International Journal of Technical Research and Applications e-ISSN: 2320-8163,
www.ijtra.com Volume 3, Issue4 (July-August 2015), PP. 390-397

PARTIAL PURIFICATION OF A BIOCHEMICALLY ACTIVE AND STABLE LIPASE FROM A MICROORGANISM ISOLATED FROM DIESEL CONTAMINATED SOIL

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***Abstract*— Lipases are defined as triacylglycerol acylhydrolases (EC 3.1.1.3) and are involved in the hydrolysis of fats and oils to yield glycerol and free fatty acids. A bacterial strain, isolated from the soil contaminated with diesel oil exhibited lipolytic activity on tributyrin agar plate with remarkable zone of clearance. The isolated strain was identified as gram positive, rod shaped bacteria, while the biochemical result showed that the isolate was catalase, urease, citrate utilization and methyl red positive; VP test was negative. The bacterial strain was found to produce extracellular as well as intracellular lipase. Maximum extracellular lipase activity in the culture supernatant was found to be 28.5 U/ml and the maximum intracellular lipase activity obtained from pellet was 5.6 U/ml upon enzyme assay. Lipase was partially purified using ammonium sulphate precipitation and the maximum lipase activity was recorded in the fraction precipitated by 30-60% saturation. Protein content in purified lipase was measured by lowry method and was found to be 0.5 mg/ml. Partially purified lipase showed fairly appreciable thermostablity activity up to 55-60ºC but a marked decrease was observed in the activity above this temperature. The lipase activity remained stable in the pH range of 2.0–10.0. The effect of detergents were studied after incubating the purified lipase with 1% detergents like tween-20, tween-80 and SDS. Addition of detergents, like tween-20, tween-80 did not alter the lipase activity, while SDS resulted in the loss of enzymatic activity. Incubation of the isolated lipase with organic solvents like isopropanol, toluene, hexane, ethanol, methanol and xylene, showed that 90% activity remained even after 12 hrs incubation; thus indicating its chemically stable nature. The isolated strain was found to esterify oleic acid with ethanol resulting in the formation of ethyl oleate which was confirmed by thin layer chromatography**

***Index Terms*— Ammonium sulphate, detergents, esterification, lipolytic, olive oil.**

I. INTRODUCTION

The use of enzyme -mediated processes can be traced to ancient civilization. Today, nearly 4000 enzyme are known, and of these, about 200 are in commercial use. Lipases are defined as triacylglycerol acylhydrolases (EC 3.1.1.3) and are involved in the hydrolysis of fats and oils to yield glycerol and free fatty acids[1]. Lipases belong to the class of serine hydrolases and do not require any cofactor. Under natural condition lipases catalyze the hydrolysis of ester bonds at the interface between an insoluble substrate phase and the aqueous phase where the enzyme remains dissolved. Apart from hydrolysis, they bring about a wide range of conversion reaction that include interesterifications, alocholysis and aminolysis. The unique characteristics of lipase include substrate specificity, stereospecificity, regiospecificity and the ability to catalyse a heterogeneous reaction at the interface of water soluble and water insoluble systems[2].

Lipases are produced by plants, animals, bacteria, yeast and molds. The microbial enzyme is advantageous over the enzyme of the plants and animals because of the vast variety of catalytic activities, stability, possible high yields, genetic manipulation and rapid production using inexpensive media[3]. Bacterial strains are generally more used as sources of lipases because they offer higher activities compared to yeast[4]. Although a number of lipase-producing bacterial sources are available, only a few are commercially exploited as wild or recombinant strains[5]. Of these, the important ones are: *Achromobacler*, *Alcaligenes*, *Arthrobacter*, *Bacillus*,

Chromobacterium and *Pseudomonas*. Of these, the lipases from *Pseudomonas* bacteria are widely used for a variety of biotechnological applications[6]. Microbes being ubiquitous in distribution, are highly successful at surviving in a wide range of environmental conditions owing to their great plasticity and physiological versatility and have been the subject of several reviews[2]. The physico-chemical fermentation parameters such as pH of the medium, particle size, nature of particles and microbial inoculum's level play crucial role in lipase production[7]. Lipase production by microorganisms is highly influenced by medium components like nitrogen and carbon sources such as fatty acids, triglycerides, sugars or complex polysaccharides like glycogen and surfactants which can stimulate or repress lipase production.

