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Optimization of α-Amylase Extraction Parameters of Rice Bran Oil (RBO) by Response Surface Methodology

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Abstract. Rice bran oil (RBO) is known as a vegetable oil that has various health benefits; it is extracted from the outer layer (bran) of rice grains using an enzymatic extraction process (Aqueous Enzymatic Extraction, AEE) in the form of α-amylase. The research goals of this study are to determine the optimal processing condition for RBO extraction with α-amylase. The processing parameters were optimized using Response Surface Methodology (RSM) in conjunction with Central Composite Design (CCD). RBO extraction with α–amylase parameters such as the incubation temperature (A) 35, 50, and 65 °C and incubation time (B) 2, 3, and 4 hours to optimize the processing condition. RBO yield (%) and free fatty acid (FFA) were analyzed. A statistical model predicted that the highest conversion yield of RBO would be 1.533% at the following optimized reaction conditions: reaction temperature of 50 °C and time of 4.41 h. Experiments performed at the predicted optimum conditions yielded 1.663% better than the predicted value. A quadratic model was selected to estimate the RBO extraction with α-amylase based on the analysis of variance (ANOVA) of the findings from several models. The linear regression coefficient (R^2) between experiments and different response values in the model was 0.97 for RBO yield and 0.8929 for FFA of RBO. The optimal conditions based on all process variables (incubation temperature of 51 °C and incubation time of 4 h) were determined by Derringer's desired function methodology. Under the conditions mentioned, the yield and FFA of RBO were 1.6% and 7.3%, respectively. All the optimizing parameters and results were validated by regression analysis model fit data using p-value (<0.05), R^2 value (yield and FFA of RBO were 0.9749 and 0.8929), and desirability value (>0.05).

Keywords: Rice Bran Oil, α-amylase, Response Surface Methodology, Yield, Free Fatty Acid

INTRODUCTION

Rice is a food ingredient that produces rice, a staple food for 90% of the Indonesian population. Aziz *et al.* (2014) stated that rising rice output impacts production waste such as rice bran. Rice bran contains 23–28% crude fiber, 15–20% fat, 7–8% ash, 12–16% protein,

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minerals, vitamins, phenolic compounds, and essential unsaturated fatty acids, making rice bran oil (RBO) superior to other edible oils (Yan *et al.*, 2020).

According to estimates, approximately 8 million tons of RBO might be produced globally. However, fewer than ten percent of crude RBO is converted into edible oil. Usually, RBO has a lot of free fatty acids (FFA). According to Wang *et al.* (2017), the principal obstacle to the commercial production of RBO as edible oil is the high FFA level of crude RBO.

The process of extracting crude rice bran oil (CRBO) with its techniques includes cold press extraction cold press extraction (Mingyai *et al.*, 2017), solvent extraction (Mingyai *et al.*, 2017), microwave-assisted extraction (MAE) (Pandey and Shrivastava, 2018), and aqueous enzymatic extraction (AEE) (Xu *et al.*, 2020).

The most used technique for extracting oil is solvent extraction, which includes hexane. However, solvent extraction has a lot of drawbacks, including the hazardous substances created and environmental problems (Karthika, 2020). According to Xu *et al.* (2020), MAE has numerous issues, including a high residual oil rate, high labor intensity and power consumption, high cost, and easily caused protein denaturation. AEE is thought to be among the most ecologically friendly techniques for extracting RBO. Enzymes are employed in vegetable oil extraction procedures to help liberate the oil and, in certain situations, to remove the need for hexane in aqueous extraction procedures.

α-amylase has recently been used to make extracting RBO easier. To help break down complex carbs into simpler sugars and improve oil extraction, α-Amylase catalyzes the hydrolysis of the starches found in rice bran (Singh and Kumar, 2019).

