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# Purification and Biochemical Characterization of an Extracellular Lipase from *Pseudomonas fluorescens* MTCC 2421

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An extracellular lipase produced by *Pseudomonas fluorescens* MTCC 2421 was purified 184.37-fold with a specific activity of 424.04 LU/mg after anion exchange and gel exclusion chromatography. The enzyme is a homomeric protein with an apparent molecular mass of 65.3 kDa. The lipase exhibited hydrolytic resistance toward triglycerides with longer fatty acyl chain length containing unsaturation as evident from the lower $V_{max}$ (0.23 mM/mg/min) of the lipase toward glycerol trioleate ($C_{18:1n9}$) compared with the fatty acid triglycerides having short to medium carbon chain lengths ($C_{18:0-12:0}$, $V_{max}$ 0.32−0.51 mM/mg/min). This indicates a preferential specificity of the lipase toward cleaving shorter carbon chain length fatty acid triglycerides. The lipase exhibited optimum activity at 40 °C and pH 8.0, respectively. A combination of $Ca^{2+}$ and sorbitol induced a synergistic effect on the thermostability of lipase with a significantly high residual activity (100%) after 30 min at 40 °C, as compared to 90.6% after incubation with $Ca^{2+}$ alone. The lipase activity was inhibited by $Cu^{2+}$ and $Fe^{2+}$ (42 and 48%, respectively) at 10 mM. The enzyme lost 31% of its initial activity by 0.001 mM EDTA and 42% by 0.1 mM EDTA. Significant reduction in lipase activity was apparent by 2-mercaptoethanol and phenylmethanesulfonyl fluoride at diluted concentration (0.001 mM), thereby indicating an important role of sulfhydryl groups in the catalytic mechanism.

**KEYWORDS: Lipase; *P. fluorescens* MTCC 2421; sorbitol; $Ca^{2+}$; fatty acids; glycerol trioleate**

## INTRODUCTION

Lipases are a specific class of esterases responsible for the hydrolytic cleavage of carboxyl esters such as triacylglycerols and are used to concentrate unsaturated fatty acids, which are known to possess pharmacological effects on animal and human nutrition (*1*). The majority of the animal kingdom lacks essential enzymes required for synthesizing them in sufficient quantity from the precursor molecules. Therefore, they require unsaturated fatty acids in high concentration for their survival, and therefore, the preparation of unsaturated fatty acid concentrates as food supplements is an emerging research area (*2*). Among several techniques, the use of lipases to concentrate a specific class of fatty acids is highly preferred by virtue of their high specificity and mild reaction conditions (*3*, *4*). There are reports of lipases purified from animals, plants, and microorganisms (*5*, *6*). The vast majority of microbial lipases reported in the literature are of bacterial origin. The bacterial lipases were proved to be valuable by virtue of their higher stability compared with animal or plant lipases (*7*). In recent years, a number of bacteria producing thermophilic and alkalophilic lipases have been purified and characterized (*8*). The bacterial genus *Pseudomonas* was reported to secrete a number of extracellular enzymes, lipases being the major class. There are reports of *Pseudomonas fluorescens* producing an alkaline lipase (*9*, *10*). *P. fluorescens* SIK W1 was found to produce a thermostable lipase, which has high lipolytic activity for short- to medium-chain triacylglycerols (*11*). Lipases were found to discriminate between different fatty acids and exhibit less hydrolytic activity toward ester bonds of polyunsaturated fatty acids (PUFAs) (*3*, *12*). The unique substrate and/or stereospecificity of bacterial lipase was used for selective enhancement of targeted fatty acids in triglycerides from fish oils (*13*).

Biochemical characterization of lipases is a preliminary requirement to identify their unique position and/or stereospecificity, which will guide us to use them as candidate biocatalysts for concentrating a specific class of fatty acids. In the present study, *P. fluorescens* MTCC 2421 was chosen for purification and characterization of an extracellular lipase. We report methods for the purification of an extracellular metallolipase from *P. fluorescens* MTCC 2421 culture broth by physical precipitation followed by chromatography on anion exchanger and molecular sieve and its biochemical characterization. The said lipase has been characterized with respect to its substrate specificity intended for use to fractionate specific fatty acids with respect to different acyl lengths ($C_{12-18}$) and unsaturation in a triglyceride mixture.

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## MATERIALS AND METHODS

**Chemicals and Reagents.** All solvents used for sample preparation were of analytical grade (E-Merck, Darmstadt, Germany). Anion exchanger Amberlite IRA 410 ($Cl^-$ form), bovine serum albumin, and phenylmethanesulfonyl fluoride (PMSF) were procured from HiMedia (Mumbai, India); Sephadex G-100 and other chemicals were from Sisco Research Laboratories (Mumbai, India). *P. fluorescens* MTCC 2421 culture was procured from Institute of Microbial Technology, Chandigarh, India. Electrophoresis grade acrylamide, bisacrylamide, medium-range molecular marker proteins, and Coomassie brilliant blue R-250 were procured from Bangalore Genei (Bangalore, India).

**Preparation of Crude Lipase from *P. fluorescens* MTCC 2421.** *P. fluorescens* MTCC 2421 was grown on tributyrin slants (0.3% meat extract, 0.5% peptone, 0.9% NaCl, 0.25% tributyrin, and 0.001% $CaCl_2 \cdot 2H_2O$) and inoculated in an Erlenmeyer flask containing nutrient broth (50 mL). The content was then incubated at 37 °C for 24 h under shaking (150 rpm) to raise the inoculum for the enzyme production. The seed culture (100 mL) (1% v/v) of *P. fluorescens* MTCC 2421 was grown in a broth containing (g $L^{-1}$) $NaNO_3$, 3; $K_2HPO_4$, 0.1; $MgSO_4 \cdot 7H_2O$, 0.5; KCl, 0.5; $FeSO_4 \cdot 7H_2O$, 0.05; and yeast extract, 5.0, and cod liver oil (1%, v/v) at 37 °C in a 1000 mL Erlenmeyer flask with shaking (150 rpm) for up to 72 h. The culture broth was harvested at different time intervals (0, 5, 10, 24, 29, 34, 48, 53, 58, and 72 h) to determine the optimum time for maximum lipase production. Cell growth was measured by recording the OD of the cultures of different time intervals at 600 nm. The increase in optical density at 600 nm was considered to be an indication of the increase in cellular growth of the bacteria. The crude broth was clarified by centrifugation (10000*g* for 20 min at 4 °C, Superspin R-V/FM Plasto Crafts, Mumbai, India) to obtain a supernatant that was filtered (0.2 $\mu$m). This concentrated filtrate was referred to as the crude lipase.

