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ORIGINAL PAPER

Detergent compatible alkaline lipase produced by marine *Bacillus smithii* BTMS 11

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Abstract *Bacillus smithii* BTMS 11, isolated from marine sediment, produced alkaline and thermostable lipase. The enzyme was purified to homogeneity by ammonium sulfate precipitation and ion exchange chromatography which resulted in 0.51 % final yield and a 4.33 fold of purification. The purified enzyme was found to have a specific activity of 360 IU/mg protein. SDS-PAGE analyses, under non-reducing and reducing conditions, yielded a single band of 45 kDa indicating the single polypeptide nature of the enzyme and zymogram analysis using methylumbelliferyl butyrate as substrate confirmed the lipolytic activity of the protein band. The enzyme was found to have 50 °C and pH 8.0 as optimum conditions for maximal activity. However, the enzyme was active over wide range of temperatures (30–80 °C) and pH (7.0–10.0). Effect of a number of metal salts, solvents, surfactants, and other typical enzyme inhibitors on lipase activity was studied to determine the novel characteristics of the enzyme. More than 90 % of the enzyme activity was observed even after 3 h of incubation in the presence of commercial detergents Surf, Sunlight, Ariel, Henko, Tide and Ujala indicating the detergent compatibility of *B. smithii* lipase. The enzyme was also found to be efficient in stain removal from cotton cloths. Further it was observed that the enzyme could catalyse ester synthesis between fatty acids of varying carbon chain lengths and methanol with high preference for medium to long chain fatty acids showing 70 % of esterification. Results of the study indicated scope for application of this marine bacterial lipase in various industries.

Keywords Marine lipase · *Bacillus smithii* · Detergent compatible · Characterization

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Introduction

Lipases (triacylglycerol acylhydrolase EC 3.1.1.3) are serine hydrolases that catalyze both the hydrolysis and the synthesis of esters from glycerol and long chain fatty acids. They are considered as the third largest enzyme group, after proteases and carbohydrases, based on their market value. There is a large potential for lipases in industrial applications such as additives in foods, pharmaceuticals (naproxen, ibuprofen), medical assay, cosmetics, leather, dairy industry (cheese ripening, flavour development, EMC technology), fine chemicals, detergents, paper manufacture and waste-water treatment (Hasan et al. 2006). The enantioselective and regioselective nature of lipases have been utilized for the resolution of chiral drugs, fat modification, synthesis of cocoa butter substituent, biofuels, and for synthesis of personal care products and flavor enhancers (Hasan et al. 2006; Treichel et al. 2010). Detergent enzymes make up nearly 32 % of the total lipase sales. Lipase for use in detergents needs to be thermostable and remain active in the alkaline environment of a typical machine wash. An estimated 1,000 tons of lipases are added to approximately 13 billion tons of detergents produced each year (Jaeger and Reetz 1998).

Although lipases are obtained from different sources, lipases of microbial origin are preferred over the plants and animal resources owing to their multifaceted applications in industries, easy production on cheap media components, easy downstream processing and unlimited supply due to renewable nature. Lipases from a large number of bacterial, fungal, plant and animal sources have been purified to homogeneity (Saxena et al. 2003) and lipases isolated from different sources have been reported to hold a wide range of properties depending on their sources with respect to positional specificity, fatty acid specificity, thermostability, pH optimum, etc. (Huang 1984). Most lipases can act in a wide range of pH and temperature, though alkaline bacterial lipases are more common and are always in high demand in the industrial sector. Because of their high specificity, stability in organic solvents, chemoselectivity, regioselectivity and enantioselectivity, lipases have gained a distinguished platform contrary to its contemporary hydrolases especially in the area of organic synthesis.

Lipases are produced by many microorganisms, including bacteria, fungi, yeasts and actinomycetes, although *Candida*, *Pseudomonas*, *Rhizomucor* and *Rhizopus* sp. stand out nowadays as sources of most commercially available enzyme preparations (Arpigny and Jaeger 1999; Sharma et al. 2001). Several species of *Bacillus* were also reported to produce lipolytic enzymes under alkaline conditions (Lindsay et al. 2000). Alkali tolerance and thermo stability of lipases are the most desirable characteristics for their commercial exploitation.

In spite of several reports on isolation of lipolytic enzymes from microbial sources, the search for new enzyme sources and improved lipases are continually pursued (Berto et al. 1997; Kulkarni and Gadre 1999) owing to the continuous demand for highly active enzymes with appropriate properties and substrate specificities. Particularly the search for new lipase/esterase sources is of interest, because it could provide new biocatalysts that could either promote novel industrial applications, or be used to achieve a better understanding of enzyme mechanism and structure–function relationships. Although microbial lipase is one among the major subject of intensive research on industrial enzymes at global level, marine microbial lipases remains yet unexploited. Considering the importance of lipase for several applications and need for exploration of new and additional source for industrial production of lipases, an attempt was made to explore the prospect of deriving potential lipase producing microorganisms from marine environment capable of producing lipase with desirable properties for industrial applications. Herein we report the characterization of detergent compatible lipase isolated from marine bacterium *Bacillus smithii* and its probable application as an additive in detergent formulations and in esterification process.