Previous research on the use of α-amylase in RBO extraction was Hernandez *et al.* (2001), who asserted that utilizing amylase with Centrifugation speed (15 min at 12,000 rpm), 50℃ with yield 5%. Sharma *et al.* (2001) used protease, α-amylase, and cellulase. pH = 7, 65 ℃, 18 hr with yield 76%. Huang *et al.* (2013) used protease, α-amylase, and cellulase. pH = 4,5 ℃ and 55 ℃ with a yield 92.63%. Hanmoungjai *et al.* (2002) optimized EAAE with a commercial protease (Alcalase 0.6 L) incubated for 1–4 h with the highest Alcalase yield (80%) compared to Celluclast, Hemicellulase, Pectinex Ultra-SPL and Viscozyme L (30-50%). The incubating temperature used in the experiments was fixed at 50 °C. Xu *et al.* (2020) used a combination of enzymes, Celluclast, Hemicellulase, Pectinex Ultra SP-L, Viscozyme L, and Alcalase, not determining yield. The samples were incubated at the optimum temperatures (50, 50, 50, 45, 60) of various enzymes for 120 min.

In the AEE process, the parameters of temperature and incubation time substantially impact the production of RBO (Mounika *et al.*, 2020; Mwaurah *et al.*, 2020). The temperature parameter impacts enzyme activity since low temperatures result in protein denaturation (Mwaurah *et al.*, 2020).

The research on the extraction of RBO by AEE is still in the process of parameter optimization, and there are no systematic studies on the percentage of yield and FFA of RBO. Besides, temperature and time are important parameters for their processing and utilization and these characteristics of RBO still need to be reported in any literature. According to Balvardi *et al.* (2015), the Response Surface Methodology (RSM) is a technique that easily finds areas with ideal operating conditions. Central Composite Design (CCD) is widely used in response surface modeling and optimization (Bhattacharya, 2021).

Therefore, this study aimed to determine the optimal processing condition for RBO extraction with α-amylase.

MATERIALS AND METHODS

Raw Material and Reagents

The source of the rice bran was Muntilan, Central Java, Indonesia. The α-amylase enzyme was purchased from Novozymes Bagsvaerd, Denmark. NaOH (99%, Sigma-Aldrich, Germany) and HCl (37%, Sigma-Aldrich, Germany) were purchased from PT. Hepilab Sukses Bersama (Indonesia).

Preparation of RB

The methods of Mounika *et al.* (2020) and Zigoneanu *et al.* (2008) were adapted to create the fresh rice bran pretreatment process. Physical pretreatment involved sifting fresh rice bran through a 30-grit screen. The powders were enclosed in plastic and stored in a freezer before use. Before usage, the RB was dried at 110°C for 20 minutes.

The Use of α-amylase in the AEE Extraction of RBO

A 500-ml beaker glass filled with 50 grams of dried RB and 300 ml of distilled water. The mixture is mixed while being heated for five minutes to 90ºC. In addition, the solution's temperature has dropped to 32ºC, and 2N HCl or 2N NaOH has been added to neutralize the pH. The neutral solution was then supplemented with 64.8 ml of the α -amylase enzyme, which was thoroughly mixed. Additionally, the mixture was incubated in different periods (2, 3, and 4 hours) and temperatures (35, 50, and 65ºC). The mixture was centrifuged (Ohaus FC5706, America) at 6000 rpm for 20 minutes to remove the emulsion phase, which was the top layer. A micropipette (Dragon Lab brand, manufactured in China) was used to collect the top layer of oil (Li *et al.*, 2017) and the yield was then determined using Equation 1 (Mounika *et al.*, 2020).

$$\%yield = \frac{\text{Mass of container and oil(g)} - \text{Mass of container (g)}}{\text{RB mass (g)}} \text{ x } 100\% \quad (1)$$

Free Fatty Acid (FFA) Levels in RBO Measurement

Titration techniques were used to analyze FFA levels (Noureen *et al.*, 2021). The analysis has gone through in the following manner: 25 ml of Erlenmeyer was combined with 5 ml of 95% ethanol, 0.1 N KOH, and 0.5 g of RBO before being heated on a hotplate until it boiled. After thoroughly agitation, three drops of the 1% v/v phenolphthalein (PP) indicator have been added to the hot solution. The solution was then titrated with 0.1 N KOH until pink was achieved. The amount of KOH under these circumstances has been meticulously recorded. The American Oil Chemist Society's (AOCS) Ca 5a-40 technique has been used to determine the free fatty acid level using Equation 2.

$$\text{FFA (\%)} = \frac{\text{V} \times \text{N} \times 28.2}{\text{m}} \quad (2)$$

where V is the necessary volume of KOH (ml), N is the normality of KOH solution, m is sample mass (grams), and 28.2 is the oleic acid's molecular weight (g/mol).

