



Optimization of solid-state fermentation condition for crude protein enrichment of rice bran using *Rhizopus oryzae* in tray bioreactor

Andhika Sukma^{1,*}, Herawati Oktaviany², Siswo Sumardiono³

¹Department of Chemical Engineering, Faculty of Engineering, University of Lampung, Jl. Prof. Sumantri Brojonegoro No 1, Bandar Lampung 35145, Indonesia

²Department of Agricultural Product Technology, Faculty of Agricultural Technology, INSTIPER, Jl. Petung Road No 2, Sleman, Yogyakarta 55281, Indonesia

³Department of Chemical Engineering, Faculty of Engineering, Diponegoro University, Jl. Prof. Sudarto No 13, Semarang 50275, Indonesia

*Corresponding author: andhika.cahaya@eng.unila.ac.id

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ABSTRACT Enhancement of crude protein content in rice bran with the solid-state fermentation method in tray bioreactor using *Rhizopus oryzae* FNCC 6011 has been investigated. This research aimed to optimize the fermentation condition using the response surface methodology (RSM). The central composite design (CCD) with three independent variables, including substrate thickness (1 to 3 cm), fermentation temperature (28 to 32 °C), and nutrient concentration of KH_2PO_4 (2 to 6 g/L) used to determine the crude protein enrichment. The quadratic model has successfully described the effect of variable interactions on responses very well as indicated by the F value and p-value are 11.20 and 0.0041, respectively. The multiple correlation coefficients (R^2) of 0.9438 indicated that 94.38% of the model data has approached the actual data with a deviation of 5.62%. The interaction between the variable substrate thickness and the fermentation temperature is the most influential variable on the crude protein enrichment of rice bran, indicated by the highest F value of 24.08 and the lowest p-value of 0.0027. The highest protein increase of 62.51% was obtained at 2 cm substrate thickness, fermentation temperature of 30 °C, and KH_2PO_4 concentration of 4 g/L.

KEYWORDS Rice bran; solid-state fermentation; *Rhizopus oryzae*; response surface methodology

1. Introduction

Ritala et al. (2017) has predicted that the global need for protein in 2050, in case of animal protein such as meat and milk, is estimated to reach 1,250 million tons. However, the increase in animal protein needs will not be met continuously due to the low conversion rate of feed into meat and dairy products Ritala et al. (2017). To produce 1 kg of animal protein, it requires more resources, which are eighteen times more land and ten times more water than to produce 1 kg of vegetable protein (Sabaté et al. 2014). The protein from fungi is the best protein alternative to meet the global needs in the future since it requires little resources, including land and water. Several fungal strains have been used for the development of protein production, including *Aspergillus*, *Saccharomyces*, *Fusarium*, *Candida*, *Trichoderma*, and *Rhizopus* (Anupama and Ravindra 2001; Nasser et al. 2011). *Rhizopus oryzae* is a Zygomycetes filamentous fungus commonly found in Indonesia, Japan, and China to produce food or alcoholic beverages. The fungus *R. oryzae* is very suitable for the production of high protein foods with a low nutrient substrate because its abil-

ity to produce polysaccharide-breaking enzymes including cellulase, xylanase, pectinase, and amylase (Chui Yu and Hang 1990; Bakir et al. 2001; Hamdy 2006; Chellapandi and Jani 2008; Karmakar and Ray 2011). The content of the essential amino acids lysine, valine, leucine, isoleucine, threonine, arginine, and omega 3 fatty acids in rice bran substrates and various other agro-industrial by-products fermented using *R. oryzae* is known to have increased (Ibarruri and Hernández 2018; Denardi-Souza et al. 2018).

Rice bran is the outermost layer of rice grains that are released during the rice milling process. Rice bran is used as an animal feed with a high proportion of 90% and the remainder is extracted to produce rice bran oil (Schramm et al. 2007). Besides containing macronutrients such as protein, fat, and dietary fibre, rice bran is also known to contain micronutrients such as minerals and vitamin E, so it is suitable to be used as a substrate in the production of high protein foods (Xu et al. 2001). Utilization of rice bran as a high protein food is still rare. Hence, the substitution of food is a maximum of 10% due to the insoluble fibre content of rice bran >20% consist-

ing of cellulose and hemicellulose in almost the same ratio so that it affects the aroma, flavour, and texture of food (Qi et al. 2015). One of the attempts for increasing rice bran protein content while decreasing insoluble fibre content is by solid-state fermentation. This technique has been proven to produce higher yields lower energy, less wastewater, and environmentally friendly compared to submerged fermentation (Pandey 2003; Martins et al. 2011). Fermented rice bran by *R. oryzae* with solid-state fermentation method has been shown to have increased protein content, aroma, flavour, and texture so that it can be widely used as food (da Silveira and Badiale-Furlong 2009; Oliveira et al. 2010; Sukma et al. 2018).

