International Journal of Bio-Technology
and Research (IJBTR)
ISSN 2249-6858
Vol. 3, Issue 1, Mar 2013, 59-66

TRANS STELLAR
•Journal Publications •Research Consultancy

OPTIMIZATION OF LIPASE PRODUCTION BY *SACCHAROMONOSPORA AZUREA* AP 11/18 USING PLACKETT-BURMAN DESIGN AND RESPONSE SURFACE METHODOLOGY

PADHIAR A. R.[1] & MODI H. A.[2]

[1]Department of Biotechnology, Kadi Sarva Vishwavidyalaya, Gandhinagar, Gujarat, India

[2]Department of Life Sciences, University School of Sciences, Gujarat University, Ahmadabad, Gujarat, India

ABSTRACT

Optimization of lipase production by *Saccharomonospora azurea* AP 11/18 was carried out using response surface methodology (RSM). Eight various nutritional parameters were screened using Plackett–Burman experimental design were further optimized by central composite design of response surface methodology for lipase production in submerged fermentation. Among the different carbon sources supplemented, castor oil was most suitable for lipase production while in nitrogen sources, Meat extract was most suitable. The maximum Lipase production of 1041.46 U were achieved in the medium containing Caster oil (1.65g %), Meat extract (1.10g%) and Ammonium sulphate (0.06g%) using response surface plots and point prediction tool of Design Expert 8.04 (Stat-Ease Inc.) software.

KEYWORDS: Optimization, Lipase, *Saccharomonospora Azurea* AP 11/18, Plackett–Burman Design, Response Surface Methodology, Submerged Fermentation

INTRODUCTION

Lipases are ubiquitous enzymes which are found in animals, plants, fungi and bacteria (Wooley and Petersen, 1994). Lipases which hydrolyze esters of fatty acids, are carboxyester hydrolases, and classified as 3.1.1 (Patil *et al.,* 2011). Industrially important enzymes have traditionally been obtained from submerged fermentation (SmF) because of the ease of handling and greater control of environmental factors such as temperature and pH.

Traditional approach to optimization of biological systems based on *One Factor At a Time*, commonly abbreviated OFAT, is not as scientific as are Plackett-Burman experimental design and 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. Hence, in the present study, optimum fermentation medium for lipase production is formulated using Plackett-Burman experimental design and RSM (Kumari *et al*., 2009).

Plackett-Burman design is a "screening design" traditionally used for identifying important factors among many potential factors. It is a screening technique used to examine the effects of several variables in one experiment and avoid multiple runs of the same basic test. This method allows checking the main effect of various compounds (Stanbury *et al*., 2003).

Response surface methodology was used to optimize important nutritional factors screened by Plackett–Burman design. Response surface methodology (RSM) is an empirical modeling system for developing, improving, and optimizing of complex processes (Manohar and Divakar, 2004). RSM assesses the relationships between the response(s) and the

independent variables and defines the effect of the independent variables, alone or in combination, in the processes (Afshin *et al*., 2008).

The isolate *Saccharomonospora azurea* AP 11/18 was found to be the best extracellular lipase producer among actinomycetes. This chapter deals with designing a suitable media for lipase production. The numerous media combinations for the lipase production were checked. The present work was undertaken to screen these various components for getting best combination of medium component for higher lipase production using Plackett-Burman design as well as RSM.

MATERIALS AND METHODS

Microorganism

Saccharomonospora azurea AP 11/18, identified as a good lipase producer was isolated from the soil of oil industry units at Kadi (Gujarat, India). The culture was grown on modified Bennet's agar incubated at 40ºC for 96 hrs, and was maintained on Bennet's agar slants at 4°C.

LIPASE PRODUCTION IN SHAKE FLASK CULTURE

50 ml of sterilized Bennet's broth in 250ml Erlenmeyer flasks was inoculated with 6 mm disc culture and incubated at orbital shaker at 140 rpm and 40ºC for 96 hrs. When the optimum growth (O.D 2.0 at 600 nm) was achieved, 1.0% of this growth was used for inoculating 50 ml lipase production Bennet's broth medium supplemented with 1% Tributyrene oil in 250 ml flasks, keeping composition and growth conditions the same as in growth phase. After 96 hrs the broth were filtered through Whatman filter paper (No. 1). The culture filtrate was used as enzyme source.

