RESEARCH ARTICLE
doi: 10.2306/scienceasia1513-1874.2010.36.118

ScienceAsia 36 (2010): **118–124**

Optimization of silk degumming protease production from *Bacillus subtilis* C4 using Plackett-Burman design and response surface methodology

Nisa Romsomsa[a], **Patoomporn Chim-anagae**[a,*], **Anuvat Jangchud**[b]

[a] Department of Microbiology, Faculty of Science, Kasetsart University, 50 Phahonyothin Rd, Chatuchak Bangkok 10900, Thailand

[b] Department of Product Development, Faculty of Agro-Industry, Kasetsart University, 50 Phahonyothin Rd, Chatuchak Bangkok 10900, Thailand

*Corresponding author, e-mail: fscippc@ku.ac.th

Received 28 Jul 2009
Accepted 26 Mar 2010

ABSTRACT: *Bacillus subtilis* C4 was isolated from waste water of a Thai-silk dyeing factory, and identified as a potent strain for producing silk-degumming protease. To optimize the protease production of this bacterial strain, seven fermentation variables were screened using a Plackett-Burman design, and were then further optimized via response surface methodology based on a central composite design. Three significant variables, i.e., soy flour, skimmed milk, and shaker speed, were selected. The optimal values were 2.0% soy flour, 0.1% skimmed milk, and a shaker speed of 280 rpm. The experimental result (1537 units/ml) in a medium optimized for protease production was in good agreement with the predicted value of a quadratic model (1576 units/ml), thus confirming its validity. In addition, the adequacy of the model was supported by a coefficient of determination (R^2) of 0.912. Protease production in the optimized medium (1537 units/ml) increased 2.2-fold over that of the non-optimized medium (729 units/ml) in the shaken flask culture. When the experiment was scaled up in a stirred tank reactor, 1891 units/ml protease activity was achieved at 27 h of cultivation, which was an overall 2.6-fold increase over the basal medium.

KEYWORDS: removal of sericin, statistical approaches, optimized medium

INTRODUCTION

Proteases are one of the most important industrial enzymes, accounting for nearly 60% of the total worldwide enzyme production[1]. The use of alkaline proteases has increased remarkably in various industrial processes such as the production of detergents, processing food and animal feed, and production of X-ray films[2].

In recent years, studies have dealt with the removal of sericin (gum) from silk yarn with protease, which is milder than soap or alkaline treatments[3,4] and saves expenses in terms of water, energy, chemicals, and effluent treatment. Alkaline and neutral proteases effectively degum silk fabrics. Almost complete sericin removal has been obtained when raw silk fabric was the substrate[5]. It is well known that extracellular protease production in microorganisms is greatly influenced by media components, especially carbon and nitrogen sources, and by physical factors such as temperature, pH, incubation time, agitation, and inoculum density[6,7]. Medium composition is one of the most important parameters when enzymes are produced for industrial purposes because around 30–40% of the production cost is estimated to be the cost of the growth medium[8]. Statistical approaches have helped to enhance product yield and reduce the cost of production, thereby making the fermentation process economical and cost effective[9].

In the optimization of media compounds, Plackett-Burman designs are used as a screening method in order to select the variables that influence a system. However, they do not give an optimum value for each variable[10] and further optimization is needed. Response surface methodology (RSM) has been widely used to evaluate and understand the interactions between different physiological and nutritional parameters[11]. It is an efficient mathematical approach widely applied in the optimization of the fermentation process and media component, e.g., production of enzymes, biomass, spore, and other metabolites[12–15]. RSM, which includes factorial design and regression analysis, can be used to help evaluate the effective factors

and build models[16]. It can give information about the interaction between variables and can be used to select optimum conditions of variables for a desirable response and multiple responses at the same time. Finally, after model building and optimization, the predicted model is verified.

In this investigation, an attempt has been made to improve silk degumming protease production from *Bacillus subtilis* C4 by statistical approaches using a Plackett-Burman design and RSM in submerged culture. The proteases obtained from the optimized medium using either shaken flasks or a stirred tank reactor were also compared.

MATERIAL AND METHODS

Microorganisms

The organism used in this study was *Bacillus* sp. C4 isolated from the waste water of a Thai-silk factory, and selected for its potential in producing protease for silk degumming. It was previously identified as *Bacillus subtilis* based on partial 16 s rRNA gene sequence homology and a standard method for bacterial identification[17]. The culture was maintained on modified basal medium with skimmed milk (BMSM) medium[18] with 20% glycerol at $-20\,^{\circ}$C. A single colony, obtained from modified Davis minimal agar[19] supplemented with 0.1% sericin, was activated twice before the starter was prepared.

