International Journal of Biochemistry Research & Review
5(3): 178-186, 2015, Article no.IJBcRR.2015.021
ISSN: 2231-086X

SCIENCEDOMAIN *international*
www.sciencedomain.org

Optimization of Starch and Tryptone Concentrations in Medium Composition for Lipase Production by Submerged Fermentation of *Rhizopus arrhizus* Using Response Surface Methodology

H. Strinska[1*], V. Dobreva[2], B. Zhekova[1] and G. Dobrev[1]

[1]Department of Biochemistry and Molecular Biology, University of Food Technologies, Plovdiv, Bulgaria.
[2]Department of Ecology Engineering, University of Food Technologies, Plovdiv, Bulgaria.

Authors' contributions

This work was carried out in collaboration between all authors. Author GD designed the study, wrote the protocol and supervised the work. Author HS carried out all laboratories work and performed the statistical analysis. Author GD managed the analyses of the study. Authors HS and VD wrote the first draft of the manuscript. Authors HS and BZ managed the literature searches and edited the manuscript. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/IJBcRR/2015/14439
Editor(s):
(1) Carmen Lúcia de Oliveira Petkowicz, Federal University of Parana, Curitiba, Parana, Brazil.
Reviewers:
(1) Anonymous, University of Cincinnati, USA.
(2) Anonymous, University of Murcia, Spain.
Complete Peer review History: http://www.sciencedomain.org/review-history.php?iid=844&id=3&aid=6939

Original Research Article

Received 30th September 2014
Accepted 23rd October 2014
Published 15th November 2014

ABSTRACT

Aims: The aim of this study is optimization of the concentrations of carbon and nitrogen sources for lipase production by *Rhizopus arrhizus* using response surface methodology.
Study Design: For this work optimal 2^2 composite design was used for studying the optimal concentrations of corn starch and tryptone for lipase production by submerged fermentation. A series of planned experiments in three replications was carried out and a mathematical model was developed which was used to describe the process. Optimal levels of studied independent variables were calculated by using the model and the conversion rate.
Place and Duration of Study: This study is a part of PhD dissertation developed in University of

**Corresponding author: E-mail: hstrinska@gmail.com;*

Food Technologies, Bulgaria, Department of Biochemistry and Molecular Biology.
Methodology: Maximum lipase activity was achieved by an optimization of some components of the fermentation medium. Corn starch (in concentrations 5.0, 10.0, 15.0 $g.dm^{-3}$) and tryptone (2.0, 5.0, 8.0 $g.dm^{-3}$) as independent variables were chosen. Lipase activity was determined by a spectrophotometric assay using synthetic substrate p-nitrophenyl palmitate.
Results: A planned mathematical experiment was carried out and a regression model was developed. The value of R^2 was 95.65% which showed that the model had high correlation with the experimental results. The effects of every independent variable had an optimal value while the interaction effects led toenhancement of lipolytical activity. In this case the enzyme activity increased rapidly to 1100 $U.dm^{-3}$. For lipase activity above that value, large enhancements of the corn starch and tryptone concentrations were needed. In order to use the medium substrates properly there the conversion rate was calculated and it was also considered for the optimization.
Conclusion: By carrying out an optimal composite design a mathematical model was derived, with the aid of which, optimum values of tryptone (6.6 $g.dm^{-3}$) and corn starch (10.5 $g.dm^{-3}$) were determined, when the conversion rate and the first derivative of enzyme activity were considered. Those results were confirmed by triplicate experiments at the optimal concentrations. Lipase activity of $\bar{Y}$=1340.74 $U.dm^{-3}$ was achieved, which was very close to the predicted one $\hat{Y}$=1235.26 $U.dm^{-3}$.

Keywords: Rhizopus arrhizus; lipase; submerged fermentation; response surface methodology; optimal composite design.

1. INTRODUCTION

Lipases (E.C. 3.1.1.3) are enzymes which catalyze the hydrolysis of long chain triglycerides into free fatty acids and glycerol at the water-lipid interface and can catalyze the reverse reaction in non-aqueous medium. Those enzymes attract a great attention because of their biotechnological potential. They have a large market share because of their wide application in different industries. That leads to the necessity of developing new production schemes [1].

Lipase production is highly affected by the medium composition and cultivation factors. Carbon and organic nitrogen sources have significant influences on the lipase activity [2].

