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Screening, Optimization and Process Scale up for Pilot Scale Production of Lipase by *Aspergillus niger*

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Abstract An enzyme with various commercial purpose, lipase is a carboxy esterase enzyme with many uses in different industries. Multiple isolates of *Aspergillus niger* were isolated from oil contaminated soil samples and screened for lipase producing ability on tributyrin medium. The isolate showing maximum activity was identified and subjected to growth parameters optimization in attempt to increase the enzyme producing ability of the isolate in larger scale. Different media with varying composition were examined for best lipase production. The activities of the lipase produced by the fungus at various pH were assessed. The enzyme activity was determined by the titration method. Maximum lipase activity of 2.4 U/ml was achieved with organic nitrogen rich media (designated as PM II) at pH 7 on the sixth day of culture. The lipase production was scaled up on a pilot scale in a 5 Liter fermenter maintaining growth parameters of pH 7, temperature at 28°C, stirrer rate at 120 rpm, airflow rate at 30 L/hr, O2 saturation 50% and pressure 0.05 MPa. The crude enzyme was extracted for further assays. Optimization of the parameters can improve the productivity as well as the quality of the enzyme produced.

Keywords: *lipase, Aspergillus niger, enzyme activity, pH, scale up*

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1. Introduction

The use of lipases in industries is enormous and increasing. Since lipase is an enzyme with various applications, finding out effective lipase producing microorganism (fungus) and supporting production methods can aid in the large scale industrial production in higher proportion with reduced economic expenditures. Lipase or triacylglycerol (TAG) acylhydrolases (E.C. 3.1.1.3) is a kind of carboxy esterase [1]. Under physiological conditions, this enzyme catalyzes hydrolysis of oils and fats, so the biological role of lipase is metabolism of lipids. The capability of catalyzing various reactions makes lipases very useful biocatalysts for modification of oils and fats, and other synthetic chemistry. The catalytic action of lipases is reversible. They catalyze hydrolysis in an aqueous system, but also esterification (reverse reaction of hydrolysis) in a micro aqueous system, where water content is very low. The lipase-catalyzed reactions are specific (or selective) for particular acyl groups, particular positions of the substrates, or particular stereoisomers. This specificity enables the modification of oils and fats in more sophisticated way.

Lipases are produced by many microorganisms either alone or together with other members of the hydrolases family, like esterases. Among microorganisms, fungi are widely recognized as preferable lipase sources because they generally produce the enzyme in an extracellular manner, which facilitates the enzyme recovery from the fermentation broth.

Aspergillus niger is one of the most important microorganisms used in biotechnology. It has already been in use for decades to produce many extracellular enzymes that are considered Generally Regarded as Safe (GRAS) by the Food and Drug Administration of the United States of America (FDA) [2]. Fungal lipases have been studied since 1950s, and Lawrence, Brockerhoff and Jensen have presented comprehensive reviews [3,18]. The fungal lipase is being exploited due to its low cost of extraction, thermal and pH stability, substrate specificity, and activity in organic solvents. It is a specific enzyme that digests fat and is characterized by its ability to hydrolyze fat over a wide range of temperatures and pH. It also regulates the levels of Cholesterol and Triglycerides and is also helpful in different dietary regime for weight management. It is ideal for use in leather, detergent and cosmetic industries. We therefore focused our attention in isolation of a potential isolate of genus Aspergillus from soil and optimize pH for the production of Lipase using suitable culture medium.

2. Materials and Methods

2.1. Isolation and Identification

Soil samples from various collection sites were taken from Lalitpur district, Nepal. The sample sites also included oil mill (Sunakoti and Thecho, Lalitpur, Nepal) and an automobile repair shop (Gwarko, Lalitpur, Nepal). After serial dilution of the soil sample (up to 10^{-6}) it was inoculated following pour plate method on Potato Dextrose Agar (HIMEDIA) for the initial isolation step and the plates were incubated at 28°C for 6 consecutive days. All of the growing fungus was subjected to identification studying colony morphology and microscopic observation (Lacto Phenol cotton blue staining). The identified *A. niger* were sub-cultured multiple times until pure culture was obtained.

2.2. Screening for Lipase Producers

2.2.1. Precipitation Test (Using T80 Agar Plates)

Agar plates containing Tween 80 (T80) were prepared (Table 1). Then organisms were inoculated in T80 agar plates and incubated at 28°C for 4 days. White precipitate along the boundary of the colony was observed.