**Purification of Lipase from *P. fluorescens* MTCC 2421.** The crude lipase thus obtained was purified to homogeneity by ammonium sulfate precipitation, Amberlite IRA 410 ($Cl^-$ form) anion exchange, and Sephadex G-100 gel exclusion chromatography.

*Ammonium Sulfate Precipitation*. Solid ammonium sulfate was added to the crude enzyme extract in increments of 5% until 70% saturation (w/v) with stirring and allowed to stand overnight to obtain the precipitate, which was thereafter harvested by centrifugation (10000*g*, 30 min, 4 °C) (*3*). The supernatant was discarded to obtain the pellet that was dissolved in Tris-HCl buffer (50 mM, pH 8.0), dialyzed against the same buffer for 18 h to remove residual salts, and assayed for lipase activity.

*Chromatographic Purification of the Lipase*. The enzyme concentrate obtained from dialysis was loaded on an Amberlite IRA 410 (Cl- form) column (1.5 × 15 cm) pre-equilibrated with Tris-HCl buffer (50 mM, pH 8.0), and the bound proteins were eluted in the same buffer with a linear gradient of NaCl (0−0.5 M). The active fractions were pooled and concentrated by lyophilization. The lyophilized protein was dissolved in distilled water (5 mL) and dialyzed against Tris-HCl buffer (50 mM, pH 8.0) to obtain the concentrated protein, which was rechromatographed on a Sephadex G-100 gel exclusion column (2.5 × 120 cm). The column was equilibrated and eluted with Tris-HCl buffer (50 mM, pH 8.0) supplemented with $CaCl_2$ (1.0 mM). The eluant fractions showing lipase activity were pooled together and analyzed for lipase activity. The active enzyme fractions were stored at 4 °C until use for polyacrylamide gel electrophoresis and biochemical characterization.

**Lipase Activity Assay.** The lipase activity of the enzyme was estimated spectrophotometrically (Varian Cary 50 Conc.) using 4-nitrophenyl palmitate (4-NPP) as substrate following an established procedure with modification (*14*). Briefly, a reaction mixture consisting of 4-NPP stock solution (20 mM, 100 $\mu$L) in Tris-HCl buffer (50 mM, pH 8.0, 1.0 mL) containing $CaCl_2$ (10 mM, pH 8) was dissolved in isopropanol to obtain a final volume of 3 mL, to which the enzyme (10 $\mu$L) was added. The reaction mixture was incubated at 45 °C for 20 min, and the reaction was terminated by adding chilled acetone/ethanol (1:1 v/v). The aliquot was clarified by centrifugation (5000*g*, 10 min) to furnish a clear supernatant. The absorbance of the supernatant containing released 4-nitrophenol (4-NP) was measured at 410 nm ($A_{410}$). One activity unit of lipase (LU) was defined as the micromolar concentration of 4-NP released from hydrolysis of 4-NPP/ mL/min by 1 mL of enzyme under standard assay conditions. The protein concentration was determined by measuring the absorbance at 590 nm (Varian Cary 50 Conc.) using bovine serum albumin (20−150 $\mu$g) as standard (*15*).

**Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS-PAGE).** SDS-PAGE of the purified enzyme solutions (20 $\mu$L) was carried out using 0.1% w/v sodium dodecyl sulfate on a 12% polyacrylamide gel (with 6% stacking gel) (*16*) on a vertical slab midi-gel apparatus (model PS-500; Bangalore Genei). After migration, the gels were fixed with acetic acid/methanol (7% v/v), and the protein bands were visualized by alternative staining (with Coomassie brilliant blue R-250, 0.1% w/v) and destaining (with acetic acid/methanol, 14% v/v). The apparent molecular mass of the purified lipase was determined with reference to the medium-range markers (14.4−94.0 kDa, Bangalore Genei). The active lipase fractions were identified by analytical SDS-PAGE of proteins using 12% gels stained with Coomassie brilliant blue R-250. The gels were incubated overnight in the refolding buffer (20 mM Tris-HCl, pH 8.0, 10 mM $CaCl_2$, 4 °C) and were transferred to a solution of Tris-HCl (100 mM, pH 8.0) containing $\beta$-naphthyl acetate and fast blue RR (*17*). The gels were rinsed with water and stored in acetic acid (7% v/v) (*18*) to develop a dark yellow color. To further validate the approximate molecular weight of the purified lipase, gel filtration chromatography with Sephadex G-75 was used. The elution volume of the lipase was compared with those of known protein standards (horse heart cytochrome C, 12.4 kDa; soybean trypsin inhibitor, 20.1 kDa; bovine serum albumin, 66 kDa; $\beta$-phosphorylase, 97.4 kDa), and the molecular masses of standard proteins were plotted against their respective ratios of elution volume to column void volume ($V_e/V_0$) to draw the calibration curve, the column void volume ($V_0$) being the elution volume of blue dextran (2000 kDa).

