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STATISTICAL OPTIMIZATION OF PHYSICOCHEMICAL FACTORS FOR PROTEASE PRODUCTION BY *BACILLUS LICHENIFORMIS* ON SKIM LATEX SERUM FORTIFIED MEDIA

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Abstract

In this study Response Surface Methodology has been applied to optimize the physicochemical factors in maximizing the production of protease by *Bacillus licheniformis* in a medium fortified with skim latex serum. The results showed that the production increased approximately a nine fold to the original medium. The predicted values of the model were in agreement with the experimental values and validation steps verify its adequacy and accuracy. Thus, this study presents a holistic approach for the bioconversion of agro-industries waste to value-added protease enzyme, potentially useful in many industries and thus minimizing the release of polluted water into the environment by the natural rubber industries.

Keywords: Protease production, Skim latex serum effluent, *Bacillus licheniformis*, Fermentation optimization, Response surface methodology.

1. Introduction

Proteases (EC 3.4.21-24) are one of the most significant commercial enzymes that represent about 60% of global sale of industrial enzymes [1-3].The industrial sale of proteases is estimated at more than $350 million annually [4]. The global demand of enzymes reached $3.3 billion in 2010 and will rise to $4.4 billion in 2015 [3, 4]. This potential enzyme can be applied in textile, leather, baking, brewing, animal feed, pharmaceutical, silver recovery, waste treatment, and detergent industries [1, 5-7]. The high demand of this enzyme has attracted researches for simple and efficient method to produce them using an abundant and inexpensive raw material such as skim latex serum effluent from Natural Rubber (NR) industry.

Nomenclatures

A	Initial pH
B	Agitation
M^+	Responses (protease production) at higher level
M^-	Responses (protease production) at lower level
N	Number of experiments performed
R^2	Determination coefficient
Y	Protease activity

Greek Symbols

ε	Coefficient tested variable

Abbreviations

FCCCD	Face Centered Central Composite Design
NR	Natural Rubber
OFAT	One factor at a time
PB	Plackett-Burmann
RSM	Response Surface Methodology

NR from *Hevea brasiliensis* is a strategic crop in Malaysia since it generates foreign exchange revenue of about 30% per year [8], contributing employment opportunities for more than 68,700 people and providing raw materials for local industries [9]. Although the rapid expansion of this sector plays an important role in developing country, at the same time it produced large quantities effluent since the production of rubber product from NR need large amount of water for its operation [8, 10]. The effluent from rubber factories estimated about 100 million litres every day [11], and Malaysia alone produces 75,000 litres of natural rubber waste serum annually [12]. This sewage could pollute environment due to high COD, BOD, and nitrogen level, that could reach 14,000, 7000, 1,800 ppm respectively [8, 13].

The main treatment carried out by businesses organizations involving 'end of pipe' solution [14, 15], but prior to this treatment; skim latex serum which is considered as a by-product or raw materials in other processes has been found to be an important growth medium for various fermentation processes [16], single cell protein production [17], pigment (carotenoids) production [18], industrial protein and quebrachitol [19], fertilizer [20, 21], animal feed [22], a medium for culturing fish [23], and enzyme production [24]. The great prospective of this waste has allowed its conversion into extra value-added natural product such as in the protease enzyme production.

Besides that, NR waste serum contains various organic compounds including proteins, lipid, carbohydrates and minerals (sulphur, potassium, phosphorus, chlorine, zinc, magnesium, sodium, calcium, aluminium and rubidium) that are required for microbial growth [12, 19, 20].

Therefore, the aim of this study is to manipulate skims latex effluent as a basal medium for extracellular protease production by *Bacillus licheniformis* (ATCC 12759) in submerged fermentation.

2. Materials and Methods

2.1. Raw material

Skim latex effluent was collected from MARDEC Industrial Latex Sdn. Bhd, Tapah, Perak, Malaysia. Skim latex serum was prepared by coagulating the rubber particle in the sample. This was carried out by decreasing the pH to 5 with glacial acetic acid and centrifuged at 10,000 xg at 4^{o}C for 10 minutes before sterilization.

2.2. Microbial culture and inoculum preparation

The *Bacillus licheniformis* (ATCC 12759) culture was maintained on nutrient agar at 37^{o}C for 24 h. A loopful of the growth was transferred to the nutrient broth media. One hundred millilitres of modified medium was inoculated with 1 to 4 % (v/v) of the inoculums (3×10^{9} cells (CFU/ml)) and was incubated for 24 h at various temperatures (based on design matrix, Table 2).

