



PRODUCTION AND APPLICATION OF LIPASE FROM *RHIZOPHORA MUCRONATA*

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ABSTRACT

The *Rhizophora mucronata* sample was collected from coastal area of Thrissur district, Kerala. The bacteria was isolated from the sample by using pour plate technique and the bacteria was tested to produce lipase by growing them on Olive oil containing agar medium for 1 day. It was screened by observing zones around the colonies. Then it was inoculated into lipase production medium. Parameters like pH, temperature, were optimized. Enzyme activity was measured by Bradford's method at 595 nm. The results showed that the optimum temperature for lipase enzyme production was 40°C and pH 7. The enzyme was partially purified by ammonium sulphate precipitation and dialysis. After partial purification, application of lipase like flavor enhancement, grease removal and oil degradation capacity were studied.

KEY WORDS

Olive agar medium, *Rhizophora mucronata*, Bradford's method, *Pseudomonas spp.*

INTRODUCTION

The demand for industrial enzymes of microbial origin is greatly increased due to their application in a wide range of processes. Lipase is one of the most useful enzymes in industry. It can be produced by plants, animals and microorganisms. Bacteria, which has high growth rate as compared to fungi has good potential to be used in lipase production. Lipases have emerged as one of the leading biocatalysts with proven potential for contributing to the multibillion dollar underexploited lipid technology bio-industry and have been used in *in situ* lipid metabolism and *ex situ* multifaceted industrial applications (Sharma C.K. and Kanwar S.S., 2012). [1] Lipases differ greatly as regards both their origins (which can be bacterial, fungal, mammalian, etc.) and their properties, and they can catalyze the hydrolysis, or synthesis, of a wide range of different carboxylic esters and liberate organic acids and glycerol. They all show highly specific activity towards glyceridic substrates

(F.Hasan *et al.*, 2006).[2] Compared to plant & fungal lipases, a relatively small number of bacterial lipases have been well studied & reviewed (Sugiura M., 1984; Brune A.K. and Godtz F., 1992).[3] Among *Pseudomonads*, three important species of *Pseudomonas* i.e. *P. fragi*, *P.fluorescens* and *P.aeruginosa* have been exploited for lipase production (Nishio T., Chikano T. and Kamimura M., 1987). [4] An endophyte is an endosymbiont, often a bacterium or fungus, that lives within a plant for at least part of its life without causing apparent disease. Endophytes are ubiquitous and have been found in all the species of plants. Endophytes promote plant growth and yield, suppress pathogens, and may help to remove contaminants, solubilize phosphate, or contribute assimilable nitrogen to plants. [5] Mangrove plants have adapted to a unique habitat with muddy saline water, anaerobic soil, brackish tidal activities and high microbial and faunal Competition.

Rhizophora mucronata is commonly known as mangrove growth in the tropical and subtropical region coastlines. It helps to maintain marine life and balances the ecosystem. It is a much-branched large shrub or moderate sized tree, up to 10 m tall, supported on adventitious prop roots from stem and branches with reddish brown bark distributed throughout largest mangrove forest. Malaysians use old leaves and roots for childbirth, Burmese use the bark for bloody diarrhoea. [6]

MATERIALS AND METHODS

(I) Sample collection

The mangrove plant samples were collected from Thrissur, Kerala. They were stored at 4°C.

(II) Preparation of inoculum

The plant leaf sample was taken, and surface sterilized with 70% ethanol and distilled water. One gram of sample was grinded along with 10 ml of sterile distilled water by using clean mortar and pestle. The slurry was filtered by Whatman's filter paper and collected in a sterile bottle. This was the inoculum for lipase enzyme production. [7]

(III) Isolation of endophytic bacteria

Nutrient agar media which contain peptone, beef extract, sodium chloride and agar for bacterial isolation was prepared and sterilized. 1 ml of inoculum was taken and serially diluted. 0.1 ml of serially diluted sample was poured into a Petri plate and evenly distributed to whole of the plate. Above the sample cooled media was poured and kept it for solidification. After the solidification the plates were kept at 37° C for 24 hours and examined for colony formation. The strains were purified by multiple streaking techniques and used for screening of endophytic bacteria for lipase production. [8]

(IV) Screening and identification of lipase producing bacteria

The isolated bacteria were screened to produce lipase by growing them on Nutrient agar medium containing Olive oil for 24 hours. (using the modified method of Kauker G and Jaeger K E, 1987). [9] The lipase producing bacteria were screened by observing zones around the colonies. The maximum zone of clearance was selected for further analysis.

