Journal of Experimental Sciences 2012, 3(8): 26-30
ISSN: 2218-1768
Available Online: http://jexpsciences.com/

journal of
experimental sciences

Enrichment, isolation and optimization of lipase-producing *Staphylococcus* sp. from oil mill waste (Oil cake)

Ajit Kumar *#, Surendra S. Parihar and Nisha Batra

P.G. Department of Life Sciences, Inst. Of Allied Sciences & Computer Applications, ITM Universe, Gwalior, M.P., 474001, India.
Centre for Bioinformatics, Maharshi Dayanand University, Rohtak, Haryana-124001, India.

Abstract
The production of commercial enzymes, including lipase from bacteria has always been the industrial choice due to its economical and commercial feasibility. Twelve samples of oil cakes were enriched to isolate lipase producing bacteria and were screened for their lipolytic activity. The best producer (OC_2) was characterized and identified as *Staphylococcus* sp. and was studied for lipase activity. The culture conditions were optimized for maximum enzyme production by isolate OC_2 and were found to be 40^0C at pH of 7.0 with agitation of 140 rpm and 48 hours of incubation time for achieving maximum enzyme activity of 13.92 U/ml in crude enzyme extracts and 34.78U/ml after partial purification of enzyme, with about 80% enzyme recovery. The purified enzyme showed maximum enzyme activity at 40^0C and pH 7.0. The study provides a good mesophilic bacterial candidate for potential industrial production of lipase.

Keywords: Lipase**,** Enrichment, Isolation, *Staphylococcus*, Mesophilic

INTRODUCTION

Lipases (acyl glycerol hydrolases EC 3.1.1.3) are a class of serine hydrolases which belongs to the α/β - hydrolases super family. They catalyze the hydrolysis of triglycerides to mono- and di-glycerides, glycerol and free fatty acids. In nature, lipases are ubiquitously produced in plants, animals and microorganisms. However, lipases of microbial origin are the most versatile and more thermostable and are known to bring about a range of bioconversions including alcoholysis, aminolysis, hydrolysis, esterification and inter-esterification (Pandey *et al*, 1999). Besides, microbes can be easily cultivated and microbial lipases are mostly extracellular and hence have received particular attention with respect to their industrial production and applications (Elibol and Ozer, 2000; Kamini *et al*., 2000; Kumar and Sharma, 2012). Lipases find their applications in various industrial sectors like processing of fats and oils, detergents and degreasing formulations, food processing, chemical and pharmaceuticals, paper mills, etc (Rubin and Dennis, 1997a,b; Kazlauskas and Bornscheuer, 1998; Masse *et al*., 2001; Takamoto *et al*., 2001).

Lipase production from microbial source, in general, and from bacteria, in particular, has always been a matter of study for finding lipases with novel and specific properties alongwith lower production and processing cost. So, the present study was carried out to enrich, isolate and characterize lipase producing bacteria from oil cake samples (oil mill waste). Also, the different bioprocess parameters were optimized for maximal enzyme activity.

Received: March 10, 2012; Revised: June12, 2012; Accepted: Aug 24, 2012.

*Corresponding Author
Ajit Kumar
P.G. Department of Life Sciences, Inst. Of Allied Sciences & Computer Applications, ITM Universe, Gwalior, M.P., India-474001.

Tel: +91-99802299176;
Email: akumar.cbt.mdu@gmail.com

MATERIALS AND METHODS

Sample Collection

For the present study 12 samples of oil mill wastes (oil cakes) were collected in sterile glass vials from different oil mills in and around Gwalior city of Madhya Pradesh, India and were stored at 4^0C till further investigations. All the reagents and media were of analytical/microbiological grade, obtained from commercial vendors.

Enrichment of lipolytic Bacteria

20 gm of each sample (OC1 to OC12) was dissolved in 100 ml of double distilled water and filtered with the help of sieve. 5 ml of the filtrate was inoculated into 45 ml of sterile enrichment media broth [5g/l $(NH_2)_2SO_4$, 0.5 g/l K_2HPO_4, 0.3 g/l $MgSO_4.7H_20$, 5% Yeast extract, 5.0 g/l Olive oil; pH 7.2]. The inoculated broth was incubated for 48 hours at 37^0C and 120 rpm in rotary shaker incubator (CIS 24BL Remi, Mumbai).

