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Optimization of protein extraction from "Cam" rice bran by response surface methodology

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ABSTRACT

"Cam" rice bran was considered a waste product from rice, which is rich in natural compounds and protein owing to its outstanding nutritional value. This study aimed to establish an optimization model for extracting protein from rice bran, with two responses: extraction yield (%) and protein content (%). The variable parameters included were pH (8.5-9.5), stirring time (3.5-4.5 h), and enzyme incubation temperature (85-95°C). The coefficient of determination for both models were above 0.95, indicating a high correlation between the actual and estimated values. The maximum extraction yield and protein content were achieved when the conditions were set at pH of 9.02, stirring time of 4.02 h, and extraction temperature of 90.6°C. Under these optimum conditions, the predicted protein extracted from rice bran was 43.03% (moisture <13.0%), with an extraction yield of 15.9%. The findings of this study suggested that this protocol can enhance the utilization of rice bran and might be employed on a large scale in the food industry to exploit the nutritional source.

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1 Introduction

Rice (*Oryza sativa* L.) is a staple food consumed daily in almost Asia countries. Numerous rice varieties differ in nutritional value and natural compounds (Kushwaha 2016), especially in pigmented rice varieties such as black, brown, purple, and red rice, containing exceptionally high levels of natural compounds (Veni 2019; Hue et al. 2018). Further, the rice bran layer and endosperm contain many compounds of high nutritional value (Pengkumsri et al. 2015; Eng and Mohd Rozalli 2022), making rice bran a rich source of these compounds that can be optimized for use in food products (Majzoobi et al. 2013). Rice bran has a higher lysine content (approximately 3-4%) than rice endosperm, other grains, or legumes. Further, lysine with a molecular weight greater than 0.5 kDa derived from alkaline-assisted extraction can exhibit anti-cancer activity without affecting normal cells. Rice bran protein (RBP) contains a significant amount of hydrolysate, including bioactive peptides (Boonla et al. 2015), which are highly digestible (> 90%) (Wang et al. 2015). Additionally, rice bran is gluten-free and does not generally contain allergens (Ngoc et al. 2019; Kaur et al. 2022). These properties make it suitable for manufacturing instant food formulas, gluten-free products, and cosmetics. Moreover, RBPs with a molecular weight of 57 kDa have been reported to exhibit cell-adhesion activity against lung carcinoma cells in Lewis mice (Shoji et al. 2001). However, rice bran is often considered a by-product of the milling process or agricultural waste (Chiou et al. 2013).

"Cam" rice is a popular Vietnamese native cultivar grown in the Cai Lay district of Tien Giang province. This cultivar is a nourishing staple food, containing more nutrients than other rice cultivars (Loan and Thuy 2019), and is especially rich in protein content. It is essential to extract protein from this rice to remove redundant starch content using amylase for a higher yield and purity (Acton 2013). Additionally, the extraction protocols should be adequate to obtain the best possible product. For the apparent reason of having various controlling factors, using the response surface methodology seems to be the most feasible way to examine the variables and predict the optimal response with a limited number of trials (Phongthai et al. 2017; Van Tai et al. 2021; Thuy et al. 2022a). However, very few comprehensive studies on the optimization of protein extraction under alkaline and enzyme-assisted environment concerning various factors are available. Therefore, this study aimed to optimize the rice bran's protein extraction protocol by investigating three parameters, including pH, stirring time (h), and incubation temperature (^oC). This protocol may enhance the quantity and quality of protein extraction to use rice bran effectively for food products.

2 Materials and Methods

2.1 Materials and procedures

"Cam" rice is conventionally cultivated under conventional conditions in the Cai Lay district, Tien Giang province (10°29'49.5"N 106°03'11.0"E). After harvesting, the rice bran was collected after milling at a local company (Tien Giang Food Company). The bran was then dried at 40°C until its moisture content was less than 13% and then vacuum-packed and stored at 4°C until use. The "Cam" rice bran contained 14.1% protein, 20.5% starch, 10.1% ash, and 7.6% moisture, which were analyzed by AOAC (2005) method. After that, 25 g of rice bran was mixed with distilled water at a ratio of 1:7 (w/v). The pH of the suspension was adjusted to a range of 8.5 to 9.5 using 1N NaOH. The mixture was continuously stirred for 3.5 to 4.5 hrs with a magnetic stirrer at 500 rpm to dissolve the protein.