(V) Various tests like Gram's staining, other biochemical tests like Indole production, Methyl- red test, Voges-proskaver test, Citrate utilization test, Spore staining were carried out using suitable reagents and standard procedures to identify the bacteria. [10]

(VI) Production of lipase enzyme

The organism with a maximum productivity was selected for enzyme production and an inoculum of 24hrs NB culture was prepared. 5ml of the inoculum was added to 95ml of production medium containing waste cooking oil as substrate in submerged fermentation and incubated for 24hrs at different temperatures and pH for optimization (Modified method of Qamsari *et al*, 2011). [11]

(VII) Optimization

Influence of different temperature for lipase enzyme production

The pH of the medium was maintained at 7 and the incubation temperature was varied as 20°C, 30°C and 40 °C. Production medium was prepared in 3 conical flasks (pH kept constant at 7) and incubated at corresponding temperatures using shaker incubators. and estimated from the crude enzyme extract. [12]

Influence of different pH for cellulase enzyme production

The incubation temperature was kept constant at 30°C and three different pH were set, such as pH-6, 7 and 8, using 0.5N NaOH and 0.5N HCl for adjusting the pH. For this, production medium was prepared in 3 conical flasks, each with a different pH (6, 7, 8) and all the 3 are incubated in a shaker incubator.

(VIII) Lipase assay

The enzymatic activity of lipase was determined titrimetrically by olive oil hydrolysis method (Borkar *et al*, 2009). [13] and the optimum pH and temperature for enzyme production were determined through the enzyme activity assay. 0.5ml of 1N NaCl, 0.5ml of 50mM CaCl₂, 4ml of phosphate buffer (pH 7) were added together and mixed well using a magnetic stirrer and the pH was maintained at 7. Then 5ml of olive oil was added and an emulsion was prepared by continuous stirring followed by the addition of 1ml of crude enzyme solution.

After 15min, the pH was checked and adjusted to 7 by the addition of 50mM NaOH, and the volume of NaOH added was noted.

$$\text{Enzyme Activity} = \frac{\text{Volume of NaOH used (ml)} \times \text{Concentration of NaOH} \times 1000}{\text{Amount of enzyme used (ml)}}$$

Unit of enzyme activity is defined as 1 micro equivalent of fatty acid hydrolyzed from a triglyceride in 1 hour at pH 7 at 30°C.

Estimation of Enzyme Protein:

The amount of enzyme present in the final solution was estimated using Bradford's (1976) method of protein estimation using a series of working BSA standards and enzyme solution as test with a final volume of 1ml in test tubes.

2.5ml of Bradford's reagent was added to all the tubes, mixed thoroughly using a vortex mixer and the absorbance was read at 595nm using a spectrophotometer.

(IX) Partial purification of enzyme

Ammonium sulphate precipitation

The sample was centrifuged in a refrigerated centrifuge at 6000 rpm for 15 minutes at 4°C and the supernatant was collected. The ammonium sulphate was added for different cut offs referred from the precipitation table to known volume of supernatant placed in a cooling bath on the top of a magnetic stirrer. The pellet was resuspended in 1-2 volume of buffer and ammonium sulphate can be removed by dialysis after being spinned at 10,000 rpm at 4°C for 20 min. [14]

Dialysis

Dialysis is a process of removal of small molecules from a sample containing mixture of both large and small molecules. Semi permeable membrane can be used for dialysis.

$$\text{COD mg/l} = \frac{\text{Volume of FAS} \times \text{Normality of FAS} \times 1000 \times \text{Dilution factor}}{\text{Volume of sample used}}$$

RESULTS AND DISCUSSION

The bacteria were isolated from *Rhizophora mucronata* by pour plate method. (Fig No 1) The identification method was followed to detect the isolated organism. From the preliminary test of gram staining a pink color rod shaped organism was observed. This indicates the organism is gram negative and this organism showed negative result for endospore staining. (Fig No 2 & 3). For the confirmation of organism Biochemical tests were followed (Table 1); Based on the results, 2 colonies

(IX) Applications of lipase:

The partially purified lipase was applied for the following processes: -

- **Flavour enhancement of butter:** A small quantity of butter (approximately 2g) was taken in a clean dry petridish and 1ml of lipase was added and incubated at 37°C for 10-15min for better flavour development.
- **Removal of Grease Stain:** A small piece of white cotton cloth was stained with grease and an appropriate (based on the level of stain) quantity of lipase was added onto it and kept for 10-20min (incubated at 37°C if necessary) for the removal or reduction in the stain.
- **Oil Degradation from Effluent:** (Felice *et al*, 2004) Effluent containing oil wastes was collected from SSD Oil Mills Company Limited, Vadavalli, Coimbatore. The COD of the effluent sample was estimated by adding 10ml of 0.25N potassium dichromate solution, a pinch of mercuric sulphate and 30ml of conc. H₂SO₄ to 20ml of the sample and refluxing for 2hrs directly on the flame or a hot plate, and then cooling for 10min and adding 90ml of distilled water, then finally titrating against standardized FAS solution using ferroin indicator (the end point was the colour change from pink to green).

were found to be *Pseudomonas spp.* and were named as Psp1 and Psp2. (Fig No 4,5,6,7,8,9 & 10).

Lipase Productivity Test:

After incubation, the NA plates with 1% (w/v) olive oil were observed for the formation of clear zone around the colonies. Among the 2 colonies plated – Psp1 and Psp2, the colony Psp2 showed a more visible clear zone than that of Psp1. Therefore, Psp2 was selected for lipase production. (Fig No 11).

Lipase Assay and Optimization of Culture Conditions:(Fig No 12)

$$\text{Enzyme Activity} = \frac{\text{Volume of NaOH used (ml)} \times \text{Concentration of NaOH} \times 1000}{\text{Amount of enzyme used (ml)}}$$

pH:

From the results, it is clear that the enzyme activity of the crude extract obtained from the medium with pH 7 was relatively high (33.91 IU) when compared to that of pH 6 (15.12 IU) and pH 8 (22.30 IU). Therefore, it indicates that the concentration of enzyme presents in the medium with pH 7 is higher than that in the other flasks. (Table 2) (Fig No 13)

Temperature:

From the results (Table 3) & (Fig No 14) it was observed that the optimum temperature for enzyme production was 40°C.

Estimation of Enzyme Protein:

The concentration of lipase after partial purification was estimated as **81µg/ml**. (Fig No. 15)

Applications of Lipase: (Fig No 16 &17)

- **Flavour enhancement of butter:** After incubation at 37°C for 10min, a pleasant aroma of modified flavour of butter was developed.
- **Removal of grease stain:** A considerable reduction in the grease stain was observed after 25min of incubation.
- **Oil degradation from effluent:** The COD content of the effluent sample was set as the measurement of oil degradation occurred by the action of lipase upon it, since COD is a measurement of degradable organic compounds present in a liquid, which can be chemically oxidized by the addition of a strong oxidizing agent such as potassium dichromate. (Table No 4)

$$\text{COD mg/l} = \frac{\text{Volume of FAS} \times \text{Normality of FAS} \times 1000 \times \text{Dilution factor}}{\text{Volume of sample used}}$$

Where,

$$\text{Normality of FAS} = \frac{\text{Volume} \times \text{Normality of potassium dichromate}}{\text{Volume of FAS}}$$

From the interpretations, it can be concluded that nearly **60%** of the oil present in the effluent sample was degraded by lipase, which is evident from the difference

in COD values before and after enzymatic digestion by lipase. TABLES & FIGURES.

