JOURNAL OF PURE AND APPLIED MICROBIOLOGY, June 2012. Vol. **6**(2), p. 789-796

Optimization of Lipase Production from Rice Straw using Response Surface Methodology by *Aspergillus niger*

Masumeh Anvari

Department of Microbiology, Faculty of Sciences, Islamic Azad University, Rasht Branch, P.O. Box 41335-3516, Iran.

(Received: 05 September 2011; accepted: 27 October 2011)

The optimal conditions for lipase production were determined by response surface methodology in solid state fermentation with *Aspergilles niger*. A second-order central composite design was applied to evaluate the effects of four independent variables incubation time, pH, olive oil concentration as inducer effect and yeast extract concentration on the lipase activity. Correlation analysis of the mathematical regression model indicated that the quadratic polynomial model could be employed to optimize the lipase production. The linear term of incubation time, olive oil and yeast extract concentration had significant effects on production of lipase (*p*-value < 0.05). Maximum lipase activity from the experimental was determined to be 69.78 U/g under the optimal conditions.

Key Words: Lipase; Rice straw; Response Surface Methodology; *Aspergillus niger*.

Lipases are enzymes belonging to the group of the hydrolases, whose main biological function is to work as the catalyst of the hydrolysis of insoluble triacylglycerols to generate free fatty acids, mono- and diacylglycerols, and glycerol. Besides its natural function, lipases can catalyze esterification, interesterification, and transesterification reactions in nonaqueous media[1].

Microbial lipases are biocatalysts that have interesting characteristics, such as action under mild conditions, stability in organic solvents, high substrate specificity, and regio- and enantioselectivity[2-4]. Due to the growing importance of lipases within biotechnological perspectives, extensive research is being carried out throughout the world to exploit hyperactive strains for lipase production and to optimize the various parameters for maximizing its production.

Traditional approach to optimization of biological systems based on *One Factor At a Time*, commonly abbreviated OFAT, is not as scientific as is Response Surface Methodology (RSM). It is less efficient than a factorial screening design and can provide incorrect conclusions in case of strong interactions among the factors. Response surface methodology is a statistical method that uses quantitative data from an appropriate experimental design to determine or simultaneously solve multivariate equation [5].

Solid substrate fermentation (SSF) has built up credibility in recent years for the production of microbial products including enzymes through inexpensive media and it is an appropriate process for developing countries [6]. Although submerged fermentation is widely used

* To whom all correspondence should be addressed.
Mob: +98 9112323482 ; Fax: +98 131 4223621
E-mail : anvari@iaurasht.ac.ir

in the enzyme industry and has advantages in process control and good yields of enzymes, the products in fermentation are relatively dilute resulting in high volumes of effluents [7]. As an alternative, SSF has been developed and proved to be an economical way to produce various enzymes including lipases and esterases. Interest in lipases has greatly increased in recent years due to their various applications in food, detergent, cosmetic, organic synthesis, and pharmaceutical industries [8-10]. Microorganisms are potent lipase producers and moulds are widely recognized for higher enzyme production due to their ability to utilize various substrates with vigorous growth and sporulation on the substrate matrix.

In the present work, the objective was to improve lipase production of *Aspergillus niger* under SSF conditions with rice straw using RSM

MATERIALS AND METHODS

Microorganism

A. niger strain G14 isolated from the soil sample of the Guilan-Iran. The fungus was identified by the Iranian Research Organization for Science and Technology (IROST) and used for the experimental studies.

Inoculum and fermentation conditions

Rice straw was used as substrate for the production of lipase by *A.niger* under solid state fermentation. Fermentation was carried out in 250 ml Erlenmeyer flasks containing 10 g substrate with the moisture content adjusted to 60% with mineral salt solution. The medium pH was adjusted to the corresponding values (Table 2) using 1 M HCl and NaOH. After sterilization (autoclaved at 121°C at 15 psi pressure for 30 min), an inoculum size of 10^6 spores / g substrate was used for each flask under aseptic conditions and incubated at 30 ± 2°C.

All the experiments were carried out in triplicates and the results were expressed as the mean of them.

Enzyme extraction

Enzymes extraction was carried out by the method of Ramachandran et al. [11]. . Crude enzymes were extracted by mixing solid substrate with 20 ml of 0.1 M Tris–HCl buffer, pH 8.0, and then shaking the mixture in a shaker at 200 rpm. The suspension was then centrifuged at 12000 rpm for 10 min and the supernatant used for lipase assay and activity was recorded as response at the end of the experiments.

