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Optimization of Pectate Lyase Production from *Paenibacillus polymyxa* N10 in Submerged Fermentation using Response Surface Methodology

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ABSTRACT

The effectiveness for optimization of pectate lyase production from *Paenibacillus polymyxa* N10 was studied using central composite design with three factors: agitation speed (X_1, 100-300 rpm), temperature (X_2, 25-45°C) and pH (X_3, 5.5-9.5). It was found that the most significant factors influencing enzyme production were temperature and pH. The second order polynomial regression model obtained was fitted and found adequate, with an R^2 of 0.9600 ($p < 0.001$). From the result of this optimization, maximum pectate lyase activity at 84.5 U/ml was achieved at temperature 35°C, pH 8 and an agitation speed 200 rpm.

Key words: pectate lyase, *Paenibacillus polymyxa*, response surface methodology

INTRODUCTION

Enzymes that hydrolyze pectin substances, which contribute to the structure of plant cells, are known as pectinolytic enzymes or pectinases. Based on their mode of actions, these include polygalacturonase, pectin esterase, pectin lyase and pectate lyase (PL) (Tari *et al*., 2007). PL (EC4.2.2.2.) hydrolyzes the α-1,4-glycosidic bond of polygalacturonate and releases unsaturated soluble oligogalacturonates (Matsumoto *et al*., 2002). PL has potential applications in cotton scouring, degumming of plant fibers, improving of fiber quality, decreasing the cationic demand of pectic solutions in paper processing, treatment of effluents from food processing industries and enhancing the fermentation step for tea and coffee processing (Gummadi and Sunil, 2007). It has been reported that PL is produced from a wide variety of microbial sources such as fungi, actinomycetes and bacteria (Hoondal *et al*., 2002). The PL was produced by several varieties of bacteria according to the type of strain, cultivation conditions (pH, temperature, aeration, and agitation) and the growth medium composition. Therefore, these have to be specified individually for each and every single strain of interest.

Paenibacillus polymyxa N10 (from a mulberry bark) was the one which had been usefully in the production of PL (Sittidilokratna *et al*., 2007). The cultivation involves with many factors, such as temperature, pH, aeration and

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agitation, which are important and affect the growth and productivity. It is difficult to find the most important factors and to optimize the conditions. Response surface methodology (RSM) is an experimental strategy for seeking the optimum conditions for a multivariable system (Chen *et al*., 2002). Tari *et al*. (2007) applied the response surface design techniques in fermentation process development for improving the production of pectinase enzyme from *Aspergillus sojae* ATCC 20235. As a result of this optimization, maximum pectinase activity was achieved. A 1.5-fold increase in pectinolytic enzyme secretion by *Kluyveromyces wickerhamii* was attained, when pH, temperature and inoculation period were optimized by RSM (Moyo *et al*., 2003). A 41-fold enhancement in alkaline pectinase production by *Bacillus pumilus* was achieved by using Burman design and RSM (Sharma and Satyanarayana, 2006).

The aim of this research is to apply the central composite design for examination and optimization of the fermentation conditions for PL production of *P. polymyxa* N10 in submerged fermentation.

MATERIALS AND METHODS

Microorganism

P. polymyxa N10 was isolated from a mulberry bark by Sittidilokratna *et al*. (2007) and maintained on nutrient agar slants at 4°C and also stored as glycerol stocks at -20°C.

Pectate lyase production

All treatment combinations (Table 2) were performed in 500 ml Erlenmeyer flasks containing 200 ml basal media (1.5% pectin, 0.5% monosodium L-glutamate, 0.3% ammonium sulphate, 0.08% disodiumhydrogen phosphate, 0.05% magnesium phosphate, 0.02% calcium chloride and 0.24% potassium dihydrogen phosphate) (Kobayashi *et al*., 1985). The experiments were performed according to the central composite design (Table 1 and Table 2). After 72 h of incubation, each flask was assayed for enzyme activity. Enzyme activity was determined on supernatant obtained after the centrifugation of the broth at 11380 xg for 20 min at 4°C. PL activity was determined in the supernatants.

Enzyme assay

PL activity was determined spectrophotometrically by measuring the increase in absorbance at 235 nm (Sittidilokratna *et al*., 2007). The reaction mixture (1 ml) containing 0.1 M NH_4Cl-NH_4OH buffer pH 10, 0.4% (w/v) sodium pectate and 0.04 ml crude enzyme was incubated at 35°C for 10 min. The reaction was terminated by adding 4 ml of 0.01 M HCl to the mixtures. Inactivated crude enzyme in boiling water for 10 min was used as control in the reaction.