2.2.2. Screening Using T80 Agar Plates With Phenol Red

Organisms were inoculated in the T80 agar plates with Phenol Red indicator (Table 1) and incubated at 28°C for 4 days. The lipase activity will bring out change in coloration from pink to lemon yellow.

2.2.3. Screening Using Tributyrin Agar Plates

The fungus was inoculated in the Tributyrin agar plates (Table 1) followed by incubation at 28°C for 4 to 5 days. A clear zone of hydrolysis appears along the boundary of the colony of the fungus representing lipase activity.

Table 1. Lipase producing *A. niger* screening medium

Components	T80 lipase assay plate (w/v)	T80 with phenol red indicator (w/v)	Tributyrin agar plate (w/v)
Peptone	1.5%	1.5%	0.5%
Sodium Chloride	0.5%	0.5%	
Calcium Chloride	0.1%	0.1%	
Tween 80	1%	1%	
Yeast Extract			0.3%
Tributyrin			0.1%
Agar	1.5%	1.5%	2%
Phenol Red Indicator		0.01%	
pH	7	7	7

2.3. Medium Selection

Four Production medium (designated as PM I, PMII, PMIII, PM IV) were chosen and four lipase producing *A. niger* isolated from screening were inoculated in these four chosen medium and shake flask culture was performed for 6 days maintaining 28°C. Then crude enzymes from all the shake flask cultures were extracted by filtration and tributyrin agar plate assay was performed extracting the crude enzyme at the 6^{th} and the 7^{th} day of culture.

2.4. Shake Flask Culture

The four selected Production medium, 25 ml each in 100 ml Erlenmeyer flasks were prepared. All the four selected isolates were inoculated in the four selected medium and incubated in water bath shaker. Process parameters (120 RPM, 28°C temperature and pH 7) were maintained for shake flask culture. 2 ml amount of broth was sampled for lipase assay on tributyrin plates.

Table 2. Composition of production medium (values expressed in gm/L)

Components	PM I (Hosseinpour et al. 2011) [4]	PM II (Rajan et al. 2011) [5]	PM III (Cihangir & Sarikaya 2004) [6]	PM IV (Mala et al. 2007): [7]
KH_2PO_4	2		2	0.2
$MgSO_4.7H_2O$	0.5		0.3	0.05
KCL	0.5			
Yeast Extract	0.25	0.5		0.1
Peptone	0.25	2	10	0.5
Glucose	12.5			
Olive oil	12	1	10	1
NaCl		0.5		0.05
Na_2CO_3		0.025		
Na_2HPO_4			12	
$CaCl_2$			0.25	
$FeSO_4.7H_2O$			0.005	
$MnSO_4.7H_2O$			0.015	
$ZnSO_4.7H_2O$			0.030	
KNO_3				0.05
pH	7	7	7	7

2.5. Lipase Assay on Tributyrin Agar Plates

Wells were bored on the Tributyrin agar plates using sterile cork borer and 30 µL of the broth were withdrawn from shake flask cultures on 6^{th} and 7^{th} day of incubation and inoculated on the wells on Tributyrin agar plates. The plates were then incubated at 28°C for 18 hours for observing the zone of hydrolysis created by release of the

fatty acid molecules. The diameter of the zone of hydrolysis was measured for selecting the most potential *A. niger* and the best supporting medium. The isolate showing maximum zone of hydrolysis was selected.

2.6. pH Optimization

2.6.1. Shake Flask Experiments

25 ml of selected production medium was prepared and distributed in Erlenmeyer flasks of 100 ml capacity. The medium to flask ratio used was 1:4 to provide better aeration. The pH of the medium was adjusted to 4.0, 5.0, 6.0, 7.0, 8.0 and 9.0 using 0.1 N NaOH or 0.1 N HCl and all the other parameters were maintained unchanged. The medium was then sterilized in an autoclave at 121°C, 15 lbs. pressure for 20 minutes. Olive oil was sterilized at 160°C for 1 hour and added separately to each 25 ml medium.

2.6.2. Inoculum Preparation for Shake Flask Experiments

The spore suspension of *A. niger* (8 x10^7 spores/ml; spores count on Haemocytometer) was used as inoculum for shake flask experiments. 0.3 ml of the spore suspension was inoculated into each flask containing 25 ml of the culture medium. The inoculated flasks were then incubated in a water bath shaker at 28°C at 120 rpm. Sampling was done from 3rd days till 7 days of culture period for measurement of enzyme activity. Highest enzyme activity can be correlated with optimum pH for lipase production.