Protease production

For protease production, the starter was prepared by growing a cell suspension in a 500 ml baffled flask containing 100 ml of BMSM medium, with the pH adjusted to 7.5 and incubation for 24 h on a shaker at 200 rpm. The starter was previously adjusted to an absorbance of 0.3 (600 nm) and cultivation of 100 ml BMSM medium was conducted in shaken flasks by varying carbon, nitrogen, and inducer sources. Incubation occurred under the conditions defined by the statistical designs for 24 h. The cell-free supernatant was recovered by centrifugation (9200 g, 10 min), and used for determining extracellular alkaline protease activity.

STATISTICAL EXPERIMENTAL DESIGNS

Plackett-Burman design

Based on the results of a preliminary study on protease production by *B. subtilis* C4, hydrolysed cassava starch, soy flour, and skimmed milk were selected as the most suitable carbon, nitrogen, and inducer sources, respectively[20]. They were subsequently screened, along with other factors (initial pH, temperature, shaker speed, and inoculum size), using a Plackett-Burman design in order to identify the significant variables affecting the enzyme production. A set of 12 experiments was conducted (Table 1). Each variable was set at two levels, high and low, denoted by $(+1)$ and (-1), respectively. The significance of each variable influencing protease production was determined by Student's t test with 95% confidence levels.

Table 1 Code level of seven variables studied regarding protease production from *B. subtilis* C4 by a Plackett-Burman design.

Variable	Symbol	Level	
		$+1$	-1
Hydrolysed cassava starch (g/l)	X_1	2.00	0.50
Soy flour (g/l)	X_2	1.00	0.25
Skimmed milk (g/l)	X_3	2.00	0.20
Initial pH	X_4	9.50	6.50
Temperature (°C)	X_5	37	30
Shaker speed (rpm)	X_6	250	100
Inoculum size (% v/v)	X_7	5.00	2.00

Central composite design and response surface methodology

After identifying the significant variables for protease production through a Plackett-Burman design, a central composite design (CCD) was adopted to optimize the major variables (soy flour, skimmed milk, and shaker speed) in 17 experiments. The variables were coded according to[21]:

$$x_i = \frac{X_i - X_0}{\Delta X}, \tag{1}$$

where x_i is the dimensionless coded level of the variable, X_i is the actual value of that variable, X_0 is the average of the high and low level values of that variable, and ΔX is the high value minus the low value of that variable. The variables and levels are shown in Table 2. The roles of the three variables, their interactions, and statistical analysis to obtain the predicted yields were modelled by using

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3, \tag{2}$$

where Y is the predicted response, β_0 is an offset term, β_i is the linear effect, β_{ii} is the quadratic effect, β_{ij} is the interaction effect, and X_1, X_2, X_3, are the levels of the three variables.

Table 2 Experimental range of the three variables (SF = soy flour; SM = skimmed milk; SS = shaker speed) studied using CCD in terms of actual and coded factors.

Variable	Symbol	Coded level				
		−1.68	−1	0	+1	+1.68
SF (%w/v)	X_1	0.18	0.50	1.50	2.50	3.18
SM (%w/v)	X_2	0.03	0.10	0.20	0.30	0.37
SS (rpm)	X_3	110	150	200	250	280

The statistical significance of the coefficients and predicted protease production in (2) were evaluated using linear regression analysis (SPSS for Windows Version 12).

Validation of the experimental model

The optimized medium obtained from the response surface model was confirmed for its accuracy. The statistical model was validated with respect to all three variables within the design space. A random set of five experimental combinations was used to study the protease production under the experimental conditions described above.

Scale-up studies in a fermenter

After optimization studies in shaken flasks, protease was produced in a 2-l stirred tank fermenter BIOSTAT B, (B. Braun Biotech International, Germany). 2% of the 24 h seed culture was inoculated into the optimal medium and fermented at pH 7.5 and 30 °C with 350 rpm agitation. The dissolved oxygen concentration was maintained at 70% air saturation throughout the fermentation process.

Protease activity assay

Protease activity was determined by a modified Ferrero's method as described by Sangyon[22]. One unit of protease activity was defined as the amount of enzyme liberating 1 μg of tyrosine per min.