Lipases are considered to be one of the third largest groups of the industrial enzymes as they have found potential applications in detergents, oil, chemical, paper, cosmetics, perfumeries, pharmaceutical (chemotherapeutic agents) and agrochemical industries[8]. Because of their wide-ranging significance, lipases remain a subject of intensive study. Furthermore, novel biotechnological applications have been successfully established using lipases for the synthesis of biopolymers and biodiesel[9].

The most desired characteristics of the lipase are its ability to utilize all mono-, di-, and tri-gycerides as well as the free fatty acids in transterification, low product inhibition, high activity/yield in non aqueous media, low reaction time, resistance to altered temperature, pH, alcohol and reusability of immobilized enzymes[7]. Moreover stability of organic solvents is desirable in synthesis reaction[10]. Keeping all the desired parameters in view, our study was designed to isolate and partially purify a microbial lipase, which could effectively carry out esterification reaction without getting affected by varied physico-chemical factors including temperature, pH, detergents and organic solvents.

II. MATERIALS AND METHODS

A. Sample collection:

Samples were collected from soil contaminated with diesel oil from the railway station area of Jalandhar, Punjab, India.

B. Isolation of lipase producing bacteria (LPB):

The sample was serially diluted with sterile distilled water and spread on the nutrient agar plates by spread plate method which were incubated for 48 hours at 37 ºC for the growth of microorganisms. Microbial colonies, which appeared on nutrient agar (NA) plate were individually picked and sub-cultured on fresh NA plate to isolate pure bacterial culture.

Lipase producing bacterial strain were identified by qualitative screening on tributyrin agar (TBA; Hi Media) plate. Lipase producing bacteria (LPB) exhibiting zone of clearance after 48 hours of incubation at 37ºC on TBA medium containing peptone 5gm, beef extract 3gm, tributyrin 10ml and agar-agar 20gm per liter, was selected for further study.

C. Characterization of isolated LPB:

The isolate was identified by means of morphological characterization (Gram's staining) and biochemical characterization (catalase, methyl red, voges-proskeurs, citrate utilization test, urease) following Bergey's manual of determinative bacteriology[11].

D. Inoculums preparation for lipase production:

In 99ml of Tributyrin broth, 1ml of olive oil (substrate for lipase production and lipase activity) was added in a 250 ml clean glass conical flask. It was then autoclaved at 15 lbs pressure, 120ºC for 15 mins, was cooled before use. In order to prepare the inoculum; a loopful of cells from freshly grown slant of the isolated culture bacteria was transferred to tributyrin medium containing olive oil and incubated at 37ºC for 48 hours.

E. Production of extracellular lipase:

The isolated species from the primary inoculum culture was subjected to submerged fermentation in the production media (PM). The composition of this PM is shown in the table 1. This PM at pH 7, inoculums 5% at temperature 37ºC and incubation period 48 hours at 160 rpm was selected for fermentation for optimum growth of microbial cells and the cell biomass (LPB) was estimated by measuring absorbance at 420 nm. After incubation it was centrifuged at 10,000 rpm for 30 min in a refrigerated centrifuge (REMI 9001:2000 ISO Certified) and the supernatant was used for estimating extracellular lipase activity.

Table 1: Composition of production media

Components	Quantity in PM (g/l)
Peptone	5
Beef extract	3
Olive oil	1
Starch	3

F. Extraction of intracellular lipase:

After desired incubation period (48 hours), in the separation of centrifugation of PM containing LPB (at 10000 rpm for 30 mins) resulted in the separation of supernatant containing extracellular lipase and pellet which was collected, total cell biomass calculated and stored at -20ºC for further use.

To release the intracellular lipase, 0.2g of harvested cells in the pellet were suspended in 1ml of lysis buffer (0.005 M phosphate buffer, pH 7) and subjected to five rounds of cell disruption (1min) with the help of a sonicator (MSE Manor Roya Crawley RH 10 2QQ) at 15 KHz for the recovery of maximum enzyme. The sonicated cell suspension was centrifuged (1500x g for 30 min) and cell free extract was collected as the source of intracellular lipase.

