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Production, Purification and Characterization of Lipase Produced from *Pseudomonas* Sp. Lp1

S. Kanimozhi* and K. Perinbam[1]

*Research Scholar, Sathyabama University, Jeppiaar Nagar, Old Mamallapuram Road, Chennai - 600 119, India.
[1]Faculty of Biomedical Engineering, Sathyabama University, Jeppiaar Nagar, Old Mamallapuram Road, Chennai - 600 119, India.

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***Pseudomonas* sp.Lp.1 isolated from oil contaminated soil produced an inducible extracellular lipase. Olive oil (1%) was used as the inducer for enzyme production. The optimum conditions for the assay of enzyme activity was found to be with 4-Nitrophenyl palmitate as the substrate incubated at 45°C for 20 minutes at pH 8.0. The enzyme was purified 18 - fold by ammonium sulfate fractionation, Dialysis and Sephadex G-100 chromatography. The purified enzyme showed a prominent polypeptide band in polyacrylamide gel and the presence of lipase was confirmed by zymogram analysis. Molecular weight of the lipase was estimated to be 49.0 kDa. The optimum pH and temperature for activity of the enzyme were 8.0 and 40°C, respectively. The lipase was stable in the pH range of 7.0 - 10.0 and showed its half-life at 40°C to be 2 h. Metal ions $CaCl_2$ and KCl enhanced lipase activity, whereas Na^{2+}, Mg^{2+} and Mn^{2+} did not alter the activity and Cu^{2+}, Fe^{2+} and Zn^{2+} inhibited the stability of the enzyme. The enzyme was stable in the organic solvents ethanol and acetone whereas the methanol, hexane, chloroform and diethyl ether showed the inhibitory action on the enzyme.**

Key words: *Pseudomonas* sp.; Extracellular lipase; Enzyme characterization.

Lipases (triacylglycerol acylhydrolases, E.C. 3.1.1.3) are ubiquitous enzymes of considerable physiological significance and industrial potential. Lipases catalyze the hydrolysis of triacylglycerols to glycerol and free fatty acids. In contrast to esterases, lipases are activated only when adsorbed to an oil–water interface[1]. Although lipases have been found in many species of animals, plants, bacteria, yeast, and fungi, commercially useful lipases are usually obtained from microorganisms that produce a wide variety of extracellular lipases. Lipases find promising applications in organic chemical processing, detergent formulations, synthesis of biosurfactants, the oleochemical industry, the dairy industry, the agrochemical industry, paper manufacture, nutrition, cosmetics and pharmaceutical processing. Development of lipase-based technologies for the synthesis of novel compounds is rapidly expanding the uses of these enzymes[2-4]. Each application requires unique properties with respect to specificity, stability, temperature, and pH dependence or ability to catalyze synthetic ester reactions in organic solvents. Therefore, screening of microorganisms with lipolytic activities could facilitate the discovery of novel lipases. The extracellular bacterial lipases are of considerable commercial importance, as their bulk production is

* To whom all correspondence should be addressed.
E-mail: skanimo@gmail.com

much easier. Although a number of lipase-producing bacterial sources are available, only a few are commercially exploited as wild or recombinant strains[5,6]. Of these, the important ones are: *Achromobacter, Alcaligenes, Arthrobacter, Bacillus, Burkholderia, Chromobacterium* and *Pseudomonas*. Of these, the lipases from *Pseudomonas* bacteria are widely used for a variety of biotechnological applications[5,7,8]. Thermostable enzymes are particularly attractive for industrial applications because of their high activities at the elevated temperatures and stabilities in organic solvents[9,10]. To use lipase for any application, it is important to characterize the enzyme and study its properties. Therefore, in this paper we report production, purification, and characterization of lipase from *Pseudomonas* sp. Lp1.

MATERIAL AND METHODS

Isolation and Screening of Potential Lipase Producing Microorganism and Maintenance

Lipolytic bacteria were isolated from oil contaminated soil. For this, 1.0 g of soil was dissolved in 100 ml of sterile distilled water. Then it was serially diluted (10^{-1} to 10^{-5}) and diluted samples were plated on nutrient agar for total viable count. Then the dominant organisms were isolated and individually tested for its lipolytic potential on Tween agar amended with calcium chloride (Peptone - 10 g; NaCl - 5 g; $CaCl_2$ - 0.01 g; Agar -20 g;Tween - 10 ml; Distilled water - 1000 ml; pH - 7.5). After 24 h of incubation at 37°C, the lipolytic activity was confirmed by the formation of a precipitate zone around the colony[11]. Screening system making use of chromogenic substrates has also been used to confirm the lipase production. The medium used was Elliker agar, supplemented with olive oil and the fluorescent dye, Rhodamine b (Casein- 20 g; Yeast extract - 5 g; Gelatin - 2.5 g; Lactose - 5 g; Saccharose - 4 g; Sodium chloride - 4 g; Sodium acetate - 1.5g; Ascorbic acid - 0.5 g; Olive oil - 10 ml; Agar - 20 g; Distilled water - 1000 ml; pH-7.5). Elliker agar plates were prepared and isolated colonies were streaked. The plates were incubated at 37ºC for 24-48 hours. Strain that produced lipase emitted orange fluorescence after exposure to UV light. The isolated bacterial strain screened for lipase production was identified based on morphological, biochemical and physiological characters according to Bergey's manual of Determinative Bacteriology and further confirmative test was made on *Pseudomonas* isolation agar. The bacterial strain was identified as *Pseudomonas* sp. and designated as strain.Lp1. Pure cultures were inoculated on sterile nutrient agar slants (Agar-2.0 g; Peptone-0.5 g; Yeast extract-0.3 g; NaCl-0.5 g; Distilled water-100 ml; pH - 7.0) and were maintained by weekly transfers on Agar slants stored at 5°C between transfers for further uses.