Materials and methods

Microorganism and inoculum preparation

Bacillus smithii BTMS 11 [identified at Microbial Type Culture Collection (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, India] isolated from marine sediment and available as a stock culture in the Microbial Technology Laboratory of the Department of Biotechnology at Cochin University of Science and Technology was used. The strain was maintained on ZoBell's marine agar at 4 °C and subcultured periodically. Since lipase is an inducible enzyme, pre-induced inoculum was prepared for utilization. Initially a preculture was prepared by inoculating 10 ml of ZoBells marine broth, using a loopful of *B. smithii* cells freshly grown on ZoBells marine agar slant, and incubating at room temperature (RT, 28 ± 2 °C) on a rotary shaker (170 rpm) for 18 h. This prepared preculture was adjusted to 0.1 OD 600 nm and used for inoculating (1 % v/v) 40 ml of ZoBells marine broth taken in a 250 ml conical flask and incubated at RT on a rotary shaker (170 rpm) for 18 h. Later the cells were harvested by centrifugation (10,000 rpm, 5 min, 4 °C) and the cell pellet was resuspended in 10 ml of 0.8 % physiological saline. The prepared cell suspension (5×10^8 cfu/ml) was used as inoculum for enzyme production.

Lipase production

Lipase production was carried out under submerged fermentation (SmF) in Minimal salts medium (MSM) with the following composition: $NaHPO_4$ 6 g/l, KH_2PO_4 3 g/l, NaCl 20 g/l, NH_4Cl 1 g/l, $MgSO_4.7H_2O$ (1.0 M) 0.2 %, $CaCl_2.2H_2O$ (0.1 M) 0.1 %, and pH 8.0. This medium was supplemented with sesame oil 1.5 %, glucose 0.5 %, soybean meal 0.2 %, and additional NaCl 0.5 %. The prepared medium was inoculated with 3 % inoculum containing 5×10^8 cfu/ml, and incubated at RT (28 ± 2 °C) for 24 h under shaking on a rotary shaker (170 rpm). The cell free supernatant obtained by centrifugation (SIGMA, Germany) at 10,000 rpm, 10 min, 4 °C was considered as the crude enzyme.

Protein estimation

Protein was estimated according to the method of Lowry et al. (1951) using Bovine Serum Albumin as a standard.

Lipase assay

Lipase activity was estimated using a spectrophotometric assay with *p*-nitrophenyl esters (*p*NP-acetate or *p*NP-butyrate) as substrate with slight modifications of the original

method (Bulow and Mosbach 1987). The substrate was dissolved in acetonitrile at a concentration of 50 mM. Subsequently, ethanol and 50 mM Tris HCl buffer (pH 7.0 containing 1 mM $CaCl_2$) were added to a final composition of 1:1:95 (v/v/v) of acetonitrile/ethanol/buffer, respectively. No ethanol was added when pNPA was used as substrate. The cell-free supernatant (0.2 ml) was added to the substrate solution (2.3 ml) and then the mixture was incubated at 37 °C. After 30 min, enzyme activity was measured by monitoring the change in absorbance at 410 nm in a UV- Visible spectrophotometer (Shimadzu, Japan) against suitable enzyme free blanks that represents the amount of released *p*-nitrophenol (*p*NP). The enzyme activity was calculated as µmol of pNP released per minute per milliliter of enzyme solution under standard assay conditions.

Purification of *B. smithii* lipase

The crude enzyme extract obtained after SmF was purified by $(NH_4)_2SO_4$ precipitation (20–90 %), followed by dialysis; and ion-exchange chromatography (Basheer et al. 2011). The precipitated protein was resuspended in 50 mM Tris–HCl buffer (pH 8), dialysed against 0.01 M solution of the same buffer for 24 h, at 4° C, and assayed for lipase activity and protein content. Further purification was done by ion-exchange chromatography using DEAE-cellulose column (XK16/26-Amersham Biosciences) pre-equilibrated with phosphate buffer (pH 7.5, 0.01 M). Peak fractions obtained from the column were pooled, and assayed for lipase activity and protein content.

Characterization of purified lipase

SDS-PAGE and zymography

Enzyme characterization by SDS-PAGE and Zymography was performed by the procedures outlined by Basheer et al. (2011). Samples precipitated with $(NH_4)_2SO_4$ and active fractions collected after ion exchange chromatography, were electrophoresed by Native-PAGE and SDS-PAGE in a 10 % polyacrylamide gel according to the method of Laemmli (1970). Low molecular weight markers of Amersham Pharmacia was used as molecular mass standard and molecular weight of lipase was determined using Quantity One® Software of Biorad.

Lipase activity of the enzyme protein band was confirmed by zymogram analysis (Diaz et al. 1999) using the fluorogenic substrate 4-methylumbelliferyl butyrate (MUF-butyrate) (Sigma, St. Louis, USA). Lipase activity for the bands was visualized by the presence of fluorescence under UV light.

Effects of temperature and pH on lipase activity and stability

Optimum temperature for maximal activity of the purified *B. smithii* lipase (360 IU/mg protein) was determined by assaying the enzyme activity at different incubation temperatures (30–80 °C) using *p*-nitrophenyl esters (*p*NP-acetate or *p*NP-butyrate) as substrate (Bulow and Mosbach 1987). Temperature stability of purified enzyme was determined by incubating the enzyme sample at various temperatures (30–80 °C) and estimating residual enzyme activities after incubation for 30 min, 1, 2, 4, 6, 8, 10, 12 and 24 h. Enzyme activity of the sample kept at 4 °C was taken as control. Residual enzyme activity (expressed in percentage) is the percent enzyme activity of the sample with respect to the enzyme activity of the control sample.

