity of amorphous lamellae while possibly still maintaining an entire crystalline lamella. Consequently, they were chosen for the process of ball milling.

**3.2 Granule morphology**

The impact of acid hydrolysis and ball milling and their combination on the structure of rice starch granules was examined using scanning electron microscopy (SEM) and illustrated in Figure 3. The rice starch granules found in their natural state exhibited a polygonal shape with distinct edges, as depicted in Figure 3 a-b. Their surface had a seamless texture absent of any fissures. The surface smoothness of starch granules had been reduced because of exterior corrosion caused by 16-h acid hydrolysis (Figure 3d). Nevertheless, acid hydrolysis proved ineffective in disintegrating the granule into smaller particles (Figure 3c). Most native rice starch granules remained unchanged in size even after undergoing ball milling for a duration of 3 h (Figure 3e-f). On the other hand, ball milling had the ability to effectively disintegrate the rice starch granules that have undergone 16 h of acid hydrolysis, resulting in the formation of small, irregularly shaped particles (Figure 3 g-h). The acid hydrolyzed starch granules were more susceptible to pulverization due to their delicate structure resulting from the absence of an amorphous area, as mentioned earlier. The dimensions of these small starch granules fell within the approximate range of 0.5-0.2 micron (Figure 4). They exhibited a tendency to form clusters, resulting in agglomerates of around 1-2 microns in size.



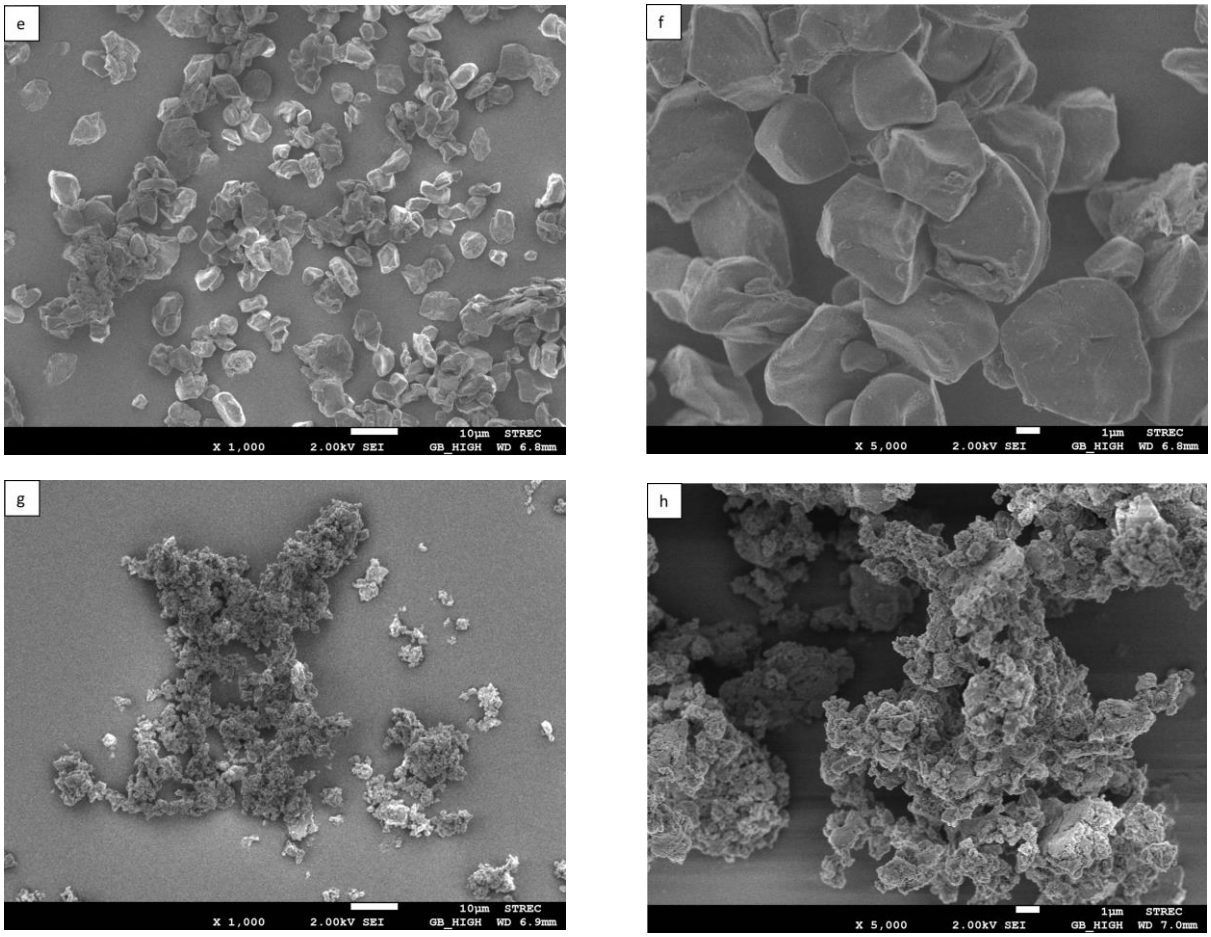



**Figure 3.** Scanning electron microscopy (SEM) at 1000x and 5000x magnification of native rice starch (a-b), 16 h acid-hydrolyzed rice starch (c-d), native rice starch ball milled at 3 h (e-f), and 16 h acid-hydrolyzed rice starch combined with ball milled at 3 h (g-h).

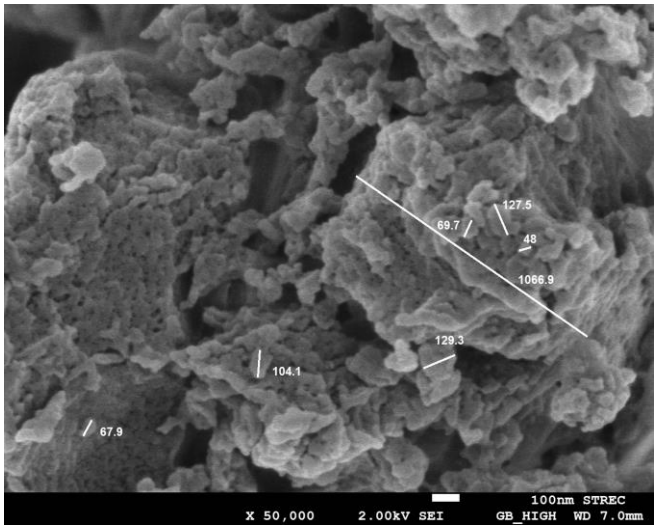



**Figure 4** Scanning electron microscopy (SEM) at 50000x magnification of 16 h acid-hydrolyzed rice starch combined with ball milled at 3 h. The size shown in nanometer unit.

### 3.3 Particle size distribution

The particle size distributions of native and modified rice starches are shown in Table 1 and Figure 5. The native rice starch granule size in terms of $10^{th}$, $50^{th}$, $90^{th}$ percentile values and volume average diameter clearly indicated a size reduction by acid hydrolysis and ball milling. The granules of starch were significantly ($P<0.05$) decreased more by ball milling than by acid hydrolysis. This was in accordance with the effect of both processes on the morphology of starch granules (Figure 3). The primary effect of the acid hydrolysis was surface corrosion, which led to a minor reduction in granule size. Conversely, granules of starch were broken apart by the forceful crushing that came from ball milling. The size reduction was more pronounced when the starches were subjected to acid hydrolysis following with ball milling.

The 16-h acid hydrolyzed sequentially 3-hours ball milled starches possessed granules with D [4,3] of 5.16 µm and 10% of the granules possessed a size smaller than 1.34 µm (Table 1). According to their morphology (Figure 4), their granules mostly were smaller than 1 µm in diameter. Although with the assistance of sonication, the starch granules still tended to agglomerate. The granule agglomeration could mislead the result analyzed by the laser particle size analyzer. The analyzed size might be belonged to the aggregated granules instead of the single granule. It is necessary to develop a better sample preparation method to reduce starch aggregation.

The high efficiency in starch particle size reduction of the combining between acid hydrolysis and ball milling is consistent with the previous report, where the application of ball milling following acid pretreatment has proven effective in breaking down corn starch granules [(6)]. Starch granules is organized in concentric growth rings that alternate between amorphous and semicrystalline layers. The semicrystalline layer contains crystalline lamellae formed by amylopectin double helices tightly packed in a parallel arrangement. Acid treatment selectively hydrolyzes the amorphous regions of starch granules [(12)], generating fragile remnants that are more susceptible to mechanical breakdown.

**Table 1.** Percentile values and volume average diameter of rice starches

| Rice starch | $10^{th}$ percentile $d_{10}$ (µm) | $50^{th}$ percentile $d_{50}$ (µm) | $90^{th}$ percentile $d_{90}$ (µm) | Volume average diameter D[4,3] (µm) |
|---|---|---|---|---|
| Native | 3.29 ± 0.02[a] | 8.36 ± 0.03[a] | 17.4 ± 0.00[a] | 9.54 ± 0.01[a] |
| RH16 | 2.17 ± 0.01[b] | 7.27 ± 0.04[b] | 15.4 ± 0.17[b] | 8.92 ± 0.02[b] |
| RM3 | 1.80 ± 0.01[c] | 5.26 ± 0.01[c] | 10.13 ± 0.06[c] | 6.07 ± 0.02[c] |
| RH16M3 | 1.34 ± 0.00[d] | 4.97 ± 0.01[d] | 9.02 ± 0.06[d] | 5.16 ± 0.03[d] |

[a] Results shown correspond to mean values ± standard deviation.
[b] Data are averages of three replications.
[c] Different superscripts in the same column indicate significant difference ($P\leq0.05$).
[d] RH16, RM3, RH16M3 indicate 16-h acid hydrolyzed, 3-h ball milled, and 16-h acid hydrolyzed combined with 3-h ball milled rice starches, respectively.

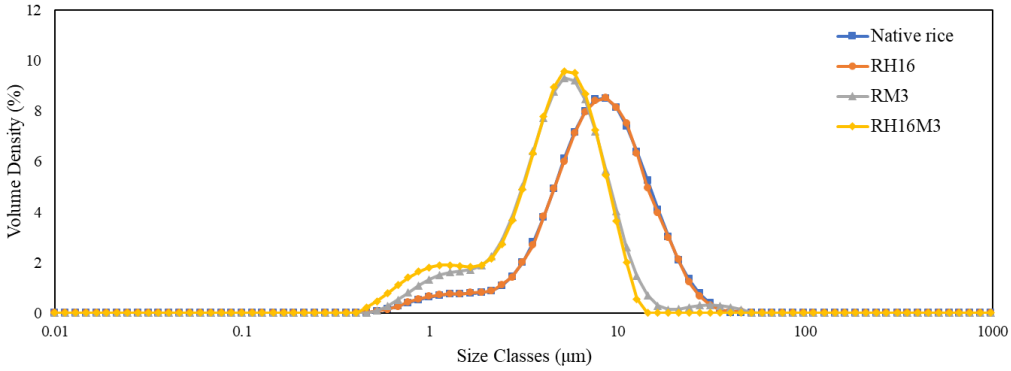



**Figure 5.** Particle size distribution of native, 16-h acid hydrolyzed (RH16), 3-h ball milled (RM3), and 16-hours acid hydrolyzed combined with 3-h ball milled (RH16M3) rice starches.

## 4. Conclusion

To create small fragments from rice starch granules, an acid hydrolysis and ball milling procedure can be used in combination. The goal of acid hydrolysis is to completely remove or minimize amorphous lamellae in starch granules. Temperature, reaction time, and acid concentration must all be precisely regulated to accomplish this. Rice starch granules become more brittle as the amorphous lamellae diminish and are efficiently pulverized by ball milling. These small rice starch granules could be used in a variety of industrial applications.

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# Screening and Selection of β-Mannanase Producing *Bacillus* spp. for Biofilm Removal Application

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## Abstract

The cultivation of edible mushrooms such as oyster mushrooms in Thailand has been gaining attention. It can grow on several lignocellulosic residues such as agro-forestry wastes and agro-industrial wastes. Mushrooms are the fruiting bodies of certain types of fungi. It is well appreciated for its exquisite taste and flavor and rich in fiber. The study aimed to observe the effect of different levels of dried oyster mushroom on physicochemical properties and sensory characteristics of cakes. Wheat flour was replaced with dried oyster mushrooms at 0% (as a control), 10%, 20% and 30 % by weight. Results demonstrated that the increase in dried oyster mushroom tended to decrease water activity of cakes but increase 1.54 to 2.41 folds in fiber content compared to control. Increasing the level of dried oyster mushrooms significantly increased specific volume and redness (*b**) of cakes meanwhile decreasing in lightness (*L**) of crumb. The cake containing 20% dried mushroom was found to be similarly acceptable to control. The quality change during storage 0, 2, 4, 6 days was evaluated. The moisture content of mushroom cake decreased during storage. Meanwhile, water activity and sensory score values of dried oyster mushroom cake presented similar qualities during 6 days. The presence of total plate count and yeast and mold were lower than the Thai Community Product Standard (459/2549). Additionally, consumer acceptance and factors affecting consumer's purchase decisions were also investigated. The overall acceptance of the final product was 4.16 from a total score of 5 indicating like slightly. The results revealed that the most important factor for a purchase decision was the taste of the product. It can be concluded that the cake replacement wheat flour with 20% dried oyster mushroom could be used for cake preparation to achieve higher fiber content and acceptable cake quality to produce an alternative product for consumers.

***Keywords****: Bacillus* spp., β-Mannanase, Biofilm degradation

## 1. Introduction

β-Mannanase (endo-1,4-β-mannan mannanohydrolase, EC 3.2.1.78) randomly hydrolyzes β-1,4 mannosidic linkages in mannan, glucomannan, galactomannan and galacto glucomannan to produce mainly oligomers along with a small amount of mannose [(1).] β-Mannanase has wide applications in the bio-bleaching of pulp and paper, as a hydrolytic agent in the detergent industry, in the improvement of animal feed, as a fish feed additive, as a slime control agent and in the pharmaceutical industry [(2)]. This enzyme has been isolated

from plants, animals and microorganisms [3]. Most of the commercial β-mannanases have been produced from microbes and being increased their market value according to their higher stability, rapid production, space and cost effectiveness that makes them suitable candidates for applications in industry. Among bacteria, most of the mannan degraders are Gram-positive bacteria particularly *Bacillus subtilis* which is GRAS microbe and has found applications as commercial enzyme producers. In addition, *Bacillus* sp. has a high ability to survive in harsh conditions thereby making most of the enzymes produced by *Bacillus* species resistant to chemicals [4]. Miang is a traditional fermented food product of north Thailand that has been produced and consumed for centuries [5]. The investigation on microbial population in the Miang samples revealed that this local fermented food produced by using tea leaves as raw material is the attractive microbial resources a variety of microorganisms including fungi, yeast, and bacteria have been reported [6]. In addition, *Bacillus* isolated from the Miang samples were able to produce various hydrolytic enzymes such as cellulase, β-mannanase, pectinase, xylanase, tannase, and amylase [7]. In this regard, isolating β-mannanase producing *Bacillus* spp. from fermented Miang for possible application in biofilm removal became a research pursuit of interest in this study.

The biofilm structure is predominantly covered in an extracellular polymeric substance (EPS) made up of mostly polysaccharides then proteins and fats. The EPS layer acts as a barrier to protect microorganisms from harmful substances such as antibiotics, detergent, etc. These biofilms cause some critical problems such as contamination of food and medical devices. Therefore, various hydrolytic enzymes have been explored for their ability in biofilm degradation. β-Mannanase is an important enzyme capable of mainly hydrolyzing polysaccharides structure of biofilm. Blakeman *et al.* [8] reported that biofilm was treated with β-mannanase decreased the adhesive force of the model bacterium *Micrococcus luteus* with the surface while also eliminating 50% of the biofilm components thus establishing a key structural role for mannan. This study focuses on isolation and screening of *Bacillus* spp. capable of hydrolytic enzyme production especially β-mannanase for application in biofilms removal.

## 2. Materials and Methods

### 2.1 Isolation and screening of β-mannanase producing *Bacillus*

Five grams of Miang sample was mixed with 20 mL sterile 0.85% (w/v) NaCl by using a stomacher for 1 min. One milliliter of each mixed sample was transferred to 9 mL of sterile 0.85% (w/v) NaCl. Samples were incubated at 80°C for 20 min to eliminate other groups of microorganisms before spreading the solution on nutrient agar (NA), incubated at 37ºC for 18 h. The single bacterial colony formed was assumed to be *Bacillus* sp. [9]. A single colony of *Bacillus* sp. was transferred to NA medium containing 0.5% (w/v) locust bean gum (LBG) by replica plating technique and incubated at 37ºC for 24 h. The hydrolyzing zones on LBG agar were visualized by flooding with 0.5% (w/v) Congo red stain [10]. The clear zones surrounding colony on LBG agar were assumed to be the mannan-degrading enzyme producer and were selected for further experiments.

### 2.2 Production of β-mannanase in liquid media

The selected isolates were cultivated in nutrient broth (NB) containing 0.5% (w/v) LBG on 180 rpm rotary shaker at 37°C for 24 h and the cell−free culture supernatants were separated and determined for β-mannanase activity. The isolate produced the highest activity of β-mannanase was selected to time course cultivation. The inoculum size of 10% (v/v) was transferred to 250-mL Erlenmeyer flask containing 100 mL of the basal medium containing gram per liter of 5.0 LBG, 10.0 NaCl, 20.0 $K_2HPO_4$, 5.0 $NaNO_3$, 1.0 KCl, 2.0 $MgSO_4 \cdot 7H_2O$ and 5.0 Yeast extract pH adjusted to 7.0. Then, the culture was incubated at 37°C on 180 rpm rotary shaker for 48 h The culture broth was taken out with 3 h interval and determined for

β-mannanase activity and the viable cell count by the methods described by Khatthongngam *et al.*[5].

**2.3 β-Mannanase activity assay**

β-Mannanase activity was determined by measuring the amount of reducing sugars released by using the dinitrosalicylic acid (DNS) method[11]. Briefly, the reaction mixture containing 0.125 mL of the desired dilution of enzyme and 0.125 mL of 0.5% (w/v) LBG in 0.1 M phosphate buffer (pH 7.0) was incubated at 37°C for 10 min. The reaction was stopped by addition of 0.25 mL of DNS solution (Sigma-Aldrich, St. Louis, MO, USA), then boiled for 10 min, and 5 mL of distilled water was added. The absorbance was measured at 540 nm. One unit of β-mannanase activity was defined as the amount of enzyme that liberated 1 µmole of reducing sugar per minute under the assay condition.

**2.4 Biofilm formation**

The biofilm formation ability of the tested pathogens (*P. aeruginosa* and *S. mutans*) was carried out in the screw cap test tube [12]. Briefly, seed culture of the bacterial pathogens overnight in Luria-Bertani medium (LB) at 37°C. Then, 100 µL of seed culture into fresh Tryptic Soy Broth (TSB) in screw cap test tube for five replicates of each treatment and incubated for 24 h at 37°C. After incubation, the liquid was dumped out by turning the tube over. The tubes were rinsed 2 times with sodium phosphate buffer pH 7 and 0.1% (w/v) crystal violet (CV) was added 1.25 mL to each tube, then incubated at room temperature for 10 min. The tube was turned over for liquid draining and dried overnight at room temperature. The biofilm formation was determined by added 1.25 mL of 30% acetic was into each tube and incubated at room temperature for 10 min for solubilize the CV. Then, the absorbance of sample was measured at 600 nm using 30% acetic acid as blank.

**2.5 Investigation of biofilm degradation by crude β-mannanase**

Bacterial pathogens, *P. seudomonas aeruginosa* and *Streptococcus mutans* were cultured in TSB medium 24 h for biofilm forming, the supernatant was discarded, and tube were washed with Sodium phosphate buffer pH 7, then each bacterial cultivated tube was incubated with the final concentration of 2 U/mL crude β-mannanase. A well without enzymes was used as control. Tubes were incubated for 1 h at 37°C. After incubation, tubes were emptied and washed with Sodium phosphate buffer pH 7. The remaining cells allowed to drying. Crystal violet solution was added into each well. Tubes were washed with sterile distilled water and dried overnight. Tubes were washed with 30% glacial acetic acid and the absorbance was measured at 600 nm.

## 3. Results & Discussion

**3.1 Isolation and screening of β-mannanase producing *Bacillus* strain**

From the Miang samples, a total of 108 isolates of the *Bacillus* group were isolated. *Bacillus* bacteria that produced the β-mannanase were selected using plate screening on NA medium with 0.5% (w/v) locust bean gum incubated at 37°C for 24 h, then recording the size of the clear zone. The clear zone of β-mannanase production is showed in Figure 1. It was found that 108 isolates of the *Bacillus* group had the potential to produce β−mannanase 73 isolates (Table 1).

**Table 1.** Screening of the *Bacillus* strains for β-mannanase activity on NA medium with 0.5% (w/v) locust bean gum incubated at 37°C for 24 h.

| symbol | Number of *Bacillus* bacteria obtained β-Mannanase clear zone |
|---|---|
| - | 35 |
| + | 45 |
| ++ | 21 |
| +++ | 7 |
| total | 108 |

*The hydrolysis zone 1-4 mm as the one plus positive, 5-8 mm as two plus positive and the colony that gave the clear zone bigger than 9 mm as three plus positive.

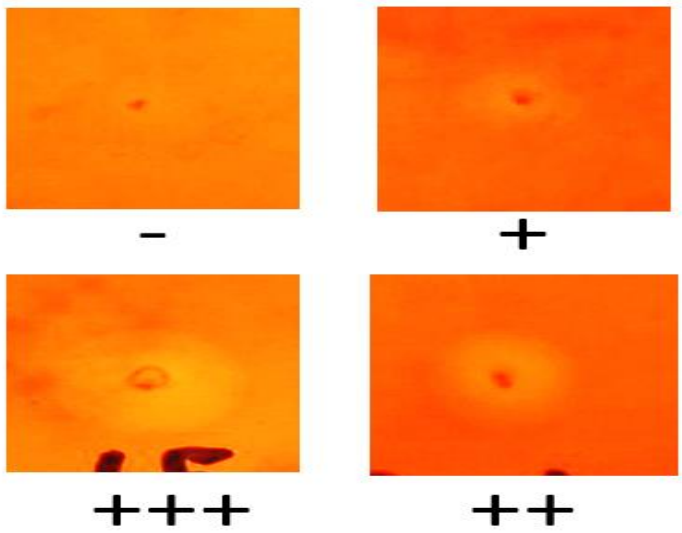


**Figure 1.** Example clear zone size of β-mannanase enzyme production on NA with a carbon source of locust bean gum (The hydrolysis zone 1 - 4 mm as the one plus positive, 5-8 mm as two plus positive and the colony that gave the clear zone bigger than 9 mm as three plus positive).

### 3.2 Production of β-mannanase enzyme in liquid media

A total of 73 isolates of *Bacillus* sp. were screened through their hydrolysis zones obtained by plate screening assay on NA with 0.5% (w/v) LBG. The total 28 isolates were selected from hydrolysis zone bigger than 5 mm were then cultured in the NB with 0.5% (w/v) LBG for the confirmation of β-mannanase producing strain. The isolate K9.1 produced the highest β-mannanase activity at 5.36 U/mL (Figure 2) after cultivation in LBG medium at 37°C for 24 h. Therefore, the *Bacillus* sp. K9.1 was selected for β-mannanase production in basal medium at 37°C for 48 h. It was found that the *Bacillus* sp. K9.1 produced the highest β-mannanase activity at 14.5 U/mL after cultivated in basal medium, which was 2.7 times higher than cultured in the NB containing 0.5% (w/v) LBG. The result is presented in Figure 3. *Bacillus* sp. K9.1 proliferated and entered the log phase within 3 h and reached the stationary phase after 18 h. The pH value decreased to pH 6 at 3 h, then constantly increased from pH 6 to 7.5 in log phase, then almost stable after 18 h. As the result showed *Bacillus* sp. K9.1 growth in associate with an increased pH, the β-mannanase activity was rapidly increased until reaching the maximum activity at 24 h. However, β-mannanase activity

continuously decreased after 24 h. A similar finding has been reported that the highest β-mannanase activity was obtained in the stationary phase of *B. nealsonii* PN-11 on minimal media [13]. In the present work, an increase in β-mannanase activity of *B. cirulans* NT6.7 was comparable with those observed by Pangsri *et al.* [14] and even higher than that of Zurmiati *et al.* [15].

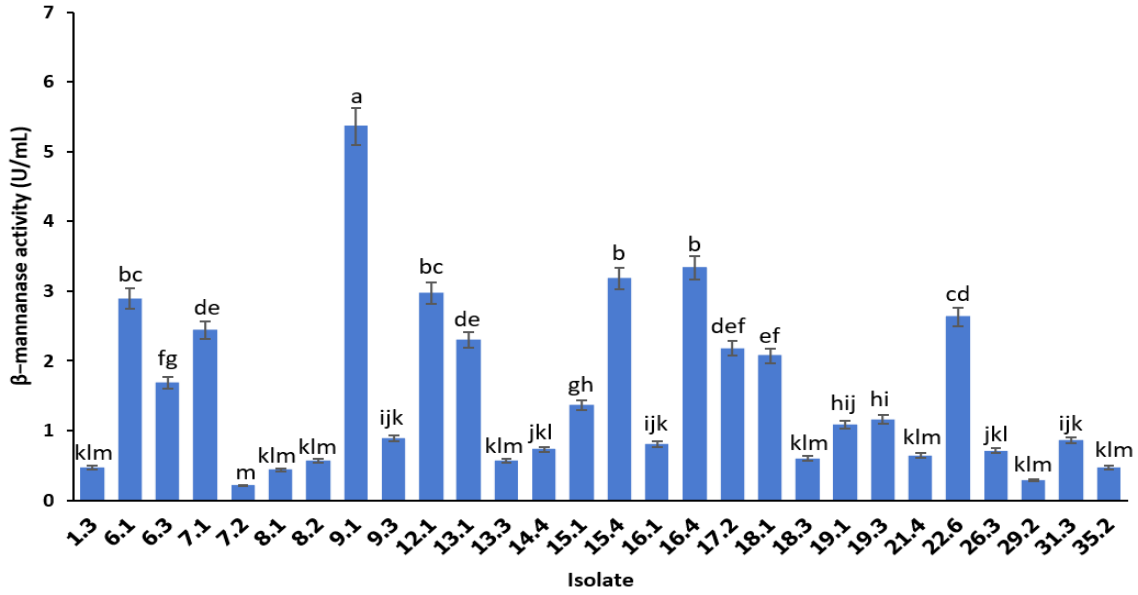


**Figure 2.** β-Mannanase activity of 28 isolates incubated in on NB with 0.5%(w/v) LBG at 37ºC for 24 h**.**

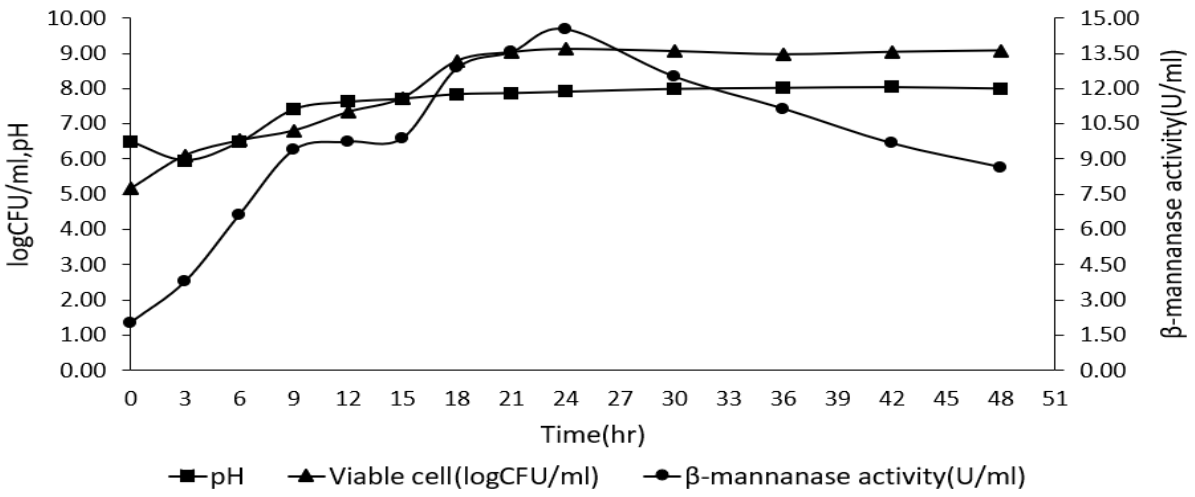


**Figure 3.** Time course of viable cells, pH, and β-mannanase activity production by *Bacillus* sp. K9.1 at 37°C in the basal medium.

### 3.3 Biofilm degradation using crude β-mannanase

The biofilms of *P. aeruginosa* and *S. mutans* were successfully formed in test tube after grown for 24 h (Figure 4). *P. aeruginosa* biofilm was adhered to the inner side of test tube on the broth surface while *S. mutans* biofilm was found at the bottom of the culture. Crude β-mannanase obtained from this experiment showed the inhibitory effect on biofilms both of *P. aeruginosa* and *S. mutans*. The quantitative data for biofilm degradation of β-mannanase was presented in Figure 5. It was found that the *P. aeruginosa* and *S. mutans* biofilm was removed 33% and 30%, respectively, after 2 U/mL of crude β-mannanase was applied. The role of the enzyme in antibiofilm results directly depended on the composition and structure of the biofilm [16].

In this study, crude β-mannanase showed the ability to degrade both bacterial biofilms, however, crude β-mannanase (2.06 U/mL) obtained from this experiment also showed other polysaccharide-degrading enzymes includes amylase (0.19 U/mL), xylanase (0.18 U/mL), and CMCase (0.10 U/mL). Therefore, the positive result in bacterial biofilm degradation might be associated with the assistance from other polysaccharide degrading enzymes mentioned previously. Lequette *et al.* [17] had investigated a variety of enzymes including proteases and α-amylase concluded that the antibiofilm effectiveness of hydrolases such as amylases could be assigned to the hydrolysis of a substrate involved in bacterial adhesion like EPS and these hydrolases were effective in digesting slime layers produced by cultures of pure and mixed strains of bacteria. Nagraj and Gokhale [18] also reported that extracellular enzyme preparation of *Penicillium janthinellum* EU2D-21 containing cellulase, protease and amylase activities showed the capability to degrade the selected bacterial biofilms cellulase, protease and amylase, within 1 h of incubation. There has been comparative study using the partially purified enzyme and crude enzymes for biofilm degradation by Lahiri *et al.* [19] and found that the partially purified α-amylase obtained from *Bacillus subtilis* brought about the best reduction of the EPS components in comparison to crude α-amylase. Regarding to the results from this research, the assessment for the influence of other polysaccharide degrading enzymes on bacterial biofilm degradation must be elucidated and confirmed by bacterial biofilm degrading experiment using the purified β-mannanase from *Bacillus* sp. K9.1.

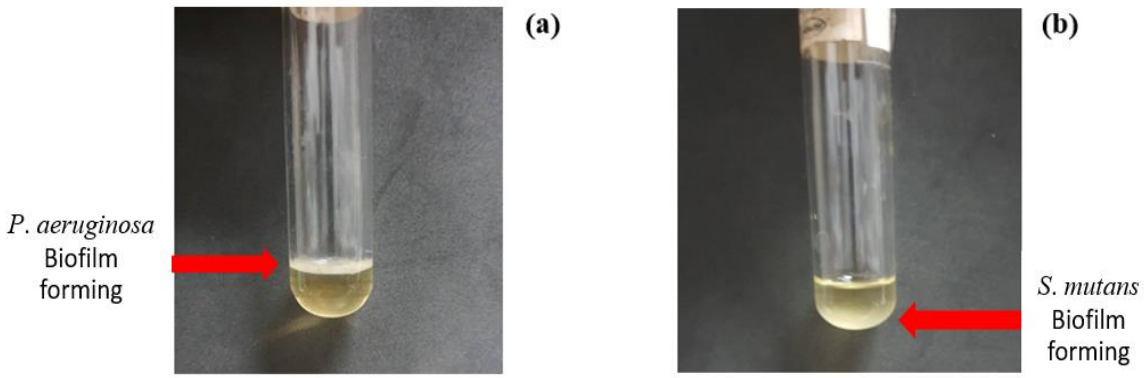


**Figure 4.** The biofilms of *P. aeruginosa* (a) and *S. mutans* (b) were successfully established in test tube screw cap at 37°C for 1 h**.**

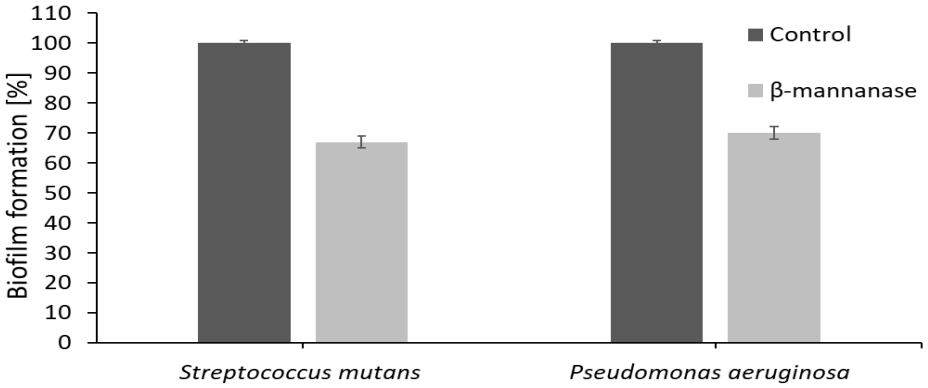


**Figure 5.** The effect of β-mannanase degrading biofilm formation by *S. mutan* and *P. aeruginosa* were incubated with the final concentration of 2 U/mL crude β-mannanase at 37°C for 1 h and quantified CV staining at 600 nm**.**

## 4. Conclusion

The isolate *Bacillus* sp. K9.1 produced the highest β-mannanase activity of 14.5 U/mL when cultivating on basal medium containing. Crude β-mannanase achieved from this experiment was demonstrated the capability in degradation of biofilms from pathogens *S. mutans* and *P. aeruginosa*.

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# Unlocking Nutritional Potential: Extended Germination Boosts Nutrition and Phytochemicals of Yellow Soybean Sprouts

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**Abstract**

Soybeans are known as one of the most economical crops in the world. Despite their popularity, their use as a functional ingredient seldom extends to soybean sprouts. Soybean sprouts typically are a product of short-time germination and are mainly used for fresh consumption. This research investigated the effect of an extended germination period of 9 days on the physical and chemical qualities of yellow soybean seeds and sprouts. Soybean seeds were grown under a controlled environment and collected on days 0, 1, 3, 5, 7, and 9. Freeze-dried samples were analyzed on major nutrients, allergen (ELIZA test), total phenolic content, total flavonoid content, and antioxidant activities (DPPH and FRAP assays). The results showed that protein content, phytochemicals, and antioxidant activities significantly increased with germination time while the allergen levels decreased ($P<0.05$). All sprouts showed higher nutrients, phytochemicals, and antioxidant activities than soybean seeds. Sprouts on day 7 had the highest protein content (39.90 ± 0.0 g/100g) while total phenolic content (308.4 ± 2.5 mg GAE/100g), DPPH value (133.5 ± 0.6 mg TE/100g) and FRAP value (123.0 ± 0.9 mg TE/100g) were highest on day 9. The allergen level of sprouts at the end of the germination period decreased by 92% compared to the starting soybean seeds (from 128.17 to 10.08 g/100g). Therefore, yellow soybean sprouts could be developed as a functional ingredient for the food and supplement industry.