2.3. Enzyme assay

Protease assay was determined by casein digestion method as suggested by Padmapriya et al. [25]. A 2.5 ml of 1 % (w/v) casein (prepared in 50 mM of tris buffer, pH 8) was equilibrated to 37^{o}C for 5 min prior adding 0.5 ml of crude enzyme. The solution was mixed by swirling and incubated for 10 min in 37^{o}C water bath. The enzyme reaction was stopped by adding 2.5 ml of 0.11 M trichloroacetic acid, then allowed to stand at room temperature for 45 min. The supernatant was collected by centrifugation at 10,000 xg for 10 min at 4^{o}C. Subsequently, colour development was achieved by mixing 1 ml supernatant with 2.5 ml of 0.5 M Na_2CO_3 and 0.5 ml of 0.1 N Folin reagent for 30 min. Ultimately, the OD of the mixed solution was measured at 660 nm against the blank sample without incubation. One unit of protease activity is defined as the amount of enzyme required to liberate 1 µg tyrosine per millilitre in 1 min under the assay condition.

2.4. Statistical optimization experiments

2.4.1. Plackett-Burman design

Screening signifant variables that influence the protease production was performed by Plackett-Burmann (PB) design. Eleven independent variables were evaluated at two level (high and low) and designated as level +1 and -1 respectively (Table 1). The significant variables were screened in 12 combinations in accordance with the design matrix (Table 2) and the response was measured by the protease activity. Design Expert 7.1.6. (State-Ease, Inc., Minneapolis, USA) was applied to generate and analyze the experimental design of Plackett-Burmann.

2.4.2. One factor at a time (OFAT) analysis

Following PB design, the classical OFAT approach was employed to determine the centre points of variables for the next optimization step. These included: pH, agitation and incubation time.

Table 1. Physicochemical components used in Plackett-Burmann.

Variables	Factors	(-1)	(+1)
X_1	**Lactose % (w/v)**	0.0	1.0
X_2	**Galactose % (w/v)**	0.0	1.0
X_3	**Casein % (w/v)**	0.0	0.5
X_4	**KH_2PO_4 % (g/l)**	0.0	0.5
X_5	**$MgSO_4.7H_2O$ % (g/l)**	0.0	0.5
X_6	**LB broth % (w/v)**	0.0	1.0
X_7	**Skim latex serum % (v/v)**	10	50
X_8	**Inoculums size % (v/v)**	1.0	4.0
X_9	**Agitation (rpm)**	50	250
X_{10}	**pH**	7.0	11
X_{11}	**Temperature (^oC)**	25	35

2.4.3. Response surface methodology

Face Centered Central Composite Design (FCCCD) under Response Surface Methodology (RSM) was adopted to optimized four significant factors, viz. galactose, skim latex serum effluent, pH and shaker's rotating speed, which was studied at three levels with six replicates at a midpoint and thirty experimental runs. A second order polynomial equation was applied for the analysis of this proteolytic enzyme production, and the data were fitted in the equation by multiple regression procedure. The outcome of this, is an empiricel model that relate the response measured to the independent variables of the experiment. In addition, for four variables system (A, B, C and D), the model equation is:

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_4 D + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 + \beta_{44} D^2 + \beta_{12} AB + \beta_{13} AC + \beta_{14} AD + \beta_{23} BC + \beta_{24} BD + \beta_{34} CD \quad (1)$$

where Y is predicted response, β_0 is intercept, $\beta_1, \beta_2, \beta_3, \beta_4$ are linear coefficient, $\beta_{11}, \beta_{22}, \beta_{33}, \beta_{44}$ are squared coefficients, $\beta_{12}, \beta_{13}, \beta_{14}, \beta_{23}, \beta_{24}, \beta_{34}$ are interaction coefficients [26, 27].

2.4.4. Verification of the quadratic model

The fermentation of the validation experiments were conducted at the optimized condition to confirm the result from the response surface model. Three sets of experiments were carried out within the design space (Table 5) for 24 h.

