



## Formula Optimization and Characterization of Soybeans and Pearl Millet (*Penisetum glaucum*) Tempeh Using Simplex Lattice Design Methods

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**Abstract**—Tempeh is a traditional Indonesian fermented food produced through the fermentation of soybeans with mold. Despite Indonesia's reliance on imported soybeans, the country possesses a wealth of locally available grains that have the potential to serve as alternative raw materials for tempeh production. These alternatives remain underutilized and insufficiently explored. The objectives of this research are (1) to optimize formulas using Indonesian indigenous grains (enjelai, sorghum, and pearl millet) as soybean tempeh substitutes, and (2) to characterize the chemical, microbiological, and antioxidant properties of tempeh. The primary response variable used to determine the optimal formulation is protein content, which must comply with the standards outlined in SNI 3144:2015. A Design of Experiments (DOE) was conducted using Mixture Design on Design-Expert 13 software with the Simplex Lattice Design (SLD) method. The optimal formula was selected based on a degree of desirability close to 1, and the model was validated by producing substituted tempeh products according to the resulting equation. The results showed that an 80:20 ratio of soybeans to pearl millet produces tempeh that meets SNI standards. The cubic model adequately explains the relationship between the experimental and predicted data,  $Y = 0.158375X_1 + 0.033704X_2 + 0.0000650X_1X_2 + 0.000016X_1X_2 (X_1 - X_2)$ , where Y is tempeh protein content,  $X_1$  is soybean content, and  $X_2$  is pearl millet content. Pearl millet tempeh contained 15.94% protein, 12.8% fat, 2.47% fiber, 3.05 APM/g of coliform bacteria, and no detectable *E. coli*. However, pearl millet tempeh exhibited low antioxidant properties, as shown by IC<sub>50</sub> values of 2,395.22 µg/mL and phenolic content of 0.43 mg GAE/g.

**Keywords**— enjelai; pearl millet; sorghum; soybean; tempeh substitute

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### I. INTRODUCTION

Tempeh is a traditional fermented product indigenous to Indonesia that has gained global recognition for its various benefits for human health. Often referred to as a 'superfood,' tempeh has been scientifically proven to contribute significantly to human nutrition and well-being. The high content of bioactive components such as isoflavones, proteins, and peptides [1]–[4] has the potential to be hypolipidemic, anti-diabetic, anti-hypertensive, anti-cancer, antioxidant, and anti-inflammatory [5], and modulate intestinal microflora to improve the immune system and nervous system [6]–[8].

According to research by [1] in 2018, tempeh has the potential to be used in handling obesity cases through the mechanism of weight loss and body fat conditions.

Several research have shown that the microbial consortium involved in tempeh fermentation process is dominated by lactic acid bacteria (LAB), yeasts, molds, and nonpathogenic *Klebsiella pneumoniae* shows potential as a paraprobiotic or natural oral vaccine that will stimulate IgA in the human body [6], [9], [10], in addition to other functions as probiotics and prebiotics. The growth of microbial consortium during the tempeh fermentation process also produces GABA (gamma-

aminobutyric acid),  $\beta$ -glucan, and antibacterial components [11], [12].

As an indigenous Indonesian fermented product, knowledge of the tempeh fermentation process has been passed down from the ancestors since 1815, as documented in the classic Javanese literature *Serat Chentini*. Tempe is produced through a solid-state fermentation process with the help of *Rhizopus* spp. mold starter. The processes involved include soaking, epidermis removal, washing, boiling, draining, cooling, inoculation, and incubation at room temperature. During the soaking process, LAB that grow spontaneously begin to participate in the subsequent fermentation involving molds and yeasts, following inoculation with tempeh starter culture. At this stage, the mold mycelium envelops soybeans, altering their texture to become compact and cohesive. In general, tempeh has pleasant characteristics, a savory, distinctive aroma, with a dense and compact texture. This product's origin was able to pass through all boundaries circles regardless of age, gender, economic level, and certain social status.

Soybean as a raw material for tempeh fermentation naturally has many advantages, specifically due to the content of isoflavones, protein, and other important nutrients such as phytic acid and lecithin [13]. The numerous benefits of soybeans, such as medicinal properties, have contributed to their status as one of the most important leguminous crops globally. In Indonesia, this crop is one of the main food commodities after rice and corn. However, the problem that occurs in Indonesia is the limited production of soybeans, thereby relying on importation to meet the needs of processed products such as tofu and tempeh. The production of soybeans in Indonesia in 2020 was 290.78 thousand tons of dry beans, and decreased to only about 212.86 thousand tons in 2021 [14]. The consumption of soybeans in Indonesia in 2021 was 13 thousand tons, and for industrial needs reached 2.8 million tons [15]. The high level of demand has led to high imports of this commodity.

