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# An extra-cellular alkaline metallolipase from *Bacillus licheniformis* MTCC 6824: Purification and biochemical characterization

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**Abstract**

An extra-cellular lipase produced by *Bacillus licheniformis* MTCC 6824 was purified to homogeneity by ammonium sulphate fractionation, ethanol/ether precipitation, dialysis, followed by anion-exchange chromatography on Amberlite IRA 410 ($Cl^-$ form) and gel exclusion chromatography on Sephadex G 100 using Tris–HCl buffer (pH 8.0). The crude lipase extract had an activity of 41.7 LU/ml of culture medium when the bacterium was cultured for 48 h at 37 °C and pH 8.0 with nutrient broth supplemented with sardine oil as carbon source. The enzyme was purified 208-fold with 8.36% recovery and a specific activity of 520 LU/mg after gel exclusion chromatography. The pure enzyme is a monomeric protein and has an apparent molecular mass of 74.8 kDa. The lipase had a $V_{max}$ and $K_m$ of 0.64 mM/mg/min and 29 mM, respectively, with 4-nitro phenylpalmitate as a substrate, as calculated from the Lineweaver–Burk plot. The lipase exhibited optimum activity at 45 °C and pH 8.0, respectively. The enzyme had half-lives ($T_{1/2}$) of 82 min at 45 °C, and 48 min at 55 °C. The catalytic activity was enhanced by $Ca^{2+}$ (18%) and $Mg^{2+}$ (12%) at 30 mM. The lipase was inhibited by $Co^{2+}$, $Cu^{2+}$, $Zn^{2+}$, $Fe^2$ even at low concentration (10 mM). EDTA, at 70 mM concentration, significantly inhibited the activity of lipase. Phenyl methyl sulfonyl fluoride (PMSF, 70 mM) completely inactivated the original lipase. A combination of $Ca^{2+}$ and sorbitol induced a synergistic effect on the activity of lipase with a significantly high residual activity (100%), even after 45 min, as compared to 91.5% when incubated with $Ca^{2+}$ alone. The lipase was found to be hydrolytically resistant toward triacylglycerols with more double bonds.


*Keywords*: Alkaline lipase; *Bacillus licheniformis* MTCC 6824; Anion exchange chromatography; Sephadex G 100 gel exclusion chromatography

## 1. Introduction

Lipases (triacylglycerol acyl hydrolases, E.C. 3.1.1.3) are biotechnologically valuable enzymes, which specifically hydrolyze carboxyl esters of triglycerides into fatty acids, and are being used as animal feed supplements to increase bioavailability of n3 or n6 polyunsaturated fatty acids (PUFAs) (Chakraborty & Paul Raj, 2008). Lipases also catalyse several industrially significant catalytic biotransformation reactions namely, esterification and transesterification (Akoh, Jennings, & Lillard, 1996; Huang & Akoh, 1994; Kosugi & Azuma, 1994). Lipases occur widely in microorganisms, plants, and animals (Jaeger & Eggert, 2002). Among the microbial sources, bacterial lipases are the most widely used class of enzymes in biotechnological applications, due to their resistance to high temperature and other operational and/or storage conditions (Jaeger & Eggert, 2002). There are reports on purification and biochemical characterization of various bacterial lipases (Jaeger, Ransac, Dijkstra, van Henrel, & Misset, 1994; Sugihara, Ueshima, Shimada, Tsunasawa, & Tominaga, 1992). Alkalophilic and thermophilic lipases have been the focus of a number of investigations, because of their potential biotechnological applications in the food industry and pharmaceuticals that rely on higher temperature (45–50 °C) and (pH > 8.0) (Schmidt-Dannert, Sztajer,

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Stocklein, Menge, & Schmid, 1994). Alkalophilic (pH > 9.5) intracellular lipase was reported from *Bacillus subtilis* (Lesuisse, Schanck, & Colson, 1993). A lipase produced by a recombinant *B. licheniformis* was found to be stable at alkaline pH of 12.0 (Nthangeni, Patterton, van Tonder, Verger, & Litthauer, 2001). These results are in contrast to thermotolerant lipases from *B. thermoacetenulatus* and *B. thermoleovorans* (Lee et al., 1999; Rua, Schmidt-Dannert, Wahl, Spraner, & Schmidt, 1997), displaying maximum activity at pH 8.0. *B. coagulans* NCIMB 9365 has been reported to possess an intracellular carboxyesterase (Molinari, Brenna, Valenti, & Aragozzini, 1996). Isolates of *Pseudomonas fluorescens* have been found to produce enzymes active on lipolytic substrates under alkaline conditions (Kojima & Shimizu, 2003). Some lipase genes from *Pseudomonas* have been cloned and expressed in *Escherichia coli* due to their potential industrial applications (Kojima, Kobayashi, & Shimizu, 2003). The unique substrate specificity of microbial lipases has been utilized for the enhancement of PUFA content in fish oils by several groups (Bottino, Vandenburg, & Reiser, 1967; Harris, 1989; Matori, Asahara, & Ota, 1991). It was found that lipases were efficient in hydrolytic and esterification reactions, because of their high stereospecificity and mild reaction conditions within low temperature and organic solvents (Jaeger & Eggert, 2002).

Although lipases are widely available in several species, the enzymes from bacteria are valuable, because of their potential use in various biotechnological applications, such as foods and pharmaceuticals (Akoh et al., 1996). Each application requires unique properties with respect to specificity, stability, temperature, and pH-dependence. Among different bacteria, *Bacillus* sp. constitutes a major source of lipase activity. To use any lipase for hydrolysis, esterification, or any other application, it is essential to biochemically characterize the enzyme. In the present study, *B. licheniformis* MTCC 6824, isolated from a mangrove ecosystem situated at the south-western coast of India, was chosen as the starting material for purification and characterization of an extracellular lipase. We report methods for lipase purification, to homogeneity, from *B. licheniformis* MTCC 6824 culture broth, and characterization with respect to its biochemical properties intended for use in biotechnological application.

## 2. Materials and methods

### 2.1. Chemicals and reagents

The solvents used for sample preparation and analyses were of analytical grade (E-Merck, Darmstadt, Germany), and were redistilled in an all-glass system, and degassed with $N_2$ prior to use. Double-distilled water was used throughout this work. Thiourea, urea, $CaCl_2$, Amberlite IRA 410 ($Cl^-$ form), β-naphthyl acetate, bovine serum albumin (BSA), phenyl methyl sulfonyl fluoride (PMSF), 4-nitrophenyl acetate, glycerol triacetate, glycerol tributyrate, glycerol trilaurate, glycerol tristearate, and glycerol trioleate were obtained from HiMedia; Sephadex G-100 and other supports of chromatography were from Sisco Research Laboratories (SRL, Mumbai, India). Electrophoresis grade acrylamide, bis-acrylamide, medium range molecular marker proteins, and Coomassie Brilliant Blue R-250 were procured from Bangalore Genei (Bangalore, India).