***Keywords****: Glycine max*, Allergen, Legume, Nutrition, Food safety

## 1. Introduction

Soybean (*Glycine max* L.) is an important economic crop in Thailand, and the main growing regions are the northern and central parts of Thailand. Yellow soybean is popular for consumption and processing because it contains a rich source of nutrients and many bioactive compounds with health benefit [(1)]. Yellow soybean is well known for its high-quality protein with all essential amino acids and valuable unsaturated fatty acids such as alpha-linolenic acid, which have been reported to reduce the risk of heart disease and thromboembolism [(2)]. Bioactive compounds reported in yellow soybeans include isoflavones, phenolics, flavonoids, saponin, phytate, gramma-aminobutyric acid (GABA), and anthocyanins [(3)].

Despite their popularity, the use of soybeans in the food industry as a functional ingredient rarely extends to soybean sprouts. Soybean sprouts typically are a product of short-time germination and are mainly used for fresh consumption. A few research have reported changes in macronutrient and bioactive compounds during the germination of soybean seeds. This is because seeds' nutrients play a part in various metabolisms, leading to changes in nutritional composition from seeds to sprouts. Some nutrients, such as proteins, are changed in structure or digested into smaller molecules to enable seed development [(4, 5)]. Wu *et al.* [(6)] noticed a significant reduction in the level of soybean allergens after 72 h of germination. According to Chen and Chang[(4)], germination also influences soybean sprouts'

phytochemicals and antioxidant activities. However, no study focuses on the qualities of soybean sprouts beyond the typical germination period of fresh consumption. Therefore, this study aimed to investigate the possibility of enhancing yellow soybean sprouts' nutritional and phytochemical potential by using extended germination to assess their potential as an alternative functional ingredient. This knowledge would be beneficial, especially when the utilization of soybeans in the food industry is limited due to its allergen [7].

## 2. Materials and Methods

### 2.1 Raw materials and chemicals

Yellow soybean seeds were obtained from the Chiang Mai Field Crops Research Center (Chiang Mai, Thailand) in April 2022. Seeds were cleaned and stored in a plastic bag at 5-10°C. All chemicals used in this study were analytical grade. Alpha-tocopherol, 2,2-diphenyl, 1 picrylhydrazyl (DPPH), Trolox, and gallic acid were purchased from Sigma-Aldrich (Beijing, China). All other chemicals and reagents were purchased from RCI Labscan Limited (Bangkok, Thailand). Distilled water was obtained from Pole Star Co, Ltd. (Chiang Mai, Thailand).

### 2.2 Germination procedure

Yellow soybean seeds were prepared according to the method of Kerdsirichairat[8] and Xue *et al.*[9] with some modifications. Yellow soybean seeds (150 g) were washed and soaked in filtered water at a controlled temperature of 35°C using a water bath for 4 h. The ratio between yellow soybean seeds (g) and water (mL) was 1:3. Afterward, the seeds were transferred to plastic baskets and placed in a dark germination chamber at room temperature (30-35°C). The seeds were watered every 4 h by an automatic watering system. Samples were collected on days 0, 1, 3, 5, 7, and 9, then dried by a freeze-dryer (Model Labconco FreeZone 7750000, Labconco Corporation, Kansas City, MO, USA) until the final moisture of the sample was under 5%. Each dried sample was ground, packed in a vacuum-sealed bag, and kept under -20°C until use. Samples were analyzed for physical and chemical qualities. Experiments were done in triplicate.

### 2.3 Physical analysis

Fresh samples of each batch were weighed and recorded. The size of the samples was reported as an average length of 10 random seeds or sprouts measured by a ruler. The color of the powder sample was measured using a Chroma Meter (Model CR-410, Konica Minolta, Inc., Tokyo, Japan) in $L^*$ (lightness), $a^*$ (red/green coordinate), and $b^*$ (yellow/blue coordinate) values. Each sample was measured 5 times.

### 2.4 Composition and allergen analysis

Samples were analyzed for moisture, protein, ash, lipid, and crude fiber, and carbohydrate content was calculated according to AOAC methods [10] and reported as g per 100 g powder sample. Samples were analyzed for allergen by the Food Quality Assurance Service Center (FQA), the Institute of Food Research and Product Development (IFRPD), Bangkok, Thailand. Allergen content was quantified using a Food allergen ELIZA kit for soya (Cat.# M2117, Morinaka Institute of Biological Science, Inc., Yokohama-shi, Japan) and reported as mg allergen per 100 g powder sample.

### 2.5 Phytochemical and antioxidant determination

Five g of powder sample were put in a 125 mL Erlenmeyer flask, added with 40 mL of 80% ethanol, and mixed well. The mixture was sonicated for 5 min to increase extraction efficiency before being filtrated with Whatman No.1 filter paper and evaporated to dryness. The final volume was adjusted to 25 mL with 80% ethanol and kept at -18°C until analysis. [11]

*2.5.1 Total phenolic analysis*

Total phenolic content was analyzed using the Folin-Ciocalteu method [11]. A mixture of 7.5 mL distilled water, 0.5 mL Folin ciocalteu reagent, and 0.5 mL sample extract was mixed using a Vortex mixer (Model VTX-3000L, LMS CO., LTD., Tokyo, Japan) and held in the dark at room temperature for 5 min. The mixture was added with 1.5 mL of 20% sodium carbonate solution ($Na_2CO_3$) and kept in the dark for another 2 h. Distilled water was used as a blank. Absorbance was read at 755 nm using a UV-VIS Spectrophotometer (Model UVmini-1240, Shimadzu Corporation, Tokyo, Japan). A standard curve was constructed using gallic acid solutions (50-500 ppm). The results were reported as mg gallic acid equivalent per 100 g powder sample.

*2.5.2 Total Flavonoid analysis*

Total flavonoid content was analyzed using the aluminum chloride colorimetric method [12]. A 1.5 mL sample extract and 450 µL of 5% NaNO2 were mixed and incubated for 5 min. Then 450 µL of 10% $AlCl_3$ was added and incubated for another 5 min. Three mL of 1 M NaOH was added, and the final volume was adjusted to 6 mL with distilled water. The final mixture was incubated for 15 min before measuring absorbance at 510 nm using a UV-VIS Spectrophotometer (Model G10S, Thermo Fisher Scientific, Waltham, MA, USA). A standard curve was constructed using quercetin solutions (50-500 ppm). The results were reported as mg quercetin equivalent per 100 g powder sample.

*2.5.3 DPPH assay*

Antioxidant activity determination was performed using the adapted method of Xu and Chang[3]. A mixture of 0.1 mL sample extract and 0.5 mL of DPPH (0.04 mM) was mixed using a Vortex mixer and kept in the dark at room temperature for 30 min. Absorbance was read at 765 nm using a UV-VIS Spectrophotometer with ethanol as a blank. Trolox solutions (50-500 ppm) were used to construct a standard curve. The results were reported as mg Trolox per 100 g powder sample.

*2.5.4 Ferric reducing antioxidant power (FRAP) assay*

FRAP assay was performed according to the method of Benzie and Strain [13]. The FRAP reagent was prepared by mixing 300 mM acetate buffer pH 3.6 with 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ) and 20 mM ferric chloride. A mixture of 1 mL sample extract and 2 mL FRAP reagent was mixed using a Vortex mixer and incubated in the dark for 10 min. Absorbance was read at 593 nm using a UV-VIS Spectrophotometer with ethanol as a blank. Trolox solutions (50-500 ppm) were used to construct a standard curve. The results were reported as mg Trolox per 100 g powder sample.

**2.4 Statistical analysis**

Results were presented as mean ± standard deviation. The difference in germination time was tested at 95% significance ($P<0.05$) using one-way ANOVA and Duncan's post hoc test (SPSS Statistics 25, IBM Company, New York, USA).

## 3. Results & Discussion

### 3.1 Physical quality

After soaking for 4 h, the weight of soybean seeds increased from 150 to 300 g. Once the germination process started, the length and weight of soybeans rapidly increased since day 1 and positively correlated with germination time (Table 1). On day 9, the length and weight of soybean samples were 96.9 and 4.35 times higher than that of Day 0. The growth rate based on the soybean length on days 1, 3, 5, 7, and 9 declined in a quick-to-slow trend from 699% to 131%, 26%, and 22%, respectively. This was in agreement with a study by Huang *et al.* [14], who studied kinetic changes of germinated yellow soybean and mung bean for 5 d. They reported similar trends for a growth rate of soybeans on days 1, 3, and 5 at 375% and 100%.

**Table 1.** Weight, length, and color values (*L*a*b**) of yellow soybean seeds and sprouts during germination

| Sample | Weight (g)* | Length (cm)* | *L** | *a** | *b** |
|---|---|---|---|---|---|
| Day 0 | 307.39$^{e}$ ± 2.76 | 0.49$^{e}$ ± 0.02 | 32.59$^{ab}$ ± 1.14 | -0.90$^{a}$ ± 0.25 | 10.03$^{d}$ ±0.51 |
| Day 1 | 332.76$^{d}$ ± 7.20 | 1.66$^{e}$ ± 0.12 | 32.64$^{ab}$ ± 0.17 | -1.18$^{ab}$ ± 0.14 | 11.54$^{cd}$ ± 0.52 |
| Day 3 | 431.62$^{c}$ ± 36.85 | 13.27$^{d}$ ± 1.60 | 33.60$^{a}$ ± 0.72 | -1.75$^{bc}$ ± 0.17 | 13.56$^{bc}$ ± 0.83 |
| Day 5 | 678.66$^{b}$ ± 46.34 | 30.75$^{c}$ ± 0.13 | 30.59$^{c}$ ± 0.57 | -2.14$^{c}$ ± 0.48 | 17.48$^{a}$ ± 0.54 |
| Day 7 | 731.58$^{b}$ ± 52.33 | 38.76$^{b}$ ± 2.01 | 31.85$^{b}$ ± 0.33 | -1.09$^{a}$ ± 0.53 | 17.01$^{a}$ ± 1.14 |
| Day 9 | 1303.92$^{a}$ ± 49.36 | 47.46$^{a}$ ± 1.28 | 32.38$^{b}$ ± 0.29 | -1.06$^{a}$ ± 0.33 | 15.58$^{ab}$ ± 3.20 |

Superscript letters within each column indicate significant differences between samples (P<0.05).
*Fresh sample

The color of soybean samples also changed during germination (P<0.05), but there was no apparent trend. The final soybean sprout powders were yellow, most likely from the soybean cotyledons. This was because soybean seeds were traditionally germinated in the dark; therefore, chlorophyll formation was restricted [15]. Sufficient water absorption at the soaking stage and during germination was one of the crucial keys to starting soybeans' physiological and biochemical activities besides the quality of the seeds, oxygen level, light, growing temperature, and time [8, 16, 17].

### 3.2 Composition and allergen

The nutritional composition of soybean seeds changed during germination (Table 2). Ash and protein content positively correlated with germination time, while lipid, carbohydrate, and allergen declined (P<0.05). Ash content, an indicator of total minerals in food, was highest in soybean sprouts on day 9 (7.28 ± 0.16 g/100 g). Minerals were present in both cotyledon and hypocotyl of commercial soy sprouts, with K, Ca, Mg, and Na being the main minerals reported [18]. Bau *et al.* [19] mentioned that the increase of Ca and Mg during germination might come from the water used in the experiment.

**Table 2.** Chemical composition (g/100 g) and allergen content (g/kg) of powder yellow soybean seeds and sprouts during germination

| Sample | Moisture (%) | Ash (%) | Crude fiber (%) | Protein (%) | Lipid (%) | Carbohydrate (%) | Allergen (g/kg) |
|---|---|---|---|---|---|---|---|
| Day 0 | 3.06$^{bc}$±0.09 | 5.40$^{d}$±0.17 | 8.50$^{bc}$±0.76 | 33.80$^{f}$±0.03 | 19.13$^{ab}$±0.4 | 30.11$^{a}$±0.8 | 128.2 |
| Day 1 | 2.09$^{c}$±0.29 | 5.34$^{d}$±0.23 | 7.54$^{d}$±0.11 | 34.73$^{e}$±0.04 | 20.81$^{a}$±0.1 | 29.49$^{a}$±0.4 | 130.2 |
| Day 3 | 3.63$^{b}$±1.08 | 5.58$^{c}$±0.09 | 7.97$^{c}$±0.51 | 35.73$^{d}$±0.02 | 17.86$^{b}$±1.6 | 29.23$^{a}$±1.0 | 59.1 |
| Day 5 | 3.87$^{b}$±0.34 | 6.60$^{b}$±0.04 | 7.70$^{d}$±0.35 | 37.78$^{c}$±0.07 | 18.80$^{ab}$±1.3 | 25.25$^{b}$±1.3 | 24.9 |
| Day 7 | 4.13$^{ab}$±0.55 | 6.55$^{b}$±0.07 | 8.90$^{b}$±0.52 | 39.87$^{a}$±0.03 | 17.60$^{b}$±0.3 | 22.95$^{c}$±1.0 | 11.9 |
| Day 9 | 5.04$^{a}$±0.14 | 7.29$^{a}$±0.16 | 11.43$^{a}$±0.18 | 39.48$^{b}$±0.01 | 12.29$^{c}$±1.3 | 24.47$^{bc}$±1.2 | 10.1 |

Superscript letters within each column indicate significant differences between samples (P<0.05).

Crude fiber content in soybean seeds decreased during days 1-5 of germination before surpassing the control on day 7 and was highest on day 9 (11.42 ± 0.18 g/100 g). This rise-and-fall trend was also mentioned by Lu *et al.* [16]; however, there was no research currently available on how germination impacts soybean fiber structure and composition. The carbohydrate content of soybean significantly decreased with germination time ($P<0.05$) and was lowest on day 7 (22.9 ± 1.0 g/100 g). This differed from the findings of Chen and Chang (4), who reported a significant increase in carbohydrate content to a maximum of 40.5 ± 1.0 g/100 g on day 7. Nonetheless, the difference may be attributed to the change in other components.

The lipid content of soybean sprouts dropped to the lowest on day 9 (12.3 ± 1.3 g/100 g), 35.6% lower than that of the control soybean seeds (19.1 ± 0.4 g/100 g). Chen and Chang[4] reported a similar decrease in lipid content (38.7%) when germinated yellow soybean (variety NDO4 10637, North Dakota State University, USA) for 7 d. This reduction corresponded to soybean energy consumption for growth [4]. Changes in the fatty acid profile of soybeans after germination have been reported to depend on the type of fatty acid, soybean variety, time, and germination environment [20]. On the other hand, the protein content of soybeans significantly increased with germination time, with the highest on day 7 (39.86 ± 0.03 g/100 g). Bau *et al.* [19] and Chen and Chang [4] credited an increase in protein content to enzyme synthesis or degradation of other components (such as lipids) during germination. Hafez *et al.* [21] also reported that germination enhanced the protein content and protein digestibility of the soybean variety Clark (Field Crops Research Institute, ARC. Giza, Egypt). This might be due to increased peptide content generated by endogenous proteases during germination. Similar to lipids, the protein content and free amino acid profile of soybean sprouts were influenced by soybean genotypes, germination environment, and time [20].

Changes in nutrient composition were expected as germination involved various lipid hydrolysis, proteolysis, and synthesis processes via endogenous metabolism [16]. The breakdown of macromolecules into smaller molecules and by-products of metabolism during germination improved soybeans' nutritional and functional quality. In addition, our study revealed that extended germination of 9 d positively impacted soybean allergen reduction by up to 92.1% (from 128.16 to 10.08 g/kg sample powder). Three major allergens (Gly m Bd 28K, Gly m Bd 30K, and Gly m Bd 60K) were previously reported in soybean and present mainly in cotyledon, followed by embryonic axes of soybean sprouts [6]. Li *et al.* [22] also reported that germination naturally reduced peanut allergenic proteins called Ara h1 and Ara h3. A rapid reduction of Ara h1 was observed after 84 h, and its activities were reduced by almost 30% after 132 h of germination (20-30°C), based on ELISA and Western blotting results.

**3.3 Phytochemical and antioxidant activities**

Germination time influenced soybean sprouts' phytochemicals and antioxidant activities, as shown in Table 3. The total phenolic content continued to increase with germination time and reached the highest on days 7-9 (300.2-308.4 mg GAE/100 g). This finding agreed with previous studies of Huang *et al.* [14] and Khang *et al.* [23], which reported that the germination period of 5 d enhanced the phenolic content of soybeans 1.91 and 2.33 times compared to the controls. However, the optimum germination time for enhancement seemed to be impacted by genotypes and germplasm lines of soybeans. Miglani and Sharma [24] reported the total phenolic content of yellow soybean cultivars SL525, SL744, and SL958 (procured from Punjab Agricultural University, Ludhiana) after 3 d of germination was significantly higher than those of 6 d ($P<0.05$). According to Khang *et al.* [23], there were 10 phenolic compounds found in soybeans, with the major ones being syringic acid (327.5 ± 14.9 µg/g dw), sinapic acid (218.1 ± 7.5 µg/g dw), and vanillic acid (189.2 ± 10.1 µg/g dw).

According to Lemmens *et al.* [25], an activity of cell-wall degrading enzymes synthesized during cereal seed germination may contribute to an increase in free phenolic content.

Total flavonoids were also affected by germination time but to a lesser extent than total phenolic (Table 3). Although soybean sprouts on day 3 showed the highest total flavonoid content (236.0 ± 17.6 mg QE/100 g), no apparent trend was observed. Chen and Chang [4] also reported no significant change in total flavonoids during 1-7 d of yellow soybean germination (ranging from 223.8-262.5 mg/g); however, significant change was identified in isoflavone profiles, especially daidzin, malonylglycitin, malonylgenistin, and aglycones. According to Lee *et al.* [15], yellow soybean sprouts accumulated isoflavones mainly in the root (2.85 mg/g), followed by cotyledon (2.54 mg/g) and hypocotyl (1.13 mg/g).

**Table 3.**Total phenolic content, total flavonoid content, and antioxidant activities of powder yellow soybean seeds and sprouts during germination

| Sample | Total phenolic (mg GAE/100 g) | Total flavonoid (mg QE/100 g) | DPPH (mg TE/100 g) | FRAP (mg TE/100 g) |
|---|---|---|---|---|
| Day 0 | $159.7^{e} \pm 2.9$ | $162.0^{c} \pm 15.5$ | $50.1^{d} \pm 13.9$ | $68.1^{d} \pm 3.0$ |
| Day 1 | $181.7^{d} \pm 2.4$ | $222.3^{a} \pm 19.7$ | $114.8^{c} \pm 1.0$ | $90.9^{c} \pm 2.1$ |
| Day 3 | $243.5^{c} \pm 6.4$ | $236.0^{a} \pm 17.6$ | $120.9^{bc} \pm 2.3$ | $94.7^{c} \pm 5.8$ |
| Day 5 | $293.1^{b} \pm 9.4$ | $183.6^{bc} \pm 16.9$ | $126.5^{ab} \pm 3.5$ | $102.3^{b} \pm 2.9$ |
| Day 7 | $300.2^{ab} \pm 11.3$ | $212.3^{ab} \pm 24.9$ | $130.0^{ab} \pm 2.2$ | $117.3^{a} \pm 7.3$ |
| Day 9 | $308.4^{a} \pm 2.5$ | $207.3^{ab} \pm 24.2$ | $133.5^{a} \pm 0.6$ | $123.0^{a} \pm 0.9$ |

Superscript letters within each column indicate significant differences between samples ($P<0.05$).
GAE, gallic acid equivalent, QE, quercetin equivalent, TE, Trolox equivalent.

In this study, soybean sprouts on day 9 had the highest antioxidant activities, with DPPH and FRAP values being 2.66 and 1.81 times higher than soybean seeds on day 0. The DPPH and FRAP values increased with germination time and closely corresponded to the total phenolic content. This agreed with the previous study of Xue *et al.* [9], who studied soybean, mung bean, and black bean germination. They found that antioxidant activities on total reducing power, hydroxyl radical scavenging capacity, DPPH radical scavenging capacity, and ABTS radical scavenging capacity of soybean sprouts were related to phenolics more than flavonoids with relative contribution rate to the antioxidant activity of 42.38-63.06% and 2.36-6.64%, respectively.

## 4. Conclusion

Extended germination significantly enhanced the nutrition value, bioactive compounds, and lower allergens of yellow soybean seeds compared to short-time germination. After 9 d of germination, yellow soybean sprouts contained the highest weight, ash, and fiber contents while being the lowest in lipid and allergens. These findings provide insight into a natural process to manipulate beneficial compounds and unlock the potential of yellow soybean sprouts beyond typical fresh consumption.

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# Green Alga *Chlorella* sp. KLSc61 Tended to Partially Remove 3-Amino-5-Nitrosalicylic Acid (ANS) from 3,5-Dinitrosalicylic Acid (DNS) Wastewater

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**Abstract**

Currently, numerous reports focus on green algae and green ecosystems, with one noteworthy discovery being their application in wastewater treatment. Additionally, various studies show that biomass from algae can be used to produce biofuels, nutritional supplements, animal feed, etc. One of particular interest in this study is the utilization of green algae for the treatment of 3,5-dinitrosalicylic acid (DNS), a representative phenolic compound, commonly used in various experiments, including reducing sugar determination. This reaction often results in the generation of wastewater, containing the remaining substrate DNS and the final product, 3-amino-5-nitrosalicylic acid (ANS). Both substances are known to be toxic with an LD50 of 860 mg/L in rat (for DNS) and 3,400 mg/L (for ANS). Our research is focused on the use of Chlorella sp. KLSC61 to remediate these compounds, contributing to the advancement of green algae applications. Chlorella sp. KLSc61 has initially demonstrated a 30% reduction in ANS, with no observable treatment effect for DNS. For further our next aim is to further enhance this percentage ANS treatment, utilizing carbon sources from ANS to promote the growth of Chlorella sp. KLSc61 for utilization in food supplements, animal feed, ecosystem development, and pharmaceuticals, as well as other various phenolic compounds.

***Keywords**:* Green algae; 3,5-dinitrosalicylic acid; 3-amino-5-nitrosalicylic acid; green ecosystems, *Chlorella* sp. KLSc61

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## 1. Introduction

3,5-Dinitrosalicylic (DNS) is aromatic compound, which is a member of the phenolic compounds [(1)]. DNS can also be used to determine the reducing sugar concentration via redox reaction [(2)]. DNS is an oxidizer, whereas sugar is a reducer. DNS will be reduced at $-NO_2$ group by aldehyde group of glucose and converted the structure to $-NH_2$, forming 3-amino-5-dinitrosalicylic acid (ANS). Subsequently, glucose will undergo a transformation from an aldehyde group to a carboxylic group, forming gluconic acid [(3)]. ANS exhibits the ability to absorb light at 540 nm [(4)], that the standard protocol uses this wavelength to determine sugar concentration.

After reacting, the reaction produced ANS ($LD_{50}$ of 3,400 mg/L in rats), including the remaining substrate, DNS ($LD_{50}$ of 840 mg/L in rats, left over KNa-tartrate (this substance does not meet the criteria for classification in accordance with Regulation No 1272/ 2008/EC.), and NaOH ($LD_{50}$ of 40 mg/L in rats). All $LD_{50}$ data are sourced from Material Safety Data Sheets (MSDS) from different databases. Studies have consistently shown that

neither ANS, a product derived from DNS, nor DNS itself, exhibit toxic properties that may impact the environment.

DNS reagents have been found extensive utilized in various studies, as reported in previous research [4-7]. Consequently, these research experiments have generated substantial DNA and ANS wastewater. Green algae have demonstrated the capacity for phenolic compound remediation, including *p*-nitrophenols [8], phenol [9], triclosan [10], 2,4-dichlorophenol [11], and *p*-chlorophenol [12]. Therefore, we firmly believe that green algae hold potential for the treatment of ANS and DNS.

Algae possess the ability to capture $CO_2$ as a carbon source, as demonstrated by Meta *et al*.[13]. Algal cells utilize this naturally fixed carbon source through the Calvin cycle, along with ATP and reducing equivalent NADPH in the photosynthesis process, to produce a diverse biomass. Consequently, algae can potentially utilize phenolic compounds as a carbon source for biomass production, reducing their reliance on acquiring $CO_2$ from natural sources. The high-value product produced from algae biomass is biofuel, and the by-products include animal feed and food supplements [14].

This concept has been supported by numerous reports, as reviewed by Wu *et al.* [15], highlighting the efficacy of algae in treating phenolic compounds. This study investigates the potential of the green alga Chlorella sp. KLSc61 to bioremediate wastewater containing 3,5-dinitrosalicylic acid (DNS) and its derivative, 3-amino-5-nitrosalicylic acid (ANS).

## 2. Materials and Methods

### 2.1 Phenolic compound monitoring

DNS and ANS were monitored using a UV-Vis spectrophotometer in spectrum scanning and photometry modes.

*2.1.1 Preparation of $DNS_{solution}$*

An improved method, as described by Saqib *et al.*[4], was used, firstly preparing by a 2N NaOH solution (dissolving 16 g of NaOH in 200 mL of distilled water). 300 g of Potassium sodium tartrate (KNa-tartrate) were later added to 2N NaOH solution and mixed till they fully dissolved. Ten grams of 3,5-dinitrosalicylic acid (DNS) were finally added to the mixture and adjusted the final volume to 1,000 mL. This solution was called DNS reagent ($DNS_{solution}$). Pured DNS reagent ($DNS_{pure}$) was also used for comparison purpose, preparing by dissolving 0.4 g of 3,5-dinitrosalicylic acid in 40 mL of distilled water, stored in the dark for future use.

*2.1.2 Preparation of ANS*

A 40 mL stock solution of glucose was prepared at a concentration of 7.9 mg/mL (molar equivalent to DNS concentration in $DNS_{solution}$). This glucose solution was used to fully react with $DNS_{solution}$, to form a hundred percent ANS. One milliliter of glucose solution and a milliliter of $DNS_{solution}$ were mixed and boiled for 5 min, subsequently, added 3 mL of sterile distilled water. This mixture was used for the experiments, along with $DNS_{solution}$ and $DNS_{pure}$, diluted with equal volume of water.

*2.1.3 Light absorption spectrum scanning*

Three solutions of ANS, $DNS_{solution}$, and $DNS_{pure}$ were used to scan the UV-Vis light absorption spectrum from 190 to 1,000 nm using spectrophotometer. For ANS, $DNS_{solution}$ was used as a blank, whereas water was used as a blank for $DNS_{solution}$, and $DNS_{pure}$.

### 2.2 Cell culture

Green alga *Chlorella* sp. KLSc61 was cultivated in Tris-Acetate-Phosphate (TAP) medium [16] at pH 7.2 under the following optimal growth conditions [17] 120 rpm shaking, a temperature of 35℃, light intensity of 55.5 $\mu mol \cdot m^{-2} \cdot s^{-1}$ from the LED lamp light source, grown for 24 hours. This culture was used as the inoculum in any experiment. After 1 day of cultivation, corresponding to the mid-log phase, the cells were harvested by centrifugation at

4,000x*g* at 25°C. The cells were washed once with sterile distilled water, and then inoculated in 40 mL of TAP or TAP-Ac (TAP without acetate) media (see the composition in Table 1) to obtain an initial $OD_{750}$ of 0.1. In each medium, 1 mL of ANS, $DNS_{pure}$, or $DNS_{solution}$ were separately added to the culture. Samples of 1 ml were collected daily for 12 days, placed in 1.5 ml Eppendorf tubes, and then measured the turbidity at 750 nm using a spectrophotometer and measured the ANS and DNS using the $\lambda_{max}$, obtained from section 2.1.3.

**Table 1.** The composition of Tris-Acetate-Phosphate (TAP) and TAP-Ac (TAP without acetate) media.

| Composition | TAP | TAP-Ac |
|---|---|---|
| Tris-base | ✓ | ✓ |
| TAP-salt | ✓ | ✓ |
| Phosphate solution | ✓ | ✓ |
| Trace element | ✓ | ✓ |
| Glacial acetic acid | ✓ | Replaced with HCl |

Remark: adjusted the pH of TAP-Ac to 7.2 using 2M HCl.

### 2.3 Wastewater treatment monitoring

As the cultures were prepared according to section 2.2, the final pH of those inoculum cultures was 7.3. We also evaluated the treatment at the pH of 4.3 using HCl titration before cells addition. During cell growth, one milliliter of each sample was collected every 2 days for monitoring. Cells were removed by centrifugation, and the supernatant of ANS and DNS was measured using spectrophotometry according to the $\lambda_{max}$, obtained from section 2.1.3.

## 3. Results & Discussion

### 3.1 Spectrum scanning

In previous reports, DNS detection had not been reported, unlike ANS detection, which can be monitored at 540 nm [(4)]. Consequently, the need arose to establish a method for DNS detection and confirm ANS detection using our systems. Spectrum scanning was conducted within the range of 190-1,000 nm, as determined by the instrument coverage range. Various dilutions of each solution were prepared to prevent detector saturation. As shown in Figure 1., obviously, it was evident that no dilution of DNS exhibited saturation absorption (above 2.0) by the instrument detector, while undiluted ANS fell within the normal range of absorption values (approximately 0.16). Ten-fold diluted DNS monitoring was evident when distilled water was used as a blank. The $\lambda_{max}$ for DNS detection was observed at 376 nm (left), whereas ANS detection was found the λ max at 522 nm (right). Our results provide the first report of the $\lambda_{max}$ for DNS, although the $\lambda_{max}$ for ANS was slightly different from other reports, which cited the ANS $\lambda_{max}$ as 540 nm [(18)]. This variation may be attributed to the dilution factor and different instruments, causing a blue shift in this detection. Based on our findings, we recommend scanning the absorption spectrum for each detection in different systems to account for wavelength shifts.

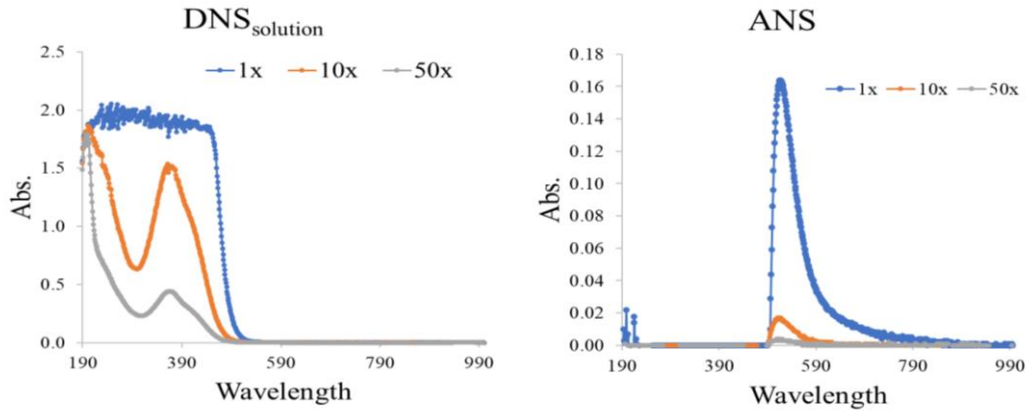


**Figure 1.** UV-Vis light absorption spectra of $DNS_{solution}$ (left). No dilution (1X), 10-fold dilution (10X), and 50-fold dilution (50X) were used as a solution for scanning against water as a blank. $\lambda_{max}$ of 376 nm was found to be the maximum absorption wavelength for detection. The absorption spectra of ANS (no dilution, 10-fold, and 50-fold dilution) were shown in right panel with $\lambda_{max}$ of 522 nm, against $DNS_{solution}$ at the same dilution as a blank.

### 3.2 Growth determination

When observed the cell morphology of *Chlorella* sp. KLSc61 under light microscope, this alga has the unicellular rounded shape (Figure 2.) with average cell diameter of 5 micrometer.

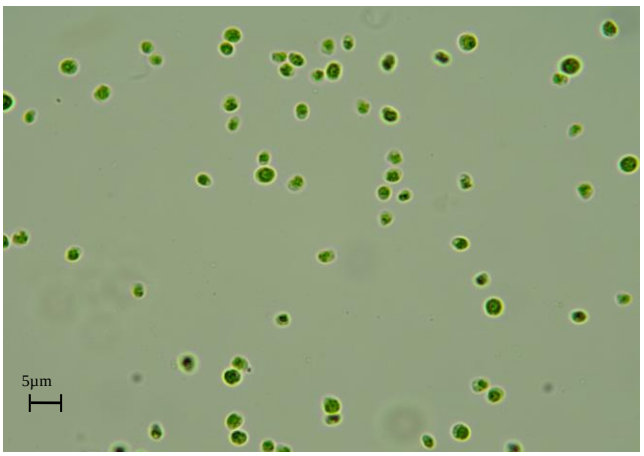


**Figure 2.** Morphology of *Chlorella* sp. KLSc61 cells in normal TAP from optical microscopy at magnification of 400X.

Based on the investigation of *Chlorella* sp. KLSc61 growth, as outlined in section 2.2, samples were collected over a span of 0-12 days, and the cell turbidity was measured at a wavelength of 750 nm. Figure 3 presents the results of the culture growth of *Chlorella* sp. KLSc61 in TAP-Ac medium at a pH of 7.2, adding ANS, $DNS_{solution}$, and $DNS_{pure}$, using normal TAP and TAP-Ac as 2 types of control. The normal TAP medium (black dashed line) contains complete nutrient sources, allowing the cells to grow at the maximum growth rate. In contrast, TAP without acetate (TAP-Ac) (green dashed line) could lead to a lower growth rate since it lacks a carbon source in the broth. Therefore, cells need to fix $CO_2$ from the atmosphere as their carbon source. ANS, $DNS_{solution}$, and $DNS_{pure}$ were separately added to the TAP-Ac culture to allow the cells to use these solutions as a carbon source. As expected,

if the cells could use these chemicals as a carbon source, the growth curve should fall somewhere between TAP and TAP-Ac. However, the results did not align with our hypothesis. Only ANS (blue solid line) was found to enhance growth after nine days of incubation. The growth under this condition was slightly above that of TAP-Ac, suggesting the ability of algal cells to use ANS as a carbon source during the late log phase. In contrast, DNS solution (grey solid line) did not enhance growth, and interestingly, $DNS_{pure}$ resulted in a slower growth rate than TAP-Ac. The DNS molecule itself may be detrimental to cell survival, as suggested in a report by [19].

