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Research Article

Organic Solvent Tolerant Lipase from *Pseudomonas aeruginosa* FW_SH-1: Purification and Characterization

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Abstract

We have aimed to isolate a new strain which produces organic solvent tolerant lipase, the strain was designated as FW_SH-1, that was identified as a *Pseudomonas* sp. The extracellular lipase from *Pseudomonas aeruginosa* FW_SH-1 was purified by Q - sepharose anion exchange chromatography. A final specific activity of 572 U/mg was achieved with 26.12 fold purification and an overall recovery of 10.21%. The molecular weight of the purified lipase was found to be 66 kDa using SDS polyacrylamide gel electrophoresis. The optimum pH and temperature for the activity of the purified lipase was found to be 8.0 and 45°C. The purified lipase was stable in the presence of hydrophilic solvents such as methanol, ethanol and propanol. The activity of the purified lipase was stimulated in the presence of Ca^{2+}, Mg^{2+}, Na^{+}, K^{+} and Tween 20.

ABBREVIATIONS

rRNA: Ribosome Ribonucleic Acid; RPM: Revolution Per Minute; p-NPP: Para - Nitrophenyl Palmitate; BSA, Bovine Serum Albumin; SDS - PAGE: Sodium Dodecyl Sulfate - Polyacrylamide Gel Electrophoresis; (v/v): Volume/Volume; EDTA: Ethylene Diamine Tetra-acetic Acid; SDS: Sodium Dodecyl Sulfate

INTRODUCTION

Pseudomonas genus, commonly present in soil and water [1] has considerable scientific and technological significance and consists of variety of organisms with ability to utilize wide range of simple and complex organic compounds [2]. Recently significant consideration has been given to explore *Pseudomonas* species because of their importance in the field of medicine, environmental microbiology, food technology, bio - energy and phytopathology [3]. They are known to bring about the biodegradation of natural and man - made toxic chemical compounds. Apart from that, the bacterial genus *Pseudomonas* is a remarkable producer of a number of extracellular enzymes, including lipase [1,4,5]. Lipases (E.C.3.1.1.3) are interfacial enzymes with tendency to catalyze the hydrolysis of ester bonds in long chain triacylglycerols to release free fatty acids [6]. Due to their unique biotechnological versatility and their capability to catalyze wide range of biochemical reactions, such as the synthesis of agrochemicals, pharmaceutical intermediates and flavor compounds, they are in focus recently [7,8]. Microbial lipases are more stable than the one attained from animals and plants, while those produced from *Acinetobacter, Bacillus, Staphylococcus* and *Pseudomonas* have revealed high stereo and region - selectivity, making them important enzymes in Biocatalysis [8,9]. The reactions being catalyzed by the lipases in the presence of organic solvents have many advantages. However lipases tends to deactivate in the presence of organic solvents, especially hydrophilic ones, due to the ability of the solvent to strip off water molecules from enzyme surface, thus leading to deactivation of the enzyme [10]. In order to reduce this problem certain strategies such as immobilization, chemical modification and protein engineering have been utilized for the stabilization of enzymes for use in such solvents [11]. It has been proposed to screen for naturally evolved solvent tolerant lipases instead of modified lipases.

The lipases obtained from microbial origin are utilized in variety of applications including food, dairy, detergents,

pharmaceutical, cosmetics and biodiesel industries [12]. Each application requires particular properties of lipases with respect to temperature, pH and stability in organic solvents [13]. The lipase activity is highly dependent on pH and any change in the pH of the reaction mixture can affect the reaction kinetics. The enzymes stable at high temperatures are also very useful in industrial applications due to high reaction rates at elevated temperatures, while stability of enzymes in the presence of metal ions and organic solvents provides extra advantage in the overall outcome of the process [14]. In the present work, a novel solvent tolerant extracellular lipase from newly isolated *Pseudomonas aeruginosa* FW_SH-1 was purified by acetone precipitation and ion exchange chromatography. The purified lipase was characterized and compared with lipases obtained from other bacterial strains.