Experimental Design for α-Amylase Extraction

RSM was given a platform to evaluate and optimize the extraction of RBO to incubation temperature and time using the Design-Expert Version 13 (Stat-Ease, Inc., Minneapolis, MN 55413) software. The correlation between the process factors and the responses was assessed using CCD, the most used RSM technique (Leong *et al.* 2017). According to Bhattacharya (2021), CCD is a useful design tool perfect for sequential experimentation because, when there are enough experimental values, it allows for a respectable amount of data to test the lack of fit. As shown in Table 1, incubation temperature (A) and incubation time (B) were the important process variables considered in this study. The real value range identified from the preliminary studies was used to numerically vary each process variable over three levels between –1 and +1. The yield (%) and FFA (%) of RBO were the tracked responses from the interactive impacts of process variables. Thirteen tests were conducted using the two process variables that resulted. Eight experiments were supplemented with five replications at the center to fit the second-order polynomial response surface model of a quadratic equation and evaluate the pure error.

The second-order polynomial response surface model based on the CCD design is mainly utilized for process optimization (Myers *et al.*, 2016). In essence, the following describes how the input of the process variable and the response are related (Equation 3)

$$Y = f(x_1, x_2, x_3 \ldots x_k) + \varepsilon \quad (3)$$

where Y is the response, f is the unknown function of the response, $x_1, x_2, x_3 \ldots x_k$ is the input of process variables that may impact the response, and k is the number of process variables. It is the random error component that represents additional sources of variability not taken into account by f (Montgomery, 2020).

Since each process variable was divided into three levels (Table 1), CCD recommended using the quadratic model (Equation 4) to predict the ideal conditions and evaluate the interplay between process variables and the resulting responses.

$$Y = \beta_0 + \sum_{i=1}^{n} \beta_i X_i + \sum_{i=1}^{n} \beta_{ii} X_i^2 + \sum_{i=1}^{n} \sum_{j=i+1}^{n} \beta_{ij} X_i X_j + \varepsilon \quad (4)$$

So, based on Table 1, Equation (4) is to be Equation (5).

$$Y = \beta_0 + \beta_1 A + \beta_2 A^2 + \beta_3 B + \beta_4 B^2 + \beta_5 AB \quad (5)$$

where n is the number of factors studied and optimized in the experiment, X_i and X_j the coded values of the variable parameters β_0 (constant term); β_1 and β_3(linear effect); β_2 and β_4(quadratic effect); and β_5 (interaction effects) were the coefficients of the polynomial.

Statistical Analysis for α-Amylase Extraction

The Design-Expert Statistical Software package version 13 (Stat Ease Inc., Minneapolis, USA) was used for the statistical study. The least squares approach was used for multiple regression analysis of the experimental data. Two tests were performed on the experimental data to determine whether different models were adequate: model summary statistics and sequential sum of squares. The analysis of variance (ANOVA) was used to examine the regression coefficients and effects of each of the variables (linear, quadratic, and interaction) that were part of the model, and ANOVA tables were produced. The F-test was used to statistically assess and validate each of the model's terms at probability levels ($p < 0.05$).

The coefficient of determination (R^2), adjusted coefficient of determination (R^2_{adj}), and anticipated coefficient of determination (R^2_{pre}) were used to assess the adequacy of the created models. Surfaces and contour plots were created to forecast the relationship between the independent variables and responses following model fitting.

Table 1. CCD with coding level for independent variables

Factor	Upper (+1)	Center (0)	Lower (–1)
(A) Incubation temperature (°C)	65	50	35
(B) Incubation time (hour)	4	3	2

The Mean Absolute Percentage Error (MAPE) (Equation 6) is used to calculate the mean deviation from the prediction model (Moreno *et al.*, 2013).

$$\text{MAPE} = \frac{1}{n}\sum_{t=1}^{n}\left|\frac{A_t - F_t}{A_t}\right| \times 100\% \qquad (6)$$

where N is the number of data points, At and Ft are the actual and predicted values at data point t, respectively.

According to Moreno *et al.* (2013), a number has considerable accuracy if MAPE is less than 10%, good accuracy between 10% and 20%, decent accuracy between 20% and 50%, and greater accuracy if it is over 50%.