To hydrolyze the starch of rice bran, the pH of the solution was adjusted to 7.0 using $(NH_4)_2SO_4$. Next, 0.25% α-amylase was added to the mixture and heated for 20 min at 85^{o}C to 95^{o}C. Afterwards, the mixture was centrifuged at 3000 g to remove any remaining residues and collect the soluble protein fraction. The protein precipitation process was carried out at pH 3.5-4.5 by 1N HCl. The mixture was recentrifuged at 3000 g to yield the precipitated protein, which was then washed with sterile distilled water (2 times). Finally, the precipitated protein was dried until its moisture content was less than 13% and stored in vacuum-sealed PA packaging for later use. The enzyme Termamyl (Termamyl 120L, liquid endo-alpha amylase-1 gallon/3.785 liters) was purchased from Novozyme company. It had a pH range of 5.5 – 7.0, 120 KNU-T/g (Kilo Novo α-amylase unit), and was resistant to heat, retaining its activity even at 105 °C.

2.2 Optimization design

The extraction process optimization was designed using the Box-Behnken model with 15 experimental units, which included three central experiments and three replicates with selected variables. Each factor was surveyed with three levels (-1, 0, and +1), including the bran fluid pH (8.5 - 9.5), stirring time (3.5 to 4.5 h) for dissolving proteins, and suitable temperature (85 ^{o}C to 95 ^{o}C)

Table 1 Range and levels of the independent variables' experiments

Variables (extraction parameter)	Levels of code		
	-1	0	1
A: pH	8.5	9.0	9.5
B: Stirring time (h)	3.5	4.0	4.5
C: Incubation temperature (^oC)	85	90	95

for amylase enzyme activity to hydrolyze starch, as shown in Table 1. The general equation of a response surface Y that depends on factors A, B, and C, is indicated below (Thuy et al. 2022b; Thuy et al. 2022c):

$$Y= b_o+b_1A+ b_2B+ b_3C+ b_4AB+ b_5AC+ b_6BC+b_7A^2+b_8B^2+b_9C^2 \quad (1)$$

Where A, B, C are independent variables, while b_{0-9} represent model term effects. The selection criteria of the model was based on the regression's R^2 value.

2.3 Determination of protein content

The protein content of rice bran extract (%) was determined by the Kjeldahl method (AOAC 2005). The protein extraction yield was calculated as the below formula (Eze et al. 2022):

$$\text{Protein extraction yield (Y1)(\%)} = \frac{\text{Total protein (rice bran + enzyme) − residual protein (in meal)}}{\text{Total protein (rice bran + enzyme)}}$$

2.4 Statistical analysis

The optimum levels of the components in the formulation for protein extraction from "Cam" rice bran were determined with RSM using Statgraphics Centurion 16. The data obtained were statistically treated by analysis of variance (ANOVA), and the means were compared by the Fisher LSD test at a significance level of 0.05. Data were presented as the mean of sample sets. Statistical analysis of the results to assess significant differences among samples was performed

3 Results and Discussion

3.1 Impact of single factors on protein extraction yield and protein content

This study focused on three factors that directly affect the rice bran's protein extraction process: pH, stirring time, and temperature to protein extraction yield (%) and protein content (%) (Figure 1). Cell disruption is critical for maximum protein acquisition since the rice bran is deep inside plant cells. The stirring motion aids in cell breakdown (Mittal and Ranade 2023). Hence, we applied a mixing time of 3.5 to 4.5 h for further investigation. Tang et al. (2002) stated that stirring is a crucial agitated factor commonly used to disrupt cell structure. Temperature also affects protein extraction efficiency, as it determines enzyme activity, including that of α-amylase. Since rice grain contains numerous carbohydrates, removing these redundant residues is necessary for the highest and purest possible protein content, and the enzyme α-amylase performs this procedure.