ENZYME ASSAY

Extracellular lipase activity was assayed spectrophotometrically using *p*-nitro phenyl palmitate (*p*-NPP) as substrate according to the method reported by Savitha *et al.*, (2007). One lipase unit (U) is equal to the amount of enzyme that liberated 1 µ mole p-nitro phenol per 60 min.

PLACKETT-BURMAN EXPERIMENTAL DESIGN

The purpose of this optimization step was to identify which ingredients of the medium had a significant effect on lipase activity. The Plackett–Burman (Sarat *et al*., 2010), Statistical experimental design is a versatile method for screening the important variables. The total number of experiments to be carried out is n + 1, where n is the number of variables.

For each variable, a high (+1) and low (-1) level was tested. All trials were performed in triplicate. The statistical software package Design- Expert software 8.04 (Stat-Ease Inc.) was used for analyzing the experimental data.

The ingredients studied by Placket-Burman design were carbon sources (glucose, castor oil), nitrogen sources (casein hydrolysate, meat extract, yeast extract and ammonium sulphate), minerals (magnesium sulphate) and surfactant (triton-x-100).

These eight independent variables with three dummy variables in twelve combinations were organized according to the Plackett-Burman design matrix. High and low concentration of all 8 ingredients was shown in table 1.

The pH of all the flasks was adjusted to 8 and sterilized by autoclaving. The inoculation was done with pre inoculated culture with 8 mm disc of the culture grown on Bennet's agar medium for 96 hours. The response for the design was measured in the terms of enzyme activity.

Table 1: High and Low Concentrations of 8 Ingredients Used in Plackett-Burman Design for Optimization of Media for Lipase Production

Components		Glucose	Castor Oil	Casein Hydro-Lysate	Yeast Extract	Meat Extract	Triton-X-100	$MgSO_4$	$(NH_4)_2SO_4$
Concentration gm/ml	High	5.0	2.5	2.5	1.0	1.0	0.8	0.5	1.0
	Low	0.5	0.5	0.5	0.1	0.1	0.1	0.1	0.1

EFFECT OF DIFFERENT NITROGEN SOURCE ON LIPASE PRODUCTION

Plackett-Burman design shows positive effects of various nitrogen sources on lipase production. So different combination of nitrogen sources were studied including control. Substitution of a total of +2% organic nitrogen source (from Bennet's broth) by 0.5 % standalone Nitrogen sources like Casein hydrolysate, Yeast extract, Meat extract, $(NH_4)_2SO_4$ and KNO_3 was provided for lipase production by *Saccharomonospora azurea* AP 11/18 at 96 hours. A combination of casein hydrolysate, yeast extract and meat extract was used as Control.

RSM-SECOND LEVEL EXPERIMENTAL DESIGN

RSM consist of a group of empirical techniques used for evaluation of relationship between cluster of controlled experimental factors and measured response. Plackett-Burman Design was used to pick factors that influence lipase production significantly and insignificant ones were eliminated in order to obtain a smaller, manageable set of factors. Once critical factor were identified via screening, the central composite design (CCD) was proceeded to obtain a quadratic model, consisting of factorial trails and star points to estimate quadratic effects and central points to estimate the pure process variability with lipase production as response.

The linear quadratic model with 3 variables expressed as:

$$Y_{EA}=\beta_o+\beta_1X_1+\beta_2X_2+\beta_3X_3+\beta_{11}X_1^2+\beta_{22}X_2^2+\beta_{33}X_3^2+\beta_{12}X_1X_2+\beta_{13}X_1X_3+\beta_{23}X_2X_3$$

Where Y is the measured response, β_0 is the intercept term, β_1,β_2 and β_3 are linear coefficient, β_{11}, β_{22} and β_{33} are quadratic coefficient, β_{12},β_{13},β_{23} are interaction coefficient and X_1,X_2 ,X_3 are coded independent variables. Low and high factor settings are coded as –1 and +1; the midpoint is coded as 0. Design- Expert software 8.04 (Stat-Ease Inc.) was used for regression and graphical analyses of the obtained data. The optimal concentrations of the critical variables were obtained by analyzing contour plots. The statistical analysis of the model was represented in the form of analysis of variance (ANOVA). The coded and actual values of each variable are listed in Table 2.