MATERIALS AND METHODS

Microorganism and chemicals *Pseudomonas aeruginosa* FW_SH-1, which produced the alkaline and organic solvent tolerant lipase, was used in the present study. The strain was isolated and identified in our laboratory; the partial 16S rRNA gene sequence from strain (FW_SH-1) was deposited in Gen Bank database under accession number KJ510652. The Phylogenetic tree was constructed by using molecular evolutionary genetics analysis (MEGA-6) software [15] which confirms that the isolated strain belongs to the genus Pseudomonas (Figure 1). *P. aeruginosa* strain was cultivated in a medium that was previously optimized nutritionally and physically for lipase production in our laboratory [16]. All of the media components were of analytic grade. Para- nitrophenyl palmitate (p - NPP) was obtained from sigma chemicals, USA; Q - Sepharose columns were purchased from GE healthcare bioscience, Sweden. Molecular weight markers were obtained from Ding Guo, Shanghai, China.

Inoculum preparation

P. aeruginosa was maintained on agar slant and kept at 4°C. A loopful of stock culture was transferred into a nutrient medium and utilized as inoculum. The inoculum culture was cultivated at 37°C and 150 rpm for overnight.

Lipase production

The lipase production medium contained peanut oil (15 mL/L), sucrose (3.5 g/L), tryptone (1 g/L), $(NH_4)_2SO_4$ (2.8 g/L), $MgSO_4$ (0.5 g/L), K_2HPO_4 (2 g/L), KH_2PO_4 (2 g/L) and NaCl (0.2 g/L) that were used to cultivate lipase from *P. aeruginosa*. The cultivation was carried out at 37°C and 150 rpm for 48 h using 3% (v/v) inoculum. Samples were collected at regular intervals and centrifuged at 12,000 × g at 4°C for 20 min. The cell free supernatant was used as a crude enzyme to determine the lipase activity from it by following standard lipase assay conditions.

Measurement of lipase activity

Extracellular lipase activity was measured spectrophotometrically by Winkler and Stuckman (1979) method with slight modifications. The substrate solution containing 10 mL of isopropanol with 30 mg of *p*-NPP was mixed with 90 mL of Tris-HCl buffer (0.05 M, pH 7.2), containing 0.4 % Triton-X-100 and 0.1% of gum Arabic [17]. Freshly prepared substrate solution (2.5 mL) was incubated at 37°C with 50 µL of lipase for 10 min. After incubation absorbance was measured at 410 nm by using a spectrophotometer against a control without enzyme. One unit of enzyme is defined as the amount of enzyme liberating 1 µmole of *p*-nitrophenol (*p*-NP) mL^{-1} min^{-1} under the assay conditions.

Purification of lipase

The cell free supernatant (crude lipase) was used for the purification of lipase. The crude lipase medium was first introduced to acetone precipitation. The ice cooled acetone was added to the tubes containing crude lipase mixture. The acetone added were four times the volume of supernatant. The mixture was than vortexed and incubated for 60 min at -20°C. The precipitated mixture was then centrifuged for 15 min at 12,000 × g at 4°C. The pellets obtained were dissolved in 0.5 M Tris-HCl (pH 8.0) buffer. The solution was analyzed for lipase activity and protein concentration.

The partially purified lipase in buffer solution was then treated with anion exchange chromatography. The column was packed with 5 ml of anion exchange Q - Sepharose sorbent slurry (size 3 × 1.5 cm) (GE Biosciences, Uppsala, Sweden). The column was first equilibrated with a three column volume of 50 mM Tris-HCl buffer (pH 8.0) containing the enzyme. Then the column was washed with five column volume of the same buffer to eliminate the unbound lipase. The bounded lipase was eluted by the elution buffer with the step wise increment of NaCl from 50 mM to 150 mM. All of the eluted fractions were analyzed for lipase activity. The fractions with highest lipase activity were pooled and analyzed for protein content. Thus, the specific activity of the