G. Lipase Assay by titrimetric method:

The activity of intracellular and extracellular lipase was measured using modified titrimetric method described by[6]. The titrable solution containing 13.5 ml of 1 % (v/v) tributyrin (substrate) in 1% gum acacia solution, 500 µL of 2% calcium chloride and 1.0 ml of 1M NaCl was used. The reaction mixture was titrated against 10mM NaOH. Appearance of pink colour was taken as the end point. The lipase was calculated using the following formula:-

$$\text{Lipase activity} = \frac{\text{Volume of NaOH consumed (ml)} \times \text{Molarity of NaOH}}{\text{Volume of lipase (ml)} \times \text{Reaction time (min).}}$$

One unit of lipase activity is defined as the amount of enzymes that liberated 1µmol fatty acid per minute at 30ºC and pH 7 under the assay condition and the lipase production was determined by spectrophotomertric method by taking O.D. at 420 nm[6].

H. Partial purification of extracellular lipase:

Since appreciable amount of extracellular lipase present in the culture filtrate was obtained, hence it was subjected to partially purification by ammonium sulphate fractionation method [7]. In this technique, the culture filtrate was subjected to protein fractionation by addition of small increments of solid ammonium sulphate (differential precipitation) at 4ºC with constant stirring to obtain three fractions i.e 0-30%, 30-60% and 60-90%. When all the ammonium sulphate was dissolved at the end of each fractionation range, the mixture was allowed to stand for 30 minutes to 1 h. The mixture was centrifuged at 10,000 x g for 30 minutes at 4ºC. The pellet was collected and supernatant was used as starting material for next fractionation. The collected precipitate of each fractionation range was resuspended in small volume of 0.05M phosphate buffer pH 7.0 and checked for the enzyme activity spectrophotomertically (as mentioned under heading "lipase assay") using tween 20 and olive oil as substrate. The

protein content of lipase obtained was determined by Folin lowry method[12].

I. Thermostability of purified lipase:

The optimum temperature for enzyme activity was determined by incubating the reaction mixture at various temperatures. Themostability of lipase was determined by incubating aliquots of semipurified lipase solution in 20 mM Tris buffer (pH 7.0) at various temperature (40ºC, 60ºC, 80ºC) for 0, 1, 2 and 3 hours when tween-20 was used as substrate. Activity was measured spectrophotometrically at 400nm using the sample at 37ºC as control.

J. pH stability of purified lipase:

Stability assay was done by incubating purified extract at 37 °C for 1 h in 100 mM buffers of different pH values (KCl-HCl, pH=2.0; glycine-HCl, pH=2.5; citrate-phosphate, pH=3.0, 4.0, 5.0 and 6.0; phosphate, pH=7.0; and borate-HCl pH=9.0 and 10.0). Residual activity was then calculated considering the enzyme activity at time zero as 100 %.

K. Effect of detergents on purified lipase:

The effect of various detergents on lipase activity was analyzed by incubating the enzyme at 30ºC in 20 mM Tris buffer containing 1% (w/v) detergent viz. SDS, tween-20 and tween-80 in separate test tubes, for various time intervals (0, 0.5, 1, 1.5, 2 hrs); the control contained no detergent. Lipase activity was measured spectrophotometrically at 400nm.

L. Effect of the organic solvents on purified lipase:

The effect of organic solvents on lipase activity was analyzed by incubating the enzyme in different solvents (25% v/v) viz. isopropanol, toluene, hexane, ethanol, methanol and xylene at 30ºC for 12 hrs. The effect of different solvents was determined spectrophotometrically by determining residual activity of lipase using the sample without any organic solvent as control.

M.Esterification of lipase:

The partially purified lipase was used as biocatalyst for esterification of oleic acid and ethanol in 1:1 (v/v) ratio in hexane[7]. The reaction was carried out at 37ºC with shaking at 100rpm for 24 hrs using heat inactivated free enzyme (incubated at 100ºC for 5 min) as control. The ester formation was identified by analytical thin layer chromatography (TLC). After chromatography, TLC plates were visualized by immersion in 10% (v/v) H_2SO_4 in ethanol solution followed by heating on a hot plate.

III. RESULTS AND DISCUSSION

A. Isolation of microorganism producing lipase:

Culture containing lipase producing bacterial strains, collected from diesel contaminated soil was plated on tributyrin agar to differentiate between the lipase producing bacteria (lipolytic bacteria) and non lipase producing bacteria (non lipolytic bacteria) (Figure 1a), and the bacterial strain with maximum zone of clearance (Figure 1b) was selected for identification and characterization.