Table 2: Design Summary

Study Type Response Surface	Runs 20
Design Type Central Composite	Blocks No Blocks
Design Model Quadratic	Build Time (ms) 3.11

Factor	Name	Units	Type	Subtype	Min.	Maxi.	-1 Actual	+1 Actual	Mean	Std. Dev.
A	Caster oil	ml	Numeric	Continuous	-0.62	3.92	0.30	3.00	1.65	1.12
B	Meat extract	gm	Numeric	Continuous	-0.41	2.61	0.20	2.00	1.10	0.74
C	Ammonium Sulphate	gm	Numeric	Continuous	-0.02	0.13	0.01	0.10	0.06	0.04

RESULTS AND DISCUSSIONS

Determination of Important Medium Components Using Plackett-Burman Design

The application of a complete factorial design would require 2^n experiments if 'n' factors have to be investigated. Thus, eleven variables including 3 dummy variables and eight effective variables would lead to 256 trials (without dummy), a huge number. However, the use of the factorial design considerably reduces the number of experiments without losing information about the main effect of variables. Eleven levels of culture variables were examined in the Plackett-Burman design matrix with 12 different trials.

The results of Plackett-Burman screening design for increased Lipase production by *Saccharomonospora azurea* AP 11/18, shown in Table 3 revealed that Castor oil is helpful in lipase production as carbon sources and Yeast extract, Meat extract and Ammonium sulphate as nitrogen source. $MgSO_4$, a common medium ingredient was selected on the basis of earlier reports also showed a positive effect. The response of the factors was studied in the form of one variables enzyme activity.

Table 3: Results for Plackett-Burman Screening Design for Lipase Production

Run	Enzyme Activity	Run	Enzyme Activity
1	64	7	26.59
2	235.24	8	89.94
3	24.64	9	25.94
4	14.91	10	37.405
5	25.29	11	27.89
6	24.86	12	24

Castor oil, Yeast extract, Meat extract, $MgSO_4$ and Ammonium sulphate exhibited positive effect on the production of lipase. This indicates that the enzyme production was enhanced by adding higher concentration of these ingredients. Glucose, Casein Hydrolysate and Triton X-100 had a negative effect which indicate that increasing the concentration of these ingredients, decreases the production of enzyme. The results were depicted in the half normal plot was presented in Fig. 1.

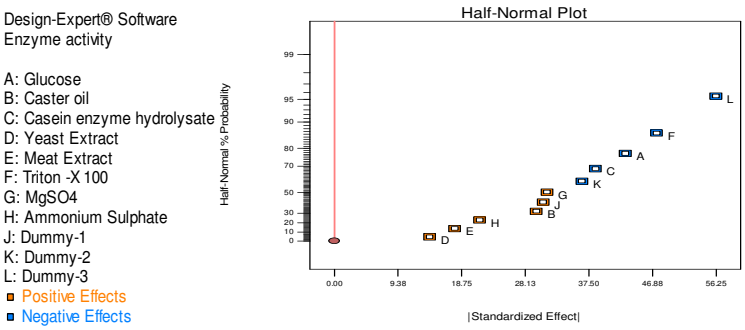

Figure 1: The Half Normal Plot of Placket-Barman Design

Abdulaziz *et al.* (2006) isolated fungal strain *Monascus ruber* which produced an antibacterial substance citrinin using batch cultures. They used Plackett-Burman experimental design to optimize the components of medium to improve citrinin production for non-food applications. They observed 1.75-fold improvement of the antibacterial activity using Plackett-Burman.