2.6.3. Determination of the Lipase Activity

Lipase activity was assayed by titrimetric method (Yamada et al) [8].

Lipase Unit Activity (U/ml/min):

$$\text{Unit Activity} = \frac{\text{N}\left[\text{NaOH}\right] \text{ x Vol of NaOH titrated x } 1000}{\text{Time of Incubation } \left[20 \text{ min}\right]}$$

One unit of lipase activity is defined as the amount of enzyme liberating one Micro mole of fatty acid per minute under standard assay conditions.

2.7. Protein Estimation

Protein estimation was done by Lowry method (Lowry *et al* 1951) [9]**.** The pH in which the enzyme showed maximum enzyme activity was selected.

2.8. Pilot Scale Production of Lipase in 5L Fermenter

The fermenter used for the experiment was biotech-5BG fermenter {Baoxing Bio-engineering equipment co.,ltd (BXBIO)}. The fermenter vessel was filled with production medium (3L). Then the head plate consisting of the probes and agitator module was fixed. The port opening (sampling, acid, alkali and antifoam supply, pH electrode, DO probe, cooling coil, condensor) was covered with cotton and brown craft paper. Finally the whole fermenter vessel along with the medium inside was placed inside autoclave (YXQ-LS-SII vertical type autoclave) and sterilized. The media was allowed to cool down. For the inoculum, first a flask culture was initiated with 0.3 ml of 8 x 10^7 spores /ml spores in a 25 ml of the optimized media. The culture was incubated for 6 days and was used to inoculate the 3 liter media of the bioreactor. The parameters for sustaining the culture were set in the fermentor's program. The parameters used were including the parameters optimized in the optimization stage earlier. The fermenter was programmed to maintain the initially set parameters value in case of fluctuations. The major parameters maintained were:

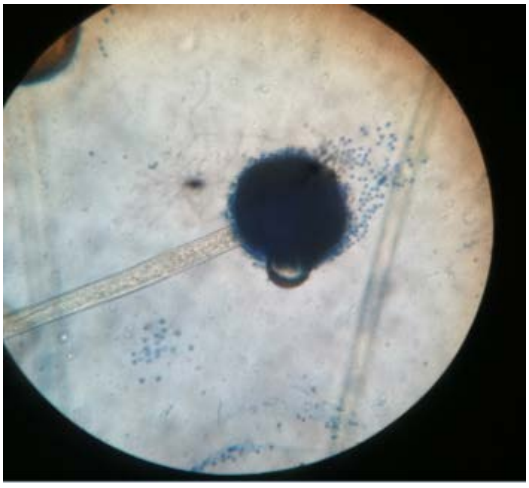
Table 3. Setting fermenter parameters

S.N.	Parameters	Set value
1.	Pressure	0.05 MPa
2.	Air Flow Rate	30 ltr/ hr
3.	Rate of Stirring	120 RPM
4.	O_2 Saturation	50%
5.	pH	7
6.	Temperature	28 ^{o} C

3. Results

In this study we were concerned with isolating *A. niger* which is generally regarded as safe for industrial production of Lipase and so we isolated a potential Lipase producing strain. Different isolates of *A. niger* was named as "A", "B", "Z" and "G". The isolates of *A. niger* was identified from colony morphology and the microscopic observation.

Photograph 1. Pure culture of *A. niger*



Photograph 2. *A. niger* at 40X magnification

3.1. Screening of *A. niger* for Lipase Production

3.1.1. Precipitation Test (Using T80 Agar Plates)

White precipitation along the periphery of the colony was observed. Quantitatively more precipitation was found in *A. niger* isolates designated as "A". The screening method suggested by Shukla and Gupta turned out to be effective leading to selection of better lipase producers [10].