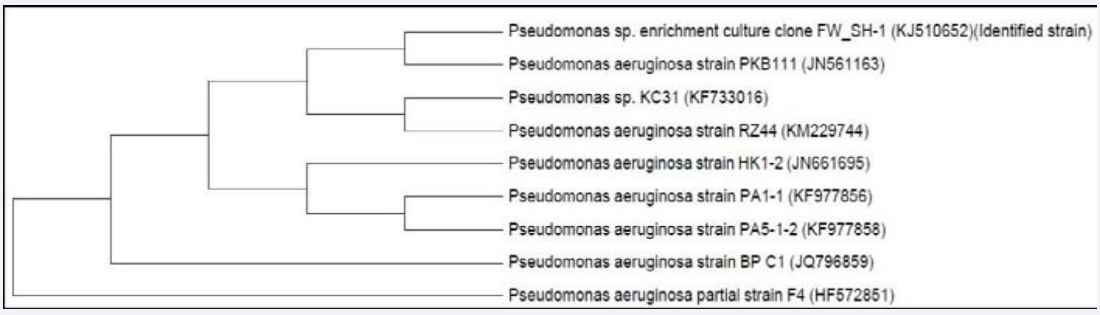

Figure 1 Phylogenetic tree showing the relationship of isolate *Pseudomonas* sp. FW_SH-1 (KJ510652) to other *Pseudomonas* sp. values shown in the parenthesis are accession number.

purified lipase was compared with that of the crude lipase and the purification fold was determined.

Protein estimation

The protein concentration was determined, according to the Bradford method [18], utilizing the Bradford reagent and bovine serum albumin (BSA) as the standard.

Determination of molecular weight: Sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE) was used to determine the molecular weight of the purified lipase as described by Laemmli [19]. The relative molecular mass was determined by comparing with molecular markers (20 - 97 kDa). Purified lipase was subjected to SDS - PAGE after boiling for 10 min. Then upon electrophoresis the gel was dyed for overnight. The protein bands were visualized by coomassie brilliant blue R-250. Then the gel was washed with decolorizing solution, followed by immersing the gel in water in order to swell the gel before being visualized for occurrence of protein bands.

Characterization of purified lipase

Effect of pH and temperature on lipase activity: The effect of pH on lipase activity was investigated by using different buffer systems in the pH range (4-9) at concentration of 50 mM: sodium acetate buffer (pH 4-5), sodium phosphate buffer (pH 6-7) and Tris-HCl buffer (pH 8-9). To conduct the lipase stability studies, 300 µL of the purified lipase solution was mixed with 700 µL of 50 mM buffer at specific pH value. The mixture was incubated at 37°C for 60 min and then the residual activity was determined. The effect of temperature on lipase activity was studied at different temperatures (25, 35, 45, 55 and 65°C) under optimized pH 8.0. The lipase thermal stability was analyzed by incubation of 100 µL of purified lipase solution at desired temperatures for 60 min. The remaining lipase activity was measured by using standard lipase assay conditions as described previously.

Effect of organic solvents on lipase activity: The effect of organic solvents on the lipase activity of the purified lipase was monitored. The organic solvents included methanol, ethanol, propanol, benzene, toluene, hexane, butanol, acetone, methyl acetate, heptane and octane that were used to analyze solvents effect on purified lipase. The concentrations of all the organic solvents were kept at 25% (v/v). 50 µL of organic solvents was added to 150 µL of the purified lipase solution. The mixture was incubated for 60 min at 45°C and 150 rpm. The remaining lipase activity was determined by following standard lipase assay conditions, while the residual activities were calculated by comparing the enzyme activities of purified lipase with and without solvent. The effect of propanol on purified lipase with different concentrations (50, 75, and 100 % v/v) by using the same method as described above was also studied.

Effect of metal ions and inhibitors on lipase activity: The effects of different metal ions such as Ca^{2+}, Mg^{2+}, Zn^{2+}, Mn^{2+}, Fe^{3+}, Cu^{2+}, Na^{+}, K^{+} and Al^{3+} ions ($CaCl_2$, $MgCl_2$, $ZnSO_4$, $MnCl_2$, $FeCl_3$, $CuSO_4$, NaCl, KCl and $AlCl_3$ respectively) on lipase activity were analyzed. All the salt - ions solutions were prepared and maintained at the final concentration of 1 and 5 mM. The mixture containing 500 µL of lipase and 500 µL of salt - ions was incubated for 60 min at 45°C and 150 rpm. The effects of various inhibitors such as EDTA, SDS, Triton X-100 and Tween-80 on lipase activity were also studied. All of the inhibitors were separately maintained at concentration of 1 and 5 mM. The mixture containing 500 µL of lipase and 500 µL of inhibitors was incubated for 60 min at 45°C and 150 rpm. Then the lipase activity was assayed and the residual lipase activity was calculated by comparing the lipase activities in the presence and absence of the effectors. All of the experiments were performed in triplicates.

