Annuaire de l'Université de Sofia "St. Kliment Ohridski"
Faculte de Biologie
2015, volume 100, livre 4, pp. 32-40
First National Conference of Biotechnology, Sofia 2014

RESPONSE SURFACE METHODOLOGY OPTIMIZATION OF PROCESS PARAMETERS FOR LIPASE PRODUCTION BY *RHIZOPUS ARRHIZUS*

HRISTINA STRINSKA[1*], BORIANA ZHEKOVA[1],
VALENTINA DOBREVA[2], GEORGI DOBREV[1]

1 – Department of Biochemistry and Molecular Biology, Technological Faculty, University of Food Technologies, Plovdiv, Bulgaria
2 – Engineering Ecology, Faculty of Economics, University of Food Technologies, Plovdiv, Bulgaria
**Corresponding author: hstrinska@gmail.com*

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

Abstract: The initial pH of the nutrient medium and the temperature of cultivation had a major impact on lipase biosynthesis by *Rhizopus arrhizus*.

The optimal values of the factors were defined by using response surface methodology. Planned mathematical design was carried out and regression model was developed. It was used for prediction of the optimal values of independent variables – 30°C for cultivation temperature and 7.0 for initial pH of fermentation medium. The maximum predicted activity from the design was 4749.91 U. dm^{-3}. The mathematical model was characterized with high value of the coefficient of determination R^2=95. 75%, which showed high correlation with the experimental results.

Three consecutive experiments at the predicted optimal values of the factors were carried out. A mean value of 4658.45 U.dm^{-3} for lipase activity was achieved, which experimentally confirmed the results obtained from the mathematical model.

INTRODUCTION

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) are important industrial enzymes due to their versatile application (Salihu, 2012). Lipases are enzymes from class α/β-hydrolase which catalyse the hydrolysis or the formation of lipids (Svendsen, 2000).

The recent interest in the production of lipases is associated with their applications as additives in food (flavour modification), fine chemicals (ester

synthesis), detergents (hydrolysis of fats), waste water treatment (decomposing and removal of oily contaminations), cosmetics (removal of lipids), pharmaceuticals, leather processing and biochemical assays (Salihu et al., 2012).

Their multiple applications are the reason for the increased interest on their production. Most of the commercially available lipases are from microbial origin because of their ease production, higher temperature and pH stability, wide substrate specificity and organic solvent resistance. There is a great interest to the fungal *Rhizopus* lipases because of their high 1,3-specificity. (Haq et al., 2002; Hasan et al., 2006).

Optimization of the culture condition is of a great importance for lipases biosynthesis. Usually the fermentation parameters such as pH and temperature are dependent on the producer strain requirements (Maldonado et al., 2012; Ghosh et al., 1996).

The lipase biosynthesis is highly dependent on the initial pH of the fermentation medium. The optimal pH value for maximum yield is different for every strain. Development of acidity in the presence of fermentable carbohydrates usually leads to lipase activity reduction (Ghosh et al., 1996). Most of the microbial producers have a pH optimum between 6.0 and 8.0 but there are strains which need alkali medium – pH 10.0 for lipase production by *Bacillus alcalophilus* (Gupta et al., 2004) or acidic – pH 4.0 for *Penicillium* P58 (Rigo et al., 2010).

The cultivation temperature is of a great importance and it is specific for every producer as well. The optimal temperature for lipase production is between 20 and 45°C. However, it corresponds with the growth temperature of the respective microorganism (Ghosh et al., 1996).

Response surface methodology (RSM) is a useful tool 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. Performing a factorial design and regression analysis helps in building models to study interactions and select optimum conditions of variables (Papagora et al., 2013; Liu et al., 2006). Khoramnia et al. (2010) increased lipase production 3.5 fold using central composite design for the parameters of lipase production (temperature, pH, inoculum size and agitation. Sharma et al. (2009) used central composite design for fermentation temperature, initial pH of the medium and incubation period. As a result of the mathematical model they achieved 1.6 fold increase in the lipase activity from *Arthrobacter* sp. BGCC#490 at the optimal conditions (T 40°C, pH 10.0 and incubation time 48 h).

The aim of this study is optimization of the cultivation temperature and initial pH of the fermentation medium for lipase production by *Rhizopus arrhizus* using response surface methodology.

MATERIALS AND METHODS

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°C for 14 days and stored at 4°C.

Vegetative inoculum preparation

0.5 cm^3 spore suspension (2×10^8 spores.cm^{-3}) was added to 100 cm^3 sterilized (at 121°C 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°C for 24 h.