Preparation of Inoculum and Lipase Production

The inoculum for production of lipase and other studies was prepared using broth medium. The pure culture was inoculated into sterile Luria Bertani (LB) broth (Yeast extract - 5 g; Peptone - 10 g; NaCl - 10 g; Distilled water- 1000 ml; pH - 8.0) and was incubated at 37°C in a rotary shaker for overnight. The fresh over night culture was used as an inoculum for production of enzyme[11]. Bacterium was cultivated in 250 ml Erlenmeyer flask containing 50ml medium composed of peptone (0.5%), yeast extract (0.5%), NaCl (0.05%), $CaCl_2$ (0.005%), and olive oil (1.0%, emulsified with gum acacia 0.5% w/v), pH 8.5. The production broth was inoculated with seed culture (3.0% v/v) and incubated at 37- 42 °C under shaking (170 rpm, 48 h) conditions. Culture broth was harvested by centrifugation (10,000g, 10 min, and 4 °C). The enzyme activity was determined in the cell-free broth thereof.

Enzyme Assay

Lipase activity was assayed by the colorimetric method of Winkler and Stuckmann, 1979[12]. Lipase activity was assayed by measuring the micromoles of 4-nitrophenol released from 4-nitrophenyl palmitate. A stock solution (20mM) of 4-Nitrophenyl palmitate (4-NPP) was prepared in HPLC grade iso-propanol. The reaction mixture contained 150 µl of 4-NPP stock solution, 100 µl enzyme, and Tris buffer (0.1M, pH 8.5) to make a final volume of 3 ml. The reaction mixture was incubated at 55°C for 10 min in water bath. Chilling at 20°C for 8 min was employed to stop the reaction. Control containing heat-inactivated enzyme was also incubated with each assay. The absorbance of 4-nitrophenol released was measured at 410 nm using UV-Vis spectrophotometer (Elico). The unknown

concentration of 4-nitrophenol released was determined from a reference curve of 4-Nitrophenol (2–20µg/ml) in 0.1M Tris–HCl buffer, pH 8.5). One unit of lipase activity was defined as micromoles of 4-nitrophenol released from 4-NPP per ml per min under standard assay conditions.

Standardization of Lipase Assay Conditions

Each enzyme has its own optimum assay conditions under which it shows its optimum activity. To optimize the assay conditions for *Pseudomonas* sp.Lp1, each of the factors was standardized as follows.

Effect of incubation time

To determine the optimum incubation time for assay, the reaction mixture was incubated for varying times (5, 10, 15, 20, 25, 30, 35, 40 and 45 min) at 55°C in a water-bath shaker and enzyme activity was measured under standard assay conditions.

Effect of temperature

To evaluate the effect of reaction temperature on lipase activity, the lipase activity was assayed at various temperatures (25°C, 30°C, 35°C, 40°C, 45°C, 50°C, 55°C and 60°C for 10 min.) and incubated for 20 minutes and lipase activity was assayed under standard assay conditions.

Effect of pH

To evaluate the effect of reaction pH on lipase activity, the reaction mixture was prepared with different pH (4-10) values in separate tubes and each tube was incubated at 45°C for 20 min. The lipase activity was assayed under standard assay conditions.

Substrate specificity of lipase

To study the substrate specificity of the enzyme, different substrates 4-Nitrophenyl palmitate, 4-Nitrophenyl formate, 4-Nitrophenyl laurate and 4-Nitrophenyl acetate (Sigma,USA) were used. The substrate concentration used was 20mM and the enzyme activity was measured under the conditions of temperature 45°C for 20 minutes at pH 8.0.

Determination of Protein Concentration

Protein analysis of the different supernatants was determined spectrophotometrically according to Lowery *et al*., 1957[13]. The different protein concentrations were derived from Bovine serum Albumin standard curve.

Purification of extracelluar Lipase

Culture supernatant was obtained by centrifugation of the culture broth after extraction at 10000 g, 4°C, for 15 min. This preparation was termed as crude extract.

Ammonium sulphate fractionation

Ammonium sulphate precipitation of proteins was carried out at three different saturations of ammonium sulphate (w/v) viz., 20-40%, 40-60% and 40-80% and the mixtures were stored in cold room for 24 hours to precipitate the proteins. Then the precipitate was separated by centrifugation at 4ºC and the supernatant was discarded. The precipitate was dissolved in phosphate buffer (pH.7.2). The mixture was subjected to dialysis.