Optimum pH for maximal activity of the purified *B. smithii* lipase (360 IU/mg protein) was determined by assaying the enzyme activity over a pH range of 3-10 using *p*-nitrophenyl esters (*p*NP-acetate or *p*NP-butyrate) as the substrate (Bulow and Mosbach 1987). Citrate–phosphate buffer (pH 3 to 6), Tris–HCl buffer (pH 7.0 and 8.0), and Carbonate–bicarbonate buffer (pH 9 and 10) were used as buffer systems. Stability of the purified enzyme over a range of pH was also determined by measuring the residual activity at pH 7.0 after incubating 0.2 ml of the purified enzyme in 1.8 ml of the above mentioned different buffer systems (pH 3–10) for, 24 h at RT (28 ± 2 °C) and at 4 °C.

Substrate specificity and kinetic constants

The substrate specificity of lipase (360 IU/mg protein) was evaluated with *p*-nitrophenyl derivatives namely *p*-nitrophenyl acetate, *p*-nitrophenyl butyrate, *p*-nitrophenyl caprylate, *p*-nitrophenyl laurate and *p*-nitrophenyl palmitate with 10 mM solution of respective substrates prepared in acetonitrile. Enzyme activity was expressed in U/ml.

The purified lipase was incubated with different concentrations (10–200 µM) of *p*-nitrophenyl butyrate. Enzyme assay was essentially the same as described above. The initial velocity data were plotted as the function of the concentration of substrate by the linear transformation of the Michaelis–Menten equation, and the resulting graph (Lineweaver–Burk double-reciprocal plot) was used to calculate the K_m and V_{max} of the reaction.

Positional specificity

Positional specificity of the purified *B. smithii* lipase (360 IU/mg protein) was determined by the method described by Lesuisse et al. (1993) with slight modifications. Pure triolein (40 mg) was sonicated in 2 ml of 0.1 M

Tris–HCl (pH 8.0) containing 1 mM calcium chloride for 3 min. The enzyme sample (200 µg) was then added and the reaction mixture was incubated at 65 °C for 2 h. After incubation, the reaction products were extracted by the addition of 1.5 ml diethyl ether. The extract was concentrated by evaporation and applied to a silica gel plate (Merck Co.). Plates were developed with a 96:4:1 mixture (by volume) of chloroform/acetone/acetic acid. The spots of glycerides and fatty acids were visualized by exposure to iodine vapor.

Effects of various metal ions, inhibitors, detergents, chelators, oxidizing agent, reducing agents and solvents on enzyme activity

Effects of various metal ions on lipase activity was evaluated by incubating the purified enzyme (360 IU/mg protein) with different concentrations (1, 5, 10 mM final concentrations) of sodium chloride, calcium chloride, magnesium sulphate, zinc sulphate, potassium chloride, cupric sulphate, ferric chloride, manganese chloride, nickel chloride, and cobalt chloride which contributed Na^{+}, Ca^{2+}, Mg^{2+}, Zn^{2+}, K^{+}, Cu^{2+}, Fe^{3+}, Mn^{2+}, Ni^{2+} and Co^{2+}, respectively, at 1:1 (v/v) ratio, in the enzyme reaction mixture for 30 min and the residual enzyme activity was estimated (Beena et al. 2010).

The effect of enzyme inhibitors on lipase activity was studied using different concentrations of phenyl methyl sulfonyl fluoride (PMSF) (1, 5 and 10 mM), sodium dodecyl sulphate (SDS) (50, 100, and 150 mM), and diethyl pyrocarbonate (DEPC) (1, 5 and 10 mM). The enzyme assay mixture containing the enzyme and the inhibitor, at 1:1 (v/v) ratio, was incubated for 30 min and the residual enzyme activities were determined. Similarly activity and stability of the enzyme in the presence of different concentrations of EDTA (1, 5 and 10 mM), hydrogen peroxide (as an oxidizing agent), and β-mercaptoethanol (as a reducing agent), were studied by incubating the enzyme assay mixture for 30 min. Impact of methanol, isopropanol, ethanol and hexane on lipase was also studied at 1, 2, 5, 10 % (v/v) level. The enzyme assay mixture containing the enzyme and organic solvent was incubated for 1 h and the residual enzyme activities were calculated (Beena et al. 2010).

Application studies

Evaluation of enzyme for use in detergent formulations

Suitability of enzyme for use in detergents was determined in terms of its compatibility in various commercial detergents by directly incorporating them into the enzyme assay mixture (Chellappan 2006). The stability of the enzyme in the presence of commercial detergents was determined using Ujala washing powder, Surf excel, Ariel compact, Henko stain champion, Tide, Sunlight extra bright with colour lock, at 7 mg/ml (w/v) concentration. Enzymes already present in the detergents were first heat inactivated by boiling for 10 min and, 2 ml of purified enzyme (134 IU/mg protein) was added to 50 ml solution of detergent. The assay mixture was incubated for 3 h at RT (28 ± 2 °C) and the residual activity was determined.

Wash performance of lipase

Wash performance of the purified lipase (134 IU/mg protein) was studied using white cotton cloth pieces (5 cm × 5 cm) stained with oily stain. The stained cloth pieces were taken in separate flasks and subjected to the following wash treatment studies. (a) 100 ml heat inactivated detergent (7 mg/ml) + stained cloth piece, (b) 100 ml heat inactivated detergent (7 mg/ml) +1 ml enzyme solution + stained cloth piece, (c) 100 ml distilled water + 1 ml enzyme solution + stained cloth piece, and (d) 100 ml distilled water + stained cloth piece. After 30 min of incubation at RT (28 ± 2 °C) in a water bath shaker, the cloth pieces were taken out rinsed with tap water, dried and visual examination was done in order to assess the effectiveness of stain removal (Chellappan 2006).

