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

Molnapat Songpim[1], Pilanee Vaithanomsat[*,1] and Sawitri Chuntranuluck[2]

[1]*Kasetsart Agricultural and Agro-Industrial Product Improvement Institute (KAPI), Kasetsart University, 50, Chatuchak, Bangkok, 10900, Thailand*

[2]*Department of Biotechnology, Faculty of Agro-Industry, Kasetsart University, 50, Chatuchak, Bangkok, 10900, Thailand*

Abstract: The parameters affecting the production of pectate lyase from *P. polymyxa* N10 were studied using the response surface methodology agitation rate (X_1, 100-300 rpm), temperature (X_2, 25-45 ^oC) and pH (X_3, 5.5-9.5). 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 ($p < 0.001$). A maximum pectate lyase activity of 84.5 U/ml was attained in 72 h of cultivation at agitation rate 200 rpm, temperature 35 ^oC and pH 8. Optimizations of agitation rate and aeration on pectate lyase production were also carried out in a 5-l stirred-tank bioreactor. The aeration rate was varied in the range of 0.5-2 vvm at agitation rate of 200 rpm (temperature 35^oC and initial pH 8). At agitation rate of 200 rpm, the shear force was high and then decreased the pectate lyase activity due to its negative effect on the enzyme structure. A maximum pectate lyase activity of 110.42 U/ml in the bioreactor was close to that obtained from the shake flask fermentation study.

Keywords: 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) [1]. PL (EC4.2.2.2.) hydrolyzes the α-1,4-glycosidic bond of polygalacturonate and releases unsaturated soluble oligogalacturonates [2]. 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 [3]. It has been reported that PL is produced from a wide variety of microbial sources such as fungi, actinomycetes and bacteria [4]. 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 [5]. The cultivation involves with many factors, such as temperature, pH, aeration rate and agitation rate, 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 [6]. 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 [7]. A 41-fold enhancement in alkaline pectinase production by *Bacillus pumilus* was achieved by using Burman design and RSM [8].

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 shake flask as well as in lab-scale bioreactor.

MATERIALS AND METHODOLOGY

Microorganism

P. polymyxa N10 was isolated from a mulberry bark by [5] and maintained on nutrient agar (NA) slants at 4 ^oC and also stored as glycerol stocks at -20 ^oC.

Pectate Lyase Production

All treatment combinations (Table **2**) were performed in 500 ml Erlenmeyer flasks containing 200 ml basal media

*Address correspondence to this author at the Kasetsart Agricultural and Agro-Industrial Product Improvement Institute (KAPI), Kasetsart University, 50, Chatuchak, Bangkok, 10900, Thailand; Tel./Fax. 66-2942-8599; E-mail: aappln@ku.ac.th, p_vaithanomsat@yahoo.com

1874-1967/10

(1.5% pectin, 0.5% monosodium L-glutamate, 0.3% ammonium sulphate, 0.08% disodium hydrogen phosphate, 0.05% magnesium phosphate, 0.02% calcium chloride and 0.24% potassium dihydrogen phosphate) [9]. The experiments were performed according to the central composite design (Tables **1** and **2**). After 72 h of incubation, each flask was assayed for enzyme activity. PL activity was determined on supernatant obtained after the centrifugation at 11380 xg for 20 min at 4 ^{o}C.

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 rate	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 rate-200)/50, X_2= (Temperature-35)/5.0 and X_3= (pH-6.5)/0.5.
[b]Levels based on the Central Composite Design.