It can be inferred that pH is the most impactful factor as long as the ideal value is maintained. The high protein extraction yield was under alkaline conditions (Ahlström et al. 2022). Furthermore, increasing pH, stirring time, or working temperature can gradually decrease protein extraction productivity. The findings of this study agree with the previous research by Chich et al. (2014), which reported that the optimal temperature range for the enzyme termamyl of seaweed is between 80-95°C.

The alkaline medium is the most effective for protein extraction as it breaks the hydrogen and peptide bonds between proteins. In contrast, an acidic environment is not optimal for protein collection as the protein isoelectric point is within this pH range. This study estimated the optimal pH range for protein extraction was from 8.5 to 9.5. Additionally, a more basic environment did not correlate with increased protein extraction efficiency, and a higher pH only decreased yield, consistent with findings by Silventoinen et al. (2019).

3.2 Regression equation of yield (%) and extracted protein content (%)

Table 2 presents the statistical analysis results for the studied factors, including pH, stirring time, and temperature, and their effects on extract yield and protein content. Most factors showed a statistically significant difference at a 95% confidence level with a p-value less than 0.05, except for the quadratic function of temperature in the regression equation of extract yield. The coefficients for determined and adjusted yield (%) were 95.3% and 94.1%, respectively, and for protein content, the coefficients were 94.4% and 94.0%. A high coefficient of determination value

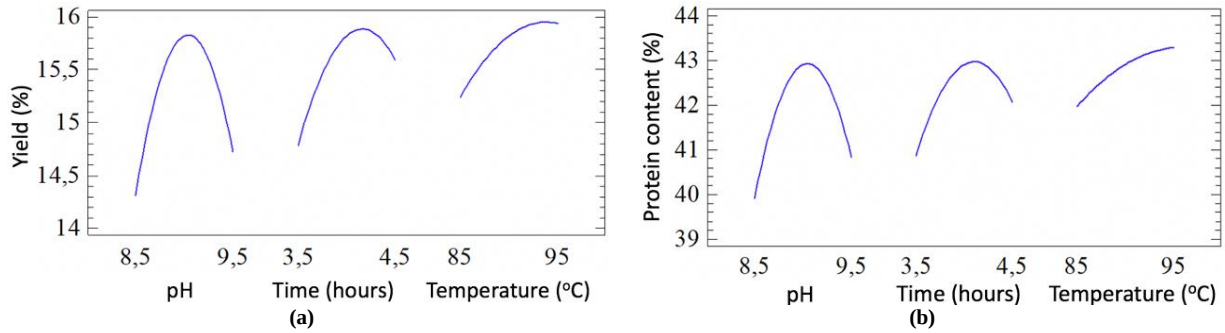

Figure 1 The effects of pH, stirring time, and temperature for α-amylase activity on the yield (%) and obtained protein content (%)

Table 2 Analysis results of the impact of coefficients on the regression equation of yield (%) and obtained protein content (%)

Model term	Protein content (%)		Yield (%)	
	P-value	Significant difference	P-value	Significant difference
A: pH	0.0001	Significant	0.0000	Significant
B: Stirring time (hour)	0.0000	Significant	0.0000	Significant
C: Temperature (^oC)	0.0000	Significant	0.0000	Significant
A^2	0.0000	Significant	0.0000	Significant
AB	0.0000	Significant	0.0000	Significant
AC	0.0047	Significant	0.0108	Significant
B^2	0.0000	Significant	0.0000	Significant
BC	0.0017	Significant	0.0003	Significant
C^2	0.0019	Significant	0.0552	Insignificant
	R^2 = 95.3%; $R^2_{adjusted}$= 94.1%		R^2 = 94.4%; $R^2_{adjusted}$= 94.0%	

indicates the best fit between actual and predicted data considered. The determined coefficients indicate the effects of variables on the model and are sequentially converted into the adjusted values. Overall, the results suggest that the studied parameters apply to protein production.