Dialysis

To remove the residual ammonium sulphate dialysis of the precipitated protein was performed. The pre treatment of the dialysis membrane (Sigma,USA) was done by immersing the membrane into the warm phosphate buffer (pH.7.2) for 10 minutes. One end of the dialysis membrane tube was closed tightly by fixing the clip. The precipitated protein was then transferred in the dialysis tube and the other end was tied with a thread. The pack was suspended freely into a large beaker, which contained around 500 ml of 0.05 M Sodium Phosphate buffer solution (pH 7.2). The buffer was stirred slowly using magnetic stirrer. The entire setup was placed in the cold room for 48 hours. Every 12 hours the buffer was changed for better dialysis. After dialysis the clip from one end of the membrane was removed and the sample was transferred into a clean sterile container for further analysis.

Sephadex G-100 gel filtration chromatography

Dialyzed enzyme solution was applied onto a column packed with Sephadex G -100 (Sigma,USA), which was previously equilibrated with 0.1M Tris- HCl (pH 7.0). The column was eluted with the same buffer with a flow rate of 30 ml/h. All eluted fractions were assayed both for lipase activity as well as Total protein (*A*280). The fractions showing highest lipase activity were pooled and assayed for lipase activity to check the purification fold and characterization of the enzyme.

Protein Analysis by Sodium Dodecyl Sulphate - Polyacrylamide Gel Electrophoresis (SDS

- PAGE) and Zymogram Analysis.

SDS PAGE

The SDS- PAGE (10%) assay was conducted according to the procedure of Laemmli (1970). Samples were loaded along with the molecular weight marker (Genei, India) on a 10% polyacrylamide gel containing 0.1% SDS with stacking gel. Electrophoresis was performed on a vertical slab mini-gel apparatus (Biotech, India) at 100 V for 5-6 h. The gel was stained using using Coomassie Brilliant blue R -250 staining solution and destaining was done using alcohol - acetic acid mixture solution.

Zymogram Analysis

To confirm the lipolytic activity of the protein and to determine the exact molecular weight of the produced lipase, a Zymogram was performed as follows: an SDS gel was prepared and the finally purified fraction of lipase was loaded in two wells. After migration, part of gel was stained for protein detection with Coomassie brilliant Blue R-250.The other part was cut and washed by agitating with 250 ml of renaturation buffer (20 mM Tris HCL buffer, pH 8.0 containing 2.5% of (w/v) of Triton X-100). The gel was overlaid with the molten chromogenic substrate (Tributyrin -1.0%; $CaCl_2$ - 0.1%; Phenol red - 0.01%; Agar- 2.0% which was then allowed to solidify. The gel with the substrate was incubated at 45ºC. The lipase activity was observed as yellow bands against a pink background.

Characterization of Purified Lipase

pH stability

The pH stability of the enzyme was determined by keeping the enzyme solution at 4ºC for 24 h in equal volumes of buffers with varying pH values viz.,4, 5, 6, 7, 8, 9, 10 ,11 .The described assay was carried out to determine its stability.

Thermo stability of lipase and its half-life at 40°C

To determine the half-life of the enzyme, the enzyme was incubated at 40°C and the enzyme activity was determined periodically up to 6 h. For the determination of thermostability, the enzyme was incubated at various temperatures viz., 30ºC, 40ºC, 50ºC, 60ºC, , 70ºC, 80ºC.The residual enzyme activity was determined after every 15 minutes.

Effect of Metal ions and EDTA on enzyme stability

The effect of metal ions on lipase activity was determined by measuring residual activity by pre incubating the purified enzyme with various metal ions (1.0mM) viz., Fe^{2+}, Cu^{2+} ,Fe^{3+} , Mn^{2+}, Mg^{2+}, Zn^{2+}, K^{+} , Ca^{2+} and EDTA separately in equal volumes and incubated at 40ºC. The residual enzyme activity was determined after 30 minutes.

Effect of Surfactants on enzyme stability

The purified lipase was incubated with 1% concentration (v/v or w/v) of some of the surfactants viz., sodium dodecyl sulphate (SDS), Tween 20, Tween 60,Tween 80, Triton X100 separately in Tris buffer (0.1 M, pH.8) at 40ºC . The residual enzyme activity was determined after 30 minutes.

Effect of Organic Solvents on enzyme stability

The stability of the purified enzyme in the organic solvents such as methanol, ethanol, acetone, isopropanol, isooctane, hexane, diethyl ether, chloroform and butanol was determined. The purified enzyme was pre incubated with equal volume of organic solvents at 40ºC separately .The residual enzyme activity was determined after 30 minutes.