3.1.2. Screening Using T80 Agar Plates With Phenol Red Indicator

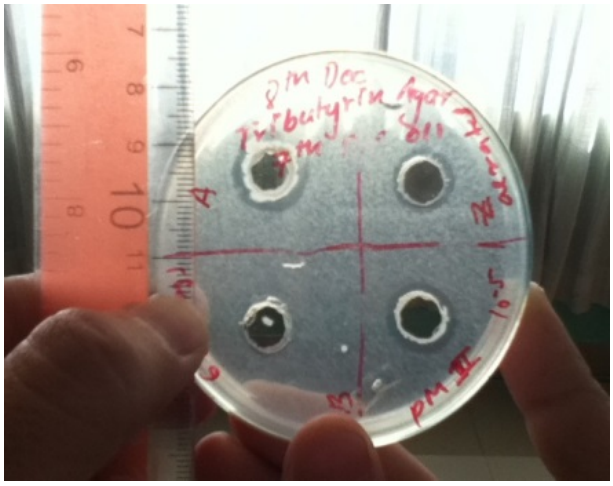
We used the phenol red indicator along with Tween 80 screening medium for the screening proved to be effective [10,11]. Yellowish lime green coloration was observed at the boundary of the colony. The isolate designated as "A" was found to give out the most prominent coloration.

3.1.3. Screening Using Tributyrin Agar Plates

Largest clearing zone of hydrolysis was shown by culture "A" in comparison with other selected three isolates (B, Z and G). The clearing zone of hydrolysis is seen as a result of lipase action on the tributyrin. Tributyrin is considered as a gold standard for screening of lipase producing microorganisms [12].

Photograph 3. Change in coloration of medium indicating fatty acid release

Photograph 4. Precipitation observed along the periphery of the colony



Photograph 5. Zone of hydrolysis seen on Tributyrin agar plate

The production medium (PM II) selected for pilot scale production showed growth of effective lipase producers with its enzyme showing high activity in comparison with other enzymes from other production medium. The crude enzymes from the four isolates each grown on four selected medium were extracted by filtration and subjected to tributyrin agar plate assay**.** The highest enzyme activity was obtained from isolate "A" grown on PM II (Table 4 and Table 5).

Table 4. Zone of hydrolysis by crude enzyme on tributyrin agar plate (Day 6)

Isolate	18 hours incubation in tributyrin agar plate 28°C			
	Length of clear zone of Hydrolysis(mm)			
	PM I	PM II	PM III	PM IV
A	2	3	2	2
B	1	1	1	1
Z	1	1	1	0
G	1	0	1	1

Table 5. Length of zone of hydrolysis by crude enzyme on tributyrin agar plate (Day 7)

Isolate	18 hours incubation in tributyrin agar plate at 28°C			
	Length of clear zone of Hydrolysis(mm)			
	PM I	PM II	PM III	PM IV
A	No distinct zone of hydrolysis	5	2	2
B		0	1	1
Z		1	1	0
G		0	1	

3.2. Optimization of the Process Parameter pH

Our study shows the highest enzyme activity of the enzyme produced at pH 7 (Figure 1). And it also was selected to be optimum by determining lipase activity by titration assay and protein concentration by Lowery method which relates with the evidence put forward by Ghosh et. al 1996 that most lipases, show profound stability around pH 6.0–7.5 with considerable stability at acidic pH up to 4.0 and at alkaline pH up to 8.0. The pH 7.0 effectively supported the *A. niger* growth and in correspondingly supported the production of lipase [13].

Statistical data presentation (graph chart) was done using SPSS 16.0 software (Figure 1).

