World Journal of Pharmaceutical and Life Sciences
WJPLS
www.wjpls.org
SJIF Impact Factor: 6.129

STATISTICAL OPTIMIZATION OF LIPASE PRODUCTION USING USED COOKING OIL (UCO) AS A CHEAP CARBON SOURCE FROM *PSEUDOMONAS AERUGINOSA* SJ2

Joshi Swapnil Satish*

Department of Microbiology S.S.V.P. Sanstha's Late Karmaveer Dr. P R Ghogrey Science College, Dhule-424005.

***Corresponding Author: Joshi Swapnil Satish**
Department of Microbiology S.S.V.P. Sanstha's Late Karmaveer Dr. P R Ghogrey Science College, Dhule-424005.
DOI: https://doi.org/10.56716/4/4235

Article Received on 21/09/2023 **Article Revised on 11/10/2023** **Article Accepted on 01/11/2023**

ABSTRACT

Pseudomonas aeruginosa SJ2 isolated and characterized in previous studies was used for lipase production using used cooking oil (UCO) as a cheap carbon source. Before optimization *Pseudomonas aeruginosa* SJ2 was able to produce 300 ± 4 U/mL of lipase in production medium consisting of (g/L; yeast extract (2.0), UCO (40.0 mL/L), NaCl (2.0), $MgSO_4$ (0.4), $(NH_4)_2SO_4$ (0.5), K_2HPO_4 (0.3), and KH_2PO_4 (0.3), pH 8.0 ± 0.2), glucose (0.5%), triton-X 100 (0.05%), temperature 30 °C, incubation time 48 h). Statistical optimization using central composite design (CCD) resulted in production of 329 ± 3 U/mL of lipase under optimized conditions viz. (g/L; yeast extract (2.33), UCO (49.0 mL/L), NaCl (2.0), $MgSO_4$ (0.4), $(NH_4)_2SO_4$ (0.5), K_2HPO_4 (0.3), and KH_2PO_4 (0.3), pH 8.0 ± 0.2), glucose (0.5%), triton-X 100 (0.05%), temperature 32 °C, incubation time 48 h). Scale-up studies were performed in a 3.0 L bench top fermenter (Systronics India Ltd., Ahmedabad) with 1.5 L working volume where lipase production dropped marginally to 321 ± 4 U/mL. Overall optimization resulted in 1.1-fold increase in lipase production by *Pseudomonas aeruginosa* SJ2 using cost effective medium.

KEYWORDS: Lipase, Central composite design (CCD), *Pseudomonas aeruginosa* SJ2, used cooking oil.

INTRODUCTION

Lipases (triacylglycerol acyl hydrolases, E. C. 3. 1. 1. 3) are enzymes that catalyse the hydrolysis of triacylglycerols to fatty acids and glycerol. Unlike esterases, lipases are activated only when adsorbed to an oil-water interface (Martinelle et al., 1995) and do not hydrolyse substrates present in high volume fluid. Lipases have many industrial applications like detergent preparations, organic chemical processing, oleochemical industries, paper making industries, synthesis of biosurfactants, dairy industries, agrochemical industries, nutrition, cosmetics, and pharmaceutical preparations. Synthesis of new compounds using lipase-based technologies is expanding the uses of these enzymes (Liese et al., 2000).

Many cost-effective carbon sources can be used for lipase production. One such cost effective carbon source is used cooking oil. Used cooking oils (UCOs) include vegetable oils like canola oil, soybean oil, and corn oil, and animal-based fats like lard and tallow that have already been used for frying or cooking food previously. Estimates suggest that around 200 million tons of waste cooking oil (WCO) are produced annually worldwide. This figure is expected to increase in the coming years due to the growing global population and rising consumption of vegetable oils (De Feo et al., 2020). In the present study, *Pseudomonas aeruginosa* SJ2 isolated and characterized in previous studies (GenBank accession number of MN700061) was used for lipase production using used cooking oil (UCO) as a cheap carbon source. Optimization studies were performed using central composite design (CCD).