This is certainly worth paying attention to, due to similarly the third strategic commodity after rice and corn, soybeans are a potential source of vegetable protein to maintain food security stability, specifically in fulfilling the need for better nutritious food. Stability in the availability of this commodity and its derivative products, such as tofu and tempeh, is very important to avoid political and economic turmoil for the Indonesian people, who always include tofu and tempeh products in their daily diet.

Indonesia is very rich in sources of beans and grains other than soybeans that have not been well explored, while their nutritional content is equal to soybeans. This is certainly a big challenge to use these commodities as raw materials or soybean substitutes in tempeh fermentation. Indonesian indigenous grain crops that have quite potential to be explored as raw materials for tempeh fermentation consist of enjelai (*Coix lacryma-jobi* var. *ma-yuen*), barley or pearl millet

(*Penisetum glaucum*), and sorghum (*Sorghum* spp.) These grains are widely grown and cultivated in Eastern Indonesia as well as East Nusa Tenggara, Sulawesi, Buru Island, and parts of Java. The utilization of these plants as food ingredients is still very limited, rather than as animal feed. The nutritional content of these grains is similar to soybeans, with the amount of carbohydrates approximately 61-73.4 g, 9.7-11 g protein, 3.3-3.5 g% fat, and dietary fiber 0.9-6.3 g per 100 grams [16].

Previous investigations on non-soybean-like tempeh have shown that various grains can become pure or mixed tempeh ingredients. These non-soybean tempehs are usually termed as "substitute tempeh" and referred to by mentioning the grains before the word "tempeh". Non-soybean tempehs that have been studied include lupin seed (*Lupinus angustifolius*) [17], enjelai seeds (*Coix lacryma-jobi* var. *ma-yuen*) [18], lamtoro seeds (*Leucaena leucocephala*) [11], [19], melinjo seeds (*Gnetum gnemon*) [20], winged bean seeds (*Psophocarpus tetragonolobus* L) [21], pumpkin seeds (*Cucurbita moschata*) and sunflower seeds (*Helianthus annuus*) [22], and ketapang seeds (*Terminalia catappa*) [23].

The objectives of this research are (1) formula optimization of Indonesian indigenous grains (enjelai, sorghum, and pearl millet) as substitutes for soybean tempeh (2) characterizing the chemical, microbiological, and antioxidant properties of tempeh substitutes.

## II. MATERIALS AND METHODS

### A. Material

This research was conducted at the Laboratory of Politeknik Negeri Lampung from May to September 2024. The materials used include soybeans, enjelai, sorghum, and pearl millet seeds, chemical reagents for protein, fat, crude fiber, antioxidant activity, phenolic content, and enumeration media for total coliform and *E.coli*.

### B. Methods

#### Sample preparation

The processing of modified tempeh was carried out based on the procedure outlined by [24], which was modified. The first stage started with sorting soybeans and grains used as substitute materials. Impurities such as gravel, leaves, and twigs, as well as empty/damaged seeds, were removed. Furthermore, the ingredients were washed and boiled. The boiling process of soybeans was carried out for 20-30 minutes at a temperature of 100 °C. Soybeans were soaked in a separate container for 24 hours, and the epidermis was peeled and discarded, then steamed using boiling water vapor for 10 minutes. Meanwhile, other grains are initially boiled with a small amount of water for 5-10 minutes, after which they are steamed following the same procedure used for soybeans. Both ingredients are then cooled and weighed according to the percentage ratio of the treatment that has been determined. The next step was to inoculate 1% commercial tempeh culture starter and stir to spread the mold.

The bean mixtures were then packaged in PP plastic that had been pierced in several parts and fermented for 48 hours.

### Design of Experiment (DOE)

Formula optimization was conducted on the main components, namely enjelai, sorghum, and pearl millet, which will be mixed with soybeans in tempeh formula. DOE was conducted using Mixture Design in Design Expert 13 software with the SLD method. In this research, the fixed variables were protein content, and the independent variables were the types of grains, namely soybean ( $X_1$ ), enjelai ( $X_2$ ), sorghum ( $X_3$ ), and pearl millet ( $X_4$ ) (Table 1).