Submerged cultivation

Submerged cultivation was carried out in 500 cm^3 flasks containing 100 cm^3 medium with composition, ($g.cm^{-3}$): corn starch 10.5; tryptone 6.6; $NH_4H_2PO_4$ 6.5; $(NH_4)_2C_2O_4$ 0.90; $MgSO_4$ 0.95 and KCl 0.95. The medium was sterilized at 121°C for 30 min. The temperature and pH were defined according to the planned experiments (Table 1.). 5.0 cm^3 vegetative inoculum was used for inoculating of each flask and cultivation was carried out for 64 h at a rotary shaker (180 min^{-1}).

Response surface methodology

Optimal composite design 2^2 was used to find the optimal process parameters 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:

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

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 (Wang et al., 2008).

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

Independent variables	Levels		
	-1	0	1
X_1 – T, °C	26	30	34
X_2 – pH	4.0	6.5	9.0

Lipase assay

Lipase activity was measured by spectrophotometric method using p-nitrophenyl palmitate as substrate buffered with Tris-HCl pH 9.0 (Kaushik et al., 2006). The reaction mixture, containing 2.4 cm^{-3} of 0.8 mM substrate and 0.1 cm^{-3} of cell-free culture supernatant, was incubated for 15 min at 35°C. The enzyme reaction was stopped by adding 1.0 cm^3 saturated solution of plumbeous acetate. After centrifugation absorbance was measured at 405 nm. 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.

RESULTS AND DISCUSSION

The influence of temperature on lipase production was investigated (Fig. 1.).

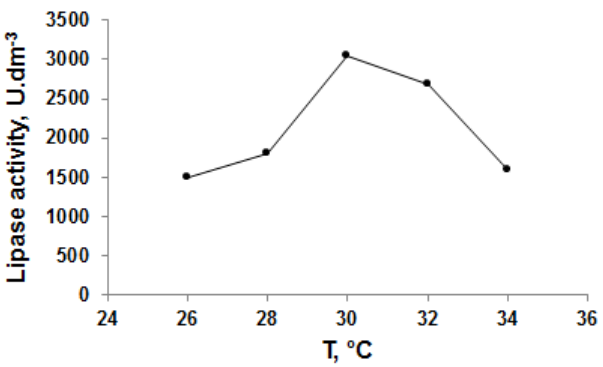

Figure 1. Influence of cultivation temperature on lipase production.

The cultivation temperature had a great impact on the biosynthesis of lipase. As seen from the chart maximum lipase activity was achieved at 30°C. Similar results were found by Rajendran and Thangavelu (2009) when using *Rhizopus arrhizus* MTCC 2233 for lipase production. The fermentation temperature was different for every producer strain and it must be experimentally discovered. According to the literature the optimal temperature varied in a wide range – between 15 and 70°C (Gupta 2004).

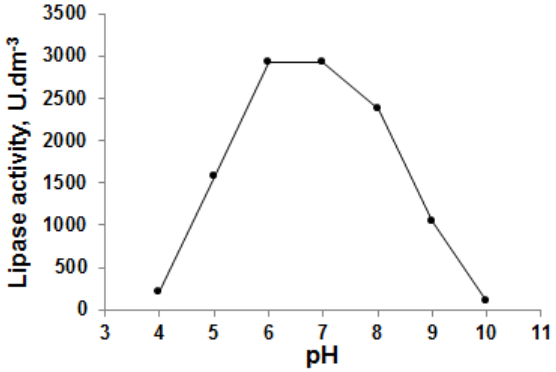

Figure 2. Influence of initial pH on lipase production.

The pH value of initial medium had also a great influence on the enzyme biosynthesis (Fig. 2.).

Significant lypolytic activity was measured in the range of pH 6.0-8.0 but enzyme activity was also noticed in pH 5.0 and 9.0. Fermentation medium with initial pH 4.0 and 10.0 was not appropriate for lipase production.

In order to determine the optimal values of the initial pH of the nutrient medium and the fermentation temperature, taking into consideration their interaction effects, an optimal composite design 2^2 was performed (Table 2).

Table 2. Experimental data and results of the optimal composite design 2^2.

Run Number	Codded level		Lipase activity, U.dm^{-3}	
	X_1	X_2	Y^a	$\hat{Y}$
1	1	1	2118.35	2492.29
2	1	-1	251.45	434.78
3	-1	1	2378.33	2392.60
4	-1	-1	67.48	-108.88
5	1	0	3973.69	3416.42
6	-1	0	2932.65	3094.75
7	0	1	4155.57	3767.36
8	0	-1	1494.84	1487.87
9	0	0	4185.32	4580.50

X_1 – cultivation temperature;
X_2 – initial pH of the nutrient medium;
[a] results are a mean value of three replications

The results from the regression analysis are presented in Table 3.

Table 3. Regression analysis results.