MATERIALS AND METHODS

Pseudomonas aeruginosa SJ2 isolated and characterized in previous studies (GenBank accession number of MN700061) was used for lipase production using used cooking oil (UCO) as a cheap carbon source.

Chemicals

All the chemicals, reagents and culture media used in this study were of analytical grade and purchased from HiMedia (Mumbai, India) unless otherwise mentioned.

Protein estimation

The protein was estimated by colorimetric method by Lowry et al., (1951) using bovine serum albumin as the standard protein.

Lipase assay

Lipase activity was determined as described in the literature (Winkler and Stuckman, 1979) using p-nitrophenyl palmitate (pNPP) as substrate with some modifications. The substrate solution was prepared by mixing solution A (30 mg of pNPP in 10 mL of 2-propanol) with solution B (0.1 g of gum Arabic in 90 mL of 50mM Tris–HCl buffer of pH 7.5 while stirring until all was dissolved. 2,700 µL of substrate solution and 300 µL of enzyme solution were preincubated at 40 °C for 5 min and then were mixed and incubated at 40 °C for 10 min and after 10min, 50 µL Triton-X 100 was added to dissolve the released free fatty acids. Tubes were immediately kept on ice until the absorbance was measured at 410 nm. Blank was also prepared in the similar way except enzyme was added after the incubation period. Molar extinction coefficient (ε) for p-nitrophenol in the given reaction system was found to $5.3*10^{3}M^{-1}cm^{-1}$. One unit of enzyme activity was expressed as one nanomole of p-nitrophenol released per minute under the assay conditions (Maia et al., 2001).

Used cooking oil (UCO) as a cheap carbon source for lipase production

Lipase production was carried out in 250 mL Erlenmeyer flasks using 50 mL basal production medium and 1% inoculum. The basal production medium used for lipase production using UCO consisted of (yeast extract, 2.0 g/L; UCO, 40.0 mL/L; NaCl, 2.0 g/L ; $MgSO_4$, 0.4 g/L; $(NH_4)_2SO_4$, 0.5 g/L; K_2HPO_4, 0.3 g/L; KH_2PO_4, 0.3 g/L; pH 8.0 ± 0.2; glucose, 0.5%; triton-X 100, 0.05%. Effect of concentration of UCO as a carbon source was studied by varying the concentration of UCO between 20 mL/L to 100 mL/L. After finding the optimum concentration, three important variables viz. A: UCO, B: yeast extract and C: temperature were optimized using RSM.

Optimization of the selected components using CCD

RSM is generally used to determine the optimum concentration and interactive effects of different variables used in the production medium. Central Composite Design (CCD) was used to find out the optimum concentration and interactive effects of the selected variables. The variables were examined at five levels (−1.68179, lowest; -1; medium, 0; 1 and highest, +1.68179). **Table 01** shows the CCD experimental setup. **Table 02** shows the actual concentration levels of variables used. Minitab version 19 (Minitab Inc., USA) was used to generate and analyse the experimental design.

Table 01: CCD design for the optimization of screened variables using UCO.

Run Order	A	B	C
1.	-1.00000	-1.00000	1.00000
2.	0.00000	-1.68179	0.00000
3.	1.68179	0.00000	0.00000
4.	0.00000	1.68179	0.00000
5.	-1.00000	-1.00000	-1.00000
6.	1.00000	-1.00000	1.00000
7.	0.00000	0.00000	-1.68179
8.	-1.00000	1.00000	-1.00000
9.	0.00000	0.00000	0.00000
10.	1.00000	1.00000	1.00000
11.	-1.00000	1.00000	1.00000
12.	1.00000	-1.00000	-1.00000
13.	-1.68179	0.00000	0.00000
14.	0.00000	0.00000	0.00000
15.	0.00000	0.00000	0.00000
16.	0.00000	0.00000	0.00000
17.	0.00000	0.00000	0.00000
18.	0.00000	0.00000	1.68179
19	1.00000	1.00000	-1.00000
20.	0.00000	0.00000	0.00000

Table 02: Concentration of the variables used in CCD using UCO.

