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Research Article

Profiles of volatile metabolite compounds of lotus tempeh: In-RSM-Boxbehken approach

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Abstract: The newest fermented product that uses lotus seeds is tempeh. Processing conditions that vary in generating tempeh products are considered in determining quantity and quality. This research aimed to optimize processing conditions (yeast ratio, soaking, and fermentation time) on lotus tempeh. In this research, the program design used RSM Box-Behnken Design to investigate and select the best combination process for lotus tempeh making. The response of protein and tannin was seen in the range protein value was 5.60% up to 9.80%, while the tannin content was 14.71 to 21.59 % w/w GAE. It was established that the highest protein content was achieved in a 1 g starter, 15 h soaking time, and 36 h fermentation time, whereas the condition of 0.75 g starter, 15 h soaking time, and 48 h fermentation time led to the highest tannin content. The ideal conditions for the production of lotus tempeh have been identified as those that yield a total of 17 amino acids and 46 volatile compounds. Indeed, the findings of this study have indicated that the fabrication of lotus tempeh is most efficiently achieved under specific conditions. Furthermore, the research has suggested that other components hold significant potential as protein and tannin sources, warranting further investigation.

1. INTRODUCTION

The seed of *Nelumbo nucifera* Gaertn, an amphibian plant in the *Nelumbonaceae* that is usually cultivated and eaten in China, is known as Lotus seed or Fragrant pink/white blossoms with expansive and round leaves (Bangar *et al*., 2022; Nainggolan *et al*., 2022). The organisms under consideration inhabit a milieu characterized by oceanic verdure, wherein stagnant or lethargic freshwater conditions prevail. In reality, the lotus can adjust to different sea-going natural surroundings, going from shallow lakes to far-reaching lakes (Gowthami *et al*., 2021). The diet of the subjects had a significant impact on their nutrient levels. A range of nutrients, including dietary fiber, carbohydrates, amino acids, phenols and other trace components, have been identified in Lotus seeds (Nainggolan *et al*., 2022). One of the newest fermented products that used lotus seeds was natto and tempeh. Tempeh is a traditional food which commonly made from soybeans through a fermentation process.

The fermentation process has been demonstrated to enhance the phytochemical content of foodstuffs, including free isoflavones, complex proteins and carbohydrates, and to produce

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probiotics that may prove beneficial to the gastrointestinal tract (Dhull *et al*., 2022; Salman *et al*., 2022). A plethora of studies have been conducted on the health-promoting potential of lotus, encompassing antioxidant, cytotoxic, and immunostimulatory properties (Bangar *et al*., 2022). Ridhowati *et al*. (2022) determined that the isoflavones contained in lotus tempeh have potential as anticancer breasts. According to Reddy *et al*. (2022), there is an overhaul of not only protein, fat, and carbohydrate but also bioactive compounds during tempeh processing. A previous study concluded the stage and time of processing to make lotus tempeh could affect the lotus tempeh quality (Ridhowati *et al*., 2022).

The processing conditions which vary in their capacity to generate tempeh fermentation must be given full consideration when determining quantity and quality. In this research, the program design used RSM Box-Behnken Design for investigating and selecting the best combination process for lotus tempeh making. The advantages of this program can be used for analyzing and modeling a problem with one or more treatments in research (Montgomery, 2012; Ridhowati *et al*., 2022). This method not only defines the effect of the independent variable but also produces a mathematical model, which describes a chemical or biochemical process. In addition, the method has the advantage that it does not require a large number of trials or a protracted period of time (Montgomery, 2012). This research aimed to optimize processing conditions (yeast ratio, soaking, and fermentation time) on tempeh processing by using Response Surface Methodology (RSM)-Box Behnken. This research is expected to be basic information in the process scale-up for lotus tempeh processing with optimized protein, tannin, and volatile profile compounds.