3. Results and Discussion

The influence of eleven medium compositions and four culture conditions namely lactose, galactose, casein, KH_2PO_4, $MgSO_4.7H_2O$, LB broth, skim latex serum effluent, inoculum size, agitation, pH and temperature of incubation were examined in 12 runs with triplicates using Plackett-Burmann design and their settings are shown in Table 2. The enzyme activity was calculated by calorimetric method with L-tyrosine as a standard curve and variations ranging from 0.4 U/ml

to 2 U/ml were observed in 12 trials (Table 2). Moreover, the effect of individual component for protease production was calculated using the following equation:

$$\epsilon = (\sum M^{+} - \sum M^{-})/N \quad (2)$$

where ϵ is the 'Main Effect' of tested variable, M^{+} and M^{-} are responses (protease production) of trials at which the parameter was at its higher and lower level respectively and N is the number of experiments performed [28, 29].

The Pareto graph was used to reveal the 'Main Effect' of all variables on protease production (Fig. 1). A large coefficient tested variable (ϵ), either positive or negative indicates a large impact on the response; while a coefficient close to zero indicates little or no effect on the response. Based on the Pareto graph below and statistical analysis (F-value=8.43 and *p*-value=0.0285) (data is not shown), significant effect (*p*-value < 0.05) were contributed by four factors namely galactose (*p*=0.0220), skim latex serum (*p*=0.0437), pH (*p*=0.0120) and agitation (*p*=0.0178), thus they were included and varied in the next optimization strategy. LB broth, inoculum size and temperature of incubation were kept constant at their optimum level at PB design, because their functions in the fermentation medium could not be replaced. On the other hand, due to low coefficient values of lactose, casein, KH_2PO_4 and $MgSO_4.7H_2O$ and their function in fermentation medium may be replaced by skim latex serum, the basal medium, thus those variables were excluded for further optimization.

Table 2. Plackett-Burmann design with coded values along with the observed results.

Trial No.	Coded variables level											Protease activity (U/ml)
	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	X_{10}	X_{11}	
1	-	-	-	-	-	-	-	-	-	-	-	1.00
2	+	+	-	+	+	-	+	-	-	-	+	2.00
3	+	+	-	+	-	-	-	+	+	+	-	0.50
4	-	-	-	-	+	+	-	+	+	-	+	0.60
5	-	-	+	+	+	-	+	+	-	+	-	0.60
6	+	-	+	-	-	-	+	+	+	-	+	1.30
7	-	+	+	-	+	-	-	-	+	+	+	0.40
8	+	-	+	+	-	+	-	-	-	+	+	0.80
9	+	-	-	-	+	+	+	-	+	+	-	0.50
10	-	+	-	-	-	+	+	+	-	+	+	1.70
11	+	+	+	-	+	+	-	+	-	-	-	1.80
12	-	+	+	+	-	+	+	-	+	-	-	1.40

X_1= Lactose, X_2= Galactose, X_3= Casein, X_4= KH_2PO_4, X_5= $MgSO_4.7H_2O$, X_6= LB broth, X_7= Skim latex serum, X_8= inoculum size, X_9= Agitation, X_{10}= pH, X_{11}= Temperature of Incubation.

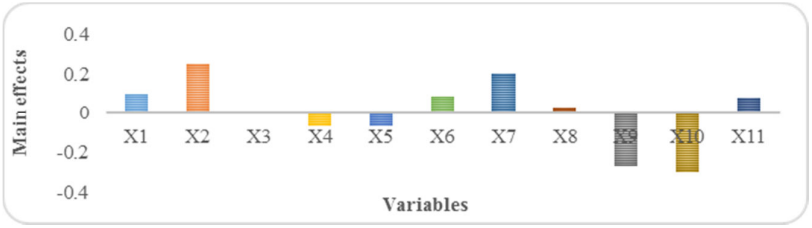


Fig. 1. Pareto graph showing the main effect result of the 11 components for protease production based on Plackett-Burman experimental results.

Since the PB design on its own does not determine the range of values for further optimization, and no report is available in literature on which the optimum level of these factors will enhance the protease production with respect to sewage from rubber factory as the major growth medium, thus screening using the classical method, OFAT study was first conducted for pH, shaker's rotating speed and incubation time, but the results are not shown here.