Esterification by lipase

Purified lipase was evaluated for its esterification ability towards catalyzing esterification reactions between fatty acids (C4: 0 to C18: 0) and methanol in n-hexane (Gulati et al. 1999). The esterification reaction was done by mixing 100 mM of the fatty acid with 50 mM of the alcoholic donor methanol in 4 ml n-hexane in 15-ml screw-capped vials. To this reaction mixture one ml of purified enzyme (360 IU/mg protein) was added and incubated at 50 °C for 24 h at 150 rpm. Ester synthesis was quantified by titrating the remaining fatty acids in the reaction mixture with 0.1 N NaOH. The ester content was determined by calculating the amount of residual fatty acid in the reaction mixture and expressed in terms of per cent conversion of fatty acid to ester.

Results

Purification of lipase

The lipase isolated from *B. smithii* BTMS 11 was purified by ammonium sulfate fractionation followed by anion exchange chromatography on DEAE Cellulose. The

enzyme could be precipitated with 80 % ammonium sulfate saturation. Enzyme, with specific activity of 134 IU/mg protein, a 1.34 % yield, and 1.6-fold purification, was obtained after ammonium sulfate fractionation. Further purification by ion exchange chromatography yielded active enzyme with a specific activity of 360 IU/mg protein, a 0.1 % yield, and 4.33-fold purification (Table 1). A major peak indicating lipase activity (Fig. 1) was recorded in the elution profile.

Characterization of purified lipase

Analysis of lipase by gel electrophoresis and zymography

SDS-PAGE analysis showed a single band on staining with Coomassie Brilliant blue R250, indicating homogeneity in the enzyme protein. Further SDS–PAGE performed under non-reducing and reducing conditions yielded a single band of 45 kDa (Fig. 2a) confirming the purity of the enzyme. Zymogram obtained showed a UV-fluorescent band (Fig. 2b) indicating lipase activity.

Effects of temperature and pH on enzyme activity and stability

Lipase activity determined at different incubation temperatures (30–80 °C), using *p*-nitrophenyl esters (pNP-acetate or *p*NP-butyrate) as substrate indicated that the enzyme preferred a higher temperature of 50 °C, as optimum, for maximal activity although the enzyme was active over a broad range of incubation temperatures varying from 30 °C to 70 °C (Fig. 3a). However, marked decline in enzyme activity was observed along with increase in temperatures above 60 °C. In fact, the lipase activity showed a linear increase along with increase in temperature and recorded a rapid increase at 40 to 50 °C. Thermostability studies indicated that the enzyme was stable at 30 to 80 °C and retained 50 % of activity even after 12 h (Fig. 3b). From the data presented in Fig. 4a it was inferred that pH 8.0 was the optimum for lipase activity and the enzyme was active over a pH range of 7–10. It was also observed that there was no enzyme activity at pH 3-6. These observations indicated the alkaline nature of this enzyme. Results of pH

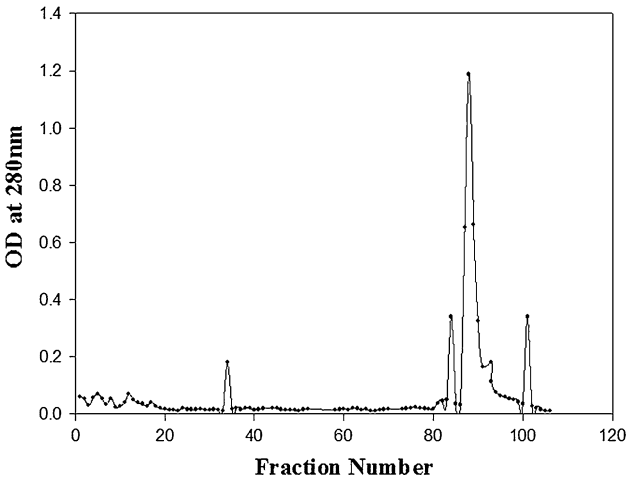

Fig. 1 Elution profile of lipase isolated from *Bacillus smithii* BTMS 11 during purification by ion exchange chromatography using DEAE Cellulose

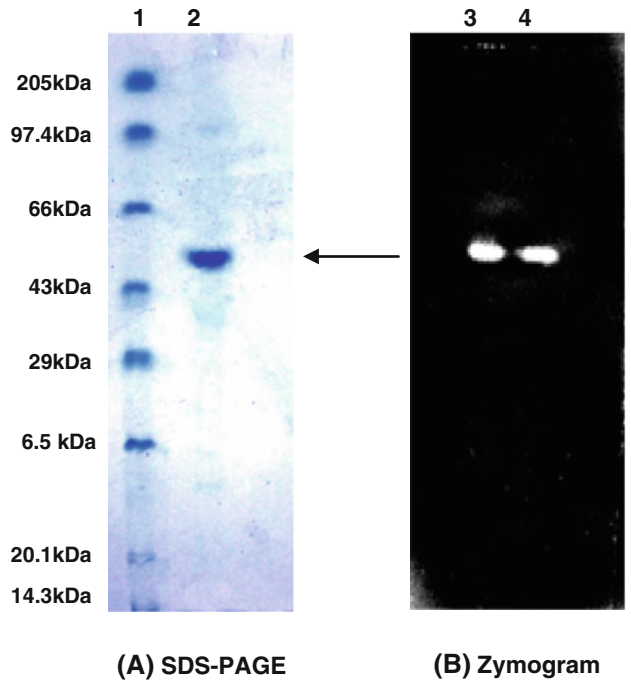

Fig. 2 SDS-PAGE analysis of lipase isolated from *Bacillus smithii* BTMS 11 **a** Coomassie blue staining and **b** Zymogram analysis. *Lane 1* protein marker (kDa). *Lane 2* ion exchange fraction. *Lane 3* Ammonium sulfate fraction (Zymogram with MUF Butyrate). *Lane 4* Ion exchange fraction (Zymogram with MUF Butyrate)