The response surface methodology (RSM) is used to model a certain number of variables aimed at optimizing the response. To describe biochemical processes using mathematical models, RSM provides accurate results in a short time with a few experiments (Baş and Boyaci 2007; Firatligil-Durmus and Evranuz 2010). RSM has been used in the fermentation process, among others, to produce L-asparaginase, laccase, and protease enzymes with high productivity through several influential variables so that the production process becomes more efficient (da Cunha et al. 2018; Sondhi and Saini 2019; Suberu et al. 2019). There are limited studies on the optimization of crude protein enhancement in rice bran using the *R. oryzae* fungus with the RSM method, so it is not known the exact parameters for scale-up production. Previous research only study on the kinetics of rice bran fermentation where there was an increase in crude protein content of rice bran by 58.5% after 120 h of fermentation using *R. oryzae* (Sukma et al. 2018). To produce high crude protein on a large scale and reduce production costs, studies are needed to optimize the operating conditions for fermentation. This information is needed as a basis for consideration for developing rice bran on a commercial scale to be widely applied as a functional food ingredient. Therefore, this study aims to optimize the enrichment crude protein of rice bran using *R. oryzae* with the solid-state fermentation method. The variables to be investigated are substrate thickness, fermentation temperature, and nutrient concentration of KH_2PO_4 . These parameters affect the mass and heat transfer rates in the fermentation medium, the biomass growth rate, and the amount of product on a larger production scale (Mitchell et al. 2006).

2. Materials and Methods

2.1. Rice Bran

The material used was fresh rice bran obtained from the rice milling machine in Teter Village, Simo Subdistrict, Boyolali Regency, Central Java, Indonesia. To eliminate the rice husk and broken rice that follow, rice bran was sieved using a 35 mesh sieve (Retsch AS200, Haan, Germany). The rice bran was put into a 3 L polypropylene box and stored at 4 °C to prevent enzymatic reactions (Sukma et al. 2018).

2.2. Inoculum Preparation

R. oryzae (FNCC 6011) was obtained from the Food and Nutrition Culture Collection (FNCC) of Food and Nutrition PAU, Universitas Gadjah Mada, Indonesia. Culture in ampoules was transferred into a petri dish containing potato dextrose agar (PDA, Merck KGaA, Darmstadt, Germany) and incubated for seven days at 30 °C (Oliveira et al. 2010). Fungal culture and formed spores were taken using sterile tweezers and dissolved in 100 mL of Tween 80 (0.2%) solution (Merck KGaA, Darmstadt, Germany) to produce spore suspension. Spore concentration was measured using the total plate count (TPC) method. The spore suspension was put into a 100 mL sterile glass bottle and stored at 4 °C until used.

2.3. Fermentation

Fermentation was carried out with the solid-state fermentation method using the tray bioreactor (plastic material, unperforated, unagitated, and unmixed). The tray bioreactor with a size of 21 cm × 12 cm × 4 cm, 16 cm × 8 cm × 4 cm, and 14 cm × 6 cm × 5 cm was used in this study. Each 100 g of rice bran was put into the tray bioreactor of different sizes so that the thickness of the rice bran substrates 1, 2, and 3 cm was produced. The tray bioreactor containing rice bran was sterilized in an autoclave (Hirayama HVE-50, Tokyo, Japan) at 121 °C for 30 min. Two grams of sample was taken from each tray bioreactor for protein content analysis followed by an additional of 45 mL of nutrient solution (KH_2PO_4 2, 4, and 6 g/L; MgSO_4 1 g/L; and $(\text{NH}_4)_2\text{SO}_4$ 8 g/L in 0.4 N HCl) (Merck KGaA, Darmstadt, Germany) into tray bioreactor which contains rice bran substrate. *Rhizopus oryzae* spore suspension was added to the rice bran substrate with an initial concentration of 4×10^6 spores/g of rice bran (Oliveira et al. 2010). The sterile distilled water was added to the rice bran substrate until the moisture content becomes 55% (Yunus et al. 2015). The rice bran substrate was further incubated in an incubator (Heraeus B6060, Hanau, Germany) for 120 h with varying temperatures 28, 30, and 32 °C using forced air circulation. Samples were taken after fermentation was completed for analysis of protein content.