Effect	*Coefficient*	P-value
constant	4580.50	
X_1	160.84	0.5125
X_2	**1139.75**	**0.0135**
$X_1.X_2$	**-110.99**	**0.0388**
X_1^2	-1324.92	0.7045
X_2^2	**-1952.88**	**0.0139**

The ANOVA analysis partitions the variability in the composite plan into separate pieces for each of the effects and then tests the statistical significance of each effect (Table 3.). In this case, three effects had $P<0.05$ – X_2, $X_1.X_2$ and X_2^2, indicating that they were significantly different from zero at the 95.0 % confidence level.

Standardized Pareto Chart (Fig. 3.) is a histogram with the effects plotted in decreasing order of their 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 effects were pH and the squared value of pH, while the temperature and the interaction effect were non-significant factors.

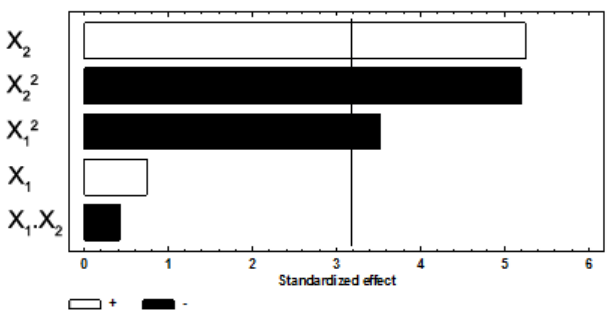

Figure 3. Standardized Pareto Chart.

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

$$\hat{Y} = 4580.50 + 1139.75.X_2 - 1324.92.X_1^2 - 1952.88.X_2^2 \qquad (2)$$

The R-Squared statistic indicates that the model as fitted explains 95.75 % of the variability in this model. The standard error of the estimate showed the standard deviation of the residuals to be 531.82. The mean absolute error (MAE) of 250.85 is the average value of the residuals. In this case Durbin-Watson statistics are 0.39 which showed that the model had positive correlation.

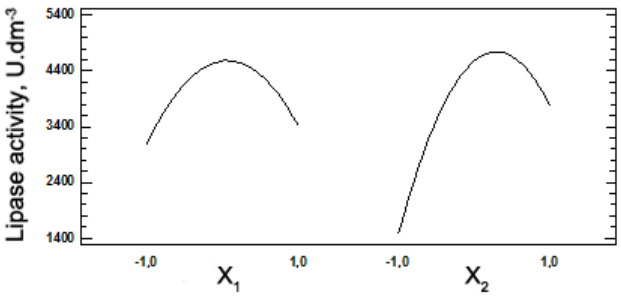

Figure 4. Main Effects Plot (X_1 – T, °C; X_2 – pH).

Fig. 4 reveals the influence of each independent variable on lipase production by *Rhizopus arrhizus* when the interaction effects were ignored. As shown every variable had an optimal value with maximum lipase activity. The presence of an optimum in the graph was a proof that the chosen levels were appropriate for planned experiments.

The interaction effect of cultivation temperature and initial pH of the fermentation medium is shown in Fig. 5.

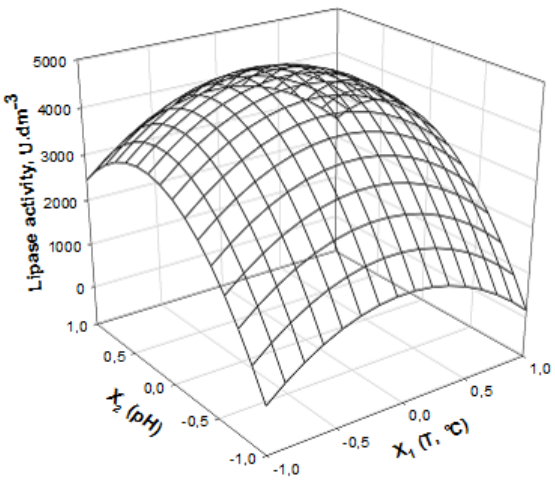

Figure 5. Response surface plot indicating interaction effects of cultivation temperature and initial pH of the fermentation medium on lipase biosynthesis.

As seen from the plot the pH optimum was the same in every studied temperature. The reason was that the interaction effect was not significant as seen from Fig. 3 and Table 3.

The regression equation (2) was studied and maximum predicted lipase activity was found to be 4749.91 $U.dm^{-3}$. This activity was achieved at the following code values of the factors: X_1 (T, °C) 0.05 and X_2 (pH) 0.29. After decoding of the levels of the factors, their optimal values were determined –

cultivation temperature 30.1°C and initial pH of the fermentation medium pH 7.2 but for easier application in industrial lipase production we choose T 30°C pH 7.0 for optimum.