Table 1 Purification of lipase isolated from *B. smithii* BTMS 11

Purification step	Volume (ml)	Total protein (mg)	Total activity (U/ml)	Specific activity (U/mg)	Protein yield (%)	Activity yield (%)	Fold of purification
Crude extract	760	1,900	158,330	83	100	100	1.00
$(NH_4)_2SO_4$ fractionation	18	25.56	3,420	134	1.34	2.16	1.60
DEAE cellulose	6	2.00	720	360	0.10	0.45	4.33

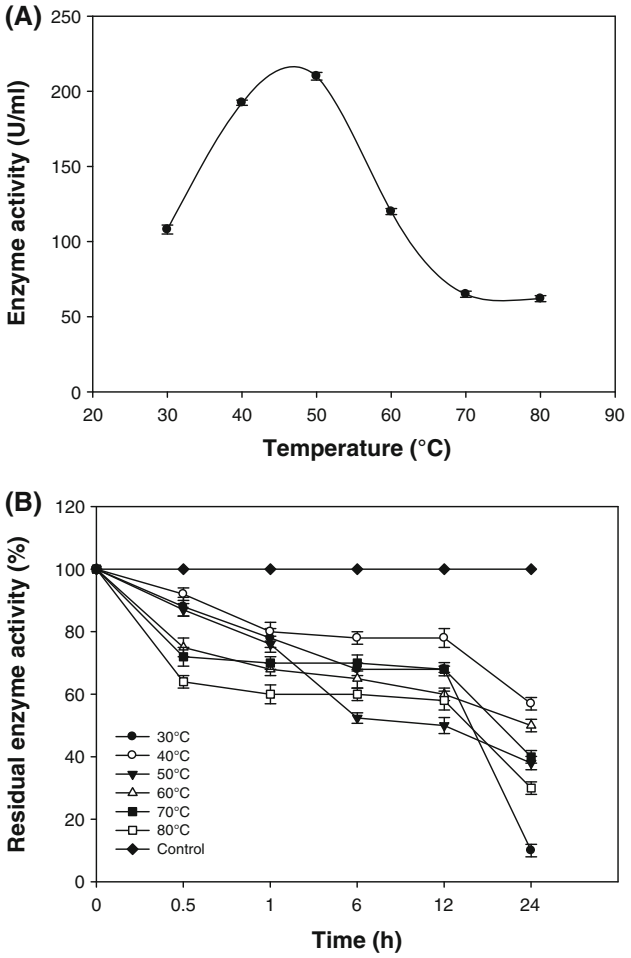

Fig. 3 Effect of temperature on activity (**a**) and stability (**b**) of lipase isolated from *Bacillus smithii* BTMS 11

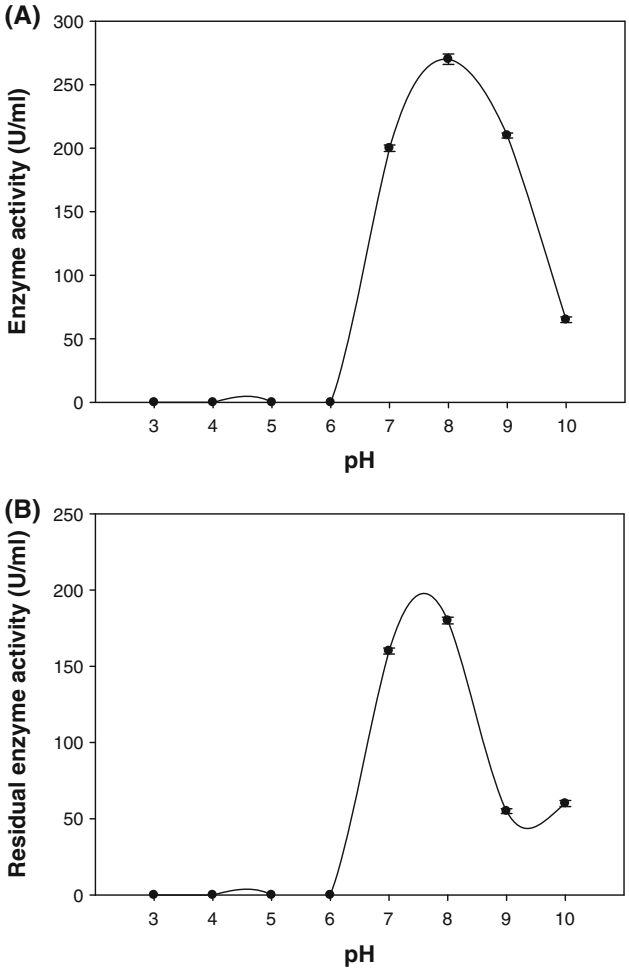

Fig. 4 Effect of pH on activity (**a**) and stability (**b**) of lipase isolated from *Bacillus smithii* BTMS 11

stability studies (Fig. 4b) showed that the enzyme was stable in the pH range of 7–9.