TABLE 1.  
DOE OF TEMPEH SOYBEAN:GRAINS RATIO

Run	$X_1:X_2$	Protein content (%)	$X_1:X_3$	Protein content (%)	$X_1:X_4$	Protein content (%)
1	0:100	3.8	33.3:66.7	8	0:100	3.45
2	50:50	10.5	75:25	13.16	50:50	11.77
3	100:0	15.9	0:100	3.8	25:75	4.93
4	75:25	12.37	25:75	6.31	0:100	3.50
5	100:0	15.87	50:50	11.19	100:0	15.9
6	66.7:33.3	11.96	66.7:33.3	12.29	100:0	15.88
7	33.3:66.7	8.37	100:0	15.86	100:0	15.89
8	100:0	15.89	100:0	15.89	66.7:33.3	13.44
9	100:0	15.89	100:0	15.87	75:25	15.76
10	25:75	6.31	0:100	3.79	33.3:66.7	8.85

The formula with the main response fit tempeh quality standard was then built as the mathematical model, and the obtained equation was validated in the next experiment

## III. RESULT AND DISCUSSION

### C. Protein content of soybean tempeh substituted

Tempeh formulation was determined using the SLD method, and the protein content is presented in Table 2.

TABLE 2.  
AVERAGE PROTEIN CONTENT

Formulation Soybean: grain ratio	Protein content (%)		
	$X_1:X_2$	$X_1: X_3$	$X_1: X_4$
100:0	15.88	15.76	15.90
75:25	12.37	13.16	15.76
66.7:33.3	11.96	12.29	13.44
50:50	10.50	11.19	11.77
33.3:67.7	8.37	8.00	8.85
25:75	6.31	5.27	4.93
0:100	3.80	3.09	3.45

Table 2 shows the tendency that the higher the number ratio of grain substitution, the lower the tempeh protein content. However, tempeh with pearl millet substitution in a 75:25 ratio produces tempeh protein content that still meets the quality standard, i.e., min 15%. Furthermore, the protein content data

was then analyzed and calculated to predict response variables at optimum formulation using Design Expert 13 software. Subsequently, formula optimization and the construction predicted curve response in the form of a 2D plot were analyzed. Significance tests of response variables and models were conducted at  $P < 0.05$ . After optimization by the SLD method, experiments were added to verify and validate the equation model obtained.

### D. Model analysis of tempeh formulation

The results of the analysis of variance (Table 3) showed that tempeh response variable was significantly influenced the cubic model that used to explain the relationship between experimental data and the prediction data to be performed. This is supported by the high coefficient of determination ( $R^2 = 0.98$ ).

TABLE 3.  
ANALYSIS OF VARIANCE ON TEMPEH PROTEIN CONTENT

Source	Sum of squares	df	Mean squares	F value	P value
Model	251.20	3	83.73	127.69	<0.0001 Significant
Mixture					
A					
soybean	239.21	1	239.21	364.79	<0.0001
B pearl millet					
AB	4.85	1	4.85	7.39	0.037
AB(A-B)	6.54	1	6.54	9.97	0.0196
Std.Dev.	0.8098				
$R^2$	0.9846				
Predicted $R^2$	0.9531				

The following is the equation/model generated to predict the optimum protein content in soybean and pearl millet substituted tempeh.

$$Y = 0.158375X_1 + 0.033704X_2 + 0.0000650X_1X_2 + 0.000016 X_1X_2 (X_1 - X_2)$$

$Y$  = tempeh protein content;  $X_1$  = soybean;  $X_2$  = pearl millet

The positive value (+) in the equation above shows that the components of soybean and pearl millet have a positive influence on the tempeh protein content response. The protein content of tempeh is influenced both by the individual effects of soybeans and pearl millet, as well as by the interaction between the two ingredients. The higher soybean ratio in the formulation will increase the protein content. Dry soybean seeds contain  $\pm 36\%$  protein, 15% carbohydrate, 15% fiber, and 18% fat [25] while pearl millet seed contains 11.6-11.8% protein, 4.8-5% fat, 67% carbohydrate, and 11.3% fiber [26]. During the fermentation process, there will be a decrease in protein content due to the activity of protease enzymes produced by *Rhizopus* spp. molds which will break down tempeh protein peptide bonds into amino acids and short chain peptides [27], [28]. This result is in line with previous research on soybean-enjelai tempeh substitution [18], where a lower

soybean ratio, a lower tempeh protein content were observed. This phenomenon, as well, can be seen in the 2D contour tempeh protein content in **Figure 1**.