**Effect of pH and Temperature on Lipase Activity.** The pH profiling of the purified lipase was performed at different pH values (from 2 to 12, with 1.0 difference). The enzyme (5 $\mu$L) was added with the reaction mixture containing 4-NPP as substrate and Tris-HCl buffer (50 mM, 50 $\mu$L) as reaction medium at each of the above pH values, and the reaction mixture was incubated at 40 °C for 20 min. The residual lipase activity was assayed under standard assay conditions (*14*). The optimum temperature for lipase activity was determined by incubating the assay mixture over different temperatures (20−90 °C for 20 min at 5 °C intervals) in Tris-HCl buffer (50 mM, pH 8.0), and the resulting enzyme activity was assayed under standard assay conditions (*14*). The thermostability of lipase was tested by preincubating the enzyme (0.2 mL) at four different temperatures (35−50 °C with 5 °C differences) for 0−120 min. The enzyme (5 $\mu$L) was sampled at intervals of 15 min and assayed for residual lipase activity. The effect of alkali metal cations $Ca^{2+}$ and $Mg^{2+}$ and polyhydric alcohols (ethylene glycol, propylene glycol, glycerol, and sorbitol), individually and in combination, on the thermostability of lipase was also studied by incubating lipase (50 $\mu$L) at 40 °C for various durations (0−120 min).

**Substrate Specificity and Kinetic Parameters of Lipase from *P. fluorescens* MTCC 2421.** To study the substrate specificity of the enzyme, each of the five fatty acid triglycerides, namely, glyceryl esters of tributyrate ($C_{4:0}$), trilaurate ($C_{12:0}$), tripalmitate ($C_{16:0}$), tristearate ($C_{18:0}$), and trioleate ($C_{18:1n9}$) (50 mM, 100 $\mu$L), were included in the reaction mixture

containing isopropanol, Tris-HCl buffer (20 mL, 50 mM), and lipase (10 $\mu$L) maintained at pH 8.0. The reaction mixture was incubated for various durations (5−60 min) at 40 °C under shaking to determine the optimum incubation time. Samples were withdrawn at regular intervals to determine the residual lipase activity following the hydrolysis of substrates at 40 °C. The affinity of the purified lipase toward different fatty acid triglycerides was studied by determining the maximum velocity for the reaction ($V_{max}$) of lipase and Michaelis−Menten constant ($K_M$) using Lineweaver−Burk double-reciprocal plots. Briefly, assays with purified lipase (100 $\mu$L) were performed in Tris-HCl buffer (pH 8.0) at 40 °C with increasing concentration of fatty acid triglycerides (10−50 mM) to calculate $K_M$ and $V_{max}$ following the reported literature (*7*).

**Effects of Metal Ions and Enzyme Modulators on Lipase Activity and Stability.** To evaluate the effect of metal ions ($Na^+$, $K^+$, $Ca^{2+}$, $Mg^{2+}$, $Cu^{2+}$, $Mn^{2+}$, $Co^{2+}$, and $Fe^{2+}$) and enzyme modulators for protein secondary structure (urea, 2-mercaptoethanol, phenylmethanesulfonyl fluoride, and ethylenediaminetetraacetic acid) on the lipase activity, stock solutions (100 mM) of the metal ion and enzyme modulators were prepared in Tris-HCl buffer (100 mM, pH 8.0), and serial dilutions were made to arrive at different concentrations ranging from 0.001 to 50 mM. The purified enzyme (0.20 mg/mL, 50 $\mu$L) was added to the solutions containing substrate and inhibitors (100 $\mu$L) at different concentrations, and the residual lipase activity was assayed after 20 min of incubation at 40 °C under standard assay condition (*14*).

**Statistical Analyses.** Data were expressed as mean ± standard deviation of three different experiments ($n$ = 3) and subjected to one-way analysis of variance (ANOVA) using SPSS (ver. 10.0) software. A significance level of 95% ($p$ = 0.05) was used throughout.

## RESULTS AND DISCUSSION

**Time Course Dependent Lipase Activity of a Culture of *P. fluorescens* MTCC 2421.** The lipase-producing *P. fluorescens* MTCC 2421 was detected using rhodamine B−triolein agar plates. The time course of lipase production followed at 37 °C with typical cell growth is shown in **Figure 1**. The lipase activity appeared to couple to growth. The lipase activity was observed to start soon after incubation, and the crude enzyme extract obtained after 48 h of growth in the culture medium exhibited highest activity (11.85 LU/mL) at the beginning of the stationary phase. A decrease in lipase activity was apparent during the late stationary phase (after 53 h of incubation) presumably due to the presence of proteases in the culture medium. The lipase activities were found to be significantly reduced after 58 h of cultivation (9.15 LU/mL) (**Figure 1**). These results are in agreement with earlier results (*7*, *19*), which reported maximum lipase activities at the onset of the stationary phase of bacterial growth.

**Purification of *P. fluorescens* MTCC 2421 Lipase and Molecular Mass Studies.** The results of the purification profile of the extracellular lipase secreted by *P. fluorescens* MTCC 2421 are summarized in **Table 1** and illustrated in **Figure 2A**,**B**. The enzyme was purified 4.30-fold over the crude extract by ammonium sulfate precipitation with 35.18% yield. The enzyme solution, obtained after anion exchange chromatography, was purified 24.78-fold with 13.04% recovery. Further purification by gel exclusion chromatography on Sephadex G-100 resulted in fractions with 184.37-fold purity and 6.27% yield (**Table 1**). The recovery of the lipase activity was marginally lower in this study, although not significantly, than those reported for the purification of other *Pseudomonas* lipases (8−12%) (*20*, *21*). This might be due to the tendency of the enzyme to adsorb on