Experimental Design

The RSM was used to investigate the effects of independent variables: agitation rate, temperature and initial pH on the responses of PL activity. Using central composite design (CCD) for 3 factors (k=3), 17 treatment combinations were generated. 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) were designed at a distance of 1.682 ($2^{n/4}$ = 1.682 for n = 3) from the design center. The remaining levels were identified using CCD [7]. Table 1 contained the actual factor levels corresponding to the coded factor levels as followed: X_1= (agitation rate-200)/50, X_2= (temperature-35)/5 and X_3= (pH-7.5)/0.5. Table **2** showed the treatment combinations and responses. 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 coefficient

b_{ii} = the quadratic coefficient

b_{ij} = the cross product coefficient

£ = the model constant

Bioreactor and k_La

A 5-l stirred tank bioreactor (STR) (BEMT-T-5L, Marubishi Co., LTD., Thailand) containing 2-l production medium and 10% pre-cultured inoculation was used to study the optimum aeration and agitation rate. Three levels of aeration rate: 0.5, 1 and 2 vvm were studied at optimum agitation rate. Estimations of biomass, pectin and PL

Table 2. Treatment Combinations and Mean Response

Treatment	Coded Variable Level[a]			Mean Response (Y) (U/ml)
	X^b_1	X_2	X_3	
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 rate-200)/50, X_2= (Temperature-35)/5.0 and X_3= (pH-7.5)/0.5.
[b]Levels based on the Central Composite Design.

production were carried out at every 3 h interval. The dynamic technique without microorganism was performed to calculate the k_La and nitrogen was used to elimination oxygen. The dissolved oxygen was measured with polarographic sensor. The k_La was calculated by static gassing out method using following Eq. (2).

$$\ln\left(\frac{C^*_{AL} - C_{AL1}}{C^*_{AL} - C_{AL2}}\right) = k_L at \quad (2)$$

Therefore, a plot of $\ln\left(\frac{C^*_{AL} - C_{AL1}}{C^*_{AL} - C_{AL2}}\right)$ *vs*. t should result in a straight line of slope k_La.

Pectate Lyase Assay

Pectate lyase activity was determined spectrophotometrically by measuring the increase in absorbance at 235 nm [5]. 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 of crude enzyme was incubated at 35 ^oC 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 ^oC [5].

Analysis

Total pectin content was determined according to the m-hydroxydiphenyl method [10]. Cell dry weight was estimated as follow. Two milliliter sample was collected in a preweighed eppendorf tube and centrifuged at 5000 rpm for 10 min. Supernatant was discarded and the pellet was washed twice with sterile distilled water, followed by drying the pellets at 95 °C till constant weight was obtained. The result was expressed in DCW (mg/ml) [11]. Dissolved oxygen (DO) was determined using polarographic type, METTLER TOLEDO, >98% response in less than 90s.

RESULTS AND DISCUSSION

Data was analyzed using SPSS 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**.

Table 3. ANOVA Table for PL Activity: Effect of Agitation Rate, 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
Agit. rate	1.669	0.004
Temp.	20.581	0.003
pH	194.581	0.000
Agit. rate x Temp.	-0.005	0.503
Temp. x pH	0.932	0.024
Agit. rate x Agit. rate	-0.004	0.000
Temp. x Temp.	-0.388	0.000
pH x pH	-.14.824	0.000

After the treatment combinations all linear terms of the independent variables, quadratic term of agitation rate, 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 might reflect their true influence to the response. The optimum pH and temperature were also consistent with values those found for pectinase production at pH 8.0-8.5 and 30-37 ^oC, respectively [7, 8, 12]. Even though interaction terms of agitation rate with temperature (X_1X_2) and interaction terms of agitation rate 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:

$$\text{PL production} = -1171.362 + 1.669X_1 + 20.581X_2 + 194.581X_3 - 0.005X_1X_2 + 0.932X_2X_3 - 0.004X^2_1 - 0.388X^2_2 - 14.824X^2_3 \ (R^2=0.9600) \quad (3)$$

Table 5. Result of Experimental and Predicted Value for PL Activity at Optimum Condition

Agitation Rate (rpm)	Temperature (^oC)	pH	PL Activity (U/ml)	
			Predicted	Experimental
200	35	8	81.3	84.5