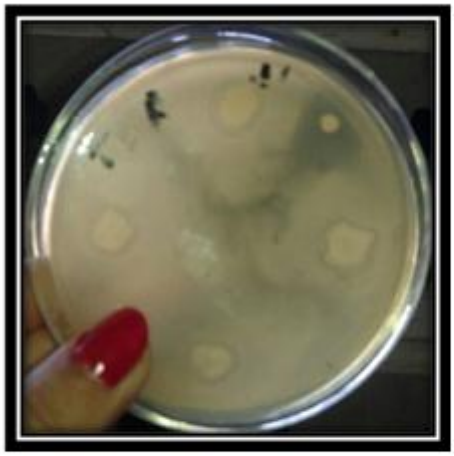

Fig. 1a: Zone of clearance on TBA plate

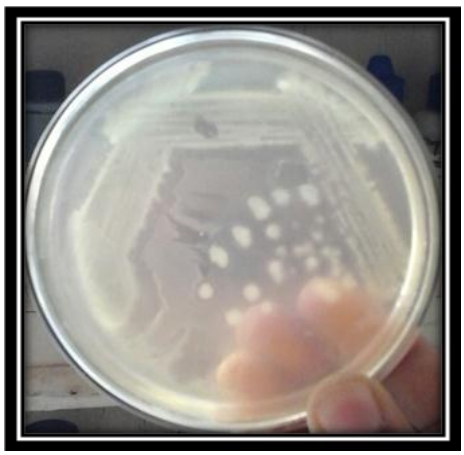

Fig. 1b: Streaking of LPB on TBA plate

B. Morphological and biochemical identification of LPB:

Morphologically, the isolated strain was identified as Gram positive, bacilli shaped colonies upon gram's staining (Figure 2).

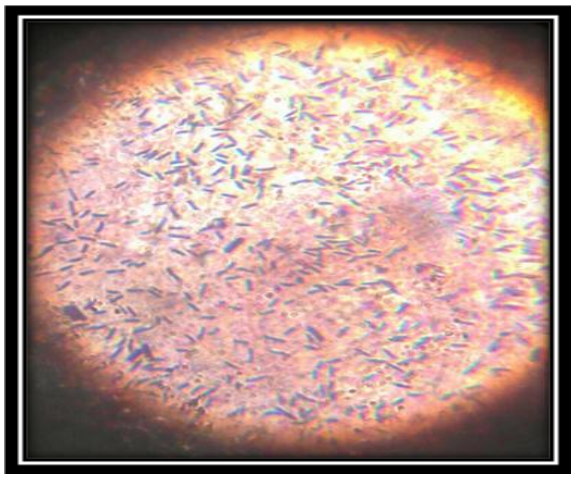

Fig. 2: Gram's staining of LPB (Gram's positive rods)

The isolated LPB was further identified using various biochemical tests like catalase, urease, methyl red, voges-proskauer, citrate utilization tests and the results are shown in table 2.

Table 2: Biochemical identification of LPB strain

Test	Results
Catalase	Positive
Citrate utilization test	Positive
Methyl red	Positive
Voges-Proskauer test	Negative
Urease test	Positive

In an earlier report on LPBs, *Bacillus* sp. isolated from similar source (agricultural soil), have shown similar characteristics i.e., catalase, citrate utilization, methyl red and urease test positive and V-P test to be negative[13].

C. Estimation of extracellular & intracellular activity:

The isolate subjected to submerged fermentation in production media was subjected to centrifugation after incubation. Using titrimetric method, maximum lipase activity of extracellular lipase present in the supernatant was found to be 28.5 U/ml whereas the intracellular lipase activity obtained from pellet was estimated to be 5.6 U /ml.

D. Partial purification and protein content determination of lipase:

Extracellular lipase which showed appreciable activity was partially purified using ammonium sulphate precipitation and the maximum lipase activity was recorded in the fraction precipitated by 30-60% saturation. Similar to our study in an earlier report on lipase from *Bacillus* sp., maximum activity was obtained at 30-60%[7]. Lipase from *Pseudomonas aeruginosa* MTCC 2488 also showed the maximum activity at 30-60% saturation[14].

The protein content in partial purified lipase was measured by Folin-lowry method (Figure.3) was determined to be 0.5 mg/ml. In an earlier report also the protein content in purified lipase obtained from *Bacillus pumilus* RK31 was found to be 0.5 mg/ml[7].