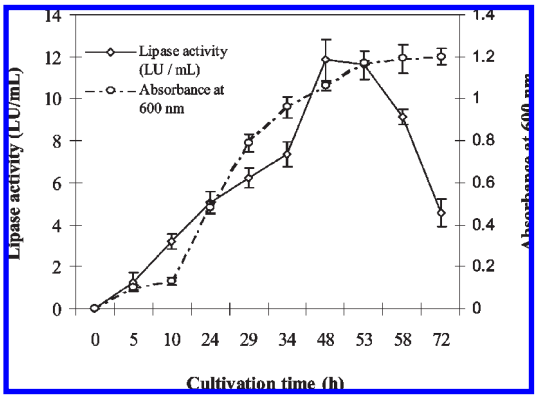


**Figure 1.** Time course dependent lipase activity and cell growth curve of a culture of *P. fluorescens* MTCC 2421. Culture conditions are described in the text. Extracellular activity of lipase was assayed in the growth medium at various intervals using 4-nitrophenyl palmitate as substrate. Bacterial cell growth was monitored by measuring the absorbance at 600 nm.

the molecular sieve or to be inactivated by accompanying protease. The homogeneity of the purified lipase was checked by the presence of a single band corresponding to an apparent molecular mass of 65.3 kDa on SDS-PAGE gels, suggesting it to be a homomeric protein (**Figure 2C**). However, one band in SDS-PAGE is insufficient to conclude that the enzyme is monomeric; it could be an oligomer consisting of identical subunits. Gel filtration chromatography using Sephadex G-75 was also used to determine the approximate molecular weight of the lipase as 66.5 kDa. Activity staining confirmed the presence of the purified lipase. The principal theory of activity staining lies in the function of $Ca^{2+}$ ion that is essential for lipase refolding in its native form (*17*). Bacterial lipases were reported to possess a characteristic α-helix and $\beta$-sheet, and the conformational change of lipases containing α,$\beta$-esterase fold in the presence of $Ca^{2+}$, indicating the structural transition from non-native to native conformation (*22*).

**Effect of pH on Lipase Activity.** The effect of pH on lipase activity with 4-NPP as substrate was examined at various pH values at 40 °C. The enzyme was active in the range of 7.0−9.0, and the optimum activity was exhibited at pH 8.0 (**Figure 3A**). The lipase retained 80.74% of its initial activity at a pH of 9.0, and at still higher pH (pH 10.0) the enzyme lost 64.16% of its original activity. This high activity at pH 8.0 makes the lipase applicable at alkaline pH conditions. Significant reduction in lipase activity was apparent in the acidic range. For example, the enzyme retained only 27.44 and 18.89% of the maximum activity at pH 6.0 and 7.0, respectively. These findings are in agreement with the results obtained for the purified lipase from *Pseudomonas fragi*, which had an optimum for lipase activity at pH 9.0 (*23*). An optimal pH of 8.6−8.7 for the exolipase purified from *P. fragi* NRRL B-25 was also reported (*24*). Industrial demand for lipases active at alkaline pH is essential for running bioprocesses, for their use to concentrate target polyunsaturated fatty acids, and other biotechnological applications.

**Effect of Temperature on Activity of Lipase.** The effect of temperature on lipase activity is shown in **Figure 3B**. The lipase was active in the temperature range of 35−50 °C, with maximal activity at 40 °C (376.19 LU/mg). The lipase activity was found to be reduced to 50.71% of its initial value at 30 °C, and at 20 °C, it lost over 93% of its initial activity. At higher temperature (50 °C) the enzyme lost

**Table 1.** Summary of Various Steps Involved in the Lipase Purification Scheme from *P. fluorescens* MTCC 2421 Culture Broth (48 h, 37 °C)

| step | volume (mL) | total activity (LU)[b] | total protein (mg)[c] | specific activity (LU/mg) | yield (%) | purification (-fold) |
|---|---|---|---|---|---|---|
| crude culture broth (48 h, 37 °C)[a] | 500 | 5926.18 | 2576.20 | 2.30 | 100 | 1 |
| $(NH_4)_2SO_4$ precipitation | 50 | 2169.33 | 219.64 | 9.84 | 35.18 | 4.30 |
| anion exchange chromatography [Tris-HCl buffer (50 mM, pH 8.0)] | 10 | 1225.48 | 21.58 | 57.25 | 13.04 | 24.78 |
| gel exclusion chromatography [Tris-HCl buffer (50 mM, pH 8.0)] | 10 | 839.59 | 1.98 | 424.04 | 6.27 | 184.37 |

[a] *P. fluorescens* MTCC 2421 was grown in selective broth (see Materials and Methods), and the harvested broth was clarified to obtain a supernatant that was termed the crude lipase. The crude lipase was purified by ammonium sulfate salt precipitation, Amberlite IRA 410 ($Cl^-$ form) anion exchange, and Sephadex G-100 gel exclusion chromatography; [b] The lipase activity was assayed by spectrophotometric method by monitoring 4-nitrophenyl palmitate hydrolysis. [c] Total protein was estimated according to the Bradford method.

