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Optimization of Protein Production from Banana Peel Flour by *Rhizopus oryzae* through Solid-State Fermentation Using Response Surface Methodology

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Abstract. Banana peel is one of the wastes from bananas with a composition of 35-40% of the weight of fresh bananas. The use of banana peels as feed and food ingredients is very limited due to the low protein content in banana peels. This study aimed to increase the protein content of banana peel flour using the solid-state fermentation method by *Rhizopus oryzae FNCC 6157* in a tray bioreactor. Optimization of protein production of banana peel flour was done using the central composite design by response surface methodology. The variables used in this study were substrate thickness (1 to 3 cm), aeration rate (2 to 4 L/min), and moisture content (50 to 70%). Statistical analysis was performed using ANOVA and resulted in an excellent quadratic equation model as indicated by the F-value and p-value of 17.77 and 0.0012, respectively, and was statistically significant ($p \leq 0.05$). Fermentation conditions at the substrate thickness of 2 cm, aeration rate of 3 L/min, and moisture content of 60% resulted in the highest enhancement of protein content in banana peel flour of 54.08%.

INTRODUCTION

The increasing world population has increased food consumption, including the consumption of fruit. Increased consumption of fruit causes an increase in the volume of fruit waste [1]. Waste generated by fruits consisting of skins and seeds is 40% of the total mass of the fruit [2]. The available fruit waste is very abundant, mostly only dumped into the environment, and can cause pollution [3]. Fruit peels can be used as fermentation substrates because they contain high levels of carbon and nitrogen. Banana peels can be processed into high nutritional animal feed and alternative food by fermentation because they contain carbohydrates, proteins, minerals, dietary fiber, amino acids, phenolic compounds, and antioxidants [4].

The use of banana peels for alternative food is still rare. Banana peels can be processed into banana peel flour and used as a food additive for a maximum of 10%. Food products added with banana peel flour more than 10% experienced a decrease in sensory characteristics [5]. The use of banana peel flour for food additives is seldom because banana peels contain lignocellulose. Banana peel contains cellulose, hemicellulose, lignin, and pectin so that the addition to food products can reduce the sensory characteristics of the resulting food products [6]. In addition to containing lignocellulose, banana peels also contain hydrogen cyanide and saponins as anti-nutritional compounds that are dangerous if they enter the body in large quantities [7].

Solid-state fermentation is known to increase the nutrient content of the fermented substrate. Nutrients on the substrate increased due to fungal metabolism that degrades lignocellulose compounds into simple sugars through enzymatic reactions. Fermentation can also reduce the content of antinutrients such as hydrogen cyanide and saponins of fermented substrates [9,10]. *R. oryzae* is a Zygomycetes fungus that can produce cellulase, xylanase, pectinase, and amylase enzymes that degrade polysaccharide compounds in the substrate into simple sugars [11]. *R. oryzae* improved the crude protein content in rice bran substrate, making it suitable for fermenting banana peel flour [12].

The most popular objective of solid-state fermentation is to increase the protein content of low-nutrient substrates [13]. Parameters in solid-state fermentation affect the efficiency of the fermentation process in terms of productivity,

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time, and production costs [14]. The maximum yield of fermented products can be obtained by optimizing the fermentation process. This process aims to reduce costs and speed up the production process. Optimization of the fermentation process using response surface methodology (RSM) can generate accurate conclusions quickly. RSM produces a mathematical equation with several variables that affect the response. Besides the short time, the advantage of RSM is that it requires few trials runs to optimize response [15,16]. The results from the optimization made the basis of consideration for designing the bioreactors on a commercial scale [17]. There is no research on the optimization of banana peel fermentation using a tray bioreactor. The purpose of this study was to determine the effect of substrate thickness, moisture content, and aeration rate on the protein content of banana peel flour in the tray bioreactor.

