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Purification and Characterization of Extracellular Lipase from *Staphylococcus epidermidis* (MTCC 10656)

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ABSTRACT

Lipases are hydrolytic enzymes with wide range of industrial applications. In the present study *Staphylococcus epidermidis* strain L2 with ability to produce extracellular lipase enzyme has been isolated from cloth samples collected from the diary industry. The extracellular lipase enzyme has been purified to homogeneity. The purified enzyme has shown a specific activity of 123.95U/mg. The molecular weight of the purified lipase enzyme was found to be 28KDa. The pH and temperature optima of the enzyme were recorded as 7.5 and 40°C respectively. The lipase enzyme has shown stability and activity in presence of varying concentrations of emulsifier and different substrates.

INTRODUCTION

Lipids constitute a large part of the earth's biomass and lipolytic enzymes play an important role in their degradation. Traditionally pancreatic lipases have been used for applications such as cheese ripening and flavor enhancement (Mark, 2002). Nonetheless lipase or Triacylglycerol lipase (EC 3.1.1.3) was isolated and purified to homogeneity from wide range of sources (Saxena *et al.*, 2003). In recent years, microbial lipases have gained importance over plant and animal lipases for commercial and industrial applications due to their multifold properties (Akoh *et al.*, 2007; Antczak *et al.*, 2009). Among microbial sources, only a few bacterial lipases were studied and purified (Gupta *et al.*, 2004). The most important among the lipase producing bacteria are *Bacillus* sp (Lee *et al.*, 2015), *Achromobacter* sp (Toshifumi *et al.*, 2001), *Alcaligenes* sp. (Masahiro *et al.*, 2009), *Arthrobacter* sp.(Chaubey *et al.*, 2006), *Pseudomonos* sp.(Dong *et al.*, 1999), *Staphylococcus* sp. (Xie et al., 2012), and *Chromobacterium* sp. (Petra *et al.*, 2001). Most of the bacterial lipases are glycoproteins, but few extracellular bacterial lipases are lipoproteins. Lipases are used in many industries (Berhanu and Amare Gessesse, 2012).

They are used in several industries such as fat oleo-chemical industry for initiation of hydrolysis and glycoclysis of mixed substrates (Gupta et al. 2003), detergent industry to remove fat stains, food processing and bakery industry for flavor development and to improve quality of food by lipolytic hydrolysis of fats and cream (Sharma *et al.*, 2001; Ghosh *et al.*, 1996; Kirk *et al.*, 2002;), paper making industry to remove hydrophilic compounds from pulp (Gutierrez *et al.*, 2009) and textile industry to increase fabric absorbability (Rita *et al.*, 2008). Lipases are also commercially used in biodegradable polymer production, cosmetics (Taneja *et al.*, 2005), cheese flavoring, tea processing and resolution of the racemic mixtures (Talon *et al.*, 1995; Abraham *et al.*, 2011; Shivika Sharma, 2014).

The industrial market for lipases was reported as 1.5 billion US dollars in 2002 (Alain Houde et al. 2004). In view of increasing demand for lipase enzyme and its potential applications in varied industries, the present study was carried out to isolate and purify a stable, cost effective and extracellular lipase and to evaluate its commercial applications.

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MATERIALS AND METHODS

Microorganism

Cloth samples collected from dairy industry (Jyothi Dairy Private Limited, RR district, Hyderabad, India). The cloth samples were soaked in sterile distilled water for 10 min and the water was squeezed from the cloth. The water samples thus obtained were serially diluted and plated on to tributyrin agar base containing 0.5% (w/v) peptone, 0.3% (w/v) yeast extract, 1% (v/v) Tributyrin and 2% agar, pH 7.0 by spread plate method. Plates were incubated at 37°C for two days. Pure cultures of the isolates were maintained on minimal agar media slants (yeast extract, NaCl, Peptone and 2% agar, pH 7.0) and were subcultured every 15 days.