Enzyme assay

Lipase assay was done spectrophotometrically using *p*-nitrophenyl palmitate (procured from Sigma) as the substrate. The assay mixture contained 1ml of 16.5 mM solution of *p*-nitrophenyl palmitate in 2-propanol along with Tris-HCl buffer, pH 8 (supplemented with 0.1% arabic gum and 0.4% Triton X-100) in a ratio of 1:9. The enzyme solution (0.025 ml) was added to it and incubated in water bath at 37°C for 10 min. *p*-nitrophenol was liberated from p-nitrophenyl palmitate by lipase mediated hydrolysis imparting a yellow color to the reaction mixture. After incubation, 2 ml of distilled water was added and the absorbance was measured at 410 nm [12]. Absorbance of control was also recorded. One unit (U) of lipase activity was defined as the amount of enzyme that liberates one micromole of *p*-nitrophenol, per min under the assay conditions.

Experimental design and statistical analysis

RSM was used to determine the optimum condition for lipase production. The effect of four independent variables: incubation time (x_1), pH (x_2), olive oil concentration (x_3) as inducer effect and yeast extract concentration (x_4) on the response variable (lipase activity) was evaluated using central composite design (CCD) (Table 1). The five coded levels of each variable were incorporated into the design and were analyzed in 31 experimental trails (Table 2). The central point of the design was repeated seven times to calculate the reproducibility of the method [13]. For each experimental trail of the independent variables in the experimental design, the dependent parameter the lipase activity was determined. The effect of these independent variables x_1, x_2, x_3 and x_4 on the response was investigated using the second-order polynomial regression equation. This equation, derived using RSM for the evaluation of the response variable, is as follows:

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x^2 + \sum_{i \neq j}^{k} \beta_{ij} x_i x_j$$

where β_0 is defined as the constant, β_i the linear coefficient, β_{ii} the quadratic coefficient and β_{ij} the interaction coefficient. x_i and x_j are the independent variables while *k* equals to the number

of the tested factors (k =4). The analysis of variance (ANOVA) tables were generated and the effect and regression coefficients of individual linear, quadratic and interaction terms were determined. The significances of all terms in the polynomial were judged statistically by computing the p-value at a probability of 0.05. The regression coefficients were then used to make statistical calculations to generate response surface and contour maps from the regression models. The analysis of data and the optimizing process were generated using Minitab statistical software version 15.

Verification of model

The optimum conditions of lipase production were obtained using RSM. For verification of the model, lipase activity under optimal conditions in experimental stage was determined. The experimental and predicted values were compared in order to determine the validity of the model.

Effect of pH and temperature on the enzyme activity and stability

The pH stability of the lipase was determined for 1 h at 37ºC by incubating the enzymes-substrate at suitable buffer with various pH from 4 to 11 in the following buffer systems: 0.1 M sodium acetate (pH 4.0-5.5); 0.1 M sodium phosphate (pH 6.0-7.5); 0.1 M Tris-HCl (pH 8.0-9.0); 0.1 M glycine-NaOH (pH 9.5-11); and 0.1 M sodium carbonate (pH 11.5-12.0), respectively. To examine the thermal stability of the lipase solution was in Tris-HCl buffer (pH 8.0) was allowed to stand for 1 h at different temperatures in the range of 30–70ºC, the reaction stopped in ice-cold water and then the residual activity was measured as described above.

RESULTS AND DISCUSSION

Fitting the models

The study utilized RSM to develop a prediction model for lipase production. The experimental conditions and the corresponding response values from the experimental design are presented in Table 2. The independent and dependent variables were analyzed to obtain a regression equation that could predict the response within the given range. The values of the coefficients in the equation are presented in Table 3. The regression equation for lipase activity is as follows:

$$\text{Lipase activity} = 56.2637 + 3.0853x_1 + 2.0213x_2 + 10.3422x_3 - 3.2933x_4 - 1.0407x_1^2 - 0.93x_2^2 - 4.494x_3^2 - 3.5084x_4^2 + 0.8133x_1x_2 - 0.6504x_1x_3 + 0.5643x_1x_4 + 0.8041x_2x_3 - 0.063x_2x_4 + 0.8041x_3x_4 \quad (2)$$

where x_1, x_2, x_3 and x_4 are the independent variables for incubation time, pH, oil and yeast extract concentration respectively.