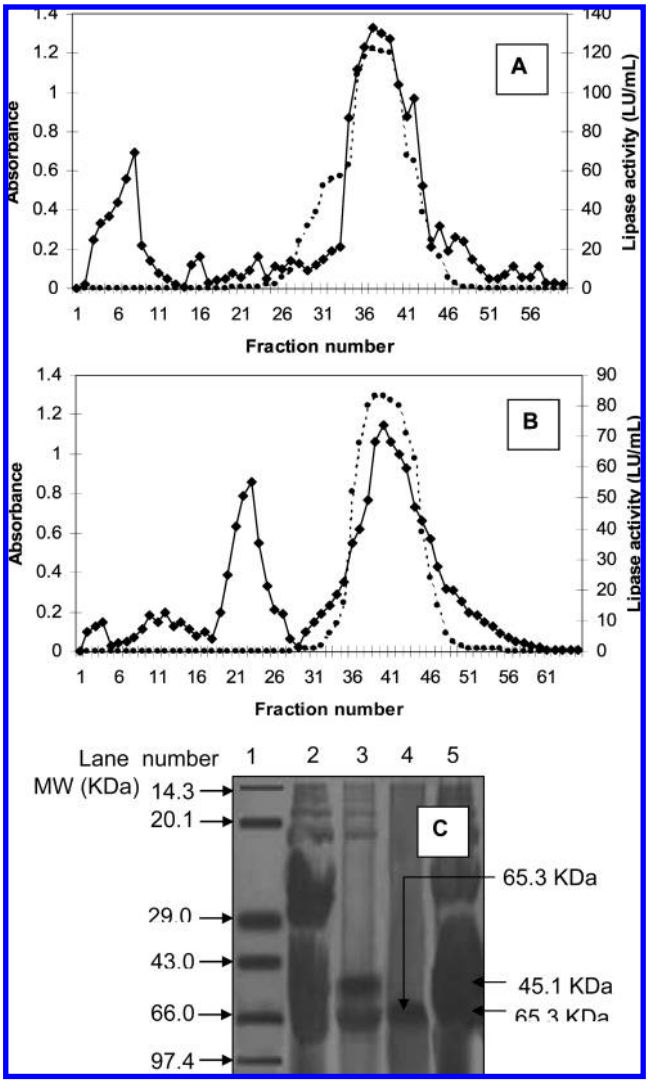


**Figure 2.** Chromatographic purification profile of the lipase on (**A**) anion exchanger (Amberlite IRA 410 $Cl^-$ form) and (**B**) Sephadex G-100 gel exclusion column. (**C**) SDS-PAGE of the lipase on 12% resolving gel: lane 1, molecular mass markers [phosphorylase $\beta$ (97.4 kDa), bovine serum albumin (66.0 kDa), ovalbumin (43.0 kDa), carbonic anhydrase (29.0 kDa), soybean trypsin inhibitor (20.1 kDa), and $\alpha$-lactalbumin (14.3 kDa)]; lane 2, crude culture broth supernatant (20 $\mu$g); lane 3, proteins (20 $\mu$g) after anion exchange chromatography; lane 4, purified lipase (10 $\mu$g) after gel exclusion chromatography (a homomeric band corresponding to a molecular mass of about 65.3 kDa represented the purified lipase); lane 5, proteins (20 $\mu$g) after electrolytic precipitation.

43.76% of the maximal activity, and the lipase activity dropped off rapidly above 55 °C, with only 13% of the activity remaining at 60 °C (**Figure 3B**). These findings are in agreement with those reported for the lipase from *Pseudomonas* sp., with optimal activity at 40−45 °C (*25*, *26*).

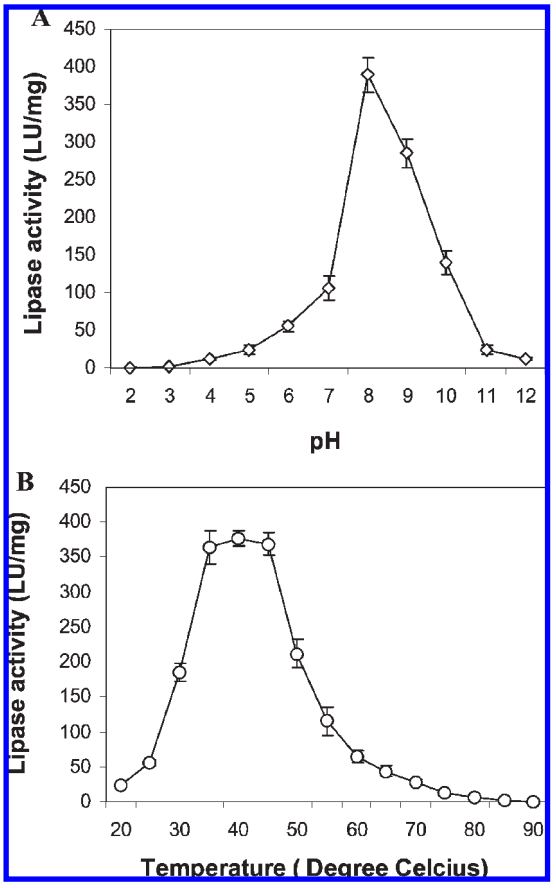


**Figure 3.** Effect of (**A**) pH and (**B**) temperature on the lipase activity. The experiments were conducted in triplicate, and standard errors are reported.

The advantages of running bioprocesses at elevated temperatures and favorable changes in most physical properties of lipids at increased temperature determine the increased interest in new thermostable enzymes for new applications (*27*).

**Thermostability of Lipase.** The thermostability of the lipase was examined by measuring the residual activity for 0−120 min at four different temperatures (35−50 °C with 5 °C difference) at pH 8.0 (**Figure 4A**). The enzyme was found to be stable at 35−45 °C with > 75% of the residual activity after 30 min of incubation. After incubation for 45 min, the enzyme was stable at 35−45 °C with residual activity greater than 60% of its initial activity. At higher temperatures (50 °C), the lipase retained 68.1% of its maximum activity after 30 min of incubation and 41.5% after 1 h of incubation (**Figure 4A**). The enzyme exhibited half-lives ($t_{1/2}$) of 70 min at 40 °C and 62 min at 45 °C. A lipase in thermophilic *Bacillus* sp. was found to have a $t_{1/2}$ of 30 min at 65 °C (*28*), whereas a thermostable lipase from *B. thermocatenulatus* and *B. stearothermophilus* recorded $t_{1/2}$ of 30 min at 60−62 °C (*29*). The higher stability of the lipase in alkaline pH and at high