MATERIALS & METHODS

Banana Peel Flour Preparation

The Kepok banana peel was obtained from the banana chip processing industry in Sungai Langka Village, Gedong Tataan Sub-District, Pesawaran District, and Lampung Province, Indonesia. Banana peels are then cut into 3 x 3 cm sizes and soaked in a solution containing 0.2% sodium metabisulfite ($Na_2S_2O_5$) and 0.2% citric acid ($C_6H_5O_7$) (MerckKGaA, Darmstadt, Germany) for an hour to prevent enzymatic browning reactions. The banana peel is then dried in an oven (Heraeus ST50, Hanau, Germany) at 60ºC for 12 h [18]. The dried banana peels were then crushed using a blender (Phillips HR2115, Batam, Indonesia) and sieved using a 35 mesh sieve (Retsch AS200, Haan, Germany). The banana peel flour that passes the sieve is then stored in a 3-L polypropylene box at 4ºC until used.

Inoculum Preparation

The pure culture of *Rhizopus oryzae (FNCC 6157)* was obtained from the Food and Nutrition Culture Collection (FNCC) of PAU Food and Nutrition, Gadjah Mada University, Indonesia in an agar slant tube. The cultures were transferred in a Petri dish containing potato dextrose agar (PDA, MerckKGaA, Darmstadt, Germany) and incubated for 7 days at 30ºC (Heraeus B6060, Hanau, Germany) [19]. The spores that formed were taken and dissolved in Tween 80 (0.2%). The spore suspension was homogenized using a vortex mixer (DLAB MX-S, Beijing, China). The concentration of spore suspension was measured using a hemocytometer. The spore suspension was stored at 4ºC until used.

Fermentation

The tray bioreactor had selected for use in the fermentation process using the solid-state method. The type of tray bioreactor used is made of plastic material and unperforated so that the substrate is unagitated and unmixed during the fermentation process. This study used the tray bioreactors with three variations in size, 16 cm × 8 cm × 2 cm, 12 cm × 6 cm × 4 cm, and 8 cm × 6 cm × 4 cm. In each tray bioreactors, 50 g of banana peel flour had added to produce the substrate thickness of 1, 2, and 3 cm, respectively. The tray bioreactor containing banana peel flour was then sterilized in an autoclave at 121ºC for 30 min (ALP KT-30LDP, Tokyo, Japan). The Banana peel flour without fermentation was analyzed for protein content. The spore suspension of *R. oryzae* was added to the banana peel flour substrate with an initial concentration of 4 x 10^6 spores/g medium [19], then sterile distilled water was added until the water content of the substrate reached 50, 60, and 70%. The banana peel flour substrate was further incubated in the 50-L incubator for 120 h at 30°C using forced air circulation with various aeration rates 2, 3, and 4 L/min. After fermentation was complete, samples had taken for analysis of protein content.

Protein Content Determination

Analysis of protein content has used the Kjeldahl method (AOAC. 2000, no. 955.04C) with a conversion factor of 6.25 to describe the total nitrogen content in a substance [19].

Experimental Design

Optimization of the fermentation of banana peel flour using the central composite design (CCD). CCD is very suitable for optimizing the parameters of the fermentation process with a minimum number of experiments. CCD can predict mathematical models accurately [20-22] and has been widely used in fermentation processes to determine the effect of interaction between the variables. The CCD levels used in this study were three levels, high, medium, and low (-1, 0, and +1). The variables selected in this study were substrate thickness, moisture content, and bioreactor aeration flow rate, while the observed response was the protein content of fermented banana peel flour. These variables are crucial and affect the solid-state fermentation process [14].

Table 1. Experimental range and coded levels of process variables

Process variables	Range and Levels		
	-1	0	1
Substrate thickness (X_1)	1	2	3
Aeration rate (X_2)	2	3	4
Moisture content (X_3)	50	60	70

The result of optimization using RSM is a mathematical equation model that describes the effect of variables on the response. The mathematical equation model had tested by analysis of variance (ANOVA) with a 99% confidence level. RSM produces a 3D contour image that shows the optimal and most influential variables on the protein content of the substrate. The total number of trials based on CCD is $2^k + 2k + n_c$, where k is the total number of independent variables and n_c is the number of runs at the center point. Based on this formula, 16 experimental runs were obtained from a combination of three variables, substrate thickness, moisture content, and aeration rate. Testing the effect of interaction between variables on the response has been carried out using a second-order equation model. The resulting polynomial equation is shown by equation 1, where Y is the response, β_0 is the intercept, β_i is the linear coefficient, β_{ii} is the quadratic coefficient, β_{ij} is the interaction coefficient, and X_i X_j is the independent variable [23].