Imandi *et al.* (2007) observed maximum lipase activity of 18.58 units per gram of dry fermented substrate in four days of fermentation by *Yarrowia lipolytica* in Solid State Fermentation (SSF) using Plackett-Burman design.

EFFECT OF COMBINATION OF DIFFERENT NITROGEN SOURCES ON LIPASE PRODUCTION

The effect of individual nitrogen source and combination of nitrogen source on lipase activity by *Saccharomonospora azurea* AP 11/18 was checked.Substitution of a total of +2% organic nitrogen source (from Bennet's broth) by 0.5 % standalone Nitrogen sources like Casein enzyme hydrolysate, Yeast extract, Meat extract, $(NH_4)_2SO_4$ & KNO_3 was provided for lipase production by *Saccharomonospora azurea* at 96 hours (Fig.2). Control was kept containing combination of Casein hydrolysate, Yeast extract and Meat extract as N source. Combination of Caster oil (as Carbon source) with Meat Extract (as Nitrogen source) gave best lipase production as compared to other combinations. So for further study of the relative importance of medium components on lipase production by *Saccharomonospora azurea* AP 11/18 using RSM-Second level Experimental Design, meat extract was selected as the Nitrogen source.

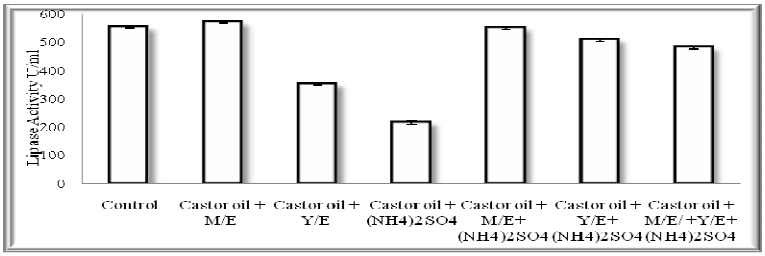

Figure 2: Effect of Different Combination of Nitrogen Source on Lipase Production

RESPONSE SURFACE METHODOLOGY ANALYSIS

Based on the results obtained, Castor oil was selected as the carbon source, Meat extract as the nitrogen source and $MgSO_4$ as the minerals for the future experiments to optimize lipase production in *Saccharomonospora azurea* AP 11/18. The different combinations of Castor oil, Meat extract and $MgSO_4$ were designed using Central Composite design (CCD). Total of 20 experiments were analyzed using the analysis of variance (ANOVA) (Table 4).

Table 4: Central Composite Design (CCD) of Factors in Coded Levels with Lipase Activity as Response

STD	Run	Caster Oil	Meat Extract	Ammonium Sulphate	Lipase Activity
6	1	+1	-1	+1	756
1	2	-1	-1	-1	651
10	3	3.92	0	0	812
12	4	0	2.61	0	990
4	5	+1	+1	-1	925
9	6	-0.62	0	0	408
14	7	0	0	+13	760
11	8	0	-0.41	0	840
8	9	+1	+1	+1	780
7	10	-1	+1	+1	956
18	11	0	0	0	1041.46
13	12	0	0	-0.02	412
19	13	0	0	0	1038
16	14	0	0	0	1039
5	15	-1	-1	+1	689
17	16	0	0	0	855
3	17	-1	+1	-1	492
2	18	+1	-1	-1	740
15	19	0	0	0	1025
20	20	0	0	0	1034

The results of the second order Response Surface Model fitting in the form of analysis of variance (ANOVA) are shown in Table 5. The Model F-value of 6.97 implies the model is significant. There is only a 0.27% chance that, such a large "Model F-Value" could occur due to noise. Values of "Prob > F" less than 0.0500 indicate model terms were significant. The "Lack of Fit F-value" of 3.01 implies the “Lack of Fit” is not significant relative to the pure error. There is a 12.60% chance that a "Lack of Fit F-value" this large could occur due to noise. Non-significant lack of fit is good -- for the model to fit. The "Pred R-Squared" of 0.0825 is close to the "Adj R-Squared" of 0.07388 as one might normally expect, which implies that the overall mean is a better predictor of response. "Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. The ratio of 7.170 in this experiment indicates an adequate signal. This model can be used to navigate the design space.