2. MATERIAL and METHODS

2.1. Preparation Lotus Tempeh

The making of lotus tempeh processing was determined by the methods of Ridhowati *et al*. (2022). Lotus seeds (250 g in 2 L of aquadest) were subjected to boiling for 15 minutes, following which they were soaked in soy acid (pH 4-5). The duration of this soaking process was customized according to the parameters outlined in Table 1. The loose seed hulls were removed. The dehulled seeds were washed and weighed with the sample proportion (w/w). The dehulled beans were boiled using aquadest (2 L) for 15 min. All of the formula samples were inoculated exploitation flora spores (*Rhizopus sp*.) to the cooled beans employing a quantitative relation (w/w) in Table 1, and sporulated beans were later on placed in perforated plastic baggage (13 cm x 20 cm) wherein its thickness was 1.5 cm for fungal development. The samples were subjected to the designated formulation during the incubation process (Table 1).

2.2. Proximate Analysis

The proximate and amino acid analyses were conducted according to Horwitz and Latimer (2005).

2.2.1. *Moisture content*

A total of 5 g of sample was weighed quickly into a dry test cup, then homogenized, and dried in an oven thermogravimetry (Memmert UN30) at 105°C for 6 h. Then, it was cooled in a desiccator until it was balanced. The sample was put back in the oven until a constant weight was obtained. The moisture content was calculated using the following formula:

$$\text{Moisture content } (\%) = \frac{b-(c-a)}{b} x100\%$$

where

a = the constant weight of the dry test cup (g)
b = the sample weight (g)
c = the constant weight of dry test cup and dry sample (g)

2.2.2. *Ash content*

A total of 5g samples were weighed and subsequently placed in the furnace (SNOL Merck, SNOL 3/1100) at a temperature of 300°C. The temperature was then increased to 550°C at a rate appropriate to the material characteristics (generally 5-7 hours) until the oxidation of all carbon had been completed. The sample was subjected to a process of evaporation, continuing until the desired consistency of dryness was achieved (a grayish-white appearance). Next, the furnace was turned off and can be opened after the temperature reaches 250°C or less. The ash content in the sample can be calculated using the formula:

$$\text{Ash content } (\%) = \frac{W2-W0}{W1-W0} x100\%$$

where

W_2 = the porcelain cup + sample after ashing (g)
W_0 = the porcelain cup (g)
W_2 = the porcelain cup + sample before ashing (g)

2.2.3. *Fat content*

A total of 5 g of the sample was wrapped in paper Strain, and then covered with cotton wool that had been deprived of fat. The filter paper containing the sample was inserted into Soxhlet extraction kits, and a condenser was installed above it, and the flask fat underneath. A quantity of 80 milliliters of hexane (purchased from Sigma-Aldrich) was added to the flask containing the fat. Subsequently, a minimum of five hours of refluxing was undertaken until the solvent receded into the fat flask and a clear color was achieved. The solvent in the fat flask was then distilled and stored. Then the fat flask containing the extraction results was heated in an oven (Memmert UN30) at 105°C, to evaporate the remaining solvent. Then, it was cooled and weighed until a constant weight was obtained. The calculation of the percentage of fat in the sample can be performed using the following formula:

$$\text{Fat content } (\%) = \frac{Wc-Wa}{Wb} x100$$

where

Wc = the fat flask + fat after extraction (g)
Wa = the fat flask (g)
Wb = Sample (g)

2.2.4. *Protein content*

Protein content was analyzed using Micro-Kjeldahl; 0.5 g samples were added 1.2 g mixed catalyst (Kjeldahl powder, Merck) and 10 mL H_2SO4 (Merck), then boiled in Kjeldahl flask until the solution colored greenish and clear. The solution was subjected to a ten-fold dilution process, whereby 5 mL of the diluted sample was introduced into a distilled flask containing 30% NaOH (Merck) and 1 mL of phenolphthalein (Merck). The neutralization process was conducted for 10 minutes, after which the ammonia gas was collected in a flask containing 2% boric acid (Merck). The nitrogen content of the protein samples was then estimated using titration with 0.01 N HCl (Merck), calculated using the following formula:

$$\text{Protein content } (\%) = \frac{(v1-v2)x\ N\ x\ 14.007\ x\ 6.25}{W} x\ 100\%$$

Where, V_1 and V_2 = titration volume of sample and blank; 14.007 gram is molecular weight (MW) of Nitrogen; N is normality of HCl; and W is the weight of the sample; 6.25 is protein factor for food.