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

RESULTS AND DISCUSSION

Central Composite Design

TABLE 2. Experimental design and response using CCD.

Run	Substrate Thickness (cm)	Aeration Rate (L/min)	Moisture Content (%)	Protein (%)
1	2.0	1.3	60	8.12 ± 0.12
2	1.0	4.0	70	8.08 ± 0.10
3	2.0	3.0	60	9.35 ± 0.16
4	1.0	4.0	50	7.92 ± 0.08
5	3.0	2.0	50	8.33 ± 0.10
6	3.0	2.0	70	8.87 ± 0.13
7	3.7	3.0	60	8.44 ± 0.07
8	2.0	3.0	76.8	8.82 ± 0.15
9	2.0	4.7	60	7.86 ± 0.12
10	3.0	4.0	50	7.76 ± 0.16
11	1.0	2.0	50	7.19 ± 0.10
12	2.0	3.0	43.2	7.66 ± 0.09
13	0.3	3.0	60	7.24 ± 0.15
14	3.0	4.0	70	7.81 ± 0.12
15	1.0	2.0	70	8.29 ± 0.08
16	2.0	3.0	60	9.43 ± 0.11

Values are expressed as means ± standard deviation

The central composite design has successfully evaluated the protein production of banana peel flour as a function of substrate thickness, aeration rate, and moisture content. All experiments of fermentation were carried out at 30°C for 120 hours. The protein content of banana peel flour before fermentation was 6.12%. It is the same for all experimental runs. The design experiment and the response of protein content at fermented banana peel flour are shown in Table 2. The mathematical model was obtained using the design expert 12.0.1.

TABLE 3. ANOVA for the quadratic model, Responses: protein content (%).

Source	Sum of Squares	df	Mean Square	F-value	p-value
Model	6.31	9	0.7009	17.77	0.0012
A (Substrate Thickness)	0.8013	1	0.8013	20.31	0.0041
B (Aeration Rate)	0.1753	1	0.1753	4.44	0.0796
C (Moisture Content)	1.06	1	1.06	26.81	0.0021
AB	0.5778	1	0.5778	14.65	0.0087
AC	0.0561	1	0.0561	1.42	0.2780
BC	0.2556	1	0.2556	6.48	0.0438
A^2	2.57	1	2.57	65.18	0.0002
B^2	2.08	1	2.08	52.72	0.0003
C^2	1.38	1	1.38	34.88	0.0010
Residual	0.2367	6	0.0395		
Lack of fit	0.2335	5	0.0467		
Pure Error	0.0032	1	0.0032		
Cor Total	6.55	15			