### 2.2. Maintenance of Bacillus licheniformis MTCC 6824 and lipase production

*B. licheniformis* MTCC 6824, isolated from a mangrove ecosystem of Kerala, India, 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 a 100 ml 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 lipase production broth (500 ml; 2 g/l of yeast extract, 5 g/l of peptone, and 5 g/l of NaCl) supplemented with $CaCl_2$ (0.05% w/v), and different vegetable and fish oils (1.0% v/v, emulsified with Tween 80) as lipidic carbon sources, namely, sardine oil, cod liver oil, olive oil, sunflower oil, gingily oil, groundnut oil and coconut oil, was inoculated with seed culture (150 ml) (inoculum size, 1% v/v; $2.0 \times 10^7$ CFU) in a 1000 ml Erlenmeyer flask and incubated at 37 °C with shaking (150 rpm) for 48 h. The culture broth was clarified by centrifugation (10,000 rpm for 20 min at 4 °C, Superspin R–V/FM Plasto Crafts, Plasto Crafts Mumbai) to recover the supernatants, which were analyzed for lipase activity. The cell pellets harvested by centrifugation were vacuum-dried, and the pellets kept at −20 °C until they were used as inocula. The cell-free supernatant (500 ml) was concentrated to 50 ml by lyophilization (Model Alpha 1-4LD, Martin Christ, Germany), followed by dialysis against Tris–HCl buffer (10 mM, pH 8.0). This concentrated liquid, referred to as the crude extracellular lipase solution, was used for further purification.

### 2.3. Lipase purification and activity

#### 2.3.1. General

All purification steps were carried out at 0 to 4 °C, unless otherwise mentioned. The purification method consisted of sequential combination of ammonium sulphate fractionation, ethanol/ether precipitation, and purification on Amberlite IRA 410 ($Cl^-$ form) anion-exchange chromatography and gel exclusion chromatography on Sephadex G-100.

#### 2.3.2. Ammonium sulphate and ethanol/ether precipitation

Solid ammonium sulphate was added to the crude enzyme solution under continuous stirring, until 70% saturation, for 120 min at 4 °C. The precipitate was separated by centrifugation (15,000 rpm, 30 min, 4 °C) and dissolved

in Tris–HCl (50 ml, 50 mM, pH 8.0). A mixture of ice-cold ethanol and ether (100 ml, 1:1 v/v) was slowly added to the aliquot with continuous stirring at 0 °C for 1 h. The precipitate was collected after centrifugation (15,000 rpm, 30 min, 4 °C), and excess solvent was evaporated by drying *in vacuo* at 0 °C over anhydrous $P_2O_5$. The residue was dissolved in Tris–HCl (100 ml, 50 mM, pH 8.0) and dialyzed extensively against the same buffer at 4 °C for 18 h. The dialyzed material was centrifuged (10,000 rpm, 30 min, 4 °C), and the supernatant (40 ml) was retained for further chromatographic purification.

#### 2.3.3. Amberlite IRA 410 ($Cl^-$ form) anion-exchange chromatography

The supernatant, after dialysis, containing the enzyme, was loaded onto an Amberlite IRA 410 ($Cl^-$ form) column (1.5 × 15 cm) equilibrated with 50 mM Tris–HCl buffer (pH 8.0) for chromatographic purification. The column was washed with two bed volumes of the same buffer and the enzyme was eluted with 0–0.5 M NaCl −50 mM Tris–HCl (pH 8.0) in a linear gradient (200 ml). The flow rate was adjusted to 0.5 ml/min, and fractions (each of 2 ml) were collected and assayed for lipase activity and total protein content ($A_{280}$). The fractions showing lipase activity (10 ml) were pooled and concentrated by lyophilization. The lyophilized lipase was reconstituted in distilled water (5 ml), and dialyzed against Tris–HCl buffer (50 mM, pH 8.0). The specific activity of the purified enzyme was compared with that of the initial crude enzyme and the purification factor was calculated.

#### 2.3.4. Sephadex G-100 gel exclusion column chromatography

The pooled and concentrated active fractions (3 ml) obtained after Amberlite IRA 410 ($Cl^-$ form) anion-exchange chromatography were rechromatogaphed on a Sephadex G-100 gel exclusion column (2.5 × 30 cm, 20 ml gel) previously equilibrated with Tris–HCl buffer (50 mM, pH 8.0) containing $CaCl_2$ (1.0 mM). The lipase was eluted with the same buffer at a flow rate of 0.5 ml/min, and the fractions (2.0 ml) were collected. The eluants from the chromatographic column were analyzed for total protein ($A_{280}$) and lipase activity. The fractions showing the highest lipase activity were pooled (10 ml), and assayed for protein content. The specific activity of the purified enzyme was compared with that of the crude enzyme, and the purification factor was calculated. The active fractions were stored at 4 °C until used for polyacrylamide gel electrophoresis and further enzyme characterization.

### 2.4. Lipase activity

The lipolytic activity of the purified enzyme was estimated by a spectrophotometric method carried out in a UV–VIS spectrophotometer (Varian Cary 50 Conc., USA) using 4-nitrophenyl palmitate (4-NPP) as substrate, following established procedures (Winkler & Stuckmann, 1979) with minor modifications. Lipase activity was assayed by measuring the μM concentration of 4-nitrophenol (4-NP) released from 4-nitrophenyl palmitate. A stock solution (20 mM) of 4-NPP was prepared in iso-propanol. The reaction mixture consisted of 4-NPP stock solution (20 mM, 100 μl) in Tris–HCl buffer (50 mM, pH 8.0, 1.0 ml) containing $CaCl_2$ (10 mM, pH 8) and dissolved in iso-propanol, to make a final volume of 3 ml, to which enzyme sample (10 μl) was added. The hydrolysis reaction was allowed to proceed by incubation for 15 min at 45 °C in a water bath under shaking (150 rpm), and lipase reaction was stopped by adding chilled acetone–ethanol (1 ml, 1:1 v/v, −20 C). The reaction mixture was kept on ice for 15 min before being centrifuged (5000*g* for 10 min). The absorbance of the supernatant containing released 4-NP was monitored at 410 nm ($A_{410}$) in the UV–VIS spectrophotometer. The unknown concentration of 4-NP released was determined from a reference curve of the same (2–50 μg/ml in 50 mM Tris–HCl buffer, pH 8.0). Each assay was performed in triplicate and mean values were determined. One activity unit of lipase (LU) was defined as μmole of 4-NP released from the hydrolysis of 4-NPP/ml/min by one ml of enzyme at 45 °C under standard assay conditions. The protein concentration was determined by measuring the absorbance at 280 nm (Varian Cary 50 Conc., USA), using bovine serum albumin (20–150 μg) as a standard.