Sr. No.	Variable	Code	Level				
			-1.68179	-1.0	0.0	+1.0	+1.68179
1.	UCO (ml/L)	A	14.77	25.0	40.0	55.0	64.26
2.	Yeast Extract (g/L)	C	0.32	1.0	2.0	3.0	3.68
3.	Temp. (^{o}C)	H	21.6	25.0	30.0	35	38.40

Verification of the model

In the recommended optimal model, parametric settings of UCO of 49mL/L, yeast extract of 2.63 g/L and temperature of 32 °C, were set and the response for this set of values for lipase enzyme production was recorded.

Scale-up studies

Scale-up studies were performed with the optimized cost-effective medium using 3.0 L bench top fermenter (Systronics India Ltd., Ahmedabad) with 1.5 L working volume. Aeration was maintained at 0.4 L/min. Samples were withdrawn after every 12 h and lipase activity was measured up-to 60 h.

Results and discussion

The optimum concentration of used cooking oil as a carbon source was determined by varying the concentration of oil from 20 mL/L to 100 mL/L. Maximum lipase production of 300 ± 4 U/ml was achieved at the concentration of 40 mL/L of used cooking oil.

Optimization of significant components by Central Composite Design

Central Composite Design (CCD) of five center points with twenty experiments was used to determine the optimal levels of the three significant factors (carbon source, yeast extract, and temperature) that affected lipase production as shown in **Table: 03**. According to the regression analysis of CCD (**Table: 04**), the model terms A, B, C, A^2, B^2, C^2, AB, AC, and BC all were significant ($p < 0.05$). The linear, interactive, and second-order interactions between the variables all were significant ($p < 0.0001$). The regression equation coefficients were studied by putting data in the 2nd order polynomial equation as follows:

U/mL = 296.55 + 51.68 A + 44.86 B
+ 29.94 C - 64.05 A*A - 33.43 B*B -
35.44 C*C + 18.49 A*B
+ 34.11 A*C - 30.85 B*C

The outcomes of ANOVA for the second-order polynomial model as given in **Table: 05** showed that R^2 was 0.9902 which means that model could explain 99.02% of the variation in response (lipase production). The square of the linear correlation is termed as the coefficient of determination (R^2), which is the measure of the strength of the linear relationship between the experimental and the predicted values. The predicted R^2 of 0.9536 was in reasonable agreement with the adjusted R^2 of 0.9814 indicating good adjustment between the observed and predicted values. i.e., the regression model could be used to analyse trends of responses. The high model F-value 112.17 and low p-value < 0.0001 implies that lipase production by *P. aeruginosa SJ2* has a good fit with the model. The contour plots (**Figure: 01 A**) and their corresponding three-dimensional surface plots (**Figure: 01 B**) provided information about the interaction between the variables and interpreted the optimum experimental conditions. These plots were generated for the pairwise combination of the three variables while keeping the third one at its center point level.

Table 03: CCD for optimization of lipase production using cheap carbon source.

Sr. No.	A	C	H	U/mL
1.	-1.00000	-1.00000	1.00000	108 ± 2
2.	0.00000	-1.68179	0.00000	127 ± 2
3.	1.68179	0.00000	0.00000	191 ± 4
4.	0.00000	1.68179	0.00000	277 ± 5
5.	-1.00000	-1.00000	-1.00000	52 ± 1
6.	1.00000	-1.00000	1.00000	259 ± 5
7.	0.00000	0.00000	-1.68179	156 ± 3
8.	-1.00000	1.00000	-1.00000	160 ± 4
9.	0.00000	0.00000	0.00000	285 ± 5
10.	1.00000	1.00000	1.00000	318 ± 3
11.	-1.00000	1.00000	1.00000	106 ± 1
12.	1.00000	-1.00000	-1.00000	53 ± 1
13.	-1.68179	0.00000	0.00000	40 ± 1
14.	0.00000	0.00000	0.00000	286 ± 5
15.	0.00000	0.00000	0.00000	313 ± 5
16.	0.00000	0.00000	0.00000	312 ± 4
17.	0.00000	0.00000	0.00000	289 ± 6
18.	0.00000	0.00000	1.68179	236 ± 4
19.	0.00000	0.00000	0.00000	291 ± 5
20.	1.00000	1.00000	-1.00000	248 ± 4

Table 04: Estimated regression coefficients and t-values of variables for yield.