2.2.5. *Carbohydrates by differences*

Carbohydrate content was calculated by the formula
Carbohydrate (%) = 100 % – (protein + fat + moisture + ash)

2.2.6. *Amino Acids analysis*

Fifty milligrams of samples were added to 10 mL HCl 6 M and incubated for 24 h at 100ºC. The aliquot of the sample was filtered and the extract was diluted 200 times with milli-Q water. A 300 mL extract was dried and derivatized with 300 mL of 9-fluorenyl methyl-chloroformate (FMOC). A 20 mL aliquot was analyzed using an analytical scale (4.6 x 250 mm^2) SGE Hypersil ODS C18 column (SGE, Dandenong, Australia) kept at 38ºC and connected to an HPLC system (GBC, Dandenong, Australia) equipped with a fluorescence detector LC 5100. The mobile phases used were as follows: A: 30 mM ammonium phosphate (pH 6.5) in 15: 85 (v/v) methanol/water; B: 15: 85 (v/v) methanol/water; and C: 90: 10 (v/v) acetonitrile/water. The flow rate was 1.2 mL/min and the gradient, wavelength for Fluorescence detection was 270 and 316 nm. A calibration curve was constructed using a mixture of standard amino acids.

2.3. Total Tannin

The tannin contents were measured as Gallic Acid Equivalents (GAE) based on the method of Ahnan (2020); Horwitz and Latimer (2005). Tannin content analysis was carried out using the Folin-ciocalteu method. The principle of the Folin-Ciocalteu method, namely the oxidation of phenolic compounds by the Folin-Ciocalteu reagent produces a blue solution. Standard solutions were prepared at concentrations ranging from 300 to 800 parts per million (ppm) by combining 1 milligram (mg) of gallic acid with 5 milliliters (ml) of methanol, followed by vortexing. The sample solution was ready. The test was made according to the dilution, 1 ml samples were added with distilled water, 1.5 ml 35% Na_2CO_3, and 0.5 ml of 50% Folin-Ciocalteu reagent up to 5 mL dilution, vortexed, and incubated for 30 min in a closed room, and then, the absorbance was measured using a UV-VIS spectrophotometer Wavelength 760 nm.

2.4. The Volatile Compound

The samples were analyzed for their volatile compounds not only lotus tempeh but also commercial tempeh, based on Kustyawati (2017). Then, they were extracted using a headspace solid phase microextraction (SPME)-GC (Gas Chromatography)- MS (Mass Spectrophotometry) method. 50 grams of sample was put into an Erlenmeyer flask, sealed with aluminum foil with a protective seal, and fitted with a space solid phase microextraction gas-tight syringe. The flask was placed in a water bath at 50ºC, wherein the samples would release their volatiles and be absorbed onto SPME for 30 minutes at 50ºC. The HP 5890A gas chromatograph associated with an HP 5970 mass-specific indicator (Hewlett Packard) was used to dissect the unpredictable examples. GC-MS was worked at 70 eV in the EI mode over the reach 35-450 amu, section utilized was BP-5x segment (30 mx0.25 mm) with 0.25 μm film thickness to determine the volatiles (Supelco, Sigma-Aldrich Co.). Helium was utilized as a transporter at a stream pace of 1 ml/minute. The volatiles thus collected were subjected to thermal desorption at a temperature of 250°C for two minutes. Thereafter, the temperature of the desorption apparatus was rapidly increased to 60°C and maintained at this level for five minutes. This was followed by a decrease in temperature to 5°C, at which point the process was continued until the temperature had reached 220°C, a process which took ten minutes. The constituents of the tests were likely distinguished by coordinating their mass spectra with those stored in the PC library (NIST98 and Wiley library).

2.5. Experimental Design

The first research started with the quality of material dried lotus seeds including the proximate, amino acid, and tannin analysis. All of the parameter analyses in this research were replicated 3 times. Then, the second stage determined the optimization of tempeh processing; 1) a

formulation design and response, 2) formulation, 3) response analysis, and 4) optimization (Montgomery, 2012; Ridhowati *et al*., 2022). The subsequent stage of the process is verification, which serves to validate the predicted value of the optimum solution response formula (see Table 1).