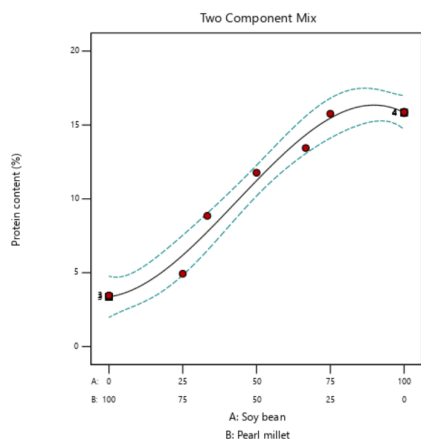


Fig 1. Cubic models on tempeh protein content

**Figure 1** shows the response of protein content from 2 raw materials mixtures in tempeh substitution, where a greater soybean ratio to pearl millet (75:25) will produce higher protein content.

*E. Optimization and validation of soybean and pearl millet substitute tempeh formulation*

The formula optimization obtained the optimum ratio of soybean and pearl millet that produces optimum protein content with desirability level = 1, as shown in **Table 3**.

TABLE 3.

TEMPEH FORMULA OPTIMIZATION

Soybean ratio (w/w)	Pearl millet ratio (w/w)	Protein content (%)	Desirability	
80	20	15.94	1	Selected

The formula optimization with desirability value = 1 or 100% (**Table 3**) shows that the ratio of 80 (w/w) soybean and 20 (w/w) pearl millet was predicted to produce tempeh with 15.94% protein content. Desirability value is the function of optimization objective that describes software's ability to meet the research objectives based on the criteria that have been set for the final product [29]. In this research, the desirability value = 1.00 shows that the tempeh formulation (80:20) ratio was the optimum formula that will produce 15.94% protein content and meet the quality standard.

**Table 4** shows that the experimental results to validate the optimum tempeh formula resulted in 16.03% protein content with 95% accuracy. This value is within a 95% accuracy range between 14.01% to 17.86% of protein content.

TABLE 4.  
 VALIDATION RESULT OF SOYBEAN AND PEARL MILLET TEMPEH

Response variable	Optimum formula (w/w)	Predicted protein content (%)	Validated protein content (%)	accuracy
Protein content	80 soybean 20 pearl millet	15.94	16.035	95%

The appearance of this formulation of tempeh can be seen in **Figure 2**.

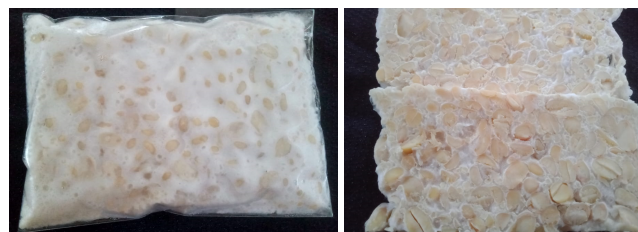


Fig 2. Tempeh with soybean and pearl millet substitution (80:20)

*F. Soybean and pearl millet substitute for tempeh characteristics*

Tempeh made of optimum formulation (80:20 ratio) was analyzed for chemical (protein, fat, fiber content) and microbiological characteristics (total *coliform* and *E. coli*), as well as bioactive components (antioxidant and phenolic content). These attributes were compared with the tempeh quality standard. The results are presented in **Table 5**.

TABLE 5.

CHEMICAL, MICROBIOLOGICAL, AND BIOACTIVE COMPONENT CHARACTERISTICS

Variables	Result	SNI 3144:2015
Protein (%)	16.03	Min. 15
Fat (%)	12.8	Min. 7
Crude fiber (%)	2.47	Max 2.5
IC <sub>50</sub> Antioxidant activity (µg/ml)	2395,22	623.14[30]
Phenolic (mgGAE/g eks)	0.43	43.91[30]
<i>coliform</i> (APM/g)	3.05	Max. 10
<i>E. coli</i> (APM/g)	0	0

**Protein, fat, and fiber content**

The protein content of tempeh is 16.035% and still meets the tempeh quality standard (min 15%), and the content is strongly influenced by the ratio of soybean and pearl millet mixtures. The protein content of soybean seeds ranges from 34%-36% [25] while pearl millet seed contains protein around 9.5%-14.1%% [31]. Soybean protein content decreases during tempeh processing by around 18%-20%. Some factors that

cause a decrease in protein content include the soaking and cooking stages, as well as the fermentation process.