The enriched cultures thus obtained, were subjected to subsequent 7 rounds of sub-culturing using the same enrichment media and incubation conditions as for the 1st round but the concentration of yeast extract (carbon source) was serially decreased to 0.1% (w/v) and that of pectin was increased serially by 2.5g/l at every round of sub-culturing. The microbial growth was measured spectrophotometrically by measuring optical density (O.D.) at wavelength of 590nm using spectrophotometer (Systronic 106). All the experiments were carried in triplicates with control.

Isolation and Screening of Lipolytic Bacteria

The Round 7th enriched cultures were serially diluted and were plated onto Tributyrin agar base containing 0.5% (w/v) peptone, 0.3% (w/v) yeast extract, 1% (v/v) Tributyrin and 2% agar with pH 7.0 by spread plate method (Sarda *et al*., 1998). The inoculated plates were incubated for 48 hours at 37^0C and zone of clearance were observed due to hydrolysis of tributyrin by lipase (Sztajer *et al*.,

1988). The isolate (OC_2) showing the maximum zone of clearance was selected for further analysis. The isolate OC_2 was analysed for morphological and biochemical characteristics for its identification, according to the Bergey's Manual of determinative bacteriology (Holt *et al*., 1996).

Lipase Assay

The selected bacterium (OC2) was assayed for extracellular lipase production using titrimetric method using olive oil as a substrate. Olive oil (10% v/v) was emulsified with gum Arabic (5% w/v) in 100 mM potassium phosphate buffer with pH 7.0. 10 µl of cellular extract/partially purified lipase was added to the emulsion and incubated for 15 min. at 37^{0}C. The reaction was stopped and fatty acids were extracted by addition of 1.0 ml of acetone: ethanol solution (1:1). The amount of fatty acid liberated was estimated by titrating with 0.05M NaOH until pH 10.5 using phenophathelin as indicator (Jensen, 1983). One unit of lipase activity is defined as the amount of enzyme required to hydrolyse µmol of fatty acids from triglycerides.

$$\text{Lipase Activity (Units/ml)} = \frac{\text{Vol of alkali consumed} \times \text{Normality of NaOH}}{\text{Time of incubation} \times \text{Vol. Of enzyme solution}}$$

Optimization of Bioprocess parameters for Lipase production by Isolate OC_2

a) Incubation period: Isolate OC_2 was cultured in Tributyrin broth and 1% (w/v) olive oil at 37^{0}C in orbital shaker at a rotary speed of 130 rpm for 4 days (96 hours). The crude broth was harvested, aseptically, at every 12 hours interval by high speed cooling centrifugation at 10,000 g for 30 min at 4^{0}C. The supernatant collected was used as crude enzyme solution and was assayed for enzyme activity.

b) Temperature: For selecting optimum temperature for lipase production by isolate OC_2, the incubation temperatures varying from 25^{0}C -45^{0}C were selected, keeping the remaining parameters same, except the incubation period as standardized above.

c) pH: A range of medium pH from 5.0 to 9.0 was scanned for determining the optimal pH for lipase production by the isolate OC_2, keeping other parameters unchanged except for the incubation time and temperature, as optimized.

d) Agitation speed: To optimize the agitation speed for the maximum production of lipase by OC_2, the isolate was cultured in orbital shaker incubator at varying rotary speed from 110-160 rpm alongwith a stationary culture. The other parameters were as per optimized conditions or kept unaltered.

e) Carbon source: To evaluate different C-source for maximum lipase production by the isolate OC_2, olive oil (1% w/v) present in the culture media was replaced with different oils like sunflower oil, mustard oil, soyabean oil, coconut oil, groundnut oil, palm oil and ghee, with the respective final concentration of 1% (w/v). The other parameters were as per their respective optimized value.

Lipase purification

The partial purification of lipase, produced by the isolate OC_2 under optimized condition, was carried out by salting out precipitation method. The supernatant of the fermented broth culture of OC_2, containing extracellular lipase, was treated with0.4M $CaCl_2$ for precipitating the fatty acids. It was followed by centrifugation at 15,000 rpm and 4^{0}C for 30 min. The supernatant was collected in a beaker and chilled acetone was added slowly to it, with continuous stirring, to obtain 70% (v/v) final concentration. It was then kept at 20^{0}C for 6 hours to allow protein precipitation. The precipitate was harvested by centrifugation at 15,000 rpm and 4^{0}C for 30 min. The pellets thus obtained were re-suspended in 35 ml of 20nM Tris-HCl buffer (pH 8.0) to allow the solubilization of proteins. The unsolubilized proteins were removed by centrifugation at 15,000 rpm and 4^{0}C for 30 min. The supernatant was then subjected to overnight dialysis against the same buffer. The protein content [estimated by Folin's Lowry Method (Lowry *et al,* 1951; Hartree, 1972)] and lipase activity (as described earlier) were determined after each step.