2.4. Protein Content Determination

The total protein content can be described as total nitrogen content using the Kjeldahl method (AOAC. 2000, no. 955.04C). The conversion factor used is 6.25 (Oliveira et al. 2010). The increase in protein content is calculated using the following equation.

$$PE = \frac{(FRB - UFRB)}{UFRB} \times 100\% \quad (1)$$

Where, PE : Protein Enrichment (%), UFRB : Protein content of unfermented rice bran (%), FRB : Protein content of fermented rice bran (%).

2.5. Experimental design

Experimental design of protein enrichment in rice bran by *R. oryzae* using the solid-state fermentation method was

obtained based on the determination of influential variables such as substrate thickness, fermentation temperature, and nutrient concentration KH_2PO_4 . These parameters were chosen because they are considered the most important in the process of protein enrichment by the solid-state fermentation method. Central composite design (CCD) at three levels (-1, 0, and +1) designated as high, medium, and low was used for this study. This study was conducted using the Design Expert 7.0.0 program. Central composite design (CCD) has been used to test the effect of interaction between the variable in the fermentation process. This method is also very suitable for matching quadratic surfaces, for optimizing parameters that affect the minimum number of experiments, and the ability to predict model data with sufficient accuracy compared to Box-Benken, Plackett-Burman, and Taguchi designs (Myers et al. 2002; Rakić et al. 2014; Marrubini et al. 2020).

Three critical steps to carry out optimization based on RSM (response surface methodology) were experimental design with a statistical approach, mathematical modeling, and prediction of responses, which then produced the desired model. Mathematical equation models were tested using an analysis of variance (ANOVA) with a 99% confidence level. The output produced by RSM was contour images and 3D graphics that show the optimal variables and the variables that most influence protein enrichment. According to CCD, the total number of combination experiments is $2^k + 2k + n_c$, where k is the total number of independent variables and n_c is the number of experiments at the center point. The combination of different variables produces 16 trial runs consisting of 8 factorial points (1-8), 6 axial points (9-14), and 2 repetitions at the center point (15,16). The second-order model was created to test the interaction of independent variables on response. The basis for the formation of the polynomial equation is shown in Equation 1, where Y is the response, β_0 is the intercept, β_i is a linear coefficient, β_{ii} is the quadratic coefficient, β_{ij} is the interaction coefficient, and $X_i X_j$ is the independent variable (Singh et al. 2020).

$$Y = \beta_0 + \sum_{i=1}^3 \beta_i X_i + \sum_{i=1}^3 \beta_{ii} X_i^2 + \dots \sum_{i < j} \beta_{ij} X_i X_j \quad (2)$$

TABLE 1 Experimental range and coded levels of process variables for crude protein enrichment.

Process variables	Range and Levels		
	-1	0	1
Substrate thickness (X_1)	1	2	3
Fermentation temperature (X_2)	28	30	32
Nutrient concentration of KH_2PO_4 (X_3)	2	4	6

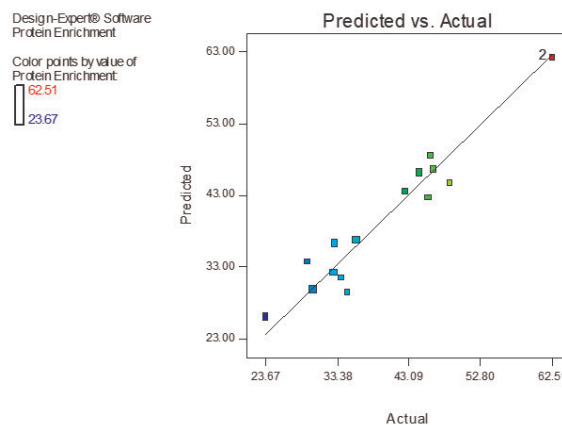


FIGURE 1 The correlation coefficient between model data and experimental data.