Table 1. Experimental design of lotus tempeh processing.

Formula	Factor 1	Factor 2	Factor 3
	Total of starter (g)	Soaking time (h)	Fermentation time (h)
1	1	15	36
2	0.5	15	60
3	0.75	15	48
4	0.75	15	48
5	0.75	15	48
6	0.75	24	60
7	0.5	15	36
8	0.75	24	36
9	1	24	48
10	0.5	6	48
11	0.5	24	48
12	1	15	60
13	0.75	6	36
14	1	6	48
15	0.75	6	60

Total of starter (g) : 0.5 (min)- 1 (max)
Soaking time (h) : 6 (min) - 24 (max)
Fermentation time (h) : 36 (min) - 60 (max)

2.6. Statistical Analysis

All stages of the process were analyzed, and each response (protein and tannin contents) was determined for the purpose of optimization in the Minitab 19.0® program. The program is capable of performing optimization procedures according to both variable and measurement data entered into the response.

Once the optimum process conditions were established, the verification steps for lotus tempeh processing were initiated. This process was analyzed in accordance with the formulation that had been optimized through the utilization of the RSM-BoxBehnken design. The verification process was conducted with two repetitions, followed by a comparison with the predicted values of the response variables from the RSM-Box Behnken design. These values were equipped with a prediction of the value of each response, thereby enabling the assessment of the suitability of the verification steps (Ahnan, 2020; Montgomery, 2012).

3. FINDINGS

3.1. Proximate Analysis

The quality substances of raw materials were determined in Table 2. Based on Table 2, the water content of lotus seed as a sample in this study was 12.48%, the value was slightly higher than standardized in Indonesia SNI 3144:2009 and FAO-WHO CODEX STAN 313-2013 for standards tempeh using soybean seed.

Table 2. Results of analysis and quality requirements of dried lotus seeds (% w/w, wet basis).

Parameters	Soybean Tempeh	Lotus Tempeh	Lotus Seeds
Moisture	61.09 ± 0.55	67.23 ± 0.56	12.48 ± 0.09
Ash	0.84 ± 0.04	1.22 ± 0.02	4.36 ± 0.03
Carbohydrates	5.16±1.18	19.54±0.36	71.02 ± 1.64
Protein	17.23±0.86	9.36±0.12	7.67 ± 1.15
Fat	5.05±0.21	5.79±1.88	4.46 ± 0.36

3.2. Optimization of Processing Conditions with RSM-BoxBehnken Design

The total starter, soaking, and fermentation time were selected as optimization conditions for making this product. As demonstrated in Table 3, the range of protein values was from 5.60% to 9.80%, while the tannin content ranged from 14.71% to 21.59% w/w GAE. The highest protein content was observed in the 1 g starter, 15 h soaking time, and 36 h fermentation time condition, while the condition of 0.75 g starter, 15 h soaking time, and 48 h fermentation time yielded the highest tannin content.

Table 3. The protein and tannin contents of lotus tempeh based on process conditions (% w/w, wet basis).

Formulas	Total of starter (g)	Soaking time (h)	Fermentation time (h)	Protein (%w/w)	Tannin (%w/w GAE)
1	1	15	36	9.80 ± 0.01	21.43 ± 0.23
2	0.5	15	60	6.61 ± 0.01	18.94 ± 0.01
3	0.75	15	48	9.36 ±0.00	21.59 ± 0.16
4	0.75	15	48	9.19 ± 0.01	21.45 ± 0.52
5	0.75	15	48	8.75 ± 0.01	20.44 ± 0.54
6	0.75	24	60	5.08 ± 0.00	15.29 ± 0.06
7	0.5	15	36	8.14 ± 0.00	18.69 ± 0.37
8	0.75	24	36	6.30 ± 0.00	16.61 ± 0.04
9	1	24	48	9.19 ±0.01	19.64 ± 0.04
10	0.5	6	48	7.88 ± 0.01	17.42 ± 0.14
11	0.5	24	48	9.14 ± 0.00	19.36 ± 0.04
12	1	15	60	4.68 ± 0.01	14.71 ± 0.25
13	0.75	6	36	5.43 ± 0.00	15.58 ± 0.42
14	1	6	48	7.22 ±0.00	17.49 ± 0.05
15	0.75	6	60	5.60 ± 0.00	15.74 ± 0.05