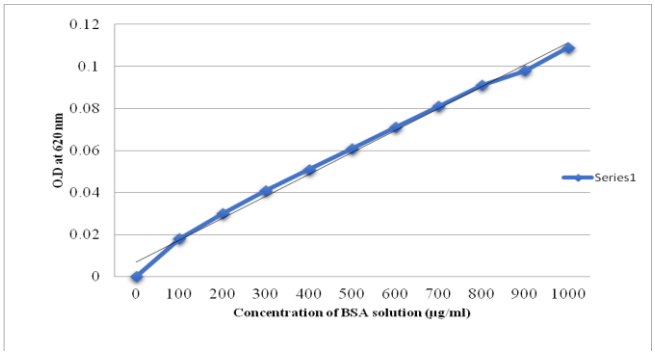

Fig. 3: Standard curve of BSA stock solution

E. Thermostability of purified lipase:

Partially purified lipase showed stable activity up to 55-60ºC, when tween-20 was used as substrate but a marked decrease was observed above this temperature (Figure 4). Earlier report have also shown that purified lipase from *Geobacillus thermodenitrificans* IBRL-nra have optimum temperature at 60ºC and activity dropped sharply above 70º[15]. Another similar report on lipase from *Pseudomonas aeruginosa* MTCC 2488 have also shown optimum temperature at 55-60ºC beyond which the activity decreased[14].

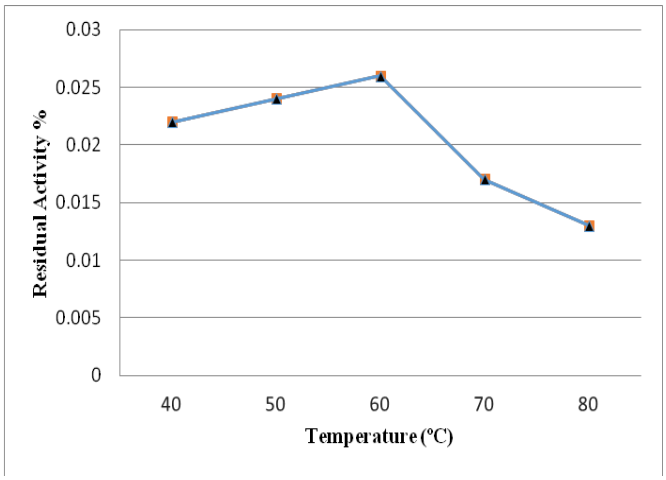

Fig. 4: Effect of temperature on lipase activity

F. pH stability of purified lipase:

The pH stability pattern of the lipolytic extract produced in the presence of olive oil. The lipase activity remained stable in the pH range of 2.0–10.0 during preincubation for 1 h at 37 °C, with residual activities in general above 100 % (Figure 5). Results show a different pH stability behavior of lipolytic extract activities. Remaining activity was compared with the control (C) without incubation.

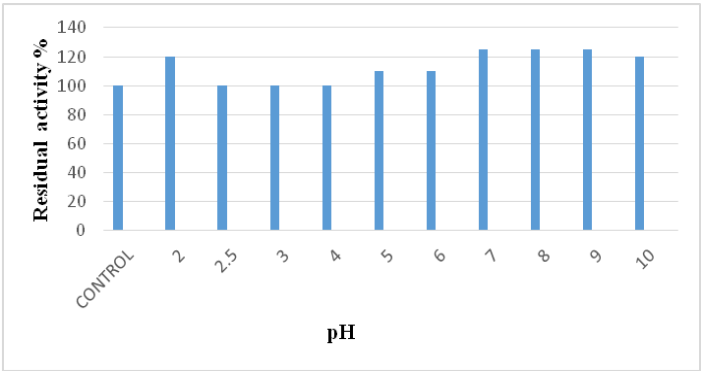

Fig. 5: Effect of pH on lipase activity

In an earlier report, observed that lipases produced by *A. niger* NCIM 1207 had maximal activity at pH 2.5, [16] and demonstrated lipase stability at a pH range of 2.0 to 10.0 for *A. niger* [17].

G. Effect of detergents on purified lipase:

The effect of detergents was studied after incubating the purified lipase with various detergents (conc.1%) at 30ºC for varying time interval. Addition of non-ionic detergent, like tween-20, tween-80 did not alter the lipase activity, while anionic detergent SDS resulted in loss of enzymatic activity (Table 3). SDS decreased lipase activity due to its effect upon the disulphide linkages of enzyme hence causing its inactivation.