Substrate specificity, kinetic constants and positional specificity

Data obtained for the studies on the substrate specificity of purified lipase evaluated with the various *p*-nitrophenyl derivatives indicated that this lipase had a wide range of substrate specificity and showed activity with all the *p*-nitrophenyl substrates tested (Fig. 5). Maximum activity was observed with *p*-nitrophenyl butyrate (C_4) followed by *p*-nitrophenyl caprylate (C_8) and *p*-nitrophenyl acetate (C_2). Comparatively less activity was observed for *p*-nitrophenyl laurate (C_{12}) and *p*-nitrophenyl palmitate (C_{16}). Hence the *B. smithii* lipase can be considered to have more preference for short to medium chain fattyacids (C_4 to C_8) than to the long chain fatty acids.

The reaction kinetics determined for the purified enzyme with *p*NPB is presented as Lineweaver–Burk double

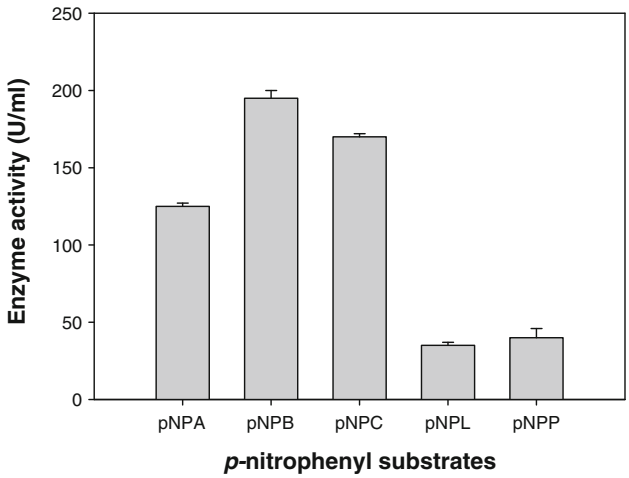

Fig. 5 Substrate specificity of lipase isolated from *Bacillus smithii* BTMS 11

reciprocal plot (Fig. 6). The enzyme was found to have an apparent K_m value of 0.1 mM, and a V_{max} value of 100 U/ml for the hydrolysis of *p*NPB. It was observed that the

lipase could hydrolyze the three positions (*sn*-1, 2 and 3) and all the ester bonds of triolein there by causing the complete breakdown of the triolein molecule. The hydrolytic products visualized in thin layer chromatogram (Fig. 7) testified that the *B. smithii* lipase is non-regiospecific in nature.

Effects of various metal ions, inhibitors, chelators, oxidizing agent, reducing agent and solvents on enzyme activity

Data depicted in Table 2 for the studies conducted on the effect of various metal ions on lipase activity, indicated that all the metal ions tested led to an enhancement in lipase activity at all the three concentrations tested. Among the metal ions tested Cobalt chloride was observed to support maximal lipase activity, at all the three concentrations tested, and compared to other metals. However Cu^{2+} was found to have an inhibitory effect on the lipase activity at higher concentrations (10 mM) while reduction in enzyme activity was observed at 1 and 5 mM concentrations. Similarly Fe^{2+} ions were observed to enhance maximal lipase activity at 1 mM concentrations while recording inhibition of the lipase activity at concentrations above 1 mM.

Results presented in Table 3 for group-specific potential inhibitors SDS, PMSF, DEPC and the hydrogen peroxide, clearly evidenced that their addition resulted in considerable levels of enzyme inhibition at all the concentrations tested. Increasing concentrations of EDTA was found to result in the decline of lipase activity and relatively very low level of enzyme activity (18 % residual activity) was recorded at 1 mM concentration. Whereas, β-mercaptoethanol was found to have a positive effect on the lipase activity along with increase in its concentration. The residual lipase activity was found to be increased from 68 % at 1 mM concentration to 120 % at 10 mM concentration.

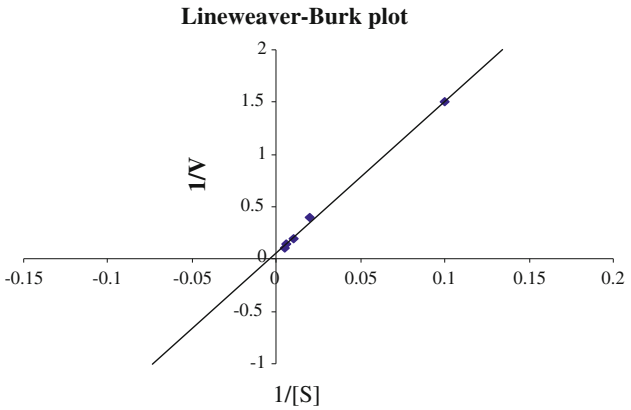

Fig. 6 Lineweaver-Burk plot obtained for the purified lipase isolated from *Bacillus smithii* BTMS 11