Carbon source has a crucial role on the lipase production and microorganism growth. Widely used carbon sources are maltose [3,4], sucrose [5], glucose [6] etc. According to many authors lipases are inducible enzymes and need lipid inducers in the medium [3,4]. There are also contradictory data that lipids can inhibit biosynthesis of the enzyme [7,8].

Most of the microbial producers of lipase are saprophytes which determinate the importance of the organic nitrogen source and its concentration on the lipase production [2]. Peptone [3,9], soy bean flour [10], yeast extract [6,11] etc. are widely used in a variety of lipase productions.

The conventional approach of optimization (one-factor-at-a-time) is a series of experiments in which only one factor varies while the others are constant. This method is rarely used because it is laborious and time consuming and it cannot provide the information on interaction effects of the parameters on the desired outcome. These disadvantages can be eliminated by using the response surface methodology (RSM) [12,13]. RSM is a useful statistical technique for optimization of complex processes. It is a collection of mathematical and statistical techniques, widely used in different biotechnological processes to study the effects of several factors influencing on the studied process [14]. Performing a factorial design and regression analysis helps in building models to study interaction and select optimum conditions of variables [15].

The aim of this study is investigation of the interaction effect of the *carbon* and nitrogen sources concentrations on lipase production in submerged fermentation of *Rhizopus arrhizus* by response surface methodology.

2. MATERIALS AND METHODS

2.1 Microorganism Maintenance and Storage

The studied *Rhizopus arrhizus* strain used in this study was provided by Biovet® Peshtera. It was grown in the following medium, $g.dm^{-3}$: malt

extract, 10.0; yeast extract, 4.0; glucose, 4.0; agar-agar 20.0. pH was adjusted to 7.0. The strain was cultivated at 28℃ for 14 days and stored at 4℃.

2.2 Vegetative Inoculum Preparation

0.5 cm^3 spore suspension was added to 100 cm^3 sterilized (at 121℃ for 30 min) medium with pH 7.0 with the following composition ($g.dm^{-3}$): glucose, 30.0; peptone, 20.0; $MgSO_4.7H_2O$, 0.5; KH_2PO_4, 1.0; $NaNO_3$, 1.0; $CaCO_3$, 1.0. The strain was cultivated on a rotary shaker (180 min^{-1}) at 28℃ for 24 h.

2.3 Submerged Cultivation

Submerged cultivation was carried out in 500 cm^3 flasks containing 100 cm^3 medium with composition, ($g.dm^{-3}$): corn starch; tryptone; $NH_4H_2PO_4$, 3.0; $(NH_4)_2C_2O_4$, 0.5; $MgSO_4$, 0.5 and KCl, 0.5. Corn starch and tryptone concentrations were defined according to the mathematical model. pH of the medium was adjusted to 7.0, then the medium was sterilized at 121℃ for 30 min. 5.0 cm^3 vegetative inoculum was used for inoculating each flask and cultivation was carried out at 28℃ for 64 h at a rotary shaker (180 min^{-1}).

2.4 Lipase Assay

Lipase activity was measured by spectrophotometric method using p-nitrophenyl palmitate as substrate buffered with Tris-HCl pH 9.0 [16]. The reaction mixture, containing2.4 cm^3 of 0.8 mM the substrate and 0.1 cm^3 of cell-free culture supernatant, was incubated for 15 min at 35℃. The enzyme reaction was stopped by adding 1.0 cm^3 supersaturated solution of plumbous acetate. After centrifugation the amount of liberated p-nitrophenol was determined by measuring the absorbance at 405 nm. An extinction coefficient at 405 nm of 5105.3 $M^{-1}.cm^{-1}$ was used for p-nitrophenol at pH 9.0. One unit of enzyme activity was defined as the amount of enzyme that released one µmol of p-nitrophenol per minute under the assay conditions described.

2.5 Response Surface Methodology

Optimal composite design 2^2 was used to find the optimal concentration of corn starch and tryptone for lipase biosynthesis by *Rhizopus arrhizus*. Independent variables participating in the design and their values are presented in Table 1. According to the obtained results a regression analysis was accomplished and a quadratic regression model was expressed as follows (eq. 1):

$$\hat{Y} = b_0 + \sum_{i=1}^{m} b_i . x_i + \sum_{i=1, j=i+1}^{m} b_{ij} . x_i . x_j + \sum_{i=1}^{m} b_{ii} . x_i^2 \quad (1)$$

Where $\hat{Y}$ is the response variable, b_0, b_j, b_{ij}, b_{ii} – the regression coefficients of the model, and x_i and x_j – coded levels of the independent variables [3].