### 2.5. Biochemical characterization of lipase

#### 2.5.1. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and activity staining

Electrophoresis of proteins (PAGE) in the presence of sodium dodecyl sulphate (0.1% w/v SDS–PAGE) was carried out on a vertical slab midi-gel apparatus (Model PS-500; Bangalore Genei, India) at 50 mA for 10 h to establish the purity of the lipase protein. The enzyme solutions (20 μl) diluted with electrophoresis sample buffer (125 mM Tris/HCl buffer at pH 6.8, 4% (w/v) SDS, 7.5% 2-mercaptoethanol, 20% (v/v) glycerol and 0.0022% (w/v) bromphenol blue) were boiled for 3 min on a water bath. The SDS–PAGE was performed on 12% polyacrylamide gel (with 6% stacking gel), and the relative molecular mass of proteins was determined with reference to the medium range molecular mass markers (14.4–94.0 kDa, Bangalore Genei, India). The molecular mass markers used were phosphorylase β (97.4 kDa), bovine serum albumin (66.0 kDa), ovalbumin (43.0 kDa), carbonic anhydrase (29.0 kDa), soyabean trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.3 kDa). After migration, the gels were fixed using 7% (v/v) $CH_3COOH/CH_3OH$, and submitted to a cycle of staining/destaining with Coomassie Brilliant Blue R-250 (stainer) and 14% (v/v) $CH_3COOH/CH_3OH$ (destainer), respectively to allow determination of molecular mass of proteins. Molecular mass was determined from the plots of log molecular mass (log M) vs. migration ($R_f$) for a series of known protein standards. The active lipase fractions were identified by analytical SDS–PAGE of

proteins using 12% gels, which were stained with Coomassie brilliant blue R-250. This was carried out by overnight incubation of gels in refolding buffer (20 mM Tris–HCl, pH 8.0, supplemented with 10 mM $CaCl_2$) at 4 °C overnight. The gels were then transferred to a solution of Tris–HCl (100 mM, pH 8.0) containing β-naphthyl acetate and fast blue RR. After 30 min of agitation, the gels were rinsed with water and stored in 7% acetic acid. A dark yellow colour appeared within 30 min.

#### 2.5.2. Effect of pH on lipase activity

To evaluate the effect of pH on lipase activity, the lipase was assayed at different pH (3–11, with 1.0 unit difference) values. The pH of Tris–HCl buffer (50 mM) was adjusted separately to furnish the targeted pH values. The enzyme (5 μl) was preincubated with the reaction mixture containing 4-NPP as substrate and Tris–HCl buffer (50 μl) supplemented with $CaCl_2$ (10 mM) as reaction medium at each of the above pH values, and the reaction was carried out at 45 °C for 20 min. Reactions were stopped after incubation, and the residual lipase activity was assayed under standard assay conditions. Protein concentrations were measured as described earlier, and the specific activities of the enzyme were calculated at various pH values.

#### 2.5.3. Effect of temperature on activity of lipase

The effect of temperature on lipase activity was studied by carrying out the enzyme reaction at different temperatures ranging from 25–90 °C with 5 °C differences in Tris–HCl buffer (50 mM, pH 8.0) containing $CaCl_2$ (10 mM), and the residual lipase activity was assayed under standard assay conditions. Briefly, the enzyme (0.2 ml) was treated with Tris–HCl buffer (50 mM, pH 8.0), and preincubated at the selected temperatures in a water bath for 20 min. Thereafter, the enzyme (10 μl) was sampled, and assayed for residual lipase activity. The thermostability of lipase was tested by preincubating the enzyme (0.2 ml) taken in an Eppendorf tube at six different temperatures (40–65 °C with 5 °C differences) for 0–120 min. The enzyme (5 μl) was sampled at intervals of 15 min, and assayed for residual lipase activity. To determine the half-life of the enzyme, the enzyme was incubated at 45 °C, and the enzyme activity was determined periodically up to 2 h.

#### 2.5.4. Effect of divalent ions and polyalcohols on lipase thermostability

The effect of $Ca^{2+}$ and $Mg^{2+}$ and polyhydric alcohols (ethylene glycol, propylene glycol, glycerol and sorbitol), individually and in combination, on the thermostability of lipase was studied by incubating lipase at 45 °C for various durations (0–120 min), and at different concentrations ranging from 10–70 mM with 10 mM differences. A stock solution (100 mM) of $Ca^{2+}$ and $Mg^{2+}$ was separately prepared in Tris–HCl buffer (100 mM, pH 8.0), and serial dilutions were made to arrive at desired concentrations (10–70 mM), to which the purified enzyme (50 μl) was added, and lipase activity was assayed.

### 2.6. Substrate specificity and kinetic characterization of lipase

To study the substrate specificity of the enzyme, each of the six triacylglycerol esters, namely glycerides of triacetate, tributyrate, trilaurate, tripalmitate, tristearate, and trioleate (50 mM, 100 μl) was separately included in the reaction mixture containing iso-propanol and Tris–HCl buffer (20 ml, 50 mM) maintained at pH 8.0. Lipase (10 μl) was added to the reaction mixture and vortexed, followed by incubation at 45 °C for 20 min in a water-bath shaker. To determine the optimum incubation time, the reaction mixture was incubated for varying durations (5–60 min) with lipase at 45 °C. Samples were withdrawn at regular intervals to determine the residual activity following the hydrolysis of substrates at 45 °C. The lipase activity was assayed by standard assay procedure (Winkler & Stuckmann, 1979). The specificity, as well as affinity, of the lipase towards various different esters was studied by determining $V_{max}$ and $K_m$ values for each of the selected substrate. The Lineweaver–Burk plots were used to determine Michaelis–Menten constant ($K_M$) and the maximum velocity for the reaction ($V_{max}$) of lipase for various substrate esters at pH 8.0, using a spectrophotometric method. Briefly, assays with purified lipase (10 μl) were performed in Tris–HCl buffer, pH 8.0 at 45 °C with increasing concentrations of substrates from 0.5 to 5.0 mg/ml, to calculate $K_M$ and $V_{max}$. The hydrolysis products of the reaction catalyzed by lipase were determined by thin-layer chromatography using silica gel as adsorbent. Briefly, glycerides of tripalmitate, tristearate, and trioleate (20 mg) were sonicated in Tris–HCl (10 ml, 50 mM, pH 8.0) supplemented with $CaCl_2$ (1 mM) for 5 min. The enzyme sample (100 μl) was added to the reaction mixture and incubated at 60 °C for 2 h. After incubation, the reaction products were extracted with diethyl ether (5 ml × 2), concentrated and applied to the TLC plates, which were developed with $CHCl_3/(CH_3)_2C{=}O/CH_3COOH$ (95:4:1, v/v/v). The spots of glycerides and hydrolyzed products were visualized by exposure to iodine vapour.

### 2.7. Stability to organic solvents

The stability in organic solvents was determined by mixing enzyme aliquots in different solvents (1:1, v/v) under standard assay procedure. Samples were taken periodically to determine the residual activity with 4-NPP as substrate.