Lipolytic organisms were screened by qualitative plate assay. The isolates were cultured on Tributyrin agar base plates and incubated at 36° C for 2 days and the zone of clearance was observed due to hydrolysis of tributyrin. Based on morphological, physiological and biochemical tests, the isolate was identified as *Staphylococcus epidermidis*. The culture was sent for further identification to IMTECH, Chandigarh where it was identified and designated as *Staphylococcus epidermidis* strain L2 (MTCC 10656).

Enzyme Assay

Enzyme activity of lipase is assayed by alkali titration method using olive oil as substrate (Pinsirodom and Parkin, 2001). Olive oil (10%v/v) was emulsified with gum Arabica (5%m/v) in 100 mM sodium phosphate buffer pH 7.2. Enzyme sample of 0.1 ml was added to 0.9 ml of this emulsion. After incubation for 15 minutes at 37°C, the reaction was stopped and the fatty acids were extracted by addition of 0.1 ml of an acetone/ethanol solution (1:1v/v).The amount of fatty acids liberated was then titrated with 0.05M NaOH until pH10.5 using a phenophathelin indicator. Reaction blank was run in the same way, but by adding the sample after the addition of acetone/ethanol solution. The activity of lipase was calculated using the following formula.

$$\frac{(\text{M of NaOH})\ (\text{vol of NaOH consumed})\ (\text{Reaction time in sec})}{(\text{time required to consume NaOH in sec})(\text{Vol of enzyme used})}$$

One unit of enzyme activity was defined as the amount of enzyme that will hydrolyze µmol of fatty acid from triglycerides.

Enzyme Preparation

Tributyrin broth containing 1% olive oil was inoculated with a 48 h old culture of *Staphylococcus epidermidis* strain L2 with 1% inoculums. The culture was incubated for 48 hours at 37°C, 150 rpm. The culture was centrifuged at 10,000 rpm for 10 min at 4°C. The supernatant obtained was lyophilized and used as crude enzyme preparation for further purification and analysis.

Protein Estimation

Lowry method was used for the determination of total protein content of the 48h old culture supernatant with bovine serum albumin as standard (Lowry *et al.*, 1951).

Enzyme purification

Fractionation by acetone precipitation method

The 48 h old culture supernatant was precipitated with cold acetone in saturation ranges of 50% and 100%. These suspensions were then centrifuged at 12,000 rpm for 30 min in cooling centrifuge at 4°C. The pellet obtained was dried completely so as to remove any traces of acetone and it was later dissolved in a minimal volume of 20 mM phosphate buffer (pH 7.2) and stored. The protein samples were assayed for enzyme activity by the standard assay method.

Ion Exchange Chromatography

The fraction of protein obtained from acetone precipitation was subjected to ion exchange chromatography (macro High-Q-anion exchanger column, 1x10 cm) for further purification. This column was connected to Biological duo flow (FPLC) system. The column was equilibrated using a flow rate of (1ml/min) with equilibration buffer (20 mM phosphate buffer pH 7.2). The protein fraction obtained from acetone precipitation was loaded on to the Macro High Q column with a flow rate of (1ml/1min). The column was washed with equilibration buffer of three column volumes to elute unbound protein.

The bound protein was eluted at the flow rate of 0.5ml/min using a linear continuous gradient of NaCl in equilibration buffer ranging from 0-500 mM. The fractions of 2 ml volume were collected and screened for lipolytic activity. Lipase fractions containing activity were pooled, concentrated and loaded onto gel filtration matrix.

Gel filtration chromatography

The active fractions obtained from Ion exchange chromatography were concentrated and loaded onto gel filtration chromatography column connected to FPLC. Sephacryl S-200 matrix was loaded with the concentrated protein sample. Isocratic pattern of elution was ensured with a flow rate of 0.1.ml/min using double filtered phosphate buffer (pH 7.2). The samples of 0.5 ml fraction size were collected and their protein concentration was determined by reading the absorbance at 280nm. Fractions were assayed for lipolytic activity. All the fractions with activity were pooled.