After excluding the insignificant factor, we acquire formulas that show the correlation between recovery yield (%) and protein content (%):

Yield (%) = -717.6 + 111.1A + 50.1B + 2.8C – $5.2A^2$ – 2.3 AB – 0.1AC – $2.5B^2$ – 0.1BC

Protein (%) = -1267.5 + 206.8A + 90.4B + 4.4C – $10.1A^2$ – 2.6AB – 0.2AC – $5.6B^2$ – 0.2BC – $0.01C^2$

3.3 The optimization results of two response surfaces of yield and protein content (%)

Table 3 describes the optimization results of two response surfaces regarding each factor being considered with the other ones sequentially. It was found that a high recovery yield (16.0%) can be obtained by setting the pH at 9.0, stirring time at 4.12 h, and temperature at 93.2°C. On the other hand, the maximum protein content (43.3%) can be achieved by setting the pH at 9.01, stirring time at 4.01 h, and temperature at 95°C. Simultaneous optimization for maximum recovery yield and protein content can be achieved by setting the pH at 9.02, stirring time at 4.02 h, and temperature at 90.6°C. It was also observed that the temperature was the most significant factor that varied the most among the three optimization factors.

Figures 2a and 2b illustrate the interactions between pH and two additional factors, stirring time and temperature, and their impact on protein extraction efficiency. The data indicates that as the pH increased from 8.5 to 9.0, protein extraction efficiency increased, with the maximum efficiency observed at pH 9.0. This finding is consistent with research by Hou et al. (2017), who reported a positive correlation between protein solubility and pH. This suggests that higher pH levels can lead to greater protein solubility, which may explain the increased extraction efficiency (Zhang et al. 2023).

In addition, Wang et al. (2015) have further elucidated that protein has a negative charge at a pH of 9.0, which increases its

Table 3 The optimal pH value, stirring time, and temperature for α-amylase to achieve the highest yield (%) and obtained protein content (%)

Factors	Optimization on each surface		Optimum condition of both surfaces
	Protein content (%)	Yield (%)	
pH	9.01	9.00	9.02
Stirring time (hour)	4.01	4.12	4.02
Temperature (^oC)	95.0	93.2	90.6
Optimal results for each surface (%)	43.3	16.0	
Optimal results for both surfaces (%)	43.02	15.9	