RESULTS

Isolation, Screening and Identification of Potential Strain

Microbiological analysis of soil samples from oil contaminated sites contained high bacterial count. Each of the isolate was screened for lipase production in Tween Agar. Among fifty two isolates, four isolates showed positive towards lipase production by showing white precipitate around the growth. The isolate that showed comparatively more precipitation zone was selected for the study (Fig.1)**.** Based on biochemical, cultural and morphological (Fig.2) characteristics the isolate was identified as *Pseudomonas* sp. and designated as strain Lp1. Lipase production by the organism was confirmed on Elliker agar medium in which the strain emitted orange fluorescence after exposure to UV light .This could be due to the formation of complex between cationic Rhodamine B and the uranyl fatty acid ion released by lipase (Fig.3)**.**

Standardization of Lipase Assay Conditions

The reaction mixtures when incubated at 45ºC, for 20 minutes at pH 8.0 showed more expression of lipase activity was observed. The

Table 1. Summary of extracellular lipase purification from *Pseudomonas* sp.Lp.1

Purification Method	Total Activity (U/Ml)	Total Protein (Mg)	Specific Activit Y (U/Mg)	Purificati On Fold
Crude enzyme solution	78.0	2.78	28.05	1.0
Ammonium sulfate precipitation	112.3	1.95	57.58	2.05
Sephadex G- 100	284.7	0.28	1016.78	17.65

Table 2. Effect of organic solvents on purified lipase from *Pseudomonas* sp.Lp.1

Organic Solvents	Relative Activity (%)
Ethanol	96.6
Methanol	35.0
Acetone	92.4
Isopropanol	57.1
Isooctane	43.0
Hexane	15.0
Diethyl ether	22.0
Chloroform	10.0
Butanol	39.0

substrates examined were 4- Nitro phenyl palmitate , 4-Nitrophenyl formate,4-Nitrophenyl laurate and 4-Ntrophenyl acetate) .The enzyme reaction was evident when substrate 4-Nnitro phenyl palmitate was used in which bright fluorescent yellow colour was developed, whereas in other substrates the lipolytic activity was less (Fig. 4).

Purification of extracellular Lipase and Determination of Molecular Weight

Cell-free supernatant was prepared by centrifugation (10,000g, 10 min, 1and 4°C) of the culture broth. The extracellular enzyme produced by *Pseudomonas* sp.Lp.1 was purified by Ammonium sulfate precipitation, dialysis and gel filtration chromatographic techniques. The purity of the enzyme was confirmed by SDS-PAGE and the lipolytic activity of the protein band was confirmed by Zymogram analysis.

In three different saturations (20-40%, 40-60% and 40-80%) of culture filtrate with ammonium sulphate the maximum lipase activity were found in the precipitate obtained with 40 - 60% saturation. The enzyme was purified to 2.05 fold by this purification step. The specific activity of lipase was 28.05 U/mg (Table.1).

The excess ammonium sulphate bound to the enzyme was removed by dialysis. The dialyzed enzyme was subjected to Sephadex G - 100 gel filtration column for further purification. The highest lipase activity was obtained when the fractions of 7 to 11 were pooled. The specific activity of lipase was 1016.78 U/mg. Nearly 18 fold purity was achieved by gel filtration chromatography using Sephadex G -100.

The purified lipase solution after each purification step was studied using SDS-PAGE (10%). The crude enzyme solution showed six bands on the gel while the partially purified enzyme mixture obtained by ammonium sulphate precipitation and dialysis exhibited three protein bands. The purified enzyme fraction obtained from gel filtration showed a single distinct band on the gel. The molecular mass was found to be around 49.0 kDa. To confirm the lipolytic activity of the band obtained, zymogram was conducted using tributyrin as a substrate. A turbid zone was appeared corresponding to the protein molecular weight of about 49.0 kDa, which matched to the purified lipase band (Fig. 5).

Characterization of Purified Lipase

The purified enzyme from *Pseudomonas* sp.Lp.1 showed maximum stability at pH 8.0 by retaining its activity of 85% and was stable between the pH 7.0-10.0.The enzymes retained 55% and 30% of its activity at pH 10.0 and pH 11.0 respectively (Fig. 6).

Our enzyme showed its half-life at 40°C to be 2 h and exhibited 20% of its activity when kept for 3 hours (Fig.7). The enzyme from *Pseudomonas* sp.Lp.1 showed maximum activity of 95% at 40°C and retained nearly 50% of its activity when kept for one hour. The enzyme was stable between the temperatures of 30°C to 50°C with 43% and 18% of relative activity for 45 minutes respectively (Fig.8).