RESULTS AND DISCUSSION

Plackett-Burman designs

The first optimization step identified the significant factors for protease production from *B. subtilis* C4 using a 12-run Plackett-Burman design (Table 1 and Table 3). Table 4 represents the effect of each variable along with the mean squares, F-values, and p-values. The observed protease production varied from 233 to 1283 units/ml, reflecting the importance of medium optimization to attain higher yields. Variables having a probability value (p-value) less than 0.05 were considered significant. The analysed data in Table 4

Table 3 Plackett-Burman design for selection of significant variables of silk degumming protease production by *B. subtilis* C4.

Run	Experimental variable							Protease activity
	X_1	X_2	X_3	X_4	X_5	X_6	X_7	(units/ml)[a]
1	+1	+1	−1	+1	+1	+1	−1	1171 ± 9
2	+1	−1	+1	+1	+1	−1	−1	366 ± 0
3	−1	+1	+1	+1	−1	−1	−1	540 ± 1
4	+1	+1	+1	−1	−1	−1	+1	429 ± 0
5	+1	+1	−1	−1	−1	+1	−1	1283 ± 50
6	+1	−1	−1	−1	+1	−1	+1	233 ± 1
7	−1	−1	−1	+1	−1	+1	+1	799 ± 2
8	−1	−1	+1	−1	+1	+1	−1	203 ± 2
9	−1	+1	−1	+1	+1	−1	+1	586 ± 2
10	+1	−1	+1	+1	−1	+1	+1	777 ± 2
11	−1	+1	+1	−1	+1	+1	+1	711 ± 2
12	−1	−1	−1	−1	−1	−1	−1	562 ± 3

[a] Values are indicated as mean ± SD ($n = 2$).

Table 4 Results of ANOVA for the Plackett-Burman design of silk degumming protease production by *B. subtilis* C4.

Variable	Mean square	F-value	p-value
Hydrolysed cassava starch	61 233	2.56	0.19
Soy flour	263 796	11.03	0.03
skimmed milk	214 883	8.99	0.04
Initial pH	55 842	2.34	0.20
Temperature	104 757	4.38	0.10
Shaker speed	412 997	17.27	0.14
Inoculum size	29 087	1.22	0.33

$R^2 = 0.923$; adjusted $R^2 = 0.790$.

suggests that protease production was affected by soy flour and skimmed milk, which had p-values of 0.03 and 0.04, respectively. The significant effect of soy flour as a nitrogen source for protease production by *B. subtilis* C4 corroborates a report of Joo and Chang[23] showing the preference of *Bacillus* spp. for soy flour, which has also been reported as an inducer for alkaline protease from *Conidiobolus coronatus*[24] as well. Casein, a component of skimmed milk, has also been reported as an inducer of this enzyme[25].

Although shaker speed was grouped among the insignificant variables, we used it as one of the major factors for further study, due to its importance in terms of oxygen and nutrient transfer into the liquid medium, especially for the growth of aerobic bacteria like *Bacillus* spp[26]. Thus, soy flour, skimmed milk, and shaker speed were chosen and their possible interactive effects on enzyme production were evaluated by response surface methodology.

Table 5 Experimental designs used in RSM studies with three independent variables showing observed and predicted values of protease production.

Run	Variable			Protease activity (units/ml)	
	Soy flour (X_1)	Skim milk (X_2)	Shaker speed (X_3)	Mean Observed[a]	Predicted
1	−1	−1	−1	173 ± 3	150
2	+1	−1	−1	201 ± 3	125
3	−1	+1	−1	486 ± 4	610
4	+1	+1	−1	692 ± 12	795
5	−1	−1	+1	1239 ± 16	1074
6	+1	−1	+1	1639 ± 16	1453
7	−1	+1	+1	535 ± 12	549
8	+1	+1	+1	1176 ± 8	1138
9	0	0	0	1175 ± 6	1146
10	0	0	0	1159 ± 22	1146
11	0	0	0	1153 ± 31	1146
12	−1.68	0	0	632 ± 3	632
13	0	−1.68	0	417 ± 3	655
14	0	0	−1.68	444 ± 4	352
15	+1.68	0	0	764 ± 3	852
16	0	+1.68	0	928 ± 12	777
17	0	0	+1.68	1263 ± 46	1476

[a] Values are mean ± SD ($n = 3$).