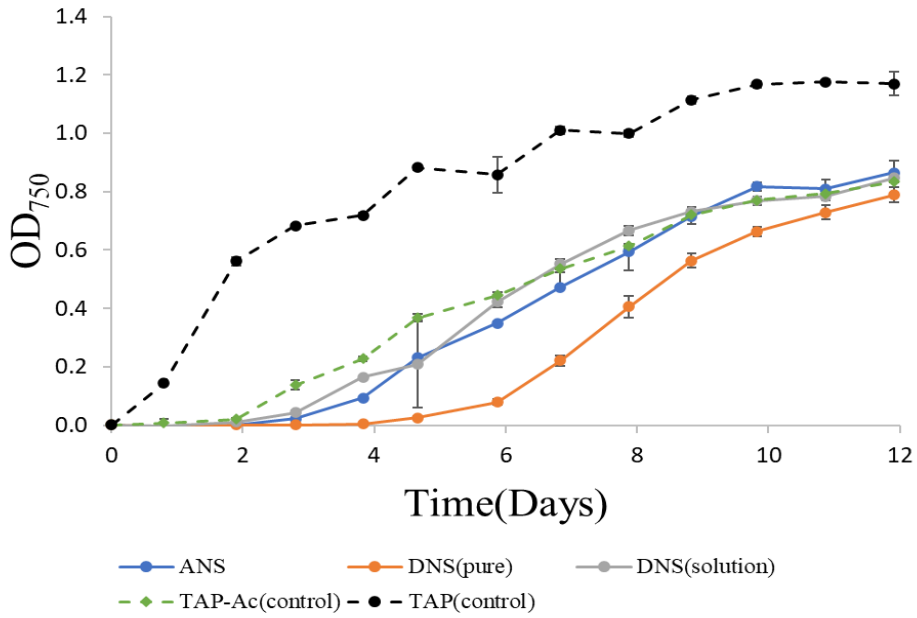


**Figure 3.** Growth curves of green alga *Chlorella* sp. KLSc61 in TAP, TAP-Ac, TAP-Ac with ANS (ANS), TAP-Ac with $DNS_{solutions}$ ($DNS_{solutions}$), and TAP-Ac with $DNS_{pure}$ ($DNS_{pure}$).

### 3.3 Treatment of DNS and ANS using *Chlorella* sp. KLSc61

In the treatment monitoring, DNS and ANS were monitored using their maximum wavelengths determined from the experimental results in section 3.1. DNS in both systems ($DNS_{solution}$ and $DNS_{pure}$) was monitored at 376 nm, whereas ANS was monitored at 522 nm. It is worth noting that [11] also reported that pH plays a significant role in ionization of chemical structure, regulating the entry and exit of substances into cells, as demonstrated in their experiments with 2,4-dichlorophenol. Thus, pH adjustments were made in the ANS and DNS treatment experiments. The predicted $pK_a$ of DNS is 1.57 ± 0.10 while it is 1.42 ± 0.10 ($pK_a$ of DNS and ANS Referenced from the website: Chemical Book [21,22] for ANS. Titrating the pH below the $pK_a$ value would protonate the chemical structure in its nonpolar form, allowing it to freely move from the broth into the cytoplasm of the algal cell. However, it was impossible to lower the pH of the culture to below the $pK_a$ value, as such pH levels would not support cell growth. Therefore, we decided to use the lowest feasible pH [3.4] to test cell survival and chemical treatment.

*3.1.1 DNS Treatment*

A wavelength of 376 nm was utilized to monitor the remaining DNS in TAP-Ac system for both $DNS_{solution}$ and $DNS_{pure}$ cultures, using distilled water as a blank. In this study, DNS treatment was divided into two experiments: one involved treating with $DNS_{solution}$ (DNS mixed with KNa-tartrate, NaOH, and DNS), and the another involved $DNS_{pure}$ (DNS without any additional substances). The results, presented in Figure 4, show that *Chlorella* sp.

KLSc61 was not respond to pH 3 for either $DNS_{solution}$ (black solid line) or $DNS_{pure}$ (yellow solid line), suggesting that the algae were unable to uptake DNS into their cells. This could be due to the fact that a pH of 4 could not convert the chemical structure into a nonpolar form as expected. Thus, the polar DNS molecules remained outside the cell in the broth. Even though a previous study found that low pH levels can impact the accumulation of phenol, as indicated by Nipper *et al.* [20].

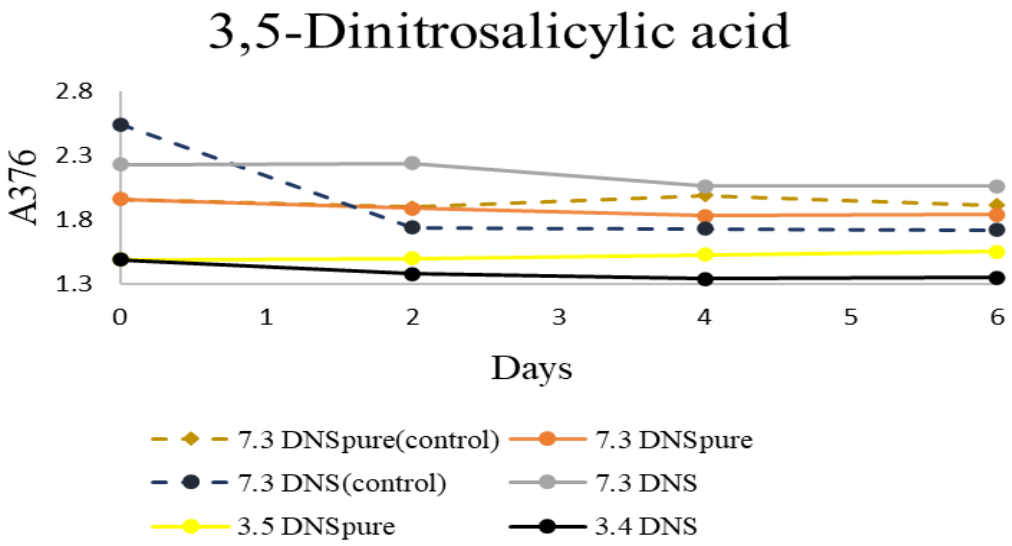


**Figure 4.** DNS Treatment monitoring using spectrophotometry at 376 nm, during 6 days of treatment by *Chlorella* sp. KLSc61; control = DNS without algae. The number in front of the line legend represents the pH of the test culture.

*3.1.2 ANS Treatment*

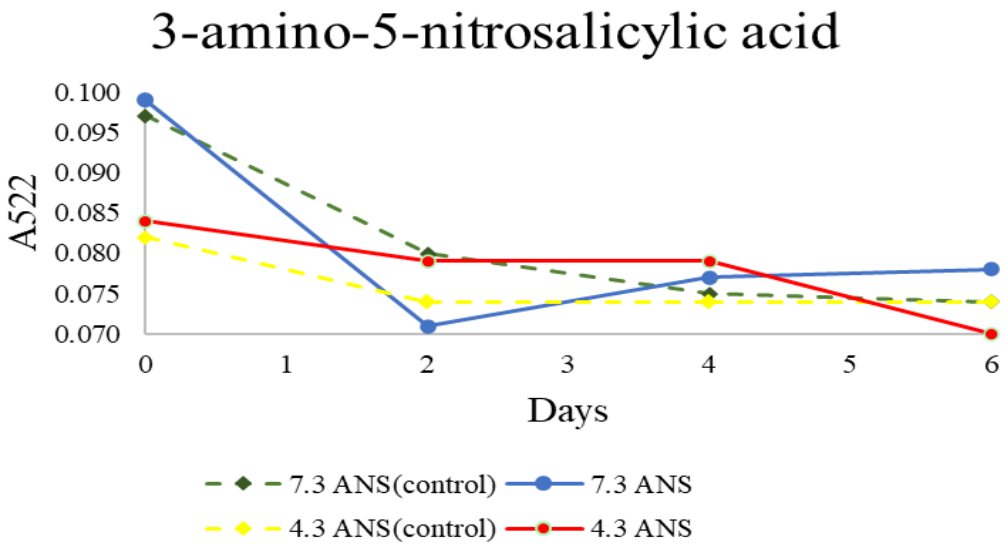


**Figure 5.** ANS Treatment monitoring using spectrophotometry at 522 nm, during 6 days of treatment by *Chlorella* sp. KLSc61; control = DNS without algae. The number in front of the line legend represents the pH of the test culture.

When monitoring ANS remaining in the system at 522 nm, $DNS_{solution}$ was used as a blank. We had previously tried using water as a blank, but reliable results were not obtained as when using DNS as a blank. The stability of ANS was tested using the system without algal cells (two dashed lines). Interestingly, figure 5 indicates that when ANS was introduced to the algae, there was a slight decrease in both ANS levels at pH 7.3 (blue solid line) and 4.3 (red solid line) after 2 days, representing 30% and 6% reduction, respectively. This suggests that the cells may be more effective at treating ANS than DNS, as found in Figure 4. Moreover, a higher treatment rate was observed when pH 7.3 was used for treatment. Conversely, at pH 4.3, ANS levels showed a continuous decrease over 4-6 days compared to the control at pH 4.3 (yellow dashed line). It is speculated that the algae may facilitate the transport of ANS into the cell more efficiently at pH 4.3 than at pH 7.3.

## 4. Conclusion

Green algae *Chlorella* sp. KLSc61 have demonstrated an initial capability to remove 30% of ANS at pH of 7.3, while DNS could not be treated by our observation, performed in this study. In the future, we will continue our efforts to enhance this removal percentage and further explore treatment methods for various other types of phenolic compounds, with the aim of establishing a sustainable ecosystem for both our country and the international community.

## Acknowledgements

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# Determination of *Cordyceps Militaris* Extraction with Ultrapure Water, Time and Temperature on the Amount of Important Substances, Polysaccharide and Antioxidant Activity of Extracts

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**Abstract**

*Cordyceps militaris* has a long history of use in traditional medicine and healthcare in East Asia due to its diverse pharmacological activities. This study aimed to investigate the effects of sonicate and incubate extraction methods, along with variations in time and temperature, on the extraction of important substances such as cordycepin, adenosine, polysaccharides, and antioxidant activity of *C. militaris* extracts. Additionally, the research aimed to support the purification of these substances and explore their potential applications in healthcare and the development of health food products. Fruiting bodies of *C. militaris*, cultured from the Biology Laboratory at the School of Science, King Mongkut's Institute of Technology Ladkrabang, Thailand, were utilized. Analysis revealed that the incubated extraction method employed a wider range of time and temperature parameters compared to sonicated extraction. High-performance liquid chromatography (HPLC) analysis of the extracts demonstrated the highest cordycepin content (6.39 ± 0.02 mg/g DW) and adenosine content (1.62 ± 0.02 mg/g DW) with sonicated extraction at 45°C for 60 min. Polysaccharide content was measured at 14.31 mg/g for the sonicated extract and 14.07 mg/g for the incubated extract at 80°C for 2 h. Both extraction methods exhibited antioxidant activity within the concentration range of 0.01-0.3 mg/mL, with $IC_{50}$ values of 0.16 mg/mL for the sonicated extract on the DPPH assay and 0.18 mg/ml on the ABTS assay. For the incubated extract, $IC_{50}$ values of 0.16 mg/mL were observed on both DPPH and ABTS assays. These findings contribute to our understanding of optimal extraction methods for harnessing the pharmacological potential of *C. militaris* and may guide further research into its healthcare applications and product development.

***Keywords**: Cordyceps militaris*; Antioxidants; Extraction; Cordycepin; Adenosine

## 1. Introduction

*Cordyceps militaris*, an edible mushroom classified in the phylum Ascomycota, class Ascomycetes, order Hypocerales, and family Clavicipitaceae, exhibits a wide array of pharmacological activities, making it a subject of significant scientific interest. Originating mostly from Asian countries such as Korea, Japan, Nepal, and China [(1-2)]. Its medicinal properties have long been recognized, contributing to its utilization in traditional medicine for health maintenance and the treatment of various ailments. Documented pharmacological activities include blood glucose control, hypolipidemic effects, anti-tumor properties, antimicrobial activity against a range of microorganisms such as *Bacillus cereus, Micrococcus flavus, Escherichia coli, S. aureus, Aspergillus fumigatus* and *Aspergillus niger*[(3)], antiviral actions, anti-inflammatory effects, neuroprotective properties, antioxidant activity, and immune modulation.

Cordycepin, a prominent bioactive compound present in *C. militaris*, demonstrates broad-spectrum antimicrobial activity against various pathogens. The medicinal and biotechnological significance of *C. militaris* stems from its rich composition of bioactive compounds, encompassing small molecules, secondary metabolites, polysaccharides, proteins, adenosine, cordymin, cordycepic acid, carotenoids, enzymes, organic selenium, ergosterol, sterol, superoxide dismutase (SOD), and multivitamins. Moreover, bioactive peptides derived from *C. militaris* exhibit diverse physiological activities, including anti-hypertensive, antibiotic, antimicrobial, antioxidant, anti-aging, antibacterial, antifungal, anti-proliferative, anti-inflammatory, and Angiotensin-I-converting enzyme (ACE) inhibitory properties [4-7].

The objective of this study is to investigate the effect of time and temperature on both sonicate and incubate extraction methods of *C. militaris* to optimize the extraction conditions for maximizing the yields of important bioactive substances such as cordycepin, adenosine, polysaccharides, and antioxidant compounds. This research aims to contribute to the development of optimal extraction protocols to support the purification of bioactive compounds from *C. militaris*. Furthermore, the study seeks to provide support for the applications of *C. militaris* in healthcare products and health food development. With the goal of expanding its utilization as a functional food ingredient, to cater to a broader demographic, encompassing consumers of all genders and age groups.

## 2. Materials and Methods

### 2.1 Preparation of *C. militaris* Extract

The fruiting bodies of *C. militaris* fungi were sourced from the Biology Laboratory, School of Science at King Mongkut's Institute of Technology Ladkrabang, Thailand. Following harvest, the samples underwent a drying process and were subsequently ground into a fine powder, which was then sieved through a Micro-Mesh sieve size of 200 mesh. For extraction, the *C. militaris* powder was soaked in a solvent at a ratio of 1:10 (w/v), with ultrapure water used as the solvent. Two extraction methods were employed: sonication and incubation. In the sonication method, the samples were sonicated at temperatures of 45°C, 55°C, and 65°C for 3 h, with samples collected every 1 h. Similarly, in the incubation method, the samples were incubated at the same temperatures for the same duration, with samples collected at hourly intervals. Following extraction, the samples were centrifuged at 9,000 rpm at 10°C for 40 min. The supernatant obtained from the centrifugation was subjected to further analysis. The amount of important substances, such as cordycepin and adenosine, in the *C. militaris* extracts was determined using high-performance liquid chromatography (HPLC). Additionally, the polysaccharide content was determined using the Anthrone method, while the antioxidant activities of the extracts were evaluated using the DPPH and ABTS methods. To prepare the crude extracts for Anthrone method and DPPH and ABTS methods, the supernatant obtained after filtering was mixed with 95% ethanol in a 1:4 ratio and incubated at 4°C for 24 h. Following this incubation period, the sample was centrifuged again at 9,000 rpm at 10°C for 40 min, and the supernatant was discarded. The crude extracts were then dried using nitrogen and further dried at 50°C for 30 min. Finally, the dried crude extracts were stored in amber glass bottles at 4°C until further use.

### 2.2. Determination of cordycepin and adenosine in *C. militaris* extracts by High Performance Liquid Chromatography (HPLC)

The determination of cordycepin and adenosine in *C. militaris* extracts was conducted using high-performance liquid chromatography (HPLC). The samples were initially filtered through a filter paper with a pore size of 0.45 µm to remove any particulate matter. HPLC analysis was performed using a C18 column with a particle size of 3.5 µm and dimensions of 250 × 4.6 mm. A UV detector set at a wavelength of 254 nm was employed for compound

detection. The flow rate of the HPLC system was maintained at 1.0 mL/min. For each sample, 0.1 microliters were injected into the column, and the column temperature was set to 40°C. The HPLC system ran for a total of 20 min. The mobile phase used for chromatographic separation consisted of methanol and ultrapure water in a ratio of 15:85 (v/v). The content of cordycepin and adenosine in the *C. militaris* extracts was quantified by comparing the peak areas of the compounds in the samples to standard calibration curves generated for each compound. These calibration curves were constructed using known concentrations of pure cordycepin and adenosine standards. By analyzing the peak areas of the sample peaks and comparing them to those of the standards, the concentrations of cordycepin and adenosine in the extracts were accurately determined.

**2.3 Determination of Polysaccharides of *C. militaris* Extracts**

The determination of polysaccharides in *C. militaris* extracts was performed using an Anthrone method with slight modifications based on the method described by Dreywood [(8)]. To prepare the reagents, a 75% sulfuric acid solution was prepared by adding 100 mL of distilled water to 390 mL of concentrated sulfuric acid (95-97%). An anthrone solution was then prepared by dissolving 0.5 grams of anthrone in 75% sulfuric acid and adjusting the volume with 75% sulfuric acid until reaching a total volume of 250 mL. For the analysis, 1 mL of *C. militaris* extract samples at a concentration of 0.02 mg/mL was added to a test tube, which was then placed in a cold water bath. Subsequently, 5 mL of the Anthrone solution was added to the test tube, and the mixture was shaken well. The test tube was then transferred to a boiling water bath and allowed to boil for 10 min. After boiling, the test tube was cooled in an ice bath. The absorbance of the cooled solution was measured at 620 nm using a spectrophotometer. The absorbance values obtained were compared with a standard glucose solution to quantify the polysaccharide content in the *C. militaris* extracts.

**2.4. Determination of DPPH radical cation scavenging activity**

The antioxidant activity of *C. militaris* extracts was assessed using a DPPH radical scavenging method with slight modifications [(9).] The extracts were prepared at various concentrations ranging from 0.01 to 2.5 mg/mL. Subsequently, these extracts were mixed with a 0.1 mM solution of DPPH (Sigma-Aldich, St. Louis, MO, USA). The mixture was then incubated at room temperature for 30 min under dark conditions to allow for the reaction to occur. After incubation, the absorbance of the mixture was measured at a wavelength of 517 nm using a spectrophotometer (Genesys 20, Thermo Scientific, Dreieich, Germany). The absorbance of a DPPH solution mixed with distilled water served as the control, while the absorbance of the DPPH solution mixed with the extract was measured as the sample. The percentage of DPPH radical inhibition was calculated using the following equation: Percentage inhibition = ([(Acontrol - Atest sample)/Acontrol] × 100. and calculate the IC50 (inhibitory concentration) value.Where Acontrol is the absorbance of the DPPH solution mixed with distilled water (control) and Atest sample is the absorbance of the DPPH solution mixed with the extract (sample). Furthermore, the IC50 ( inhibitory concentration) value, representing the concentration at which 50% of the DPPH radicals are inhibited, was calculated from the dose-response curve generated by plotting the percentage inhibition against the concentration of the *C. militaris* extract.

**2.5. Determination of ABTS radical cation scavenging activity**

The ABTS radical scavenging activity of the *C. militaris* extracts was evaluated using an ABTS radical cation decolorization assay, based on a previously reported method [(10)], with some modifications. To prepare the ABTS radical solution, 7 mM ABTS (2,20-azinobis (3-ethylbenzothiazoline-6-sulfonic acid)) and 2.45 mM potassium persulfate were mixed in water. This solution was then incubated in the dark at room temperature for 16 h. Subsequently, the solution was diluted with distilled water to achieve an absorbance of 0.70 ± 0.05 at 734 nm. The *C. militaris* extracts, prepared at various concentrations ranging

from 0.01 to 2.5 mg/mL, were mixed with the ABTS solution. The mixture was then incubated in the dark at room temperature for 30 min. Following incubation, the absorbance of the reaction mixture was measured at 734 nm using a microplate reader. The percentage of ABTS radical inhibition was calculated using the equation: [(Acontrol-Atest sample)/Acontrol] × 100. Where Acontrol represents the absorbance of the ABTS solution mixed with distilled water (control), and Atest sample represents the absorbance of the ABTS solution mixed with the *C. militaris* extract (test sample). Additionally, the IC50 (inhibitory concentration) value, indicating the concentration at which 50% of the ABTS radicals are inhibited, was determined by plotting the dose-response curve of percentage inhibition against the concentration of the *C. militaris* extract.

**2.6. Statistical Analysis**

The experimental results were presented as means ± standard deviations (SDs) of three independent experiments. Statistical analysis was performed using one-way analysis of variance (ANOVA). Differences between groups were assessed using Tukey's Honestly Significant Difference (HSD) tests. A significance level of $P<0.05$ was considered statistically significant. Statistical analyses were conducted using Minitab 18 software.

## 3. Results & Discussion

### 3.1 Analysis of Cordycepin and Adenosine in *C. militaris* extracts by HPLC

The HPLC analysis of *C. militaris* extracts revealed findings, as depicted in Table 1, Table 2, and Figure 1. The highest cordycepin content was observed in the sample subjected to sonication at 55°C for 2 h, yielding values of 6.99 ± 0.40 mg/g. Conversely, the extraction resulting in the highest adenosine content was achieved at 45°C for 2 h, with a value of 1.67 ± 0.05 mg/g. Notably, the difference in cordycepin concentration between extractions at 55°C for 2 h and 45°C for 1 h, as well as the disparity in adenosine concentration between extractions at 45°C for 2 h and 45°C for 1 h, was not statistically significant. Further investigations revealed that incubated extraction at 45°C for 2 h exhibited the highest cordycepin content (6.02 ± 0.29 mg/g), while the highest adenosine content (2.13 mg/g) was observed at 45°C for 3 h. However, with the exception of extractions at 55°C and 65°C for 3 h, the concentration of cordycepin did not significantly differ across all treatments.

In the comparison between sonicated and incubated extractions, it was found that for cordycepin and adenosine extracts, employing a temperature of 45°C for 1 h in sonicated extraction, and 45°C for 2 h for cordycepin extraction and 55°C for 2 h for adenosine extraction, respectively, yielded significantly different adenosine content. However, the cordycepin content remained consistent across both conditions. In light of these findings, the research team opted for sonicated extraction conditions at 45°C for 1 h, resulting in cordycepin and adenosine contents of 6.39 ± 0.02 mg/g and 1.62 ± 0.02 mg/g, respectively. This method produced higher quantities of important substances compared to the research conducted by Nguyen NQ. *et al.* [(11)], which reported a cordycepin content of 2938.97 ± 94.66 µg/gDW at 70°C for 120 min. The concentration of cordycepin at the mark of 60°C and 70°C was not significantly different, that is, choose one of the two conditions to conduct the concentration to have the same quality

**Table 1.** Cordycepin and adenosine contents of *C. militaris* extracts obtained through sonicated extraction at different time intervals (1 h, 2 h, and 3 h) and temperatures (45°C, 55°C, and 65°C). The values are expressed as mean ± standard deviation.

| Time | Cordycepin | | | Adenosine | | |
|---|---|---|---|---|---|---|
| | Temperature | | | | | |
| | 45°C | 55°C | 65°C | 45°C | 55°C | 65°C |
| 1 h | 6.39±0.02$^{ab}$ | 5.71±0.02$^{bc}$ | 4.85±1.11$^{c}$ | 1.62±0.02$^{ab}$ | 1.02±0.04$^{d}$ | 0.85±0.01$^{e}$ |
| 2 h | 5.36±0.12$^{bc}$ | 6.99±0.40$^{a}$ | 5.60±0.29$^{bc}$ | 1.67±0.05$^{a}$ | 1.47±0.02$^{c}$ | 0.90±0.004$^{e}$ |
| 3 h | 5.15±0.03$^{c}$ | 5.70±0.05$^{bc}$ | 5.06±0.06$^{c}$ | 1.57±0.01$^{b}$ | 1.47±0.01$^{c}$ | 0.84±0.04$^{e}$ |

$^{a,b,c,d,e}$, The data of different superscript letters (a,b,c,d,e) are revealed significantly different values for each extracts (ANOVA followed by Tukey's HSD tests, (P<0.05).

**Table 2.** Cordycepin and adenosine contents of *C. militaris* extracts obtained through incubated extraction at different time intervals (1 h, 2 h, and 3 h) and temperatures (45°C, 55°C, and 65°C). The values are expressed as mean ± standard deviation.

| Time | Cordycepin | | | Adenosine | | |
|---|---|---|---|---|---|---|
| | Temperature | | | | | |
| | 45°C | 55°C | 65°C | 45°C | 55°C | 65°C |
| 1 h | 5.25±0.09$^{ab}$ | 5.39±0.08$^{ab}$ | 5.40±0.15$^{ab}$ | 1.91±0.03$^{b}$ | 1.57±0.05$^{d}$ | 1.15±0.03$^{d}$ |
| 2 h | 6.02±0.29$^{a}$ | 5.63±0.17$^{ab}$ | 5.53±0.15$^{ab}$ | 2.13±0.01$^{a}$ | 1.79±0.007$^{c}$ | 1.38±0.01$^{f}$ |
| 3 h | 5.34±0.37$^{ab}$ | 4.96±0.53$^{b}$ | 5.07±0.37$^{b}$ | 2.13±0.007$^{a}$ | 1.75±0.05$^{c}$ | 1.50±0.02$^{f}$ |

$^{a,b,c,d,e,f}$, The data of different superscript letters (a,b,c,d,e,f) are revealed significantly different values for each extracts (ANOVA followed by Tukey's HSD tests, P<0.05).

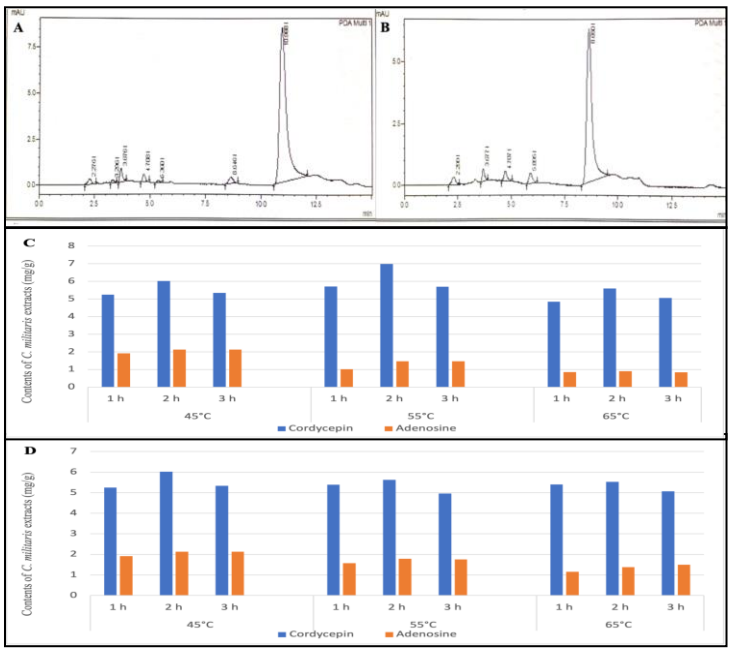


**Figure 1.** illustrates the HPLC chromatograms depicting cordycepin and adenosine present in *C. militaris* extracts, utilizing the HPLC method. Sub-figures include: (A) Standard compounds of cordycepin, (B) Standard compounds of adenosine, (C) The impact of temperature and time on the concentration of cordycepin and adenosine contents (mg/g) in sonicated extraction and (D) The effect of temperature and time on the concentration of cordycepin and adenosine contents (mg/g) in incubated extraction. These figures serve to elucidate the chromatographic profiles and variations in compound concentrations under different extraction conditions, providing valuable insights into the extraction process and compound yields.

### 3.2 Antioxidant Activities on DPPH and ABTS assay of *C. militaris* Extracts

The effect of inhibiting DPPH free radicals was investigated, and the results are presented in Fig. 2. The graph depicts the relationship between the percentage of free radical inhibition and the concentration of *C. militaris* extracts at various concentrations. Notably, both sonicated and incubated extraction conditions exhibited antioxidant activity within the concentration range of 0.01-0.3 mg/mL. Sonicated extracts demonstrated the ability to scavenge 84.22% of DPPH radicals and 69.82% of ABTS radicals, whereas incubated extracts showed scavenging capacities of 82.25% for DPPH radicals and 91.09% for ABTS radicals. Upon calculating the IC50 value, it was found that the concentration required to inhibit free radicals by half was 0.16 mg/mL in the DPPH assay and 0.18 mg/ml in the ABTS assay for sonicated extract, and 0.16 mg/mL in both DPPH and ABTS assays for incubated extract. These values suggest a superior inhibitory efficiency compared to the research conducted by Monthira [(11)], which utilized a boiling extraction method followed by solvent evaporation and re-dissolution.

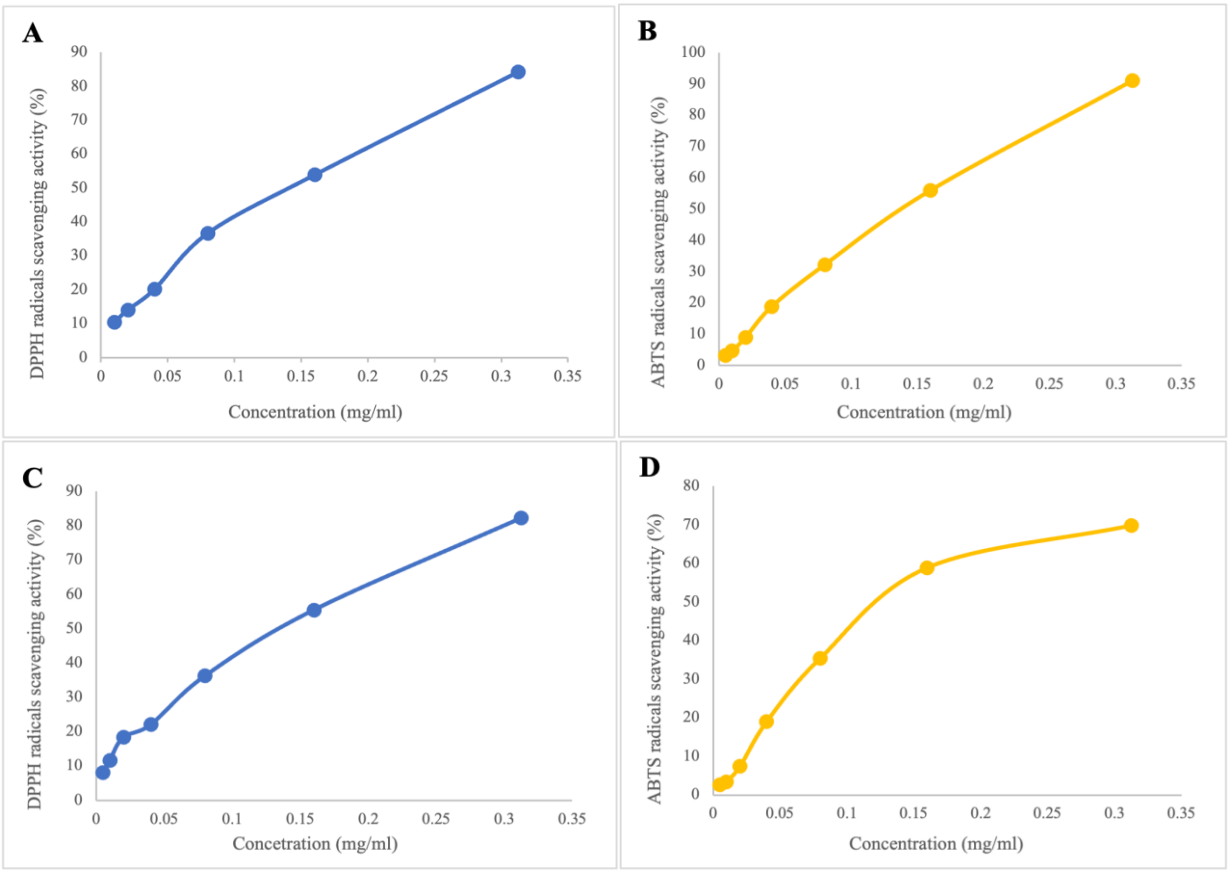


**Figure 2.** illustrates the relationship between the percentage of free radical inhibition and the concentration of *C. militaris* extracts (mg/mL) of both incubated and sonicated extractions. Subfigure (A) displays the DPPH radical scavenging activity (%) of the incubated extraction, while Subfigure (B) shows the ABTS radical scavenging activity (%) of the incubated extraction. Subfigure (C) illustrates the DPPH radical scavenging activity (%) of the sonicated extraction, while Subfigure (D) presents the ABTS radical scavenging activity (%) of the sonicated extraction.

Furthermore, the use of a rotary evaporator in the extraction process may have contributed to the higher average IC50 value of 0.48 mg/mL observed in the DPPH assay, potentially due to the loss of biologically important substances during extraction. Additionally, our results outperform those reported by Sukalya [12], who studied the antioxidant activity of *C. militaris* extracts grown on riceberry and jasmine rice. In their study, the IC50 values for extracts from the fruiting body of *C. militaris* and fiber grown on jasmine rice were reported as 0.22 and 0.56 mg/mL, respectively.

**3.3 Polysaccharides content analysis using the Anthrone method.**

The polysaccharide content of *C. militaris* was quantified using the Anthrone method, with samples prepared at a concentration of 0.02 mg/mL. Each sample underwent three independent experiments. Results indicated that the polysaccharide content in *C. militaris* extracts was 14.31 mg/g for sonicated extraction and 14.07 mg/g for incubated extraction. It's noteworthy that the composition of chemical components in *C. militaris* can vary significantly due to various factors, including the species of mushroom, ingredients and pH of the growth media, environmental conditions, drying techniques, and extraction methods employed. These findings highlight the importance of understanding the multitude of factors that influence the chemical composition of *C. militaris* extracts, thereby emphasizing the necessity for rigorous experimentation and analysis to accurately quantify and characterize its bioactive components.

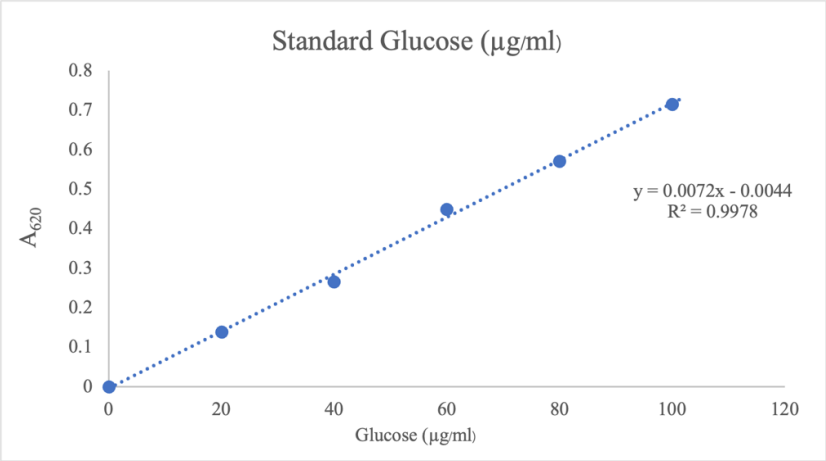


**Figure 3.** illustrates the relationship between the concentrations of standard glucose solutions (ranging from 0 to 100 µg/mL) and the corresponding absorbance values at a wavelength of 620 nm.