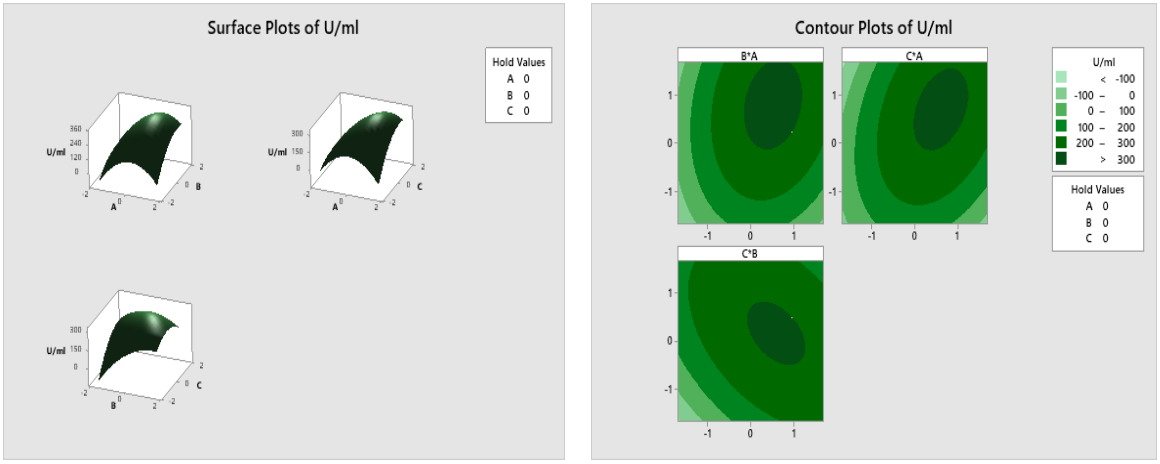
Term	Coeff	SE Coeff	T-Value	P-Value
Constant	296.55	5.39	55.06	<0.0001
A	51.68	3.57	14.46	<0.0001
B	44.86	3.57	12.55	<0.0001
C	29.94	3.57	8.38	<0.0001
A*A	-64.05	3.48	-18.41	<0.0001
B*B	-33.43	3.48	-9.61	<0.0001
C*C	-35.44	3.48	-10.19	<0.0001
A*B	18.49	4.67	3.96	0.003
A*C	34.11	4.67	7.31	<0.0001
B*C	-30.85	4.67	-6.61	<0.0001

Table 05: Model Summary for CCD.

S	R^2	R^2(adj)	R^2(pred)
13.2055	99.02%	98.14%	95.36%

Table 06: Analysis of variance of variables for yield.

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	9	176052	19561.4	112.17	<0.0001
Linear	3	76196	25398.8	145.65	<0.0001
A	1	36477	36476.6	209.17	<0.0001
B	1	27480	27480.0	157.58	<0.0001
C	1	12240	12239.6	70.19	<0.0001
Square	3	80197	26732.2	153.29	<0.0001
A*A	1	59122	59121.5	339.03	<0.0001
B*B	1	16101	16101.2	92.33	<0.0001
C*C	1	18103	18103.1	103.81	<0.0001
2-Way Interaction	3	19659	6553.1	37.58	<0.0001
A*B	1	2735	2735.3	15.69	0.003
A*C	1	9310	9309.8	53.39	<0.0001
B*C	1	7614	7614.2	43.66	<0.0001
Error	10	1744	174.4		
Lack-of-Fit	5	913	182.7	1.10	0.460
Pure Error	5	831	166.1		
Total	19	177796			



A B

Figure 01: A. Contour plots showing the effect of variables B. Response surface plots showing the interaction between variables.