Characterization of Lipase

Molecular weight determination: SDS-PAGE analysis

Molecular weight of the purified protein sample obtained from gel filtration chromatography was determined by carrying out Sodium dodecyl sulphate polyacrylamide gel electrophoresis according to the method of Laemmli (1970) using 12% cross linked polyacrylamide gel. Electrophoresis was carried out at constant voltage of 300V and 40mA for 100 min at room temperature and silver staining was used to visualize protein bands on the gels (Morrissey, 1981). Molecular weight of the lipase was estimated by comparing the relative mobility of proteins with the standard molecular marker.

Determination of pH optima and stability of enzyme

Lipase enzyme was assayed at different pH values to obtain optimum pH for enzyme. The enzyme activity was measured by standard assay method with 5% w/v gum acacia emulsified olive oil as the substrate in different buffer systems such as citrate buffer (pH 3.5-6.5), phosphate buffer (pH 7.5-8.5), glycine-NaOH (pH 9.5) and carbonate buffer (pH 10.5). The reaction mixture was incubated for 15 minutes at 37° C with 1ml of the respective buffer systems as blanks. The activity of the enzyme was measured by following the standard enzyme assay.

Determination of temperature optima and stability of enzyme

The effect of temperature on the enzyme was studied by conducting the assay at different temperatures including 30°C, 40°C, 50°C, 60°C, 70°C and 80°C in phosphate buffer at pH 7.2 for 15 minutes using 0.1ml of olive oil as substrate to obtain optimum temperature.

Effect of emulsifier on enzyme activity

Lipase activity was assayed with different emulsions made by using varying percentages of gum acacia such as 3%, 5%, 7% and 9% in 0.1 ml of olive oil and 0.8 ml of phosphate buffer (pH 7.2). To each emulsion, 0.1 ml of crude enzyme was added and the reaction was conducted for 15 minutes at 37°C with 0.1ml of phosphate buffer as blank.

Effect of substrate on enzyme activity

The effect of substrates was studied by incubating 0.1ml of lipase enzyme with different substrates such as olive oil, groundnut, coconut oil, sesame oil and sunflower oil each emulsified with 5%w/v gum acacia for 15min at 37°C.

Effect of metal ions on enzyme activity

The effect of various different metal ions such as Ca^{+2}, Mg^{+2}, Mn^{+2}, Hg^{+2}, Ni^{+2}, Fe^{+2}, EDTA and SDS was studied by pre-incubating the lipase enzyme with each metal ion (5mM concentration) at 36°C for 1h. The activity of the pre-incubated enzyme was determined by following the standard enzyme assay.

RESULTS AND DISCUSSION

Microorganism

Initially screening studies were conducted to isolate lipase producing bacteria from cloth samples. Water squeezed from cloth samples collected from dairy industry, Hyderabad, Telangana, India were serially diluted and plated on to nutrient agar plates at a pH of 7.3 at 36°C for 2 days. In total, 12 isolates were obtained from the cloth samples. The isolates were repeatedly subcultured to obtain pure cultures and were maintained on nutrient agar slants. All the isolates were screened for lipolytic activity by culturing on Tributyrin agar base plates (pH 7.0) at 36°C for 2 days. The zone of clearance formed due to hydrolysis of tributyrin indicated the lipolytic activity. Among the isolates one culture that showed profound lipolytic activity was designated as LSP-1and was chosen for further studies. Based on morphological, physiological and biochemical tests, the isolate was identified as *Staphylococcus epidermidis*.

Table 1: Morphological, Physiological and Biochemical Characterization of *Staphylococcus epidermis.*