**4. Conclusion**

In conclusion, both sonicated and incubated extraction methods demonstrated antioxidant activity in *C. militaris* extracts within the concentration range of 0.01-0.3 mg/mL. The calculated IC50 values indicated that the extracts possess notable efficacy in inhibiting DPPH free radicals, with sonicated extracts exhibiting IC50 values of 0.16 mg/ml (DPPH assay) and 0.14 mg/mL (ABTS assay), and incubated extracts showing values of 0.15 mg/mL (DPPH assay) and 0.13 mg/ml (ABTS assay). Additionally, the polysaccharide content of the extracts was measured at 14.31 mg/g for sonicated extraction and 14.07 mg/g for incubated extraction.

Considering the extraction parameters, it was observed that while the content of adenosine extract differed significantly between sonicated and incubated extraction methods, the content of cordycepin remained consistent. Given the significant difference in concentration time and temperature, the research team opted for sonicated extraction

conditions at 45°C for 1 h. Moving forward, efforts will be directed towards enhancing extraction efficiency to maximize the contents of cordycepin and adenosine. These findings provide valuable support for the application of *C. militaris* in healthcare products and the development of health food.

**Acknowledgements**

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# Optimization of Cannabinoids Extraction form *Cannabis Indica* L. (Blueberry variety) Using the Combination of Ultrasound and Microwave

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**Abstract**

Microwave-ultrasound assisted extraction (MUAE) was applied to extract *Cannabis indica* L. (Blueberry cultivar) cannabinoids including cannabidiol (CBD) and delta-9-tetrahydrocannabinol (THC). The conditions of MUAE were optimized by response surface methodology based on central composite design (CCD). The influence of solid to liquid ratio (X1, A: 1:10-1:30), microwave time (X2, B: 5-20 min), and ultrasound time (X3, C: 10-30 min) was analyzed. A significant quadratic regression models were obtained and validated using the analysis of variance (ANOVA). According to the results, the optimal conditions of the UMAE extraction procedure were following solid to liquid ratio (X1, A): 1:22, microwave extraction time (X2, B): 5 minutes, and ultrasound extraction time (X3, C): 14 minutes. The maximum CBD and THC contents were 0.298 ± 0.001 mg/g dry weight and 91.35 ± 0.35 mg/g dry weight, respectively. Experimentally achieved values were in accordance with those estimated by CCD model, suggesting applicability of the utilized model and the favorable result of CCD application in optimization of UMAE extraction parameters.

***Keywords**:* Green technology; Cannabinoids; Ultrasound; Microwave

## 1. Introduction

Cannabis (*Cannabis* ssp.) is an herbaceous plant of the Cannabaceae family. It has been utilized medically, therapeutically, and spiritually. Cannabis was legally legalized for medical and recreational use in Thailand on February 18, 2019 (1). It contains many biological active materials, such as cannabinoids, terpenoids, alkaloids, and quinones (2). Δ9-Tetrahydrocannabinol (THC) is the most prominent cannabinoid in cannabis that receives attention because of its psychoactive properties, analgesic, anti-inflammatory, appetite stimulant, and antiemetic properties (3). Regular use by teenagers under the age of 30 may, however, result in cognitive impairments and neurodevelopmental abnormalities (4). Cannabidiol (CBD) can control THC's euphoric effects and has antipsychotic, neuroprotective, anticancer, antidiabetic, and other properties such as lowering anxiety caused by fear or cigarette intake in tobacco smokers (5). Despite the promise of these natural biomolecules and their prospective use in industrial areas of cannabis, developing more effective techniques for their recovery remains a challenge.

Several research studies have been conducted to enhance the extraction of these chemicals using conventional, ultrasound, or microwave-assisted extraction techniques [6-8]. Microwave and ultrasonic extractions, on the other hand, are more efficient than other extraction procedures [6-7]. Microwave and ultrasound extractions are rapid, easy, safe, and ecologically beneficial technology since it decreases solvent usage and energy consumption, as well as different environmental dangers such as chemical waste [9]. Microwave irradiation creates an electric and magnetic field, generating high-frequency motion and dipole rotation. In contrast to typical conductive heating, it may swiftly transmit energy directly to the reactants and raise internal and exterior temperatures virtually simultaneously. This enables fast breakdown of the precursors, resulting in extremely supersaturated solutions conducive to nucleation and development of the required nanocrystalline products [10]. Ultrasound is a sound wave having a frequency greater than 20 kHz. Acoustic cavitation and acoustic streaming are the two fundamental phenomena of every ultrasonic process. Ultrasound waves are made up of compression and expansion cycles. When an ultrasonic wave passes through a liquid, it exerts positive pressure during the compression cycle and negative pressure during the expansion cycle. During this period of negative pressure, molecules are drawn apart from one another. As a result, cavities occur in the liquid. These holes form and expand throughout several cycles. Finally, the cavities are imploded [10, 11]. As a result, combining these two extraction procedures might be a beneficial tool for improving cannabinoid extract.

Therefore, the main objective of this study was to optimize these two extraction methods using a response surface methodology, in order to enhance CBD and THC contents from *Cannabis indica* L. (Blueberry cultivar), a hybrid cultivar of a Purple Thai, Highland Thai, and Afghani [12].

## 2. Materials and Methods

### 2.1 Raw material and preparation

Dried *Cannabis indica* L. (Blueberry cultivar) were manually collected in May 2023 at Chiangmai, Thailand. The decarboxylation of the cannabis was performed in an oven at 150°C for 10 min [13]. Then, the samples were ground into fine powder and stored in a bag under vacuum in a cool and dry place until use.

### 2.2. Experimental Design

In this study, central composite design (CCD) was used for optimization of MAE and UAE of cannabinoids extraction from Blueberry cultivar. The design consisted of 16 randomized runs with two replicates at the central point, as presented in Table 1. The variables in the designed experiment were defined as $X_1$, A: solid to liquid ratio (1:10-1:30), $X_2$, B: microwave extraction time (5-20 min), and $X_3$, C: ultrasound extraction time (10-30 min).

**Table 1**. Central composite design of microwave-ultrasound assisted extraction (MUAE).

| No. | Factors | | |
|---|---|---|---|
| | Ratio ($X_1$, A) | Microwave time ($X_2$, B) | Ultrasonic time ($X_3$, C) |
| 1 | 30 | 5 | 10 |
| 2 | 30 | 5 | 30 |
| 3 | 30 | 10 | 10 |
| 4 | 30 | 10 | 30 |
| 5 | 20 | 7.5 | 10 |
| 6 | 20 | 7.5 | 30 |
| 7 | 40 | 7.5 | 10 |
| 8 | 40 | 7.5 | 30 |
| 9 | 20 | 12.5 | 3.2 |
| 10 | 20 | 12.5 | 36.8 |
| 11 | 20 | 0 | 20 |
| 12 | 20 | 25.1 | 20 |
| 13 | 3.2 | 12.5 | 20 |
| 14 | 36.8 | 12.5 | 20 |
| 15 | 20 | 12.5 | 20 |
| 16 | 20 | 12.5 | 20 |

### 2.3. Extraction procedure

5 g of ground *C. indica* L. (Blueberry cultivar) was weight and inserted into a microwave machine tubes. Ethanol was used as an extraction solvent. The ratio of *C. indica* L. and ethanol was used according to the experimental design (Table 1). Firstly, extractions were performed in a microwave digestion system (ETHOS UP, Milestone srl, Bergamo, Italy). The temperature and power were set at 60°C and 1000 W, respectively [(6)]. After conditions selected were reached, samples were transferred to ultrasonic extraction. Second, ultrasonic extractions were performed using an ultrasonic bath system (GT SONIC-D20, GT SONIC Technology Park, Guangdong, China) with a fixed frequency at 40 kHz and set the extraction temperature at 60°C [(6)]. Following the extraction, samples were centrifuged (5804 R centrifuge, Eppendorf, Hamburg, Germany) at 5,000 rpm for 5 min and then transferred into vials for further analysis.

### 2.4. Liquid chromatography analysis of cannabinoids content

The samples from the experimental design were filtered using a 0.45 µm nylon filter (Part No. 5191-5909, Agilent Technologies, USA) before injection into an ultra-high performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) (PerkinElmer, MA, USA). The contents of CBD and THC was determined using Quasar SPP C18 column (100 mm × 2.1 mm, 2.6 µm-PerkinElmer, Buckinghamshire, UK) at 30°C. Flow rate was set at 0.2 mL/minute and injection volume of 3 µL. The modification gradient program for mobile phase A (0.1% formic acid in a water) and mobile phase B (0.1% formic acid in a methanol) was adjusted at 0-18 min, 90% B;18.0-19.8-min, 95% B; and 19.8-20.0 min, 75% B [(3)].

### 2.5. Statistical analysis

All experimental values are done in triplicates and presented by mean ± standard derivation. The experimental data were fitted to the second-order polynomial model (Equation (1)) to obtain the regression coefficients (b) using Design-Expert software version 13 (Stat-Ease Inc., Minneapolis, MN, USA). The generalized second-order polynomial model used in the response surface analysis was the following:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 \quad (1)$$

where Y is the dependent variable (response variable) to be modelled, $\beta_0$ is a constant coefficient (intercept); $\beta_1$, $\beta_2$, and $\beta_3$ are the coefficients of the linear, quadratic, and interactive terms, respectively; $X_1$, $X_2$, and $X_3$ are the independent variables. Parametric estimation responses were collected in the form of CBD and THC contents.

## 3. Results & Discussion

### 3.1 Cannabinoids identification

Figure 1 presents the peak characteristics present in the ethanolic extracts of *C. indica* L. (Blueberry cultivar). The main cannabinoids of Blueberry cultivar are THC at a retention time of 12.70 min. Backes [14] revealed that the Blueberry variety had approximately 14-16% THC. Whereas CBD are presented at the retention time of 7.64 min.

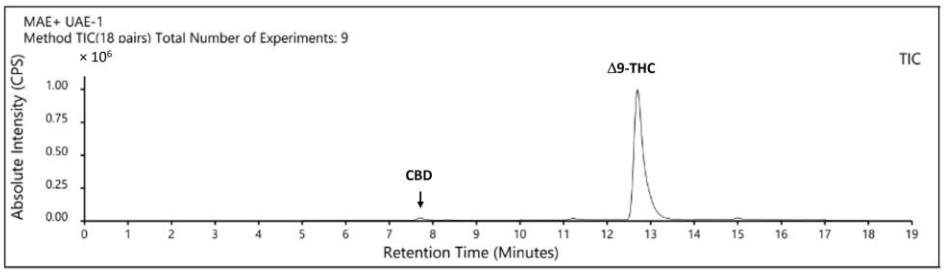


**Figure 1.** UHPLC chromatogram of *C. indica* L. (Blueberry cultivar) extracted by Microwave-ultrasound assisted extraction (MUAE).

### 3.2. CCD optimization for cannabinoids extraction

In this study, the CCD was used in the screening experiment to determine the most important factor that produced a higher content of CBD and THC. The experimental and predicted CBD and THC contents are shown in Table 2, while adequacy and fitness were evaluated by ANOVA (Table 3). By using multiple regression analysis, the polynomial model for an empirical relationship between the content of CBD and THC and test variables is expressed in a code unit by Eq. (2) and Eq. (3), respectively.

Based on Table 2, the highest concentration of THC was obtained at 1:40 of ratio, 7.5 min of MAE, and 30 minutes of UAE, run no. 8, which yielded 105.460 ± 5.433 mg/g of THC. Thus, a higher ratio had a positive effect on the content of THC. However, the results indicate that a higher CBD content (0.350 ± 0.018 mg/g) was obtained at 1:20 of ratio, 0 minutes of MAE, and 20 min of UAE, run no. 11. This indicated that MAE had a negative effect on the CBD content. This could be proved that CBD is converted into THC under high temperature [15].

**Table 2.** Experimental and predicted values of CBD and THC contents.

| No. | CBD content (mg/g) | | THC content (mg/g) | |
|---|---|---|---|---|
| | Predicted | Experimental[a] | Predicted | Experimental |
| 1 | 0.147 | 0.143 ± 0.080 | 72.29 | 74.528 ± 0.885 |
| 2 | 0.206 | 0.193 ± 0.037 | 91.04 | 90.476 ± 5.322 |
| 3 | 0.201 | 0.217 ± 0.018 | 71.61 | 74.047 ± 7.934 |
| 4 | 0.156 | 0.154 ± 0.004 | 82.52 | 82.581 ± 7.064 |
| 5 | 0.128 | 0.118 ± 0.073 | 51.08 | 53.619 ± 1.086 |
| 6 | 0.152 | 0.107 ± 0.019 | 93.52 | 93.680 ± 3.031 |
| 7 | 0.150 | 0.150 ± 0.013 | 70.50 | 73.656 ± 2.659 |
| 8 | 0.054 | 0.045 ± 0.017 | 105.11 | 105.460 ± 5.433 |
| 9 | 0.043 | 0.036 ± 0.010 | 52.01 | 47.085 ± 0.070 |
| 10 | 0.012 | 0.038 ± 0.039 | 96.84 | 98.087 ± 2.675 |
| 11 | 0.322 | 0.350 ± 0.018 | 81.00 | 79.632 ± 4.622 |
| 12 | 0.301 | 0.293 ± 0.018 | 90.09 | 87.758 ± 1.581 |
| 13 | 0.152 | 0.148 ± 0.119 | 79.97 | 78.734 ± 0.608 |
| 14 | 0.051 | 0.074 ± 0.059 | 81.13 | 78.682 ± 0.939 |
| 15 | 0.147 | 0.138 ± 0.011 | 87.38 | 86.546 ± 1.656 |
| 16 | 0.147 | 0.153 ± 0.021 | 87.38 | 88.888 ± 0.379 |

[a]The experimental values expressed as mean ± standard deviation (n=3).

The ANOVA results for the effect of parameters on CBD and THC contents (Table 3) demonstrated that the model was highly significant ($p$ = 0.0009 and $p$ = 0.0002). The value of determinations coefficient ($R^2$) was 0.9664 for CBD and 0.9786 for THC, indicating a good agreement between experimental and predicted values which can explain 96.64% and 97.86% variability of the responses. The lack-of-fit measured the failure of the model to represent data in the experimental domain at points which are not included in the regression. In this study, C, AB, $A^2$, and $B^2$ are significant model terms for CBD, and A, B, AC, BC, and $A^2$ are significant model terms for THC.

**Table 3.** Statistical analysis (ANOVA) of the central composite design in term of $p$-values.

| Source | $p$-value | |
|---|---|---|
| | CBD | THC |
| Model | 0.0009 | 0.0002 |
| A | 0.2220 | < 0.0001 |
| B | 0.3471 | 0.0269 |
| C | 0.0043 | 0.7253 |
| AB | 0.0273 | 0.1597 |
| AC | 0.1985 | 0.0028 |
| BC | 0.3984 | 0.0062 |
| $A^2$ | 0.0021 | 0.0068 |
| $B^2$ | 0.0004 | 0.5836 |
| $C^2$ | 0.0948 | 0.0769 |
| Lack of Fit | 0.2891 | 0.3261 |
| $R^2$ | 0.9664 | 0.9786 |
| Adjusted $R^2$ | 0.9168 | 0.9464 |

A full quadratic regression model represents the predicted CBD (Eq. 2) and THC (Eq. 3) of *C. indica* L. (Blueberry cultivar) (Y): solid to liquid ratio (A), microwave extraction time (B), and ultrasound extraction time (C). The model equation 2 in terms of coded factors shows that the positive coefficients for CBD are A, C, and $B^2$, which are the high levels of the factors, and the negative coefficients are B, AB, AC, BC, $A^2$, and $C^2$, the interaction of AB, AC, BC are the low levels of the factors. While the positive coefficients for THC are A, and AC.

$$\begin{aligned} CBD = {} & +0.002018 + 0.022899A - 0.017999B + 0.007410C \\ & - 0.000343AB - 0.000128AC - 0.000107BC - 0.000424A^2 \\ & + 0.001043B^2 - 0.000163C^2 \end{aligned} \quad (2)$$

$$\begin{aligned} \Delta 9\text{-}THC = {} & +59.49908 + 2.31180A - 0.160096B - 1.01927C \\ & - 0.026115AB + 0.059229AC + 0.066988BC \\ & - 0.045897A^2 - 0.011760B^2 - 0.024206C^2 \end{aligned} \quad (3)$$

Figure 1 and 2 illustrated 2D surface plots for the CBD and THC contents, respectively, as a function of solid to liquid ratio and microwave time (A), solid to liquid ratio and ultrasound time (B), and microwave time and ultrasound time (C). Based on the response 2D surface plot, it was observed that the CBD concentration in the extracted *C. indica* L. (Blueberry cultivar) increased with an increase in solid to liquid ratio but decreased with an increase in microwave extraction time (Figure 1).

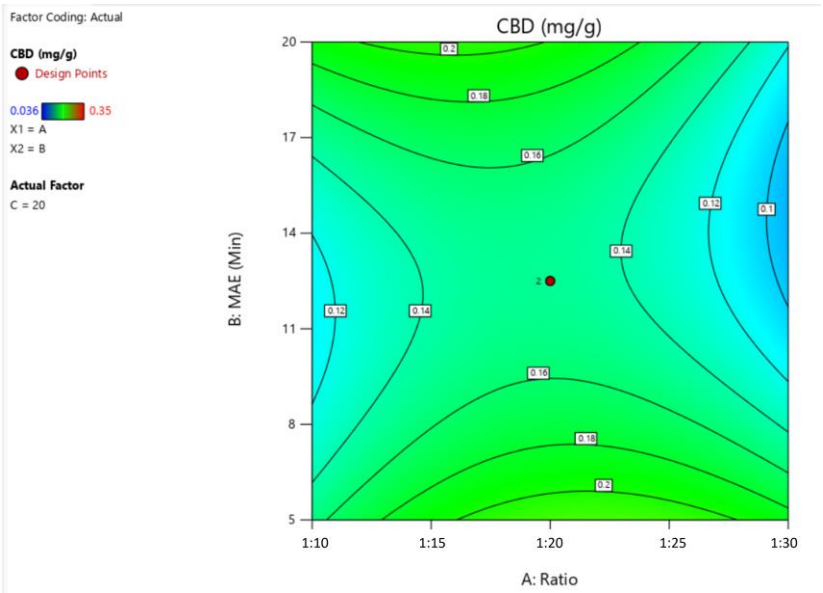


**Figure 1**. 2D surface presented the correlation of factor AB on the CBD extracted by combined MAE and UAE.

While the THC content increased with incresing the solid-to-liquid ratio (Figure 2A) and ultrasonic extraction time (Fiure 2B). Increased liquid-to-solid ratio increases the quantity of solvent diffusing into plant matrix in both microwave and ultrasonic systems, resulting in increased cannabinoid concentration [6]. Change in ethaonol concentration (polarity) affected to the density, extractability, iscosity, and dielectric constant, which resulted in increased the extraction efficiency [16]. Cheng *et al.* [17] reviewed that the polar compounds colud be extracted at lower temperatures and less polar ingredients were extracted at higher temperatures. CBD had a higher polarity than THC [18]. Therefore, the increase of solid-to-liquid ratio, microwave extraction time and ultrasonic extraction time could increase the THC content.

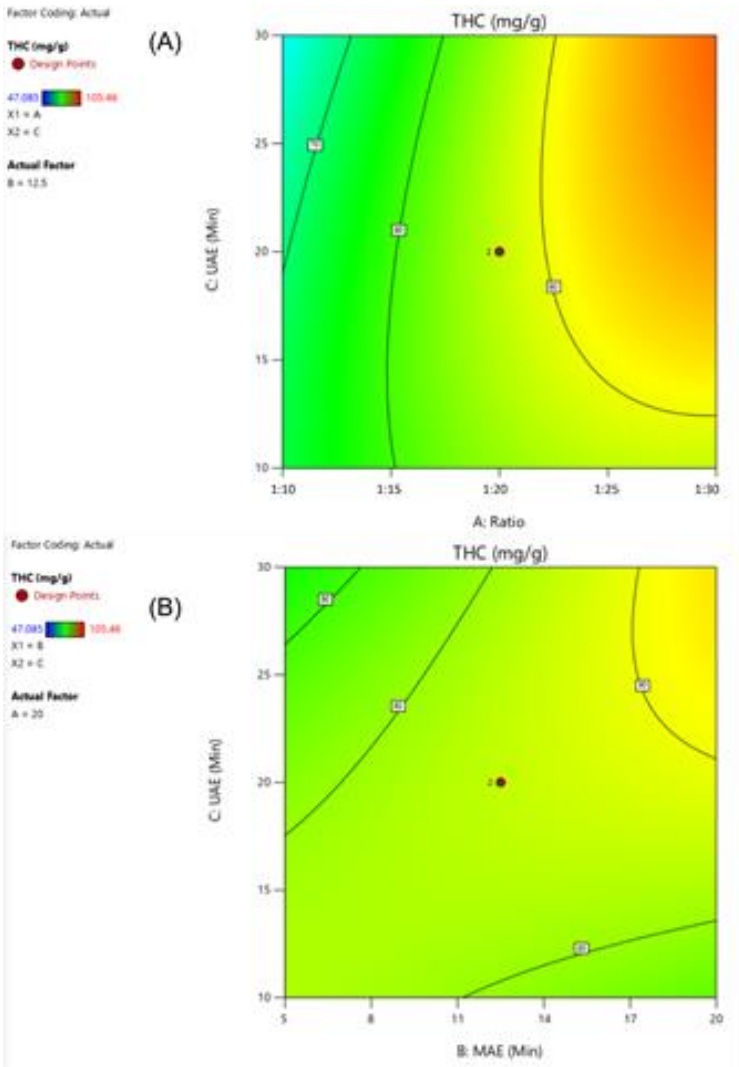


**Figure 2.** 2D surface presented the correlation of factor AB (A), AC (B), and BC (C) on the THC extracted by MAE and UAE.

By solving the inverse matrix (from Eq. (2) and Eq. (3)), the optimal values of the variables affecting the extraction of CBD and THC given by the software were solid to liquid ratio 1:22.54, microwave extraction time 5 min, and ultrasound extraction time of 14.31 minutes, with a desirability of 0.774. In view of the operating convenience, the optimal extraction parameters were determined to be solid to liquid ratio 1:22, microwave extraction time 5 minutes, and ultrasound extraction time of 14 minutes, while the predicted values of CBD and THC were 0.224 mg/g dry weight and 88.60 mg/g dry weight, respectively. Triplicate experiments were performed under the determined conditions and the concentration of CBD and THC (0.298 ± 0.001 mg/g dry weight and 91.35 ± 0.35 mg/g dry weight) was in agreement with the predicted value, indicating that the model was adequate for cannabinoid extraction process.

## 4. Conclusion

Microwave and ultrasound are both typical extraction procedures utilized for the recovery of bioactive substances from plants. In this study, the optimal microwave-ultrasound assisted extraction (MUAE) processing parameters were determined by response surface methodology with central composite design (CCD). The highest concentrations of CBD (0.298 ± 0.001 mg/g dry weight) and THC (91.35 ± 0.35 mg/g dry weight) were produced at the optimized solid to liquid ratio of 1:22, microwave extraction time of 5 minutes, and ultrasound extraction time of 14 minutes. Three factors of solid to liquid ratio, microwave extraction time, and ultrasound extraction time have significant impacts for both positive and negative effects on the CBD and THC recovery. Thus, the present study provides a green extraction process to produce CBD and THC of *C. indica* L. (Blueberry cultivar).

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# Effect of Enzymatic Hydrolysis and Hydrolysis Time on Functional Properties of Rice Bran Protein Hydrolysate

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## Abstract

Rice bran is a by-product from the rice milling industry. It is an interesting protein source because of its nutritional values, especially protein. The aim of this study is to investigate the types of enzymes (alcalase or flavourzyme) and hydrolysis time (0, 30, 60, 90 or 120 min) on emulsion and foaming properties of resulting rice bran protein hydrolysate. Rice bran was defatted before protein extraction. For the enzymatic hydrolysis, the rice bran protein mixture (1000 mg/mL) was adjusted temperature and pH to 55°C, pH 8 for alcalase or 50°C, pH 6 for flavourzyme. The hydrolysis was performed for 0, 30, 60, 90 or 120 min, then the appearance, degree of hydrolysis, emulsion and foaming properties of the hydrolysate were determined. The results showed that the darker color and degree of hydrolysis (DH) of the hydrolysate prepared by alcalase was higher than those of flavourzyme comparing in the same hydrolysis time ($P<0.05$). The highest DH value was noticeable for alcalase hydrolysis at 120 min (56.40%). Enzymatic hydrolysis improved the emulsifying properties when compared to control (without enzyme). Emulsion activity index of the hydrolysate prepared from both enzymes was significantly highest at 30 min ($P<0.05$) (alcalase = 77.71 $m^2/g$ and flavourzyme = 63.39 $m^2/g$). The highest emulsion stability index was significantly found at 90 min that hydrolyzed by alcalase (65.84 min). The hydrolysate from both alcalase and flavourzyme at 120 min showed the highest foam expansion (12.67%, 23.33%) and foam stability (120%, 119.17%), respectively. This finding showed that functional properties of protein extraction from rice bran could be improved by enzymatic hydrolysis and the optimal hydrolysis time should be considered further depending on the properties of food products.

***Keywords:*** Rice bran protein; Enzymatic hydrolysis; Alcalase; Flavourzyme; Hydrolysis time; Functional properties.

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## 1. Introduction

Recently, the demand for cheap protein sources has been growing. Plant-based protein sources are interesting as they provide good alternatives with health benefits with high bioactive compounds and lower cholesterol compared to meat based protein [(1)]. Rice bran is a by-product of the rice milling process. It is cheap and generally used in low-value products such as animal feed and fertilizer. Thailand is the world's largest producer and exporter of jasmine rice, also known as Khao Dok Mali 105 cultivar [(2)]. Rice bran is an interesting protein source as it contains 12-20% protein [(3)]. It is also a source of dietary fiber, vitamins B, vitamin E, vitamins, and antioxidants [(4)]. However, the use of rice bran protein in food products is not widespread because the functional properties of rice bran protein are not yet accepted by consumers. The use of enzymes to hydrolyze proteins is another approach to improving the functional properties of plant proteins [(5)], and it is a method that has received widespread attention and research. Since enzymes are specific to the substrate. Therefore, the type of enzyme and the hydrolysis conditions can be selected as appropriate, and there will be

no undesirable side effects in the product [6]. Commercial protease enzymes used for enzymatic hydrolysis of proteins originate from animals, plants, or microbial sources, the latter is safer, more efficient, and less expensive [7]. Alcalase is an endopeptidase enzyme that breaks peptide bonds from C-terminal amino acids and it has the highest efficacy, resulting in the highest antioxidant activity. Flavourzyme is a mixture of endo- and exo-peptidase enzyme that breaks the N-terminal of peptide chains within and at the ends of protein molecule [8]. Enzymatic hydrolysis by protease changes the structure and size of proteins and peptides by turning them into a short-chain peptide or amino acid. Thus, increasing the alpha-amino fraction and the ε-$NH_2$ group. Enzymatic hydrolysis can improve the functional properties of emulsifiers and stability of the emulsion [9]. Enzymatic hydrolysis is an efficient and non-toxic method to increase protein solubility and emulsification [10]. The functional properties of protein hydrolysates depend on many factors *e.g.* type and size of the protein, type of enzyme, digestion time, size, number, and type of amino acids. These are important factors affecting the functional properties of proteins such as solubility, gelling, emulsification, and foaming [6]. Therefore, the type of enzyme and hydrolysis time on functional properties of rice bran protein hydrolysate were investigated in this study. This finding has potential to improve the efficiency and functional properties of protein extraction from rice bran for further use in food products.

## 2. Materials and Methods

### 2.1 Materials

Rice bran (RB) was obtained from Medifoods (Thailand) Co., Ltd. (Chaiyaphum, Thailand). RB protein was self-extracted in the laboratory. Food-grade protease enzymes; alcalase and flavourzyme, were obtained from Sigma Aldrich, USA. Chemicals and reagents were analytical grade.

### 2.2 Preparation of defatted RB and RB protein

Defatted RB was prepared from jasmine rice (*Oryza sativa*, variety Khao Dawk Mali 105). RB was defatted twice using hexane: RB to hexane in a ratio of 1:3 w/v. The slurry was stirred at 800 rpm for 30 min, then centrifuged with a refrigerated centrifuge (5920 R, Eppendorf, Protronics Intertrade Co., Ltd., Phatumtani, Thailand) at 8,000 *g* 25°C for 30 min followed by air drying overnight in a fume hood [11]. The 500 g of defatted RB was distributed in 2000 mL of distilled water. The protein solution was dissolved by adjusting pH to 9.5 using 1.0 N NaOH and stirred at 800 rpm for 45 min. The mixture was centrifuged at 10,000 *g* 25°C for 30 min. The supernatant was adjusted to pH to 4.5 using 1.0 N HCl and centrifuged again at 10,500 *g* 25°C for 45 min. The precipitate was RB protein (RBP) [12].

### 2.3 Preparation of RBP hydrolysate

To conduct the enzymatic hydrolysis, 5% of RBP solution was prepared in distilled water. The optimal conditions: pH and temperature, for flavourzyme and alcalase hydrolysis were separately performed before adding the enzymes; pH was adjusted to pH 6 or 8 and temperature was adjusted to 50 or 55°C, respectively. The flavourzyme or alcalase was added into the solution and hydrolyzed for 0, 30, 60, 90 or 120 min. The hydrolysate was inactivated at 85°C for 15 min in the hot bath. Then, it was cooled down to 4°C (Modified from Humiski *et al.* [13]).

### 2.4 Determination of degree of hydrolysis (DH)

The DH was determined by measuring the soluble and total nitrogen of RBP hydrolysate with and without treated by trichloroacetic acid (TCA), respectively [14]. 10 mL of RBP hydrolysate of each hydrolysis time (0, 30, 60, 90 or 120 min) was mixed with 10 mL of 20% TCA, then centrifuged at 10,000 *g* 25°C for 15 min. The dissolved nitrogen in the supernatant and total nitrogen of RBP hydrolysate were determined by a Kjeldahl method (AOAC, 1990). The DH was calculated from the following equation:

$$DH\ (\%) = \frac{\text{soluble nitrogen in TCA solution (mg)}}{\text{Total nitrogen (mg)}} \times 100$$

**2.5 Determination of functional properties of RBP hydrolysate**

*2.5.1 Emulsifying properties*

The emulsifying activity index (EAI) and the emulsion stability index (ESI) of RBP hydrolysate at different hydrolysis times were determined by a turbidity method with some modifications [15]. 6 mL of RBP hydrolysate at different hydrolysis times was mixed with 2 mL of soybean oil. The mixture was homogenized in a mechanical homogenizer (Model T25 basic; IKA Labortecnik, Selangor, Malaysia) at 10,000 rpm for 1 min. The 50 µL of emulsion was pipetted from the middle portion of the container at 0 and 10 min after homogenization and subsequently diluted 100-fold with a 0.1% sodium dodecyl sulfate solution. The mixture was mixed for 10 sec using a vortex mixer (G-560E, Vortex-Genie 2, Scientific Industries, Inc., Bohemia, NY). The absorbance of emulsion was measured at 500 nm with a UV–VIS spectrophotometer (UV-1800, Shimadzu, Kyoto, Japan,). EAI and ESI were calculated from the following equation:

$$EAI\ (m^2/g) = (2 \times 2.303 \times A \times DF) / l\text{Ø}C$$

Where A = absorbance of emulsions at 500 nm, DF = dilution factor, l = path length of cuvette (m), Ø = oil volume fraction and C = protein concentration in aqueous phase ($g/m^3$).

$$ESI\ (min) = A_0 \times \Delta t/\Delta A$$

Where $\Delta A = A_0 - A_{10}$ ( $A_0$ = absorbance of emulsions at 500 nm for 0 min and $A_{10}$ = absorbance of emulsions at 500 nm for 10 min) and $\Delta t$ = 10 min.

*2.5.2 Foaming properties*

Foam expansion (FE) and foam stability (FS) of RBP hydrolysate at different hydrolysis times were determined using the method described by Shahidi *et al* [16]. The 20 mL of RBP hydrolysate at 0, 30, 60, 90 or 120 min was transferred into a 100 mL cylinder. The solution was homogenized at 13,400 rpm for 1 min at room temperature. The RBP hydrolysate was allowed to stand for 0 and 60 min. FE and FS were calculated from the following equation:

$$FE\ (\%) = (V_T/V_0) \times 100$$
$$FS\ (\%) = (V_t/V_0) \times 100$$

Where $V_T$ is total volume after whipping, $V_0$ is the original volume before whipping, and $V_t$ is total volume after leaving at room temperature for 60 min.

**2.6 Statistical analysis**

All measurements are performed in triplicate. One-way analysis of variance (ANOVA) and Duncan's multiple range comparison test (SPSS 28.0, IBM Inc., New York, USA) were conducted to evaluate the significant differences between samples at $P<0.05$.

## 3. Results & Discussion

### 3.1 Degree of hydrolysis

The appearance of RBP hydrolysate with different enzymes and hydrolysis times are shown in Figure 1A and 1B, respectively. The RBP hydrolysate prepared by alcalase showed darker color than those of flavourzyme. This might be due to the smaller size of peptides and amino acids of alcalase hydrolysis confirmed by the significantly higher DH value of the hydrolysate of alcalase than flavourzyme in Figure 2. The higher amount of α-free amino acid and smaller size of peptides via the hydrolysate of alcalase could act as substrate for the Maillard reaction that increased the browner or darker color from the Melanoidin or interact with carbonyl group from lipid oxidation [17]. However, longer hydrolysis time did not significantly affect the color of hydrolysate but obviously decreased viscosity of the hydrolysate. The increasing of hydrolysis time indicated that more peptide bonds are cleaved giving amino acids and short chain peptides during hydrolysis. According to this reason, the viscosity of RBP hydrolysate prepared by alcalase was lower than those of flavourzyme.

The DH of RBP hydrolysate with different enzymes and hydrolysis times are shown in Figure 2. The DH represents the number of peptide bonds that break during hydrolysis. It is used to describe the hydrolysis efficiency of proteases [18]. The DH value of RBP hydrolysate significantly increased as increased the hydrolysis time of both alcalase and flavourzyme. The duration of enzymatic hydrolysis affects the size of protein molecules and shortens the peptide chain when increases the DH [6]. The highest DH value was significantly detected at 120 min of RBP hydrolysate prepared by alcalase (56.40%) ($P<0.05$). The DH of RBP hydrolysate from alcalase and flavourzyme ranged from 2.17 to 56.40% and 2.65 to 17.53%, respectively. The hydrolysate from flavourzyme possessed a lower DH than those of alcalase across all hydrolysis times. The higher DH of alcalase hydrolysate could be supported by the higher maximum velocity ($V_{max}$) of alcalase (0.0523 mM/min) than those of flavourzyme (0.0357 mM/min) [19-20].