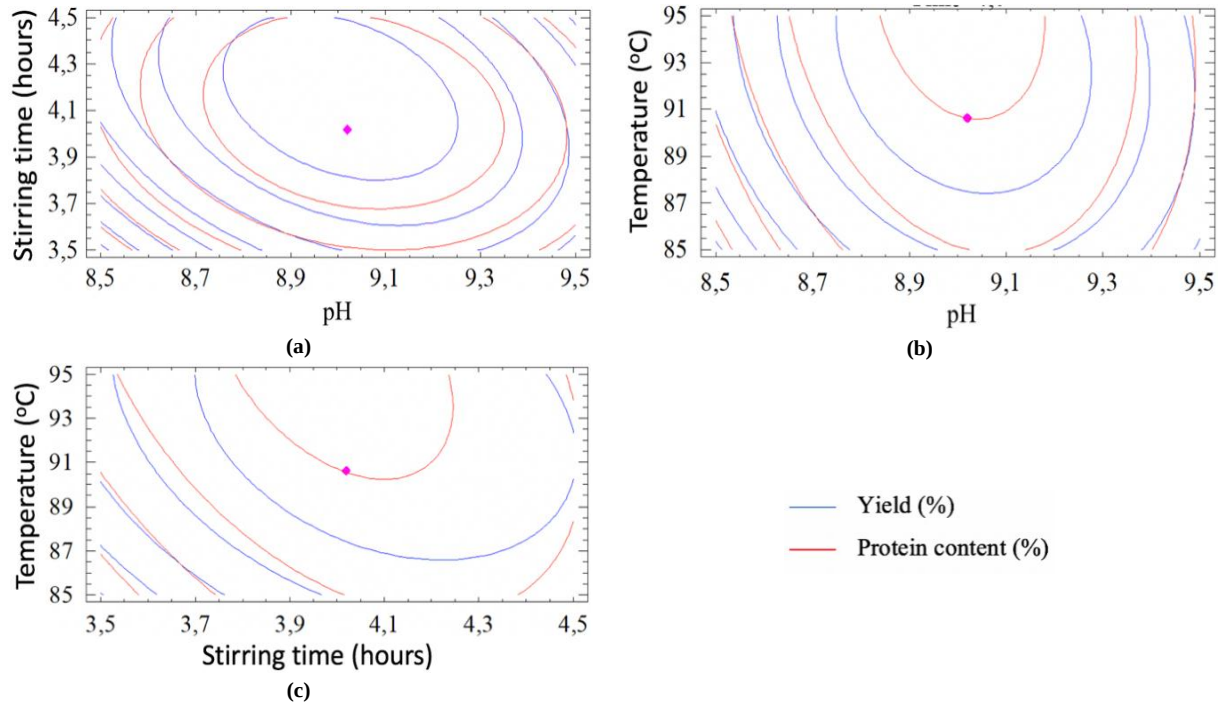

Figure 2 The response surface of each pair of factors investigated pH, stirring time, and temperature affecting the yield (%) and obtained protein content (%); (a) pH and stirring time (Temperature: 90°C); (b) pH and temperature (stirring time: 4 h); (c) Stirring time and temperature (pH: 4.0)

interaction with water molecules and enhances solubility. However, this relationship is not linear; solubility rises to a certain point and gradually decreases. Guan et al. (2017) proposed that this may be because proteins interact more with other raw materials than water, reducing the amount of protein available for extraction. This idea is supported by Theerakulkait et al. (2006), who found that pH 9.0 was more effective for protein extraction than pH 9.5. However, it is important to note that alkaline environments can cause protein degradation, leading to the formation of toxins in organisms. This degradation process can convert cysteine and serine residues into dehydroalanine and lysinoalanine, which alters their original conformation. Thus, selecting pH 9.0 may be an ideal condition for protein extraction while minimizing the risk of degradation and toxin formation. It also agreed with the study of Wang et al. (2022) on extracting protein from edible oil.

Figures 2a and 2c correlate stirring time and two other variables. The effectiveness of protein extraction generally improved as the stirring time increased from 3.5 to 4.0 h, reaching its peak at 4.0 h. According to Shen et al. (2008), extraction productivity rose sharply in the first 2.0 h, peaked at 4.0 h, and no significant growth was observed beyond 4 h. Stirring motions that occur in less than 4.0 h were necessary to break the bonds between phytic acids and proteins, which hinder protein extraction (Canan et al. 2011; Nourmohammadi et al. 2023). Surprisingly, stirring for more than 4.5 h does not yield better results than stirring for 4.0 h since proteins are partially oxidized due to prolonged immersion in the water.

Figures 2b and 2c present temperature in the relationship with the two remaining factors and prove that raising the temperature from 85 to 90°C, increased the effectiveness of protein extraction. However, this conclusion must be considered that the temperature range must be within the tolerance of the enzyme under the denaturation point (Luong 2014). If inapplicable, as soon as the temperature exceeds the limit, the enzyme activity will gradually decrease and finally disappear (Jouanneau et al. 2010). Amylase cannot function properly, leading to the high remaining amount of carbohydrates in the rice bran, causing difficulty in extracting protein (Phuong et al. 2015). As a result, attained, 90°C acts as an upper limit since exceeding this temperature will decrease the efficiency of the process.

Conclusion

In conclusion, the working pH, stirring time, and temperature affected the protein extraction protocol used with "Cam" rice bran. The optimal parameters to achieve the highest extraction efficiency were pH 9.02, a stirring time of 4.02 hrs, and a temperature of 90.6°C. These conditions yielded an extraction efficiency of 15.9% with a protein content of 43.03% (moisture <13%).

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Conflicts of Interest

The authors declare no conflict of interest

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