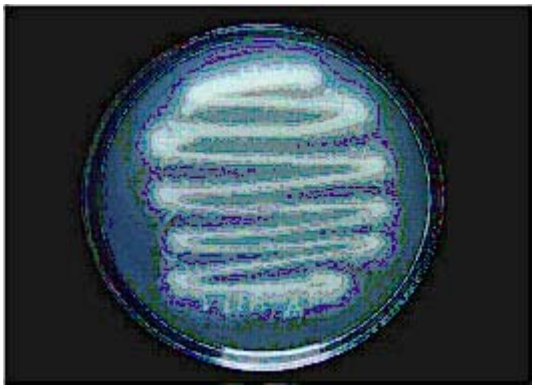

Fig. 1. Lipolytic Activity of *Pseudomonas* sp.Lp.1 in Tween Agar

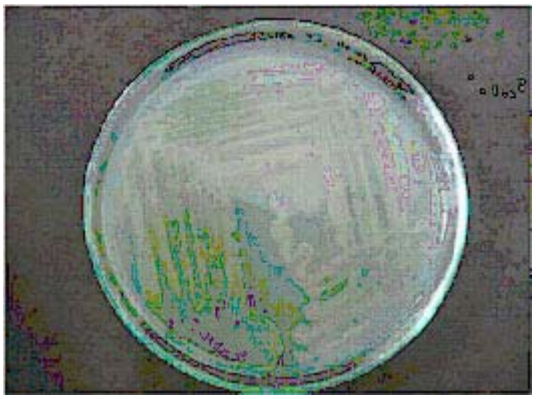

Fig. 2. Growth of *Pseudomonas* sp. Lp.1 in Nutrient agar plate

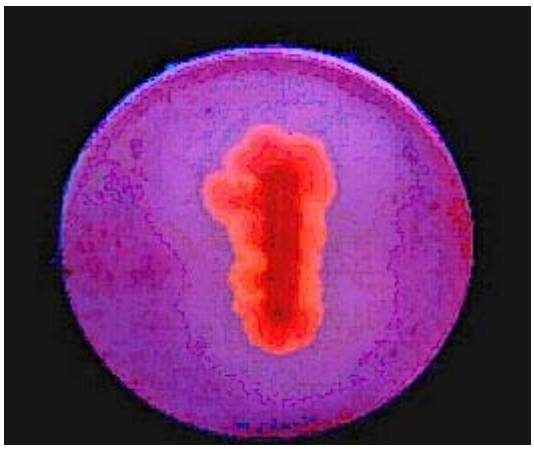

Fig. 3. Lipolytic activity of *Pseudomonas* sp.Lp1 in Elliker medium

The addition of metal ions such as Ca^{2+} and K^{+} enhanced the activity of the purified enzyme from *Pseudomonas* sp.Lp.1 after 30 minutes of incubation at 40°C with the residual activity of 134 and 112 respectively. The metal ions such as Na^{2+}, Mg^{2+} and Mn^{2+} did not alter the activity of the enzyme in which the enzyme was stable and retain its original activity. The metal ions Cu^{2+}, Fe^{2+} and Zn^{2+} inhibited the stability of the enzyme. The presence of EDTA also inhibited the enzyme activity (Fig. 9).

Lipase from *Pseudomonas* sp.Lp.1 retained its original activity in the presence of organic solvents such as ethanol (96.6%) and acetone (92.4) after incubating at 40°C for 30

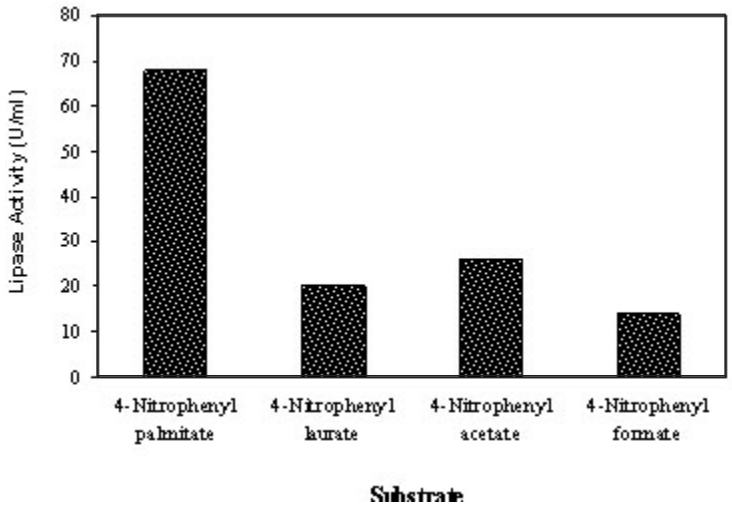

Fig. 4. Substrate specificity of lipase from *Pseudomonas* sp.Lp.1

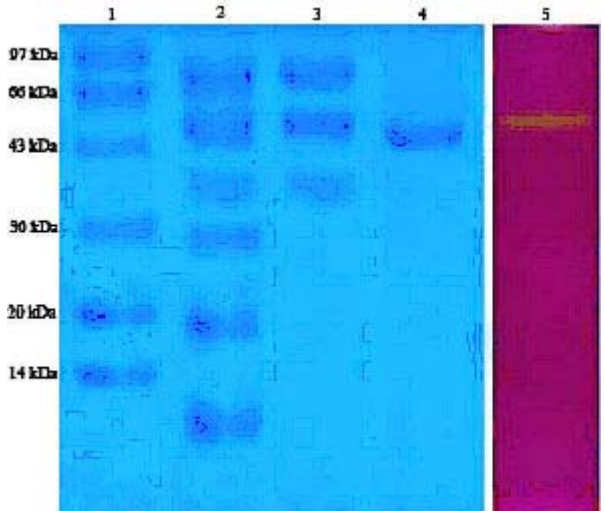

Fig. 5. Molecular weight Determination of purified lipase through SDS-PAGE and Zymogram analysis. Molecular Weight marker (Lane 1), Crude enzyme solution (Lane 2), Ammonium sulfate fractionation (Lane 3),Sephadex G 100 chromatography (Lane 4) and Zymogram confirming lipase activity (Lane 5)