Optimization for α-Amylase Extraction

One of Derringer's desirability's main benefits is that if at least one of the criteria is outside of the desired range, global desirability is unsatisfactory. The user's ability to customize the function between the desirable and non-desirable ranges to suit his tastes is an additional benefit. It is also possible to set different weights to criteria to differentiate their relevance (Bystrzanowska and Tobiszewski, 2019).

RESULTS AND DISCUSSION

Analysis Central Composite Design (CCD)

The optimal operating parameters for this study's response (yield and FFA) were determined by using the CCD to assess the interaction between the independent variables (temperature and incubation time) and the desired response (Table 2).

Using Table 2, the yield and FFA MAPEs achieved are 7.0195 and 1.1244%, respectively; these numbers represent the agreement between the actual and projected values. This MAPE value, which has the same good accuracy as using the cellulase enzyme (2.6812 and 0.4687, respectively)(Damayanti *et al.*, 2023), is < 10%. With points representing zero error between the predicted and actual values, a line of unit slope, or line of perfect fit, was displayed in Figure 1.

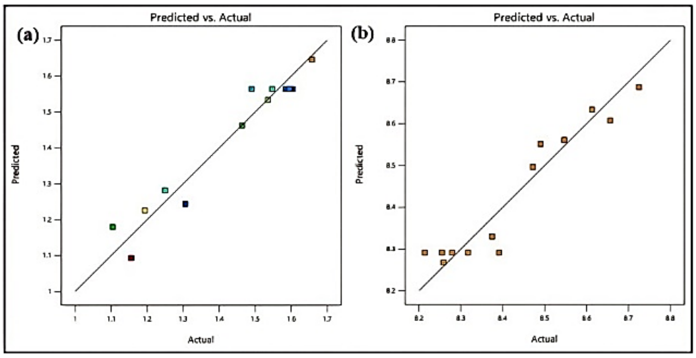


Fig. 1: Comparison of the predicted value and actual value (experiment) for yield (a) and FFA (b) of RBO using α-amylase.

This suggests that the model provides a reasonably accurate description of the experimental data about the yield (Fig. 1a) and FFA (Fig. 1b) of RBO. Some points are quite far from the perfect fit line from the graph. Therefore, there is an inadequate correlation between the predicted values and the experimental values of the independent variables, which further describes the adequacy of the model.

The experimental data has been observed to develop a suitable model for predicting reactions. Responses are predicted using linear, interactive (2FI), quadratic, and cubic polynomial models. The factors utilized to determine the best model are the adjusted R^2, lack of fit p-value, predicted R^2, and sequential p-value shown in Table 3.

The quadratic model (Table 3) is recommended for yield optimization and FFA. Because it is not advised, not even the cubic model can be utilized in this one (Aliased). Using an unrecommended model (Aliased) can result in unreliable graphics (Khelifa *et al.*, 2021).

Analysis of Optimal Conditions Statistically for Yield of RBO

The CCD modeling technique was used to assess the relationship between the experimental process factors and the yield of RBO. The response yield of RBO (Y) and the process variables, incubation temperature (A) and incubation time (B), were fitted by a second-order polynomial regression equation. The quadratic model is appropriate

Table 2. Experimental data and predictions for CCD design

Run	Incubation temperature (°C, A)	Incubation Time (Hour, B)	Yield (%)		FFA (%)		Error (%)	
			Experiment	Predicted	Experiment	Predicted	yields	FFA
1	35	4	1.0228	1.1008	7.5873	7.4643	7.6261	0.6405
2	50	4.41	1.6630	1.5333	7.2729	7.2335	6.1053	1.0849
3	35	2	0.8790	0.7151	7.3026	7.4643	18.6462	2.1061
4	71.2	3	0.7774	0.8890	7.6413	7.5942	14.3555	1.0522
5	50	1.59	0.9404	0.9437	7.3160	7.2335	0.3509	1.1277
6	65	4	1.2934	1.3145	7.4492	7.4052	1.6314	0.3437
7	50	3	1.4432	1.4948	7.2422	7.2335	3.5754	0.8547
8	50	3	1.5120	1.4948	7.1153	7.2335	1.1376	0.9135
9	50	3	1.4198	1.4948	7.3043	7.2335	5.2824	1.6976
10	50	3	1.5670	1.4948	7.0931	7.2335	4.6075	1.2294
11	50	3	1.5322	1.4948	7.1466	7.2335	2.4409	0.4716
12	28.8	3	0.5998	0.6309	7.6925	7.6779	5.1851	0.6227
13	65	2	1.0872	0.8664	7.2764	7.4052	20.3091	2.4284
					MAPE (%)		9.4702	1.0794