The four selected variables were optimized by FCCCD under RSM and the experimental design matrix (data is not shown) resulted thirty experiments that were performed in triplicates. Then, the data were analyzed using Design-Expert software. The regression equation obtained after the analysis of variance (ANOVA) (data is not shown) indicated the model is significant (p-value= <0.0001) and the F-ratio for lack of fit (0.3433) demonstrated that the lack of the regression model is insignificant. The coefficient of determinations (R^2) was 0.9012 which ensured a satisfactory adjustment of the quadratic model to the experimental data. It also explains that statistical model could explain 90.12% of variability in the protease activity (response) and only 9.88% of the total variations were not explained by the model. Additionally, the model had adjusted determination coefficient of Adj R^2= 0.8091. This value was close to R^2, suggesting a higher significant of the model used for analyzing the data. The value of the coefficient of variation was (CV = 7.71%), indicating a better precision and reliability of the experiments carried out [30].

In order to understand the interaction of each component required for maximum protease production, three dimensional response surface curve were then plotted (Fig. 2). Figure 2(a) shows the interaction between skim latex serum and galactose, while keeping the pH and agitation at the center point, pH 4 and 100 rpm respectively. Maximum enzyme yield in these conditions was predicted to be 15.4484 U/ml, corresponding to a maximum level (+1) of both skim latex serum (70 % (v/v)) and galactose (1.5 % (w/v)). However, the curve imply that the enzyme yield still increase with increasing concentration of skim latex serum and the response did not vary much as a function of galactose concentration.

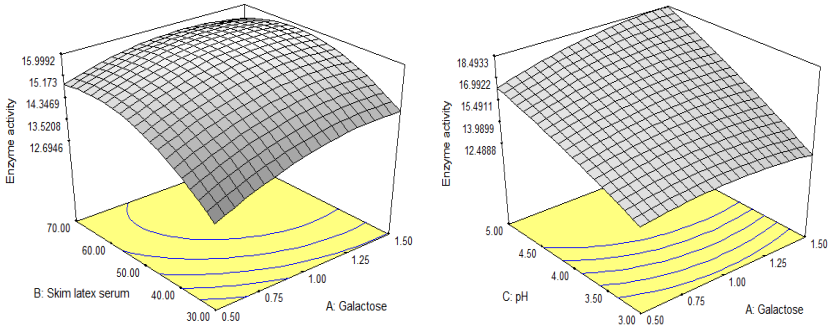
Figure 2(b) represents the interactive factors, pH and galactose, when skim latex serum was maintained at a midpoint. With the increase in the initial pH, the enzyme production further increased from 13.2454 U/ml to 15.4484 U/ml. However, the response curve did not show curvature; rather it was flattened with further points moving towards higher enzyme units. This also occurred between agitation and galactose (data is not shown).

Since the surface plots suggested demand for higher concentration of skim latex serum, increase pH and decrease agitation factors to obtain optimum protease production, thus, a second optimization was established. But, since it was important to limit the total fermentation volume to 100 ml (for comparison purposes), the second optimization was carried out with two variables combinations, pH and agitation while keeping the concentration of skim latex serum constant at maximum value of 70 % (v/v).

Two cultural conditions, initial pH and shaker's rotating speed were optimized in thirteen runs with 5 triplicates at a center point (Table 3). They were set at pH 3 – 8 and agitation of 60-100 rpm. The goodness of fit of the model was checked by determining the coefficient of determination (R^2) and adjusted R^2 through ANOVA analysis as displayed in Table 4. Analysis of variance from RSM

yielded the following final regression equation in terms of protease activity (Y) as a function of initial pH (A) and agitation (B):

$$Y = +17.98 + 0.22\,A - 0.91\,B - 4.48\,A^2 - 1.56\,B^2 - 1.10\,A\,B \quad (3)$$



(a) **(b)**

Fig. 2. Three dimensional graphs showing the effect of: (a) skim latex serum and galactose (b) pH and galactose on protease production by Bacillus licheniformis (ATCC 12759).

ANOVA of the quadratic regression model demonstrated that the model is very significant as was evident from the Fisher's F-test. ($p>F = 0.0002$). The model F-value of 28.84 implies the model is significant with lack of fit value of 0.7790. Furthermore, determination coefficient (R^2 = 0.9537), which means 95.37% variability in the response could be explained by this model. The R^2 value is always between 0 and 1.0 that imply stronger model with better prediction to response [27, 31].