The plot of experimental values of lipase activity versus those calculated from Eq. (2) indicated a good fit, as presented in Fig. 1. The results of analysis of variance (ANOVA) are shown in Table 4. For the model fitted, the coefficient of determination (R^2), which is a measure of degree of fit [14], was 0.901. This implies that 90.1% of the variations could be explained by the fitted model. The closer of R^2 value to unity, the better the empirical models fits the actual data [15]. On the other hand, the smaller of R^2 value the less relevance the dependent variables in the model have in explaining the behavior of variations [16]. The probability p-value of the regression model significance was less than 0.001. Therefore, the developed model could adequately represent the real relationship among the parameters chosen.

Effects of independent variables on responses

The effects of lipase production conditions by the regression coefficients of fitted second-order polynomial are presented in Table 3. It was evident that the linear terms except for pH, and two quadratic terms (pH and incubation time) were significant (p-value < 0.05), whereas all the cross-product terms were not insignificant (p-value > 0.05). The results indicated that the effects of inducer (oil concentration) and yeast extract were the major contributing factors to lipase production. Within the experimental range, however, pH had no significant effects (p-value > 0.05) on the lipase production.

The response surfaces and contour plots of lipase production conditions are shown in Figs. 2–4. The response surface and contour plots of the effects of incubation time and oil concentration on lipase production are presented in Fig. 2. The results indicated that incubation time displayed a linear effect on the lipase production, and it increased with an increase of incubation time. Gupta et al. [17] also reported the incubation time as significant factor for lipase production. However,

inducer (oil concentration) demonstrated a quadratic effect on the response; hence lipase activity increased up to about oil concentration 3.2 (%w/w), followed by a decline with its further increase. The effects of incubation time and yeast extract concentration on lipase production are shown in Fig. 3. Yeast extract concentration a quadratic effect on the response, maximum lipase activity at 1.8 (%w/w) yeast extract, whereas the effect of incubation time was linear. Both oil and

Table 1. Independent variables and their coded and actual levels

Independent variables (factors)	Symbol	Actual values of coded levels				
		$-\alpha$	-1	0	+1	$+\alpha^*$
Incubation time (h)	x_1	24	48	72	96	120
pH	x_2	6.5	7.0	7.5	8.0	8.5
olive oil concentration (%w/w)	x_3	1.0	2.0	3.0	4.0	5.0
yeast extract concentration (% w/w)	x_4	1.0	2.0	3.0	4.0	5.0

* α = 2.0 (star point for orthogonal CCD for the case of 4 independent variables)

Table 2. Central composite rotatable design (CCRD) and responses (lipase activity)

Run	Time	pH	oil con.	yeast ext.	Lipase activity (U/g)
1	48	6.5	1	1	37.11
2	96	6.5	1	1	45.44
3	48	7.5	1	1	38.12
4	96	7.5	1	1	46.36
5	48	6.5	3	1	63.54
6	96	6.5	3	1	62.77
7	48	7.5	3	1	64.46
8	96	7.5	3	1	67.86
9	48	6.5	1	3	28.40
10	96	6.5	1	3	31.96
11	48	7.5	1	3	25.33
12	96	7.5	1	3	35.10
13	48	6.5	3	3	51.06
14	96	6.5	3	3	58.14
15	48	7.5	3	3	54.13
16	96	7.5	3	3	62.93
17	24	7	2	2	41.81
18	120	7	2	2	54.13
19	72	6	2	2	40.50
20	72	8	2	2	56.32
21	72	7	0	2	21.62
22	72	7	4	2	46.69
23	72	7	2	0	37.96
24	72	7	2	4	38.24
25	72	7	2	2	54.96
26	72	7	2	2	57.22
27	72	7	2	2	58.27
28	72	7	2	2	56.72
29	72	7	2	2	56.41
30	72	7	2	2	58.41
31	72	7	2	2	51.87

yeast extract concentration quadratic effects on lipase production, and the maximum lipase activity was obtained at 3.2 and 1.8 (%w/w) respectively (Fig. 4).

Optimum conditions and model verification

From the model, optimum conditions for lipase productin were obtained as presented in Table 5. Under the optimum conditions of incubation time 116.12 h, pH 8, oil concentration 3.19 (%w/w) and yeast extract concentration 1.78 (%w/w) a maximum response of 68.31 U/g was predicted. The suitability of the model equation for predicting the optimum response value was tested by additional independent experiments using the recommended optimum conditions (Table 5). The results indicated that the experimental lipase activity (69.87 U/g) was not significantly different from the predicted protein value (68.31 U/g).