One unit of pectate lyase corresponds to the amount of enzyme which lyzes a 0.4% sodium pectate solution and releases products with an absorbance increase of 0.2 at 235 nm within 10 min at pH 10.0 and 35°C (Sittidilokratna *et al*., 2007).

Experimental design

The RSM was used in investigated the effects of independent variables: agitation speed, temperature and initial pH of medium on the responses of PL activity. Using central composite design (CCD) for 3 factors (k=3), 17 treatment combinations was generated (Jangchud, 2006). To set up a statistical model, five levels for each variable were chosen. The upper and lower limits of each variable were chosen to encompass the range in literature and to reflect what was done in practice after a preliminary investigation of the limits. The codes of $\pm\alpha$ ($\pm$ 1.682) was designed at a distance of 1.682 ($2^{n/4} = 1.682$ for $n = 3$) from the design center. The remaining levels were

Table 1 Process variables used in the central composite design (K=3) with actual factor levels corresponding to coded factor levels.

Factor	code[a]	Actual factor level at coded factor levels of:				
		-1.682[b]	-1	0	1	+1.682
Agitation speed	X_1	115.9	150	200	250	284.1
Temperature	X_2	26.59	30	35	40	43.41
pH	X_3	5.82	6.5	7.5	8.5	9.18

[a] Code level limits based on preliminary investigations and also to reflect what was done in practice. (X_1= (Agitation speed-200)/50, X_2= (Temperature-35)/5.0 and X_3= (pH-6.5)/0.5.

[b] Levels based on the Central Composite Design

Table 2 Treatment combinations and mean response.

Treatment	Coded variable levela			Mean response (Y) (U/ml)
	X b1	X2	X3	
1	-1	-1	-1	60.4063
2	-1	-1	+1	59.2938
3	-1	+1	-1	53.6063
4	-1	+1	+1	69.1979
5	+1	-1	-1	64.6667
6	+1	-1	+1	61.5792
7	+1	+1	-1	51.3417
8	+1	+1	+1	68.8292
9	-1.682	0	0	69.7000
10	+1.682	0	0	70.4729
11	0	-1.682	0	78.1104
12	0	+1.682	0	60.2396
13	0	0	-1.682	43.8688
14	0	0	+1.682	65.6438
15	0	0	0	96.8417
16	0	0	0	91.2958
17	0	0	0	98.5208

[a] Code level limits based on preliminary investigations and also to reflect what was done in practice. (X_1= (Agitation speed-200)/50, X_2= (Temperature-35)/5.0 and X3= (pH-7.5)/0.5.

[b] Levels based on the Central Composite Design

identified using CCD (Moyo *et al.*, 2003). Table 1 contained the actual factor levels corresponding to the coded factor levels as followed: X_1= (agitation speed-200)/50, X_2= (temperature-35)/5 and X_3= (pH-7.5)/0.5. Table 2 showed the treatment combinations and response. From the experimental data according to this design, a second order polynomial regression model was:

$$Y= b_0+ b_1X_1+ b_2X_2+ b_3X_3+ b_{11}X^2_1+ b_{22}X^2_2+ b_{33}X^2_3+ b_{12}X_1X_2+ b_{13}X_1X_3+ b_{23}X_2X_3+ £ \quad (1)$$

Where Y = PL production (U/ml)
b_i = the linear coefficients
b_{ii} = the quadratic coefficient
b_{ij} = the cross product coefficients
£ = the model constant

RESULT AND DISCUSSION

Data were analyzed using SPSS for Windows Release 11.5 to yield regression equations (Eq.1), regression coefficients and analysis of variance. The model was examined for lack of fit, adequacy, and efficiency. From the analysis of variance (ANOVA), the model was highly significant ($p < 0.001$; Table 3) and the R^2 value being the measure of the goodness of fit of the model, indicated that 96.00 % of the total variation was explained by the model. Coefficient estimates in the regression model was presented in Table 4.

After the treatment combinations, all linear terms of the independent variables, quadratic term of agitation speed, temperature, pH and interaction terms of temperature with pH (X_2X_3) were included in the model for PL production since these were significant ($p < 0.05$). Thus, the temperature and pH were important in the enzyme activity of *P. polymyxa* N10, and treating them together may reflect their true influence to the response. The optimum pH and temperature were also consistent with values those have been found for pectinase production of pH 8.0-8.5 and 30-37°C (Kashyap *et al*., 2003; Moyo *et al*., 2003; Sharma and Satyanarayana, 2006). Even though interaction terms of agitation speed with temperature (X_1X_2) and interaction terms of agitation speed with pH (X_1X_3) were not found statistically significant ($p > 0.05$). The model equations for PL activity with the coefficients in coded units of factors were given below:

$$\text{PL production} = -1171.362 + 1.669X_1 + 20.581X_2 + 194.581X_3 - 0.005X_1X_2 + 0.932X_2X_3 - 0.004X^2_1 - 0.388X^2_2 - 14.824X^2_3 \quad (R^2=0.9600) \quad (2)$$

Table 3 ANOVA table for PL activity: effect of agitation speed, temperature and pH.