### 2.8. Effect of modulators on lipase activity

The effects of various divalent metal-ions, namely $Ca^{2+}$, $Mg^{2+}$, $Cu^{2+}$, $Mn^{2+}$, $Zn^{2+}$, $Co^{2+}$, $Hg^{2+}$, and $Fe^{2+}$ as $CaCl_2$, $MgCl_2$, $CuSO_4$, $MnCl_2$, $ZnSO_4$, $CoCl_2$, $HgCl_2$, and $FeCl_2$, monovalent ions, namely $Na^+$ and $K^+$ as their chloride salts (KCl and NaCl, respectively), and modulators for protein secondary structure (urea, thiourea, 2-mercaptoethanol, phenyl methyl sulfonyl fluoride, *N*-bromo

succinamide and ethylene diamine tetraacetic acid) on lipase activity were studied. In brief, a stock solution (100 mM) of the metal-ion modulators was separately prepared in Tris–HCl buffer (100 mM, pH 8.0), and serial dilutions were made to arrive at different concentrations ranging from 10 to 70 mM with 10 mM differences. The purified enzyme (50 μl) was added to the solutions containing substrate and inhibitors (100 μl) at different concentrations, and lipase activity was assayed after 20 min of incubation at 45 °C. To study the effects of urea, thiourea, 2-mercaptoethanol, phenyl methyl sulfonyl fluoride (PMSF), *N*-bromo succinamide (NBS) and ethylene diamine tetraacetic acid (EDTA) on lipase activity, a mixture with different concentrations (100 μl; 10–70 mM in Tris – HCl buffer, pH 8.0) was treated with the lipase (50 μl), followed by 20 min of incubation at 45 °C. The reaction was terminated, and lipase activity was determined.

### 2.9. Statistical analyses

Data were expressed as means ± standard deviation of three experiments ($n = 3$), and subjected to one-way analysis of variance (ANOVA) using SPSS (ver. 13.0) software. A significance level of 95% ($p = 0.05$) was used throughout.

## 3. Results and discussion

### 3.1. Growth and lipase production by Bacillus licheniformis MTCC 6824

The lipase-producing *B. licheniformis* MTCC 6824 isolated from the mangrove ecosystem, situated at the south-western coast of India was detected using rhodamine B-triolein agar plates prepared from the culture (500 ml). Among the various carbon sources tested for lipase production in nutrient broth, sardine and cod liver oil were found to induce higher lipase activity (13.6 and 7.53 LU/ml) after 48 h of incubation, and the activity was found to gradually decrease (Fig. 1). After 72 h, the lipase activity

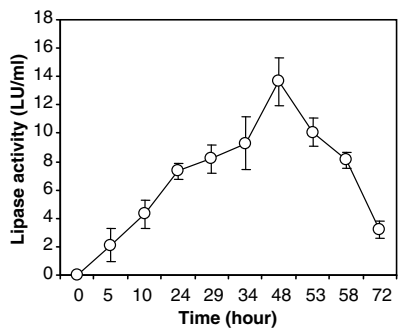


Fig. 1. Time-course of a culture of *Bacillus licheniformis* MTCC 6824. Cultivation of *Bacillus licheniformis* MTCC 4869. Cultivation was performed at 45 °C in the conditions described in the text. Extracellular activity of lipase was assayed with 4-nitrophenyl palmitate. Phenomenologically, extracellular lipolytic activity appeared coupled to the growth.

was found to be 3.54 LU/ml. The lipase hydrolytic activity on vegetable oils was found to be inferior to the action on fish oil, presumably due to the lower polyunsaturated fatty acid (PUFA) content in vegetable oil. Comparing the lipase hydrolytic activity among all the vegetable oils, sunflower oil and olive oil were hydrolyzed to great extents, because of their higher amounts of unsaturated fatty acids.

### 3.2. Purification of Bacillus licheniformis MTCC 6824 lipase

#### 3.2.1. General

An extracellular lipase produced by *B. licheniformis* MTCC 6824 was purified by employing a three-step procedure, namely chemical precipitation, chromatography on anion-exchange, and gel exclusion chromatography. The results of the purification profile are summarized in Table 1. 4-NPP was employed as a substrate in the lipolytic assay, because it gave low background colour and formed a suspension in the buffer like that of typical fat molecules (Lesuisse et al., 1993). The enzyme was purified 208-fold over the crude extract with 8.36% recovery.

#### 3.2.2. Ammonium sulphate [$(NH_4)_2SO_4$] and ethanol/ether precipitation

About 500 ml of the crude fermented broth were obtained after 48 h of fermentation (13.6 LU/ml, 5.47 mg protein/ml) of *B. licheniformis* MTCC 6824. The *B. licheniformis* MTCC 6824 culture broth was centrifuged, and the supernatant was precipitated with 70% (w/v) $(NH_4)_2SO_4$. The enzyme solution, obtained after precipitation with $(NH_4)_2SO_4$ and ethanol/ether exhibited a specific activity of 4.84 mg/ml with a recovery of 29.9% (Table 1), and an increase in the purification factor (4.26-fold). The dialyzed enzyme showed an activity of 53.8 LU/ml with a specific activity of 1.8 mg/ml (11.9-fold).

#### 3.2.3. Anion-exchange chromatography

The lipase was purified 33.5-fold with 16.4% yield by anion-exchange chromatography (Table 1). No lipolytic activity was detected in the washing flow. Anion-exchange chromatography of lipase on an Amberlite IRA 410 ($Cl^-$ form) column resulted in one prominent peak at the 36th fraction (Fig. 2). The fractions under the activity peak that possessed lipase activity (lipase activity of 166 LU/ml and specific activity of 83.8 LU/mg) were pooled. The recovery of lipase after this purification step was 16.4%.

#### 3.2.4. Gel exclusion chromatography on Sephadex G-100

The pooled fractions containing lipase activity eluted from anion-exchange chromatography were concentrated and further purified on a gel exclusion Sephadex G-100 column (2.5 × 120 cm) equilibrated and eluted with Tris–HCl buffer (50 mM, pH 8.0). The fractions containing the lipase activity eluted between 1.2 and 1.8 void volumes were pooled together. The lipase was purified 208-fold with 8.36% recovery and high specific activity (520 ± 123 LU/mg) by the Sephadex G-100 gel exclusion

Table 1
Flow sheet of lipase purification scheme from *B. licheniformis* MTCC 6824 culture broth (48 h, 37 °C)

| Step | Volume (ml) | Total activity[a] (LU) | Total protein[b] (mg) | Specific activity[c] (LU/mg) | Yield (%) | Purification (fold) |
|---|---|---|---|---|---|---|
| Culture broth[d] (48 h, 37 °C) | 500 | 6819 ± 131 | 2737 ± 209 | 2.50 ± 0.24 | 100 | 1 |
| $(NH_4)_2SO_4$ precipitation | 50 | 2524 ± 34.5 | 242 ± 46.8 | 10.7 ± 1.88 | 29.9 | 4.26 |
| Dialysis | 40 | 2150 ± 72.5 | 72.3 ± 3.51 | 29.8 ± 2.41 | 25.1 | 11.9 |
| Amberlite IRA-410 ($Cl^-$ form) anion-exchange chromatography[e] | 10 | 1656 ± 53.7 | 20.0 ± 3.00 | 83.8 ± 10.0 | 16.4 | 33.5 |
| Sephadex G-100 gel exclusion chromatography[f] | 10 | 1340 ± 22.6 | 2.67 ± 0.58 | 520 ± 123 | 8.36 | 208 |