Table 5: ANOVA for Response Surface Quadratic Model

Source	Sum of Squares	Degree of Fredom	Mean Square	F Value	p-Value Prob > F
Model	6.870E+005	9	76327.79	6.97	0.0027 significant
A-Caster oil	87387.22	1	87387.22	7.98	0.0180
B-Meat extract	23729.26	1	23729.26	2.17	0.1717
C-Ammonium Sulphate	67238.74	1	67238.74	6.14	0.0327
AB	1275.13	1	1275.13	0.12	0.7400
AC	49770.12	1	49770.12	4.55	0.0588
BC	8778.13	1	8778.13	0.80	0.3916
A^2	2.304E+005	1	2.304E+005	21.04	0.0010
B^2	4993.74	1	4993.74	0.46	0.5148
C^2	2.624E+005	1	2.624E+005	23.96	0.0006
Residual	1.095E+005	10	1094	9.52	
Lack of Fit	82180.90	5	1643	6.18	3.010.1260 Not significant
Pure Error	0.32	5	0.064		
Cor Total	**7.964E+00519**				
Std. Dev.-104.64,	R-Squared: 0.8625,	Mean: 812.17,	Adj R-Squared: 0.07388,		
C.V. %: 12.88,	Pred R-Squared: 0.0825,	PRESS: 7.307E+005,	Adeq Precision: 7.170.		

The optimal value of each variable was clearly represented in the three dimension surface plots. Figure 3 shows the function of Castor oil and Meat extract on the Enzyme activity, when variables kept at central point. Maximum lipase activity of 1041.46 U was obtained at the 1.65% Castor oil and 1.10% Meat extract. Further increase or decrease in the concentration of Castor oil and Meat extract led to the decrease in the enzyme production. Figure 4 represents the enzyme activity as a function of Castor oil and Ammonium sulphate (by keeping Meat extract at central point). According to plot, the optimal value lies toward middle range of Castor oil and Ammonium sulphate. The maximum of lipase activity was obtained at 1.65% Castor oil and 0.06% Ammonium sulphate.

Figure 5 represents the combined effect of Meat extract and Ammonium Sulphate on the enzyme activity (by keeping Castor oil at central point). Middle region of graph shows the point of maximum enzyme activity at 1.10% Meat extract and 0.06% Ammonium sulphate. Factorial design and response surface analysis were used by Aniela *et al.* (2008) to optimize lipase production by *Penicillium verrucosum* strain using soybean bran as substrate. Different inductors were evaluated and the results showed that there is no influence of this variable on the lipase production, while temperature and initial moisture were the main factors that affected enzyme production (Aniela *et al*., 2008).

Sibel and Osman (1999) reported that lipase activity and biomass concentration in *R. oryzae* were significantly higher in the presence of olive oil than glucose or lactose used as carbon source. Also *Geotrichum*–like R59 (Basidiomycete) was known to be greatly influenced by sucrose and triolein for lipase production (Gryzyna *et al*., 2007).