Table 3. Significance of regression coefficients of the fitted second-order polynomial model for lipase activity

Term	Regression coef.	Standard error	*t*- value	*p*- value
Constant	56.2637	2.386	23.583	0.000
Linear				
Time (h)	3.0853	1.288	2.395	0.029
pH	2.0213	1.288	1.569	0.136
Oil con.	10.3422	1.288	8.027	0.000
Yeast ext.	-3.2933	1.288	-2.556	0.021
Quadratic				
Time*Time	-1.0407	1.180	-0.882	0.391
pH*pH	-0.9300	1.180	-0.788	0.442
Oil con.*Oil con.	-4.4940	1.180	-3.807	0.002
Yeast ext.*Yeast ext.	-3.5084	1.180	-2.972	0.009
Cross product				
Time*pH	0.8133	1.578	0.515	0.613
Time*Oil con.	-0.6504	1.578	-0.412	0.686
Time*Yeast ext.	0.5643	1.578	0.358	0.725
pH*Oil con.	0.8041	1.578	0.510	0.617
pH*Yeast ext.	-0.0630	1.578	-0.040	0.969
Oil con.*Yeast ext.	0.8041	1.578	0.510	0.617

Table 4. Analysis of variance (ANOVA) of the regression parameters for the response surface model

Source	D. F.	Sum of Squares	Mean Square	*F*-value	*P*-value
Model	14	4042.91	288.78	7.25	Â 0.001
Linear	4	3153.90	788.48	19.79	Â 0.001
Quadratic	4	845.81	211.45	5.31	0.006
Cross product	6	43.20	7.20	0.18	0.978
Residual	16	637.47	39.84		
Pure error	6	30.78	5.13		
Total	30	4680.38			

Table 5. Optimum conditions of lipase production experimental and predicted from RSM

Optimum condition				lipase activity (U/g)	
x_1	x_2	x_3	x_4	Cal. value	Expt. value
116.12	8	3.19	1.78	68.31	69.78

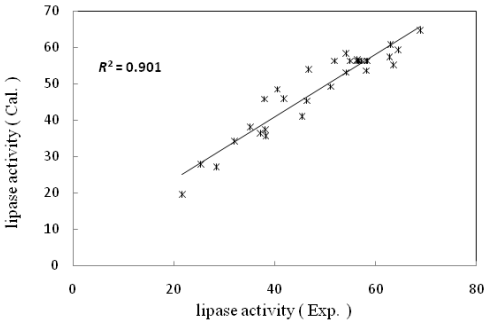

Fig. 1. Correlation of calculated with experimental lipase activity

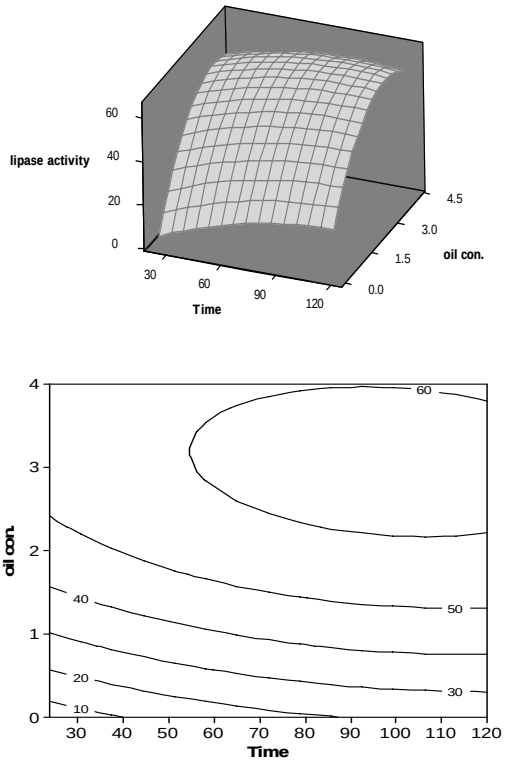

Fig. 2. Response surface plot (upper) and its contour plot (lower) for the effects of incubation time (h) and oil concentration (%w/w) on lipase activity at its center level