Optimization by response surface methodology

Response surface methodology is the most accepted statistical technique for bioprocess optimization to examine the relationship between a set of experimental factors and observed results. The design matrix and the corresponding results of the experiments to determine the effect of soy flour, skimmed milk, and shaker speed are depicted in Table 2 and Table 5. Maximum protease production (1639 units/ml) was observed in the presence of 2.5% soy flour, 0.1% skimmed milk, and a shaker speed of 250 rpm. The analysis of variance of the quadratic regression model demonstrated that the model was highly significant ($p < 0.001$). The determination coefficient (R^2) value of 0.912 indicated that 91.2% of the total variations were explained by the model and revealed good agreement between the experimental results and the predicted values calculated from the model. In addition, the value of the adjusted determination coefficient (adjusted $R^2 = 0.893$) was also very high, emphasizing the high significance of the model[27].

The significance of each term of the model was determined by p-values (Table 6). The results implied that the quadratic effect of shaker speed (β_{33}) was significant ($p < 0.05$). The linear effects of skimmed milk (β_2), shaker speed (β_3), and the quadratic effects of soy flour (β_{11}), skimmed milk (β_{22}), and the interaction effects of β_{23} and β_{13} were more significant than other factors. However, the p-value of the coefficients of the linear effects of soy flour and the interactive effect of soy flour and skimmed milk may have been significant to some extent. According to this result, skimmed milk, which is a source of casein, appeared to have the greatest effect on enzyme production (p-values of β_1, $\beta_{11} < 0.001$), since the casein provided the intact peptides necessary for the induction process of the enzyme[28]. Thus, the response of silk degumming protease production (Y) by *B. subtilis* C4 can be expressed in terms of the regression equation:

$$Y = -3780.73 + 206.06\,X_1 + 15\,603.63\,X_2 + 24.34\,X_3 - 188.69\,X_1^2 - 15\,259.87\,X_2^2 - 0.03\,X_3^2 + 513.32\,X_1X_2 - 49.50\,X_2X_3 + 1.97\,X_1X_3, \quad (3)$$

where X_1 is soy flour, X_2 is skimmed milk, and X_3 is shaker speed.

The 2D contour plots were plotted based on the model equation to investigate the interaction among variables and to determine the optimum concentration of each factor for maximum protease production by *B. subtilis* C4. The contour plots were based on the final model, holding one variable constant at its central level, while the other two were varied within their experimental ranges (Figs. 1–3). Fig. 1 shows that an increase in soy flour concentration in the range 1.3–2.7% w/v and skimmed milk in the range 0.14–0.28% w/v caused an increase in the protease production to optimum values. Any further increases in these concentrations led to a decrease in enzyme production.

Table 6 Statistical significance of the response equation developed for *B. subtilis* C4 protease production[a].

Model	Coefficient	Standard error	t-value	p-value
β_0	−3780.73	505.32	−7.48	< 0.001
β_1	206.06	158.88	1.30	0.202
β_2	15 603.63	1562.12	9.99	< 0.001
β_3	24.34	3.84	6.33	< 0.001
β_{11}	−188.69	26.76	−7.05	< 0.001
β_{22}	−15 259.87	2280.78	−6.69	< 0.001
β_{33}	−0.03	0.01	−3.12	0.003
β_{12}	513.32	281.21	1.83	0.075
β_{23}	−49.50	5.62	−8.80	< 0.001
β_{13}	1.97	0.56	3.55	0.001

[a] $R^2 = 0.912$; adjusted $R^2 = 0.893$.

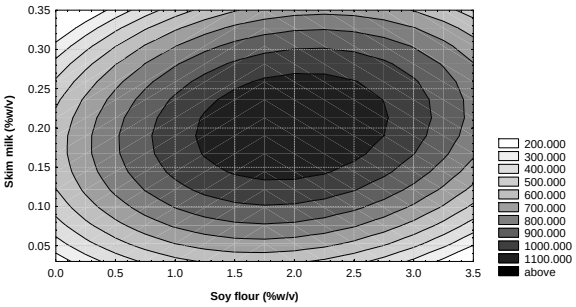

Fig. 1 Contour plots of protease production by *B. subtilis* C4 showing the effect of soy flour and skimmed milk (shaker speed was kept at 200 rpm).