3. RESULTS AND DISCUSSION

The most appropriate sources of carbon and organic nitrogen for lipase production in submerged fermentation by the studied strain *Rhizopus arrhizus* were corn starch and casein trypsic peptone (results from our previous research). Some authors also achieved similar results. Immanuel et al. [17] used 4.0 $g.dm^{-3}$ starch for lipase production by *Serratia rubidaea*; Pogaku at al. [18] achieved maximum activity using 15.0 $g.dm^{-3}$ starch for fermentation of *Staphylococcus* sp. Lp12 and Bisht at al. [19] – a combination of 20.0 $g.dm^{-3}$ starch and 2.0 $g.dm^{-3}$ castor oil. Tryptone is also widely used component of fermentation medium. Le et al. [20] used 24.2 $g.dm^{-3}$ tryptone for lipase production by *Burkholderia* sp.

As those components can be expensive it was important to study the efficiency of the process when the optimal levels were chosen.

Table 1. Values of independent variables at different levels of the optimal composite design 2^2

Independent variables	Levels		
	-1	0	1
X_1 – Corn starch concentration, $g.dm^{-3}$	5.0	10.0	15.0
X_2 – Tryptone concentration, $g.dm^{-3}$	2.0	5.0	8.0

In order to determine the optimal value of the corn starch and tryptone concentrations; taking into accounts the interaction effects an optimal composite design 2^2 was performed. The optimal composite design 2^2 and the results for lipase activity are shown in Table 2.

The results from the regression analysis are presented in Table 3. The ANOVA table partitions the variability in the composite plan into separate pieces for each of the effects and then tests the statistical significance of each effect. In this case, three effects had P-values less than 0.05 – X_1, X_2 and X_1. X_2, indicating that they were significantly different from zero at the 95.0 % confidence level.

Standardized Pareto Chart (Fig. 1) is a histogram with the effects plotted in decreasing order of significance. The line passing through the chart depends on the value of alpha and any factor with significance will extend beyond the line. As seen from the chart the most significant effect was the tryptone concentration followed by the corn starch concentration. The significance of the X_1. X_2 revealed that the interaction effect has an impact on the lipase activity.

Table 2. Optimal composite design 2^2

Run number	Coded levels		Lipase activity, U.dm^{-3}	
	X_1	X_2	$\bar{Y}$	$\hat{Y}$
1	1	1	1397.49	1436.31
2	-1	1	210.67	310.48
3	1	-1	232.07	116.54
4	-1	-1	263.40	208.86
5	1	0	1005.41	1082.12
6	-1	0	610.63	565.37
7	0	1	1283.93	1145.30
8	0	-1	264.52	434.60
9	0	0	1127.09	1095.65

results are a mean value of three replications

Table 3. Regression analysis results

Effect	Coefficient	P-Value
Average	1095.65	
X_1	258.38	0.0328
X_2	355.35	0.0140
$X_1.X_2$	304.53	0.0362
X_1^2	-271.91	0.1059
X_2^2	-305.70	0.0823

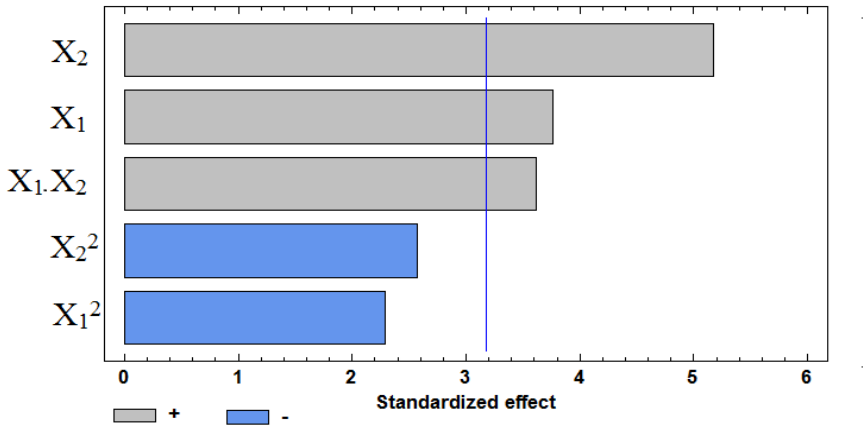

Fig. 1. Standardized Pareto chart for optimization of starch and tryptone concentrations

As a result of the optimal composite design the following mathematical model (eq. 2) was developed:

$$\hat{Y} = 1095.65 + 258.38.X_1 + 355.35.X_2 + 304.53.X_1.X_2 - 271.91.X_1^2 + 305.70.X_2^2 \quad (2)$$

The R-Squared statistic indicates that the model as fitted explains 95.65 % of the variability in this model. The standard error of the estimate showed the standard deviation of the residuals to be 168.2. The mean absolute error (MAE) of 85.6 is the average value of the residuals. In this case Durbin-Watson statistic is 2.8 which showed that the model has negative correlation.