[a] Total activity (in lipase units, LU) was determined by monitoring 4-nitrophenyl palmitate hydrolysis. The units used for total activity are μmole of 4-nitrophenol released/min.
[b] Total protein of the crude culture broth and purified fractions was expressed in mg.
[c] The units for specific activity are μmole of 4-nitrophenol released per min per mg of total protein.
[d] Crude samples were taken after fermenting 500 ml of culture and centrifuging the sample. The specific activity was attributed to lipases from *Bacillus licheniformis* MTCC 6824.
[e] Chromatographic purification of lipase on anion-exchange column (Amberlite IRA-410 anion-exchange column ($Cl^-$ form)); samples obtained from column fractions using Tris–HCl eluant buffer (pH 8.0) had been pooled and concentrated.
[f] Lipase activity (absolute and specific) was of the purified enzyme fractions (2.0 ml) as eluted in gel exclusion column (Sephadex G-100) (the data are mean values of three independent experiments with their standard deviations); samples obtained from the size-exclusion chromatography fractions using Tris–HCl eluant buffer (pH 8.0) had been pooled and concentrated.

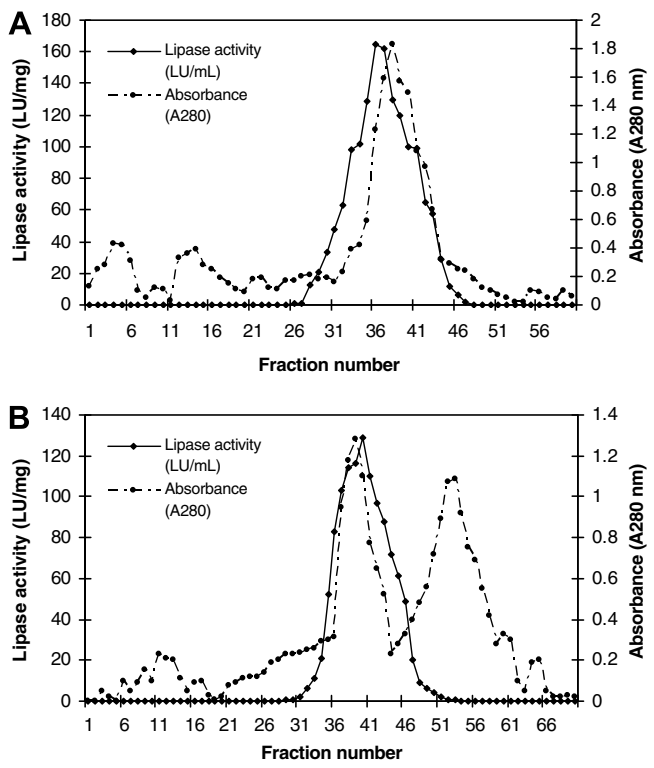


Fig. 2. Chromatographic purification profile of lipase from *Bacillus licheniformis* MTCC 6824. A: Chromatographic purification profile of lipase on anion-exchange column (Amberlite IRA-410 anion-exchange column ($Cl^-$ form)) equilibrated with Tris–HCl buffer (50 mM, pH 8.0). The lipase was eluted with a gradient of sodium chloride in Tris–HCl buffer (50 mM, pH 8.0); B: Chromatographic purification profile of lipase on Sephadex G-100 gel exclusion chromatography. Column was equilibrated with Tris–HCl buffer (50 mM, pH 8.0) containing $CaCl_2$ (1 mM) at a flow rate of 30 ml/h. The sample was loaded and eluted with the same buffer. Lipase activity (LU/mg) was determined by monitoring 4-nitrophenyl palmitate hydrolysis. Total protein was estimated by the Bradford method. The units for specific activity are μmol of 4-nitrophenol released per min per mg of total protein. The absorbance of the eluants were measured at $\lambda = 280$ nm.

chromatographic step (Table 1). The homogeneity of the purified lipase was checked by the presence of a single band corresponding to an apparent molecular mass of 74.8 kDa on non-denaturing PAGE gels, as stained by Coomassie Brilliant Blue R-250 (Fig. 3). Activity staining

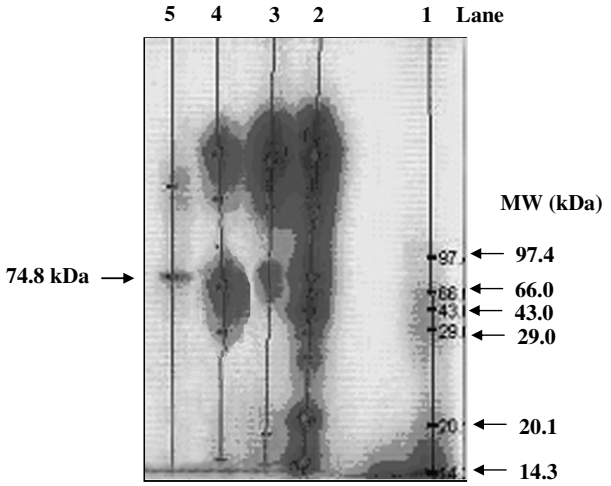


Fig. 3. Sodium dodecyl sulphate polyacrylamide gel electrophoretic analyses of (SDS–PAGE) lipase obtained at different stages of purification from culture broth of *Bacillus licheniformis* MTCC 6824 on 12% polyacrylamide gel (with 6% stacking gel). Lane 1: Medium range molecular mass markers (Bangalore Genei, India): β phosphorylase (97.4 kDa), bovine serum albumin (66.0 kDa), ovalbumin (43.0 kDa), carbonic anhydrase (29.0 kDa), soyabean trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.3 kDa); lane 2:20 μg of total proteins after ammonium sulphate precipitation; lane 3:20 μg of total proteins after dialysis; lane 4:20 μg of total proteins after Amberlite IRA-410 ($Cl^-$ form) anion-exchange chromatography; lane 5:10 μg of purified lipase after Sephadex G-100 gel exclusion chromatography. After migration, gels were submitted to a cycle of staining/distaining with Coomassie Blue R-250 to allow determinations of molecular weights of proteins. The purified lipase was represented by a single band corresponding to a molecular mass of about 74.8 kDa. This value is in agreement with that determined by gel filtration experiments under native conditions.

confirmed the presence of the purified lipase, as revealed by SDS/PAGE analysis.

### 3.2.5. Effect of pH on the enzyme activity

The purified lipase from *B. licheniformis* MTCC 6824 exhibited activity over the pH range 6–10 (Fig. 4) with maximum activity (100%) observed at pH 8.0, followed by pH 9.0 (67.9% of the maximum). A rapid decline in the enzyme activity was observed on both sides of the pH-optimum. The activity was reduced drastically at pH 5.0 (15.5%) and was 1.94% of the maximum at pH 11.0. The enzyme retained 67.9% and 22.6% of the maximum activity at pH 9.0 and 10.0, respectively, but was inactivated at acidic pH (4.0) losing 97.0% of its original activity. At pH 3.0, the enzyme lost about 99.7% of its initial activity whereas, at pH 11.0, the enzyme was found to retain about 1.94% of its initial activity (Fig. 4).