Tests	L-2
Morphological tests	
Colony morphology	
Configuration	Circular
Margin	Entire
Elevation	convex
Surface	Smooth
Pigment	Cream
Opacity	Opaque
Gram's reaction	+
Cell shape	Coccus
Size (µm)	0.5 µ
Arrangement	Bunches
Spore(s)	-
Motility	-
Physiological tests	
Growth at temperatures	
4°C	-
10°C	-
25°C	+
30°C	+
37°C	+
42°C	+
55°C	-
Growth at pH	
pH 4.0	-
pH 5.0	-
pH 6.0	+
pH 7.0	+
pH 8.0	+
pH 10.0	+
Growth on NaCl (%)	
2.0	+
4.0	+
6.0	+
8.0	+
10.0	+
Growth under anaerobic condition	+
Biochemical tests	
Growth on MacConkey	+ (LF)
Indole test	-
Methyl red test	+
Voges Proskauer test	+
Citrate utilization	-
Casein hydrolysis	-
Esculin Hydrolysis	-
H_2S production	-
Gas production from glucose	-
Gelatin hydrolysis	-
Starch hydrolysis	-
Nitrate reduction	+
Ornithine decarboxylase	-
Lysine decarboxylase	(+)
Catalase test	+
Oxidase test	-
Tween 20 hydrolysis	-
Tween 40 hydrolysis	-
Tween 60 hydrolysis	-
Tween 80 hydrolysis	-
Dextrose	+
Maltose	+
Mannitol	-
Sucrose	+

The culture was sent for further identification to IMTECH, Chandigarh where it was identified and designated as *Staphylococcus epidermidis* strain L2 (MTCC 10656) (Table 1). *Staphylococcus epidermidis* strain L2 (MTCC 10656) was cultured in tributyrin broth (pH 7.0) containing 1% olive oil at 37°C and 150 rpm for 48 hours. The culture was centrifuged at 10,000 rpm for 10 min at 4°C and the culture supernatant was used as the crude enzyme for further studies. Protein concentration of the crude extract was estimated as 11.2mg/ml by Lowry *et al.*, 1951 method.

Purification of lipase enzyme

Lipase enzyme produced by *Staphylococcus epidermidis* was purified to homogeneity. Purification was carried out in three steps viz., acetone precipitation, Ion exchange chromatography (anion) and gel filtration chromatography. The results obtained from purification studies of lipase enzyme are shown in table 2.

The 48 h old culture was centrifuged culture supernatant was precipitated with 50% and 50 to 100% cold acetone. Maximum enzyme activity was observed in 50 % acetone precipitated sample. Maximum activity of 1345 U/ml was observed in 50% acetone fraction.

The protein fraction obtained from 50% cold acetone precipitation was further purified by Macro High Q anion exchange chromatography (Figure IA). Unbound protein samples have shown lipolytic activity. Maximum enzyme activity of the samples was recorded as 812.7 U/ml with a specific activity of 94.5U/mg.

The active fraction obtained from ion exchange chromatography was loaded onto Sephacryl S-200 matrix of gel filtration column (Figure IB). The pooled samples with lipolytic activity have shown a maximum enzyme activity of 562.1U/ml. Purification process has resulted in the lipase enzyme with a specific activity of 123.95 with a yield of 7.5%.

Table 2: Summary of purification studies on Lipase extracted from *Staphylococcus epidermidis* (MTCC 10656).

Purification Steps	Volume (ml)	Total protein (mg)	Total activity (U/ml)	Specific activity (U/mg)	Yield %	Fold purified
Crude	100	1120	7492	6.7	100	1
Acetone	10	54	1345	24.9	17.9	3.72
Ion Exchange Chromatography	10	8.6	812.7	94.5	10.8	14.1
Gel Filtration Chromatography	2	4.53	562.1	123.95	7.5	18.5