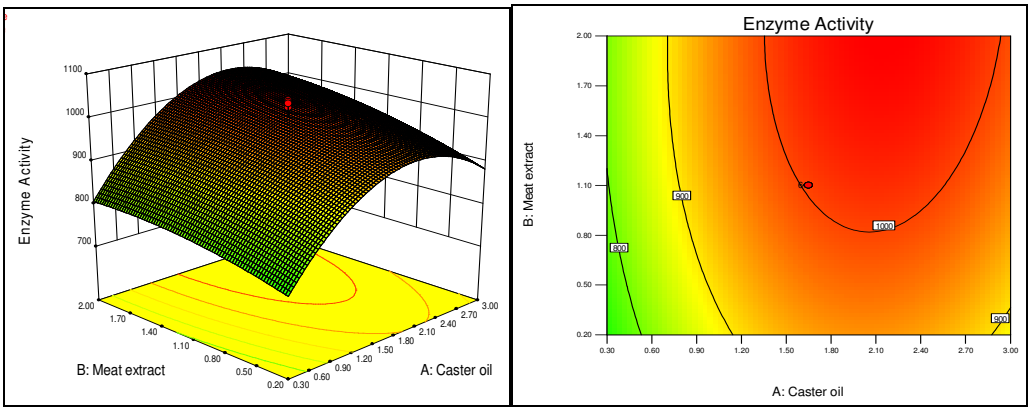

Figure 3: Response Surface and Contour Plot Showing the Effect of Castor Oil and Meat Extract on Enzyme Activity (U) with Other Variable at Zero Level

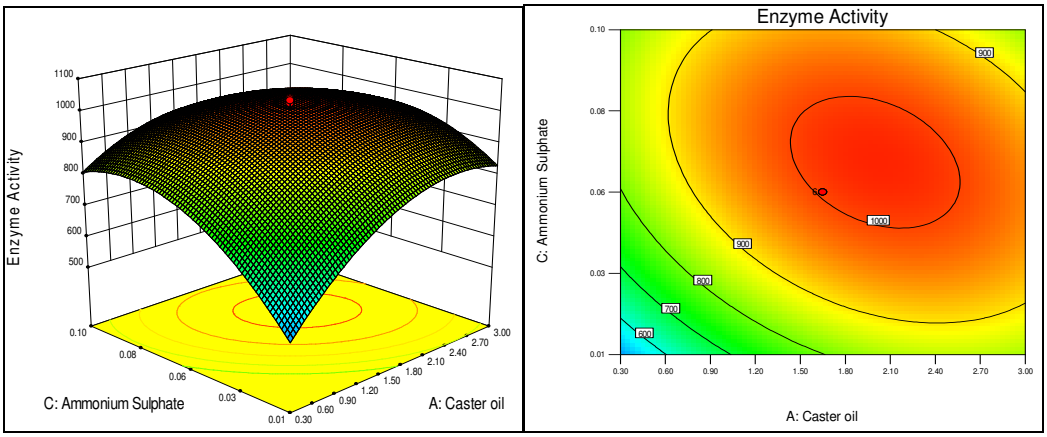

Figure 4: Response Surface & Contour Plot Showing the Effect of Castor Oil and Ammonium Sulphate on Enzyme Activity (U) with Other Variable at Zero Level

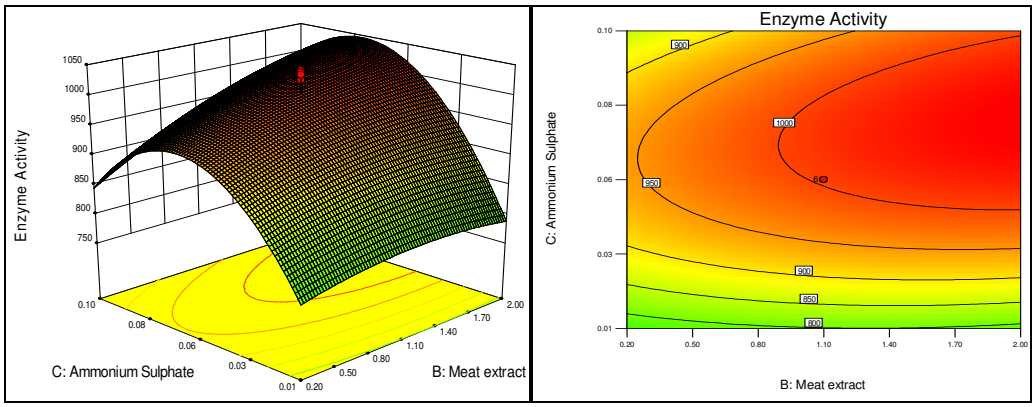

Figure 5: Response Surface and Contour Plot Showing the Effect of Meat Extract and Ammonium Sulphate on Enzyme Activity (U) with Other Variable at Zero Level

CONCLUSIONS

Factorial design and response surface analysis were used to study and optimize the conditions for lipase production by *Saccharomonospora azure* AP 11/18. The optimum conditions as stated by further numerical analysis of the responses using the Design Expert Software revealed that the maximum enzyme activity is **1041.46 U** using **Caster oil**

(1.65g %), **Meat extract (1.10g%)** and **Ammonium sulphate (0.06g%)**. Exploration of Actinomycetal diversity for improved production of lipases using statistical models by employing these approaches for novel bioprocess development makes it as a high-profile area for novel discovery with enormous potential of massive returns.