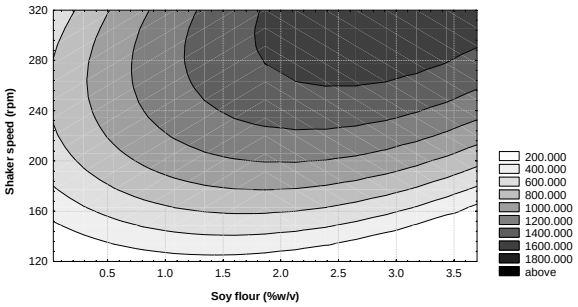

Fig. 2 Contour plots of protease production by *B. subtilis* C4 showing the effect of soy flour and shaker speed (skimmed milk was kept at 0.2% w/v).

Fig. 2 shows the response for the interactive factors of soy flour and shaker speed. It was evident that increasing the shaker speed and increasing the soy flour content (more than 1.6% w/v) had a positive influence on protease production, until an optimum value was reached. A similar response curve in Fig. 3 shows that protease activity varied as a function of shaker speed in an interactive effect along with skimmed milk, which corroborated the results reported by Dutt et al[25]. A maximum protease activity of 1639 units/ml was observed, corresponding to a high level of shaker speed (250 rpm) and a low amount of skimmed milk (0.1% w/v). On the other hand, the addition of skimmed milk to 0.3% (w/v) reduced the protease activity to 535 units/ml, indicating the negative effect of a high concentration of skimmed milk on protease production. Thus, the contour plots indicated the need for a higher shaker speed and a high concentration of soy flour along with a low amount of skimmed milk to obtain maximum protease production. This confirmed earlier reports on protease production, where it was observed that production of proteases by *Bacillus* sp. was highly dependent on the availability of oxygen[29]. Since soybean meal is an inexpensive and readily available substrate[30], it is possible to be used as an ingredient in the culture medium for the cost effective production of an extracellular protease.

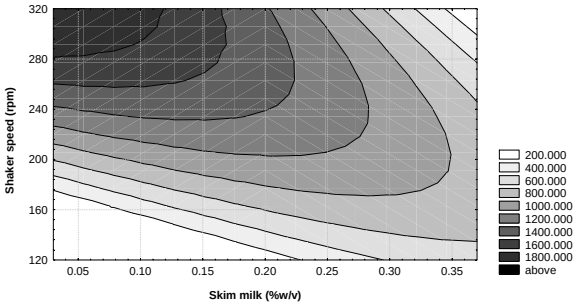

Fig. 3 Contour plots of protease production by *B. subtilis* C4 showing the effect of skimmed milk and shaker speed (soy flour was kept at 0.2% w/v).

Validation of the model

To confirm the validity of the model, experimental rechecking was performed using a random set of five production combinations to test for protease production (Table 7). Every predicted response for protease production was very close to the observed value, confirming the model's accuracy. The predicted maximum protease production was 1576 units/ml, while the observed value was 1537 units/ml and the corresponding concentrations of soy flour, skimmed milk, and the shaker speed were 2.0% (w/v), 0.1% (w/v,) and 280 rpm, respectively.

In order to solve the problem of oxygen limitation, which might occur in the shaken flasks, a stirred tank reactor was used to cultivate *B. subtilis* C4 in the optimized medium, with a dissolved oxygen (DO) concentration of about 70% air saturation maintained throughout the process. The protease activity gradually increased and reached a maximum value of

Table 7 Data for validation of the experimental model.

Run	Variables			Protease activity (units/ml)	
	soy flour (% w/v)	skimmed milk (% w/v)	shaker speed (rpm)	Observed[a]	Predicted
1	2.0	0.15	280	1500 ± 10	1522
2	2.0	0.10	280	1537 ± 17	1576
3	2.5	0.15	280	1512 ± 32	1561
4	2.5	0.10	280	1489 ± 13	1521
5	1.5	0.10	280	1436 ± 40	1496

[a] Values are indicated as mean $\pm$ SD ($n = 3$).