Table 3. Experimental data and predictions for CCD design

Model	Sequential p-value		Lack of fit		Adjusted R^2		Predicted R^2		Remarks
	Yield	FFA	Yield	FFA	Yield	FFA	Yield	FFA	
Linier	0.2844	0.9324	0.0010	0.0269	0.0668	-0.1833	-0.3879	-0.7646	
2FI	0.7006	0.5280	0.0008	0.0220	-0.0191	-0.2548	-0.6612	-09736	
Quadratic	<00001	0.0005	0.2608	0.5864	0.9569	0.8163	0.8776	0.6226	Suggested
Cubic	0.1319	0.3943	0.5586	0.6356	0.9732	0.8228	0.9182	0.6009	Aliased

for analyzing the experimental data, according to Table 4's ANOVA results. The findings of the ANOVA in Table 4 indicated that the quadratic model is appropriate for analyzing the experimental data. The model for the percentage of RBO (Y) in terms of the process variables' coded factors is provided by Equation 7.

$$\text{Yield of RBO (\%)} = -3.6702 + 0.166167\,A + 0.540614\,B + 0.004718\,AB - 0.001761\,A^2 - 0.097126\,B^2 \quad (7)$$

The p-values were used to assess the significance of the regression coefficients to create a statistically significant regression model. Insignificant coefficient terms are eliminated from the regression model when their p-values exceed 0.05. The analysis in Table 4 shows that linear terms of temperature and time and quadratic terms of temperature and time, which are A, B, A^2, and B^2, are significant model terms ($p < 0.05$). After eliminating the insignificant coefficients, the model reduces Equation 7 to 8.

$$\text{Yield of RBO (\%)} = -3{,}6702 + 0{,}166167\,A + 0{,}540614\,B - 0{,}001761\,A^2 - 0{,}097126\,B^2 \quad (8)$$

According to the ANOVA, the quadratic polynomial model was significant and sufficient to capture the valid link between the yield of RBO and the significant model variable, as evidenced by a p-value of 0.0001.

It was found that the examined extraction's lack of fit (LoF) p-values of > 0.05 were not significant for the yield of RBO, indicating that the variables and RBO extraction are correlated. Adequacy precision, comprised of the average prediction error and the expected value at the design points, is used to quantify the signal-to-noise ratio.

The adequate precision ratio in this study is 19.3159, which is good because it is higher than 4. As a result, the design space can be guided by the developed model.

A coefficient of determination (R^2) value of 0.9749 further clarified the importance and suitability of the developed model. This suggests that the model can explain 97.49% of the variance in the yield of RBO that can be ascribed to the experimental variables. The ANOVA regression model for predicting the percent yield levels using the α-amylase enzyme is presented in Table 4.

A 95% confidence level is shown by the yield of the RBO response's significant p-value of less than 0.05. The model's F value of 54.29 and p-value of less than 0.05 indicate that it is significant since it demonstrates that the model's variables (A, B, A^2, and B^2) significantly affect the response. Even though the p-value is higher than 0.05, the AB variable is insignificant. This factor barely impacts the RBO yield. The AB variable is a term stored in the model even though it is unimportant to preserve the model term hierarchy discovered by ANOVA and prevent its removal. According to the F-test for LoF, which is 1.97, the undermatch is insignificant compared to pure mistake. An "Unmatched F-value" of this magnitude could occur owing to noise with a probability of 26.08%. The F value also demonstrates that time (55.88) significantly influences the RBO yield more than temperature (6.03). "Adeq Precision" evaluates the ratio. The Adeq precision rating of 19.3159 in this model indicates the viability of the chosen model. A second-order polynomial equation represents the empirical connection between the quadratic model and the interplay of variables. Equation 7 contains the last equation for yield optimization.