Regression analysis (Table 4) was plotted to make three-dimensional response surface. Response protein contents would directly be high along with increased soaking time, fermentation time, and interaction between total starter and soaking time which were indicated by a positive constant value (Figure 1A and Table 4).

Table 4. Analysis model for the protein and tannin contents.

Response	Model	Equation	Significant (p<0.05)	Lack of fit (p<0.05)	R^2
Protein	Quadratic Polynomial	-33.6 - 0.3 A +0.578 B + 1.670 C + 8.68 A*A - 0.01438 B*B - 0.01538 C*C + 0.079 A*B - 0.299 A*C - 0.00324 B*C	0.067	0.057	88.05
Tannin	Quadratic Polynomial	- 49.4 +27.5 A +1.197 B +2.204 C - 0.36 A*A - 0.03284 B*B - 0.01872 C*C + 0.023 A*B - 0.581 A*C - 0.00343 B*C	0.031	0.234	91.2

*A = total of starter; B = soaking time; C = fermentation time

The tannin responses had a positive value (Figure 1B and Table 4) for total starter, soaking time, fermentation time, and interaction between total starter and soaking time. All of the responses would decrease if there was correlation with fermentation time that is indicated by negative constant values.

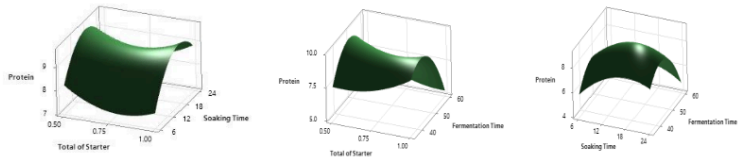

Figure 1A. Response surface plots of protein contents by Total of starter (A), soaking time (B), and fermentation time (C).

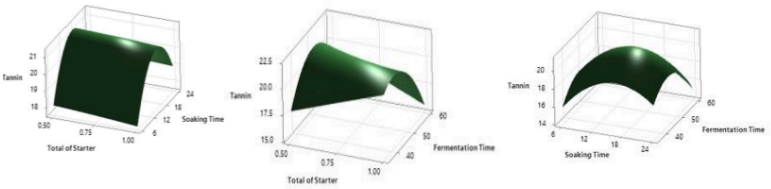

Figure 1B. Response surface plots of tannin contents by Total of starter (A), soaking time (B), and fermentation time (C).

A Box-Behnken response surface methodology (RSM) was employed to optimize the total starter, soaking, and fermentation times. The significance of model parameters was evaluated using analysis of variance (ANOVA) and regression linear (R2) values, and the responses from experimental runs were fitted into a second-order polynomial regression model. Using the desirability principle, the best conditions for protein was in 1 g starter, 15 h soaking time, and 36 h fermentation time, while the condition in 0.75 g starter, 15 h soaking time, and 48 h fermentation time was the tannin contents. The lotus tempeh made in the optimal condition resulted in 17 amino acids and 46 volatile compounds (Table 5). Ridhowati *et al*., (2022) determined that the optimal condition for getting these volatile compounds was 0.5 g starter, 24 h soaking time, and 36 h fermentation time.

Table 5. The profile's chemical lotus tempeh based on the best conditions (% w/w, wet basis).