The model was studied and maximum activity of 1436.3 U.dm^{-3} was predicted when using concentrations of the components corresponding to coded values of the levels: X_1=1 and X_2=1.

Fig. 2 reveals the influence of each one independent variable on lipase production by *Rhizopus arrhizus* when the interaction effects were ignored. As shown every variable had an optimal value for its concentration with maximum lipase activity. The presence of an optimum in the graph was a proof that the chosen concentrations were appropriate for planned experiments.

The interaction effect of starch and tryptone concentration is shown at Fig. 3. The chart reveals that simultaneously increasing of corn starch and tryptone concentrations led to enhancement of lipolytical activity. It can also be noticed that the enzyme activity increased rapidly until 1100 U.dm^{-3}. For lipase activity above that value, large enhancements of the corn starch and tryptone concentrations were needed.

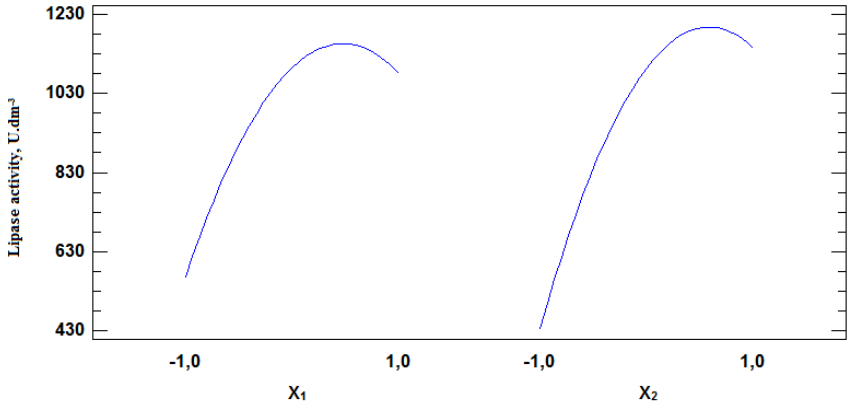

Fig. 2. Main effects plot

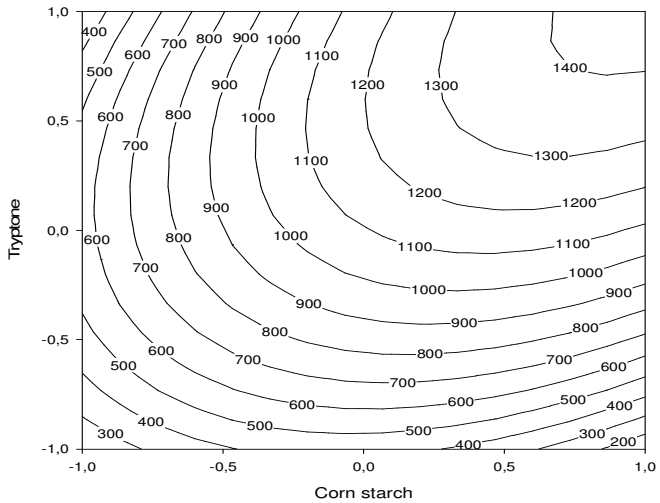

Fig. 3. Response contour plot indicating interaction effects of starch and tryptone concentrations on lipase biosynthesis

As the medium components may be expensive and in order of their properly usage the efficiency of the process was taken in consideration and the conversion rate and the first derivative of lipase activity were calculated.

The conversion rate is a characteristic of the efficiency of the process which means the quantity of fermentation product (lipase) produced by 1.0 g medium substrate. First derivative measures the rate of change of an independent variable as a function of a dependent variable which means the lipase activity enhancement per gram substrate in the fermentation medium (Fig. 4. and Fig. 5.).