### 3.2.6. Effect of temperature on activity and stability of lipase

The results of the temperature – activity profile of the purified lipase are shown in Fig. 4. The lipase, when pre-

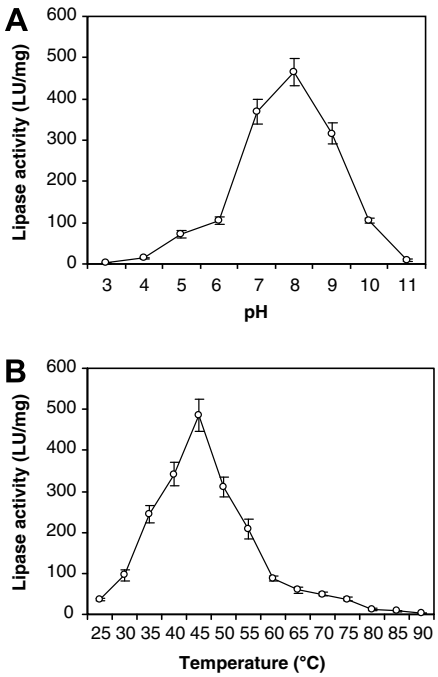


Fig. 4. Effects of pH and temperature on the activity of chromatographically purified lipase from *Bacillus licheniformis* MTCC 6824. A: Effect of pH on lipase activity. 4-NPP was used as a substrate using Tris–HCl buffer (50 mM, pH 8.0) adjusted to different pH values. Activity was measured at different pH values and 45 °C with colorimetric method. Lipase activity was measured under the reaction condition of Tris–HCl buffer (50 mM, pH 8.0) at 45 °C under standard experimental conditions (at various pH) using 4-NPP as substrate. The experiments were conducted in triplicate, and standard errors are reported. B: Effect of temperature on lipase activity. Lipase activity was tested at pH 8.0 at various temperatures using 4-NPP (20 mM) as substrate under standard conditions. The experiments were conducted in triplicate and standard errors are reported. The Tris/HCl (50 mM, pH 8) buffer containing 10 mM $CaCl_2$ was adjusted at each assayed temperature.

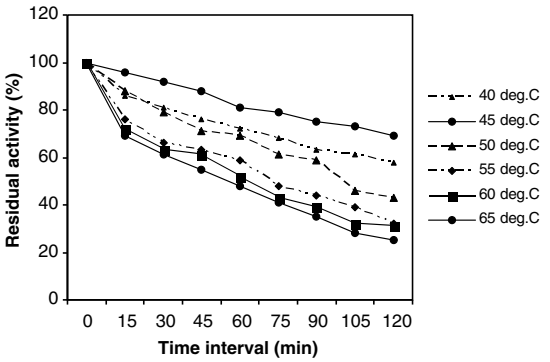


Fig. 5. Thermostability profile of chromatographically purified lipase from *Bacillus licheniformis* MTCC 6824 at six different temperatures (40–65 °C) at various time intervals (0–120 min).

incubated at 25, 35, 45, and 55 °C, exhibited 35, 244, 485, and 209 LU/mg activities, respectively, after 20 min of exposure. The lipase exhibited optimum activity ($0.48 \times 10^3$ LU/mg) at 45 °C, when exposed for 20 min, with linear increase from 25 to 45 °C (Fig. 5). The activity was reduced to 63.9% of its original value in 20 min at 50 °C (lipase activity 310 LU/mg), followed by a drastic decline. The enzyme retained 81.9% of the residual activity after 60 min of incubation at 45 °C, and 69.3% after 2 h. At higher temperatures, e.g. 50 and 55 °C, the lipase exhibited 69% and 59% of the maximum activity after 1 h of incubation, and 43% and 32% of the residual activity after 2 h of incubation (Fig. 5). Lipase exhibited significant activity even at 65 °C (48% of the residual activity) after 1 h of incubation. The enzyme showed a half-life ($T_{1/2}$) of 82 min at 45 °C, and 75 min at 50 °C. The enzyme $T_{1/2}$ at 55 °C was found to be 48 min.

### 3.2.7. Effects of divalent ions and polyalcohols on lipase thermostability

The catalytic activity of the lipase was enhanced by 18% and 12% in the presence of $Ca^{2+}$ and $Mg^{2+}$ at 30 mM; after that it exhibited a slow but gradual decrease (Fig. 6). Sorbitol was found to be an effective additive as it enhanced the thermostability of the enzyme. About 98.6% of the residual activity was apparent after 45 min of incubation

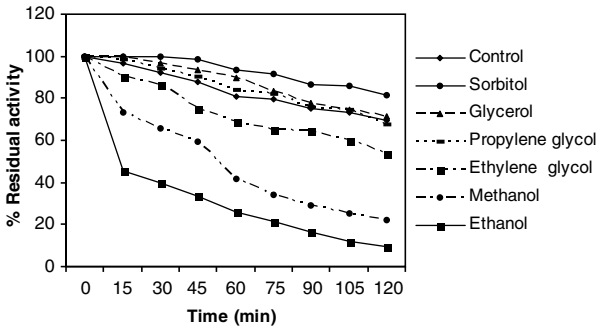


Fig. 6. Influence of alcohols (monohydric, dihydric, and polyhydric) on thermostability of chromatographically purified lipase from *Bacillus licheniformis* MTCC 6824 for different time intervals (0–120 min) at 45 °C.

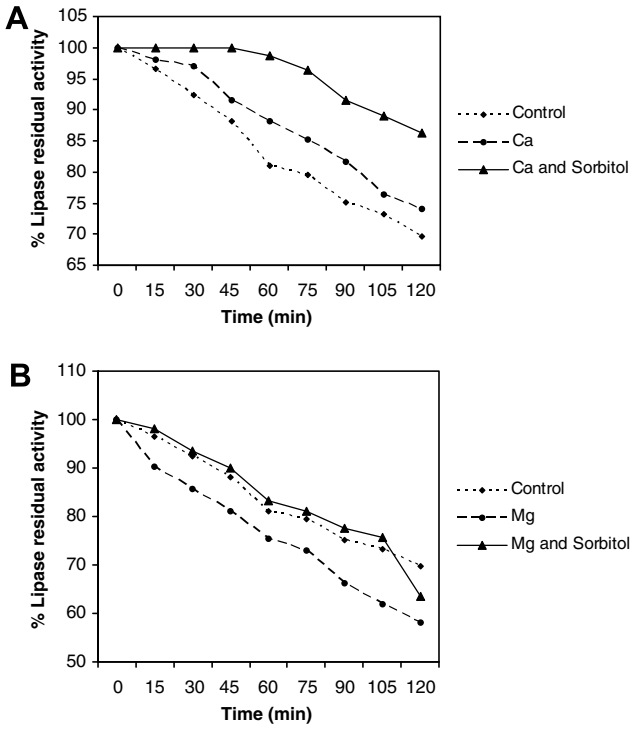


Fig. 7. Influence of divalent Ca and Mg vis-à-vis Ca and Mg combined with polyhydric alcohol sorbitol on thermostability of chromatographically purified lipase from *Bacillus licheniformis* MTCC 6824 for different time intervals (0–120 min) at 45 °C.

with sorbitol and, after 2 h, it retained 81.2% of the activity. Polyhydric alcohols having smaller numbers of free hydroxyl groups, e.g. glycerol (three –OH groups), propylene glycol, and ethylene glycol (two –OH groups) had a detrimental effect on the activity of the lipase. Lipase, when incubated with glycerol, retained 71.6% of the residual activity as compared to 67.8% and 53.8% activity, when incubated for 1 h with propylene and ethylene glycols, respectively (Fig. 7).