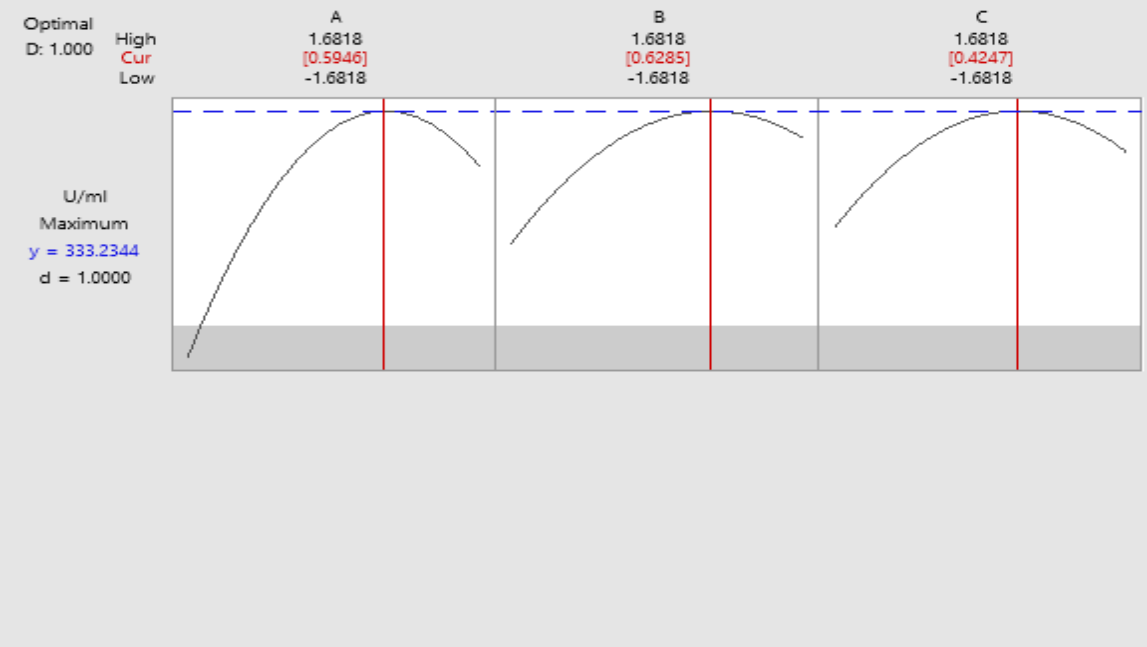


Figure 02: Optimizer plot showing optimum concentration of the selected variables.

Verification of the model

Eventually, after making the regression model, a numerical optimization method by desirability function was implied to optimize the response. The graph in **Figure: 02** indicates how individual factors in each column influence the response while the other factors are held constant. The values between high and low values optimal parametric settings were recommended by the Minitab 19 software to obtain the most suitable responses. D is the composite desirability and d is the individual desirability. The maximum values for D and d are 1.0000 (Myers and Montgomery, 1995). **Figure: 02** shows values for D and d in optimal conditions as 1.0000, confirming that the model proposed is suitable. In the recommended optimal model, parametric settings of UCO of 49 ml/L, yeast extract of 2.63 g/L and temperature of 32 °C, were set. The response for this set of values for lipase enzyme production with the desirability of 1.0000 was 333.23 U/mL. The predicted optimum condition was verified experimentally and compared with the predicted data. The measured lipase activity was 329 ± 3 U/mL. The verification revealed a high degree of accuracy of the predicted model.