This result is in line with research conducted by [32], which stated that there was a slight decrease in the nutritional value of tempeh compared to soybean seeds, although the nutritional value of tempeh becomes higher due to nutritional components that are more easily digested and absorbed by the body. At this stage, tempeh soluble protein will increase due to the activity of proteolytic enzymes produced by *Rhizopus* spp. as tempeh starter cultures. Similar results occurred in soybean tempeh substituted with enjelai and lamtoro seeds [18], [19]. However, other investigations have reported different results regarding the protein content of tempeh when soybeans are partially substituted with cowpea (*Vigna unguiculata* L. Walp) and lupin seeds (*Lupinus angustifolius*) were higher than other tempeh substitutes, i.e., 17.67% and 19.66%. This is related to the high protein content of cowpea and lupin seeds, i.e.32% and 41% [17], [33].

The fat and fiber content of tempeh was 12.8% and 2.47%, respectively, higher than the tempeh quality standard (min 7% and 2,5%). The fat content of pearl millet seeds ranges from 5%-7.3%, and 2.21%-8.21% fiber content [34]–[36]. Pearl millet substitution in tempeh would be expected to increase the fat and crude fiber content.

#### Total Coliform and *E. coli* content

Tempeh contained 3.05 APM/g *coliform* and 0 APM/g *E. coli*. This number is still below from tempeh quality standard, i.e., min 10 APM/g for total *coliform* bacteria and no detectable *E. coli*. *Coliform* bacteria are microorganisms that can be used as indicators to determine the quality of water sources used during processing. In general, these bacteria are harmless and naturally present in the aquatic environment. Total *coliform* bacteria detected in processing water show that the water is contaminated with plant debris or soil [37]. The low number of *coliform* bacteria and the absence of *E. coli* in tempeh show that the water used in the tempeh processing process is still appropriate for the requirements of water used for food processing.

#### Antioxidant and phenolic components

Tempeh IC<sub>50</sub> antioxidant activity was 2395.22 µg/ml, and the phenolic component was 0.43 mgGAE/g. The IC<sub>50</sub> value shows that it's need 2392.22 µg/ml of extract tempeh concentration is needed to reduce 50% of free radicals from 2,2-Diphenyl-1-picrylhydrazyl (DPPH). This value expresses that the tempeh antioxidant activity was very weak due to less than 500 µg/ml. The level of antioxidant activity can be classified into 4 levels, namely very strong (IC<sub>50</sub> < 50 µg/mL), strong (IC<sub>50</sub>: 50-100 µg/mL), moderate (IC<sub>50</sub>: 101-150 µg/mL), and weak (IC<sub>50</sub>: 250-500 µg/mL) [38]. The results of research by [36] showed that the IC<sub>50</sub> value of pearl millet seed was 2293.66 µg/ml, which suggests that pearl millet does not have antioxidant activity. Other researchers also showed that

the IC<sub>50</sub> value of various varieties of pearl millet ranged from 3900-5230 µg/ml [39].

This antioxidant trait was also shown by the low phenolic component, 0.43 mGAE/g. According to [40] total phenol component is classified as low if it is less than 20 mgGAE/g. The phenolic component contributes to the levels of antioxidant activity in food ingredients. The antioxidant mechanism consisted of the capacity of the phenol component to bind free radicals by contributing hydrogen atoms or electrons to free radicals and forming stable intermediates. This component binds free radicals, decomposes oxidation products, and chelates metal ions [41]. The lower phenolic content is supposedly due to the stages in tempeh processing, which will reduce the bioactive component in the final product. [42,43] state that all treatments in tempeh processing, including soaking [31], dehulling, cooking, and fermentation, led to a reduction in the total phenolic content.

#### IV. CONCLUSION

In conclusion, pearl millet substitution in the tempeh formula with a ratio of 80:20 produces tempeh that still meets the SNI 3144:2015 for soybean tempeh. It contains 15.94% protein, 12.8% fat, and 2.47% fiber, 3.05 APM/g *coliform* bacteria, and no detectable *E. coli*. Tempeh did not have antioxidant activity properties due to its low IC<sub>50</sub> value and phenolic component, namely 2395.22 µg/ml and 0.43 mgGAE/g, respectively.

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#### USE OF ARTIFICIAL INTELLIGENCE (AI) TOOLS STATEMENT

We used Grammarly (Grammarly Inc., 2025) to improve the clarity and grammar of the manuscript. The authors reviewed and approved all changes.

#### CONFLICT OF INTEREST

Authors declare no conflict of interest to disclose

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