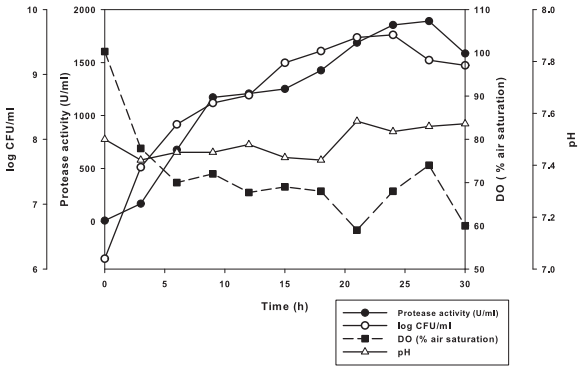

Fig. 4 Profiles of cell growth (log CFU/ml), protease activity (units/ml), dissolved oxygen concentration (%), and pH in a batch culture of *B. subtilis* C4 for silk degumming in a stirred tank reactor.

1891 units/ml after cultivation for 27 h which was an overall 2.6-fold increase over the basal medium (729 units/ml) in a shaken flask culture. Thereafter, a decline in protease activity was observed (Fig. 4) that was caused by a lack of available substrates and autoproteolysis[31,32]. The protease production of *B. subtilis* C4 was improved 1.2 fold over that in a shaken flask (1537 units/ml). Although the oxygen requirement of each bacterial strain varies and has been reported to influence the productivity[33], the DO concentration of 70% air saturation may not be the most suitable condition for *B. subtilis* C4. Therefore, further study on the DO requirement is necessary, in order to obtain the highest production of silk degumming protease by this bacterial strain.

These results support further process development to produce silk degumming protease on a large scale by varying the dissolved oxygen concentration prior to fed-batch fermentation.

Acknowledgements: This research was supported by the Kasetsart University Research and Development Institute.

REFERENCES

1. Beg KB, Sahai V, Gupta R (2003) Statistical media optimization and alkaline protease production from *Bacillus mojavensis* in a bioreactor. *Process Biochem* **39**, 203–9.
2. Joo HS, Kumar GC, Park GC, Paik SR, Chang CS (2003) Oxidant and SDS stable alkaline protease from *Bacillus clausii* I-52: production and some properties. *J Appl Microbiol* **95**, 267–72.
3. Kaeyanon C, Wongsaengchantra PY (2005) Degumming of Thai silk with baterial protease. In: Proceedings of the 31st Congress on Science and Technology, Suranaree Univ of Technology, Thailand, L0004.
4. Senatham D, Wongsaengchantra PY (2005) Preliminary characterization of protease from bacteria GR_5_4, 4TS9-001, and H1C_13_8. In: Proceedings of the 31st Congress on Science and Technology, Suranaree Univ of Technology, Thailand, L0005.
5. Freddi G, Raffaella M, Riccardo I (2003) Degumming of silk fabric with several proteases. *J Biotechnol* **106**, 101–12.
6. Çelik E, Çalik P (2004) Bioprocess parameters and oxygen transfer characteristics in beta-lactamase production by *Bacillus* species. *Biotechnol Progr* **20**, 491–9.
7. Puri S, Khali O, Gupta R (2002) Optimization of alkaline protease production from *Bacillus* sp. by response surface methodology. *Curr Microbiol* **44**, 286–90.
8. Kirk O, Borchert TV, Fuglsang CC (2002) Industrial enzyme applications. *Curr Opin Biotechnol* **13**, 345–51.
9. Kaur P, Satyanarayana T (2005) Production of cell-bound phytase by *Pichia anomala* in an economical cane molasses medium: optimization using statistical tools. *Process Biochem* **40**, (9), 3095–102.
10. Jalbani N, Kazi T, Arain B, Jamali M, Afridi H, Sarfraz R (2006) Application of factorial design in optimization of ultrasonic-assisted extraction of aluminium in juices and soft drinks. *Talanta* **70**, 307–14.
11. Bandaru VVR, Somalanka SR, Menduc DR, Madicherla NR, Chityala A (2006) Optimization of fermentation conditions for the production of ethanol from sago starch by co-immobilized amyloglucosidase and cells of *Zymomonas mobilis* using response surface methodology. *Enzym Microb Tech* **38**, 209–14.
12. Reddy LVA, Wee YJ, Yun JS, Ryu HW (2008) Optimization of alkaline protease production by batch culture of *Bacillus* sp. RKY3 through Plackett-Burman and response surface methodological approaches. *Bioresource Tech* **99**, 2242–9.
13. Hujanen M, Linko S, Linko YY, Leisola M (2001) Optimization of media and cultivation conditions for L(+)(S)-lactic acid production by *Lactobacillus casei* NRRL B-441. *Appl Biochem Microbiol* **56**, 126–30.
14. Rao YK, Tsay KJ, Wu WS, Tzeng YM (2007) Medium optimization of carbon and nitrogen sources for the production of spores from *Bacillus amyloliquefaciens* B128 using response surface methodology. *Process Biochem* **42**, 535–41.
15. Ghosalkar A, Sahai V, Srivastava A (2008) Optimization of chemically defined medium for recombinant *Pichia pastoris* for biomass. *Bioresource Tech* **99**, 7906–10.
16. de Coninck J, Bouquele S, Dumortier V, Duyme F, Verdier-Denantes I (2000) Industrial media and fermentation processes for improved growth and protease production by *Tetrahymena thermophila*. *J Ind Microbiol Biotechnol* **24**, 285–90.