Effect 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 variables at their '0' levels. 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 [7]. In predicting the response, all three-dimensional response surface graphs and

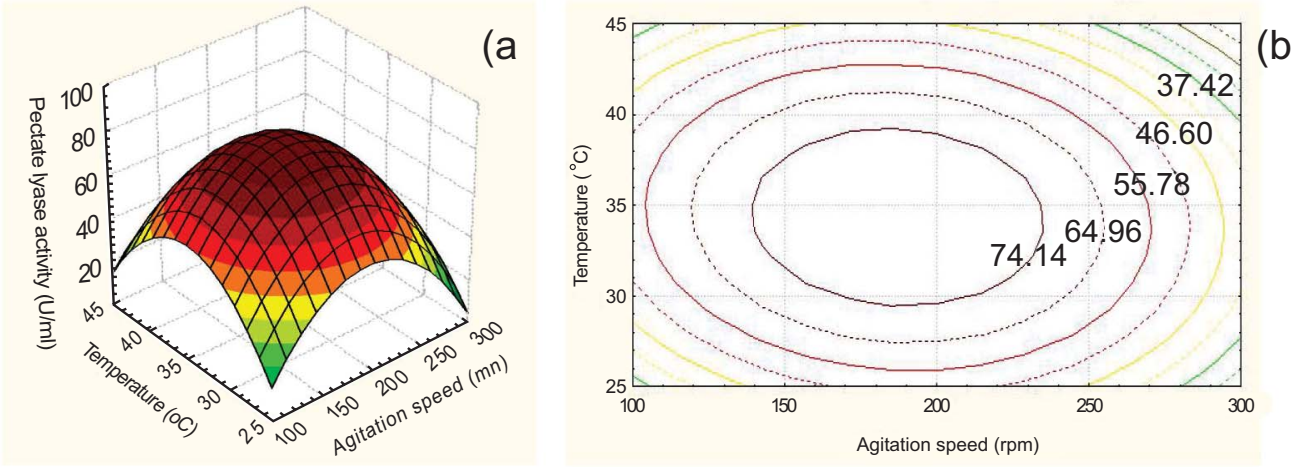

Fig. (1). Response surface (**a**) and Contour plots (**b**) for the interaction of agitation rate and temperature at pH 7.5 on pectate lyase activity of *P. polymyxa* N10 after 72 h of incubation. The values in the figure indicated the level of pectate lyase activity (U/ml).