RESULTS AND DISCUSSION

Enrichment, Screening & Isolation of Lipolytic Bacteria

5 out of 12 collected oil cake samples showed high microbial density after 7th round of enrichment (Fig.1) and were selected for screening. These samples (OC_2, OC_5, OC_7, OC_9 and OC_{10}) when plated onto the tributyrin agar base for screening of their lipolytic activity showed OC_2 with maximum zone of clearance, indicating maximum lipolytic activity. Therefore OC_2 was selected for further study and its pure culture was obtained with its glycerol stock maintained at -20^{0}C.

Identification of OC_2

The isolate OC_2 was identified as *Staphylococcus* sps., based on staining, morphological and biochemical characterization (Table 1). Lipolytic bacterial strains (*Bacillus* sps., *Staphylococcus* and *Lactobacillus*) have been reportedly isolated earlier from oil mill wastes (Mohan *et al*., 2008), oil contaminated soils (Sirisha *et al*., 2010), cocnut milk (Padmapriya *et al*., 2011) with comparative enzyme yield and activity.

Optimization of Bioprocess parameters for Lipase production by Isolate OC_2

Isolate OC_2 showed maximum lipase activity after 48 hours of incubation (Fig. 2) at 40^{0}C (Fig. 3) and pH 7.0 (Fig. 4) with 140 rpm agitation speed (Fig. 5). The enzyme activity was found to be maximum for olive oil followed by palm oil and groundnut oil (Fig. 6) while optimizing the process for C-source. The results show that lipase production by the isolate OC_2 vary widely under different growth conditions and indicates that medium supplemented with triglycerides (olive oil) enhances the enzyme production. The results are in good accordance with earlier reports where triglycerides supplementation in the medium has been shown to be indispensable for lipase production (Del Rio *et al.,* 1990; Marek and Dednarski, 1996; Cihangir and Sarikaya, 2004; Mohan *et al.,* 2008; Sirisha *et al.,* 2010; Papdampriya *et al.,* 2011). The high agitation speed of 140 rpm, required for maximum lipase production by OC_2 indicates its elevated aeration requirement. Also, shaking may also be required to create a condition for better bioavailablity of nutrients, thus confirming the earlier reports (Nahas, 1998; Rao *et al.,* 1993; Cihangir and Sarikaya, 2004; Sirisha *et al.,* 2010).

Lipase purification

The partial purification of lipase enzyme from the crude culture filtrate of isolate OC_2 resulted in almost 2.5 fold purification with about 80% recovery. The enzyme yield is good but requires further investigation for proper downstream processing, so that its commercial value could be increased.

Lipase characterization

The partially purified lipase enzyme produced by the isolate OC_2 was found to show maximum activity at pH 7.0 (Fig. 7a) and temperature of 40^0C (Fig.7b), after 1 hour of incubation with substrate. There have been earlier reports of microbial lipases with similar characteristics of being stable at temperature up to 50^0C and in pH range of 2.0-10.5 (Rajeswari *et al*., 2010; Padmapriya *et al*., 2011).

Table 1.Morphological and Biochemical characteristics of isolate OC_2

S. No.	Test/Substrate/Parameter	Result
1	Gram Staining	Positive
2	Morphology (Negative staining)	*Cocci*
3	Citrate	Negative
4	Nitrate reduction	Positive(Weak)
5	Catalase	Positive
6	Gelatin liquefaction	Negative
7	Oxidase	Negative
8	Glucose fermentation	Positive
9	Sucrose fermentation	Positive
10	Lactose fermentation	Positive
11	Methyl red	Negative
12	Indole	Negative
13	Urease	Negative
14	Voges-Proskauer	Negative