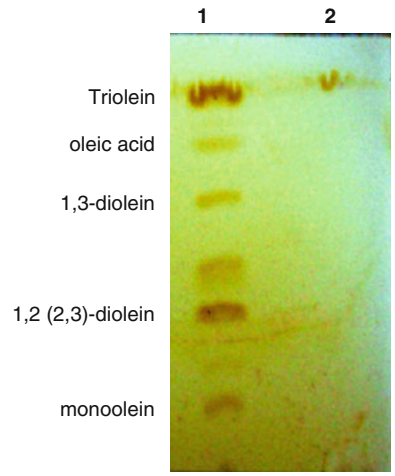

Fig. 7 TLC showing the positional specificity of lipase of *Bacillus smithii* BTMS 11. *Lane 1* triolein hydrolyzed by *B. smithii* lipase. *Lane 2* triolein

Table 2 Effect of various metal ions on lipase activity of *B. smithii* BTMS 11

Metal ions		Residual activity (%)		
		1 mM	5 mM	10 mM
Calcium chloride	Ca^{2+}	127 ± 1.58	95 ± 1.58	93 ± 1.22
Cupric sulphate	Cu^{2+}	52 ± 1.58	70 ± 1.00	0.0
Cobalt chloride	Co^{2+}	126 ± 1.58	180 ± 2.55	161 ± 2.0
Ferric chloride	Fe^{2+}	233 ± 3.00	0.0	0.0
Manganese chloride	Mn^{2+}	95 ± 1.58	98 ± 2.0	105 ± 2.0
Magnesium sulphate	Mg^{2+}	97 ± 1.41	103 ± 2.24	111 ± 3.0
Potassium chloride	K^{+}	101 ± 1.41	99 ± 1.58	68 ± 2.12
Sodium chloride	Na^{+}	90 ± 1.58	95 ± 2.00	105 ± 3.00
Zinc sulphate	Zn^{2+}	103 ± 2.00	102 ± 1.41	100 ± 2.55

The studies conducted on impact of various organic solvents on lipase activity showed that methanol, isopropanol, ethanol and hexane had significant effects on the enzyme activity (Fig. 8). All the solvents tested were found to enhance the lipase activity at a lower concentration of 1 %. However, the enzyme activity was observed to decrease gradually in response to 2–5 % and there after steeply at 10 % concentrations in the case of all the organic solvents tested.

Application studies

Evaluation of lipase for use in detergent formulation

Detergent compatibility of the enzyme was studied in order to determine the suitability of *B. smithii* lipase for use in detergent formulations. Results obtained using Ujala washing powder (pH 9.79), Surf excel (pH 9.70), Ariel

Table 3 Effect of various chemical agents on lipase activity of *B. smithii* BTMS11

Chemical agents	Concentration (mM)	Residual activity (%)
SDS	50 mM	0
	100 mM	0
	150 mM	0
β-mercaptoethanol	1 mM	68 ± 1.22
	5 mM	90 ± 5.6
	10 mM	120 ± 5.0
PMSF	1 mM	0
	5 mM	0
	10 mM	0
DEPC	1 mM	0
	5 mM	0
	10 mM	0
EDTA	1 mM	18.0 ± 1.41
	5 mM	4.7 ± 0.28
	10 mM	0.47 ± 0.01
H_2O_2	0.1	0
	0.5	0
	1.0	0

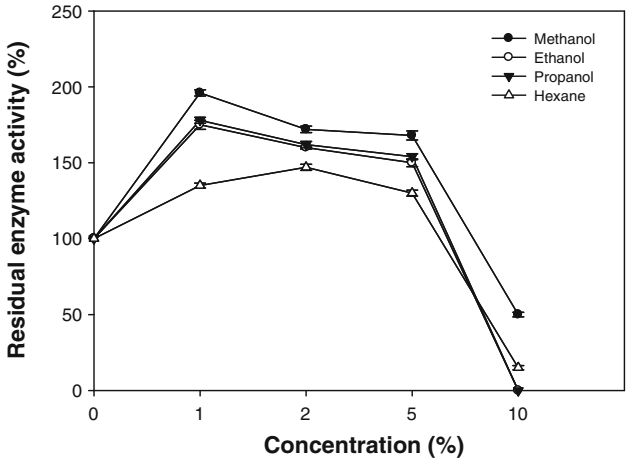

Fig. 8 Effect of organic solvents on lipase obtained from *Bacillus smithii* BTMS 11

compact (pH 9.92), Henko stain champion (pH 10.12), Tide (pH 9.89), Sunlight extra bright with colour lock (pH 10.0) indicated that the enzyme retained more than 90 % of activity, even after 3 h of incubation, in all the detergents tested (Fig. 9). Further it was found that the lipase was 100 % compatible with all the detergents tested since there was no decrease in enzyme activity. In fact enhanced enzyme activity was observed with the detergents surf excel, sunlight and Ariel compared to other detergent formulations. Results presented in Fig. 10 for the visual examination of the oil stained white cotton cloth pieces subjected to wash treatment exhibited the effectiveness of *B. smithii* lipase in removal of stains.

Esterification ability of lipase

Bacillus smithii lipase was capable of effectively catalyzing methyl–ester synthesis between fatty acids of varying carbon chain lengths and methanol in n-hexane. However, in all the esterification reactions, the lipase showed a high esterification capability, recording above 70 %, with all the fatty acids tested, except in the case of Butyric acid (Table 4). Further it was observed that the lipase preferred medium-chain to long chain fatty acids (C8: 0 to C18: 0) which were etherified at higher conversion rates (70 %) in comparison to very short chain fatty acids.