Lipase incubated with a combination of $Ca^{2+}$ (as its chloride salt) and sorbitol induced a synergistic effect, as indicated by the significantly ($p = 0.05$) higher residual activity of lipase (100%) even after 45 min, as compared to 91.5% when incubated with $Ca^{2+}$ alone (Fig. 7). After 2 h, this combination was found to retain 86.2% of the original activity as against 69.6% in the control. The Mg/sorbitol combination exhibited a marginal increase in lipase activity, inducing 89.9% after 45 min of incubation as compared to 88.1% in the control (Fig. 7). Increased thermostability of lipase due to $Ca^{2+}$ and higher polyhydric alcohols, as in sorbitol, ($CH_2OH(CHOH)_4CH_2OH$), appeared to be due to hindered denaturation, by hydration, of the catalytic site of the enzyme, resulting in charge rearrangement and $Ca^{2+}$ ion complexation. Stabilization of enzymes by $Ca^{2+}$ ion at high temperatures was due to $Ca^{2+}$ ion complexation, which was a process with a favourable entropy factor. This was because water previously bound to the hydrated $Ca^{2+}$ (as $CaCl_2 \cdot 2H_2O$) in solution was liberated when $Ca^{2+}$ ion became bound to the protein. Thus, the process was favoured at high temperatures. A number of enzymes require the presence of metal ions, such as $Ca^{2+}$ ions, for the maintenance of their stable and active structures. These ions are bound strongly to specific binding sites on the surfaces of the enzyme molecules. The binding sites of lipase are usually constructed from negatively charged carboxylate side-chain groups of aspartyl and glutamyl residues, brought together by folding of the polypeptide chain (Gray, 1995). The polypeptide chain is 'cross-linked' by the metal ion bridge and the enzyme–calcium ion complex should, therefore, be more rigid and hence more stable (Gray, 1995). The bridging by metal ions in this way is compared to that brought about by disulfide formation. In the absence of calcium ions, the binding site would represent a high local concentration of negative charges. The tendency of these groups to move apart to reduce the repulsive electrostatic interactions would contribute to the relative instability of the folded protein. Significant increase in thermostability in the presence of sorbitol/$Ca^{2+}$ combination has valuable practical importance.

### 3.2.8. The effect of organic solvents

Stability against organic solvents is very important when using enzymes for synthesis of esters. The purified lipase exhibited good tolerance to organic solvents, such as isopropanol, methanol, and ethanol. A relative activity of 62% was apparent in the presence of isopropanol and 71% in the presence of methanol (Fig. 8). However, chloroform (3%), acetonitrile (5%), and ethyl acetate (8%) inhibited the lipase activity.

## 3.3. Substrate specificity and kinetic parameters of lipase from Bacillus licheniformis MTCC 6824

### 3.3.1. General

To study the enzyme–substrate affinity, the kinetic parameters of the hydrolyses of different substrates having

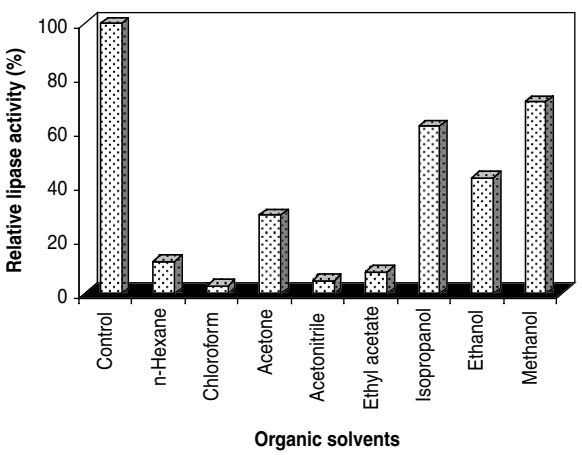


Fig. 8. Effect of organic solvents on activity of lipase purified from *Bacillus licheniformis* MTCC 6824. The enzyme was incubated in Tris–HCl buffer, pH 8.0 containing different organic solvents for 30 min at 45 °C.

ester functionality were determined for the purified fractions. The lipase showed a variable specificity/hydrolytic activity towards various triacylglycerols. The Lineweaver–Burk plots were linear and indicated that hydrolysis of various triglyceride esters by the lipase followed Michaelis–Menten kinetics. The low C-length (14:0) ester, as in glycerol tripalmitate, was more efficiently hydrolyzed than were other esters. The lipase exhibited lowest affinity towards glycerol trioleate, followed by glycerol tristearate, as observed from the measured $V_{max}$ and $K_m$ of 0.17 mM/mg/min and 9.30 mM, and 0.23 and 12.0 mM/mg/min, respectively (Table 2). The $K_m$ and $V_{max}$ values were determined, as a function of glycerol tristearate concentration, to be 15.0 mM and 0.30 mM/mg/min (Table 2). The lipase had a $V_{max}$ and $K_m$ of 0.64 mM/mg/min and 29 mM with 4-nitro phenylpalmitate as a substrate, respectively, as calculated from the Lineweaver–Burk plot. The hydrolytic activity of the lipase for hydrolysis of glycerol trioleate was approximately 34% lower than that of glycerol tripalmitate. The ability to hydrolyze triglycerides containing short-chain fatty acids determined the enzyme to be a lipase. This indicated a preferential specificity of the lipase towards shorter carbon chain length substrates.