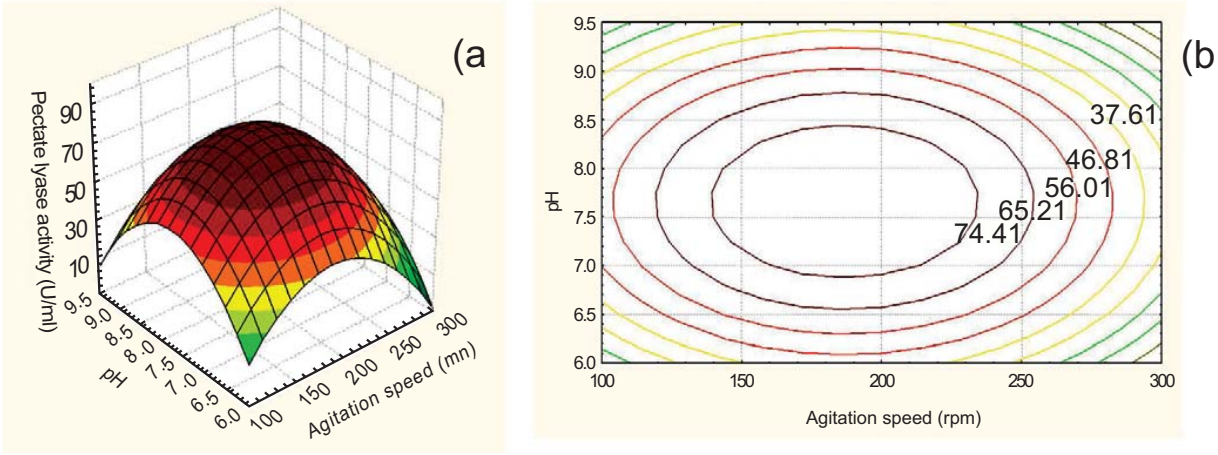

Fig. (2). Response surface (**a**) and Contour plots (**b**) for the interaction of agitation rate and pH at temperature 35 ^{o}C on pectate lyase activity of *P. polymyxa* N10 after 72 h of incubation. The values in the figure indicated the level of pectate lyase activity (U/ml).

two dimensional contour plots were generated using STATISTICA for Windows (Release 5.0, Stasoft, USA). Figs. **1**(**a**) and (**b**) depicted three dimensional curve and contour plot of the calculated response surface from the interaction between agitation rate and temperature while keeping pH at '0' level (Table **1**). The response surface plot indicated an optimum PL activity around an agitation rate 170-230 rpm and temperature 33-37 ^{o}C. When the temperature was fixed at 35 ^{o}C ('0' level), a maximum of PL activity was obtained at agitation rate 180-230 rpm and pH 7.5-8.0 (Figs. **2**(**a**) and (**b**)). Figs. **3**(**a**) and (**b**) showed the interaction of temperature and pH at agitation rate 200 rpm ('0' level), it was indicated that the maximum PL activity was achieved around temperature 33-37 ^{o}C and pH 7.5-8.0. Taken all together, in order to achieve a high PL activity, agitation rate of 200 rpm (X_1), temperature 35 ^{o}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 rate 200 rpm, temperature 35 ^{o}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 were applied for up scale experiment in 5l bioreactor. The batch STR (stir tank reactor) was run with 10% inoculum for 72 h at 35 ^{o}C and pH 8 and tested three different aeration rates: 0.5, 1 and 2 vvm at agitation rate 200 rpm. Fig. **4** (**a**) showed the optimization of PL production in STR under aeration rate 0.5 vvm. The trend of biomass growth (DCW, g/l) was increased while PL production was found to be negatively affected at agitation rate 200 rpm. An agitation rate 200 rpm increased the amount of dissolved oxygen and dispersion of macromolecules in the medium. It might, therefore, contribute to the greater growth but not for the enzyme production. The shearing effect induced by higher agitation rate on the enzyme inactivation might contribute negatively toward enzyme stability [13]. It had been reported in *Paenibacillus* sp. fermentation that increasing agitation rate might bring

about many negative effects such as change in cell morphological state, cell autolysis and then decrease in enzyme productivity [14]. The same phenomena were observed for enzyme production in this study. The shear force was high and therefore decreased the pectate lyase activity due to its negative effect on the enzyme structure. As a result, the agitation rate was decreased at 150 and 120 rpm and aeration rate at 0.5, 1 and 2 vvm. As shown in Fig. **4**(**b**), it could be