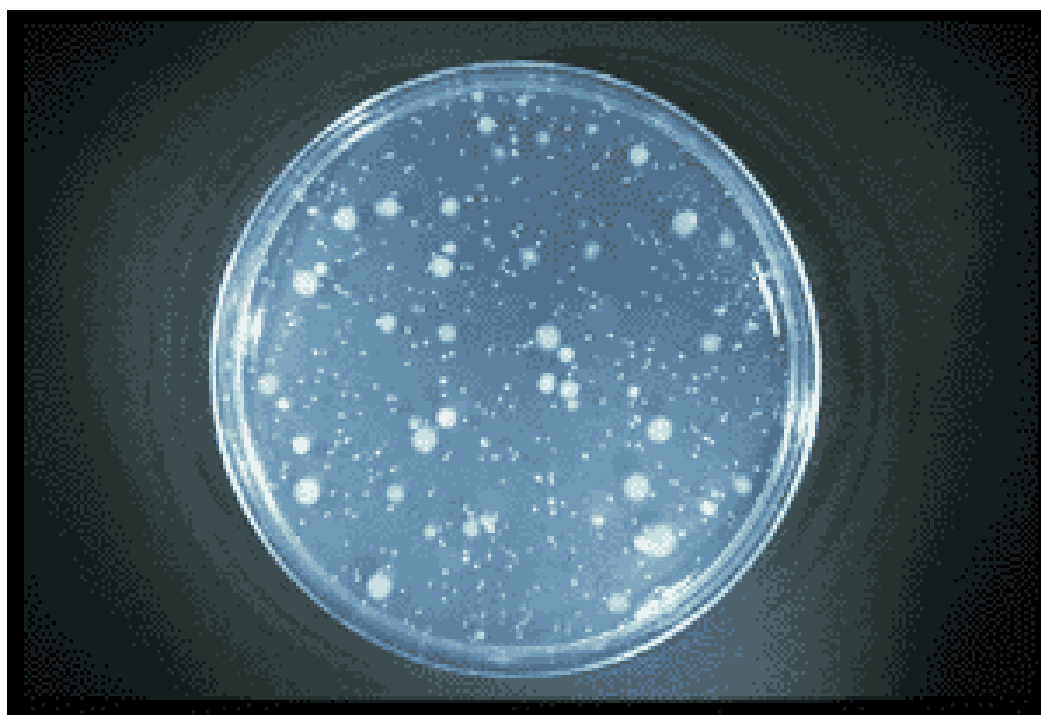


Fig No 1. Bacterial colonies obtained after pour plate method.

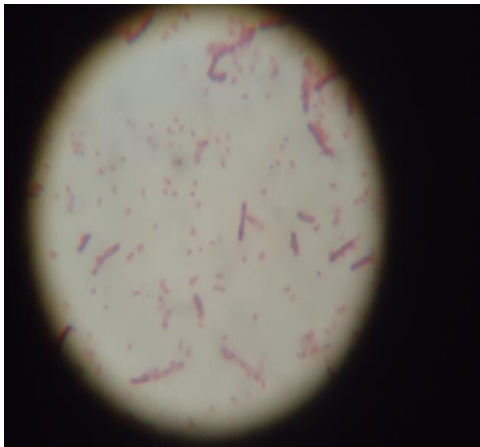


Fig No. 2. Gram staining.

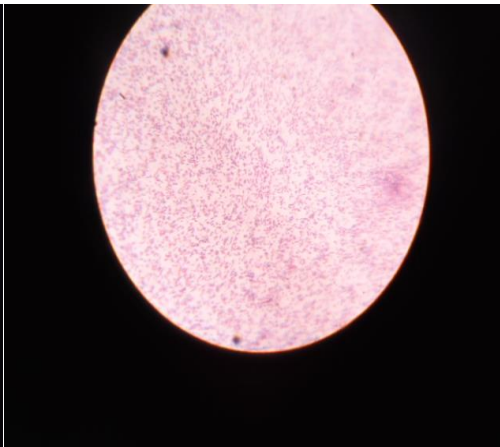


Fig No 3. Spore staining.

Test / Substrate	Result for <i>Pseudomonas</i> colonies
Motility	+
Indole	-
Methyl Red	-
Voges-Proskauer	-
Citrate	+
Oxidase	+
Urease	-
Triple Sugar Iron	-

TABLE 1: Biochemical tests and the results.



Fig No.4 Methyl red test



Fig No. 5 Indole production test



Fig No 6. Voges Proskauer test.



Fig No 7: Citrate utilization test.



Fig no. 8 Oxidase test



Fig No 9. Urease test.



Fig No 10. Triple Sugar Iron Test

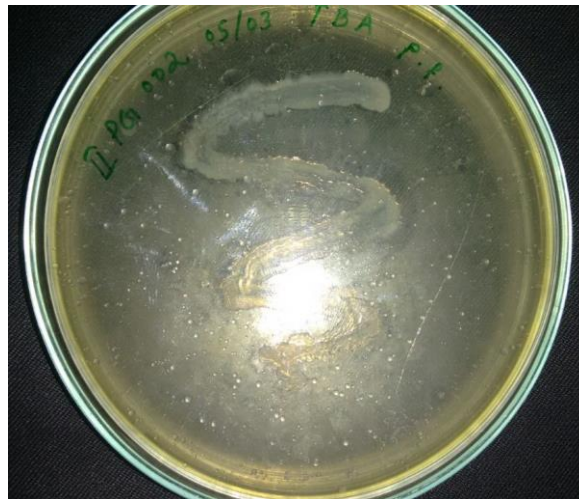


Figure 11: Clear zone formation around Psp2 culture.