Source of variation	*df*	Sum of squares	Mean square	*F*-ratio	*p*-value
Model	9	3586.932	398.548	18.898	0.0000
Residual	7	147.630	21.090		
Total	16	3734.567			

R-square = 0.9600; adjusted R- square = 0.9100

Table 4 Estimated regression coefficients for PL activity.

Term	Parameter estimates	*p*-value
Constant	-1171.362	0.000
Agitation speed	1.669	0.004
Temperature	20.581	0.003
pH	194.581	0.000
Agitation speed x Temperature	-0.005	0.503
Temperature x pH	0.932	0.024
Agitation speed x Agitation speed	-0.004	0.000
Temperature x Temperature	-0.388	0.000
pH x pH	-.14.824	0.000

Table 5 Result of experimental and predicted value for PL activity at optimum condition.

Agitation speed (rpm)	Temperature (°C)	pH	PL activity (U/ml)	
			Predicted	Experimental
200	35	8	81.3	84.5

Effects of interaction of various parameters on the PL production was studied by plotting three dimensional response curves against any two independent variables while keeping the other independent variable at its '0' level. The shapes of contour plots indicated the nature and extent of the interactions. Prominent interactions were shown by the elliptical nature of the contour plots, while less prominent or negligible interaction would otherwise be shown by the circular nature of the contour plots (Moyo *et al*., 2003). In predicting the response, all three-dimensional response surface graphs and two dimensional contour plots were generated using STATISTICA for Windows (Release 5.0, Stasoft, USA). Figures 1(a) and (b) depicted three dimensional curve and contour plot of the calculated response surface from the interaction between agitation speed and temperature while keeping pH at '0' level (Table 1). The response surface plot indicated a maximum PL activity within the agitation speed range of 170-230 rpm and the temperature range of 33-37°C. When the temperature was fixed at 35°C ('0' level), a maximum of PL activity was obtained at within the agitation speed range of 180-230 rpm and the pH range of 7.5-8.0 (Figures 2(a) and (b)). Figures 3(a) and (b) showed the interaction of temperature and pH at agitation speed 200 rpm ('0' level), it was indicated that the maximum PL activity was achieved within around the temperature range of 33-37°C and the pH range of 7.5-8.0. Taken all together, in order to achieve a high PL activity, agitation speed of 200 rpm (X_1), temperature 35°C (X_2) and pH 8 (X_3) were chosen.

This was a reconfirmation that the fitted surface had a maximum point which was agitation speed of 200 rpm, temperature 35°C and pH 8. The model predicted a maximum response of 81.3 U/ml for this point. To confirm these results, experimental rechecking was performed using a condition of fermentation representing this maximum point, and a mean value of 84.5 U/ml was obtained (Table 5). The good correlation between these two results confirmed the validity of response model and the model was proven to be adequate. After the enzyme production in laboratory scale was optimized, the obtained conditions will be further applied for up scale experiment in bioreactor.

CONCLUSION

At present, no reports are available in literature regarding the optimization of fermentation condition for PL enzyme production by *P. polymyxa* N10. Therefore, this study will serve as a base knowledge of initial studies in this field.

These optimization experiments, the optimal conditions for maximum PL enzyme activity (84.5 U/ml) were to use agitation speed at 200 rpm (X_1), temperature 35°C (X_2) and pH 8 (X_3). The experimental results clearly showed that the PL production is dependent mainly on temperature and pH. The temperature and pH had the most significant positive effect on PL production. Through the statistically designed optimization, the PL activity could be closed from average of 81.3 U/ml in predicting experiments to average of 84.5 U/ml in the optimization experiments. The model equation was useful to predict the results of experiments, as in PL activity it was shown that the experimental result and the PL predicted values were not different. These results indicate that RSM may be useful for optimization of PL production in a large scale.