In an earlier report on LPBs, *Pseudomonas aeruginosa* MTCC 2488 exhibited similar effect[14], i.e, no effect of detergents like Tween-20, Tween-80, on activity, whereas complete loss of enzymatic activity with SDS.

Table 3: Effect of detergents on lipase activity (O.D. at 400nm)

Detergents used	0 hr	0.5 hr	1hr	1.5 hr	2 hr
Control	0.021	0.021	0.022	0.024	0.025
Tween-20	0.021	0.021	0.020	0.019	0.018
Tween-80	0.021	0.020	0.020	0.019	0.019
SDS	0.019	0.015	0.013	0.010	0.007

H. Effect of the organic solvents on purified lipase:

Incubation of the lipase with organic solvents like isopropanol, toluene, hexane, ethanol, methanol and xylene, did not cause much loss of its activity. 90% activity remained even after 12 hrs incubation in strong dehydrating solvent like methanol, ethanol, hexane and xylene (Figure 6).

There are only a few reports on stability of lipase in organic solvents. In one of these studies purified lipase from *Pseudomonas aeruginosa* MTCC 2488 showed stability in some organic solvents[14].

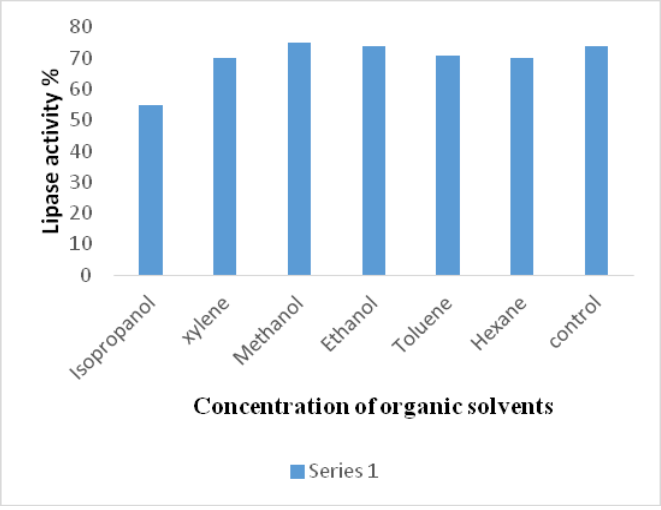

Fig. 6: Effect of organic solvents on lipase activity

I. Esterification of lipase:

The purified lipase obtained from LPB was found to esterify oleic acid with ethanol resulting in the formation of ethyl oleate which was confirmed by thin layer chromatography (Figure 7). Spot of ethyl oleate was obtained at an Rf value (retention factor) which was different from the Rf value of ethanol and oleic acid. The reaction mixture containing heat inactivated (100ºC/10 mins) lipase was loaded on TLC as control.

In an earlier report lipase from *Bacillus* sp. DVL2 was found to esterify steric acid with ethanol resulting in the formation of the ethyl stearate[7].

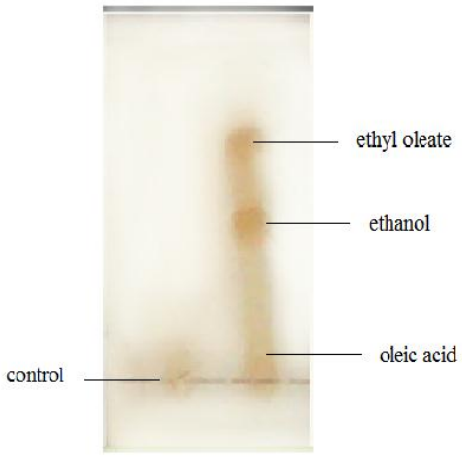

Fig. 7: TLC plate showing esterification

IV. CONCLUSION

Microbial production of lipase has a huge advantage in many of the industrial applications as there are a variety of lipases which can be produced and modified according to the requirements of the industry like detergent industry, food and dairy industry, for the synthesis of biodegradable polymer, in the synthesis of biodiesel, degreasing of leather isolate and also in pulp and paper industry. Esters of oleic acid are also useful in pharmaceutical industry. In the present study,

extraction and partial purification of lipase from microorganism was done. The study suggested that soil contaminated with diesel may serve as excellent breeding ground for the isolation of lipase producing bacteria of industrial significance and further work can be carried out for determining the mechanism of action of lipase including cloning, genetic modification, sequencing and detailed structural knowledge of the enzyme and phylogenetic study of the enzyme so that lipase with better potential and multiple applicability's can be produced.

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