Scale-up studies

Scale-up studies resulted in the marginal reduction in the amount of lipase synthesis. The maximum amount of lipase for the optimized medium was and 321 ± 4 U/mL (**Figure: 04**) with the cost-effective medium. The findings correlated with previous reports. Kulkarni and Gadre (2002) have reported lipase secretion in the late logarithmic phase with a marginal difference of lipase production in shake flasks (69.7 U/mL) as compared to a 1 L fermenter (68.7 U/mL). The results were supported by D'Annibale et al. (2006), who have studied lipase production in shake flasks and in a stirred tank (3 L), found higher production (1,230 U/L) from shaken flasks as compared to the stirred reactor (735 U/L). Similarly, Tan et al., (2003) have noticed decrease lipase production from 8,500 to 8,060 U/mL upon scale-up using Candida sp. and the authors have emphasized agitation speed as an important parameter in maintaining the level of dissolved oxygen. The possible reason for retardation in product formation is the insufficient availability of dissolved oxygen in the fermenter. The metabolic pathway for lipase biosynthesis requires oxygen. An increase in stirrer speed provides more dissolved oxygen (DO) in the medium thereby enhanced the lipase activity (Tan et al., 2003). Calik et al. (1997) have established that the amount of DO is restricted by solubility and mass transfer rate of the medium, and Lima et al. (2003) have noticed the presence of oil leads to poor oxygen transfer into the medium, concomitantly inhibit lipase synthesis. *P. aeruginosa* is aerobic bacteria; the shortage of oxygen might have repressed the respiration of cells leads to a reduction in lipase activity. Iftikhar et al., (2008) have reported that, when dissolved oxygen concentration falls below a critical level, an instant decrease in lipase biosynthesis occurs. The authors have successfully corrected a set of experiments by reducing dissolved carbon dioxide concentration. The overall productivity for the lipase largely depended on agitation than aeration. An increase in the availability of oxygen at higher airflow rates led to faster lipid uptake resulted in a high amount of enzyme release (Chen et al., 2000).

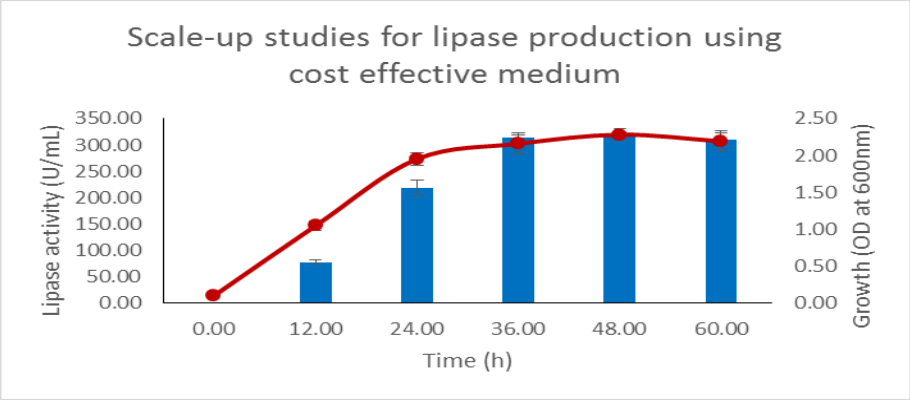


Figure 03: Scale-up studies for lipase production.

CONCLUSION

In designing of cost-effective lipase production medium used cooking oil (UCO) was used as a cheap carbon source. In unoptimized conditions production medium (g/L; yeast extract (2.0), UCO (40.0 mL/L), NaCl (2.0), $MgSO_4$ (0.4), $(NH_4)_2SO_4$ (0.5), K_2HPO_4 (0.3), and KH_2PO_4 (0.3), pH 8.0 ± 0.2), glucose (0.5%), triton-X 100 (0.05%), temperature 30 °C, incubation time 48 h) *Pseudomonas aeruginosa* SJ2 was able to produce 300 ± 4 U/mL of lipase. Statistical optimization using CCD resulted in production of 329 ± 3 U/mL of lipase under optimized conditions medium (g/L; yeast extract (2.33), UCO (49.0 mL/L), NaCl (2.0), $MgSO_4$ (0.4), $(NH_4)_2SO_4$ (0.5), K_2HPO_4 (0.3), and KH_2PO_4 (0.3), pH 8.0±0.2), glucose (0.5%), triton-X 100 (0.05%), temperature 32 °C, incubation time 48 h). In scale-up studies lipase production dropped marginally to 321± 4 U/mL. Optimization resulted in 1.1-fold increase in lipase production by *Pseudomonas aeruginosa* SJ2 using cost effective medium.