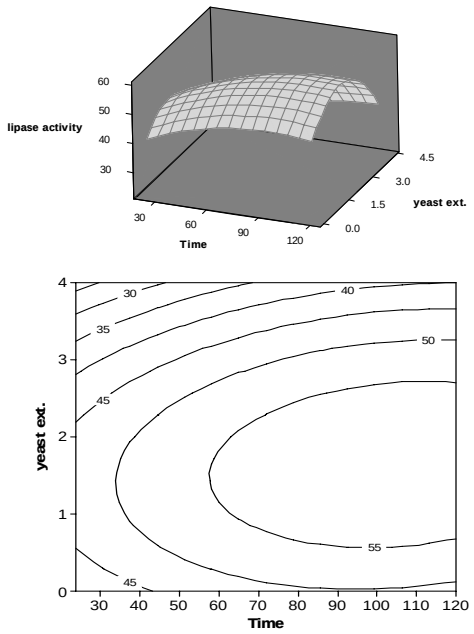

Fig. 3. Response surface plot (upper) and its contour plot (lower) for the effects of incubation time (h) and yeast extract concentration (%w/w) on lipase activity at its center level

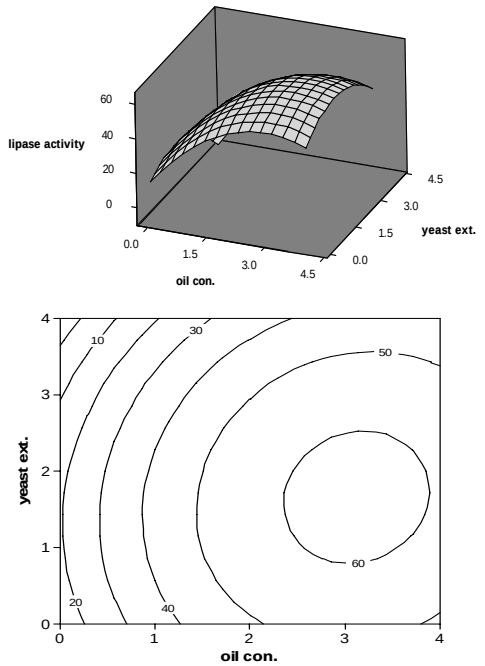

Fig. 4. Response surface plot (upper) and its contour plot (lower) for the effects of oil and yeast extract concentration (%w/w) on lipase activity at its center level

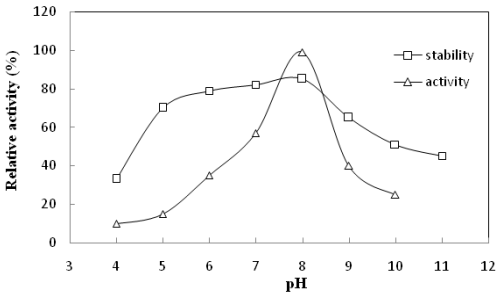

Fig. 5. Effect of pH on the stability and activity of the lipase.

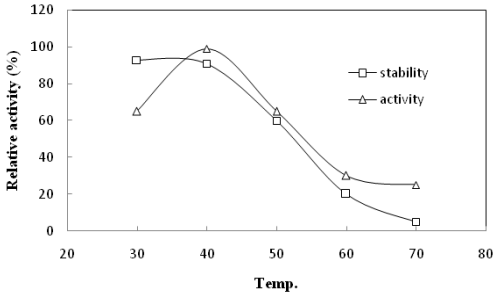

Fig. 6. Effect of temperature on the stability and activity of lipase.

Effect of pH and temperature on the activity and stability of the enzyme

The effect of pH on the catalytic activity was studied by using *p*-nitrophenyl palmitate as a substrate under the standard assay condition. The lipase exhibited maximum activity at pH 8.0. The pH stability profile of the lipase was determined by the measurement of the residual activity at pH 8.0 after incubation at various pH values at 37°C for 1 h. The lipase was stable between pH 6.0 and 8.0, and retained more than 80% of its original activity (Fig. 5).

The optimum temperature for the lipase activity was found to be 40°C. To examine the thermal stability of the lipase, the enzyme solution in Tris-HCl buffer (pH 8) was allowed to stand for 1 h at various temperatures, and the residual activity was measured. The results showed that at temperatures higher than 40°C, the residual activity was decreased significantly. At 50°C, the enzyme retained 63% of its activity (Fig. 6). The lipase activity was inactivated at 70°C.