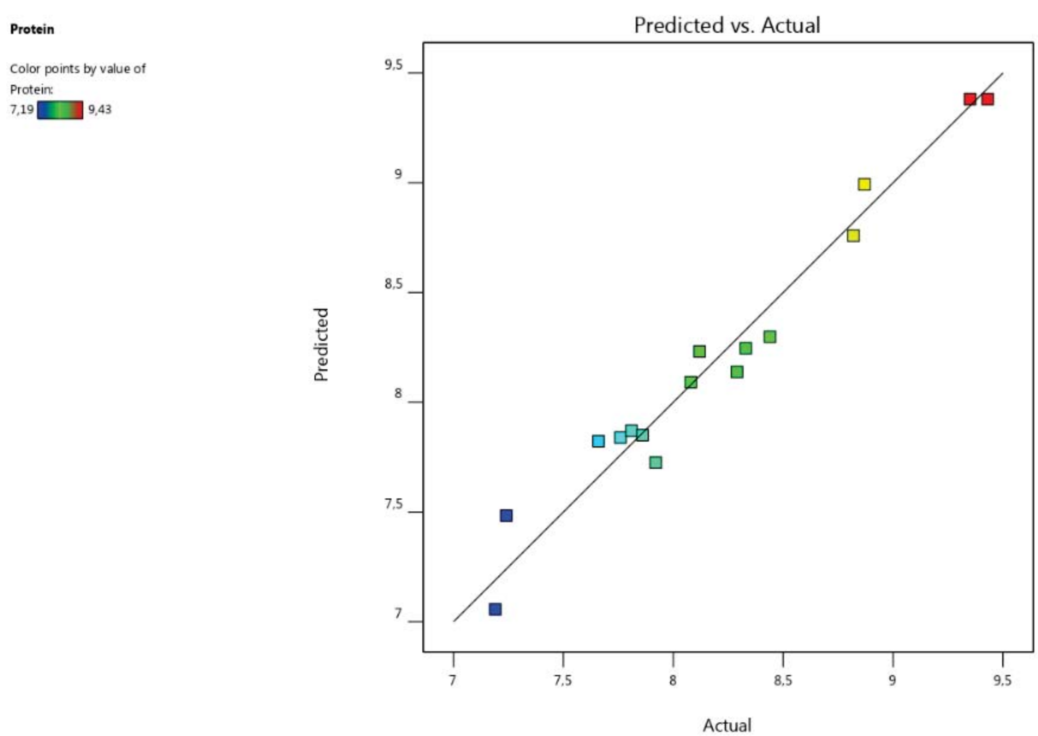

FIGURE 1. The correlation coefficient between model data and experimental data

To determine the effect of a single variable and the interaction between variables on the response used ANOVA. Design expert 12.0.1 had used to generate ANOVA in this study. ANOVA on the quadratic equation model for protein production of fermented banana peel flour by *R. oryzae* is showed in Table 3. The quadratic equation model generated using ANOVA has been significant can be seen from the F-value and p-value of 17.77 and 0.0012, respectively ($p \leq 0.05$). The multiple correlation coefficient (R^2) produced is 0.7208, which means that 72.08% of the model data from the equation is close to the actual data from the experiment. A model would be accurate if the R^2 value has more than 70% [24]. The coefficient and the quadratic equation model of the protein content of fermented banana peel flour *R. oryzae* as a function of substrate thickness, aeration rate, and moisture content are showed in equation 2.

$$Protein(\%) = 9.3812 + 0.2422X_1 - 0.1133X_2 + 0.2783X_3 - 0.2687X_1X_2 - 0.0837X_1X_3 - 0.1787X_2X_3 - 0.5268X_1^2 - 0.4738X_2^2 - 0.3854X_3^2 \quad (2)$$

Based on the quadratic equation of response to protein content, the most influential variable is moisture content with β coefficient and p-values of 0.2783 and 0.0021, respectively. While the interactions between variables that the most influence the response of protein content are substrate thickness and aeration rate with β coefficient and p-values of -0.2687 and 0.0087, respectively. The greater the absolute value of the β coefficient and the smaller the p-value, the more influential the variable on the response [24].

Response Surface Methodology

The effect of the variables of substrate thickness, aeration rate, and moisture content on the protein content of fermented banana peel flour was optimized using design expert 12.0.1. Solid-state fermentation of banana peel flour using *R. oryzae* has proven to increase protein content. Enhancement of protein content in banana peel flour was obtained from complex carbohydrates on the substrate that decompose into simple compounds by *R. oryzae* activity. *R. oryzae* produces cellulase, xylanase, pectinase, and amylase as enzymes that break down carbohydrates [11,25-27]. Simple compounds resulting from the breakdown of carbohydrates used for the growth of *R. oryzae* biomass cells and the production of extracellular compounds such as enzymes and organic acids. *R.oryzae* biomass cells are known to contain protein, chitin, and chitosan so that the protein content in the fermented substrate increases [28]. During the fermentation process, the substrate temperature will rise and produce heat because of the metabolic activity of the *R. oryzae* fungus. The heat generated is an indicator of the success of the fermentation process where the enzyme activity and the growth of *R. oryzae* biomass are at optimal levels [29]. The thinner the substrate causes the heat generated to be lost faster. The thicker the substrate causes overheating that the biomass growth is not optimal [12,30].

In addition to protein and organic acids, the metabolic products of *R. oryzae* are carbon dioxide and water vapor. Gases from metabolism also need to be removed because they can interfere with the growth of *R. oryzae* biomass. Aeration can help to transfer CO_2 and H_2O out of the fermentation medium [31]. The smaller the aeration rate, the smaller the transfer rate of metabolic gases that do not run optimally, thus disturbing the growth of *R. oryzae* biomass. Conversely, the greater the aeration rate, the greater the transfer rate of CO_2 and H_2O gases out of the medium resulting in an insufficient medium substrate to generate heat. The higher the heat loss, the lower the productivity of the complex degrading enzymes in the substrate [32,33].