### 3.3.2. Metal ions and enzyme modulators

$K^+$, $Ca^{2+}$, and $Mg^{2+}$ were found to enhance the enzyme activity while $Co^{2+}$, $Mn^{2+}$, $Cu^{2+}$and $Zn^{2+}$ ions inhibited the enzyme activity; $Na^+$ had no significant effect on the enzyme activity while $Ca^{2+}$ and $Mg^{2+}$ were found to activate the lipase at 10–30 mM and higher concentrations (Fig. 9), with the $Ca^{2+}$ ions inducing maximum lipase activity. The lipase was inhibited by metal ions such as $Cu^{2+}$ and $Fe^{2+}$ (31 and 33%) even at the lowest concentration (10 mM). $Hg^{2+}$, $Zn^{2+}$, $Fe^{2+}$, and $Co^{2+}$ at 50 mM concentration inhibited lipase by 55.3%, 69.1%, 82.7%, and 62.6%, respectively (Fig. 9). $Hg^{2+}$ had a strong (72% at 70 mM) inhibitory effect, suggesting that cysteine residue(s) participate in activity expression. Catalytic activity of this lipase was significantly inhibited by chelation (EDTA) and a serine protease inhibitor (PMSF). This indicates that this lipase also possesses a triad of three amino acids at its catalytic site just like many other lipases. The presence of PMSF (40 mM) in the reaction mixture severely decreased the hydrolytic activity of the lipase. Complete inhibition by PMSF at >60 mM concentration, a serine inhibitor, was probably caused by modification of an essential serine residue in the active site. The enzyme was sensitive to chelation by EDTA that was observed to be inhibiting at concentrations >70 mM (Fig. 9), indicating that it was a metalloenzyme. EDTA, at 70 mM concentration, drastically reduced the lipase activity (approximately 88%). The activity was strongly affected by the thiol inhibitor, NBS, which indicates an important role of SH-groups in the catalytic mechanism. Among other enzyme modulators, reducing reagents such as 2 – mercaptoethanol had inhibitory effects on the lipase,

Table 2
Substrate specificity of lipase from *Bacillus licheniformis* MTCC 6824 to triacylglycerol esters of different C-chain lengths

| Substrates | *C*-chain length | Relative activity (%) | $K_M$ (mM) | $V_{max}$ (mmol/mg/min) |
|---|---|---|---|---|
| *Triacylglycerols* | | | | |
| Glyceryl trioleate | 18:1 | 14 ± 0.12 | 9.3 ± 0.08 | 0.17 ± 0.002 |
| Glyceryl tristearate | 18:0 | 31 ± 0.09 | 12.0 ± 0.17 | 0.23 ± 0.002 |
| Glyceryl tripalmitate | 16:0 | 42 ± 0.67 | 15.0 ± 0.37 | 0.30 ± 0.003 |
| Glyceryl trilaurate | 12:0 | 66 ± 0.32 | 22.0 ± 0.46 | 0.38 ± 0.02 |
| Glyceryl tributyrate | 4:0 | 100 ± 0.18 | 34.0 ± 0.51 | 0.69 ± 0.03 |

All assays were performed in triplicate ($n = 3$) at 45 °C, and mean values are presented.[a]Percent change in the activity of enzyme is with respect to the enzyme (active) control. The 4-NPP was used as the chromogenic substrate and mean values are presented. The standard deviation was 0.1–0.5%. The substrates used were triacylglycerides of oleate, stearate, palmitate, laurate and butyrate.

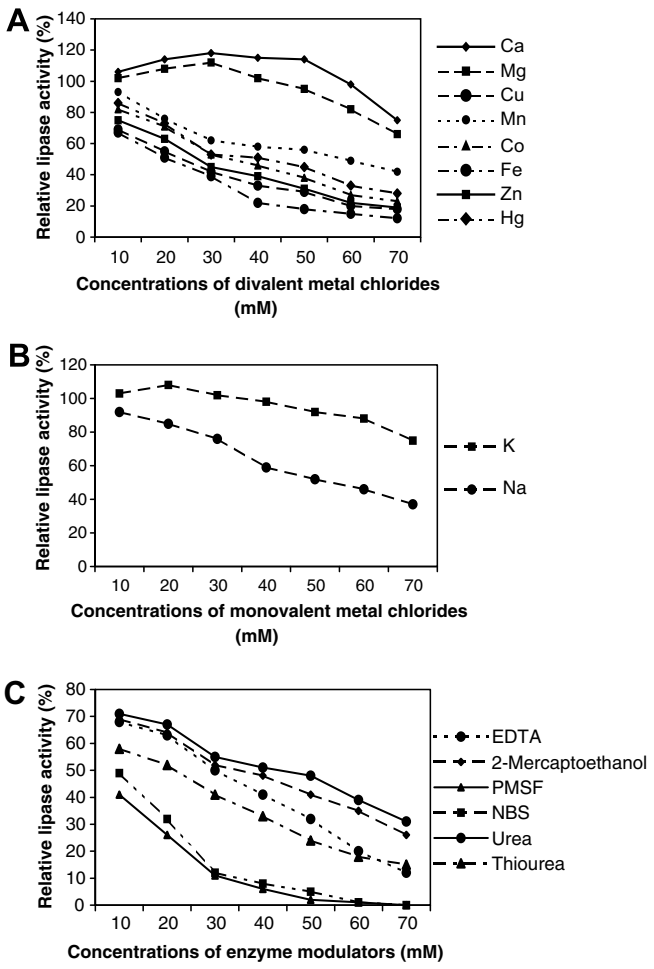


Fig. 9. Effect of metal ions and enzyme modulators on activity of lipase purified from *licheniformis* MTCC 6824. The enzyme was incubated in Tris–HCl buffer, pH 8.0, containing different ions and enzyme modulators for 30 min at 45 °C. Activity was determined spectrophotometrically using 4-NPP as substrate at pH 8.0. A: effect of divalent metal cations on lipase activity; B: effect of monovalent metal cations on lipase activity; C: effect of enzyme modulators on lipase activity.

exhibiting 74% inhibition at 70 mM concentration. There was no significant loss of activity in the presence of 10 mM urea (Fig. 9).

## 4. Conclusions

We have demonstrated the occurrence of an extracellular lipase, with molecular weight of 74.8 kDa derived from cultures of *B. licheniformis* MTCC 6824. Bacterial lipases play an important role as hydrolases. To exploit lipases of *B. licheniformis* MTCC 6824 for performing hydrolysis reactions, it is important to biochemically characterize these enzymes. The present study showed unique properties of an extra-cellular alkaline metallolipase of *B. licheniformis* MTCC 6824 purified by ion-exchange and gel exclusion chromatography. The purified lipase showed fairly good thermostability and had optimums of activity at 45 °C and alkaline pH (pH 8.0), properties that make it highly potent for future biotechnological applications. Divalent metal cation and polyhydric alcohol sorbitol combinations, particularly $Ca^{2+}$/sorbitol, play important roles in the structure and function of proteins, and calcium-stimulated lipases have been reported in this context. Of the metal ions tested, lipase was found to be activated by $Ca^{2+}$, and other divalent cations were unable to replace $Ca^{2+}$, with the exception of $Mg^{2+}$, to some extent. The presence of $Ca^{2+}$ also made lipase more stable at alkaline pH. The purified lipase showed lower affinity for medium to long C-chain length esters (e.g. triolein) than for short C-chain triglycerides. Because of its high specificity towards short C-chain esters, the potential application(s) of the lipase from *B. licheniformis* MTCC 6824 in the enrichment of long C-chain PUFAs as triglycerides will be further explored by performing enzyme-catalyzed hydrolytic reactions of oils rich in long chain polyunsaturated fatty acids.

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