Fig No 12. Optimization of culture conditions. (Ph & temperature)

Relative Activity (%)	pH of Production Medium
44.5	6
100.0	7
65.7	8

Table 2: Relative activity of lipase corresponding to pH of the medium.

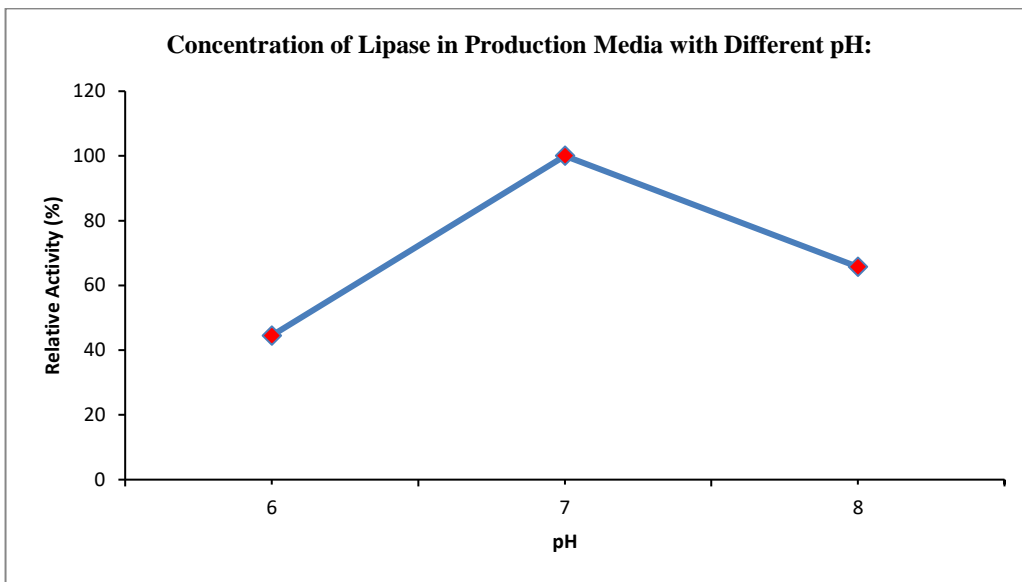


Figure 13: Effect of pH on lipase production.

Relative Activity (%)	Incubation Temperature
20.4	20°C
66.1	30°C
100	40°C

Table 3: Relative activity of lipase corresponding to the temperature of production medium.

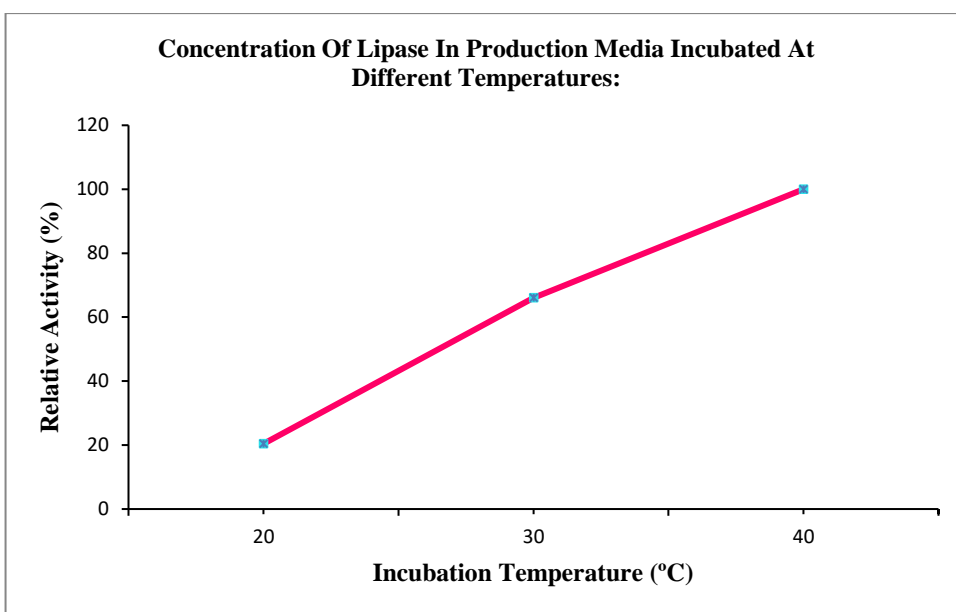


Figure 14: Effect of temperature on lipase production.