Table 3. Experimental and predicted value of protease activity for FCCCD matrix.

pH (A)	Agitation (rpm) (B)	Protease activity (U/ml)	
		experimental	Predicted
8	80	11.47	11.54
8	60	13.77	14.17
8	100	12.33	11.91
5.5	100	10.23	10.14
5.5	60	12.93	13.28
3	80	14.03	13.72
3	100	17.81	17.33
5.5	80	15.00	15.51
5.5	80	18.83	17.98
5.5	80	17.87	17.98
5.5	80	19.13	17.98
5.5	80	17.07	17.98
3	60	17.03	17.98

Table 4. Analysis of variance for protease production by Bacillus licheniformis on skim latex serum as a basal media.

Source	Sum of square	DF	Mean Square	F-value	p-value
Model	99.98	5	20.00	28.84	0.0002
A	0.28	1	0.28	0.41	0.5444
B	5.00	1	5.00	7.21	0.0313
A-A	55.41	1	55.41	79.93	<0.0001
B-B	6.71	1	6.71	9.68	0.0170
A-B	4.84	1	4.84	6.99	0.0333
Residual	4.85	7	0.69		
Lack of fit	1.06	3	0.35	0.37	0.7790
Pure error	3.80	4	0.95		
Cor total	104.83	12			

Std. Dev = 0.83, R^2 = 0.9537, Adj R^2 = 0.9206, Pred R^2 = 0.8445, Adq. Precision = 13.851, CV = 5.48.

The adjusted R^2 value corrects the R^2 value for the sample size and for the number of terms in the model. In this study the adjusted R^2 value (0.9206) was less than the R^2 value (0.9206). This is because, if there are many terms in the model and the sample size is not very large, the adjusted R^2 may be smaller than the R^2. At the same time, a relatively lower value of the coefficient of variation (CV=5.48%) indicated a better precision and reliability of the experiments executed. The adequate precision measures the signal to noise ratio. A ratio greater than 4 is desirable. Ratio of 13.851 indicates an adequate signal and this model can be used to navigate the design space.

The interaction effect between pH and agitation was determined by plotting the 3D response surface curve (Fig. 3). The curve illustrated the interaction effect of pH and agitation on protease production on skim latex serum enriched media. Maximum protease activity was produced around pH 5.5 and agitation 75 rpm. Further increase in pH and any deviation of agitation from optimal condition, decrease the enzyme production.

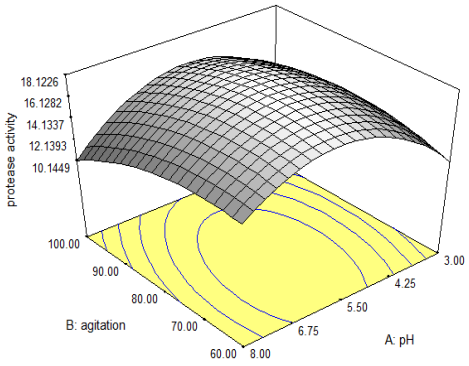


Fig. 3. Three dimensional graph showing the interaction between pH and agitation on protease production by Bacillus licheniformis (ATCC 12759).

The optimized condition was finally verified by again performing the batch shake flask experiment in triplicate as describe in Table 5. The highest experimental protease production of 19.867 U/ml was very close to 18.1103 U/ml predicted by FCCCD under RSM. Therefore, under optimized condition, the production of protease by *Bacillus licheniformis* utilizing skim latex serum effluent improved from 2 U/ml to 18.1103 U/ml using RSM.

Table 5. Validation of the response surface model.

pH	Agitation (rpm)	Enzyme activity (U/ml)		Error (%)
		Mean Experimental	Theoretical	
5.5	75	19.867	18.1103	8.84
4.5	80	20.2	17.1763	14.96
6.5	70	21.5	17.6361	17.97

4. Conclusions

The application of Plackett-Burman design and Face Centered Central Composite Design in Response Surface Methodology for determination of optimal physicochemical factors for protease production is demonstrated in the present study. The optimization resulted in about nine fold increase of protease activity. Mutual interaction between pH 5.5 and agitation rate of 80 rpm with fixed concentration of skim latex serum (70 %, v/v) reveals maximum protease production approximately 19.867 U/ml by *Bacillus licheniformis* (ATCC 12759). Therefore, the utilization of an inexpensive and abundant known pollutant (skim latex serum) by valorization strategy that was explored in this study may lead to future application of *Bacillus licheniformis* protease in industries particularly in laundry detergent formulation as well as biological treatment for the pollutant.

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