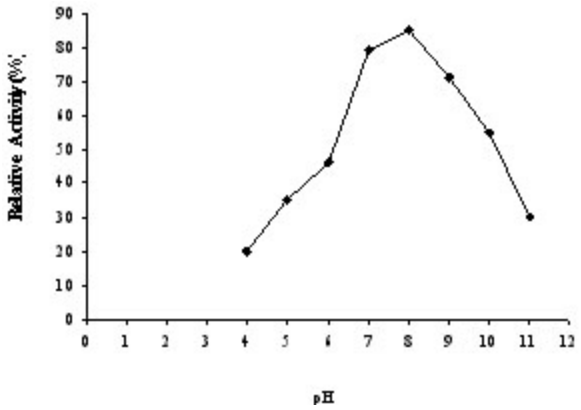

Fig. 6. pH stability of lipase from *Pseudomonas* sp.Lp.1

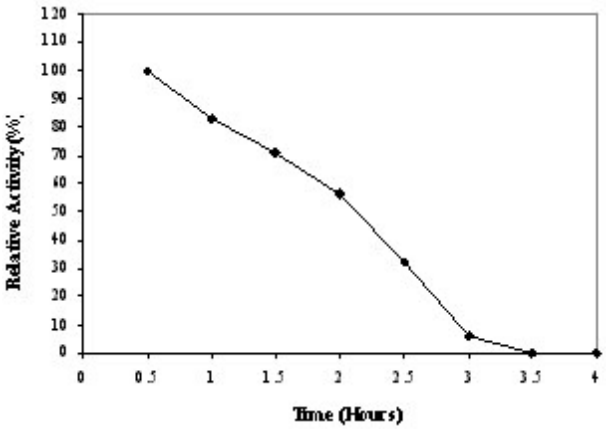

Fig. 7. Activity of lipase from *Pseudomonas* sp.Lp.1 at 40°C

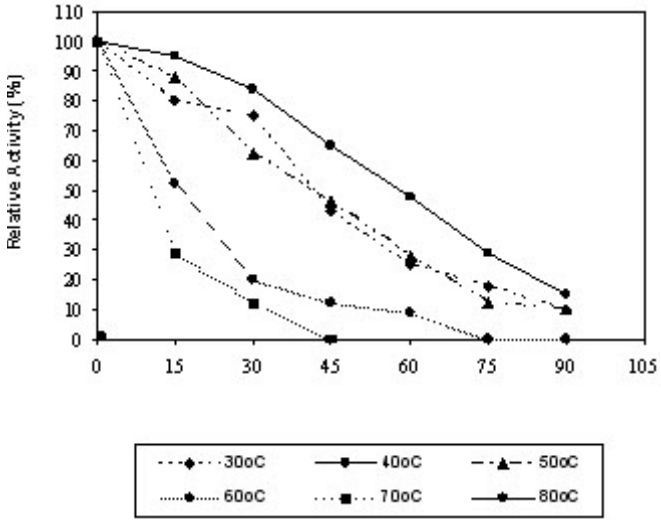

Fig. 8. Temperature stability of lipase from *Pseudomonas* sp.Lp.1

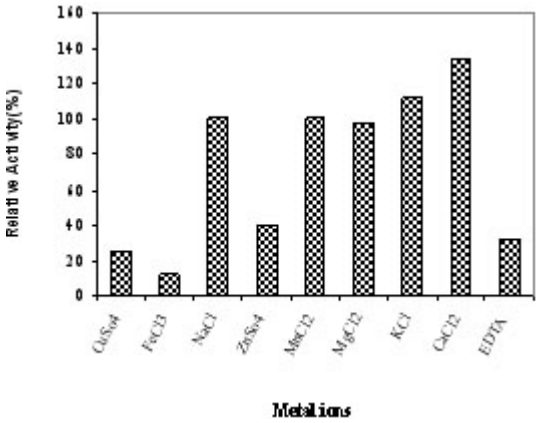

Fig. 9. Stability of lipase from *Pseudomonas* sp.Lp.1 in Metal ions

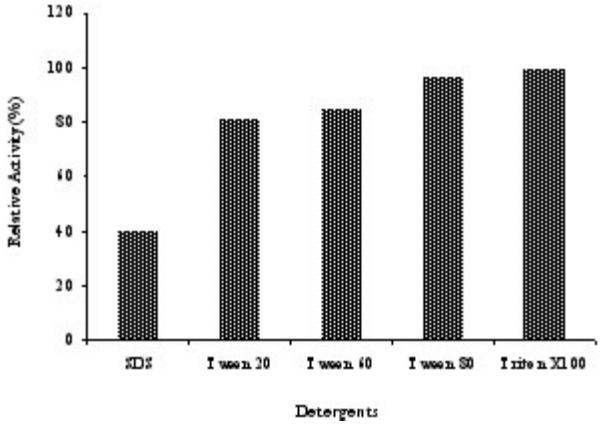

Fig. 10. Stability of lipase from *Pseudomonas* sp.Lp.1 in Detergents

minutes. In the presence of isopropanol it retained 57.1% of activity whereas in the organic solvents butanol and isooctane the enzyme retained nearly 40% of its activity where as the methanol, hexane, chloroform and diethyl ether showed the inhibitory action on the enzyme activity (Table 2).