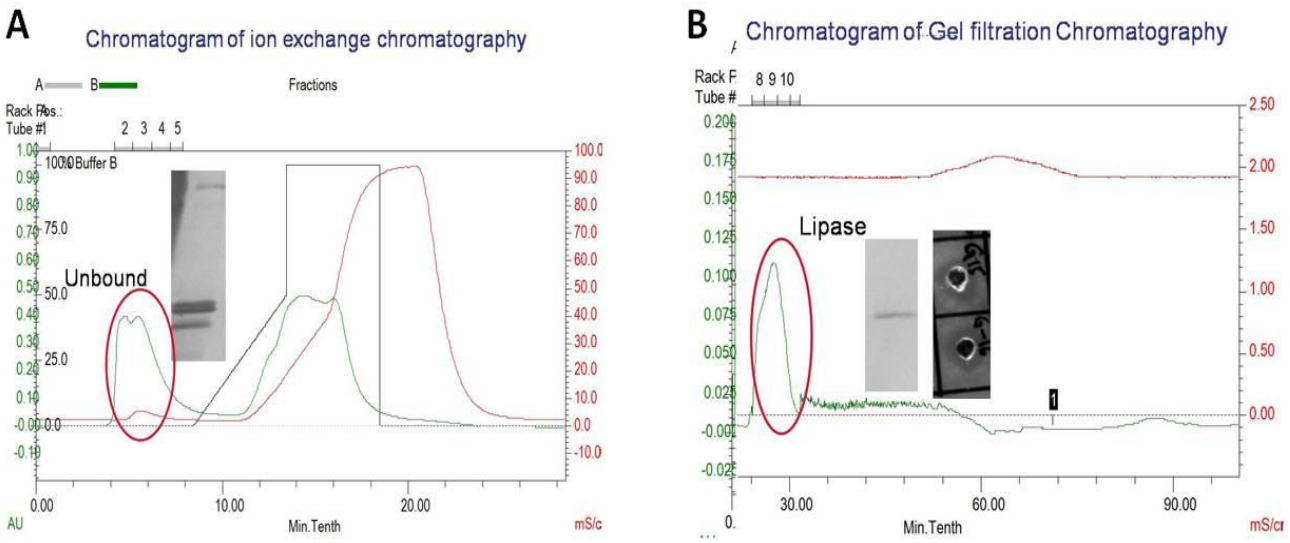

Fig. 1 A & B: A-Chromatogram of ion exchange chromatography, B-Chromatogram of gel filtration chromatography.

Figure 1 A shows the chromatogram of ion exchange chromatography conducted for the protein fraction obtained from acetone precipitation. The enzyme was eluted in unbound fraction. Figure 1 B shows the chromatogram of gel filtration chromatography of the protein sample obtained from ion exchange chromatography

Characterization studies

Determination of molecular weight of purified enzyme

Molecular weight of the enzyme was determined by SDS PAGE analysis. The gels were stained by using silver nitrate. The purified enzyme showing lipase activity was observed as one single band corresponding to the molecular weight of approximately 28KDa (Figure 2). Lipases (1&2) purified from, *staphylococcus warneri* were reported to have molecular weight of 28KDa and 40KDa and those from *staphylococcus aureus* and S. *hyicus* have molecular weights ranging from 28 – 46 KDa (Brune A.K.nad Gotz 1992; Valpato *et al.*, 2010).

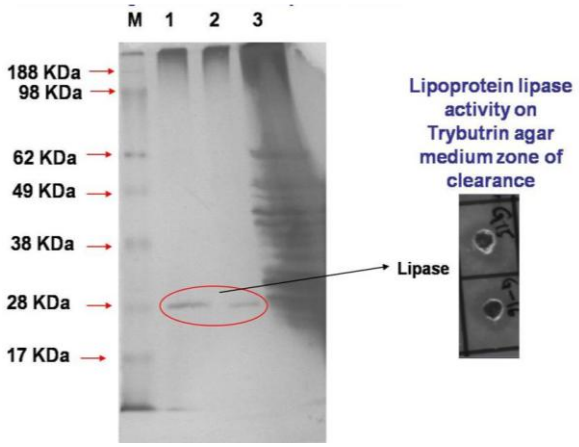

Fig. 2: SDS PAGE analysis of the purified lipase protein. L1- protein marker, L2- gel filtration sample 1, L3-gel filtration sample 2, L4- crude. SDS PAGE analysis of the protein sample obtained from gel filtration chromatography has shown that the active fraction coincides with the 28KDa band of standard molecular marker.

Optimum pH and Stability

The purified enzyme was found to be stable and active over broad pH range of 4.5 to 9.5. Maximum activity of the enzyme was observed at pH 7.5 (Figure 3).