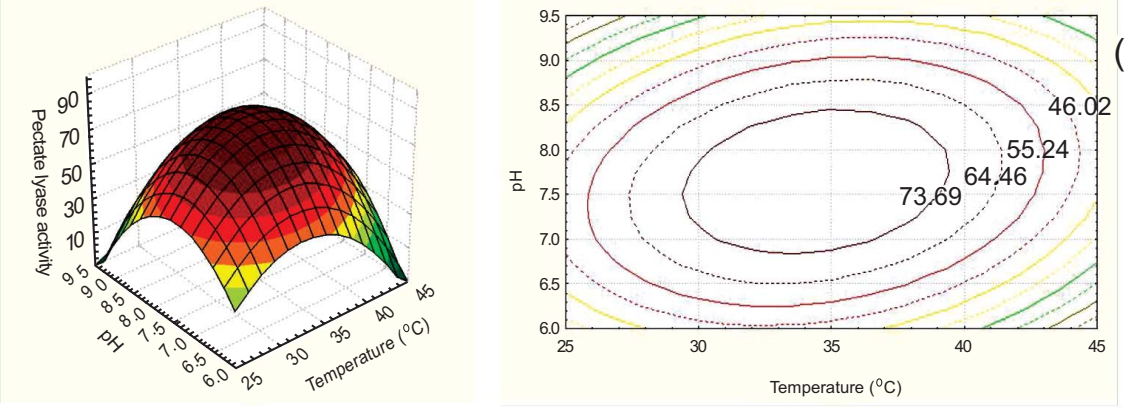

Fig. (3). Response surface (**a**) and Contour plots (**b**) for the interaction of temperature and pH at agitation rate 200 rpm on pectate lyase activity of *P. polymyxa* N10 after 72 h of incubation. The values in the figure indicated the level of pectate lyase activity (U/ml).