Samples	Profile's chemical			
	Amino acid (%w/w)		Organic acid (% w/w)*	
Lotus tempeh	Serine	0.44 ± 0.00	Cyclobutanol	2.78
	Glutamate acid	1.05 ± 0.00	Isoamyl-hydride	2.82
	Phenylalanine	0.36 ± 0.00	Acetaldehyde	3.02
	Isoleucine	0.22 ± 0.00	Pentanal	3.71
	Valine	0.26 ± 0.00	Ethyl acetate	4.49
	Alanine	0.35 ± 0.00	Ethene, ethoxy	4.62
	Arginine	0.45 ± 0.00	Ethanol	5.10
	Glycine	0.36 ± 0.00	3-Pentanone	5.62
	Lysine	0.31 ± 0.00	Acetic acid, cyano-	5.98
	Aspartate acid	0.66 ± 0.00	α-Pinene	6.41
	Leucine	0.42 ± 0.00	2-Butanol	6.67
	Tyrosine	0.20 ± 0.00	1-Propanol	6.75
	Proline	0.23 ± 0.00	Dimethylamine	7.16
	Threonine	0.29 ± 0.00	Iso-butanol	7.61
	Histidine	0.16 ± 0.00	3-Pentanol	7.87

Table 5. *Continues*.

	Cystine	0.22 ± 0.00	Isoamyl acetate	8.17
	Methionine	0.01 ± 0.00	β- Myrcene	8.92
			γ-Terpinene	8.98
			α-Ocimene	9.09
			(+)-2-Carene	9.27
			2,3-Epoxybutane	9.41
			Limonene	9.62
			Isoamyl alcohol	9.75
			Furan, 2-pentyl-	10.16
			2-Ethylcyclobutanone	10.37
			Iso-butenyl-carbinol	10.51
			3-Octanone	10.63
			m-Xylene, 5-ethyl-	10.92
			Acetoin	11.19
			Butanal	11.89
			2-Octanol, (R)-	12.15
			2,3-Butanediol	12.23
			1-Hexanol	12.35
			2-Methylcyclohexanol	13.56
			Acetic acid	13.91
			Benzene, 1,2-dichloro-	13.97
			Ethyl 3-hydroxybutyrate	14.11
			2-Anthracenamine	14.47
			Benzaldehyde	15.29
			Iso-butyric acid	15.81
			Caryophyllene	16.49
			Methyl benzoate	16.84
			Naphthalene	18.63
			2-Amino-5-methylbenzoic acid	19.79
			Benzyl alcohol	20.36
			Phenylethyl alcohol	20.88

Note: * (Ridhowati *et al*., 2022)

4. DISCUSSION and CONCLUSION

A comprehensive understanding of the variation step in tempeh production is instrumental in optimizing the quality of the final product, particularly with regard to proximate substances (see Table 2). In addition, the production step of lotus tempeh was optimized for maximum protein and tannin contents using RSM-Box Behnken with three-level-three factor (Table 3), based on Ahnan (2020) who determined that soaking, incubating, and inoculating were important factors in tempeh making.

It has been demonstrated that an increase in fermentation time is associated with a corresponding increase in protein content (Table 3). There was the activity from the fungi which broke down the protein into many free amino acid compounds during the fermentation process (Bahlawan *et al*., 2022; Erkan *et al*., 2020). On the other hand, fermentation was carried out for 60 hours resulting in decreasing tempeh protein levels, a number of proteins were used as a nitrogen source for growth by *Rhizopus sp*. However, Boutas *et al*. (2022) found no significant change in total crude protein after seven days of germination. This was the result of soaking the seeds for 12 hours and then sprouting them in the light at 20°C. Soaking was commonly processed to produce tempeh ranging from 6 to 24 hours, the lotus seed in this step made it easy

to peel due to hydration mechanism. In the fermentation stage, the temperature was maintained at 25 to 30°C for 36 to 48 hours. This process leads to the proliferation of microorganisms, which in turn stimulates the bioavailability of nutrients and eliminates anti-nutrients. Consequently, this results in the promotion of health, as evidenced by the research conducted by Bahlawan *et al*., (2022), Boutas *et al*., (2022) and Ridhowati *et al*., (2022).

The protein and tannin contents varied depending on each treatment point, which determined the importance of processing conditions for tempeh making. All of the responses had the quadratic polynomial (second-order regression equation) calculated by RSM Box-Behnken for evaluation of the correlation between variables and responses (Table 4). The relationship between the two variables of protein and tannin content was demonstrated in three-dimensional surface plots (three-dimensional responses) based on regression equations (see Figure 1A and 1B). These figures demonstrated that the model fitted the experimental data well and was suitable for optimization.