REFERENCES

1. Abdulaziz, Q. M., A-Sarrani. and Moustafa, Y. M. El-Naggar. (2006). Application of Plackett-Burman factorial design to improve citrinin production in Monascus ruber batch cultures. Botanical Studies, 47, 167-174.
2. Afshin, E., Raja, Noor. Zaliha., Raja, Abd. Rahman., Diana, Hooi. Ean. Ch'ng., Mahiran, Basri. and Abu, Bakar. Salleh. (2008). A modelling study by response surface methodology and artificial neural network on culture parameters optimization for thermostable lipase production from a newly isolated thermophilic Geobacillus sp. strain ARM. BMC Biotechnology, 8, 96.
3. Aniela, P. K., Nadia, L. L., Thais d. L. F. P., Silvana, M., Helen, T., Denise, M. G. F., Marco, D. L. and De´bora, D. O. (2008). Response surface method to optimize the production and characterization of lipase from Penicillium verrucosum in solid-state fermentation. Bioprocess Biosyst Eng, 31, 119-125.
4. Gryzyna, G., Hee–Yeon, C., Nam–Seok, C., Renata, B., Teresa , K.´O., Andrzej, L., Soo–Jeong, S. and Shoji, O. (2007). Effect of Culture Conditions on Growth and Lipase Production by A Newly Isolated Strain, Geotrichum–like R59 (Basidiomycetes). J. Fac. Agr., Kyushu Univ, 52(1), 29-34.
5. Imandi, S. B. and Garapati, H. Rao. (2007). Optimization of process parameters for the production of lipase in submerged fermentation by Yarrowia lipolytica NCIM 3589. Research Journal of Microbiology, 2(1), 88-93.
6. Kumari, A., Mahapatra, P. and Banerjee, R. (2009). Statistical optimization of culture conditions by response surface methodology for synthesis of lipase with Enterobacter aerogenes. Brazilian Archives of Biology and Technology, 52(6), 1349-1356.
7. Manohar, B. and Divakar, S. (2004). Applications of surface plots and statistical design to selected lipase-catalyzed esterification reactions. Process Biochem, 39, 847-853.
8. Patil, K. J., Chopda, M. Z. and Mahajan, R. T. (2011). Lipase biodiversity. Indian Journal of Science and Technology, 4, 971-982.
9. Sarat, B. I., Sita, K. K. and Hanumantha, R. G. (2010). Optimization of media constituents for the production of lipase in solid state fermentation by Yarrowia lipolytica from palm Kernal cake (Elaeis guineensis). Advances in Bioscience and Biotechnology, 1, 115-121.
10. Savitha, J., Srividya, S., Jagat, R., Payal, P., Priyanki, S., Rashmi, G. W., Roshini, K. T. and Shantala, Y. M. (2007). Identification of potential fungal strain(s) for the production of inducible, extracellular and alkalophilic lipase. African Journal of Biotechnology, 6(5), 564-568.
11. Sibel, F. l. and Osman, E. (1999). Lipase production by Rhizopus oryzae growing on different carbon and nitrogen sources. Journal of the Science of Food and Agriculture, 79, 1936-1938.
12. Stanbury, P. F., Whitaker, A. and Hall, S. J. (2003). Principles of Fermentation Technology, Butterworth-Heinemann Publications. Second Edition, ISBN 07506 45016.
13. Wooley, P. and Petersen, S. B. (1994). Lipases: their structure, biochemistry and applications. Cambridge (UK): Cambridge University Press.