Fig. 4. shows the relation of lipase activity, conversion rate, first derivative of the enzyme activity and the corn starch concentration while the tryptone concentration was fixed at codded value 1.0 (8.0 $g.dm^{-3}$) which was found to be the optimal concentration according to the mathematical model. As seen from the chart the increase of enzyme activity was accompanied with decreasing of the efficiency. That was the reason for studying the conversion rate and the first derivative of enzyme activity when the optimal starch concentration was chosen. Increasing starch concentration in fermentation medium led to increasing of conversion rate until it reached 115 $U.g^{-1}$ at 10 $g.dm^{-3}$ corn starch. When higher concentrations of the carbon source were used, the conversion rate decreased. Also with increasing of corn starch concentration in the medium, the first derivative of enzyme activity (the rate of lipase activity change) decreased from 214 to 10 $U.g^{-1}$. The concentration (10.5 $g.dm^{-3}$) which led to first derivative = 100 $U.g^{-1}$ was chosen as optimal value, as it led to high activity and conversion rate. When the first derivative was lower than 100 $U.g^{-1}$, the efficiency decreased which was proven by the decreasing of conversion rate.

The same characteristics were calculated for tryptone concentration as well but the corn starch concentration was fixed at 10.5 $g.dm^{-3}$. This value was chosen after studying the effect of corn starch on the conversion rate and the first derivative of lipase activity. In this case the maximum conversion rate was achieved at 3.5 $g.dm^{-3}$ tryptone but this concentration corresponded to low activity (848.99 $U.dm^{-3}$). As tryptone concentration was more significant (Fig. 1) the first derivative of enzyme activity had higher values (from 414 to 25 $U.g^{-1}$). Optimal tryptone concentration (6.6 $g.dm^{-3}$) was chosen, in which the first derivative of enzyme activity was 100 $U.g^{-1}$, lipase activity was high (1235.26 $U.dm^{-3}$) and the conversion rate was relatively high.

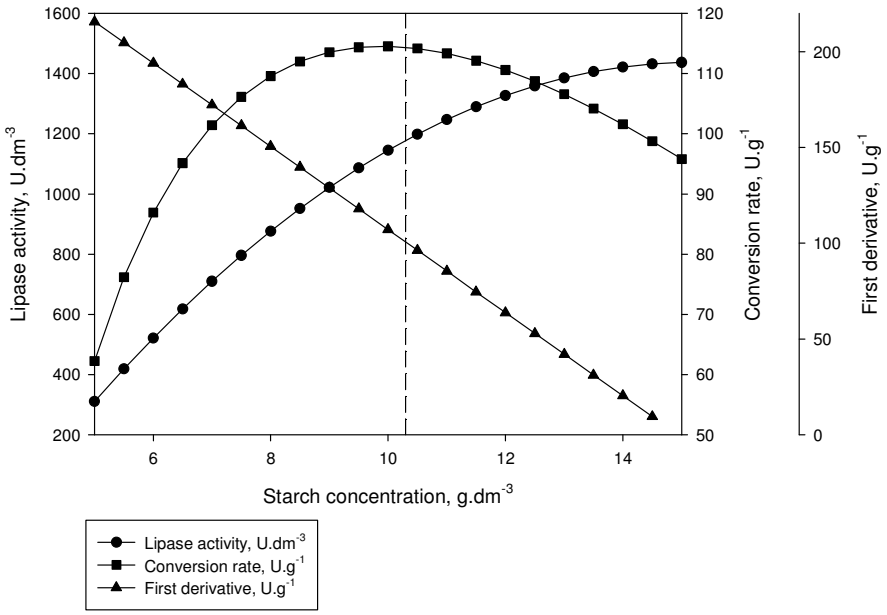

Fig. 4. Effect of corn starch concentration on lipase activity, conversion rate and first derivative of lipase activity

According to the model, maximum activity (1436.3 U.dm^{-3}) was achieved by using concentrations of the components corresponding to coded values of the levels: X_1=1 and X_2=1. When the interaction effects were studied it was noted that when the concentrations of the components reached defined values above which there was no significant enhancement in the lipase activity. This was the reason the conversion rate and first derivative to be taken in consideration when the optimal values of the independent variables were chosen.