RESULTS AND DISCUSSION

Purification of lipase

The lipase in the cell free supernatant (22.031 U/mg) was precipitated using acetone. The precipitated protein was dissolved in 50mM Tris-HCl (pH-8) buffer. The precipitated lipase showed the specific lipase activity of 105.75 U/mg with 4.77 fold purification. Lipase was further purified by the Q - sepharose anion exchange chromatography. The partially purified lipase was loaded into the ion exchange column and the binding enzyme was eluted out at 150 mM concentration of NaCl. The enzyme was purified to approximately 26 fold with a specific activity of 572.02 U/mL and an overall yield of 10.21 %. The purification results are summarized in Table (1). According to the previous reports, *Aneurinibacillus thermoaerophilus* strain HZ producing organic solvent tolerant lipase was purified to 15.62 fold with an overall yield of 19.69 % [20]. Lipase from *Pseudomonas aeruginosa* LX1 has been purified by using ammonium sulphate precipitation and DEAE - sepharose ion exchange chromatography with purification fold of 4.3 and an overall yield of 41.1% [21]. In the present work we have used two purification steps to purify the enzyme, thus obtaining purification of 26 - fold and specific activity of 572 U/mg.

Determination of Molecular weight

SDS - PAGE was used to confirm the homogeneity of the eluted protein. The purified lipase showed the single band on 10% polyacrylamide gel in the presence of SDS (Figure 2). The single protein band with a molecular weight of approximately 66 kDa indicated that the lipase was novel enzyme from *P. aeruginosa* strain. The molecular weight of 66 kDa of lipase from *Pseudomonas* sp. has not been determined before. Hence it suggests that this lipase has novel characteristics. On the other hand lower molecular weight of lipase from *Pseudomonas aeruginosa* LST-03 of around 27.1 kDa has been reported [22]. Whereas higher molecular weight of around 54 kDa has been reported from a thermo stable lipase from *Pseudomonas aeruginosa* San-ai [23]. The highest molecular weight of lipase from *P. aeruginosa* strain has been reported to be 60 kDa [24]. Mesophilic lipases from many *Pseudomonas* sp. have been found to possess a molecular weight of 44-60 kDa [21].

Characterization of lipase

Effect of pH on lipase activity: The purified lipase from *P. aeruginosa* was able to tolerate wide range of pH from 6.0 to 9.0. The maximum relative activity was recorded at pH 8.0 as shown in Figure (3). The lipase activity significantly dropped when the pH was increased up to 9.0 where relative activity approaches 74%. Also the activity was reduced considerably at pH value below 6.0 and a loss of 60% of the maximal activity occurred. The

Table 1: Summary of purification of the lipase from *Pseudomonas aeruginosa* FW_SH-1.

Step	Volume (mL)	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Culture supernatant	500	6.4	141	22.031	1	100
Acetone precipitation	100	0.452	47.8	105.75	4.77	33.89
Q - sepharose Ion exchange	25	0.025	14.3	572.02	26.12	10.21

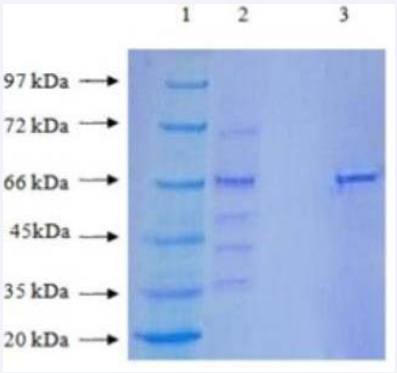

Figure 2 SDS-PAGE, (Lane 1) Standard protein marker (97 kDa-Phosphorylase; 66 kDa-BSA; 45 kDa- Ovalbumin; 35 kDa- Lactate dehydrogenase; 25 kDa-REase Bsp981 , (Lane 2) Crude enzyme, (Lane 3) Purified enzyme.