R. oryzae in carrying out its metabolic activities do not require large amounts of free water in the medium. The optimal water content for fungal growth was 50-60%, while bacteria were 80-90% free water content in the medium [30]. The higher moisture content in the medium will inhibit the transfer of oxygen into the substrate. The transfer rate of metabolic gases such as CO_2 and H_2O out from the substrate also inhibits. This situation causes the growth of *R. oryzae* biomass hampered. On the other hand, the lower the moisture content in the medium, the effectiveness of dissolution and nutrient absorption in the medium substrate by *R. oryzae* decrease [30].

The response surface has succeeded in describing the relationship between the response and the interaction between variables using 3D graphics. The results of the RSM analysis showed in Figures 2, 3, and 4. Based on the coefficient β and p-value, the most effective interaction between variables is the thickness of the substrate and the rate of aeration. The interaction between the substrate thickness and aeration rate is a crucial factor in solid-state fermentation, and it is very influential affects the transfer rate of metabolic gases. The rate of transfer of metabolic gases is the most significant issue in solid-state fermentation. Metabolic gases need to be removed, but the temperature of heat generated during the fermentation process also needs to be maintained [14,30]. The thinner the substrate media and the greater the aeration rate during the fermentation process, the greater the heat loss. On the other hand, the thicker the substrate layer and the lower the aeration rate, the lower the rate of gas transfer from the metabolism and inhibit the biomass growth of *R. oryzae*.

Based on optimization using RSM, the highest protein content of fermented banana peel flour was obtained at the substrate thickness of 2 cm, aeration rate of 3 L/min, and moisture content of 60%. To increase the production capacity of fermented banana peel flour on a commercial scale, it is necessary to optimize the design of the tray bioreactor and incubator. Further studies are needed to study mass and heat transfer phenomena in tray bioreactors.