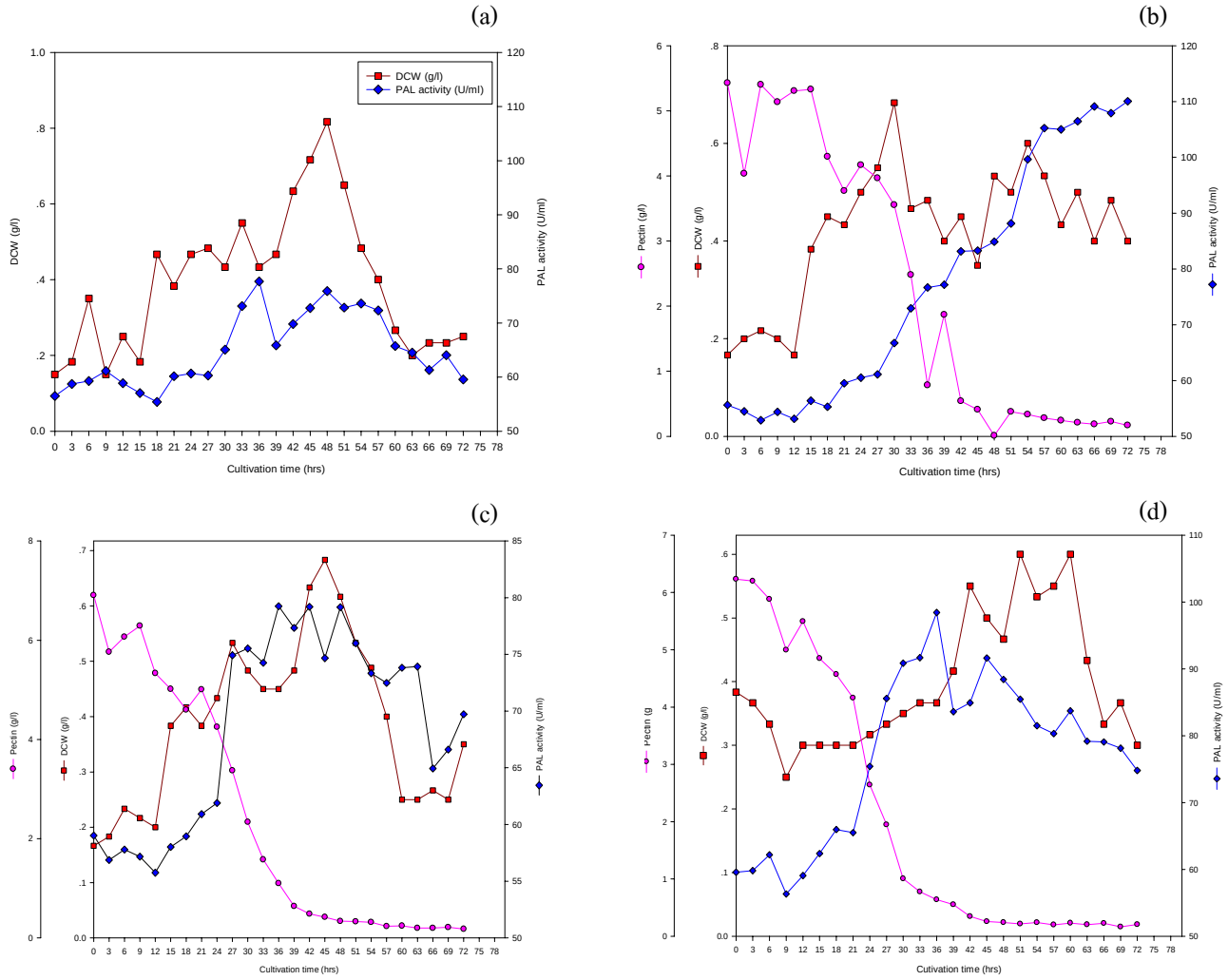

Fig. (4). Production of pectate lyase in STR uder different aeration and agitation rates. (**a**) 0.5 vvm and 200 rpm; (**b**) 0.5 vvm and 150 rpm; (**c**) 1 vvm and 120 rpm; (**d**) 2 vvm and 120 rpm. (■) DCW (g/l); (♦) PL production; (●) Pectin (g/l).

seen that there was an increase in the enzyme production with the decrease of agitation rate at 150 rpm and aeration at 0.5 vvm. The maximum achieved activity was 110.42 U/ml after 72 h of fermentation. The maximum enzyme activities at agitation rate 120 rpm and aeration rates 1.0 and 2.0 vvm were 79.25 and 98.43 U/ml, respectively (Table **6**). The trend of PL production was similar in agitation rate 120 rpm; aeration rate 1.0 vvm and 2.0 vvm, showing a growth-associated type of behavior could still be observed for the two aeration rates studied (Figs. **4**(**c**) and (**d**)). Besides, it was obvious from the given biomass growth data that aeration rate was crucial for better oxygen and nutrient transfer rate during the entire period of operation. However, high aeration rate was found to inhibit pectate lyase at aeration rate above 1.0 vvm. The scaling up of enzyme production form flask to lab scale bioreactor and from lab scale bioreactor to pilot plant and subsequently to industrial level have been generally based on the volumetric oxygen transfer coefficient (K_La) [11]. The K_La values were estimated by static gassing out method for all batches and the data was reported in Table **6**. The maximum K_La value (37.08 h^{-1}) was found at 150 rpm and 0.5 vvm. At 120 rpm of agitation rate, as the aeration rate increased from 1.0 and 2.0 vvm, the K_La values also increased to 31.68$h^{-1}$and 32.76h^{-1}, respectively.

The optimum operation condition for PL production by *P. polymyxa* N10 arising from this study was as follows: temperature 35 ^oC, pH 8, agitation rate 150 rpm and aeration rate 0.5 vvm. Under this condition, the enzyme production could be completed within 72 h and the maximum PL activity was 110.42 U/ml and K_La value was 37.08 h^{-1}.

Table 6. Pectate Lyase Production Formation in Batch STR: Effects of Aeration and Agitation Rates

	Aeration		
	0.5 vvm	**1 vvm**	**2vvm**
	150 rpm[a]	**120 rpm[a]**	**120 rpm[a]**
K_La (h^{-1})	37.08	31.68	32.76
PL activity (U/ml)	110.42	79.25	98.43

[a] Agitation.

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 rate at 200 rpm (X_1), temperature 35 ^oC (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. Though, 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 predicted PL values were not different. These results indicated that RSM was useful for optimization of PL production in stirrer tank reactor. Under this condition, the effects of agitation rate and aeration rate were optimized in 5-1 bioreactor and shown to be significant for enzyme production. The best condition found after an optimization study in a 5-l bioreactor was: 150 rpm agitation, 0.5 vvm aeration with a production of 110.42 U/ml and maximum K_La value 37.08h^1.