CONCLUSIONS

The response surface methodology is an efficient technique for the rapid screening of the significant influencing parameters and development of a polynomial model to optimize fermentation condition for the production of lipase from *A.niger*. The R^2 value of 0.901 showed a good fit of the model with the experimental data. The model, predicted accurately for maximum lipase production. The optimum fermentation conditions obtained for the production of lipase from *rice straw* were incubation time 116 h, pH 8, oil concentration 3.2 (%w/w) and yeast extract concentration 1.78 (%w/w) for obtaining a maximum lipase activity of 69.87 U/g.

REFERENCES

1. Houde, A., Kademi, A., Leblanc, D. Lipases and their industrial applications. *Appl. Biochem. Biotechnol.*, 2004;**118**: 155-170.
2. Snellman, E.A., Sullivan, E.R., Colwell, R.V. Purification and properties of the extracellular lipase, LipA, of *acinetobacter sp. Biochem. Eng. J.*, 2002; **11**: 269-274.
3. Hasan, F., Shah, A.A., Hameed, A. Industrial Applications of microbial lipases. *Enzyme Microb. Technol.*, 2006; **39**: 235- 251.
4. Jaeger, K., Reetz, M.T. Microbial lipases form versatile tools for biotechnology. *Trends Biotechnol.*, 1998; **16**: 396-403.
5. Wang, L., Yang, B., Du, X., Yang, Y., Liu, J. Optimization of conditions for extraction of acid-soluble collagen from grass carp (Ctenopharyngodon idella) by response surface methodology. *Innovative Food Sci. Emerg. Technol.*, 2008; **9**: 604–607.
6. Singhania, R.R., Patel, A.K., Soccol, C.R., Pandey, A. Recent advances in solid state fermentation. *Biochem. Eng. J.*, 2009; **44**: 13–18.
7. Nagy, V., Toke, E.R., Keong, L.C., Szatzker, G., Ibrahim, D., Omar, I.C., Szakacs, G., Poppe, L. Kinetic resolutions with novel, highly enantioselective fungal lipases produced by solid state fermentation. *J. Mol. Catal. B: Enzym.*, 2006; **39**: 141–148.
8. Edwinoliver, N.G., Thirunavukarasu, K., Purushothaman, S., Rose, C., Gowthaman,

M.K., Kamini, N.R. Corn steep liquor as a nutrition adjunct for the production of *Aspergillus niger* lipase and hydrolysis of oils thereof. *J. Agric. Food Chem.*, 2009; **57**: 10658–10663.

9. Shua, Z., Jianga, H., Lina, R., Jianga, Y., Lina, L., Huanga, J. Technical methods to improve yield, activity and stability in the development of microbial lipases. *J. Mol. Catal. B: Enzym.* 2010; **62**: 1–8.
10. Treichel, H., de Oliveira, D., Mazutti, M.A., Di Luccio, M., Oliveira, J.V. A review on microbial lipases production. *Food Bioprocess Technol.* 2010; **3**: 182–196.
11. Ramachandran, S., Patel, A.K., Nampoothiri, K.M., Francis, F., Nagy, V., Szakacs, G., Pandey, A. Coconut oil cake – a potential raw material for the production of a-amylase. *Bioresour. Technol.* 2004; **93**, 169–174.
12. Kordel, M., Hofmann, B., Schomburg, D., Schmid, R.D. Ex-tracellular lipase of *Pseudomonas sp*. strain ATCC-21808, puritication, characterization, crystallization, and preliminary X-ray diffraction data. *J Bacteriol.*, 1991; **173**: 4836-4841.
13. Montgomery, D.C. Design and analysis of experiments (5th ed.). New York: John Wiley and Sons, 2001.
14. Haber, A., Runyon, R. General statistics (3rd ed.). Reading, MA: Addison-Wesley, 1977.
15. Sin, H.N., Yusof, S., Hamid, N.S.A., Rahman, R.A. Optimization of enzymatic clarification of sapodilla juice using response surface methodology. *J. Food Eng.*, 2006; **73**: 313"319. J.
16. Lee, W.C., Yusof, S., Hamid, N.S.A., Baharin, B.S. Optimizing conditions for hot water extraction of banana juice using response surface methodology (RSM). *J. Food Eng.*, 2006; **75**: 473–479.
17. Gupta, N., Sahai, V., Gupta, R. Alkaline lipase from a novel strain *Burkholderia multivorans*: Statistical medium optimization and production in a bioreactor. *Process Biochem.*, 2007; **42**: 518-526.