Table 4. ANOVA regression model for predicting yield of RBO using the α-amylase

Source	Actual Coefficient	Sum of Squares	df	Mean Square	F value	p value	
Model	-3.6702	1.46	5	0.2919	54.29	<0.0001	significant
A-Temperature	0.166167	0.0324	1	0.0324	6.03	0.0437	
B-Time	0.540614	0.3004	1	0.3004	55.88	0.0001	
AB	0.004718	0.0200	1	0.0200	3.73	0.0948	
A^2	-0.001761	1.09	1	1.09	203.10	<0.0001	
B^2	-0.097126	0.0656	1	0.0656	12.21	0.0101	
Residual		0.0376	7	0.0054			
Lack of Fit		0.0224	3	0.0075	1.97	0.2608	not significant
Pure Error		0.0152	4	0.0038			
Cor Total		1.50	12				
Adeq Prec	19.3159						
R^2	0.9749						

Table 5. ANOVA regression model for predicting FFA of RBO with the α-amylase

Source	Actual Coefficient	Sum of Squares	df	Mean Square	F value	p value	
Model	9.45357	0.4145	5	0.0829	11.67	0.0027	significant
A-Temperature	-0.090560	0.0004	1	0.0004	0.0621	0.8104	
B-Time	-0.025332	0.0060	1	0.0060	0.8456	0.3884	
AB	-0.004820	0.0209	1	0.0209	2.94	0.1300	
A^2	0.001045	0.3848	1	0.3848	54.15	0.0002	
B^2	0.048956	0.0167	1	0.0167	2.35	0.1694	
Residual		0.0497	7	0.0071			
Lack of Fit		0.0176	3	0.0059	0.7281	0.5864	not significant
Pure Error		0.0322	4	0.0080			
Cor Total		0.4642	12				
Adeq Prec	8.3967						
R^2	0.8929						

Analysis of Optimal Conditions Statistically for FFA of RBO

The results of ANOVA for FFA of RBO are represented in Table 5. The findings of the ANOVA in Table 5 indicated that the quadratic model is appropriate for analyzing the experimental data. The model for the percentage of RBO (Y) in terms of the process variables' coded factors is provided by Equation 9.

$$\text{FFA of RBO (\%)} = 9{,}45357 - 0{,}090560\,A - 0{,}025332\,B - 0{,}004820\,AB + 0{,}001045A^2 + 0{,}048956B^2 \quad (9)$$

The p-values were used to assess the significance of the regression coefficients to create a statistically significant regression model. Insignificant coefficient terms are eliminated from the regression model when their p-values exceed 0.05. The analysis in

Table 5 shows that linear terms of quadratic terms of temperature, which is A^2, are significant model terms. After eliminating the insignificant coefficients, the model reduces Equation 9 to 10.

$$\text{FFA of RBO (\%)} = 0{,}001045A^2 \quad (10)$$

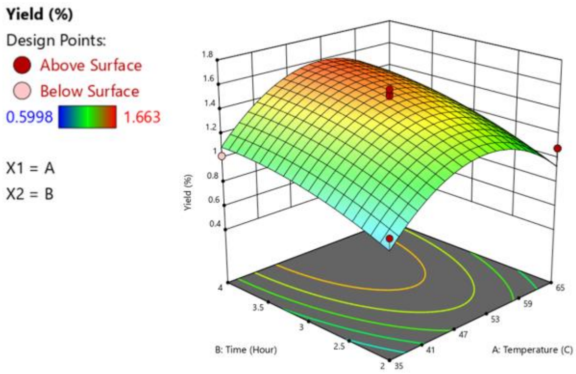
This analysis was used to regulate the model's fit and significance. The P value's significance level in the ANOVA statistical analysis establishes the model's importance for every response. The relevance of the words in the models described is indicated by p-values (< 0.0001) of FFA of RBO.

The model's lack of fit test for the FFA of RBO resulted in a non-significant result at $p > 0.05$. A coefficient of determination (R^2) value of 0.8929 further clarified the importance and suitability of the developed model. This suggests that the model can explain 89.29% of the variance in the RBO yield that can be ascribed to the experimental variables.