In the study by Riswanto *et al*. (2021), the impact of varying soybean germination times (28-72 h) on the quality of soymilk was examined. The results indicated that a germination time of 28 h resulted in higher protein and total phenolic content. Concurrent research on soybean bioactive components has indicated that a germination time of 45 hours is the optimal treatment to elevate lunasin and reduce lectin and lipoxygenase activity (Acin-Albiac *et al*., 2021; Nahar *et al*., 2023). However, the extant research is limited in its discussion of the soaking conditions required for bean sprouting, particularly in relation to other parameters. Consequently, there is a necessity to optimize the soaking and germination times to achieve the desired qualitative characteristics of soymilk (He *et al*., 2023; Roasa *et al*., 2021).

The extended germination periods may be a contributing factor to the depletion of nutrients (Feng *et al*., 2022; Roasa *et al*., 2021; Zhang *et al*., 2023). This appears reasonable, given that sprouting has a metabolic process involving the breakdown of nutrients, cotyledon expansion, and energy release (Feng *et al*., 2022; Zhang *et al*., 2023). It has been demonstrated that there is a direct correlation between the total protein content and the duration of the soaking and germination processes (Chai *et al*., 2022; Zhang *et al*., 2023). Sprouting causes the formation of a variety of enzymes, which could be involved in the biosynthesis or degradation of complex proteins. Previous research has found that sprouted soybeans and buckwheat had somewhat higher protein content after 72 hours of germination (Zhang *et al*., 2023)

As Zhang *et al*. (2023) highlighted that the soaking time constituted a pivotal process for products derived from beans. It was determined that an increase in phenolic content, including tannin, was observed in products when compared to their dormant states, with an increase of up to 30% being recorded. Furthermore, the protein content was found to be higher in the sprouted samples than in the non-sprouted ones (Ahnan, 2020; Acin-Albiac *et al*., 2021; Boutas *et al*., 2022). These conditions assisted in breaking the complex compounds until more absorbable forms, hence, enhancing nutritional content, digestibility, and overall functionality (Acin-Albiac *et al*., 2021; Boutas *et al*., 2022). Many factors, including soaking time, germination time, temperature and grain variety can affect the quality of sprouted grain (Chai *et al*., 2022; He *et al*., 2023).

Sprouting is a potent way of increasing the phenolic acids in leguminous seeds, as phenolics are biosynthesized and bioaccumulated in germinating seeds, as a response and defense mechanism of seeds to the adverse environmental stress created by sprouting conditions (Ahnan, 2020; Erkan *et al*., 2020). Sprouting had previously been linked to a decrease in amino acid content (Ahnan, 2020; Feng *et al*., 2022; Ridhowati *et al*., 2022). Hydrolysis, synthesis, and rearrangement of protein moieties were thought to be responsible for the variations in amino acid concentration found in sprouted beans during the first 72 hours of sprouting (Ahnan, 2020; Feng *et al*., 2022; Ridhowati *et al*., 2022). The extraction process in the current study by solid-state fermentation (SSF) of wheat by *Rhizopus oryzae* which helped to release the bound compounds from the matrix (Acin-Albiac *et al*., 2021; Ahnan, 2020).

Sprouting prior to processing into a desired product is a cheap and effective pre-treatment for increasing metabolic activity (Ahnan, 2020; Chai *et al*., 2022). The process of autoclaving and fermentation treatments might alter the diversity and contents of phenolic compounds, resulting in an increased release of free compounds, ferulic acid (Bahlawan *et al*., 2022; Feng *et al*., 2022). The improved release of phenolic compounds contributed to the rise in antioxidant capabilities. The pre-treatments using a weak alkali solution could eliminate the condensed tannins up to 86.92% for 10 hours. Following a 60-hour fermentation process, the levels of condensed tannins were found to have decreased to 0.24%. In conclusion, the present study has demonstrated that fermentation can indeed reduce tannins, thus facilitating an increase in phenolic compounds and crude protein (Bahlawan *et al*., 2022; Chai *et al*., 2022; Feng *et al*., 2022).