When the optimal values were established, a series of three experiments were carried out to confirm the results of the planned experiments. Submerged fermentation for lipase production by *Rhizopus arrhizus* with the chosen parameters (30°C and pH 7.0) was prepared and average lipase activity $\bar{Y}$=4658. 45 U. dm^{-3} was achieved, which was close to the predicted one $\hat{Y}$=4749. 91 U. dm^{-3}.

CONCLUSION

The initial pH of the nutrient medium and the temperature of cultivation had a major impact on lipase biosynthesis by *Rhizopus arrhizus*. The optimal values of the factors were defined by using response surface methodology. Planned mathematical design was carried out and regression model was developed. The mathematical model was characterized with high value of the coefficient of determination R^2=95. 75%, which showed high correlation with the experimental results. The optimal values of independent variables were found to be 30°C for cultivation temperature and 7.0 for initial pH of fermentation medium. The maximum predicted activity from the design was 4749.91 $U.dm^{-3}$. Three consecutive experiments at the predicted optimal values of the factors were carried out. A mean value of 4658.45 $U.dm^{-3}$ for lipase activity was achieved, which experimentally confirmed the results obtained from the mathematical model.

Acknowledgements: The authors are grateful to Biovet® Peshtera for providing us the opportunity to work with the studied strain *Rhizopus arrhizus*.

REFERENCES

1. Ghosh, P.R., Saxena, R., Gupta, R.P., Davidson, S. 1996. Microbial lipases: production and applications. *Sci Prog*, 79(2): 119-157.
2. Gupta, R., Gupta, N., Rathi, P. 2004. Bacterial lipases: an overview of production, purification and biochemical properties. *Appl Microbiol Biotechnol*, 64: 763–781.
3. Hasan, F., Shah, A. A., Hameed, A. 2006. Industrial applications of microbial lipases. *Enzyme and Microbial Technology* 39: 235-251.
4. Haq, I., Idreesa, S., Rajokab, M. I. 2002. Production of lipases by *Rhizopus oligosporous* by solid-state fermentation. *Proc Bioch* 37(6): 637-641.
5. Kaushik, R., Saurabh, S., Isar, J., Saxena, R. 2006. Statistical optimization of medium components and growth conditions by response surface methodology to enhance lipase production by *Aspergillus carneus*. *J. Mol. Catal. B: Enzym.*, 40: 121–126.

6. Khoramnia, A., Lai, O.M., Ebrahimpour, A., Tanduba, C.J., Voon, T.S., Mukhlis, S. 2010. Thermostable lipase from a newly isolated Staphylococcus xylosus strain; process optimization and characterization using RSM and ANN. *Electronic Journal of Biotechnology*, 13(5).
7. Liu, C.H., Lu, W.B., Chang, J.S. 2006. Optimizing lipase production of *Burkholderia* sp. by response surface methodology. *Process Biochemistry*, 41: 1940–1944.
8. Maldonado, R.R., Medeiros Burkert, J.F., Mazutti, M.A., Maugeri, F., Rodrigues, M.I. 2012. Evaluation of lipase production by *Geotrichum candidum* in shaken flasks and bench-scale stirred bioreactor using different impellers. *Biocatalysis and Agricultural Biotechnology*, 1: 147–151.
9. Papagora, C., Roukas, T., Kotzekidou, P. 2013. Optimization of extracellular lipase production by *Debaryomyces hansenii* isolates from dry-salted olives using response surface methodology. *Food and Bioproducts Processing*, 91: 413-420.
10. Rajendran, A., Thangavelu, V. 2009. Statistical experimental design for evaluation of medium components for lipase production by *Rhizopus arrhizus* MTCC 2233. *LWT - Food Science and Technology*, 42: 985-992.
11. Rigo, E., Ninow, J.L., Luccio, M.D., Oliveira, J.V., Polloni, A.E., Remonatto, D., Vardanega, R., Oliveira, D., Treichel, T. 2010. Lipase production by solid fermentation of soybean meal with different supplements. *LWT - Food Science and Technology*, 43: 1132-1137.
12. Salihu, A., Zahangir Alam, M., Ismail Abdul Karim, M., Salleh, H.M. 2012. Lipase production: An insight in the utilization of renewable agriculture residuals. *Resourses, Conservation and Recycling*, 58:36-44.
13. Sharma, A., Bardhan, D., Patel, R. 2009. Optimization of physical parameters for lipase production from *Arthrobacter sp.* BGCC#490. *Indian Journal of Biochemistry & Biophysics*, 46:178-183.
14. Svendsen, A. 2000. Lipase protein engineering. *Biochimica et Biophysica Acta*, 1543: 223-238.
15. Wang, D., Xu, Y., Shan, T. 2008. 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*, 41: 30-37.