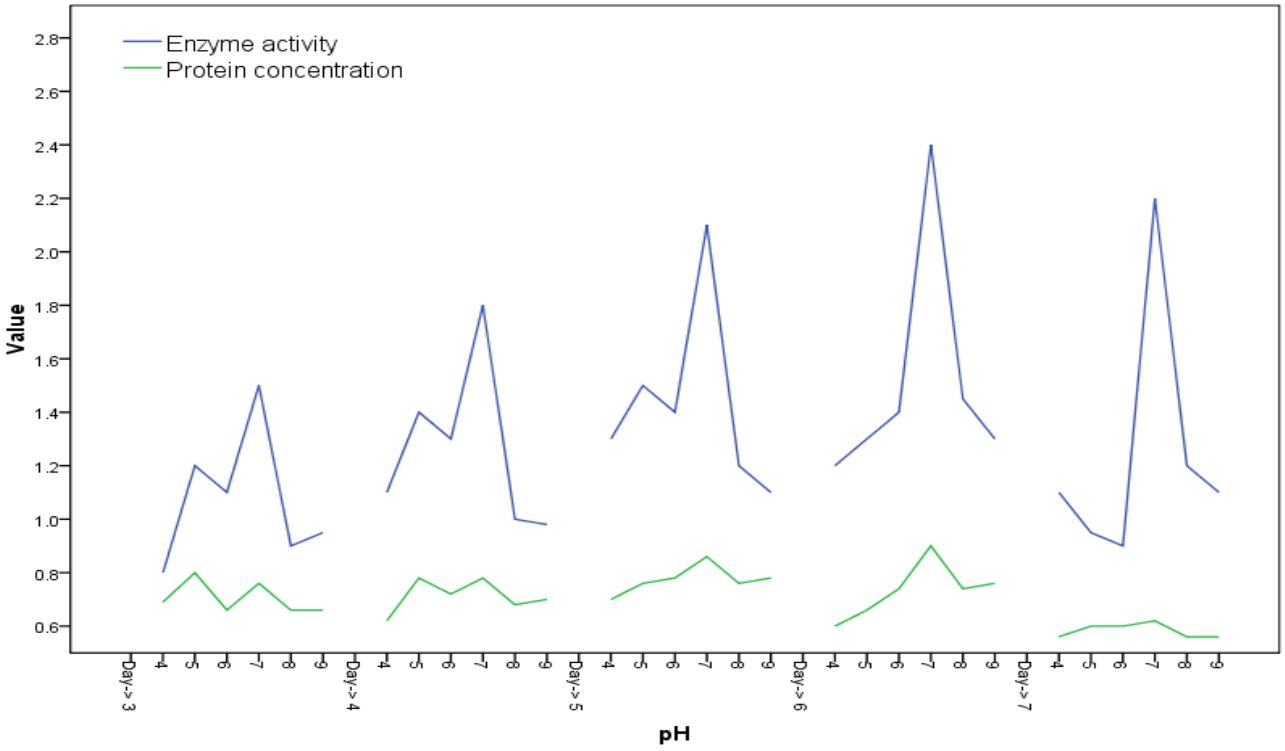


Figure 1. Enzyme concentration and Protein concentration showing at various pH in shake flask culture during pH optimization. (In Y-Axis → Enzyme Activity U/ml and X-Axis → pH)

3.3. Lipase Production in Fermenter:

The pH of 7 was maintained for 6 days culture at constant temperature of 28°C. The fermentation process was allowed to run for 6 days maintaining the initially set parameters automatically. Then crude enzyme extraction for the assay was done by filtration using Whatman No. 1 filter paper as filter membrane. The tributyrin plate assay of crude enzyme extract showed large zone of hydrolysis indicating good lipase activity.

4. Discussion

Oil contaminated sources were explored for finding a fungus with lipase enzyme producing capabilities. The lipolytic fungus was subjected to various optimization of the growth parameters for improving the lipase production. The possibilities of improving the lipolytic strain in terms of the enzyme productivity in a larger scale were targeted. All the parameters optimized were taken and the production of the enzyme was attempted at a pilot scale. There was an increase in the activity of lipase enzyme in the pilot scale production where the growth parameters used were optimized.

The search for the best lipolytic strain was initiated from the oil contaminated areas such as oil mills and automobile garages which represents an oil and lipid rich environment for the fungus to thrive on. The area is a sanctuary for lipolytes reducing the oil substrate to get glycerol as carbon source [15]. The search was intended for *A. niger*, so only the particular fungus was screened and selected only from the dominant lipolytes on the screening plate. The choice was *A. niger* since the enzyme from the fungus is Generally Regarded as Safe by the FDA (Food and Drug Administration of the United States of America) [2] which directs that the products from the fungus can be consumed if purified to the required level. Lipase from fungi has been exploited as valuable due to its thermal stability, pH stability, substrate specificity and activity in organic solvents [14].

Four of the already established media with varying compositions were used for sustaining the lipase production. The highest enzyme activity was recorded from the growth in the media with organic nitrogen sources (Yeast extract and Peptone) designated as PM II among the four media involved in the process. Reports on microorganisms providing high yields of lipase with organic nitrogen sources also exist [16,17].

The titrimetric method presented some difficulties in detecting the color change of the indicator used but the method proved competent for enzyme activity detection. Crude enzyme without precise filtration was used for the enzyme activity assay which would have presented higher activity value in case of purified enzyme sample. All the essential parameter like the pH, temperature, dissolved oxygen concentration and air supply were maintained during the pilot scale production in the fermenter. The pH used was 7 after the finding from the pH parameter optimization. The maintaining of the parameters throughout the fermentation process was essential for the optimum lipase production.