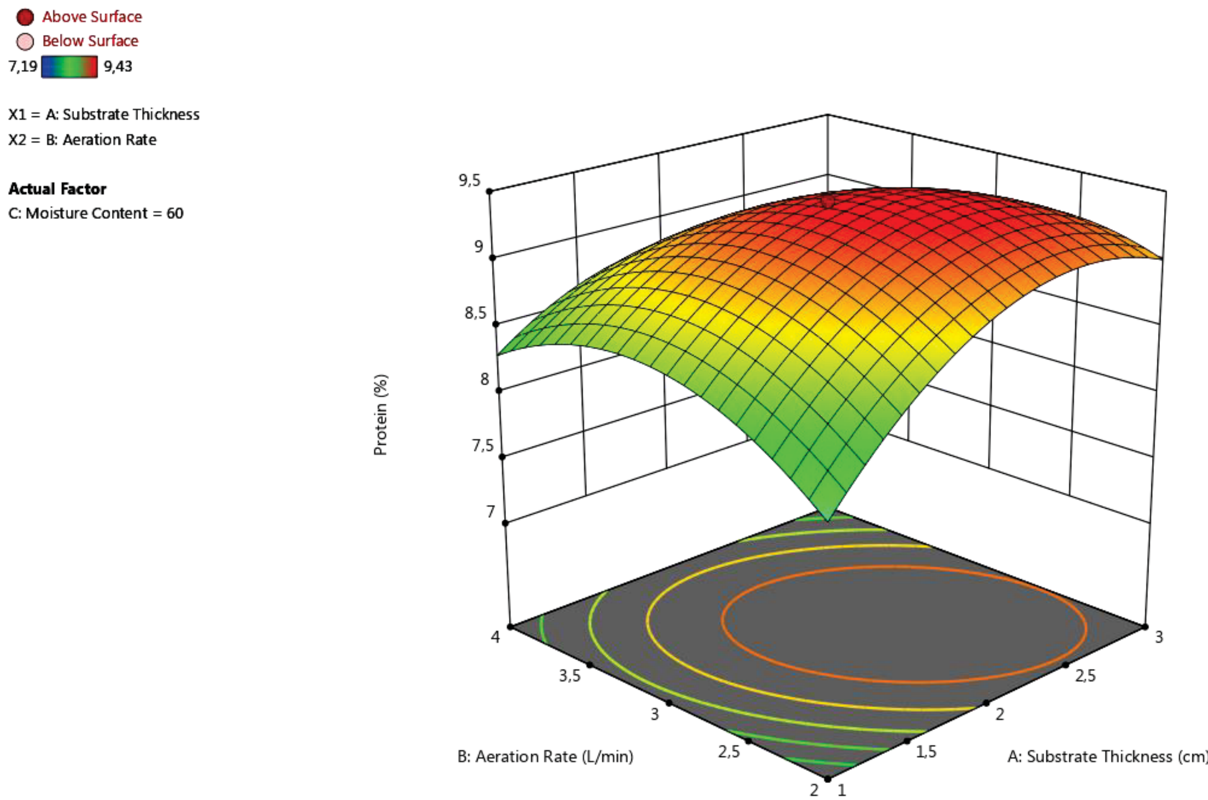

FIGURE 2. 3D graph of the effect of substrate thickness and aeration rate on protein production

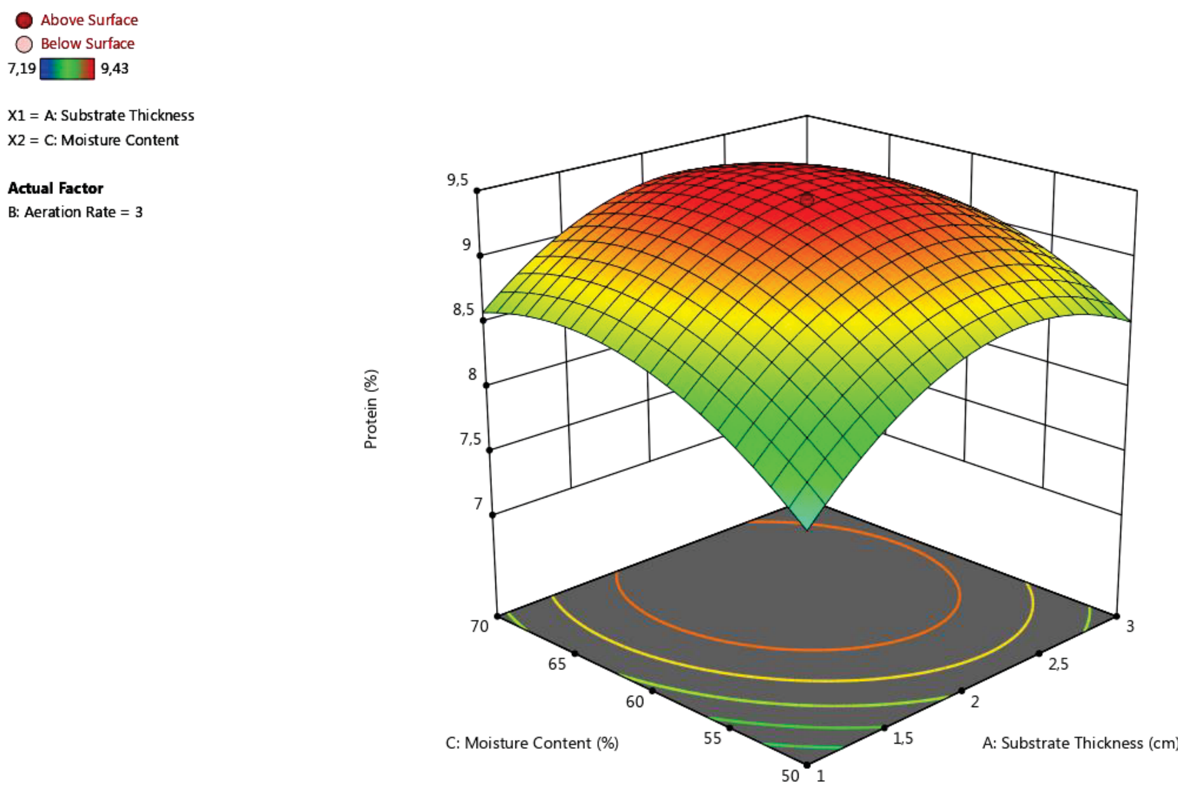

FIGURE 3. 3D graph of the effect of substrate thickness and moisture content on protein production