Glutamic acid, arginine, and aspartic acids were the highest amino acids produced during the fermentation process of lotus tempeh. *Rhizopus oligosporus* produces protease which could degrade proteins into peptides and free amino acids. Furthermore, fungi are capable of facilitating the decomposition of primary substances, thereby generating derivatives or minor compounds, including volatile substances such as ammonia, aldehydes, and ketones. The volatile compounds are instrumental in determining the flavor of the resulting tempeh. The predominant substances in lotus seeds are carbohydrates, and it has been hypothesized that the degradation of these substances may result in the production of more acids. Profile's lotus tempeh, which is produced in optimal conditions, has been found to contain ethanol and naphthalene.

In the research by Kustyawati *et al*. (2017), pointed out that ordinary tempeh produced 23 volatile compounds consisting of alcohols (7 compounds), ketones (2 compounds), furans (2 compounds), fatty acids (4 compounds), esters (1 compound), hydrocarbons (1 compound), sesquiterpenes (2 compounds), benzenoids (3 compounds), and compounds containing sulfur (1 compound). As demonstrated in Table 5, lotus tempeh has 46 volatile compounds, with cyclobutanol and ethanol being the main compounds in profile's organic acids (Ridhowati *et al*., 2022). A tempeh produced from lotus and soybeans has been found to contain 19 identical compounds; cyclobutanol, isoamyl hydride, acetaldehyde, pentanal, ethyl acetate, ethanol, 3-pentanone, 2-butanol, 1-propanol, iso-butanol, 3-pentanol, iso-amyl acetate, limonene, iso-amyl alcohol, 2-pentyl-furan, iso-butenyl-carbinol, acetone, 1-hexanol, and phenylethyl alcohol. According to Kustyawati *et al*. (2017), acids easily evaporate because the long fermentation process produces a lot of acid. The distinctive aroma of tempeh is attributable to the growth of mold and the subsequent breakdown of components in the seeds into simple volatile compounds, including ammonia, aldehydes and ketones.

The presence of fungal mycelia has been observed on the surface of soybeans used in the production of tempeh. These mycelia have been shown to degrade components in the soybeans, resulting in the formation of a specific flavour profile during the fermentation process. Nainggolan *et al*. (2022) determined that the total BAL (*Bacteria Lactate Acid*) in lotus tempeh was higher than soybean tempeh; 6.58 log cfu/gr - 8.59 log cfu/gr, thus, the levels of acid and alcohol were found to exceed the established parameters. During fermentation, microorganisms produce enzymes to hydrolyze substrates into simple materials (sugar) and then convert them into ethanol. Several studies report that ethanol production is produced by microorganisms such as mold, yeast and bacteria. This yeast can grow in media containing simple sugars such as glucose, fructose and mannose. According to Hermanto *et al*. (2020), the presence of ethanol in food products during the fermentation process is attributable to the carbohydrate fermentation stage, which is catalyzed by enzymes to convert carbohydrates into glucose and subsequently into ethanol.

Lotus seeds are widely used in the production of fermented products and tempeh, which has a functional effect. The optimal condition for achieving the maximum protein content was determined using the RSM-Box Behnken method. This condition involved the use of 1 g of

starter, 15 hours of soaking time, and 36 hours of fermentation time, as well as 0.75 g of starter, 15 hours of soaking time, and 48 hours of fermentation time for the tannin content. Consequently, this research offers a promising basis for the efficient production of lotus tempeh, with the caveat that further development is required to explore the potential of alternative components as valuable protein and tannin sources. The research has concluded that the tempeh maker should choose the optimal condition with a high protein content due to tempeh being recognized as a protein source.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors.

Authorship Contribution Statement

Sherly Ridhowati: Investigation, Resources, Visualization, Software, Formal Analysis, and Writing -original draft. **Herpandi Herpandi**: Definition of intellectual content, and manuscript review. **Indah Widiastuti**: Definition of intellectual content, and manuscript review.

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