Lipase from *Pseudomonas* sp.Lp.1 retained its activity about 90-100% in the detergents like Triton X-100 and Tween 80 whereas in Tween 20 and Tween 60 it exhibited 81.5% and 84.7% of activities respectively. The enzyme retained only 40.5% of activity in SDS (Fig. 10).

DISCUSSION

Extracellular lipase producing *Pseudomonas* sp.Lp.*1 was* isolated from the soil contaminated with oil. The lipolytic activity was checked by Tween agar test and identification of this was confirmed using confirmatory test including Elliker agar test, staining and various biochemical tests. Lipase-producing psychrotrophic bacteria belong to the genus *Pseudomonas* was isolated from soil[14]. Production of lipase by the organism was confirmed on Rhodamine B agar plate containing olive oil. The colonies showed orange colored fluorescence when exposed to UV light; this is due to the formation of complex between cationic Rhodamine B and the uranyl fatty acid ion. Similar identification method was followed for *P. aeruginosa* MTCC 2488[15] and for identification of cold active lipase producing organisms[16]**.**

Lipase production was carried out in the medium supplemented with olive oil (1% v/v) which acted as an inducer for enzyme production and carbon source. The major factor for the expression of lipase activity has always been reported as the carbon source, since lipases are inducible enzymes. These enzymes are generally produced in the presence of a lipid such as oil or any other inducer, such as triacylglycerols, fatty acids, hydrolysable esters, Tweens, bile salts, and glycerol[17]**.** Lipidic carbon sources seem to be essential for obtaining a high lipase yield. The medium with 3% olive oil enhanced the the lipase production by *Burkholderia multivorans*[18]**.**

Lipases are highly diversified in their catalytic properties. Maximum lipase activity of *Pseudomonas* sp.Lp.1 was obtained after 20 minutes of incubation at 45°C at pH 8.0.All these factors exhibited the maximum lipase activity when using 4 - Nitrophenyl palmitate as the substrate. Temperature optimum of 55 °C or higher for lipase activity has been reported in many *Bacillus* species[19]. Maximum lipase activity by *Bacillus coagulans* BTS-3 exhibited when the reaction time for assay was 10 minutes[20].

Study on the substrate specificity of lipase towards 4-Nitrophenyl palmitate, 4- Nitrophenyl formate, 4-Nitrophenyl lautate and 4-Nitrophenyl acetate showed highest specificity towards 4 - Nitrophenyl palmitate by giving bright yellow color. In other substrates the intensity of the yellow color was less.

Pre-purification steps involve concentration of the culture supernatant containing the enzyme by ultra filtration, ammonium sulfate precipitation or extraction with organic solvents. Precipitation often gives a high average yield[21]**.** In the present study precipitation by ammonium sulphate was performed in which the fraction 40-60% of saturation the lipase activity was high and yielded two fold purity.Futhur purification was done by dialysis and Sephadex G 100 gel filtration column chromatographic technique and the molecular weight determined was 49.0 kDa. The molecular weights of lipases vary considerably, ranging from 11.0 to116.0 kDa. The extracellular lipase produced by *Geobacillus stearothermophilus* was purified to 22.6 fold with 8.8% recovery and determined the molecular weight to be 61.0 kDa[22]**.** The molecular mass of *Bacillus coagulans* BTS-3 which was purified by ammonium sulphate precipitation technique and DEAE-Sepharose was found to be 31.0 kDa[20]. Molecular mass of lipase from *Pseudomonas* sp.isolated from raw meat was 60.0 kDa[23]**.** Extracelluar lipase producing *Serratia marcescens* isolated from raw milk was purified to homogeneity by ion-exchange chromatography and gel filtration with a final recovered activity of 45.42% and molecular mass was estimated by SDS-PAGE assay to be 52.0 kDa[24]**.** *Pseudomonas fluorescens* MTCC 2421 lipase was purified 184.37-fold with a specific activity of 424.04 LU/mg after anion exchange and gel exclusion chromatography with an apparent molecular mass of 65.3 kDa[25]**.**Lipase from *Candida rugosa* was purified by ammonium sulphate precipitation,

dialysis, ultra-filtration and gel filtration using Sephadex-200 to a 43-fold purification and 64.35 mg/ml specific activity. SDS-PAGE of purified enzyme revealed three distinct bands, indicating the existence of three iso-forms, Lip A, Lip B and Lip C with an apparent molecular weight about 64,000, 62,000 and 60,000 Da, respectively[26].