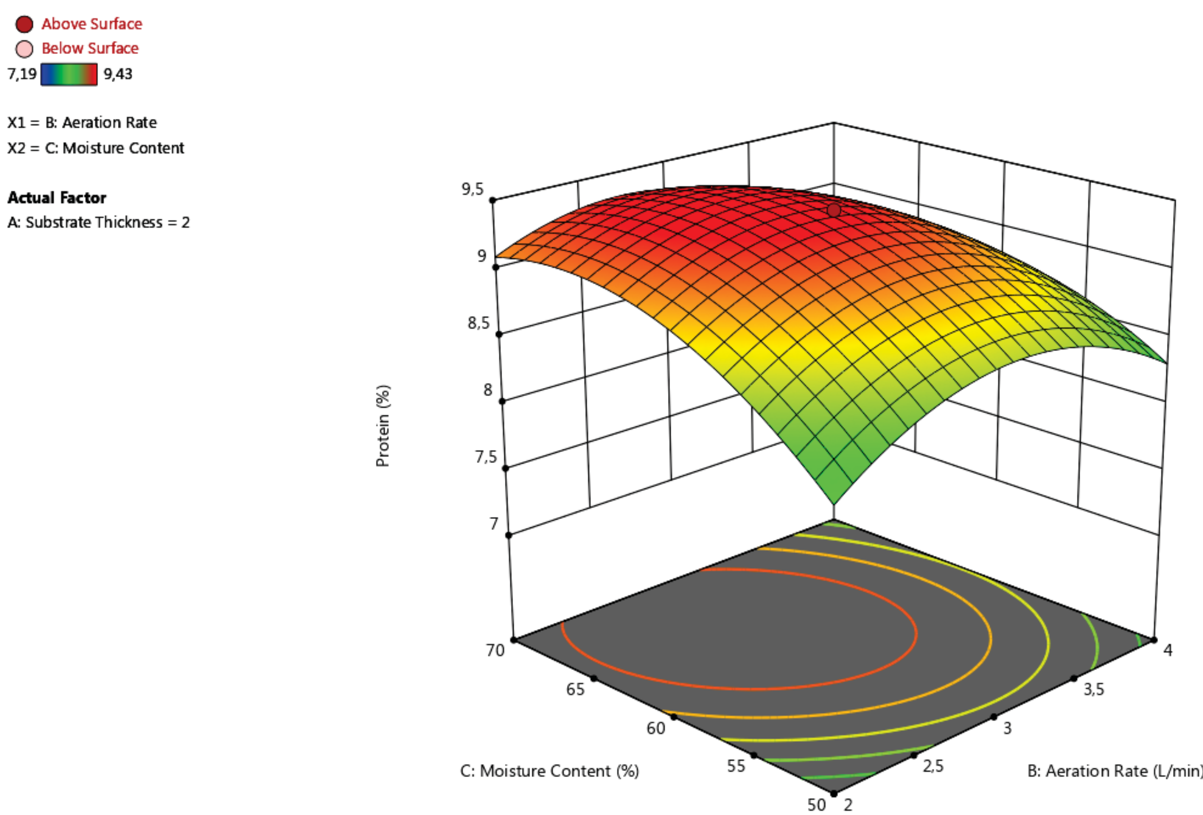

FIGURE 4. 3D graph of the effect of aeration rate and moisture content on protein production

CONCLUSION

Solid-state fermentation using *R. oryzae* in the tray bioreactor had proven to increase the protein content of banana peel flour. It is a solution to utilize banana peels for higher-value products. Optimization of solid-state fermentation of banana peel flour had been implemented successfully using the response surface methodology with a central composite design. Variables 2 cm substrate thickness, 3 L/min aeration rate, and 60% moisture content were known to produce the highest enhancement of protein content in banana peel flour of 54.08%. The most influential factor in this research is the interaction between the variables of substrate thickness and aeration rate. The substrate thickness of 2 cm was maintained for designing commercial-scale tray bioreactors. The addition of the number of trays is also required to maintain the thickness of the substrate. To maintain an aeration rate of 3 L/min can be done by increasing the size of the blower fan when using a bigger incubator.

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