ACKNOWLEDGEMENTS

The authors would like to thank Assist. Prof. Dr. Surang Suthirawut, Department of Microbiology, Faculty of Science, Kasetsart University, for the identification of *Paenibacillus polymyxa* N10 and Kasetsart University Research and Development Institute (KURDI), Kasetsart University, for financial support throughout the experiments.

REFERENCES

[1] Tari C, Gogus N, Tokatli F. Optimization of biomass, pellet size and polygalacturonase production by *Aspergillus sojae* ATCC 20235 using response surface methodology. Enzyme Microb Technol. 2007; 40: 1108-16.

[2] Matsumoto T, Daisuke K, Akihiko K, Hideki F. Efficient secretory overexpression of *Bacillus subtilis* pectate lyase in *Escherichia coli* and single-step purification. Biochem Eng 2002; 12: 175-9.

[3] Gummadi SN, Sunil KD. Batch and fed batch production of pectin lyase and pectate lyase by novel strain *Debaryomyces nepalensis* in bioreactor. Bioresour Technol 2007; 99: 874-81.

[4] Hoondal GS, Tiwari RP, Tiwari R, Dahiya N, Beg QK. Microbial alkaline pectinases and their industrial applications: a review. Appl Microbiol Biotechnol 2002; 59: 409-18.

[5] Sittidilokratna C, Suthirawut S, Chitradon L, Punsuvon V, Vaithanomsat P, Siriacha P. Screening of pectinases producing bacteria and their efficiency in biopulping of paper mulberry bark. Sci Asia 2007; 33(1): 131-5.

[6] Chen QH, He GQ, Ali Mokhtar AM. Optimization of medium composition for the production of elastase *by Bacillus sp.* EL31410 with response surface methodology. Enzyme Microb Technol 2002; 30: 667-72.

[7] Moyo S, Gashea BA, Collisona EK, Mpuchaneb S. Optimizing growth conditions for the pectinolytic activity of *Kluyveromyces wickerhamii* by using response surface methodology. Int J Food Microbiol 2003; 85: 87-100.

[8] Sharma DC, Satyanarayana T. A marked enhancement in the production of a highly alkaline and thermostable pectinase by *Bacillus pumilus* dcsr1 in submerged fermentation by using statistical methods. Bioresour Technol 2006; 97: 727-33.

[9] Kobayashi Y, Komae K, Tanabe H. Production of pectolytic enzyme from *Erwinia carotovora* and their stability. Ferment Technol 1985; 63: 451-9.

[10] Kintner PK, Buren V. Carbohydrate interference and its correction in pectin analysis using m- hydroxydiphenyl method. J Food Sci 1982; 47(3): 756-64.

[11] Potumarthi A, Subhakar Ch, Tetty A. Alkaline protease production by submerged fermentation in stirred tank reactor using *Bacillus licheniformis* NCIM-2042: effect of aeration and agitation regimes. Biochem Eng J 2007; 34: 185-92.

[12] Kashyap DR, Soni SK, Rupinder T. Enhanced production of pectinase by *Bacillus sp.* DT7 using solid state fermentation. Bioresour Technol 2003; 88: 251-4.

[13] Feng Y, He Z, Ong SL, Hu J, Zhang Z, Ng WJ. Optimization of agitation, aeration, and temperature conditions for maximum β-mannanase production. Enzyme Microb Technol 2003; 32: 282-9.

[14] Kao PM, Chin-I C, Shu-Chen H, Yung-Chi C, Po-Jen T, Yung-Chan L. Effects of shear stress and nass tranfer on chitinase production by *Paenibacillus* sp. CHE-N1. Biochem Eng J 2007; 34: 172-8.

Received: May 20, 2009 Revised: October 20, 2009 Accepted: October 29, 2009