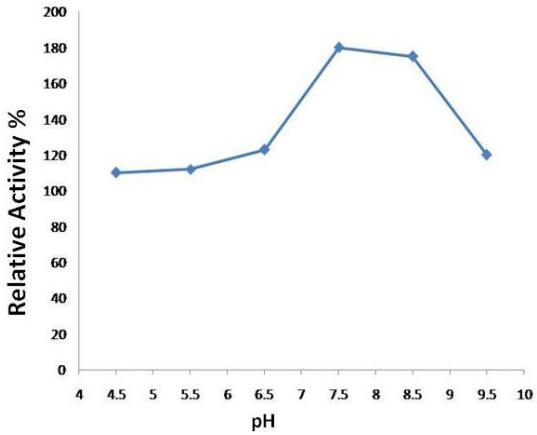

Fig. 3: Effect of pH over the lipase enzyme from *Staphylococcus epidermidis* L2. Studies on the effect of pH over the lipase enzyme activity have indicated that the enzyme is active over a broad range of pH of 4.5-10.0 with an optimum pH of 7.5

A gradual increase in lipase activity was observed from 5.5 to 7.7. Most of the bacterial lipases were reported to be active at neutral pH and were found to be stable over a broad pH range of 4 to 11 (Dharmsthiti et al. 1998; Dong et al. 1999). The optimum pH values of lipases obtained from *Staphylococcus aureus* and *Staphylococcus epidermidis* were reported as 8 and 8.5 respectively (Troller and Bozeman, 1970; Xie *et al*., 2012).

Optimum temperature and Stability

The lipase enzyme has shown activity over a temperature range of 35 to 45°C. The maximum enzyme activity was observed at 40°C (figure 4) and thereafter a sharp decrease in activity was observed up to 60°C. Temperature range for most of the bacterial lipases was reported as 30-60°C (Dharmsthiti and Luchai 1999; Sunna *et al.*, 2002). Temperature optima for lipase produced by *staphylococcus* species was reported as 32°C by Xie *et al.*, in 2012.

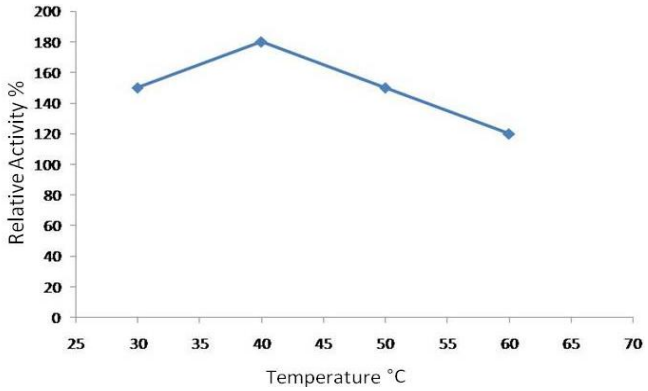

Fig. 4: Effect of temperature over the lipase enzyme from *Staphylococcus epidermidis* L2. Studies on the effect of temperature over the lipase activity have indicated that the optimum temperature for the maximum enzyme activity is 40°C.

Effect of emulsifier (Gum Acacia) on the enzyme activity

Gum Acacia was reported to affect the interfacial binding capacity and activity of the lipase enzyme (Tissa *et al.*, 2001). Concentration of emulsifier was found to directly affect the activity of the enzyme. In the present study, different percentages of the emulsifier (Gum Acacia) were used to obtain the optimum concentration of gum acacia for maximum lipase activity.

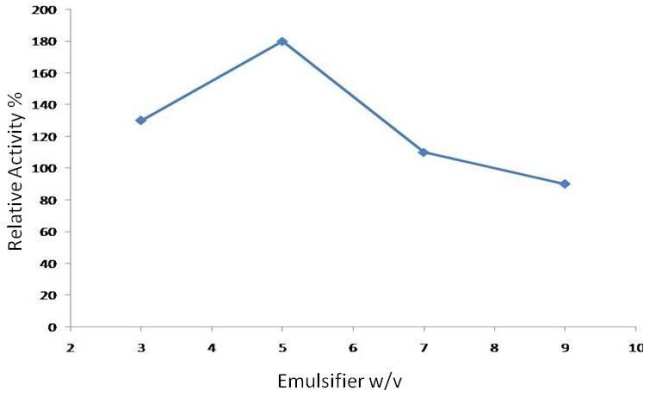

Fig. 5: Effect of emulsifier (Gum Acacia) on lipase activity. Studies on the effect of emulsifier over the lipase enzyme activity have indicated 5% gum acacia as the optimal emulsifier percentage for enzyme activity

Enzyme was stable at a range of emulsifier concentrations of 3-9% however maximum lipase activity was observed with 5% concentration of Gum acacia (figure 5).