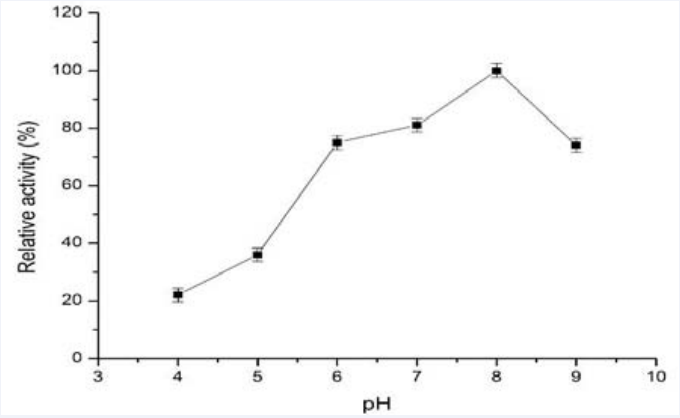

Figure 3 Effect of pH on lipase activity.

good stability of enzyme in broad pH range was due to firmness of the secondary structure of lipase in the present work and the similar sort of pH activity and stability trends were also reported previously [25,26]. Singh et al., (2007) has also found stable alkaline lipase from *P. aeruginosa* strain with excellent stability in pH range of 7-9 [27]. Whereas Ji et al., (2010) has reported a lipase which retains its maximum activity at pH 7.0 while its activity decreased sharply with increasing or decreasing the pH value even for single unit [21].

Effect of temperature on lipase activity: The purified enzyme was active in the range of temperature from 25 to 55°C with higher activity recorded at 45°C as shown in Figure (4). The maximum relative activity was observed to be 110 ± 2.42% at 45°C as compared to control. The relative activity of lipase decreased sharply from 85% to 45% in the temperature range of 55-65°C respectively. Similar trends of temperature stability of purified lipase from mesophilic strains have also been noticed previously [28,29].

Effect of organic solvents on lipase activity: The lipase has a good stability in hydrophilic solvents such as methanol, ethanol and propanol as shown in Table (2). The highest effect was observed with 25% (v/v) of propanol and ethanol showing 103.3% ± 1.86 and 97.21% ± 2.26 respectively. Closed chain organic solvents such as benzene and toluene had affected the activity of lipase in adverse way with 77.05% ± 2.63 and 79.66% ± 1.76 of relative activity recorded. Whereas hydrophobic long chain alkanes heptanes and octane negatively affected the activity and around 46% and 50% loss of activity occurred. Generally hydrophilic solvents cause more adverse enzyme denaturation as compared to hydrophobic ones [26] but in the present work the trend is different. The good stability in the polar solvents makes the lipase very useful to catalyze transesterification reaction to

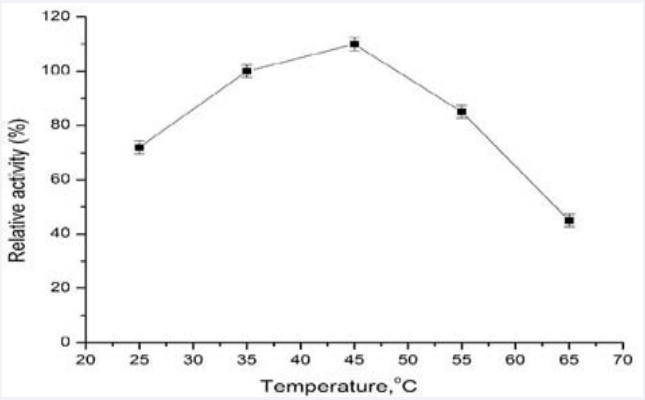

Figure 4 Effect of temperature on lipase activity.

Table 2: Effect of organic solvents on lipase activity.

Organic solvent 25 % (v/v)	Log *P*	Relative activity (%)	Organic Solvent (Propanol) (%v/v)	Relative Activity (%)
Control		100 ± 0.00	Control	100 ± 0.00
Methanol	-0.76	97.18 ± 2.42	0	100 ± 1.86
Ethanol	-0.24	97.21 ± 2026	25	102.28 ± 2.42
Propanol	0.02	103.3 ± 1.86	50	82.78 ± 2.15
Benzene	2	77.05 ± 2.63	75	66.65 ± 2.67
Toluene	2.5	79.66 ± 1.76	100	53.65 ± 2.75
Hexane	3.5	68.98 ± 2.14		
Butanol	0.83	80.50 ± 2.54		
Acetone	-0.23	81.15 ± 2.05		
Methyl	0.73	81.70 ± 1.35		
Acetate				
Heptane	4	54.33 ± 2.64		
Octane	4.9	50.62 ± 2.75		

produce biodiesel. Yoo et al., (2011) has also found hydrophilic solvent ethanol as the most stabilizing solvent for its lipase with residual activity of 108.44% ± 5.2 recorded at 25% (v/v) of ethanol in lipase [30]. As the concentration of propanol increased the relative activity further decreased as shown in Table (2).