5. Conclusion

Thus, *A. niger* was isolated from the soil sample collected from various sites likely to consist of lipase producing microorganism (oil contaminated soil samples). Only one isolate of *A. niger* was selected by various plate

assays and enzyme assay methods on the basis of lipase producing capacity from the four isolates of *A. niger*. Four production medium were selected on the basis of literature review and only one was selected for taking into production which supported optimum growth of the lipase producers. The isolate of *Aspergillus* species producing industrially significant enzyme lipase was successfully isolated from soil sources. A few growth conditions were optimized to develop a suitable protocol for the lipase enzyme production and the process was successfully scaled up and simulated in pilot scale.

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References

[1] The Enzyme List Class 3 — Hydrolases[internet]. Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NC-IUBMB); 2010 September [cited 2012 march 12]. Available from: http://www.enzyme-database.org/.html.

[2] Schuster, E. Dunn-Coleman, N. Frisvad, J. and Van Dijck, P. "On the safety of *Aspergillus niger*– A review.", *Applied Microbiology and Biotechnology*, 59: 426-35. 2002.

[3] Lawrence, R.C. *Dairy Sci. Abstr*., 29, 1-8. 1967.

[4] Hosseinpour, N.M. Najafpour, D.G. Younesi, H. and Khorrami, M. "Submerged culture studies for lipase production by *Aspergillus niger* NCIM 584 on soya flour.", *Middle-East JSci Res*.; 7(3): 362-66. 2011.

[5] Rajan, A. Kumar, S.D.R. and Nair, J.A. "Isolation of a noval alkaline lipase producing fungus *Aspergillus fumigatus* MTCC 9657 from aged and crude rice bran oil and quantification by HPTLC." *Int JBiol Chem.* 5(2): 116-26. 2011.

[6] Cihangir, N. and Sarikaya, E. "Investigation of lipase production by a new isolate of *Aspergillus* sp.", *World Journal of Microbiology & Biotechnology*. 20: 193-97. 2004.

[7] Mala, S.G.J. Edwinoliver, G.N. Kamini, R.N. and Puvanakrishnan, R. "Mixed substrate solid state fermentation for production and extraction of lipase from *Aspergillus niger* MTCC 2594.", *J Gen Appl Microbiol*.53: 247-53. 2007.

[8] Yamada, K. Ota, U. and Mochida, H. "Quantitative determination of lipases.", *Agri Bio Chem*: 26**:** 636-40. 1962.

[9] Lowry, O.H., Rosenbrough, N.J. Farr, A.L. and Randall, RJ. "Protein measurement with folin-phenol reagent.", *J Biol Chem.* ; 193: 265-75. may 28,1951.

[10] Shukla, P. and Gupta, K. "Ecological screening for lipolytic molds and process optimization for lipase production from *Rhizopus oryzae* KG-5.", *J Applied Sci Environ Sanitation.* 2: 35-42. 2007.

[11] Singh, R. Gupta, N. Goswami, V.K. and Gupta, R. "A simple activity staining protocol for lipases and esterases.", *Applied Microbial Biotechnol*. 70: 679-82. 2006.

[12] Sarkar, S.B. Sreekanthkant, S. Banerjee, R. and Bhattacharyya, B.C. "Production and optimization of microbial lipase. Bioprocess Engineering".; 19: 29-32. 1998.

[13] Ghosh PK, Saxena RK, Gupta R, Yadav RP and Davidson S. "Microbial lipases: production and applications.", *Science Progress*. 79(2): 119-57. 1996.

[14] Gulati, R. Saxena, R.K. and Gupta, R. "Fermentation and downstream processing of lipase from *Aspergillus terreus.*", *Process Biochemistry*. 36: 149-55. 2000.

[15] Farag, R., Khalil, F., Salem, H. and Ali, L. "Effects of various carbon and nitrogen sources on fungal lipid production". *Journal of the American Oil Chemists' Society.* 60(4): 795-800. 1983.

[16] Arbige, V. and Neubeck, C.E. United States Patent. 1998; 4: 726.

[17] Vulfson, E.N. Tanaka, T. Kamimura, R. Fujiwara, R. and Nakanishi, K. "Cross flow Filtration of Yeast Broth Cultivated in Molasses.", *Biotechnol Bioeng*. 43: 1094-1101. 1994.

[18] Brockerhoff, H. and Jensen, R. *Lipolytic Enzymes*, Academic Press, New York, 1974, 1-340.