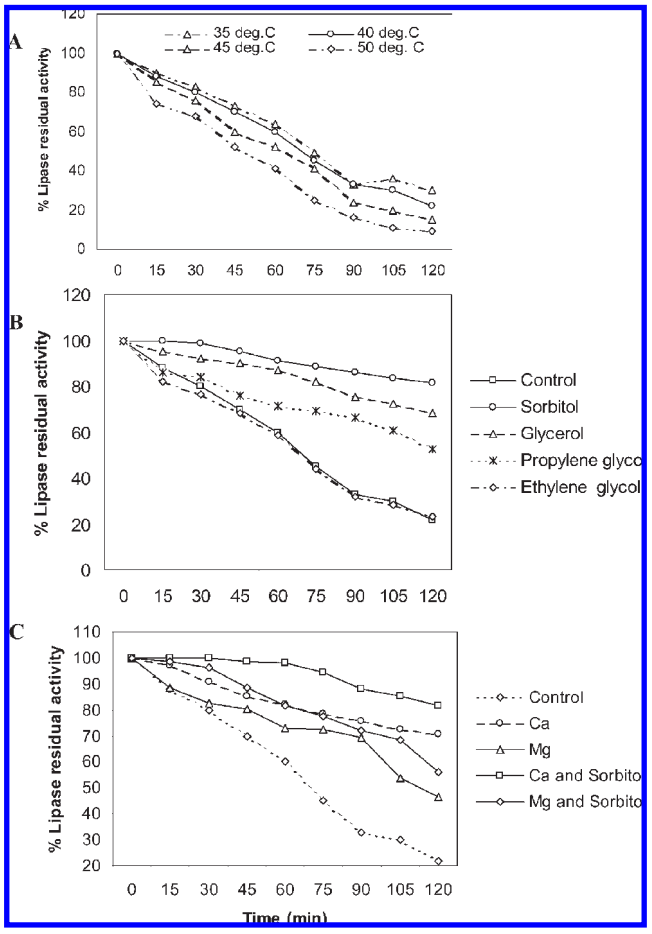


**Figure 4.** Effects of divalent ions and polyhydric alcohols on the lipase thermostability: (**A**) thermostability profile of the lipase at different temperatures (35−50 °C) at 0−120 min; (**B**) influence of polyhydric alcohols on lipase thermostability for 0−120 min at 40 °C; (**C**) influence of Ca and Mg vis-à-vis Ca and Mg combined with sorbitol on thermostability of the lipase for 0−120 min at 40 °C.

temperature indicates its potential utility in industrial application.

**Effect of Polyhydric Alcohols and Metal Ions on Lipase Activity and Stability.** Sorbitol, a polyhydric alcohol with five hydroxyl groups, was found to be an effective stabilizer of the lipase. About 95.3% of the residual activity was apparent after 45 min of incubation with sorbitol, and after 2 h, it retained 81.5% of the initial activity. Polyhydric alcohols with fewer numbers of free hydroxyl groups such as glycerol (three −OH groups), propylene glycol, and ethylene glycol (two −OH groups) had a detrimental effect on the activity of the lipase apparently due to lower interaction with negatively charged groups in the active site (*30*). Lipase when incubated with glycerol retained 86.9% of the residual activity as compared to 71.3 and 58.6% activity when incubated for 1 h with propylene and ethylene glycol, respectively (**Figure 4B**). Lipase incubated with $Ca^{2+}$/sorbitol induced a synergistic effect as indicated by the significantly higher residual lipase activity (100%) after 30 min of incubation, as compared to 90.6% when incubated with $Ca^{2+}$ alone ($p = 0.05$). The $Mg^{2+}$/sorbitol combination also exhibited significant increase in lipase activity (96.3%) after 30 min of incubation as compared to 80.6% in the control (**Figure 4C**). Enzyme stabilization at high temperatures by $Ca^{2+}$/ $Mg^{2+}$ ions and sorbitol might be due to the free −OH groups (in sorbitol) and water molecules (bound to the hydrated $Ca^{2+}$/ $Mg^{2+}$ as $CaCl_2 \cdot 2H_2O$ and $MgCl_2 \cdot 2H_2O$), which are liberated when the ions become bound to the protein. The free water molecules and −OH groups, in turn, hydrate the catalytic site of the enzyme, resulting in hindered denaturation. The catalytic triads of most lipases are constructed by negatively charged carboxylate side-chain groups of aspartyl, serine, and glutamyl residues, which are "cross-linked" by the $Ca^{2+}$/ $Mg^{2+}$ bridge, and the enzyme−$Ca^{2+}$/ $Mg^{2+}$ complex should, therefore, be more rigid and hence more stable (*31*). Significant increase in thermostability in the presence of sorbitol/$Ca^{2+}$ combination has valuable practical importance in the design of reaction media to enrich specific unsaturated fatty acids.

The catalytic activity of the lipase was found to be enhanced by 3% in the presence of $K^+$ at 30 mM (**Figure 5A**). $Na^+$ does not have any activating effect on this lipase, although at higher concentration (50 mM) significant ($p = 0.05$) inhibition of this lipase (38%) was apparent. It is well-known that nearly one-third of enzymes require the presence of metal ions as tightly bound metal ion cofactors for catalytic activity. The alkaline earth metals, $Ca^{2+}$ and $Mg^{2+}$, were found to enhance the lipase activity by 10 and 5%, respectively, at 40 mM. Lower concentrations (0.001−5 mM) of these metals were found to reduce lipase activity, although not significantly (**Figure 5B**). About 90 and 86% residual lipase activities were apparent after incubation with 0.001 mM $Ca^{2+}$ and $Mg^{2+}$, respectively, thus leading to the conclusion that these alkaline earth metals have a direct effect on the lipase activity. Among other divalent metal cations, the lipase activity was found to be significantly ($p = 0.05$) blocked by $Cu^{2+}$ and $Fe^{2+}$ (42 and 48%, respectively) at 10 mM. Using 10−50 mM metal ions, inhibitory effects were greater. The inhibitory activity of the transition metal cations at high concentrations may be due to direct inhibition of the catalytic site and/or formation of complexes between metal ions and ionized fatty acids (*32*). Extracellular lipases from *Pseudomonas* sp. were reported to be inhibited by $Cu^{2+}$ at higher concentration (*33*). However, this lipase was unaffected by most of the metal ions tested at lower concentration (0.1−0.001 mM), and the relative activity was recorded to be more than 70%.