3. Results and Discussion

3.1. Central composite design (CCD)

The response of protein enrichment (%) as a function of substrate thickness, fermentation temperature, and nutrient concentration of KH_2PO_4 has been evaluated using CCD. The study design and response are shown in Table 2. Mathematical models are obtained using Design Expert 7.0.0. The crude protein content in rice bran before fermentation has been obtained at 12.59% so that the crude protein enrichment as a response is determined based on Equation 1.

Analysis of variance (ANOVA) was carried out using Design Expert 7.0.0 to evaluate the effect of interactions between variables. Table 3 shows the ANOVA for the quadratic equation model on the response surface (partial sum of squares) and the response for protein enrichment. From Table 3 it can be seen that the F value and $\text{prob} > F$ are 11.20 and 0.0041, respectively, indicating that the model is significant. The multiple correlation coefficients (R^2) of 0.9438 indicated that 94.38% of the model data had approached the actual data and only a deviation of 5.62% of the variables was used. The model indicates to be accurate if the R^2 value exceeds 70%. Therefore, it can be concluded that the value estimated by the model is close to the value obtained from the experimental results (Haaland 1989). The most dominant factor in this study was the interaction between thickness substrate and fermentation temperature with the highest F value of 24.08 and the lowest p -value of 0.0027. The regression line in Figure 1 shows the best prediction of the predicted value against the actual value. In fact, there is always a residual value which is a deviation of certain points from the regression line (predictive value) (Anggoro et al. 2019).

Experimental equations of protein enrichment as a function of substrate thickness, fermentation temperature, and nutrients concentration of KH_2PO_4 are presented below:

$$\begin{aligned}
 \text{Protein Enrichment (\%)} = & -2298.1399 + 154.8928X_1 \\
 & + 144.2289X_2 + 20.5514X_3 \\
 & - 3.6337X_1X_2 - 0.4562X_1X_3 \\
 & - 0.2931X_2X_3 - 10.7274X_1^2 \\
 & - 2.2642X_2^2 - 1.3127X_3^2
 \end{aligned} \quad (3)$$

3.2. Response surface methodology (RSM)

The relationship between responses and variables is described using response surfaces by following the model that has been made. Figures 2, 3, and 4 show the interactions between variables on the response of protein enrichment using 3-D graphs.

Figure 2 shows the interaction variable between a substrate thickness and fermentation temperature on the protein enrichment of rice bran. The interaction variable between the substrate thickness and the fermentation tem-

perature is the most influential factor to increase the protein content of rice bran. Rice bran substrate during the fermentation process has an increase in temperature this is due to the metabolic activity of the fungus *R. oryzae*. The highest protein enrichment of rice bran was obtained at the substrate thickness of 2 cm and the fermentation temperature of 30 °C with the nutrient concentration of KH_2PO_4 4 g/L (Mitchell et al. 2006; Chen 2013). In this condition, the organic compound produced by *R. oryzae* which consists of amino acids and polysaccharide degrading enzymes such as cellulase, xylanase, pectinase and, amylase reach a maximum limit (Chui Yu and Hang 1990; Bakir et al. 2001; Chellapandi and Jani 2008; Karmakar and Ray 2011). In addition to extracellular compounds, the metabolic products of *R. oryzae* are water vapour and carbon dioxide gas. These gases will diffuse from the sub-

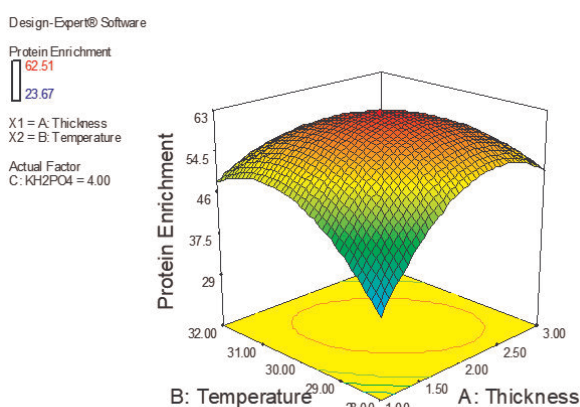


FIGURE 2 3D graph of the effect of substrate thickness and fermentation temperature on protein enrichment.