Generally, alcalase has a very broad specificity to break peptides. Alcalase is an endopeptidase that breaks peptide bonds within the protein molecules, while flavourzyme is a mixture of exo- and endopeptidase that cleaves the peptide bonds within and at the ends of protein molecules [8]. These DH values confirmed that cleavage protein inside the molecule of alcalase was faster than those of flavourzyme.

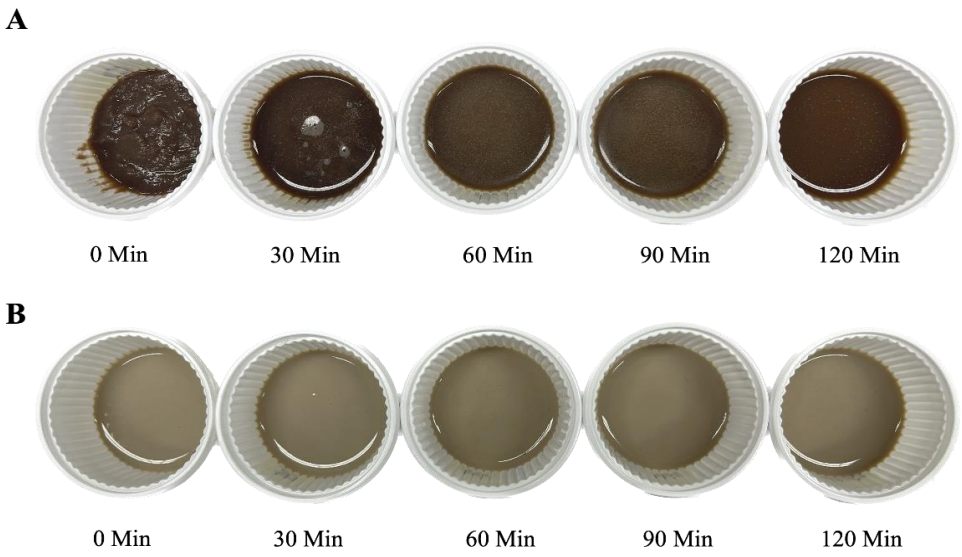


**Figure 1**. The appearance of RBP hydrolysate with different enzymes and hydrolysis times: Alcalase (A) and flavourzyme (B)

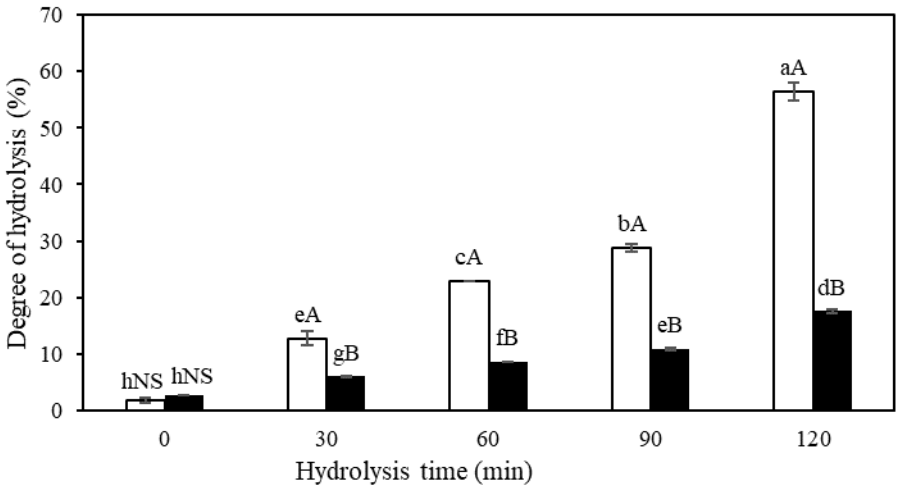


**Figure 2**. Degree of hydrolysis of RBP hydrolysate at different hydrolysis time using alcalase (□) and flavourzyme (■).
[a-h] Different small letters indicate significant difference between difference hydrolysis time (P<0.05).
[A-B] Different capital letters indicate significant difference between enzymes (P<0.05).
[NS] Not significant difference between enzymes (P<0.05).

### 3.2 Emulsifying properties

Emulsion activity index (EAI) and emulsion stability index (ESI) of RBP hydrolysate with different enzymes and hydrolysis times are shown in Figure 3A and 3B, respectively. The highest EAI was significantly found at 30 min for both RBP hydrolysate that hydrolyzed by alcalase (77.71 $m^2/g$) and flavourzyme (63.39 $m^2/g$). Enzymatic hydrolysis of both alcalase and flavourzyme had higher EAI than the control (P<0.05); however, the increasing of hydrolysis time did not significantly increase the EAI of the hydrolysate (P≥0.05).

This was due to the progress of hydrolysis, protein turned into short chain peptides and free amino acids, which related to the increasing of DH value as increasing the hydrolysis time. These increased the flexibility of the molecules and makes the arrangement at the oil-water interface more orderly. The emulsifying activity was related to the exposure of hydrophobic groups inside the molecule, and proteins with high hydrophobicity have higher emulsifying activity [(21)]. Kato and Nakai [(22)] also reported that the more hydrophobic groups, the lower the interfacial tension of the protein led to the stronger emulsifying ability. Moreover, RBP composed of both hydrophobic and hydrophilic amino acids and the proportion of hydrophilic amino acids were higher than those of hydrophilic amino acids [(23)].

Enzymatic hydrolysis of both alcalase and flavourzyme also had higher ESI than the control (P<0.05). The highest ESI was significantly found at 90 min of the RBP hydrolysate that hydrolyzed by alcalase (65.84 min); however, there was not significantly different for 60 and 120 min (P≥0.05). The longer hydrolysis time resulted in the loss of emulsifying properties because of lower molecular weight of peptides and amphiphilicity. Generally, high amphiphilicity of proteins is responsible for good emulsion system. The lower amphiphilicity makes it less likely to form a protective film around the oil droplets [(24)]. Thus, excessive hydrolysis greatly reduces the emulsifying properties of proteins. Moreover, the small deamidation improves emulsifying activity and emulsion stability [(21)].

The RBP hydrolysate prepared by alcalase hydrolysis showed higher EAI and ESI than those of flavourzyme (P<0.05). This because alcalase is endoproteases. It attack peptide bonds within the polypeptide chain which is quicker hydrolyzed than flavourzyme that is the mixture of endo- and exoprotease [(25)].

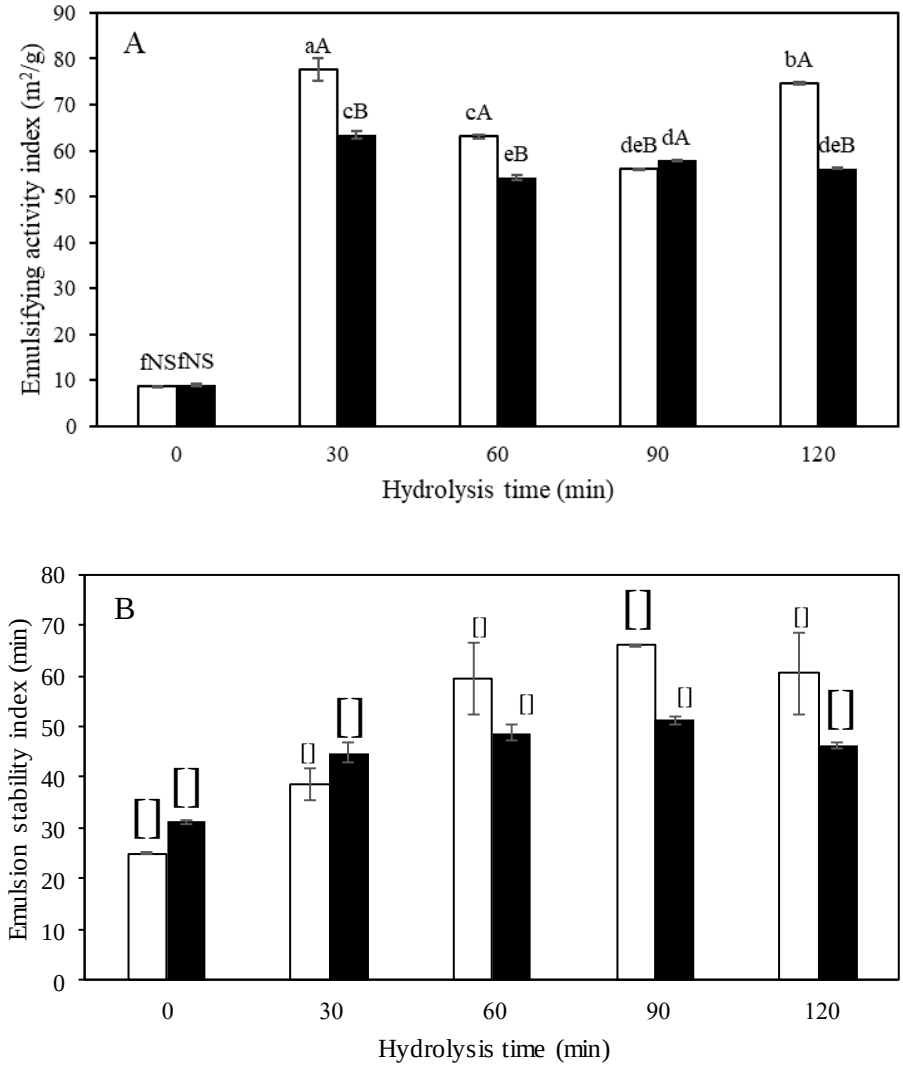


**Figure 3**. Emulsion activity index (A) and emulsion stability index (B) of RBP hydrolysate at different hydrolysis time using alcalase (□) and flavourzyme (■).
[a-g] Different small letters indicate significant difference between difference hydrolysis time ($P<0.05$).
[A-B] Different capital letters indicate significant difference between enzymes ($P<0.05$)

### 3.3 Foaming properties

Foaming properties are physicochemical characteristics of proteins to form and stabilize foams [(26)]. Foam expansion (FE) and foam stability (FS) of RBP hydrolysate with different enzymes and hydrolysis times are shown in Figure 4A and 4B, respectively. The highest FE and FS were significantly detected at 120 min of RBP hydrolysate prepared by alcalase (12.67%, 120%) and flavourzyme (23.33%, 119.17%). As a result of high surface hydrophobicity of RBP hydrolysate, there may be more hydrophobic groups at the air-water interface, which form an intermolecular polymer and a gel like layers that could prevent the collapse of bubbles [(27)]. The increasing in hydrolysis time tended to increase both FE and FS because short-chain peptides, from enzymatic hydrolysis, could bind more air bubbles denser, resulting in a stronger film surrounding the air bubbles and increased foam stability 90 [(28)]. Moreover, the smaller peptide move rapidly to the air-liquid interface and protein unfold to rearrange at the interface [(29)], resulting in good foaming properties. It has high potential to apply of RBP in food products depending on the property of food products. RBP was used as an ingredient in the production of plant meat, beverages, and biofilms [(30)]. Moreover, it could

be used as a binder in sausages or beverage [31]. From this study, RPB could be utilized in the products that require emulsion and foaming properties such as milk shake, whipping cream or ice-cream.

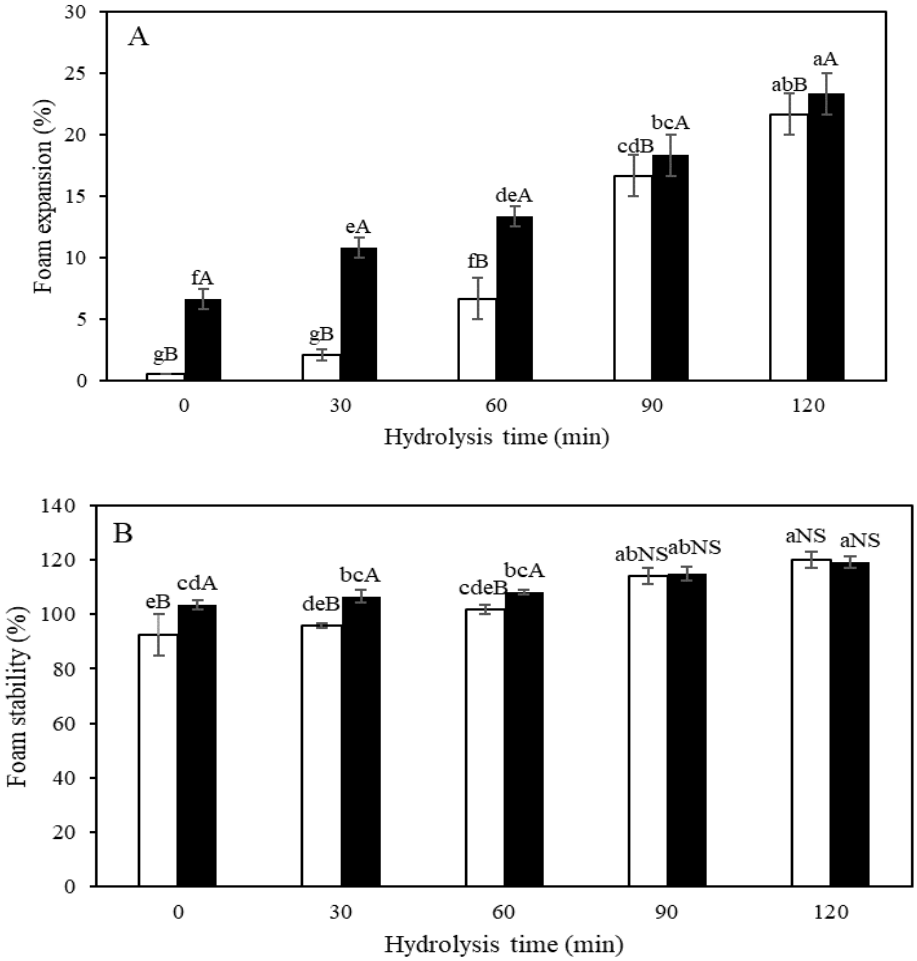


**Figure 4**. Foam expansion (A) and foam stability (B) of RBP hydrolysate at different hydrolysis time using alcalase (□) and flavourzyme (■)
[a-g] Different small letters indicate significant difference between difference hydrolysis time (P< 0.05).
[A-B] Different capital letters indicate significant difference between enzymes (P<0.05).
[NS] Not significant difference between enzymes (P<0.05).

## 4. Conclusion

Rice bran protein was hydrolyzed by alcalase and flavourzyme with different hydrolysis time. The degree of hydrolysis increased as increasing hydrolysis time. The highest degree of hydrolysis was significantly detected at 120 min of the hydrolysate prepared by alcalase (56.40) (P<0.05). The degree of hydrolysis of the hydrolysate from alcalase was higher than those of flavourzyme (P<0.05). Enzymatic hydrolysis improved the emulsifying properties when compared to control (without enzyme). The highest emulsion activity index was significantly found at 30 min for both enzymes (alcalase = 77.71$m^2$/g and flavourzyme = 63.39 $m^2$ / g). The hydrolysate from alcalase and flavourzyme had higher emulsion stability index than the control (P<0.05) and the highest emulsion stability index was significantly found at 90 min that hydrolyzed by alcalase (65.84 min). The highest foam expansion and foam stability were significantly detected at 120 min of RBP hydrolysate prepared by alcalase (12.67%, 120%) and flavourzyme (23.33%, 119.17%). This finding

showed that functional properties of protein extraction from rice bran could be improved by enzymatic hydrolysis.

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# Comparative Study on Nutritional Values and Amino Acid Profile, Physical and Functional Properties of Different Sources of Protein Powder

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## Abstract

Whey protein isolate (WPI) is known for being a highly digestible animal protein. However, the global shift toward plant-based diets is driven by increased awareness of the impact of food choices on health, environment, and animal welfare. Soy protein isolate (SPI), a dominant plant protein source, is rich in phytochemicals and widely used. However, interest is growing in alternative sources such as pea protein isolates (PPI) and sunflower protein concentrates (SfPC), known for their hypoallergenic properties. This research aimed to examine and compare the nutritional values and amino acid profile, physical and functional properties of SPI, PPI, WPI and SfPC. The results showed that WPI contained the highest protein content (84.21 g/100 g) followed by SPI (81.88 g/100g), PPI (74.04 g/100g), and SfPC (56.65 g/100g), respectively. However, the highest content of total fat, carbohydrate, ash was observed in SfPC. Leucine (6.12-10.62 g/100 g) and glutamic acid (11.02-13.62 g/100 g) were the major amino acids in all protein powder samples. SPI, PPI, WPI and SfPC showed a higher level of valine, isoleucine, and leucine than FAO reference pattern on essential amino acid for adults except for a slightly lower level of leucine in PPI. WPI also had higher lysine and threonine than FAO reference pattern. For the physical properties, water activity and pH ranged of 0.13-0.49 and 6.07-7.27, respectively. WPI was lighter than other samples, while the lowest $L^*$ (lightness) was found in SfPC. Functional properties exhibited that WPI had better water and oil holding capacity and solubility, while PPI had better foaming capacity and foaming stability than other samples. WPI exhibited the highest solubility except at 95°C while SPI solubility increased with temperature and highest at 95°C. SfPC maintained consistent solubility across temperatures while PPI demonstrated the lowest solubility with slight variations as temperatures increased. Overall, this study might help processors select the appropriate protein source for their target application in food products.

***Keywords:*** Soy protein isolate; Pea protein isolate; Whey protein isolate; Sunflower protein concentrate

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## 1. Introduction

The global demand for plant-based products is rising due to increased public awareness of the impact of food choices on human health, animal welfare, environmental sustainability, and a growing number of flexitarians, vegetarians, and vegans [(1-2)]. Although it is currently popular in Western countries, there is also a growing interest in Asian countries, making it a promising market in the near future [(2)]. Krungthai compass research center expects the annual Thai Plant-Based market growth from the current 2-10% per year to 10-35% per year, expected to reach US$ 1.5 billion by 2024 [(3)].

Soybean is an extraordinary source of high-quality plant protein that offers not just technological benefits for several food applications but also a great source of phytochemicals with positive impact to animal and human health. Soy protein isolate (SPI, ≥ 90% protein) is the most common soy protein commercial ingredient. The main components of SPI are glycinin (300-380 kDa) and β-conglycinin (150-200 kDa), which represent 34% and 27%, respectively [4]. While soybean remains a predominant source of vegetable proteins for nutritional formulations, recent significant interest has emerged in alternative sources such as chickpeas and peas.

Pea is a rich source of hypoallergenic protein and it makes advantage over soybean and milk-abundant proteins [5]

Pea protein isolates (PPI) are cost-effective, nutritionally rich and alternative to soybean in Europe because of their functional properties, and bioactive components [6]. The main proteins in pea are albumins (68.5 kDa) and globulins (15-410 kDa), accounting for 18-25% and 55-65% of the total protein, respectively [7].

Moreover, Whey protein is a highly beneficial animal protein known for its excellent digestibility and high biological value [8]. Whey protein concentrates (WPC) contain 30-80% of protein, while whey protein isolates (WPI) contain > 90% of protein, with a lower concentration of carbohydrates (10-55% in concentrates and less than 1% in isolates) (8, 9). The major protein of WPI are β-lactoglobulin (18.3 kDa), α-lactalbumin (14.1 kDa), immunoglobulin (20-70 kDa) and serum albumin (66.4 kDa), which account for 50%, 25%, 9% and 6%, respectively [10]. However, people also are looking for other sources of protein.

Sunflower protein concentrates (SfPC) are by-products of sunflower oil production. They contain high levels of minerals, phenolic compounds, and antioxidant activity, coupled with low antinutritional compounds. Apart from nutritional benefits, it also demonstrates potential applications in functional foods, acting as a source of proteins with favorable technological properties [11]. The main protein components are albumins (3-10 kDa) and globulins (190-440 kDa) which accounts for 10 to 30% and 40% to 90%, respectively [12].

Considering the increasing interest in plant-based diets and the thriving plant-based food market, food manufacturers have chosen from a wide range of protein ingredients, with a lack information on characterize real world ingredients available to food manufacturers, in terms of variability, functionality, and ways that the protein ingredients affect the final food product. Therefore, this study aimed to examine and compare the nutritional values and amino acid profile, physical and functional properties of commercial protein powder including SPI, PPI, WPI and SfPC. The results from our study could provide a better understanding on the variability of commercial protein powder and how the raw material quality affects the final protein functionalities, and also supporting the proper selection of commercial protein powder for food product development.

## 2. Materials and Methods

### 2.1 Materials

Commercial protein isolates (soy, pea, and whey) were purchased from club protein Co., Ltd., Bangkok, Thailand. Sunflower protein concentrate (Wisamin brand) was obtained from Max Global Marketing Co., Ltd., Bangkok, Thailand. All reagents used in this study were obtained from Sigma-Aldrich (St. Louis, MO, USA).

### 2.2 Methods

*2.2.1 Proximate analysis*

The proximate compositions of samples including moisture, ash, fiber, protein, and fat were analyzed according to Association of Official Analytical Chemists (AOAC) [13]. The calorie content was calculated based on protein, carbohydrate, and fat content.

All samples were determined in the Accredited Laboratory, complying with ISO/IEC 17025:2017, at the Institute of Nutrition, Mahidol University (Nakhon Pathom, Thailand).

*2.2.2 Amino acid profile*

The amino acid profile was determined using high-performance liquid chromatography (HPLC) according to an in-house method TE-CH-372 adapted from the Official Journal of the European Communities, L257/16 [14]. Amino acid profile analyses were performed by the Central Laboratory (Thailand) Co., Ltd., Bangkok, Thailand.

*2.2.3 Physical properties*

Physical properties were analyzed in term of water activity (Aw) using a water activity measurement instrument (model ms1-1M, Novasina, Lachen, Switzerland) and pH using a pH meter (Ohaus Corporation, Morris Country, NJ, USA). Color of samples also were determined using Hunter Lab ColorFlex EZ (Hunter Lab, Reston, VA, U.S.A.). The results were expressed as color values of *L** (lightness=100, darkness=0), a* (+ redness, – greenness) and *b** (+ yellowness, - blueness). Moreover, Bulk density (BD) was analyzed according to Oladele and Aina [15]. The sample (50 g) was placed into a 100 mL measuring cylinder, followed by tapping the cylinder continuously until a constant volume was achieved. The BD was calculated using the following equation:

$$\text{Bulk density (g/cm3)} = \frac{\text{Weight of the sample}}{\text{Volume of the sample after tapping}} \quad (1)$$

*2.2.4 Functional properties*

Water holding capacity (WHC) and oil holding capacity (OHC) were determined using the method described previously [16] with some modification. Sample (1 g) was added in 10 mL of distilled water (or sunflower oil) in a pre-weighed centrifuge tube. The mixture was vortexed for 1 min and centrifuged at 3000 ×g for 30 min at room temperature. The free water (or oil) was poured out and the excess water (or oil) in the upper phase was drained for 10 min. The tube containing the protein residue was weighed again to determine the amount of water (or oil) retained per gram of sample. WHC and OHC was calculated according to the following equations:

$$\text{WAI and OAI (g/g)} = \frac{\text{Weight of the remaining wet sediment sample}}{\text{Original sample weight}} \quad (2)$$

Foaming capacity (FC) and Foaming stability (FS) were also determined according to previous study [16] with some modification. Samples (4 g) were dispersed in distilled water (100 mL). The solution was mixed with vortex for 3 min. Volumes were recorded before (V0) and after (VT) mixing were measured and recorded. The percentage of volume increase was calculated as the FC. Moreover, FS was determined from the volume of the foam after 30 min at room temperature which was recorded as V30. FS was calculated following equation:

$$\text{FC (\%)} = \frac{V_0 - V_r}{V_T} \times 100$$

$$\text{FS (\%)} = \frac{V_{30} - V_T}{V_T} \times 100$$

Solubility of samples were determined according to method of Sopawong *et al.* [17]. Sample (0.6 g) was dispersed in distilled water (30 mL), then heat in a temperature-controlled water bath at 55, 65, 75, 85 and 95°C for 30 min with mixing interval at every 5 min.

The heated samples were cooled to room temperature prior to centrifuge at 4000× g for 20 min. The supernatant was carefully removed, dried for 2 h at 105°C and weighed. Solubility was calculated according to the following equations:

*2.2.5 Statistical analysis*

All samples were performed 3 replicates of each analysis. Data were presented as mean ± SD, and data were analyzed by one-way ANOVA followed by Duncan's multiple-range test using SPSS software version 19 (SPSS Inc., Chicago, IL, USA). A value of $P<0.05$ were considered statistically significant.

## 3. Results & Discussion

### 3.1 Proximate analysis

The proximate compositions of SPI, PPI, WPI and SfPC are shown in Table 1. The range of energy contribution was between 408.27 and 355.54 kcal/100 g. The major component in all protein powders was protein (84.21-56.65 g/100 g). Carbohydrate and fat contents varied between 26.67-3.07 and 8.33-0.28 g/100 g, respectively, and ash content ranged from 5.67-2.83 g/100 g. All samples contain low moisture content. Therefore, they could be less susceptible to attack by microorganisms [18]. Among the four protein powders, WPI contained the highest protein (84.21% of total nutrients), followed by SPI (81.88%), PPI (74.04%) and SfPC (56.65%). These protein values were high and may enhance the use as a good protein source in food formulation [18]. In contrast, SfPC showed higher carbohydrate contents than those powders. Since protein and carbohydrate provide 4 kcal/g and fat provides 9 kcal/g, SfPC with higher carbohydrate and fat contents provide higher energy than other protein powder. In general, the differences in chemical composition could be attributed by the difference protein source and extraction methods [19]. The similar results were reported by Solorio, Aguilar and Toro [20].

**Table 1.** Chemical composition of SPI, PPI, WPI and SfPC (per 100 g protein powder).

| Component | SPI | PPI | WPI | SfPC |
|---|---|---|---|---|
| Energy (kcal) | $358.06 \pm 0.63^{c}$ | $399.71 \pm 0.82^{b}$ | $355.54 \pm 0.86^{d}$ | $408.27 \pm 1.01^{a}$ |
| Moisture (g) | $8.34 \pm 0.1^{a}$ | $6.68 \pm 0.12^{b}$ | $5.78 \pm 0.14^{c}$ | $2.94 \pm 0.03^{d}$ |
| Protein (g) | $81.88 \pm 0.24^{b}$ | $74.04 \pm 0.22^{c}$ | $84.21 \pm 0.17^{a}$ | $56.65 \pm 0.09^{d}$ |
| Total Fat (g) | $2.02 \pm 0.02^{c}$ | $7.55 \pm 0.11^{b}$ | $0.28 \pm 0.01^{d}$ | $8.33 \pm 0.03^{a}$ |
| Total Carb (g) | $3.07 \pm 0.02^{d}$ | $8.88 \pm 0.08^{b}$ | $4.04 \pm 0.04^{c}$ | $26.67 \pm 0.08^{a}$ |
| Ash (g) | $4.67 \pm 0.08^{c}$ | $2.83 \pm 0.08^{d}$ | $5.67 \pm 0.06^{a}$ | $5.40 \pm 0.24^{b}$ |

Values are expressed as mean ± SD. Values with different superscript letters in the same row indicate significant difference at $P<0.05$ using one-way ANOVA followed by Duncan's test.

### 3.2 Amino acid profile

The nutritional values of protein powder were based on the amino acid composition. Table 2 lists amino acid composition for SPI, PPI, WPI and SfPC. Sulphur amino acids (cystine and methionine) were the most limiting amino acid in SPI, PPI and SfPC due to the limiting amino acid in pulses, lentil and horse gram [18]. The different content of amino acid was observed among the different protein powders. The different amino acid compositions of samples could be due to source of protein and the modification of amino acid during the extraction process [19]. Leucine (6.12-10.62 g/100 g) and glutamic acid (11.02-13.62 g/100 g) were the major amino acids in all protein powder samples. The higher levels of valine, isoleucine, and leucine were than those reported in FAO reference pattern on essential amino acid for adults were found in all samples except for a slightly lower level of leucine in PPI. SPI and SfPC had higher levels of histidine than those reported in FAO reference pattern on essential amino acid for adults while PPI and WPI had lower levels than the reference pattern.

However, WPI presented higher levels of lysine and threonine than other protein power and the reference pattern [21]. According to the results, it might be said that the combination of protein powder could help to get adequate nutrients.

**Table 2.** Amino acid composition of SPI, PPI, WPI, and SfPC (g/ 100 g protein powder).

| Amino acid | SPI | PPI | WPI | SfPC | FAO/WHO reference* |
|---|---|---|---|---|---|
| **Essential** | | | | | |
| Methionine | 1.01 ± 0.01$^{b}$ | 0.81 ± 0.01$^{c}$ | 2.02 ± 0.02$^{a}$ | 1.03 ± 0.04$^{b}$ | |
| Valine | 4.02 ± 0.02$^{b}$ | 3.51 ± 0.01$^{b}$ | 5.84 ± 0.05$^{a}$ | 4.03 ± 0.04$^{b}$ | 3.5 |
| Isoleucine | 3.74 ± 0.06$^{c}$ | 3.31 ± 0.01$^{d}$ | 6.02 ± 0.02$^{a}$ | 4.05 ± 0.06$^{b}$ | 2.8 |
| Leucine | 6.63 ± 0.04$^{c}$ | 6.12 ± 0.02$^{d}$ | 10.62 ± 0.02$^{a}$ | 7.07 ± 0.09$^{b}$ | 6.6 |
| Phenylalanine | 4.31 ± 0.01 $^{b}$ | 4.21 ± 0.01 $^{c}$ | 3.02 ± 0.03 $^{d}$ | 5.01 ±0.01$^{a}$ | |
| Histidine | 2.12 ± 0.03 $^{a}$ | 1.61 ± 0.01 $^{b}$ | 1.53 ± 0.04 $^{b}$ | 2.06 ±0.08$^{a}$ | 1.9 |
| Lysine | 5.22 ± 0.02 $^{c}$ | 5.41 ± 0.01 $^{b}$ | 9.04 ± 0.05 $^{a}$ | 5.06 ±0.08$^{d}$ | 5.8 |
| Threonine | 3.21 ± 0.01 $^{b}$ | 2.92 ± 0.02 $^{c}$ | 6.07 ± 0.09 $^{a}$ | 3.05 ±0.06$^{c}$ | 3.4 |
| **Nonessential** | | | | | |
| Cystine | 0.72 ± 0.02 $^{c}$ | 0.82 ± 0.02 $^{b}$ | 1.11 ± 0.01 $^{a}$ | 0.52 ± 0.03 $^{d}$ | |
| Tyrosine | 3.31 ± 0.01 $^{b}$ | 3.02 ± 0.03 $^{c}$ | 3.04 ± 0.05 $^{c}$ | 4.03 ±0.04 $^{a}$ | |
| Aspartic acid | 6.02 ± 0.03 $^{a}$ | 4.46 ± 0.04 $^{d}$ | 5.04 ± 0.05 $^{b}$ | 4.60 ± 0.02 $^{c}$ | |
| Proline | 4.44 ± 0.06 $^{a}$ | 3.81 ± 0.01 $^{b}$ | 2.81 ± 0.01 $^{d}$ | 3.04 ± 0.05 $^{c}$ | |
| Serine | 4.04 ± 0.06 $^{a}$ | 3.62 ± 0.02 $^{b}$ | 2.03 ± 0.04 $^{c}$ | 4.03 ± 0.04 $^{a}$ | |
| Glutamic acid | 11.41 ± 0.01$^{b}$ | 13.62 ± 0.03$^{a}$ | 11.05 ± 0.06$^{c}$ | 11.02 ± 0.02$^{c}$ | |
| Glycine | 4.03 ± 0.04 $^{a}$ | 4.03 ± 0.04 $^{a}$ | 1.64 ± 0.05 $^{c}$ | 3.03 ± 0.04 $^{b}$ | |
| Alanine | 4.42 ± 0.02 $^{a}$ | 4.32 ± 0.02 $^{a}$ | 4.03 ± 0.04 $^{b}$ | 4.06 ± 0.08 $^{b}$ | |
| Arginine | 7.21 ± 0.01 $^{c}$ | 7.72 ± 0.02 $^{b}$ | 2.05 ± 0.06 $^{d}$ | 8.07 ± 0.10 $^{a}$ | |

Values are expressed as mean ± SD. Values with different superscript letters in the same row indicate significant difference at P<0.05 using one-way ANOVA followed by Duncan's test. *Essential amino acid for adults according to FAO/WHO recommended pattern.

### 3.3 Physical properties

The physical properties of samples are shown in Table 3. The water activity ($a_w$) of all samples ranged from 0.13-0.49. The lower $a_w$ means a lower chance that bacteria and yeast & mold can grow [22]. The pH values ranged from 6.07-7.27. It seems that protein source impacted the color of all samples [23, 24]. WPI exhibited that highest lightness ($L^*$) values with lowest redness ($a^*$) values. While SPI and PPI had higher yellowness ($b^*$) than WPI and SfPC. Bulk density is associated with powder flowability and storage stability. The bulk density of samples ranged from 0.35-0.63 g/cm3, which was correlated with the size of the powder particles. The higher bulk density is desirable because it signifies reduced packaging, storage, and transport cost [25].

**Table 3.** Physical properties of SPI, PPI, WPI and SfPC.

| Functional properties | SPI | PPI | WPI | SfPC |
|---|---|---|---|---|
| WHC (g/g) | 4.71 ± 0.10 $^{a}$ | 3.43 ± 0.19 $^{b}$ | 4.82 ± 0.10 $^{a}$ | 1.15 ± 0.01 $^{c}$ |
| OHC (g/g) | 0.93 ± 0.03 $^{c}$ | 1.44 ± 0.08 $^{b}$ | 1.88 ± 0.19 $^{a}$ | 0.67 ± 0.09 $^{d}$ |
| Foaming Capacity % | 21.66 ± 2.88 $^{b}$ | 43.33 ± 2.88 $^{a}$ | 15.00 ± 0.00 $^{c}$ | 10.00 ± 0.00 $^{d}$ |
| Foaming Stability % | 5.00 ± 0.00 $^{b}$ | 40.00 ± 5.0 $^{a}$ | 8.33 ± 2.89 $^{b}$ | 5.00 ± 0.00 $^{b}$ |

Each value was represented as mean ± SD (n=3). Different superscript letters in the same row indicate significant differences (P<0.05) among the average values using one-way ANOVA followed by Duncan's test.