**Effect of Enzyme Modulators on Lipase Activity.** The lipase was found to retain 58% of its initial activity after incubation with 0.1 mM EDTA and 69% by using 0.001 mM of the same modulator. As reported earlier, a concentration as low as 1 mM EDTA was found to inhibit the lipase activity (*33*, *34*). The enzyme exhibited a significant reduction in activity by PMSF, a thiol, and serine protease inhibitor, thus indicating an important role of sulfhydryl groups in catalytic mechanism (*27*). The lipase was found to retain 61% of its initial activity when incubated with 0.001 mM PMSF and 42% by using 0.1 mM PMSF (**Figure 5C**). Complete inhibition by PMSF at higher concentration (> 1 mM) might be due to the modification of an essential serine residue in the active site (*27*). Thiol inhibitor 2-mercaptoethanol ($CH_3CH_2SH$) was found to inhibit the lipase even at diluted concentration (0.001 mM), thereby indicating an important role of sulfhydryl groups in the catalytic mechanism. A thermostable lipase from *B. stearothermophilus* was reported to be inhibited by 2-mercaptoethanol (25%) at a concentration of 1 mM (*27*). Urea was found to exhibit least reduction in lipase activity (84% at 0.001 mM and 78% at 0.1 mM) (**Figure 5C**).

**Fatty Acid Specificity of Lipase from *P. fluorescens* MTCC 2421.** To study the substrate specificity of the lipase, the hydrolytic kinetic variables of fatty acyl triglycerides with

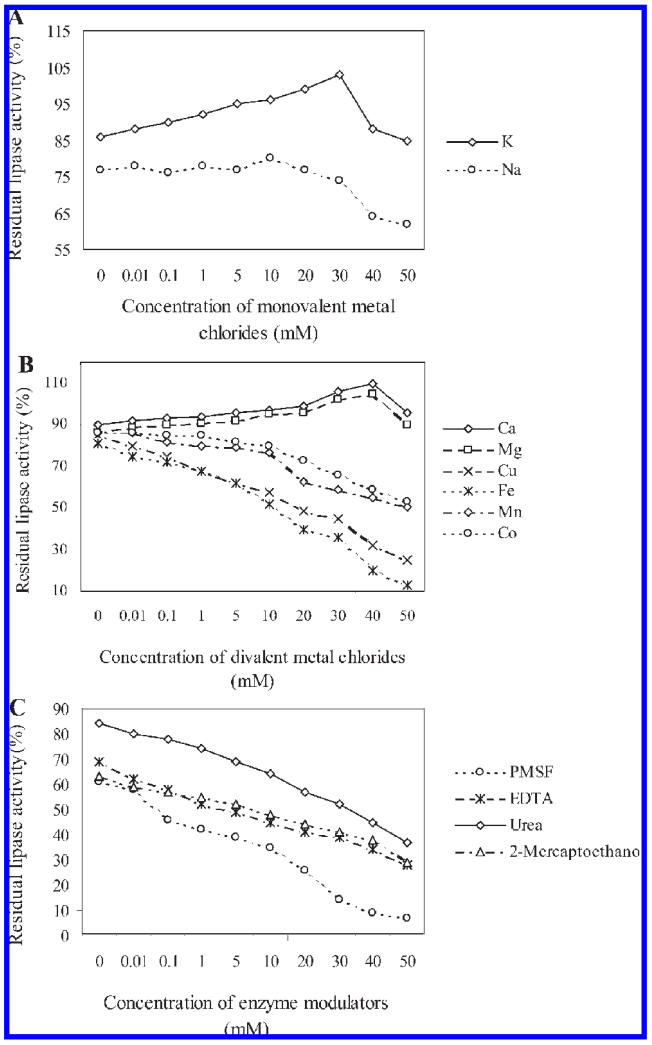


**Figure 5.** Effect of metal cations: (**A**) monovalent, (**B**) divalent, and (**C**) enzyme modulators on the lipase activity. A stock solution (100 mM) of the metal ion and enzyme modulators were prepared in Tris-HCl buffer (100 mM, pH 8.0), and serial dilutions were made to arrive at 0.001−50 mM.

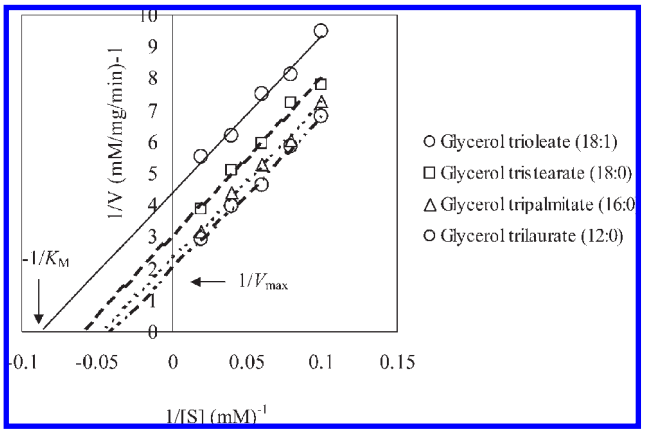


**Figure 6.** Lineweaver−Burk double-reciprocal plots for the purified bacterial lipase using glyceryl esters of trioleate, tristearate, tripalmitate, and trilaurate as substrates. $1/V_{max}$ values (intercept of the straight lines to *Y* axis of the plot) were recorded as 1.96, 2.17, 3.13, and 4.35 (mM acid/mg/min)$^{-1}$ when glycerol trilaurate ($C_{12:0}$), tripalmitate ($C_{16:0}$), tristearate ($C_{18:0}$), and trioleate ($C_{18:1n9}$), respectively, were used as substrates toward the lipase. The corresponding $-1/K_M$ values (intercept of the straight lines to *X* axis) were found to be 0.043, 0.048, 0.058, and 0. 086 mM$^{-1}$, respectively, for these fatty acid triglycerides. The Lineweaver−Burk equation of glyceryl esters of trilaurate ($C_{12:0}$), tripalmitate ($C_{16:0}$), tristearate ($C_{18:0}$), and trioleate ($C_{18:1n9}$) were determined as $1/V_0 = 48.29/[S] + 1.93$, $1/V_0 = 49.25/[S] + 2.28$, $1/V_0 = 50.0/[S] + 2.98$, and $1/V_0 = 49.69/[S] + 4.38$, respectively.