When the optimal values were established, a series of three experiments were carried out to confirm the results from the planned experiments. Submerged fermentation for lipase production by *Rhizopus arrhizus* with the chosen concentrations (10.5 g.dm^{-3} for corn starch and 6.6 g.dm^{-3} for tryptone) was prepared and average lipase activity Ȳ=1340.74 U.dm^{-3} was reached. A comparison between *Rhizopus arrhizus* lipase and different *Rhizopus* lipases produced by submerged fermentation is presented on Table 4.

As seen from the table the lipase activity reached in this study is higher than the enzyme activities reached by Prabhakar et al. [21] and comparable with the one achieved by Açikel et al. [5] while Rajendran et al. [6] and Li et al. [10] achieved higher lipase activity as absolute values. However such a comparison cannot be conclusive because of the differences in methods, assay conditions and substrates.

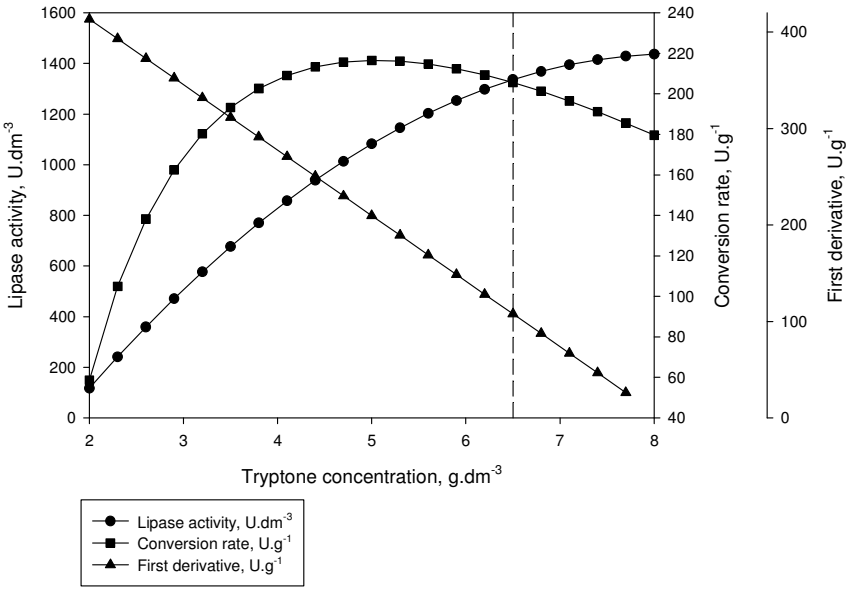

Fig. 5. Effect of tryptone concentration on lipase activity, conversion rate and first derivative of lipase activity

Table 4. Comparison between lipase from *Rhizopus arrhizus* used in this study and different Rhizopus lipases from literature

Strain	Substrate for lipase assay	Assay	Lipase activity	References
R. arrhizus	p-nitrophenyl palmitate	Spectrophotometric	1 340.74 U.dm^{-3}	Present study
R. arrhizus MTCC 2233	Olive oil	Titrimetric	3.98 U.cm^{-3}	[6]
R. arrhizus BUCT	Olive oil	Titrimetric	260 U.cm^{-3}	[10]
R. delemar	p-nitrophenyl palmitate	Spectrophotometric	1 585 µmol.dm^{-3}.min^{-1}	[5]
Rhizopus sp.	p-nitrophenyl palmitate	Spectrophotometric	0.155 U.cm^{-3}.min^{-1} 0.128 U.cm^{-3}.min^{-1}	[21]