17. Fufeungsombut E, Chim-anage P, Promboon A, Suwannaphan S (2009) Isolation and selection of silk degumming protease producing bacteria from Thailand. In: Proceedings of the 47th Kasetsart University Annual Conference, Bangkok, pp 456–63.
18. Horikoshi K (1991) *Microorganisms in Alkaline Environments*, Kodansha, Tokyo, pp 5–6.
19. Davis BD, Mingioli ES (1950) Mutants of *Escherichia coli* requiring methionine of vitamin B12. *J Bacteriol* **60**, 17–28.
20. Romsomsa N, Chim-anage P, Jangchud A (2008) Optimization medium and culture conditions for silk degumming protease production from *Bacillus* sp. C4 by Plackett-Burman design. In: Proceedings of the 34th Congress on Science and Technology, Bangkok, p 77.
21. Neter J, Kutner MH, Nachtsheim CJ, Wasserman W (1996) *Applied Linear Statistical Models*, 4th edn, McGraw-Hill, New York.
22. Sangyon S (2005) Properties of alkaline protease in bacteria isolated from soil. MSc thesis, Kasetsart Univ, pp 115–6.
23. Joo HS, Chang CS (2005) Production of protease from a new alkalophilic *Bacillus* sp. I-312 grown on soybean meal: optimization and some properties. *Process Biochem* **40**, 1263–70.
24. Laxman RS, Sonawane AP, More SV, Rao BS, Rele MV, Jogdand VV, Rao MB (2005) Optimization and scale up of production of alkaline protease from *Conidiobolus coronatus*. *Process Biochem* **40**, 3152–8.
25. Dutt K, Gupta P, Saran S, Misra S, Saxena RK (2009) Production of milk-clotting protease from *Bacillus subtilis*. *Appl Biochem Biotechnol* **158**, 761–72
26. Hameed A, Keshavarz T, Evans CS (1999) Effect of dissolved oxygen tension and pH on the production of extracellular protease from a new isolate of *Bacillus subtilis* K2, for use in leather processing. *J Chem Tech Biotechnol* **74**, 5–8.
27. Khuri AI, Cornell JA (1987) *Response Surfaces: Design and Analysis*, Marcel Decker, New York.
28. Wang Q, Hou Y, Xu Z, Miao J, Li G (2008) Optimization of cold-active protease production by the psychrophilic bacterium *Colwellia* sp. NJ341 with response surface methodology. *Bioresource Tech* **99**, 1926–31.
29. El Enshasy H, Enein AA, Helmy S, El Azaly Y (2008) Optimization of the industrial production of alkaline protease by *Bacillus licheniformis* in different production scales. *Aust J Basic Appl Sci* **2**, 583–93.
30. Gattinger LD, Duvnjak Z, Khan AW (1990) The use of canola meal as a substrate for xylanase production by *Trichoderma reesei*. *Appl Microbiol Biotechnol* **33**, 21–5.
31. Jang JW, Jun HK, Kim EK, Jang WH, Kang JH, Yoo OJ (2001) Enhanced thermal stability of an alkaline protease, prP, isolated from a *Pseudomonas* sp. by mutation at an autoproteolysis site, Ser-331. *Biotechnol Appl Biochem* **34**, 81–4.
32. Chu IM, Lee C, Li TS (1992) Production and degradation of alkaline protease in batch culture of *Bacillus subtilis* ATCC 14416. *Enzym Microb Tech* **14**, 755–61.
33. Tang YJ, Zhong JJ (2003) Role of oxygen supply in submerged fermentation of *Ganoderma lucidum* for production of Ganoderma polysaccharide and ganoderic acid. *Enzym Microb Tech* **32**, 478–84.