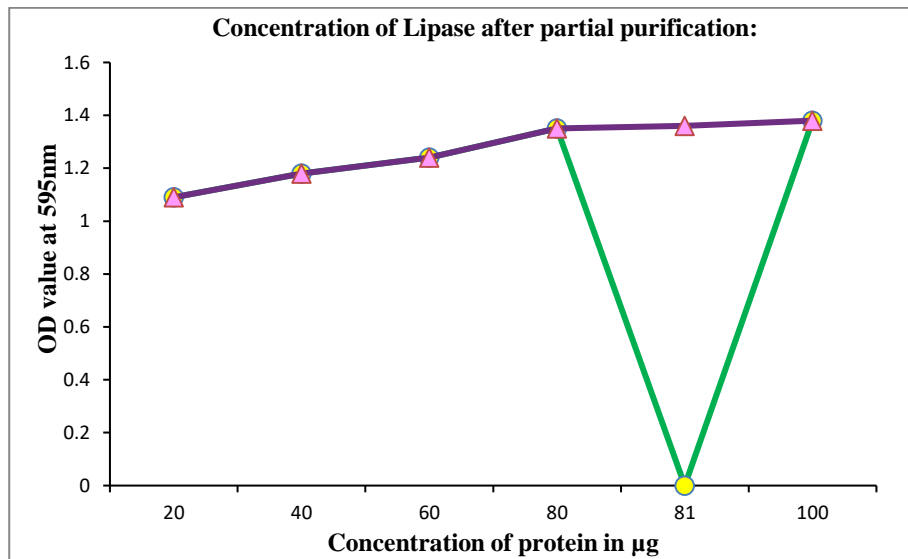


Figure 15: Protein estimation by Bradford's method.



Figure 16: Flavour enhancement of butter.

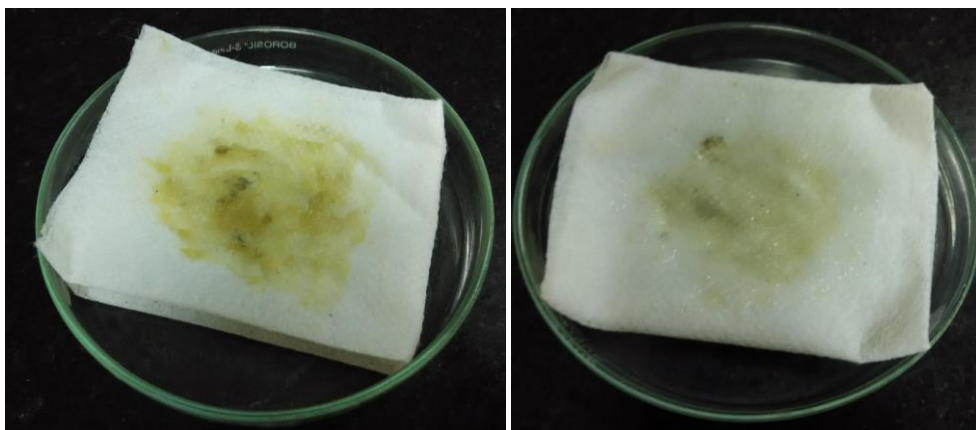


Figure 17: Removal of grease stain (Before and after enzyme application).

Estimation	Calculation	Result
Normality of FAS (N)	5x0.25/12.1	0.1033
COD before addition of lipase (mg/l)	20.2x0.1033x1000x100/20.0	10433.3
COD after lipase addition and incubation (at 37°C with shaking for 15min) (mg/l)	8.0x0.1033x1000x100/20.0	4132.0

Table 4: Oil degradation from effluent by lipase.

CONCLUSION

Many attempts have been made to isolate lipase producing microorganisms since this enzyme is used in numerous biotechnological processes including food, leather, cosmetic, detergents and pharmaceutical industries and industrial wastes management etc. Microbes are the never-ending sources of enzymes and are considered far more reliable than plant and animal sources. Bacterial lipases are mostly extracellular and are greatly influenced by nutritional and physico-chemical factors, such as temperature, pH, nitrogen and carbon sources, inorganic salts, agitation and dissolved oxygen concentration. Among bacterial lipases, attention has usually been focused on particular classes of enzymes such as the lipases from the genus *Pseudomonas*, which are especially interesting for biotechnology.

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