4. CONCLUSION

As a result of the study we can conclude that we have chosen appropriate levels for independent variables which are very important for every optimization process. The mathematical model inferred describes the process properly and we were able to establish the optimum concentrations for carbon and organic nitrogen sources – 10.5 $g.dm^{-3}$ for corn starch and 6.6 $g.dm^{-3}$ for tryptone, taking in consideration the conversion rate. Those values were not the optimal according to the regression model but they resulted in activity which was very close to the highest one. The results were confirmed by triplicate experiments at the optimal concentrations. A lipase activity $\bar{Y}$=1340.74 $U.dm^{-3}$ was achieved, which was very close to the predicted one $\hat{Y}$=1235.26 $U.dm^{-3}$.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Hasan F, Shah A, Hameed A. Industrial application of microbial lipases. Enzyme and Microbial Technology. 2006;39:235-251.
2. Ghosh PR, Saxena R, Gupta RP, Davidson S. Microbial lipases: production and applications. Sci Prog. 1996;79(2):119-157.
3. Wang D, Xu Y, Shan T. Effects of oils and oil-related substrates on the synthetic activity of membrane-bound lipase from *Rhizopus chinensis* and optimization of the lipase fermentation media. Biochemical Engineering Journal. 2008;41:30–37.
4. Teng Y, Xu Y. Culture condition improvement for whole-cell lipase production in submerged fermentation by *Rhizopus chinensis* using statistical method. Bioresource Technology. 2008;99:3900-3907.
5. Açikel Ü, Erşana M, Sağ Açikel Y. Optimization of critical medium components using response surface methodology for lipase production by *Rhizopus delemar*. Food and Bioproducts Processing. 2010;88:31-39.
6. Rajendran A, Palanisamy A, Thangavelu V. Lipase catalyzed ester synthesis for food processing industries. Brazilian Archives of Biology and Technology. 2009;52:207-219.
7. Eitenmiller RR, Vakil JR, Shahani M. Production and properties of *Penicillium roqueforti* lipase. Journal of Food Science. 1970;35(2):130-133.
8. Chander H, Sannabhadti SS, Elias J, Ranganathan B. Factors affecting lipase production by *Penicillium chrysogenum*. Journal of Food Science. 1977;42(6):1677-1679.
9. Alford JA, Pierce DA, Suggs FG. Activity of microbial lipases on natural fats and synthetic triglycerides. J Lipid Res. 1964;5(3):390–394.
10. Li D, Wang B, Tan T. Production enhancement of *Rhizopus arrhizus* lipase by feeding oleic acid. Journal of Molecular Catalysis B: Enzymatic. 2006;43:40–43.
11. Rodriguez JA, Mateos JC, Nungaray J, González V, Bhagnagar T, Roussos S et al. Improving lipase production by nutrient source modification using *Rhizopus homothallicus* cultured in solid state fermentation. Process Biochemistry. 2006;41:2264–2269.
12. He YO, Tan TW. Use of response surface methodology to optimize culture medium for production of lipase with *Candida sp*. 99-125. Journal of Molecular Catalysis B: Enzymatic. 2006;43:1-14.
13. Kishan G, Gopalakannana P, Muthukumaranb C, Thirumalai Muthukumaresanc K, Dharmendira Kumard M, Tamilarasan K. Statistical optimization of critical medium components for lipase production from *Yarrowia lipolytica* (MTCC 35). Journal of Genetic Engineering and Biotechnology. 2013;11:111-116.
14. Papagora C, Roukas T, Kotzekidou P. Optimization of extracellular lipase production by *Debaryomyces hansenii* isolates from dry-salted olives using response surface methodology. Food and Bioproducts Processing. 2013;91:413-420.
15. Adinarayana K, Raju K, Zargar M, Devi R, Ellaiah P. Optimization of process parameters for production of lipase in solid-state fermentation by newly isolated *Aspergillus species*. Indian J. Biotechnol. 2004;3:65-69.
16. Kaushik R, Saurabh S, Isar J, Saxena R. Statistical optimization of medium components and grouth conditions by response surface methodology to enhance lipase production by *Aspergillus carneus*.

J. Mol. Cat. B: Enzymatic. 2006;40(3-4):121-126.
17. Immanuel G, Esakkiraj P, Jebadhas A, Iyapparaj P, Palavesam A. Investigation of Lipase Production by Milk Isolate *Serratia rubidaea*. Food Technol Biotechnol. 2008;46(1):60–65.
18. Pogaku P, Suresh A, Srinivas P, Ram Reddy S. Optimization of lipase production by *Staphylococcus* sp. Lp12. African Journal of Biotechnology. 2010;9(6):882-886.
19. Bisht D, Yadav S K, Darmwal N S. Enhanced production of extracellular alkaline lipase by an improved strain of *Pseudomonas aeruginosa* MTCC. American Journal of Applied Sciences. 2012;9(2):158-167.
20. Lo C F, Yu C Y, Kuan I C, Lee S L. Optimization of lipase production by *Burkholderia* sp. using response surface methodology. Int. J. Mol. Sci. 2012;13:14889-14897.
21. Prabhakar T, Bhogavalli P K, Vallem P R, Srirangam V. Studies on optimization of extracellular lipase from potential fungal strain(s) isolated from oil contaminated soil. Journal of Microbiology & Biotechnology Research. 2012;2(3):418-425.

Peer-review history:
The peer review history for this paper can be accessed here:
http://www.sciencedomain.org/review-history.php?iid=844&id=3&aid=6939