Effect of substrates on enzyme activity

Bacterial lipases are 1,3-regiospecific. They hydrolyze only primary ester bonds and convert triglycerides to free fatty acids (Gupta *et al.*, 2004). Ground nut oil, sunflower oil, sesame oil and olive oil were used in 10% concentrations in the present study. Maximum enzyme activity was observed in olive oil and sesame oil whereas in coconut oil enzyme activity was profoundly decreased (figure 6).

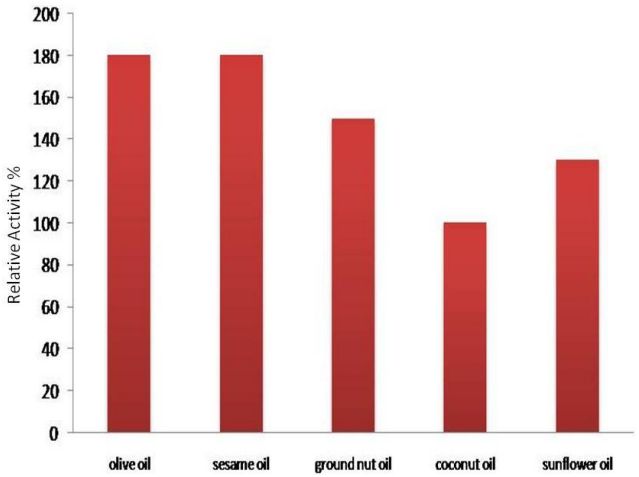

Fig. 6: Effect of different substrates on enzyme activity. Studies on the effect of substrates over enzyme activity have indicated olive oil and sesame oil as the best substrates.

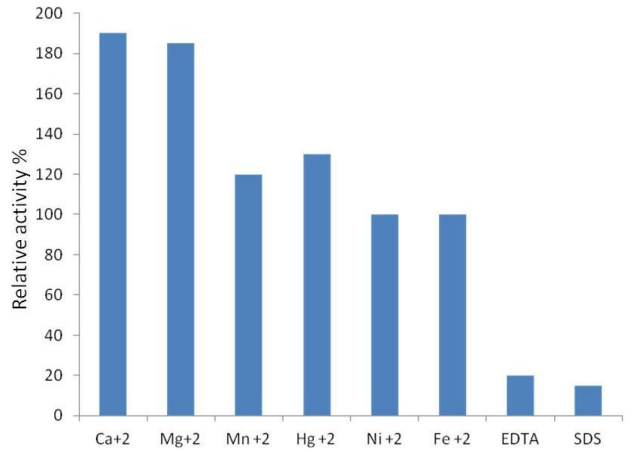

Fig. 7: Effects of metal ions on the lipase activity. Studies on the effect of different metal ions over enzyme activity have indicated Ca^{+2} and Mg^{+2} as the enhancers of enzyme activity

Effect of metal ions on enzyme activity

Maximum enzyme activity was found in presence of Ca^{+2}, Mg^{+2}. The presence of EDTA and SDS have inhibited 90% of the lipase activity. Only 50% of the activity was observed in presence of Mn^{+2}, Hg^{+2}, Ni^{+2}, Fe^{+2} (figure 7). Lipase isolated from *Burkholderia* sp. was reported to be stimulated in presence of Ca^{+2} and $Mg^{+}2$ (Rathi *et al.*, 2001).

CONCLUSION

Lipase is one of the most potential industrial enzymes. In the present study, an extracellular lipase enzyme was isolated from *Staphylococcus epidermidis* with a molecular weight of 28 KDa. The enzyme was found to be stable at 40°C, pH 7.5 and in different vegetable oils. This enzyme can be further scale-up for different commercial applications.

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Conflict of Interests: There are no conflicts of interest.

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