The majority of lipolytic bacteria have neutral to alkaline pH optima. Our enzyme also showed activity between the pH 7.0 - 10.0 with the optimum at pH 8.0. Hence lipase produced from *Pseudomonas* sp.Lp.1 is an alkaline lipase. The purified enzyme from *Bacillus coagulans* BTS-3 exhibited optimum lipolytic activity at pH 8.5 and temperature 55 °C and was stable within the pH range of 8.0 - 10.5[20]. *Pseudomonas mendocina* lipase was optimally active at pH 9.0 and was found to be stable between the pH 7.0–11.0[27]. The purified lipase of *Serratia marcescens* showed maximal stability at pH 8.0[24]. The alkaline lipase from *Pseudomonas pseudoalcaligens* F-111 was most active in the pH range between 6.0 and 10.0[28]. The lipase of *Pseudomonas fluorescens* exhibited optimum activity at pH 8.0[25].

Bacterial lipases generally have temperature optima in the range 30 - 60°C[29]. Lipase from *Pseudomonas* sp.Lp.1 was stable between the temperatures of 30°C to 50°C and showed maximum activity of 95% at 40°C and retained nearly 50% of its activity for one hour. Reports exist on bacterial lipases with temperature optima in both lower and higher ranges. Temperature optimum of 55°C or higher for lipase activity has been reported in many *Bacillus* species [30, 31, 20]. The lipase from *Pseudomonas fluorescens* exhibited optimum activity at 40°C[25]. Lipase obtained from *P. aeruginosa* MTCC 2488 found to be thermostable by having optimum activity in the range of 50-60°C.

Cofactors are generally not required for lipase activity, but divalent cations such as calcium often stimulate enzyme activity. This has been suggested to be due to the formation of the calcium salts of long-chain fatty acids[32]. In the present study also the addition of metal ions Ca^{2+} and K^{+} enhanced the activity of the purified enzyme from *Pseudomonas* sp.Lp.1 after 30 minutes of incubation at 40°C with the residual activity of 134 and 112 respectively. Calcium chloride was able to restore and enhance enzyme activity of *Bacillus licheniformis*. Calcium-stimulated lipases have been reported in the case of *B. subtilis* 168[33], *B. thermoleovorans* ID- 1[34], *P. aeruginosa* EF2[35]. Lipase activity is in general inhibited drastically by heavy metals like Co^{2+}, Ni^{2+}, $Hg^{2+}$and $Sn^{2+}$and slightly inhibited by Zn^{2+} and Mg^{2+}[36]. In the present study the metal ions Na^{2+}, Mg^{2+} and Mn^{2+} did not alter the activity of the enzyme in which the enzyme was stable and retain its original activity. The metal ions Cu^{2+}, Fe^{2+} and Zn^{2+} inhibited the stability of the enzyme. The presence of EDTA also inhibited the enzyme activity. The enzyme from *Pseudomonas fluorescens* lost 31% of its initial activity by 0.001 mM EDTA and 42% by 0.1 mM EDTA[25].

Lipases are generally stable in organic solvents, with few exceptions of stimulation or inhibition. Stability in organic solvents is desirable in synthesis reactions. Our enzyme retained 96.6% and 92.4% of activity in the presence of organic solvents such as ethanol and acetone respectively. In the organic solvents isooctane and butanol the enzyme retained nearly 40% of its activity where as the methanol , hexane, chloroform and diethyl ether showed the inhibitory action on enzyme activity. The lipase activity of *Pseudomonas pseudoalcaligens* was stable in the presence of 30% ethanol, methanol and acetone whereas inhibited by n-propanol, 2-propanol, hexane and isooctane[37]. The lipase from *Bacillus stearothermophilus* lost its half of its original activity in the presence of 1mM EDTA[22]. Acetone was inhibitory for *P. aeruginosa* YS-7 lipase and hexane for *Bacillus* sp. lipase[38].

Lipase from *Pseudomonas* sp.Lp.1 retained their activity about 90-100% in the detergents like Triton X-100 and Tween 80 whereas the Tween 20 and Tween 60 exhibited 81.5% and 84.7% of the activity respectively .The enzyme retained only 40.5% of activity in SDS. The lipase activity of *Pseudomonas pseudoalcaligens* was stable in the presence of nonionic detergents whereas the anionic detergent SDS completely inactivated the enzyme[37]. The presence of detergents such as SDS,Tween 20,Tween 80 and Triton X100 had a slight effect on the Lipolytic activity[22].

CONCLUSION

The strain isolated from oil contaminated soil in this study was identified as *Pseudomonas* sp. Lp.1, produced an extracellular lipase. Many significant conditions for production and assay of lipase activity were optimized. The enzyme was purified to homogeneity .The relative activity was particularly high, and the activity was stable high pH and treatment with some of the organic solvents. Hence the organism will be exposed for its potential esterification and transesterification reaction in organic solvents. It will be used to synthesize some esters of industrial interest. It may also be applied to treat lipid-rich industrial effluent treatments and other biotechnological applications. Experimentation is currently being performed in order to determine the complete sequence of this lipase.

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