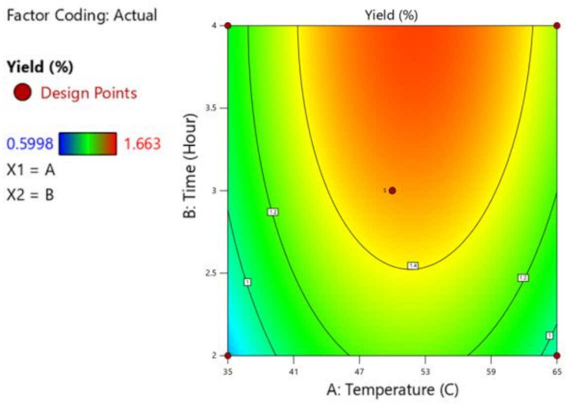
Impact of Process Parameters on Yield and FFA of RBO

RSM was used to investigate how temperature and incubation time affected the response variables yield and FFA of RBO. Using the derived model as a foundation, 3D and 2D for yield (Figure 2) and FFA (Figure 3) of RBO were used to analyze the impacts and interactions of the process variables on the response variables. The semi-elliptical shape of the model contour lines, which demonstrated a strong relationship between the POCF dosage and test temperature, was discovered. The color of the contour plot represents response levels, with blue, green, and reddish representing a lower, medium, and more optimum interaction region, respectively.

The yield value gradually increases (turns greenish blue) as the test duration increases, as the response surface plot shows test time impacts on temperature. The yield value also does as the temperature rises significantly (to a reddish red). Additionally, the ideal output zone with the maximum yield value has been depicted as a yellowish-red area on the response surface plots.



(a)



(b)

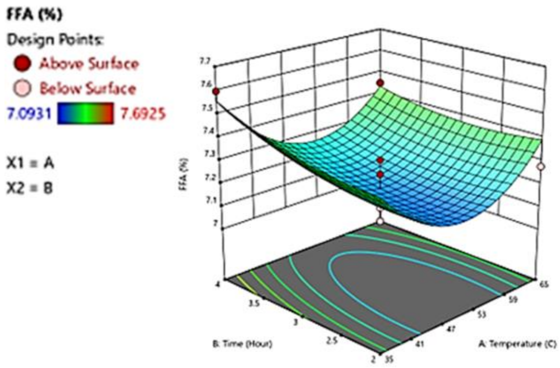
Fig. 2: The interaction between temperature and time on the yield of RBO in (a) 3D surfaces and (b) 2D contours

There were significant interaction effects among the process factors. Table 4 illustrates the favorable interaction between temperature and time on the yield of RBO. The oil's mass transfer and diffusion can be enhanced, and its viscosity can be decreased by raising the extraction temperature. Cell membranes' diffusion coefficient and permeability rise with temperature until they reach their maximum value. High temperatures cause structural changes in enzymes above the optimum temperature, reducing activity and unfavorable for oil extraction. However, overheating causes denaturation of proteins (Mwaurah *et al.*, 2020). The cell wall can degrade more quickly if the reaction time is extended. However, the oil output tends to fluctuate over time. Figure 2 displays the response surface plot of the temperature and time interacting impact. The RBO yield rose as the incubation of temperature and time were raised simultaneously to roughly 50°C and 4.41 hours, respectively, after which the yield value decreased (Gao *et al.*, 2024).

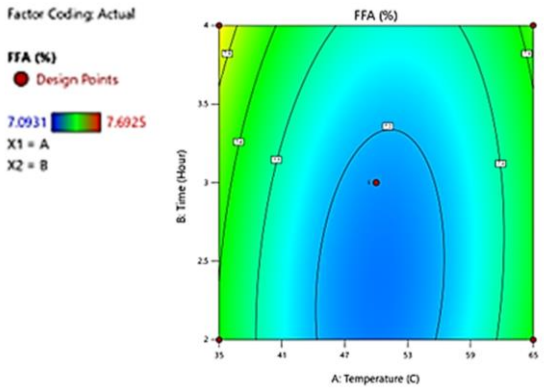
However, if the incubation period is too lengthy, the oil's quality may suffer and burn up much energy (Karthika, 2020; Qian *et al.*, 2021). According to Mounika *et al.* (2020), the ideal incubation period is between 1.4 and 3 hours.

Fresh rice bran crude RBO contains 6-8% FFA (Arora, 2016). High FFA levels are inappropriate for ingestion (Charoonratana, 2020) and result in losses during the refining process (Mahesar *et al.*, 2014). Temperature and incubation period showed no discernible impact, as shown in Table 5. It is supported by Figure 3. Because both values significantly increase (become greenish blue) as the test time increases, the response surface plot demonstrates that the test time impacts temperature and FFA.