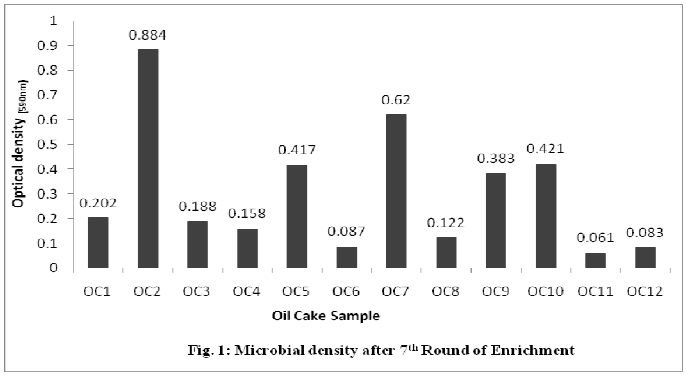

Fig. 1: Microbial density after 7th Round of Enrichment

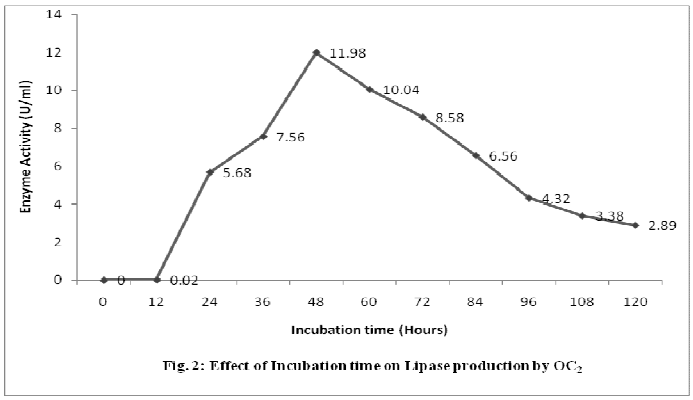

Fig. 2: Effect of Incubation time on Lipase production by OC_2

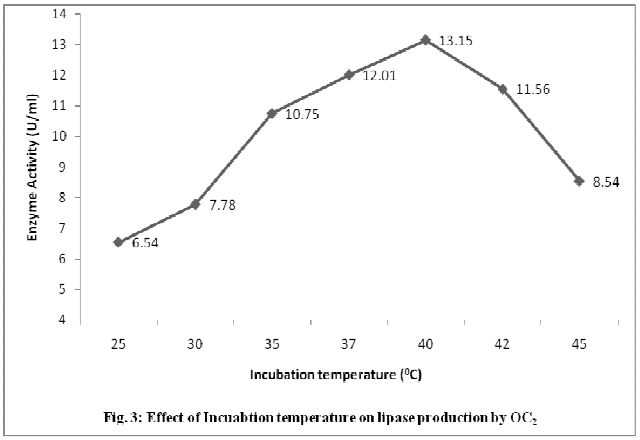

Fig. 3: Effect of Incuabtion temperature on lipase production by OC_2

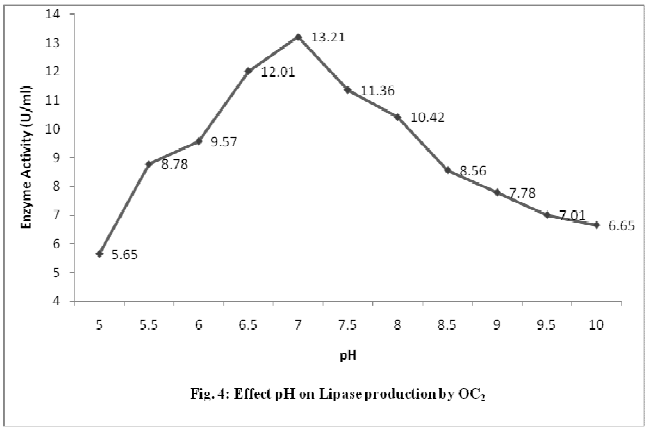

Fig. 4: Effect pH on Lipase production by OC_2

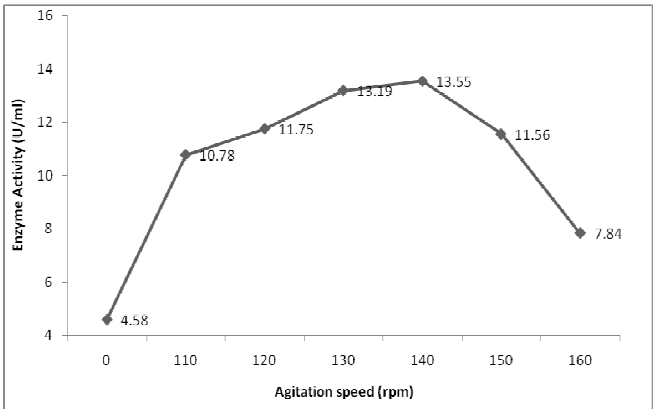

Fig. 5: Effect of Agitation speed on lipase production by OC_2

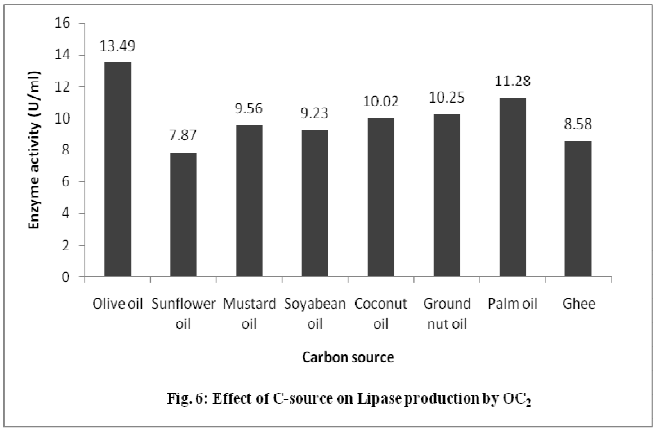

Fig. 6: Effect of C-source on Lipase production by OC_2

Table 2. Partial purification of lipase enzyme produced by isolate OC_2

Fractions	Total Vol (ml)	Enzyme activity (U/ml)	Protein (mg/ml)	Total activity (u/ml)	Specific activity (U/ml)	Purification (Fold)	Recovery (%)
Crude culture filtrate	100	13.92	6.4	1650	2.30	1.00	100
Salting out precipitation	50	34.78	4.8	1300	5.75	2.50	80.0

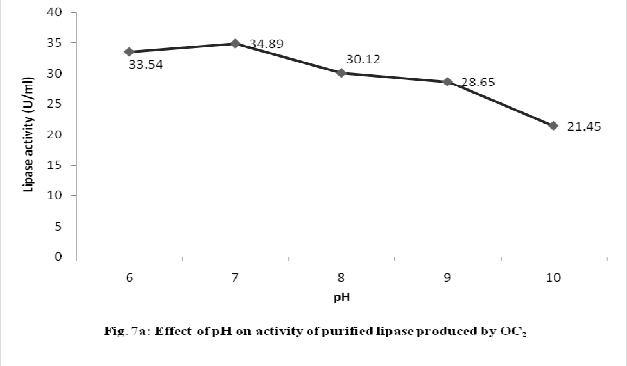

Fig. 7a: Effect of pH on activity of purified lipase produced by OC_2

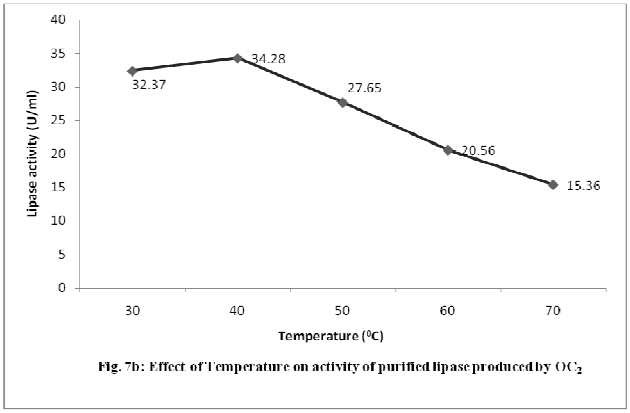

Fig. 7b: Effect of Temperature on activity of purified lipase produced by OC_2

CONCLUSION

It may be concluded from the present study that the isolate OC_2, characterized as *Staphylococcus* sps., can be used as a potential bacterial source of lipase with high thermal stability and pose to be a promising bacterial candidate for exploration at higher scales of pilot and industrial level.

ACKNOWLEDGEMENT

This research project was part of M.Sc. course in Biotechnology course and authors are thankful to the management of Instt. Of Allied Sciences & Computer Applications, ITM Universe, Gwalior, India, for providing the research facility to carry out the present piece of work.

REFERENCES

[1] Cihangir N. and E. Sarikaya. 2004. Investigation of lipase production by a new isolate of *Aspergillus* sp. *W J Microb. Biotech.* 20:193-197.

[2] Del Rio, J.L., Serra, P., Valero, F., Poch, M. and Sola, C. 1990. Reaction scheme of of lipase production by Candida rugosa growing on olive oil. *Biotechnology Letters* 12: 835–838.

[3] E. Sirisha, N. Rajasekar and M. Lakshmi Narasu. 2010. Isolation and optimization of lipase producing bacteria from oil contaminated soils. *Adv. Biol. Res*. 4 (5): 249-252.