### 3.4 Functional properties

Water holding capacity (WHC), oil holding capacity (OHC), foaming capacity (FC), and foaming stability (FS) of samples were determined and showed in Table 4. WHC and OHC are crucial functional properties as it affects a product's texture and flavor binding [26]. WPI and SPI presented a significantly higher WHC than PPI and SfPC. This could be due to their high protein content and consequently a high hydrolysis capacity. On the other hand, OHC of these samples ranged from 0.67-1.88 g/g respectively, which was similar in comparison to flour (0.84-1.4 g/g) [26]. OHC values can be affected by a protein's matrix structure, surface hydrophobicity, the type of lipid present, and the distribution and stability of lipids. It is important to understand the factors that affect OHC to maintain product quality [26]. Shevkani *et al.* [27] reported that sample with higher WHC and OHC is suitable for food products such as breads, cakes, and muffins where hydration and shortening properties are desirable while high OHC sample could be suitable for the cold meat industry where protein can bridge fat and water in the product [27]. However, the SfPC which exhibited poor WHC compared to other protein isolate, could be used in beverage applications where WHC is undesirable [28]. Protein foaming properties, which are influenced by solubility and molecular flexibility, are key indicators in the production of meringue, cakes, and ice cream [29]. It can be seen that PPI exhibited higher FC than SPI, WPI and SfPC. This was likely due to low molecular weight and high Surface hydrophobicity (Ho), a small net charge in terms of the pH of the proteins, therefore encapsulating more air particles due to their rapid diffusion on the air–water surface [6,29]. PPI showed superior foaming properties while SfPC showed less foaming properties compared to other samples. This might be due to a high fat content in SfPC [28].

**Table 4.** Functional properties of the SPI, PPI, WPI, and SfPC.

| Functional properties | SPI | PPI | WPI | SfPC |
|---|---|---|---|---|
| WHC (g/g) | $4.71 \pm 0.10^{a}$ | $3.43 \pm 0.19^{b}$ | $4.82 \pm 0.10^{a}$ | $1.15 \pm 0.01^{c}$ |
| OHC (g/g) | $0.93 \pm 0.03^{c}$ | $1.44 \pm 0.08^{b}$ | $1.88 \pm 0.19^{a}$ | $0.67 \pm 0.09^{d}$ |
| Foaming Capacity % | $21.66 \pm 2.88^{b}$ | $43.33 \pm 2.88^{a}$ | $15.00 \pm 0.00^{c}$ | $10.00 \pm 0.00^{d}$ |
| Foaming Stability % | $5.00 \pm 0.00^{b}$ | $40.00 \pm 5.0^{a}$ | $8.33 \pm 2.89^{b}$ | $5.00 \pm 0.00^{b}$ |

Each value was represented as mean ± SD (n=3). Different superscript letters in the same row indicate significant differences ($P<0.05$) among the average values using one-way ANOVA followed by Duncan's test.

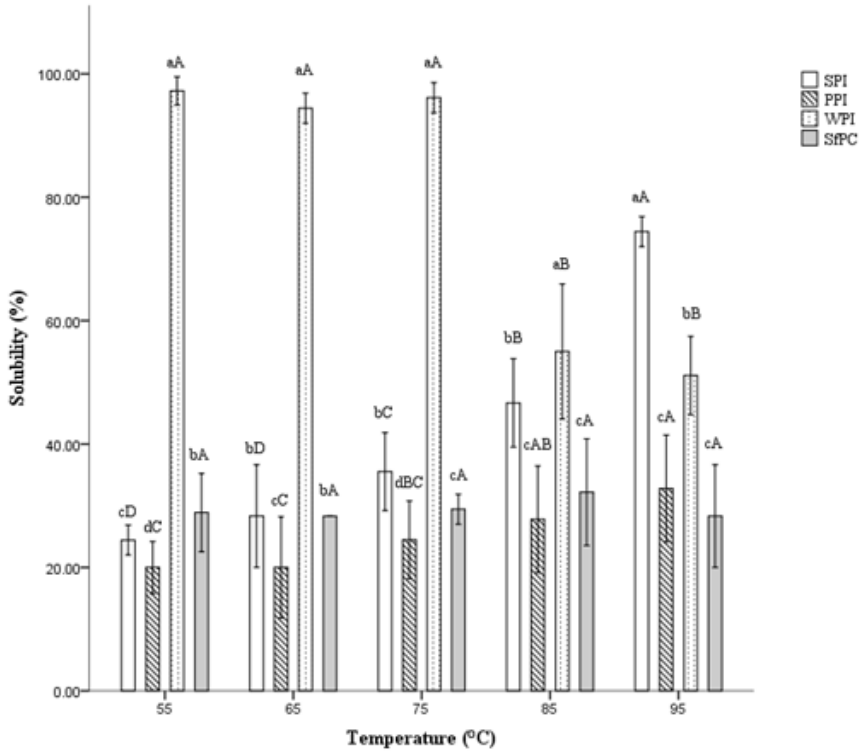


**Figure 1.** Solubility at different temperatures of SPI, PPI, WPI, and SfPC. Data are expressed as means of three replicates with error bars of standard deviations. Open bars represent Soy protein isolate (SPI), striped bars represent Pea protein isolate (PPI), dotted bars represent Whey protein isolate (WPI), and filled bars represent Sunflower protein concentrate (SfPC). Different lowercase superscripts indicated significant differences between different samples at the same temperature ($P<0.05$). Different uppercase superscripts indicated significant differences between different temperatures at same sample ($P<0.05$).

Solubility, also known as water-soluble index, of SPI, PPI, WPI, and SfPC were studied at 55, 65, 75, 85, and 95°C, and the results are shown in Figure 1. For a protein to be used in a food system, it must be hydrophilic and readily soluble [28]. At any of the temperatures except 95°C, WPI had the highest solubility in water. This indicated a presence of native protein and lower degree of denaturation [28]. The solubility of SPI increased with increasing temperatures, and it showed the highest among all samples at temperature 95°C. For SfPC, the solubility remained similar at all temperature sets. The similar results were reported by Salgado *et al.* [30]. The solubility of PPI was lowest across all temperature sets, but it slightly changed as the temperatures increased. It is important to highlight that the variations in solubility observed among the samples in this study could potentially be attributed to differences in their composition, as shown in Table 1. Moreover, it might suggest that SPI, PPI and WPI could be used in food products with mild processing condition, which the protein conformation remains intact and prevents exposure of hydrophobic amino acids [28].

## 4. Conclusion

As plant-based proteins gain popularity across diverse food applications, this study conducted a comparative analysis of nutritional compositions, amino acid profiles, physical and functional characteristics of commercially available soy, pea, and whey protein isolates, along with sunflower protein concentrate. The protein powders exhibited notable distinctions in their nutritional attributes, physical and functional. WPI contained the highest protein content followed by SPI, PPI and SfPC. Moreover, WPI also possesses the highest amount in most essential amino acids and low in non-essential amino acids. Although plant protein showed lower protein quality than WPI, the combination of different plant protein could solve this problem. The color of protein powders depends on the protein source. All protein samples are low in moisture content and water activity which make them have long shelf life. For the functional properties, WPI might be used as ingredient in bakery products. While plant-based protein (SPI, PPI, SfPC) might be suitable for sausage or patty. Moreover, WPI might be combined with plant-based protein to improve their functional properties. Thus, new studies are needed in the near future to evaluate the applicability of pretreatments to promote dairy and plant proteins' coexistence, to improve their functional and sensorial properties, and, consequently, to open a new market category of innovative food products.

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# Total Phenolic Content and Antioxidant Activities of Ethanolic Extracts from Stems and Leaves of Chinese Chives

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**Abstract**

Chinese chive (*Allium tuberosum* Rottl. ex Spreng) is one of the popular vegetables in the allium family. Allium species are rich in organosulfur compounds, flavonoids, saponins, and thioethers, exhibiting high antioxidant activity and total phenolic content. This study aimed to compare the total phenolic content and antioxidant activities of ethanolic extracts from the stems (SCC) and leaves (LCC) of Chinese chives. SCC and LCC were extracted with 70% ethanol and removed chlorophyll before determining total phenolic content using the Folin-Ciocalteu method and antioxidant activities by 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), and ferric reducing antioxidant (FRAP) method. The results indicated that LCC had a higher total phenolic content (2.48 ± 0.04 mg GAE/g fresh weight) than SCC (1.37 ± 0.04 mg GAE/g fresh weight). Antioxidant activity of LCC was two times higher than SCC, with values of 1131.79 ± 0.05, 8043.84 ± 0.11, and 1871.64 ± 0.00 µM TE/g fresh weight for DPPH, ABTS, and FRAP assays, respectively, compared to SCC with values of 582.13 ± 0.03, 2591.44 ± 0.20, and 1146.32 ± 0.01 µM TE/g fresh weight. Although LCC had higher total phenolic content and greater antioxidant activity, both extracts could be used compatibly in some food applications since the de-chlorophyllization process reduced the green color value.

***Keywords***: *Allium tuberosum* Rottl. ex Spreng; Chinese chive; Chlorophyll removal; Total phenolic content; Antioxidant activities

## 1. Introduction

Currently, there is a growing trend among consumers to look for products that contain natural extracts. This is because consumers are becoming interested in consuming foods that contain natural extracts that have antioxidant properties [1,2]. In Thailand, Chinese chives are widely cultivated and used as fresh Chinese chives with Thai noodles and cooked into dumplings (kuichai) [3], and their extracts are used to extend the shelf life of food and treat diseases [4]. These extracts have various health benefits, including anti-inflammatory, hypocholesterolemia, anticancer, and cardioprotective effects [5].

*Allium tuberosum* Rottl. ex Spreng or Chinese chive is in the Allium genus, Amaryllidaceae family, and the allium family. Studies have found that there are up to 700 species [6], such as *Allium cepa* (onion), *Allium sativum* (garlic), etc. All of these are important because of their commercial and nutritional value [7], The allium family is widely used and has been studied by medical science as an antioxidant and antimicrobial extract among its properties [8]. The study found that a group of phenolic compounds, organosulfur compounds, flavonoids, saponins [9], and thioethers, which are medicinal substances, give Chinese chives a unique aroma. A total of 40 chemical compounds of Chinese chives were isolated in the first extraction [10]. The current research on Chinese chives has numerous practical applications, including high levels of antioxidant and antibacterial activity.

There is a lack of research that has compared the effectiveness of extracting Chinese chives from their stems and leaves. This study aimed to provide a detailed comparison of the two parts and evaluated their total phenolic content and antioxidant activities. The research will use three assays, namely DPPH, ABTS, and FRAP, to accurately measure the antioxidant activities presented in both parts.

## 2. Materials and Methods

### 2.1 Chemical materials

DPPH (2,2-diphenyl-1-picrylhydrazyl), ABTS (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)), TPTZ (2,4,6-Tris(2-pyridyl)-s-triazine), Gallic acid, Trolox (6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) were purchased from Sigma-Aldrich (Milano, Italy). Folin-Ciocalteu and acetate buffer were purchased from CARLO ERBA (Milano, Italy) and all other chemicals are analytical grade.

### 2.2 Plant preparation

Chinese chive (*Allium tuberosum* Rottl. ex Spreng) was collected from Suan Phrik Thai, Mueang Pathum Thani, Pathum Thafni province, Thailand, in August 2023. The raw material was cleaned with distilled water to remove dust, and the stems and leaves were separated and then extracted immediately.

### 2.3 Plant extract preparation

Each sample was blended with 70% ethanol at a ratio of 1:3 (w/v) for 10 min in a blender. The extract was centrifuged at 8000 rpm for 10 min at 25°C. The supernatant was collected and concentrated in a rotary evaporator. Then, the chlorophyll was removed from the crude sample by adding activated charcoal powder at 10% (w/v). After that, the mixed solution was then passed through the paper filter NO.4 on a Buchner funnel and kept in an amber glass bottle in the refrigerator (4°C) until used. Chinese chive extracts from leaves and stems were named “LCC and SCC” respectively.

### 2.4 Total phenolic content (TPC)

Total phenolic content was estimated by the Folin-Ciocalteu method according to Škerget, Kotnik *et al.* [11] with slight modifications. Using a micropipette, 500 µL of each ethanolic extract was added with 2.5 mL of 10% (v/v) Folin-Ciocalteu reagent. After the vortex for 5 min, 2 mL of 7.5% (w/v) sodium carbonate was then added. The mixture was incubated for 1 h in the dark prior to reading absorbance at 760 nm. Gallic acid was used as reference standards for the calibration curve and expressed as gallic acid equivalents (mg GAE/g fresh weight).

### 2.5 Antioxidant activities

*2.5.1 DPPH free radical-scavenging assay*

The antioxidant activity of each Chinese chive extracts were determined following the method of Khorasani Esmaeili, Mat Taha *et al.* [12] with some modifications. In brief, 2 mL of each sample was mixed with 2 mL of 0.1 mM DPPH in methanol. After 30 min incubation at room temperature, the mixtures were measured at 517 nm with a UV-Vis spectrophotometer. Trolox was used as a reference standard. DPPH radical scavenging activity was expressed in µM TE/g fresh weight.

*2.5.2 ABTS radical cation decolorization assay*

The antioxidant activity of each Chinese chive ethanolic extract was determined by the free radical cation solution ABTS method modified from Nhut, An *et al.* [13]. ABTS radical cation was mixed using 7.4 mM reacting ABTS stock solution with 2.6 mM potassium persulfate ratio 1:1 (v/v) and was stored in the dark for 12 h in the dark. Before being used, ABTS radical cation solution was diluted with methanol at a ratio of 1:50 (v/v) to an absorbance of 1.1 (± 0.02) at 734 nm. 150 µL of each ethanolic extract was added with 2.85 ml ABTS radical cation. After the vortex, the mixture was incubated for 2 h in the dark and

the absorbance was immediately recorded at 734 nm. Trolox was used as a reference standard at different concentrations (0-600 µM). ABTS radical scavenging activity was calculated and expressed in µM TE/g fresh weight.

*2.5.3 FRAP assay*

The antioxidant activity of each ethanolic extract from Chinese chives was determined by the ferric reducing antioxidant power method according to Surco-Laos, García Ceccarelli *et al.* [14]. FRAP solution was prepared with 300 mM acetate buffer in water (pH 3.6), 10 mM TPTZ in 40 mM hydrochloric acid, and 20 mM ferric chloride at a ratio of 10:1:1 (v/v/v). To analyze the antioxidant activity of the ethanolic extract samples, 150 µL of each sample was mixed with 2.85 mL of FRAP solution. The mixture was then allowed to stand for 30 min in the dark. Afterward, the absorbance was measured at 593 nm using a UV-Vis spectrophotometer. Trolox was used as a reference standard at various concentrations ranging from 0-600 µM. Finally, the results were expressed in µM TE/g fresh weight.

**2.6 Color analysis**

The color of each Chinese chive extract samples was analyzed by CR-400 (Konika, Minolta, Osaka, Japan) to measure the differences between each sample and compare samples before and after chlorophyll removal. The results were expressed in CIE-LAB (*L* a* b** parameters) color scale, where *L**, *a**, and *b** indicated lightness, redness, and yellowness, respectively.

**2.7 Statistic analysis**

The experiments and analysis were conducted in triplicates, and the results were expressed as Mean ± standard deviation of the mean (SEM). The data was analyzed using SPSS (SPSS 29.0, IBM Inc., New York, USA). One-way ANOVA and independent sample t-test or Duncan new multiple range test were used to determine the level of significance. Data was considered statistically significant at a level of $P<0.05$.

## 3. Results & Discussion

**3.1 Total phenolic content (TPC)**

The total phenolic content of SCC and LCC were analyzed and reported in Table 1. It can be observed that the TPC of LCC (2.48 ± 0.04 mg GAE/g fresh weight) is higher than that of SCC (1.37 ± 0.04 mg GAE/g fresh weight) ($P<0.05$). In a previous report, the amount of TPC found in the Chinese chive extracts (*Allium tuberosum*) [1] and other allium species, such as *Allium ursinum* L. and *Allium bulgaricum* L. that used 70% ethanol to extract, were found to have a value of 0.40 ± 0.02 and 0.41 ± 0.08 mg GAE/g fresh weight, respectively [15]. Chinese chive extracts in this study had a higher value than the reports mentioned above, but it was not suitable to compare it to other experiments due to differences in location conditions and vegetation. According to studies, Chinese chives are a very important source of phytochemicals such as organosulfur compounds, flavonoids, saponins, and phenolic compounds [9], including phenolic acids and phenolic glycosides, which were likely present in higher concentrations in leaves compared to stems, contributing to antioxidant activity. Even though it is still an important source for the treatment of cancer, hepatic disorders, and impotency [16].

**Table 1.** Total phenolic content (TPC) of Chinese chives ethanolic extracts from stems (SCC) and leaves (LCC).

| Samples | Total phenolic content (mg GAE/g fresh weight) ⸸ |
|---|---|
| SCC | $1.37 \pm 0.04^{B}$ |
| LCC | $2.48 \pm 0.04^{A}$ |

⸸: Values are mean ± standard deviation (n=3). Different superscripts within the same column indicate significant differences (P<0.05)

### 3.2 Antioxidant activities

Antioxidant activities of Chinese chives ethanolic extracts determined by DPPH, ABTS, and FRAP assays are shown in Table 2. DPPH is a stable free radical with an absorption band at 517 nm. It loses this absorption when accepting electrons or free radical species. Its bleaching is used to measure the hydrogen atom or electron-donating ability of extracts and compounds [17]. The DPPH radical scavenging activity of LCC (1131.79 ± 0.05 µM TE/g fresh weight) is higher than SCC (582.13 ± 0.03 µM TE/g fresh weight). This is related to the TPC values of both stems and leaves of Chinese chive ethanolic samples (Table 1). The ABTS procedure is used for evaluating antioxidant activity, which involves the decolorization of cations from free radicals of ABTS (green) [18]. The data indicates that the antioxidant activity of LCC (8043.84 ± 0.11 µM TE/g fresh weight) is higher than that of SCC (2591.44 ± 0.20 µM TE/g fresh weight). Reducing power is an important indicator of antioxidant activity, which can be demonstrated by using $Fe^{3+}$ to $Fe^{2+}$ as a modifier. The color of the solution changes from yellow to green and blue based on the reducing power of the sample [19]. According to Table 2, the antioxidant activity on FRAP of the SCC was 1146.32 ± 0.01 µM TE/g fresh weight, which is lower than that of LCC 1871.64 ± 0.00 µM TE/g fresh weight (P<0.05).

The following analysis pertains to the TPC values of both the stems and leaves of Chinese chive ethanolic samples, as shown in Table 1. This study found that the ethanolic extract of *Allium tuberosum* contains a large amount of phenolic content. It can be inferred that these phenolic contents are responsible for the remarkable antioxidant activity [20]. Therefore, the TPC value of LCC is greater than SCC, resulting the antioxidant activities of DPPH, ABTS and FRAP of LCC is significantly higher than SCC. a previous report, the antioxidant activity of ABTS found in the Chinese chive extracts (*Allium tuberosum*) was 30.63 ± 0.34 mg TE/g sample [21] Another report of the antioxidant activities of FRAP, DPPH, and ABTS found in the Chinese chive extracts that compared with a standard curve of ascorbic acid was 0.24 ± 0.03, 0.18 ± 0.02 and 1.09 ± 0.12 mg VCE/g fresh weight, respectively [1]. Other documentation on the antioxidant activities of other *Allium* species such as ethanol extracts of *Allium ursinum* L. and *Allium bulgaricum* L., used 70% ethanol to extract samples. These studies analyzed the antioxidant activity values, DPPH, ABTS, and FRAP, with Trolox as the standard substance. with 1.86 ± 0.22, 11.37 ± 1.96 and 4.56 ± 0.04 µM TE/g FW and 4.77 ± 0.88, 5.79 ± 0.25 and 7.16 ± 0.06 µM TE/g FW, respectively [15]. The results showed that the values for *allium tuberosum* were significantly higher.

**Table 2.** Antioxidant activities of Chinese chives ethanolic extracts from stems (SCC) and leaves (LCC).

| Samples | Antioxidant activity⸸ | | |
|---|---|---|---|
| | DPPH (µM TE/g fresh weight) | ABTS (µM TE/g fresh weight) | FRAP (µM TE/g fresh weight) |
| SCC | 582.13 ± 0.03[b] | 2591.44 ± 0.20[b] | 1146.32 ± 0.01[b] |
| LCC | 1131.79 ± 0.05[a] | 8043.84 ± 0.11[a] | 1871.64 ± 0.00[a] |

⸸ : Values are mean ± standard deviation (n=3). Different superscripts within the same column indicate significant differences (P<0.05)

### 3.3 Color analysis

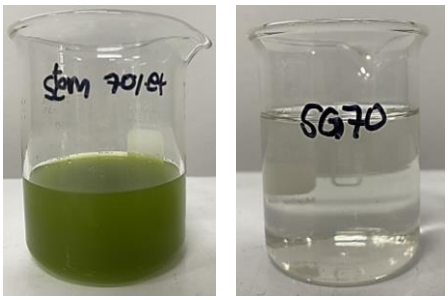
The color of the extract is also important in modifying it for use in food [22]. The color of Chinese chive ethanolic extracts was analyzed and reported as shown in Table 3. It was found that ethanol extracts before chlorophyll removal had *L**, *a**, and *b** values of 24.61 ± 0.07, -5.72 ± 0.06, and 5.83 ± 0.29 for LCC and 24.45 ± 0.39, -4.06 ± 0.13, and 5.75 ± 0.17 for SCC, respectively. Chlorophyll in stems and leaves can be extracted using organic solvents such as acetone or ethanol [23] and it's a green pigment. which is the color of the leaves [24]. Since the intense green color of some food cannot be accepted by consumers, the chlorophyll was therefore removed. The *L**, *a** and *b** values of LCC and SCC after chlorophyll removal were 31.41 ± 0.02, 1.13 ± 0.03 and -0.90 ± 0.02, and 26.31 ± 0.12, -0.66 ± 0.02 and 0.70 ± 0.04, respectively. It is evident that the green value significantly decreased after the removal of chlorophyll from the sample as shown in Figure 1 (P<0.05). Chlorophyll was removed in this extraction process. A previous study found that removing chlorophyll results in a significant decrease in TPC values [25], thereby reducing the antioxidant value but it'll be able to adapt to a wider variety of foods.

**Table 3.** Colors of Chinese chives ethanolic extracts from stems (SCC) and leaves (LCC).

| Samples† | Color value⸸ | | |
|---|---|---|---|
| | Lightness (*L**) | Redness (*a**) | Yellowness (*b**) |
| BCR-SCC | 24.45 ± 0.39[c] | -4.06 ± 0.13[d] | 5.75 ± 0.17[c] |
| BCR-LCC | 24.61 ± 0.07[c] | -5.72 ± 0.06[c] | 5.83 ± 0.29[b] |
| SCC | 26.31 ± 0.12[b] | -0.66 ± 0.02[b] | 0.70 ± 0.04[a] |
| LCC | 31.41 ± 0.02[a] | 1.13 ± 0.03[a] | -0.90 ± 0.02[a] |

⸸ Values are mean ± standard deviation (n=3). Different superscripts within the same column indicate significant differences (P<0.05)

† BCR: Before chlorophyll removal.



**(A)** **(B)**

**Figure 1.** Extracts color of Chinese chive ethanolic extracts from stems (SCC) before chlorophyll removal (A), after chlorophyll removal (B)

## 4. Conclusion

In this experiment, the stems and leaves of Chinese chives (*Allium tuberosum* Rottl. ex Spreng) were extracted by 70% ethanol to compare the total phenolic content and antioxidant activities presented in the DPPH, ABTS, and FRAP. The study found that Chinese chive leaves have higher Total Phenolic Content (TPC) and greater antioxidant activities than the stems. The color value of the extract can be seen that the extract, after removing chlorophyll, has a significantly reduced green color. Therefore, the removal of chlorophyll can make it possible to apply this method in a broader range of food products and still have the activity of TPC and antioxidant activities.

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# Effect of Physical Modification Techniques on Nutritional Values, Physical Properties and *In Vitro* Estimated Glycemic Index of Jackfruit Seed Flour

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**Abstract**

This research aims to investigate the nutritional values, physical properties, and *in vitro* estimated glycemic index (eGI) of native jackfruit seed flour (N-JSF) and four modified jackfruit seed flours (JSF), namely partial gelatinization treatment (PG), heat-moisture treatment (HMT), microwave heat-moisture treatment (MHMT), autoclave heat-moisture treatment (AHMT). The results found that there was no significant difference in protein content of HMT-JSF and AHMT-JSF with N-JSF, while PG-JSF and MHMT-JSF increased protein content to 11.58-11.83%. PG-JSF contained similar carbohydrate contents to N-JSF, while those of AHMT-JSF, HMT-JSF, and MHMT-JSF differed from their native form. Moreover, the fiber content of PG-JSF, HMT-JSF, and MHMT-JSF was not significantly different from N-JSF, while AHMT-JSF decreased fiber content to 12.73%. The determination of eGI exhibited that these modified methods resulted in increasing eGI of N-JSF from low range (32.30) to medium range (58.53-68.27) in modified JSF. For the physical properties, the result indicated that samples contained various sizes of particles ($P<0.05$). N-JSF had more lightness values but less redness and yellowness values than modified JSF samples. The water activity of the JSF samples ranged from 0.16-0.35. Water holding capacity was found to be higher in modified JSF samples, while oil holding capacity showed a similar value across all JSF samples. In terms of pasting properties, N-JSF exhibited the highest peak viscosity, trough, breakdown, final viscosity, and setback. In comparison between modified JSF samples, the lowest peak viscosity was reported in HMT-JSF. No significant differences were found in breakdown values between modified JSF samples. Moreover, HMT-JSF, MHMT-JSF and AHMT-JSF showed the lowest trough viscosity, final viscosity and setback. A comparison of various characteristics of N-JSF and Modified JSF samples is expected to be a reference for developing and applying these JSF in various fields and products.

***Keywords:*** Jackfruit; Flour; Modification; Nutritional values; Physical properties

## 1. Introduction

Diabetes mellitus is a metabolic disorder characterized by high blood glucose levels (1), may lead to various health complications if left untreated (2). The global prevalence of diabetes is expected to increase by 31.4% between 2021 and 2045 (3). According to International Diabetes Federation, 9.7% of Thais aged 20 to 79 years had diabetes in 2021, which is predicted to increase to 11% by 2045 (4). This trend will pose a significant burden on national health systems and economies by 2030 (5). The goals for medical nutrition therapy for diabetes place an emphasis on lifestyle modification to improve glucose control, lipids,

and blood pressure [6]. Among all the macronutrients, carbohydrate intake plays a crucial role in controlling and preventing type 2 diabetes [7]. It possibly becomes even more effective when considering the classification of carbohydrate-containing foods based on their glycemic index and rate of carbohydrate digestion. This approach is particularly beneficial in managing and preventing chronic diseases, including those related to insulin resistance, such as diabetes [8].

Jackfruit (*Artocarpus heterophyllus* Lam.) is a tropical tree that belongs to a family of Moracea and originates from India. Jackfruit is commonly found in Asia, Africa, and multiple regions of South America. It is well-known for its size as one of the largest fruits that can be consumed [9]. In addition to their size, jackfruits are highly nutritious, containing a wide range of essential nutrients, such as minerals, carbohydrates, volatile compounds, proteins, vitamins, and various phytoconstituents [9]. Not only the fruits but also the seeds offer nutritional benefits. The seeds contain a decent amount of both macronutrients, and micronutrients, as well as bioactive compounds [10]. The fiber content of raw jackfruit seeds contains approximately 9 g of fiber per 100 g of seeds [11] in which 28.8% is soluble fiber and 71.2% is insoluble fiber, specifically higher in the form of flour. According to the study in Malaysia and Tanzania, the fiber content in jackfruit seed flour is approximately 24-25 g in 100 g of jackfruit seed flour with the process of drying and grinding the seeds into powder [12,13]. The seeds contain around 10% to 15% of the fruit weight and are an important reservoir of starch [14]. A review article on health-promoting effects of jackfruit seeds, with special emphasis on their applications in food, concluded that the nutritional value of products enriched with jackfruit seed flour is notably higher in terms of their ash, protein, dietary fiber, and phytochemical contents. Certain value-added products have been produced successfully using jackfruit seed flour, such as bakery products (bread, biscuits, cake) and extruded products (pasta, noodles) [15].

Many previous studies have investigated various methods to enhance and broaden the application of starch in various flours, namely lotus seed flour, rice flour and yam flour. Physical modification techniques, including heat-moisture treatment, partial gelatinization, microwave moisture heat treatment, and autoclave heat treatment, have the potential to alter functional properties and modify structural aspects, making them suitable for a range of food applications. These methods are considered safe and have the capacity to influence particle size and starch polymer structure [16].

Therefore, this study aimed to modify jackfruit seed flour (JSF) using heat-moisture treatment, partial gelatinization treatment, microwave moisture heat treatment and autoclave heat treatment. The nutritional values, physical properties and *in vitro* glycemic index of these sampleswere also determined and compared with native JSF. This acquired knowledge is expected to be valuable in the food industry, presenting the jackfruit seed as an alternative flour with potential health benefits.

## 2. Materials and Methods

### 2.1 Raw materials and native jackfruit seed flour (N-JSF) preparation

Jackfruit seeds (*Artocarpus heterophyllus* Lam.) variety Thong Phraset was collected from a local market at Nakhorn Phathom province, Thailand, in October 2023. Once received, N-JSF was prepared by blanching jackfruit seeds in boiling water for 4 min, using a seed-to-water ratio of 1:4 *w/v*. Then, the seed coats were manually peeled off. The seeds were blended and dried in a hot air oven at 60°C until the moisture content was less than 10% w/w. The dried jackfruit seeds were grinding using a grinder machine, followed by sieving through a 100-mesh sieve. The N-JSF was packed in polyethylene aluminum bags and stored in a refrigerator at 4 ± 2°C until use. The production yield of N-JSF from jackfruit seeds was determined to be 33.89%.

**2.2 Physical modification of jackfruit seed flour**

*2.2.1 Heat-Moisture Treatment (HMT-JSF)*

N-JSF subjected to heat-moisture treatment was prepared with a slight modification to the method as described by Sopawong *et al.* [17]. Distilled water was added to N-JSF at a flour-to-water ratio of 1:4 *w/v*. The mixture was kept overnight at 4°C. Then, excess water was removed from the slurry before being heated at 110°C in a hot air oven for 4 h and then refrigerated (4°C) overnight. The obtained sample was oven-dried at 60°C until the moisture content was less than 10%. The HMT-JSF samples were packed in polyethylene aluminum bags and stored in a refrigerator at 4 ± 2°C until further analysis. The recovery of HMT from N-JSF was 76.42%.

*2.2.2 Patial gelatinization (PG-JSF)*

Jackfruit seeds underwent a parboiling process at 75°C for 40 min to achieve partial gelatinization of starch. Then, the parboiled seeds were blended and dried at 60°C in a hot air oven (Model FFD115/E2, Binder GmbH, Tuttlingen, Germany) until the moisture content reached 10%. After drying, the seeds were ground and sieved through a 100-mesh sieve to obtain partially gelatinized jackfruit seed flour (PG-JSF). PG-JSF samples were packaged in polyethylene aluminum bags and stored in a refrigerator at 4 ± 2°C for further analysis. The production yield from jackfruit seeds was about 38.20%.

*2.2.3 Microwave treatment (MHMT-JSF)*

N-JSF subjected to microwave heat-moisture treatment was prepared with a slight modification to the method as described by Zailani *et al.* [18]. N-JSF sample was mixed with distilled water in a ratio of 1:4 *w/v* for overnight at 4°C before the treatment. The sample was treated with a microwave power (Model: ER-ND 300C, Toshiba Thailand Co., Ltd, Bangkok, Thailand) of 500w for 10 min in microwave oven. The treated JSF was then cooled down and stored overnight at 4°C before being dried at 60°C in a hot air oven until the moisture content was 10%. The dried sample was grounded into flour by a grinder machine and passed through a 100-mesh sieve for further analysis. Samples were kept in polyethylene aluminum bags and stored in a refrigerator at 4 ± 2°C. The recovery of MHMT from N-JSF was up to 91.15%.

*2.2.4 Autoclave heat treatment (AHMT-JSF)*

Similar to MHMT-JSF, AHMT-JSF was prepared by mixing the N-JSF with distilled water (1:4 *w/v*) and stored overnight at 4°C. Then, the mixture was autoclaved (Model: HVA-110, Hirayama Manufacturing Corporation, Saitama Japan) at 121°C for 30 min. After autoclaving, the samples were cooled to room temperature in the same Duran glass bottle for 1 h before being stored overnight at 4°C. The overnight sample was dried at 60°C in a hot air oven. All samples were grinded and sieved through a 100-mesh sieve. The samples were packed in polyethylene aluminum bags and stored in a refrigerator at 4 ± 2°C for further analysis. The recovery of AHMT from N-JSF was approximately 93.17%.

**2.3 Characterization of jackfruit seed flour**

*2.3.1 Nutritional values*

Proximate composition of both native and modified jackfruit seed flours was assessed following the guidelines of the AOAC Official Methods [19]. The moisture content was determined using the gravimetric method (AOAC Method 925.45), while the protein content was analyzed as nitrogen using the Kjeldahl method, with a conversion factor of 5.70 (AOAC Method 991.20). Fat content was analyzed through acid hydrolysis (AOAC Method 922.06), and ash content was determined by incineration (AOAC Method 930.30). The measurement of dietary fiber was determined using enzyme-gravimetric method (AOAC Method 985.29). Total energy was calculated by multiplication factors: 4 for carbohydrate and protein and 9 for total fat, and carbohydrate content was calculated by subtracting the contents of moisture, protein, fat, and ash from 100.

*2.3.2 Physical properties*

Color analysis was determined using a colorimeter according to the instrument's instruction manual. The parameters under analysis are *L** (brightness or whiteness), *a** (redness to greenness), *b** (yellowness to blueness). The color of the samples was measured as replicates (*n*=3).

The water activity ($A_w$) of the samples were determined using a water activity meter that employs the chilled mirror technique at a room temperature of 25 ± 1°C. The flour was packed into the equilibrium chamber and inserted into the analyzer for a duration of 20 min, after which the water activity of the sample was measured and recorded. This process was measured in replicates (n=3).

Water holding capacity (WHC) and oil holding capacity (OHC) were determined following the methods described by Kazemi. [20]. 1 g of the flour sample was dissolved in 10 mL of distilled water. Then, the suspension was vortex for 1 min and followed with centrifugation at 3000× g for 30 min. Afterward, the supernatant was removed, and the remnants were weighed. OHC measurement is similar to WHC, except using sunflower oil instead of distilled water. WHC and OHC were calculated and reported as follows:

$$\text{WHC (g/g)} = \frac{\text{Weight of the remaining flour sediment}}{\text{Weight of initial flour}}$$

$$\text{OHC (g/g)} = \frac{\text{Weight of the remaining flour sediment}}{\text{Weight of initial flour}}$$

Water solubility index (WSI) and swelling power of the flour were assessed following the procedure as described by Sopawong *et al.* [17]. 0.6 g of flour sample was suspended in 30 mL of distilled water and then heated in a water bath at different temperatures (55, 65, 75, 85, and 95°C) for 30 min with a mixing interval every 5 min. The suspensions were cooled down to room temperature before being centrifuged at 4000× g for 10 min. The liquid supernatant was then pulled out, dried at 105°C for 2 h, and weighed. Meanwhile, the precipitates were immediately weighed. Both WSI and swelling power were calculated using the provided equations:

$$\text{WSI (\%)} = \frac{\text{Dried supernatant weight}}{\text{Weight of initial flour}} \times 100$$

$$\text{Swelling power (g/g)} = \frac{\text{Weight of precipitate}}{\text{Weight of dried sample x } [1 - \left(\frac{\text{Solubility}}{100}\right)]}$$

The Rapid Visco Analyzer (RVA) was used to evaluate the pasting properties of the flour sample, following the method described by Sopawong *et al.* [17]. Three g of the sample was weighed and placed into RVA canisters. Then, 25 mL of distilled water was added to create a slurry. This slurry was heated at 50°C for 1 min before increased to 95°C at a rate of 12°C/min. The slurry was then held for 2.5 min before returning to 50°C at the same rate. Various parameters were measured, including peak viscosity, breakdown, final viscosity,

setback, trough viscosity, peak time, and pasting temperature. The pasting properties parameters were presented as RVU units.