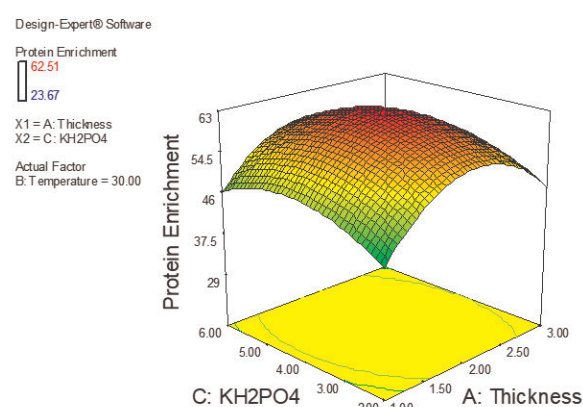


FIGURE 3 3D graph of the effect of substrate thickness and nutrients concentration of KH_2PO_4 on protein enrichment.

TABLE 2 Experimental design and response using CCD.

Run	Substrate Thickness (cm)	Fermentation Temperature (°C)	Nutrient KH_2PO_4 (g/L)	Crude Protein Enrichment (%)	Crude Protein (%)
1	2.00	30.00	7.36	45.99	18.38 ± 0,06
2	1.00	28.00	2.00	23.67	15.57 ± 0,12
3	3.68	30.00	4.00	29.39	16.29 ± 0,08
4	1.00	32.00	6.00	42.57	17.95 ± 0,06
5	3.00	28.00	6.00	46.47	18.44 ± 0,07
6	2.00	30.00	4.00	62.51	20.46 ± 0,09
7	1.00	28.00	6.00	33.92	16.86 ± 0,15
8	3.00	28.00	2.00	48.61	18.71 ± 0,12
9	2.00	30.00	4.00	62.51	20.46 ± 0,09
10	3.00	32.00	2.00	32.88	16.73 ± 0,09
11	0.32	30.00	4.00	30.10	16.38 ± 0,10
12	2.00	30.00	0.64	44.48	18.19 ± 0,11
13	2.00	33.36	4.00	32.96	16.74 ± 0,08
14	1.00	32.00	2.00	45.75	18.35 ± 0,05
15	2.00	26.64	4.00	35.98	17.12 ± 0,12
16	3.00	32.00	6.00	34.79	16.97 ± 0,15

Values are expressed as means ± standard deviation

strate into the environment consequently the water content of the substrate will decrease. In this study, the incubator was set with varying temperatures of 28, 30, and 32 °C using forced air circulation. The thinner the substrate layer, the faster the rice bran substrate temperature loss, resulting in the rate of transfer of O₂, CO₂, and H₂O gases will be greater and the water content of the substrate decreases rapidly, this affects the speed of excretion and activity of these enzymes. Likewise, if the substrate is getting thicker, the substrate temperature will increase rapidly, this causes the growth of the fungus *R. oryzae* to be inhibited, as well as the rate of transfer of O₂, CO₂, and H₂O gases to be inhibited (Chen 2013; Mitchell et al. 2006; Dutt and Kumar 2014).

R. oryzae generally grow in the temperature range of 30-35 °C, with a maximum survival limit to temperatures of 45 °C (Lennartsson et al. 2014). Rice bran is known to contain insoluble fibre >20% consisting of cellulose and hemicellulose. To convert these complex compounds into simpler compounds, cellulase and xylanase enzymes are needed. The activity of cellulase and xylanase enzymes in the agro-industrial substrate containing lignocellulose by solid-state fermentation method is known to be maximal at 30 °C (Ezeilo et al. 2020). Rice bran is an agro-industrial waste, so that the optimum temperature for breaking down cellulose and hemicellulose into simple sugars for the formation of biomass and protein is at 30 °C.

The addition of potassium and phosphate elements in fermentation media is expected to be a cofactor in several enzymes produced by microbes to degrade polysaccharides into monosaccharide and as forming elements of nucleic acids, nucleotides, and phospholipids in *R. oryzae*

TABLE 3 ANOVA for the response surface quadratic model, Responses: protein enrichment (%).