REFERENCES

1. Calik, Güzide; Vural, Halil and Ozdamar, Tunçer. Bioprocess parameters and oxygen transfer effects in the growth of Pseudomonas dacunhae for L-alanine production. *Chemical Engineering Journal*, May 1997; 65(2): 109-116.
2. Chen, J.C., & Tsai, S.W. Enantioselective synthesis of (S)-ibufrofen ester prodrug in cyclohexane by *Candida regusa* lipase immobilized on accurel MP1000. *Biotechnol Prog*, 2000; *16*: 986-92.
3. D'Annibale, A., Sermanni, G. G., Federici, F., & Petruccioli, M. Olive-mill wastewaters: a promising substrate for microbial lipase production. *Bioresource technology*, 2006; *97*(15): 1828–1833. https://doi.org/10.1016/j.biortech. 2005.09.001
4. De Feo, G., Di Domenico, A., Ferrara, C., Abate, S., & Sesti Osseo, L. Evolution of Waste Cooking Oil Collection in an Area with Long-Standing Waste Management Problems. *Sustainability*, 2020; *12*(20). doi:10.3390/su12208578
5. Iftikhar, T., Niaz, M., Afzal, M., Haq, I., & Rajoka, M. I. Maximization of Intracellular Lipase Production in a Lipase-Overproducing Mutant Derivative of Rhizopus oligosporus DGM 31: A Kinetic Study. *Food Technology Biotechnology*, 2008; *46*: 402–412.
6. Kulkarni, N. *Studies on lipase enzyme from Pseudomonas fluorescens NS2W 5I* [Doctoral Dissertation, Pune University], 2002.
7. Liese, A. (Andreas), Seelbach, K. (Karsten), & Wandrey, C. *Industrial biotransformations*. Wiley-VCH, 2000.
8. Lima, V. M. G., Krieger, N., Sarquis, M. I. M., Mitchell, D. A, Ramos, L. P., & Fontana, J. D. Effect of Nitrogen and Carbon Sources on Lipase Production by *Penicillium aurantiogriseum*. *Food Technology and Biotechnology*, 2003; *41*(2): 105–110.
9. Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. Protein measurement with the Folin phenol reagent. *The Journal of biological chemistry*, 1951; *193*(1): 265–275.
10. Maia, M. M. D., Heasley, A., Camargo De Morais, M. M., Melo, E. H. M., Morais, M. A., Ledingham, W. M., & Lima Filho, J. L. Effect of culture conditions on lipase production by *Fusarium solani* in batch fermentation. *Bioresource Technology*, 2001; *76*(1): 23–27.
11. Martinelle, M., Holmquist, M., & Hult, K. On the interfacial activation of *Candida antarctica* lipase A and B as compared with *Humicola lanuginosa* lipase. *Biochimica et Biophysica Acta*, 1995; *1258*(3): 272–276.
12. Myers, R.H., & Montgomery, D.C. Response surface methodology: process and optimization using designed experiments. 1st. Wiley Interscience, 1995.
13. Tan, T., Zhang, M., Wang, B., Ying, C., & Deng, L. Screening of high lipase producing Candida sp. and production of lipase by fermentation. *Process Biochemistry*, 2003; *39*(4): 459–465. doi:10.1016/S0032-9592(03)00091-8.
14. Winkler, U. K., & Stuckmann, M. Glycogen, hyaluronate, and some other polysaccharides greatly enhance the formation of exolipase by *Serratia marcescens*. *Journal of Bacteriology*, 1979; *138*(3): 663–670.