Temperature and time negatively affect RBO's FFA (Table 4). As both variables increased, RBO's FFA decreased, which may be related to the maximum FFA level of 5%, as oils with higher than 5% FFA are unsuitable for human consumption (Charoonratana, 2020; Punia *et al.*, 2021). This study found that the FFA levels in CRBO (7.3%) were lower than Rajam *et al.* (2005) and Van Hoed *et al.* (2010) (7.85% and 9.98%, respectively, for CRBO) but comparable to Thanonkaew *et al.* (2012) (3.17–5.58% for CBRO) and Alfaro *et al.* (2017) (3.23% for brown CRBO).



(a)



(b)

Fig. 3: The interaction between Time and Temperature on (a) 2D contours and (b) 3D surfaces for FFA of RBO

Figure 3 shows the nonsignificant interactive effects of temperature and time as a response surface plot. FFA (%) decreased as the temperature was elevated from 35 to 53°C (Figure 3). Nevertheless, it raised the temperature from 53°C to 65°C. However, FFA showed a declining trend when the duration was reduced from 4 to 2 hours.

The decrease in FFA at a temperature of 35–53 °C is thought to be influenced by the RB stabilization treatment with cooling, which only inhibits activity but cannot deactivate lipase. So, the free fatty acid content will increase rapidly after the rice bran is removed from the refrigerator. Then, suppose the lipase in RB is extracted using α–amylase at a temperature of 35 - 53 °C. In that case, it is thought that protein denaturation occurs and causes changes in the tertiary and quaternary structures so that the catalytic activity of both is reduced (Yu *et al.* 2020), although still small. On the other hand, when the temperature is above 53 °C, the reduction in the catalytic activity is more significant, so the FFA is also higher.

AEE's Optimization Results Using α-amylase to Extract RBO

We selected the intended objective for every element and response in numerical optimization. There were several options: target, within range, maximize, minimize, set to an exact value (for factors alone), and none (for replies only). Every included parameter needs to have a minimum and a maximum level. Each objective can be given weight to change the form of its desirability function. An overall desirability function is created by combining the objectives. An objective function, desirability, goes from zero outside the bounds to one at the objective. The program aims to optimize this function. Starting at a random point, the goal-seeking proceeds up the steepest slope to reach its maximum. The response surfaces' curvature and their combination in the desirability function may result in two or more maximums. It is more likely to identify the "best" local maximum if you start from multiple places in the design space (Mounika *et al.*, 2020; Xu *et al.*, 2020). The initial incubation temperature and incubation time were the two goals that were optimized using a multiple-response method.

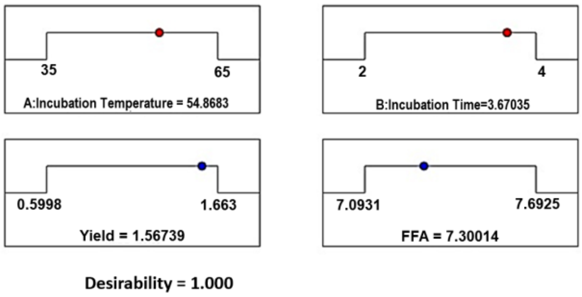


Fig. 4: Derringer method optimization results

Figure 4 shows the desirability values of the numerical optimization process, wherein the "minimum" for incubation time (2 hours) and the "maximum" for incubation temperature (65 °C) were established to analyze economically viable ideal conditions. This procedure used minimal incubation time and temperature to determine the yield and FFA of RBO %. The optimal local maximum was discovered at incubation temperature (A) = 54.8683 °C and incubation time (B) = 3.67035 h after searching from 13 starting points (Experimental Data and Predictions for CCD Design, Table 2) in the response variations. When desirability = 1, the minimum FFA of RBO is 7.30014 and the maximum yield of RBO is 1.56739% (Figure 4).

CONCLUSIONS

The yield of the rice bran enzymatic extraction process utilizing the α-amylase enzyme is influenced by temperature and incubation time. However, neither factor has a significant impact on the FFA of RBO. At 51 °C and 4 hours of incubation, the yield and FFA response determined by the optimization results were 1.6% and 7.3%.

Future developments should concentrate on enzymatic refining of rice bran oil, scaling up production, and doing techno-economic analysis to increase the viability of RBO manufacturing further.

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