*2.3.3 In vitro estimated glycemic index*

The *in vitro* estimate glycemic index was determined *in vitro* digestibility using the method reported by Sopawong *et al.* [17]. First, 100 mg of flour was placed into a 50 mL screw-capped test tube with 10 mL of HCl-KCl buffer. Then, a 200 µL pepsin solution containing 1 mg from porcine gastric mucosa was added to the sample. The sample was incubated at 37°C for 60 min in a shaking water bath, followed by 9 mL of tris-maleate buffer. For starch hydrolysis, 1 mL of tris-maleate buffer containing 1 mg of α-amylase was added to each sample. The sample was then placed in a shaking water bath at 37°C. 100 µL of aliquots was taken from the sample every 30 min for 2 h. After that, the tubes were placed in a boiling water bath for 5 min to inactivate the enzyme. The digesta was undergo centrifugation at 4600× g for 10 min to separate precipitate for RS determination. The supernatant was mixed with 0.4 M sodium-acetate buffer and amyloglucosidase. The samples were incubated at 60°C for 45 min. Glucose concentration in the sample was measured using glucose oxidase-peroxidase kit GOPOD reagent enzyme. The rate of starch digestion was expressed as a percentage of glucose at 0, 20, 30, 60, 90 and 120 min. The hydrolysis index (HI) was determined using the area under the curve from GraphPad Prism 7 software, with white bread as a reference food. The estimated glycemic index (eGI) was calculated based on the linear relationship between the hydrolysis index and the glycemic index using the equation:

$$eGI = (0.549 \times HI) + 39.71$$

**2.4 Statistical analyses**

The statistical analysis was performed using SPSS software (version 18, SPSS Inc., Chicago, II, USA). All samples were analyzed in three replicates for each analysis. Differences between N-JSF and its modified samples were presented as mean ± standard deviation (SD), and determined using one-way analysis of variance (ANOVA) with Turkey's post hoc test ($P<0.05$).

**3. Results & Discussion**

**3.1 Nutritive values of Native and Modified Jackfruit Seed Flour**

N-JSF contained the lowest moisture content of 4.65%, while all modified JSF contained higher moisture content ranging from 4.88-7.91% (Table 1). The moisture content of all JSF met the standard for flour (<14%). The protein content of MHMT-JSF exhibited the significantly higher than N-JSF and other modified JSF. MHMT could increase protein in JSF. It might be due to destruction of anti-nutrients such as tannins in the grains/seeds which led to a concomitant increase in the assayed protein. Tannins form complexes with proteins through hydrogen bonding, hydrophobic, ionic, and covalent reactions [21]. However, the complexes formed between tannin and protein can be dissociated by mild treatments. Furthermore, microwaving may also have resulted in protein denaturation and starch gelatinization which in turn may increase bioavailability of inherent nutrients as previously reported [22].Moreover, the protein content in this study was in range with Aritsara and Supattra [23]. However, the higher protein content of the modified JSF was reported by Zuwariah *et al.* [13], which could be due to the different variety of jackfruit seeds. Moreover, the native and modified JSF could be claimed as high protein [24]. Carbohydrate was the major composition of native and modified JSFN-JSF. There was no significant difference incarbohydrate content between N-JSF and PG-JSF, but carbohydrate content of these two JSF were higher than HMT-JSF, MHMT-JSF and AHMT-JSF. Fat and ash content of all

samples rangedfrom 0.47-0.62 and 3.01-3.42 g/100 g, respectively. There was no significant difference in dietary content between native and modified JSF except AHMT-JSF. The AHMT treatment resulted in decreased the fiber and carbohydrate content to 12.73% and 76.76%, respectively, which could be due to the high temperature and pressure used in this modified method. The exposure to high temperature and pressure might cause chemical bond breakage, creating smaller particles which are soluble [25]. The energyof native and modified JSF were in the range of 357.86-370.63 kcal/100 g which could be due to the higher content of carbohydrate.

*In vitro* estimate glycemic (eGI) analysis was performed and the results were presented in Table 1. eGI of N-JSF was 32.30, while modified JSF exhibited the eGI values ranging from 58.53-68.27. It seems that GI category of N-JSF was in low GI while the modified JSF samples were in medium GI. This result indicated that the modification process may have had an impact on the structure of the starch, resulting in a higher estimated glycemic index compared to N-JSF. The higher eGI in the modified JSF could be explained by Sopawong *et al.* [17] who found that the increasing of eGI in pregelatinized lotus seed flours caused by higher amount of rapid digestible starch and the lower amount of resistance starch in the samples [17].

**Table 1**. Nutritional values and *in vitro* estimate glycemic index (eGI) of native and modified jackfruit seed flours.

| Component | N-JSF | PG-JSF | HMT-JSF | MHMT-JSF | AHMT-JSF |
|---|---|---|---|---|---|
| Energy (kcal) | 370.63 ± 0.07$^{a}$ | 370.43 ± 0.11$^{a}$ | 366.96 ± 0.64$^{b}$ | 366.08 ± 0.47$^{b}$ | 357.86 ± 0.78$^{c}$ |
| Moisture (g) | 4.65 ± 0.04$^{c}$ | 4.88 ± 0.04$^{c}$ | 6.03 ± 0.18$^{b}$ | 5.79 ± 0.15$^{b}$ | 7.91 ± 0.26$^{a}$ |
| Protein (g) | 11.27 ± 0.01$^{c}$ | 11.58 ± 0.00$^{ab}$ | 11.49 ± 0.00$^{bc}$ | 11.83 ± 0.16$^{a}$ | 11.35 ± 0.17$^{bc}$ |
| Total Fat (g) | 0.53 ± 0.01$^{c}$ | 0.47 ± 0.01$^{d}$ | 0.62 ± 0.01$^{a}$ | 0.58 ± 0.01$^{b}$ | 0.61 ± 0.02$^{ab}$ |
| Total Carb (g) | 80.20 ± 0.04$^{a}$ | 79.98 ± 0.04$^{a}$ | 78.86 ± 0.19$^{b}$ | 78.39 ± 0.01$^{b}$ | 76.76 ± 0.42$^{c}$ |
| Dietary fiber (g) | 14.94 ± 0.74$^{a}$ | 14.44 ± 0.04$^{a}$ | 14.92 ± 0.02$^{a}$ | 14.49 ± 0.04$^{a}$ | 12.73 ± 0.38$^{b}$ |
| Ash (g) | 3.36 ± 0.01$^{b}$ | 3.10 ± 0.00$^{c}$ | 3.01 ± 0.01$^{d}$ | 3.42 ± 0.01$^{a}$ | 3.39 ± 0.04$^{ab}$ |
| eGI | 32.30 ± 0.46$^{d}$ | 58.53 ± 1.05$^{c}$ | 64.20 ± 2.60$^{b}$ | 63.47 ± 1.72$^{b}$ | 68.27 ± 0.46$^{a}$ |

Values are expressed as mean ± SD. Values with different superscript letters in the same row indicate significant difference at $P<0.05$ using one-way ANOVA followed by Turkey's test.

### 3.2. Physical Properties of Native and Modified Jackfruit Seed Flour

The particle sizes of N-JSF and four modified jackfruit seed flours are shown in Table 2. The result indicated that samples contained various sizes of particles. More than half of N-JSF could pass through the 100 mesh sieves for 53.80%, while only 26.58% to 35.32% of modified flour could pass through them. The higher number of mesh sizes could represent the higher fineness of the flour, which means that N-JSF had more finer particles compared to the modified flour. Many studies found that particle size has an effect on the characteristics of food products, with finer particle size generally associated with a better overall quality score compared to coarser particles [26].

**Table 2**. Particle size of native and modified jackfruit seed flour.

| Sieve mesh | Flour (%) | | | | |
|---|---|---|---|---|---|
| | N-JSF | AHMT-JSF | MHMT-JSF | HMT-JSF | PG-JSF |
| 40 | 19.39±0.79$^{a}$ | 39.10±0.82$^{c}$ | 36.59±1.31$^{c}$ | 30.51±0.71$^{b}$ | 35.64±2.81$^{c}$ |
| 60 | 17.68±0.12$^{a}$ | 23.50±0.14$^{c}$ | 23.49±0.20$^{c}$ | 24.26±0.32$^{d}$ | 18.65±0.35$^{b}$ |
| 80 | 9.22±0.32$^{a}$ | 10.92±0.29$^{c}$ | 10.93±0.31$^{c}$ | 11.18±0.06$^{c}$ | 10.12±0.31$^{b}$ |
| 100 | 7.35±1.13$^{b}$ | 5.73±0.17$^{ab}$ | 5.83±0.14$^{ab}$ | 6.26±0.09$^{ab}$ | 5.6±8.71$^{a}$ |
| 120 | 10.64±1.78$^{b}$ | 3.08±0.10$^{a}$ | 2.92±0.17$^{a}$ | 3.11±0.07$^{a}$ | 3.42±0.57$^{a}$ |
| ≥120 | 35.73±1.21$^{d}$ | 17.67±0.27$^{a}$ | 20.24±0.76$^{ab}$ | 24.67±0.43$^{bc}$ | 26.50±4.04$^{c}$ |

Values are expressed as mean±SD. Values with different superscript letters in the same row indicate significant difference at P<0.05 using one-way ANOVA followed by Turkey's test.

The physical properties of native and modified JSF are presented in Table 3. The color values of the native and modified JSF samples were significantly different from each other (P<0.05). It was observed that N-JSF has the highest lightness value (*L**) while lowest redness (*a**) and yellowness (*b**) values. It seemed that the lightness values were decreased while the redness and yellowness was increase after modified treatment. This could be due to the alteration of material surface characteristics when exposed to high temperature during the modified process. In addition, thermal treatment induced non-enzymatic browning which led to the formation of a brown color. The water activity ($a_w$) measures the availability of free water in food sample [27]. It plays a crucial role in influencing the shelf life, spoilage, and safety of food products. A lower aw indicates a lower chance for the growth of bacteria, yeast, and mold. As shown in Table 3, the $a_w$ value for the flour samples ranged from 0.160-0.354, placing them in the category of nuts and seeds, flour, as well as dry products [28].

Water holding capacity (WHC) indicates the ability of the product to bind with water, primarily dependent on the protein and starch content; similarly to WHC, Oil holding capacity (OHC) indicates the ability of products to bind with oil [29]. The WHC and OHC of the native and modified JSF samples ranged from 2.89 to 3.53 g/g and 1.83 to 1.94 g/g, respectively (Table 3). There was no significant difference on WHC between modified JSF while WHC of those modified JSF samples were higher than native JSF. It seems than modified treatment could help to improve WHC. For OHC, PG-JSF exhibited the lowest values while no significant difference was found in other JSF. The product with high WHC and OHC is suitable for food products such as breads, cakes, and muffins where hydration and shortening properties are desirable while high OHC sample could be suitable for the cold meat industry where protein can bridge fat and water in the product [30].

**Table 3**. Physical properties of native and modified jackfruit seed flours.

| Physical Properties | N-JSF | PG-JSF | HMT-JSF | MHMT-JSF | AHMT-JSF |
|---|---|---|---|---|---|
| Color | | | | | |
| *L** | 83.75±0.05$^{a}$ | 76.44±0.02$^{b}$ | 75.71±0.03$^{c}$ | 75.34±0.07$^{d}$ | 71.33±0.04$^{e}$ |
| *a** | 3.67±0.02$^{e}$ | 4.62±0.01$^{d}$ | 6.17±0.02$^{b}$ | 6.06±0.02$^{c}$ | 7.76±0.00$^{a}$ |
| *b** | 12.18±0.07$^{e}$ | 13.40±0.06$^{d}$ | 14.46±0.03$^{c}$ | 15.66±0.04$^{b}$ | 17.49±0.04$^{a}$ |
| Water activity ($a_w$) | 0.16±0.02$^{c}$ | 0.16±0.00$^{c}$ | 0.29±0.01$^{b}$ | 0.27±0.02$^{b}$ | 0.35±0.00$^{a}$ |
| WHC (g/g) | 2.89±0.14$^{b}$ | 3.53±0.04$^{a}$ | 3.37±0.04$^{a}$ | 3.34±0.07$^{a}$ | 3.40±0.01$^{a}$ |
| OHC (g/g) | 1.94±0.02$^{a}$ | 1.83±0.01$^{b}$ | 1.93±0.08$^{a}$ | 1.91±0.01$^{ab}$ | 1.94±0.01$^{a}$ |

Values are expressed as mean ± SD. Values with different superscript letters in the same row indicate significant difference at P<0.05 using one-way ANOVA followed by Turkey's test.

Solubility, also known as water-soluble index, of both native and modified jackfruit seed flours was investigated at 55, 65, 75, 85, and 95°C, as shown in Figure 1. The solubility of both native and modified flour showed a higher value with increasing temperature, particularly at 85°C and 95°C. This rise in solubility at elevated temperatures was attributed to the increased leaching of amylose from the broken starch granules. Additionally, high temperatures led to the disruption of hydrogen bonding within the crystalline region, resulting in the destruction of starch granule crystallinity. As a result, more hydroxyl groups are available for binding with water [17]. At 85°C, N-JSF showed significantly higher solubility compared to AHMT, HMT, and PG-JSF. In contrast, AHMT-JSF demonstrated higher solubility than the modified flours across all temperatures except at 85°C. This might be due to some of the starch components may still have been trapped inside the remnant swollen granules, and not solubilized within the medium. However, the difference in solubility between samples were minimized at 90°C, which was well above the gelatinization temperature [31]. Moreover, it is noteworthy that the difference in solubility of different flour samples in this study might also cause by their different composition (Table 1).

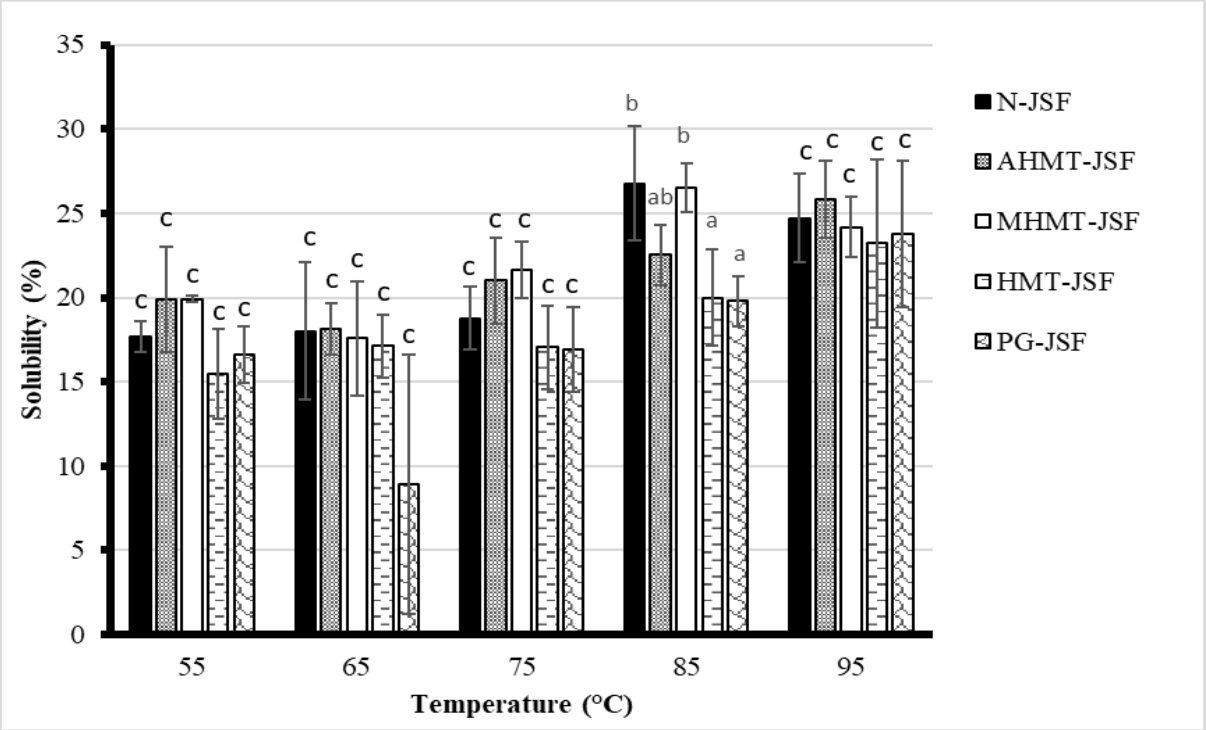


**Figure 1.** Solubility at different temperatures of native and modified jackfruit seed flours. Data are presented as means of three replicates with error bars of standard deviations. The bar with superscript indicated a significant different between different temperature and different superscripts indicated significant differences between samples at the same temperature ($P<0.05$).

The swelling power indicates the capacity of the flour to absorb and retain water (Figure 2). The results demonstrated significant differences at all samples. At 85 and 95°C, N-JSF exhibited the highest swelling power, significantly differing from all the modified JSF. This results were similar to the study of lotus seed flour [17]. Similar to solubility, the swelling power of each flour sample increased with rising temperature. Notably, N-JSF showed lower swelling power compared to all the modified flours at 55, 65, and 75°C. However, at 85 and 95°C, swelling power of N-JSF was 2-3 times higher than that of the modified JSF samples,

while all the modified flours exhibited a similar swelling power. The results also had no direct correlation between swelling and solubility of lotus seed flour samples in this study. It is worth noting that lotus seed flour consisted of not only starch but also protein, fat, and dietary fibers [17].

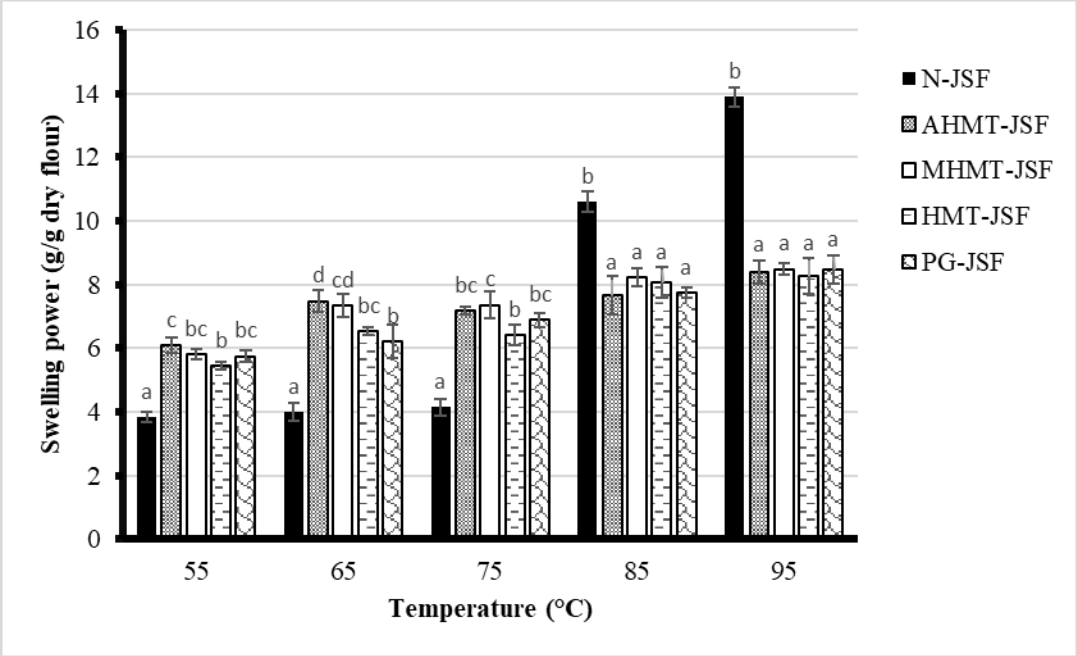


**Figure 2.** Swelling power at different temperatures of native and modified jackfruit seed flours. Data are presented as means of three replicates with error bars of standard deviations. The bar with superscript indicated a significant different between different temperature and different superscripts indicated significant differences between samples at the same temperature ($P<0.05$).

Pasting properties, including peak viscosity, trough viscosity, breakdown viscosity, setback viscosity, final viscosity, peak time, and pasting temperature, are shown in Table 4. The modified methods gave JSF with different pasting characteristics from N-JSF. N-JSF demonstrated the highest values in all the pasting properties compared to all the modified flours. In contrast, the peak time of N-JSF exhibited the lowest values. These resulted was correlated with its swelling power. Flours with higher swelling power would exhibit higher peak viscosity, but lower peak time. In addition, the pasting properties also are depending on the ratio of amylose and amylopectin, and other composition such as protein and lipids. Modified JSF also presented different pasting properties due to the different modification process. These modification processes caused different changes to the starch granules. The lowest peak viscosity was reported in HMT-JSF. This could be due to the limited starch granule dispersion and swelling after HMT process. No significant differences were found on breakdown values between modified JSF samples. The low breakdown means flour is more resistant to heat and shear force which results in more stable cooked paste [32]. Moreover, HMT-JSF, MHMT-JSF and AHMT-JSF showed the lowest trough viscosity, final viscosity and setback. The lower setback indicated their lower retrogradation tendency, which might be useful for application in thickened food products like soups and sauces [32].

**Table 4**. Pasting properties of native and modified jackfruit seed flours.

| Pasting Properties | N-JSF | PG-JSF | HMT-JSF | MHMT-JSF | AHMT-JSF |
|---|---|---|---|---|---|
| Peak viscosity (RVU) | 56.25±1.36$^{a}$ | 15.17±0.58$^{b}$ | 6.50±0.25$^{c}$ | 10.00±0.58$^{ab}$ | 9.06±1.06$^{ab}$ |
| Trough viscosity (RVU) | 42.56±0.48$^{a}$ | 13.25±1.18$^{b}$ | 4.47±0.41$^{c}$ | 7.78±0.55$^{c}$ | 7.42±0.93$^{c}$ |
| Breakdown (RVU) | 13.69±1.35$^{a}$ | 1.92±0.95$^{b}$ | 2.03±0.46$^{b}$ | 2.22±0.60$^{b}$ | 1.64±0.49$^{b}$ |
| Final viscosity (RVU) | 61.31±0.24$^{a}$ | 27.47±1.54$^{b}$ | 14.86±0.48$^{c}$ | 18.33±2.05$^{c}$ | 17.67±0.46$^{c}$ |
| Setback (RVU) | 18.75±0.42$^{a}$ | 14.22±0.65$^{b}$ | 10.39±0.41$^{c}$ | 10.56±1.55$^{c}$ | 10.25±1.23$^{c}$ |
| Peak time (min) | 5.20±0.07$^{c}$ | 6.49±0.32$^{a}$ | 6.42±0.83$^{a}$ | 6.02±0.30$^{bc}$ | 6.73±0.24$^{a}$ |
| Pasting temperature (°C)$^{ns}$ | 68.08±18.94 | 68.30±3.53 | 64.47±6.19 | 61.57±10.24 | 57.05±8.31 |

Values are expressed as mean ± SD. Values with different superscript letters in the same row indicate significant difference at $P<0.05$ using one-way ANOVA followed by Turkey's test. $^{ns}$ means no significant difference.

## 4. Conclusion

The physical modification treatment of the flour altered its nutritional values, physical properties and glycemic index when compared to its native form. All the modified flour exhibited a comparable nutrient composition in terms of protein, carbohydrates, fat, and dietary fiber compared to N-JSF. Regarding physical properties, the color of N-JSF was lighter than all modified JSF samples as well as higher in pasting properties and swelling power. However, modified method could improve water holding capacity of JSF. The study found that N-JSF had a low glycemic index as well as a high fiber content, making it an alternative flour to use in food products for individuals with diabetes. However, future studies should investigate the resistance starch, antioxidant, and bioactive compounds of the flour as well to gain a deep understanding of how each treatment impacts the starch and the additional benefits they may offer.

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# Effect of Preparation Conditions on Physicochemical Properties of Cocoa Pod Husk Powder

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**Abstract**

Cocoa pod husk (CPH) is a by-product from cocoa industry, which is 52-76% by total weight of cocoa fruit. It contains beneficial substances, but there is limited study about its preparation for applying in food products. In this study, preparation conditions such as enzyme, drying time and temperature on physicochemical properties of CPH powder were investigated. CPH was prepared with and without pectinase enzyme, then it was dried in a tray dryer at 60 or 70°C for 4, 5 or 6 h. The CPH powder treated with pectinase enzyme and higher drying temperature and longer drying time decreased the *L** and increased *a** and *b** that represented the darker, more red and yellow color in CPH powder The CPH powder treated with pectinase enzyme showed significantly lower moisture content (3.64-18.56%) with more consistent than those without enzyme (3.81-13.62%) ($P<0.05$). CPH powder prepared by using pectinase enzyme had significantly higher water activity (0.18-0.80) than those of without enzyme (0.19-0.68) ($P<0.05$). However, treated with pectinase enzyme decreased the water and oil holding capacity, but did not affect the total phenolic content of CPH powder. The CPH powder prepared with pectinase enzyme and dried at 60°C for 4 h was the best preparation conditions in this study.

***Keywords**:* Cocoa pod husk; Drying condition, Pectinase; Phenolic content

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## 1. Introduction

Cocoa is one of the ingredients for chocolate that the most popular confectionery. The International Cocoa Organization reported that the cocoa market for the global volume of chocolate confectionery is projected to grow by 1% in 2022 to 2023 [(1)]. Cocoa is also an ingredient for other industries *e.g.* cosmetic and medicine industries. However, only cocoa beans are utilized for the products that contributed waste from other parts of cocoa, especially cocoa pod husk (CPH). The CPH contains high nutritional values, *e.g.* protein, carbohydrate including fiber such as cellulose, hemicellulose, pectin, ash, and lignin. The bioactive compounds: *e.g.* flavonoids, phenolics, methylxanthine and phytosterol, are also found in CPH [(2)]. Although CPH contains high bioactive compounds, it has short shelf-life because of its high moisture content led to easily perishable. Therefore, drying is the way to preserve and extend the shelf-life of CPH for applying into products. Nowadays, there are several drying methods commonly used in the food industry *e.g.* sun drying, oven drying, tray drying, freeze drying, hot air drying etc. Tray dryers are widely used in various industries for the purpose of removing moisture with many advantages. It provides uniform drying across all trays, low cost, energy efficient, precise control over drying conditions with controllable parameters such as temperature, airflow, and humidity. Tray dryer is suitability for many products and handles with diverse materials that contributed to the decrease moisture and help prolong the shelf-life.

In addition, Valadez-Carmona *et al.* [3] has been reported that tray drying can increase total phenolic compounds, flavonoid content, and antioxidant activity of cocoa pod husk flour when compared to fresh cocoa fruit. A thermal process with low temperature (50-60°C) could release phenolic compounds, which are typically found in bound form or attached to the cell walls of fruits and vegetables. The thermal process can contribute to the liberation of these bound phenolic compounds by disrupting the cell structures resulting in phenolic compound degradation to oxidative processes or covalent bond breaking [3].

Nowadays, enzymes have been used for cocoa beans fermentation. They reduced fermentation and drying time of cocoa beans. Moreover, they contributed higher quality cocoa beans after drying [4]. In this study, pectinase was selected because the main composition of CPH is pectin, fiber and other components that related to the functional properties of CPH. Pectinase could digest pectin into small size, which affected to the functional properties. The expectations for applying pectinase in the preparation of CPH powder are to digest pectin and other components that affect the functional properties of CPH powder.

## 2. Materials and Methods

### 2.1 Materials

Fresh cocoa (*Theobroma cacao* L.), TN4 cultivar was purchased from a local farm (Rayong, Thailand). The fresh cocoa fruit was kept in the box at -18°C.

### 2.2 Preparation of cocoa pod husk powder

Cocoa pods husk was separated and cut into 2×2 cm The cocoa pod husk powder without enzyme treatment was prepared by soaking 100 g of cocoa pod husk in 200 mL of distilled water at 60°C in a water bath shaker for 24 h (Memmert, WNB29, Büchenbach, Germany). The mixture was dried in a tray dryer (Progress electronic, Thailand) at 60°C for 4, 5 or 6 h (N604H, N605H or N606H, respectively) and dried at 70°C for 4, 5 or 6 h (N704H, N705H or N706H, respectively).

The cocoa pod husk powder with enzyme (pectinase) treatment was prepared by cocoa pods husk was separated and cut into 2×2 cm. The cocoa pod husk powder with enzyme treatment was prepared by soaking 100 g of cocoa pod husk in 200 mL of 100 ppm pectinase solution at 60°C in a water bath shaker for 24 h. Then, the mixture was dried in a tray dryer (Progress, Thailand) at 60°C for 4, 5 or 6 h (P604H, P605H or P606H, respectively) and dried at 70°C for 4, 5 or 6 h (P704H, P705H or P706H, respectively).

### 2.3 Color analysis

The color of cocoa pod husk powder was analyzed by a CR-400 Chroma meter (Konika Minolta, Osaka, Japan). The Hunter degrees of lightness ($L^*$), redness ($a^*$) and yellowness ($b^*$) values of cocoa pod husk powder were measured.

### 2.4 Determination of moisture content

The moisture content of cocoa pod husk powder was analyzed by AOAC 930.15 (AOAC, 2000). 0.5 g of cocoa pod husk powder was dried at 105°C until constant weight.

### 2.5 Determination of water holding capacity (WHC)

0.3 g of cocoa pod husk powder was weighted in a centrifuge tube. Then 10 mL of distilled water was added, vortexed for 1 min and kept for 16 h. After that, the mixture was centrifuged by a centrifuge at 1,328 (Eppendorf, 5702, Hamburg, Germany) *g* for 30 min. The WHC was reported as g of water per g of cocoa pod husk powder (w/w) [5].

### 2.6 Determination of oil holding capacity (OHC)

0.5 g of cocoa pod husk powder was weighted in centrifuge tube. Then 5 grams of vegetable oil was added, vortexed for 1 min and kept for 16 h. After that, the mixture was centrifuged 1,328 *g* for 30 min. The OHC was reported as volume of oil per g of cocoa pod husk powder (v/w) [5].

**2.7 Determination of total phenolic content**

1 g of cocoa pod husk powder was weighted and 40 mL of the mixture of acetone: water: acetic acid (70:29.5:0.5) was added into a flask. The mixture was stirred on a stir plate (IKA, RT15P, Guangzhou, China) for 1 h in the dark at room temperature. After that, the mixture was centrifuged by a refrigerated centrifuge (Eppendorf, 5804R, Hamburg, Germany) at 14,758 *g* at 4°C for 30 min. The supernatant was collected and kept in amber glass vial and stored at -20°C until use [(3)].

The total phenolic content of the extract was analyzed by using the Folin-Ciocalteu method. The absorbance of the extract of cocoa pod husk powder was measured at 765 nm by a UV1800 UV-Visible spectrophotometer (Shimadzu, Kyoto, Japan). The standard curve of gallic acid concentration was range from 0 to 500 µL/mL. Total phenolic content was reported as mg of gallic acid equivalents (GAE)/1 g dry mass cocoa pod husk powder. [(3)]

**2.8 Statistical analysis**

All experiments were repeated in triplicate. The result was analyzed using One-way analysis of variance (ANOVA) and Duncan's multiple range comparison test (SPSS 26.0, IBM Inc., New York, USA) were performed for the differences between samples which were reported as significant for $P<0.05$.

**3. Results & Discussion**

**3.1 Color**

Color of CPH powder is shown in Table 1. The effect of pectinase enzyme was significantly showed on the color of CPH powder dried at 60°C. *L** and *a** of CPH powder with pectinase and drying for 4, 5 and 6 h decreased and increased ($P<0.05$), respectively. The *b** of CPH powder with pectinase significantly increased only drying at 5 and 6 h ($P<0.05$). The effect of drying temperature showed significantly higher *L** and *a** of CPH powder dried at 70°C than those dried at 60°C in some samples. The effect of drying time obviously showed in increasing *L** of the CPH powder drying at 60°C with and without pectinase. The color of CPH powder dried at 70°C did not show the significantly different on the effect of enzyme, drying temperature, and time because of their darker color. The variety of cocoa is one of the main factors that affects the color of husk. The color of CPH is also affected by the bioactive compounds such as polyphenolic compounds, which accumulate mostly in the exocarp. Moreover, the preparation conditions such as enzyme, drying temperature and time of CPH were the main factors to increase the darker, more red and yellow color in CPH powder dried at 60°C.

**Table 1.** Color parameters of CPH powder prepared by different preparation conditions.

| Samples | L* | a* | b* |
|---|---|---|---|
| N604H | 50.13 ± 0.71$^{f}$ | 8.12 ± 0.55$^{b}$ | 18.67 ± 0.73$^{e}$ |
| N605H | 55.69 ± 0.69$^{c}$ | 5.03 ± 0.20$^{e}$ | 15.53 ± 0.20$^{g}$ |
| N606H | 57.48 ± 0.84$^{b}$ | 6.21 ± 0.30$^{d}$ | 20.06 ± 0.45$^{c}$ |
| P604H | 48.47 ± 0.38$^{g}$ | 8.67 ± 0.15$^{a}$ | 18.59 ± 0.31$^{e}$ |
| P605H | 52.35 ± 0.34$^{e}$ | 6.19 ± 0.20$^{d}$ | 16.83 ± 0.42$^{f}$ |
| P606H | 57.00 ± 0.44$^{b}$ | 6.50 ± 0.35$^{cd}$ | 20.11 ± 0.54$^{c}$ |
| N704H | 54.42 ± 1.25$^{d}$ | 6.18 ± 0.57$^{d}$ | 18.80 ± 0.33$^{e}$ |
| N705H | 60.23 ± 0.95$^{a}$ | 6.40 ± 0.62$^{cd}$ | 21.18 ± 0.70$^{b}$ |
| N706H | 55.88 ± 0.56$^{c}$ | 6.78 ± 0.32$^{c}$ | 19.46 ± 0.32$^{d}$ |
| P704H | 54.31 ± 0.64$^{d}$ | 6.20 ± 0.31$^{d}$ | 18.64 ± 0.27$^{e}$ |
| P705H | 59.80 ± 0.75$^{a}$ | 6.78 ± 0.50$^{c}$ | 21.92 ± 0.61$^{a}$ |
| P706H | 57.04 ± 0.61$^{b}$ | 6.78 ± 0.36$^{c}$ | 19.51 ± 0.39$^{d}$ |

Results are expressed as means ± standard deviation. Different letters in the same column indicate significant differences (P<0.05), The CPH powder samples was dried in a tray dryer at 60°C for 4, 5 or 6 h (N604H, N605H or N606H, respectively) and dried at 70°C for 4, 5 or 6 h (N704H, N705H or N706H, respectively).