Effect of metal ions on lipase activity: The presence of metal ions such as Ca^{2+} and Mg^{2+} at concentration of 1mM and 5 mM have increased lipase activity up to 5-15% more than the control, while 5mM of Na^{+} and K^{+} ions have promoted the activity up to 12-14%. (Table 3) indicates that the purified lipase in the present work has been stable in variety of metal ions. On the other hand metal ions such as Zn^{2+}, Mn^{2+}, Cu^{2+} and Al^{3+} has adversely affected the lipase activity with zinc affecting the most with 28% loss in activity at concentration of 1mM, while aluminum affected the most with 35% loss in activity at concentration of 5mM. Jiewie et al., (2014) has also found the presence of K^{+} and Na^{+} ions to increase the relative activity [31]. Sivaramakrishnan et al., (2012) also has found Ca^{2+}, Mg^{2+} and K^{+} ions to be positively affecting the relative activity of its purified lipase obtained from bacterial strain [32]. Whereas Mander et al., (2012) has discovered that the presence of Ca^{2+} ions significantly deteriorates the relative activity up to 40% while Mg^{2+} and Na^{+} ions elevates the relative activity [33].

Effect of inhibitors on lipase activity: The lipase was sensitive to the presence of EDTA and SDS at the concentration of 1-5 mM. EDTA with a concentration of 5mM significantly reduced the lipase activity around 46% as shown in Table (4). The surfactant SDS reduced the lipase activity up to 42%. The lipase activity was more affected with higher concentration of Triton X-100; with 1mM concentration, 12% loss in activity took place, while at 5mM concentration 22% loss in activity occurred. Both Tween-20 and Tween-80 have positively affected the lipase with increase in relative activity up to 2-6% as compared to the control. Dharmsthiti et al., (1998) also has found its lipase to be inactivated by EDTA, while the enzymes activity enhanced with Tween-20 and Tween-80 [34].

CONCLUSION

Pseudomonas aeruginosa FW_SH-1 is a source of novel lipase and with high specific activity and good stability in organic solvents makes the enzyme very useful for biotechnological applications. An effective approach of purifying the enzyme using acetone precipitation and Q - sepharose anion exchange chromatography with overall yield of 10.21% was carried out. The SDS - PAGE confirmed the presence of purified lipase, with a molecular weight of 66 kDa. The purified enzyme had optimum temperature and pH of 45°C and 8.0 respectively. The metal ions

Table 3: Effect of metal ions on lipase activity.

Metal ions	Relative activity (%)	
	1 (mM)	5 (mM)
Control	100 ± 0.00	100 ± 0.00
Ca^{2+}	103 ± 1.67	108 ± 1.75
Mg^{2+}	112 ± 1.37	115 ± 1.54
Zn^{2+}	72 ± 0.86	67 ± 1.32
Mn^{2+}	85 ± 1.62	78 ± 2.01
Cu^{2+}	78 ± 1.82	70 ± 1.68
Fe^{3+}	92 ± 2.12	86 ± 1.98
Na^{+}	104 ± 1.64	114 ± 1.82
K^{+}	106 ± 1.53	112 ± 0.98
Al^{3+}	73 ± 1.61	65 ± 1.86

Table 4: Effect of inhibitors on lipase activity.

Inhibitors	Relative activity (%)	
	1 (mM)	5 (mM)
Control	100 ± 0.00	100 ± 0.00
EDTA	72 ± 1.45	54 ± 1.62
SDS	84 ± 1.23	58 ± 2.01
Triton X-100	88 ± 1.64	78 ± 2.32
Tween-20	105 ± 2.10	106 ± 1.82
Tween-80	102 ± 1.85	103 ± 2.12

such as Ca^{2+}, Mg^{2+}, Na^{+}, K^{+} and Tween (20 and 80) as surfactants were found to enhance the relative activity. The organic solvents such as methanol, ethanol and propanol had a very little effect in deactivating the lipase. The properties of purified lipase make it a very favorable biocatalyst for catalyzing various reactions in presence of short chain polar organic solvents.

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