[4] Elibol, M. and D. Ozer. 2000. Influence of Oxygen Transfer on Lipase Production by Rhizopus arrhizus. *Process Biochem*. 36: 325-329.

[5] Hartree, E.E. 1972. Determination of protein; A modification of the Lowry method that gives a linear photometric response. *Anal. Biochem*. 48, 422-427.

[6] Jensen, R.G. 1983. Detection and determination of lipase (acylglycerol hydrolase) activity from various sources. *Lipid* 18: 650-657.

[7] Kamini, N.R., T. Fuji, T. Kurosu and H. Iefuji. 2000. Production, purification and characterization of an extracellular lipase from the yeast, Cryptococcus spS2. *Process Biochem*. 36: 317-324.

[8] Kazlauskas, R.J. and U.T. Bornscheuer. 1998. Biotransformation with lipases. *Biotechnology, New York: VCH*. 8: 37-192.

[9] Kumar A. and R. Sharma. 2012. Production of alkaline pectinase by bacteria (Cocci sps.) isolated from decomposing fruit materials. *J. Phyto*. 4(1):1-5.

[10] Lee, S.Y. and J.S. Rhee.1993. Production and partial purification of a lipase from Pseudomonas putida 3SK. *Enzyme Microb Technol*. 15: 617-24.

[11] Lowry O.H., Rosebrough N.J., Farr A.L. and Randall R.J.1951. Protein measurement with the Folin phenol reagent. *J.Biol.Chem.* 193: 265-275.

[12] Marek, A. and Bednarski, W. 1996. Some factors affecting lipase production by yeasts and filamentous fungi. *Biotechnology Letters* 18: 1155–1160.

[13] Masse, L., K.J. Kennedy and S.P. Chou .2001. The effect of an enzymatic pre treatment on the hydrolysis and size reduction of fat particles in slaughterhouse wastewater. J. Chem. Technol. Biotechnol., 76: 629-35.

[14] Mohan T.S., A. Palavesam and G. Immanvel. 2008. Isolation and characterization of lipase-producing *Bacillus* strains from oil mill waste. *Af. J. Biotech*. 7(15): 2728-2735.

[15] Nahas, E. 1988. Control of lipase production by *Rhizopus oligosporus* under various growth conditions. *J. Gen. Microbiol.* 134: 227–233.

[16] Padamapriya B., T. Rajeshwari, E. Noushida, D.G. Sethupalan and C.K. Venil. 2011. Production of lipase enzyme from *lactobacillus* spp. and its application in degradation of meat. *World App. Sc. J.* 12(10): 1798-1802.

[17] Pandey, A., S. Benjamin, C.R. Soccol, N. Poonam, N. Krieger and V.T. Soccols. 1999. The realm of microbial lipases in biotechnology. *Biotechnol. Appl. Biochem*. 29: 119-131.

[18] Rajeswari, T., M. Palaniswamy, C.K. Venil, K. Nathia and P. Joyruth. 2010. Production, partial purification and characterization of lipase from Aspergillus flavus KUF108.

Pak. J. Sci. Ind. Res. 53(5): 258-264.

[19] Rao, P.V., Jayaraman, K. and Lakshmanan, C.M. 1993. Production of lipase by *Candida rugosa* in solid state fermentation: Medium optimization and effect of aeration. *Process Biochemistry* 28: 391– 395.

[20] Rubin, B. and E.A. Dennis. 1997. Lipases: Part B, *Enzyme characterization and utilization Methods in enzymology. New York: Academic Press*. 286: 1-563.

[21] Rubin, B. and E.A. Dennis, Lipases: Part A. 1997. *Biotechnology Methods in enzymology. New York: Academic Press*. 284: 1-408.

[22] Sarkar B.S., S. K. Sreekanth, R. Banerjee and B.C. Bhattacharyya. 1998. Production and optimization of microbial lipase. *Bioprocess Engineering* 19: 29-32.

[23] Sztajer, H., Maliszewska, I. and Wieczorek, J. 1988. Production of exogenous lipases by bacteria, fungi and actinomycetes. *Enzy. Microb. Tech.* 10:, 492–497.

[24] Takamoto, T., H. Shirasaka, H. Uyama and S. Kobayashi. 2001. Lipase-catalyzed hydrolytic degradation of polyurethane in organic solvent. *Chem Lett*., 6: 492-3.

[25] Walavalkar G.S. and M.M. Bapal .2002. *Staphylococcus Warneri* BW 94 A new source of lipase. *Indian J. Exp. Biol.* 40:1280-1284.