### 3.2 Moisture content and water activity

The moisture content of CPH powder is presented in Figure 1. The moisture content of CPH powder prepared with pectinase and dried at 60° C for 4, 5 and 6 h was 18.56, 9.36 and 3.64%, respectively, whereas the moisture content of CPH powder those without pectinase was 13.62, 7.80 and 3.81%, respectively. Moreover, the moisture content of CPH powder prepared with pectinase and dried at 70°C for 4, 5 and 6 h was 3.69, 4.17 and 3.79%, respectively and those without enzyme was 4.45, 4.27 and 4.25%, respectively. The effect of pectinase enzyme showed that CPH powder prepared by using pectinase enzyme had significantly lower moisture content than those of without enzyme (P<0.05). This might be due to the enzyme activity could digest structure of CPH and increasing more surface area that increased the moisture loss during drying. The effect of drying time showed significantly different than drying temperature on moisture content, especially in CPH powder prepared without enzyme. It was found that the longer drying time (6 h) reduced the moisture content much higher than the shorter drying time (4 and 5 h). Some studies reported that moisture of CPH should be 4 - 8% to extend their shelf life [(3)].

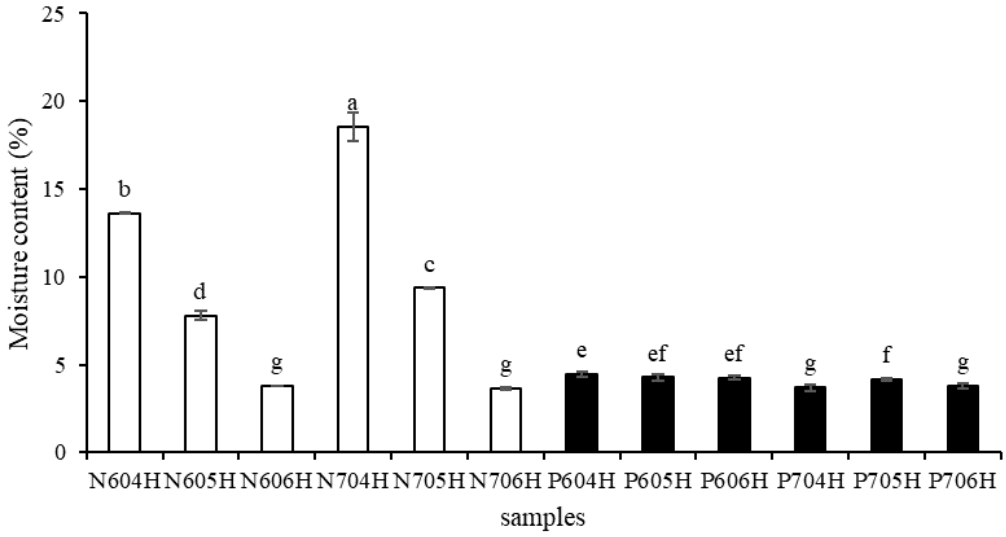


**Figure 1.** Moisture content *(%)* of CPH powder prepared by different preparation conditions. Different letters within each bar indicate significant differences (P<0.05)

The water activity of CPH powder is presented in Figure 3. The effect of pectinase enzyme showed that CPH powder prepared by using pectinase enzyme had significantly higher water activity than those of without enzyme ($P<0.05$). This is because of the 1-4 glucoside linkage in the pectin molecule in the cellulose cell wall was broken by the enzymatic hydrolysis with pectinase, led to release more fluid and increased the water activity while using enzyme compared to those without enzyme [6]. The effect of drying temperature and time tended to decrease the water activity of CPH powder when dried at higher temperature and longer time.

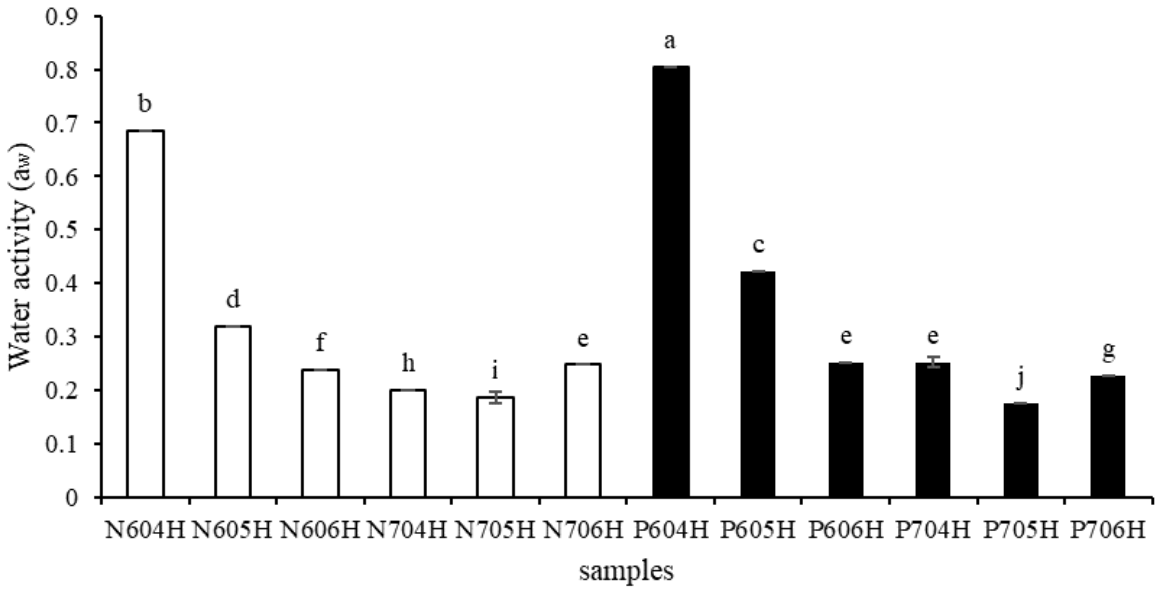


**Figure 2.** Water activity ($a_w$) of CPH powder prepared by different preparation conditions. Different letters within each bar indicate significant differences ($P<0.05$)

### 3.3 Water and oil holding capacity

Water holding capacity (WHC) of CPH powder is shown in Figure 3. The effect of pectinase enzyme showed that CPH powder prepared by using pectinase enzyme had significantly lower WHC than those of without enzyme ($P<0.05$). Data showed that WHC of the CPH has higher WHC when compared to other agricultural co-products used as a dietary fiber source, such as potato peels [7]. According to other research, dried cocoa pod husk has a high hydration capacity and thermal process caused cocoa pod husk cell collapsed and lost of turgor pressure. These resulted in the opening of cavities within the cocoa pod husk which could potentially enhance the retention capacity of the cocoa pod husk [5]. Therefore, the CPH powder showed extremely high WHC property, and it could be used as a replacement for the water-holding agents. Moreover, the increasing drying temperature and time significantly decreased WHC of CPH powder that prepared without enzyme ($P<0.05$), but it did not significantly affect the WHC of those powder prepared with enzyme.

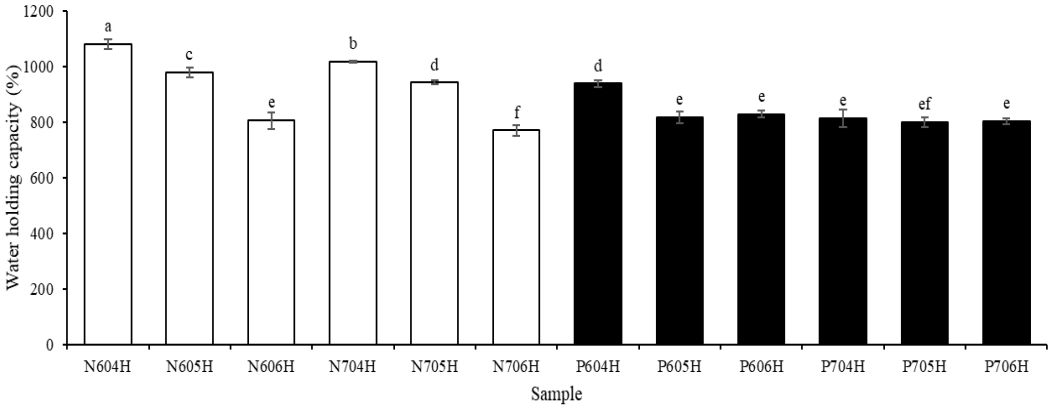


**Figure 3.** WHC of CPH powder prepared by different preparation conditions. Different letters within each bar indicate significant differences (P<0.05)

Oil holding capacity (OHC) of CPH powder is presented in Figure 4. The effect of pectinase enzyme showed that CPH powder prepared by using pectinase enzyme had significantly lower OHC than those of without enzyme (P<0.05) except for N704H. However, the increasing of drying temperature and time did not significantly affect the OHC of those powder prepared with enzyme and without enzyme obviously. According to the high WHC and OHC values of CPH powder, it has potential to apply to food products.

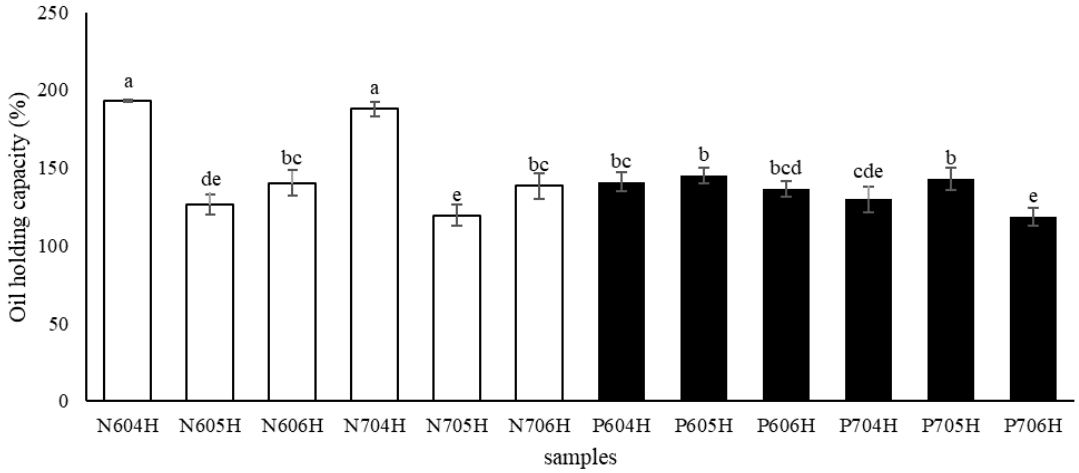


**Figure 4.** OHC of CPH powder prepared by different preparation conditions. Different letters within each bar indicate significant differences (P<0.05)

### 3.4 Total phenolic content

Total phenolic content of CPH powder prepared by different preparation conditions is presented in Figure 5. The effect of pectinase enzyme showed that CPH powder prepared by using pectinase enzyme had significantly higher phenolic content than those of without enzyme only in the CPH powder dried at 70°C for 5 and 6 h (P<0.05). Our result agreed to the study of Pardo *et al.* [8], Kunnika and Pranee [9] and Dao *et al.* (2023) [10] who reported that pectinase and pectinase with higher concentration increased the amount of phenolic content. This might be due to the action of pectinase resulted in the releasing of polyphenols from cell structure [10]. The effect of drying temperature and time significantly decreased phenolic content of CPH powder. Especially the CPH powder prepared by without enzyme and drying at 5 and 6 h, the phenolic content significantly decreased when increased drying

temperature from 60 to 70°C and increased drying time from 5 to 6 h (P<0.05). Although, a thermal process with low temperature (50-60°C) could release and break down phenolic compounds from cell wall of fruits and vegetables [3], the drying temperature and time at 70°C for 5-6 h in this study could destroy and decrease the amount of phenolic content.

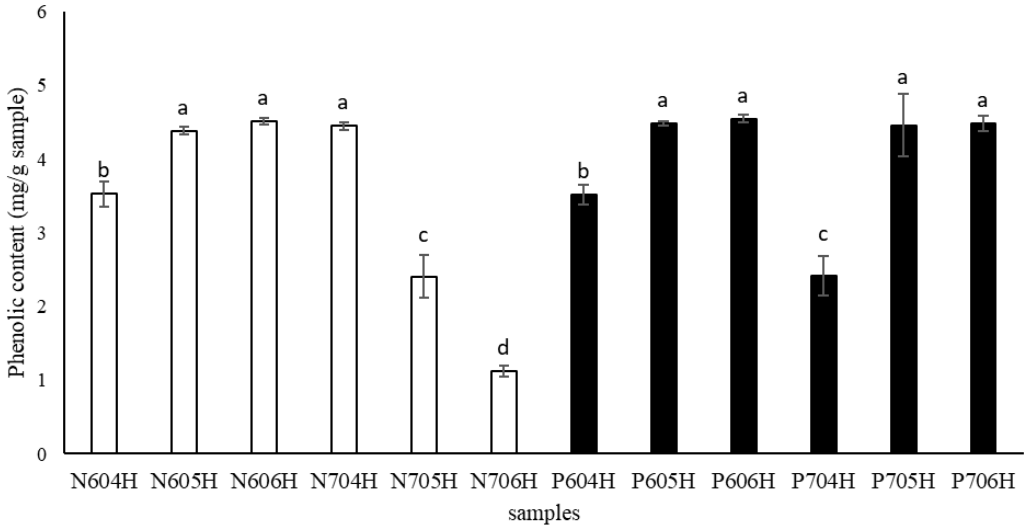


**Figure 5.** Total phenolic content of CPH powder prepared by different preparation conditions. Different letters within each bar indicate significant differences (P<0.05)

## 4. Conclusion

Cocoa pod husk (CPH) is a by-product containing health-promoting bioactive substances. The preparation conditions such as enzyme, drying time and temperature affected the physicochemical properties of CPH powder. Using enzyme and increasing drying temperature and time increased the darker, more red and yellow color in CPH powder. The CPH powder treated with pectinase enzyme showed significantly lower moisture content with more consistent than those without enzyme (P<0.05). CPH powder prepared by using pectinase enzyme had significantly higher water activity than those of without enzyme (P<0.05). However, treated with pectinase enzyme decreased the water and oil holding capacity, but did not affect the total phenolic content of CPH powder. According to the water and oil holding capacity and phenolic content treated CPH with pectinase enzyme and dried at 60°C for 4 h was the best preparation condition in this study and CPH powder has potential to apply in food products due to its high water and oil holding capacity and total phenolic content.

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# Ultrasonic Assisted Extraction of Polysaccharides from Defatted and Deproteinated Tiger Peanut (*Arachis Hypogaea*) Cake

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**Abstract**

Polysaccharides are widely used in the food industry because of their physical and chemical properties. They are the major nutrients remaining in defatted and deproteinated Tiger peanut cakes, which can be transformed into a value-added product such as a food encapsulant. Ultrasonication was used to extract polysaccharides from the Tiger peanut cake. Optimization of extraction was done using a Factorial design with independent variables being amplitude and time. The most effective extraction parameters based on yield were 20 min using an amplitude of 20%, keeping temperature below 40°C. Water activity and moisture content of the dried Tiger peanut cake powder by vacuum oven before extraction were 0.48 and 11.97%db, respectively. Protein content was 7.94% while fat content was 7.71%. The findings from this research will determine the suitability of Tiger peanut polysaccharides as a commercial food encapsulant and provide an avenue for further studies into various other applications in the food and pharmaceutical industries.

***Keywords:*** Extraction; Polysaccharide; Optimization; Tiger peanut cake; Ultrasound

## 1. Introduction

Peanuts, also known as groundnuts, originated in Brazil, South America, but are now grown worldwide primarily for the edible seeds. It plays an important role in the economy of several countries [(1)]. It is used as a major oilseed and a source of protein for vegetarians which also contains multiple health benefits including promoting heart health, antioxidant properties and promoting a healthy gut. Peanuts belong to the botanical family *Leguminosae*, commonly known as legumes [(2)] and they are known to contain a significant amount of dietary fiber which includes oligosaccharides and polysaccharides such as starch and hemicellulose [(3)].

More recently, with the advocation of sustainability and reducing the use of animal proteins, researchers have turned to defatted peanut cakes as an inexpensive source of proteins from plants [(4)]. After the extraction of oil and proteins, the major nutrient remaining is carbohydrate, and the peanut cake is considered waste. From proximate analysis, peanut cake contains a significant amount of polysaccharides, namely starch and other heteropolysaccharides. Oligosaccharides include lactose, maltose, stachyose and sucrose, with the latter having the greatest percentage and only small amounts of monosaccharides; glucose and fructose [(5)]. These carbohydrates can be extracted to obtain polysaccharides with specific properties such as large molecular weight, emulsifying and gel/film forming properties which are suitable for food industrial utilization.

There are several methods used in the extraction of plant polysaccharides: enzymatic digestion, acid hydrolysis, alkali hydrolysis, organic solvents and hot water, with the latter being the oldest and most common [6]. The method of extraction generally depends on the intended use of the extract as it determines the type of polysaccharides obtained. Ultrasonic assisted extraction has become a popular method used by researchers to extract polysaccharides because of its low energy consumption and short extraction time [7]. This type of extraction makes use of acoustic cavitation that is achieved through the production of high power, low-frequency ultrasound waves which travel through a material creating high/low pressure cycles. This leads to local hotspots, heating/cooling rates, pressure differentials and high shear force in the medium. The implosion of the cavitation bubbles results in surface peeling, erosion, particle breakdown, sonoporation and cell disruption, releasing intracellular molecules [8].

Current trends of healthier lifestyles and diets have influenced the search for polysaccharides from novel botanical sources as supplementary alternatives to traditional materials [9]. There is limited research available on the properties and applications of polysaccharides from peanuts and this study is the first about Tiger peanut polysaccharides. Therefore, defatted and deproteinated Tiger peanut cake has the potential to be transformed into a high-value product through suitable extraction.

Certain factors during ultrasound assisted extraction can affect the yield obtained and therefore it is essential to determine the correct combination of parameters to achieve maximum yield [10]. The use of factorial design can help realize because it is deemed highly efficient and allows for the detection of interactions amongst intervention components [11]. The objectives of this study include the use of a full factorial model to optimize the ultrasound assisted extraction of polysaccharide from defatted and deproteinated Tiger peanut cake and to determine the effects of time and amplitude on extraction yield.

## 2. Materials and Methods

### 2.1. Experimental materials and chemicals

Defatted and deproteinated Tiger peanut cakes were obtained from PAUL Trading Company Limited, Chiang Mai, Thailand. The moist cakes were vacuum dried at 55°C until constant weight and pulverized into a powdered form using a grinding mill with a 0.355 mm sieve (mesh number 46) and refrigerated in vacuum bags at 4°C until used [12].

### 2.2 Extraction of polysaccharides

Ultrasound assisted extraction was carried out according to Aguiló-Aguayo *et al* [13] with some modifications. Ultrasonication was performed using a Cole Parmer Ultrasonic Processor at 700 W. Extraction was performed using 2.0 g of dried Tiger peanut cake powder and distilled water (60 mL) in a 100 mL beaker. The beaker was held in the ultrasound chamber for different time periods at varying amplitudes as shown in Table 1. A water bath was used to control temperature, keeping it below 40°C during the entire extraction process.

**Table 1.** Range of independent variables and their corresponding levels.

| Variables | High (1) | Intermediate (2) | Low (3) |
|---|---|---|---|
| Time (min) (A) | 25 | 20 | 15 |
| Amplitude (%) (B) | 60 | 40 | 20 |

### 2.3 Isolation and determination of yield of Tiger peanut cake polysaccharides

The extracted slurry was centrifuged at 4000 rpm for 15 min after which the supernatant was collected and concentrated to one-fourth of its volume using a rotary evaporator at 50°C under vacuum conditions. The concentrated extract was then mixed with ethanol (95%) in a 1:4 ratio (extract: ethanol) and refrigerated at 4°C overnight. The solution was then centrifuged again at 4000 rpm for 15 min, washed with ethanol and the precipitate was collected as crude extract [7]. This was air-dried at 50°C until constant weight and percentage yield was calculated using equation 1.

$$\text{Yield (\%)} = \frac{\text{Weight of dried crude extract (g)}}{\text{Weight of Tiger peanut cake powder (g)}} \times 100 \quad (1)$$

### 2.4 Experimental design

A two variable and three level factorial design (Design Expert Software, Version 6.0.2, Stat-Ease Inc., Minneapolis, MN) was applied to determine the best combination of variables for the extraction of Tiger peanut polysaccharides. The extraction variables considered for this research were time (A) with levels 1 (25 min), 2 (20 min) and 3 (15 min). and amplitude (B) with levels 1 (60%), 2 (40%) and 3 (20%). The design consisted of nine treatments each with three replicates as shown in Table 2.

**Table 2**. Treatments to be used for ultrasonic assisted extraction.

| Std | Time (min) | Power (W) | Number of replicates |
|---|---|---|---|
| 1 | A1 | B1 | 3 |
| 2 | A2 | B1 | 3 |
| 3 | A3 | B1 | 3 |
| 4 | A1 | B2 | 3 |
| 5 | A2 | B2 | 3 |
| 6 | A3 | B2 | 3 |
| 7 | A1 | B3 | 3 |
| 8 | A2 | B3 | 3 |
| 9 | A3 | B3 | 3 |

### 2.5 Statistical analysis

Statistical analysis was done using Design Expert Software (Version 6.0.2, Stat-Ease Inc., Minneapolis, MN). The responses obtained from each treatment were subjected to multiple linear regression. The fit of the model was expressed by the coefficient of determination $R^2$ and significance was established by *F*-test and *P*-value.

## 3. Results & Discussion

### 3.1 Composition of dried Tiger peanut cake

The residual protein and fat content of the peanut cake were 7.94 ± 0.41% and 7.33 ± 0.27%, respectively (Table 3). These values were low owing to prior extraction of oil and proteins. It is necessary to remove unwanted materials such as fats and proteins which may interfere with the polysaccharide extraction process. Fat molecules are non-polar and are therefore immiscible with water. During extraction they can form an emulsion, interfering with the retrieval of the polysaccharide extract. Therefore, it is necessary to remove unwanted materials before the extraction process of Tiger peanut polysaccharides [6]. The water activity and moisture content were well below the levels that support microbial growth, thereby extending shelf life of the dried sample.

**Table 3.** Composition of dried Tiger peanut cake before extraction.

| Chemical composition | Amount (%) |
|---|---|
| Water activity | 0.49 ± 0.01 |
| Moisture content (wb.) | 11.97 ± 0.06 |
| Protein (wb.) | 7.94 ± 0.41 |
| Fat (wb.) | 7.33 ± 0.27 |

### 3.2 Extraction yield

Extraction yields of Tiger peanut polysaccharides were reported in mean values as shown in Table 4. Statistical analysis of the data showed that there were significant differences in the yield obtained between the levels of each variable. Table 4 shows the output of Duncan's multi range test of the significance of time and amplitude on extraction yield. The treatments using 60% amplitude were not significantly different ($P \leq 0.05$) from each other regardless of the time used in the extraction process. The same pattern was observed for the other two levels, 40% and 20%. Similar results could be noted for the time factor. There was no significant difference in extraction yield within a particular time level of, however, the mean yield obtained from each level of time was significantly different ($P \leq 0.05$).

**Table 4.** Effect of ultrasonic amplitude and time on extraction yield.

| Time (min) | Amplitude (%) | | |
|---|---|---|---|
| | 20 | 40 | 60 |
| 15 | $8.61 \pm 0.02^{Aa}$ | $11.28 \pm 0.17^{Ab}$ | $7.68 \pm 0.03^{Ac}$ |
| 20 | $11.65 \pm 0.04^{Ba}$ | $10.73 \pm 0.47^{Bb}$ | $7.38 \pm 0.05^{Bc}$ |
| 25 | $11.62 \pm 0.02^{Ca}$ | $10.10 \pm 0.02^{Cb}$ | $7.27 \pm 0.02^{Cc}$ |

Means with different lowercase letters in the same row and different uppercase letters in the same column represent significant differences ($P \leq 0.05$), in yield using DMRT.

### 3.3 Experimental model

In this study, a three-level full factorial design was employed to determine the parameters which affected extraction yield using ultrasonication. A multi linear regression analysis, shown in Table 5, was performed to determine if the predicted variables; time and amplitude, significantly affected extraction yield. The fitted regression model is displayed by equation 2, showing the regression coefficients, yield (Y) as the measured response associated with each factor combination, 9.59 as the intercept, A and B representing the variables time and amplitude, respectively.

$$Y = 9.59 + 0.073A[1] + 0.33A[2] - 2.15B[1] + 1.11B[2] - 0.25A[1]B[1] - 0.39A[2]B[1] - 0.68A[1]B[2] - 0.30A[2]B[2] \quad (2)$$

**Table 5.** Analysis of variance for the fitted factorial model of extraction of polysaccharides.

| Source | Sum of Squares | DF | Mean Square | F Value | Prob > F |
|---|---|---|---|---|---|
| Model | 83.07056 | 8 | 10.3838204 | 2455.019 | < 0.0001 |
| A | 2.461896 | 2 | 1.23094815 | 291.0298 | < 0.0001 |
| B | 62.34423 | 2 | 31.1721148 | 7369.94 | < 0.0001 |
| AB | 18.26444 | 4 | 4.56610926 | 1079.553 | < 0.0001 |
| Pure Error | 0.076133 | 18 | 0.00422963 | | |
| Lack of fit | 0.00 | 0 | | | |
| Cor Total | 83.1467 | 26 | | | |
| Std. Dev. | 0.065036 | | R-Squared | 0.999084 | |
| Mean | 9.58963 | | Adj R-Squared | | |
| C.V. | 0.678187 | | Pred R-Squared | 0.99794 | |

The regression model for yield of polysaccharides in Table 5 shows the coefficient of determination $R^2$ value to be 0.999084, while the adjusted coefficient of determination $R^2$ value to be 0.998677, indicating a very high degree of correlation between observed and predicted values. The lower the coefficient of variance, the smaller the difference between the residual values and predicted values. A low C.V indicates good precision and reliability of models when predicting experimental values [(7)]. The model F-value is 2455.019, indicating that the model is significant. There is only a 0.001% chance that the value could be as a result of noise. The model terms also have low Prob > F values less than 0.05, which indicates all the model terms are significant, those are amplitude, time and the interaction between them.

*3.3.1 Main and interaction effects*

The main effects are time and amplitude, both of which significantly affected the yield, individually and when combined. Figure 2 shows that yield increases as time increases and the yield obtained from each level of time were significantly different ($P \leq 0.05$). Therefore, longer time has a positive influence on the yield. However, according to Table 6, the F-value for time is 291.03, which is approximately $\frac{1}{25}$ times that of the F-value for amplitude. This indicates that amplitude has a greater effect on the extraction yield than extraction time. Amplitude shows an inverse relationship, as it increases yield decreases significantly ($P \leq 0.05$). A high amplitude could be used but only for a short period of time. Low amplitude and short time are not a favorable combination. An outlier was detected as emphasized (Figure 1). A replicate of standard run number 16 which was a combination of 40% amplitude for 15 min showed a higher value from the other replicates done at those conditions, however, there was no significant difference between the three replicates ($P > 0.05$). A possible explanation for this is the drastic increase in temperature during extraction due to the application of a higher amplitude. One of the challenges, therefore, is to maintain a steady temperature using a water bath throughout the duration of the extraction process.

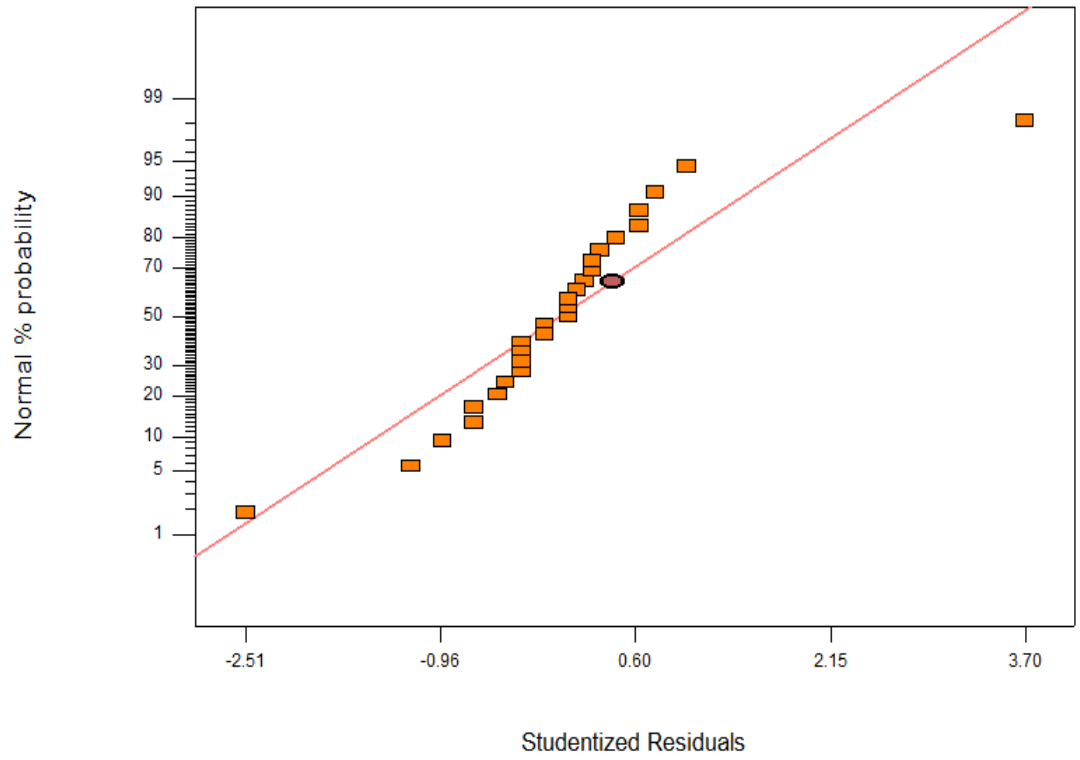


**Figure 1.** Normal plot of residuals

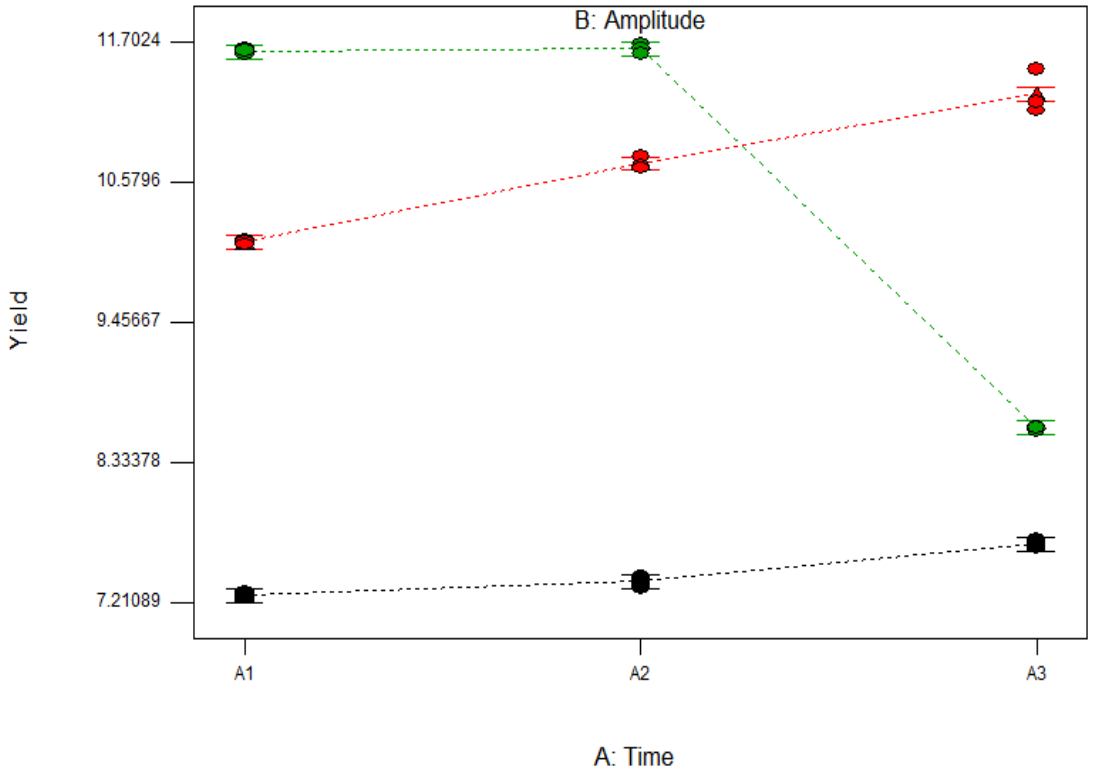


**Figure 2.** Interaction between variables

*3.3.2 Optimization*

A desirability function was applied to optimize the extraction process. This is an objective function ranging from zero to one, values closer to one show high desirability [(14)]. Results indicated that maximum value is achieved by keeping the amplitude low (20%) and the time at 20 min. The optimized yield predicted at 11.65% with a desirability of 0.992. This value is very close to the actual yield obtained from experimental data to be 11.68% as shown in Table 6.

**Table 6.** Model prediction of optimum yield compared to actual yield.

| Values | Optimized process parameters | | Yield (%) |
|---|---|---|---|
| | Time (min) | Amplitude (%) | |
| Predicted | 20 | 20 | 11.65 |
| Actual | 20 | 20 | 11.68 |

High intensity ultrasound produces larger amplitude ultrasonic waves at lower frequencies that can affect the physicochemical properties or even the structure of the material [(15)]. It was reported from previous studies that ultrasonic extraction resulted in up to 70% reduction in molecular weight as well as sonochemical degradation of polysaccharides [(16,17)]. This may explain why the yield was far less using higher amplitudes compared to the lower levels. There is a high possibility that the polysaccharides were broken into smaller structures that could not precipitate when the extract was washed with ethanol.

## 4. Conclusion

Ultrasound assisted extraction and full factorial design were successfully employed to extract polysaccharides from defatted and deproteinated Tiger peanut cakes. The model showed that the two independent variables significantly affected the response values, and this could be employed to extract polysaccharides from Tiger peanut cake using ultrasonic technology. Ultrasonic amplitude had a greater effect on extraction yield compared to the effect of extraction time. Optimum conditions for extraction of polysaccharides from Tiger peanut cake was extraction using amplitude of 20% and extraction time of 20 min. The experimental yield under these conditions was 11.65%. Temperature should be maintained within a short range for all treatments to ensure reproducibility of data. Physical and chemical analysis of the Tiger peanut polysaccharide should be conducted to determine optimum polysaccharide yield.

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