Source	Sum of Squares	DF	Mean Square	F Value	Prob >F
Model	1764.64	9	196.40	11.20	0.0041
A (Thickness)	17.92	1	17.92	1.02	0.3511
B (Temperature)	0.23	1	0.23	0.013	0.9132
C (KH ₂ PO ₄)	6.44	1	6.44	0.37	0.5667
AB	422.53	1	422.53	24.08	0.0027
AC	6.66	1	6.66	0.38	0.5604
BC	11.00	1	11.00	0.63	0.4586
A ²	1066.90	1	1066.09	60.77	0.0002
B ²	759.91	1	759.91	43.32	0.0006
C ²	255.43	1	255.43	14.56	0.0088
Residual	105.26	6	17.54		
Lack of fit	105.26	5	21.05		
Pure Error	0.000	1	0.000		
Cor Total	187.90	15			
Std.Dev.			4.19		
R ²			0.9438		
Adjusted R ²			0.8595		

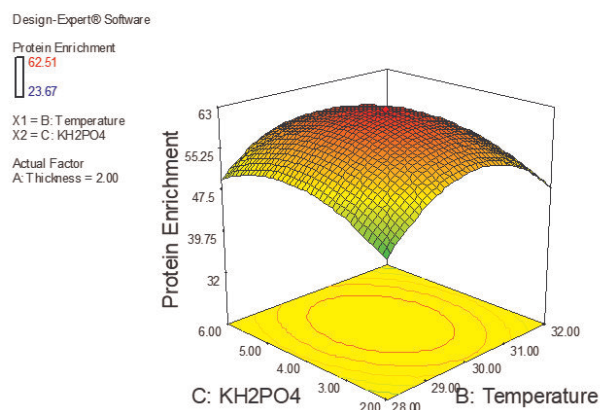


FIGURE 4 3D graph of the effect of temperature fermentation and nutrient concentration KH₂PO₄ on protein enrichment.

cells (Shuler and Kargi 2002). Phosphate is also used as an energy source for microbes, where the more phosphate is obtained, the ATP (adenosine triphosphate) formed during the fermentation process will also increase. ATP is used in the process of cell metabolism, among others, for the growth and development of biomass and the excretion of organic compounds out of cells (Rubio-Arroyo et al. 2011). Increasing nutrient concentration of KH₂PO₄ from 2 g/L to 4 g/L has been shown to increase protein content in the medium, while the addition of nutrients up to 6 g/L, an increase in protein content decreases. The phenomenon is caused by the elements of potassium and phosphate used for the initial phase of the growth of biomass cells. The more KH₂PO₄ biomass cell growth at the beginning of fermentation the faster it is so that the results of cell metabolism are preferred for the growth and development of biomass cells, compared to the excretion of organic compounds (Mohammadi et al. 2013; Das and Ghosh 2014; Deepthi and Satheeshkumar 2017).

Based on these results, it can be seen that the KH₂PO₄ concentration of 4 g/L is the best choice in the process of rice bran fermentation using the fungus *R. oryzae*. Enhancement of protein content in this study was 62.51%, indicating that it is 6.85% higher than previous studies using the same substrate. The same phenomenon also occurs in the fermentation of glucose into ethanol using *Mucor indicus*, where the highest ethanol yield is obtained by using KH₂PO₄ concentration of 3.5 g/L, the higher the KH₂PO₄ concentration the greater the energy lost during the fermentation process this makes the metabolic process to be inefficient (Aghbashlo et al. 2017). Most bacteria and fungi can live and carry out metabolic processes well at KH₂PO₄ concentrations reaching 5 g/L (Vogel and Todor 2014).

The results showed that the variables selected for the fermentation process affected the crude protein enrichment in rice bran. The interaction between the variable substrate thickness and fermentation temperature has the strongest effect in this experiment. This is the focus of

attention for further studies when using the solid-state fermentation method to increase the crude protein content in materials, especially for scale-up production on a commercial scale. Based on the optimization results using RSM, the best results were obtained using the variable thickness of the substrate 2 cm, fermentation temperature 30 °C, and the nutrient concentration of KH₂PO₄ of 4 g/L.

4. Conclusions

Response surface methodology with central composite design has succeeded in optimizing the increase in rice bran protein content using *Rhizopus oryzae* with the solid-state fermentation method. The highest increase in protein content of 62.51% was obtained with the variable substrate thickness of 2 cm, fermentation temperature of 30 °C, and KH₂PO₄ nutrient concentration of 4 g/L. The interaction between the variable substrate thickness and fermentation temperature is the most dominant factor in this study. To increase the production of rice bran protein on a large scale, we recommend expanding the tray size or increasing the number of trays while maintaining the thickness of the substrate by 2 cm.

Authors' contributions

AS, SS designed the study. AS carried out the laboratory work. HO analyzed the data. AS, HO, SS wrote the manuscript. All authors read and approved the final version of the manuscript.

Competing interests

The author declare that they have no competing interest.

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