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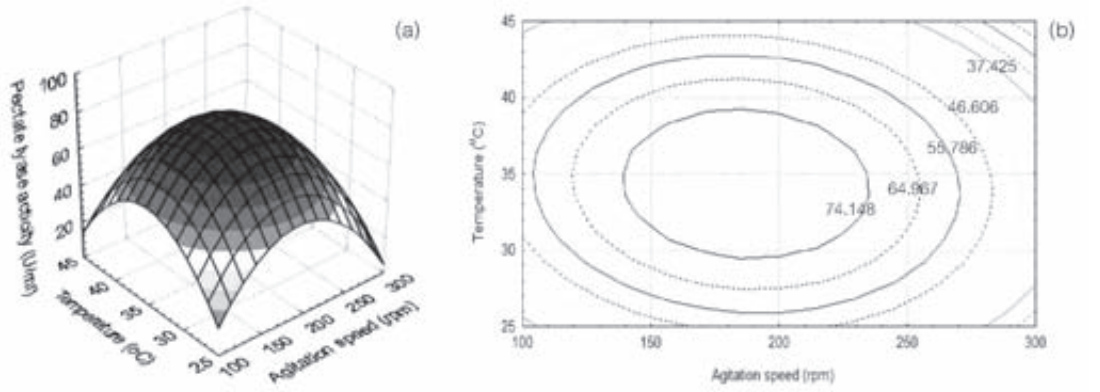

Figure 1 Response surface (a) and Contour plots (b) for the interaction of agitation speed and temperature at pH 7.5 on pectate lyase activity of *P. polymyxa* N10 after 72 h of incubation. The values in the figure indicated the level of pectate lyase activity (U/ml).

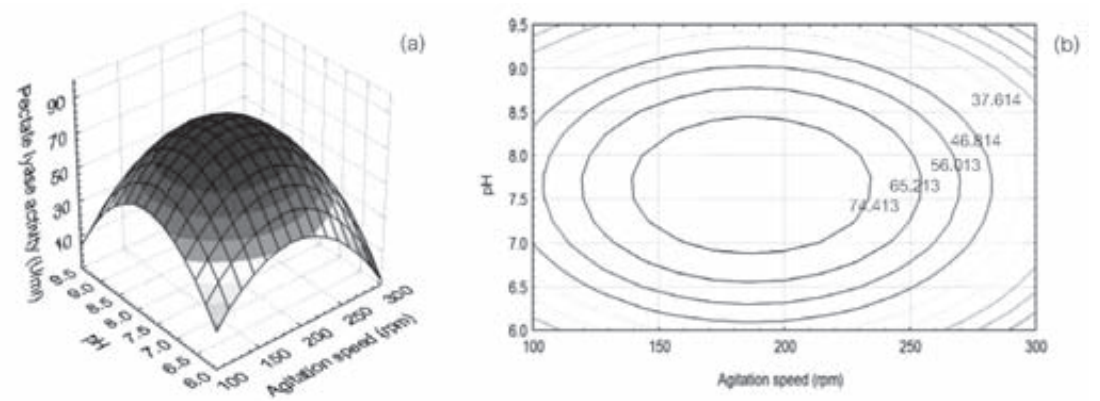

Figure 2 Response surface (a) and Contour plots (b) for the interaction of agitation speed and pH at temperature 35°C on pectate lyase activity of *P. polymyxa* N10 after 72 h of incubation. The values in the figure indicated the level of pectate lyase activity (U/ml).

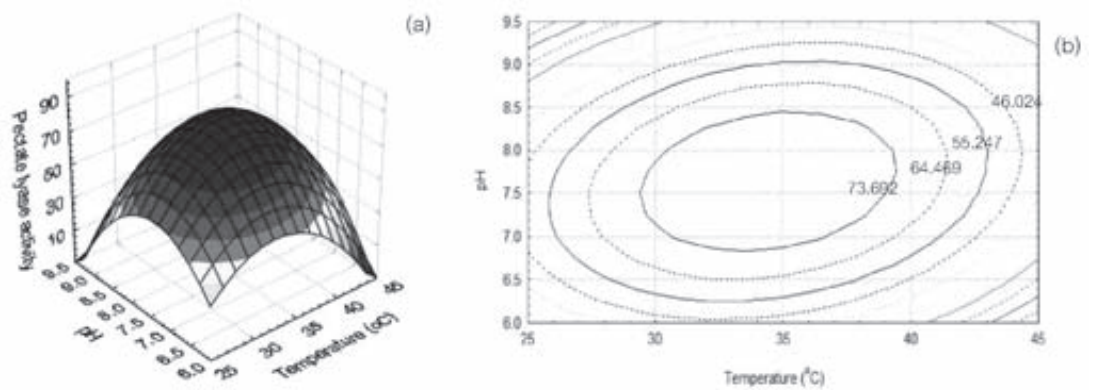

Figure 3 Response surface (a) and Contour plots (b) for the interaction of temperature and pH at agitation speed 200 rpm on pectate lyase activity of P. *polymyxa* N10 after 72 h of incubation. The values in the figure indicated the level of pectate lyase activity (U/ml).

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