**Table 2.** Substrate Specificity of the Lipase from *P. fluorescens* MTCC 2421 to Triacylglycerols of Different Fatty Acyl Chain Lengths ($C_{12\text{-}18}$).

| substrate (fatty acyl triglycerides) | relative activity[a] (%) | $K_M$ (mM) | $V_{max}$ (mM/mg/min) |
|---|---|---|---|
| glycerol trioleate ($C_{18:1n9}$) | 9 ± 0.27 | 11.6 ± 0.11 | 0.23 ± 0.04 |
| glycerol tristearate ($C_{18:0}$) | 23 ± 0.56 | 17.2 ± 0.38 | 0.32 ± 0.09 |
| glycerol tripalmitate ($C_{16:0}$) | 29 ± 1.25 | 20.7 ± 1.71 | 0.46 ± 0.12 |
| glycerol trilaurate ($C_{12:0}$) | 49 ± 1.63 | 23.3 ± 0.85 | 0.51 ± 0.07 |

[a] The relative activities were calculated by taking the activity of glyceryl tributyrate ($C_{4:0}$) as 100%.

variable chain length were determined. The Lineweaver−Burk double-reciprocal plot of activity of the *P. fluorescens* MTCC 2421 lipase incubated with substrates of variable fatty acyl chain length is shown in **Figure 6**. The linearity of the plot indicates that hydrolysis of triglyceride esters by the lipase followed Michaelis−Menten kinetics. Incubation of the lipase with glycerol trioleate ($C_{18:1n9}$) and glycerol tristearate ($C_{18:0}$) furnished $K_M$ values of 11.6 and 17.2 mM, respectively, and $K_M$ values of 20.7 and 23.3 mM were found when glycerol tripalmitate ($C_{16:0}$) and glycerol trilaurate ($C_{12:0}$) were employed as substrates (**Table 2**). The $V_{max}$ values were found to decrease with the increase in acyl chain length of the triglycerides, with $V_{max}$ values of 0.51, 0.46, 0.32, and 0.23 (mM/mg/min)$^{-1}$ after incubation with glycerol trilaurate ($C_{12:0}$), glycerol tripalmitate ($C_{16:0}$), glycerol tristearate ($C_{18:0}$), and glycerol trioleate ($C_{18:1n9}$), respectively, thus indicating that the lipase from *P. fluorescens* MTCC 2421 was highly active toward fatty acid triglycerides having short to medium carbon chain length ($< C_{18}$) resulting in rapid breakdown of ester bonds of glycerol trilaurate ($C_{12:0}$), followed by glycerol tripalmitate ($C_{16:0}$) and glycerol tristearate ($C_{18:0}$). The longer chain length unsaturated fatty acid triglyceride (as in glycerol trioleate) was found to be hydrolyzed slowly as compared to its saturated homologue ($C_{18:0}$), thus indicating its potential use to enrich long-chain unsaturated fatty acids (viz., triolein). These findings are in agreement with earlier results, which reported that a lipase from *P. fragi* CRDA 037 is most active with fatty acid triglycerides of intermediate carbon chain lengths such as tristearine with a higher $V_{max}$ value of 13.3 $\mu$M/mg/min than recorded for glycerol trioleate of 11.1 $\mu$M/mg/min (*7*). The unsaturated fatty acids such as oleic acid with a double bond at the Δ9 position apparently hinder the bacterial lipase from acting on it, resulting in enrichment of the fatty acid in the triglyceride fraction. On the contrary, saturated fatty acids are exposed to the reactive groups at the enzyme active site apparently due to the absence of an olefinic bond, resulting in their rapid breakdown (hydrolysis of esteritic linkage) from the fatty acid triglycerides. Such fatty acid selectivity of lipases, that is, hydrolysis favoring unsaturates over saturates, may therefore be useful to fractionate highly unsaturated fatty acids from marine oil.

In conclusion, an extracellular alkaline metallolipase from *P. fluorescens* MTCC 2421 has been purified by a combination of precipitation and chromatography. The homogeneity of the purified lipase was checked by the presence of a single band corresponding to an apparent molecular mass of 65.3 kDa on SDS-PAGE gels, suggesting it as a homomeric protein. The purified lipase had optimum activity at 40 °C and alkaline pH (pH 8.0). A combination of divalent metal

cation and polyhydric alcohol, particularly $Ca^{2+}$/sorbitol, appeared to play an important role in the structure and function of the lipase. $Ca^{2+}$ plays an important role in the increased activity of lipase, possibly due to the hydration of the catalytic site and cross-linking by a Ca-ion bridge with negatively charged amino acyl residues. The lipase appeared to be a metalloenzyme due to its inhibition by EDTA. The purified lipase exhibited a lower affinity for medium to long fatty acyl chain length ester bonds (as in glycerol trioleate) than short acyl chain fatty acid triglycerides. Because of its high specificity toward short carbon chain length esters, the potential application(s) of the lipase from *P. fluorescens* MTCC 2421 in the enrichment of unsaturated fatty acids as triglycerides may be further explored by performing enzyme-catalyzed hydrolytic reactions of marine oils. Further optimization of hydrolysis parameters will be taken up in future studies for obtaining targeted unsaturated fatty acids from marine sources, which will pave the way for lipase application on a commercial scale. This study has potential applications to design the specific reaction medium with suitable stabilizers and additives (such as sorbitol/$Ca^{2+}$) for selective enrichment of unsaturated fatty acids by lipase-catalyzed hydrolysis and transesterification reactions.

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