Discussion

Members of *Bacillus* genera are ubiquitous and are known to produce wide range of enzymes that have potential industrial applications. In the present study lipase produced as extracellular enzyme by *B. smithii* isolated from marine sediments was purified and characterised. The lipase activity of the purified enzyme protein was confirmed by zymogram analysis using Methyl umbelliferyl butyrate as substrate indicating a single type of extracellular lipase. Bacterial lipases are reported to span a molecular weight range of 19 to 60 kDa (Iwai et al. 1970). In the present study the molecular mass for the *B. smithii* lipase was estimated as 45 kDa which corroborates with a molecular mass of approx. 45 kDa reported for lipases isolated from *B. thermocatenulatus* and *B. stearothermophilus* (Schmidt-Dannert et al. 1996; Kim et al. 1998). In fact thermostable lipases from many *Bacillus* species have been

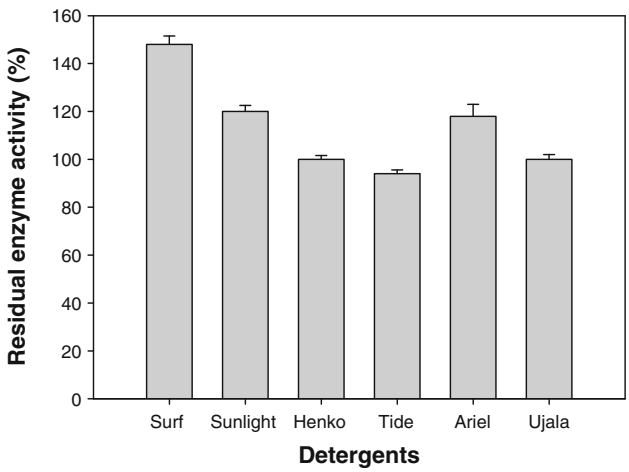

Fig. 9 Stability of lipase isolated from *Bacillus smithii* BTMS 11 in the presence of commercial detergents

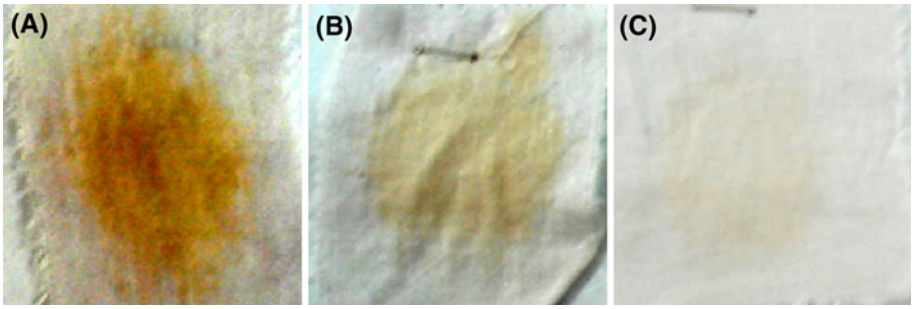

Fig. 10 Wash performance study of lipase isolated from *Bacillus smithii* BTMS 11. **a** Cotton cloth stained with oily stain. **b** Stained cloth washed with commercial detergent. **c** Stained cloth washed with commercial detergent + *B. smithii* lipase

Table 4 *B. smithii* lipase-mediated esterification of fatty acids with methanol

Fatty acids	Ester conversion (%)
Butyric	2 ± 0.08
Caprylic	80 ± 2.0
Lauric	79 ± 1.6
Mystric	78 ± 1. 8
Palmitic	73 ± 1. 8
Stearic	70 ± 1.6

found to possess a molecular mass of 43–45 kDa (Nawani and Kaur 2000; Lee et al. 2001).

Extreme pH conditions alter the structure of the surface of the enzymes modifying the interaction between active site and substrate. Because of that, under strong acidic and alkaline conditions, enzymes are denatured and as a consequence, their activity is totally or partially lost (Chellappan 2006). A change in pH will have a progressive effect on the structure of the protein and the enzyme activity (Fullbrook 1996) and at the optimal pH, the conformation of lipase is suitable to absorb to the interface of oil/water, helping open the lid that blocks the active site of the lipase to lower the activation energy of hydrolysis (Jaeger et al. 1994). Generally, bacterial lipases have neutral (Dharmsthiti and Luchai 1999; Lee et al. 1999) or alkaline pH as optimum (Kanwar and Goswami 2002; Sunna et al. 2002). In the present study *B. smithii* lipase was observed to prefer an alkaline pH 8.0 as optimum although active over a pH range of 7.0–10.0. It was also noted that the enzyme was completely inactive in the acidic pH range (pH 3.0–6.0). These results are in accordance with the earlier reports of alkaline lipases of *Bacillus* strain A30-1 (ATCC 53841) (Wang et al. 1995), *Bacillus stearothermophilus* L1 (Kim et al. 1998), *B. thermocatenulatus*, *B. subtilis*, (Schmidt-Dannert et al. 1996; Kulkarni and Gadre 2002) which showed optimal activity in the pH range of 8–10. Bacterial lipases possess pH stability over a wide range, from pH 4 to 11 (Dong et al. 1999). *B. smithii* lipase was found to be stable over a wide range of pH from 6 to 10 and this property of the enzyme indicates scope for use as a potential candidate for application in industrial process that are conducted in the alkaline range, especially in the detergent industry.

Bacterial lipases generally have temperature optimum in the range 30–60 °C (Litthauer et al. 2002). The present study revealed that *B. smithii* lipase was active over a wide range of incubation temperatures (30–70 °C) with optimum at 50 °C. Further, the results obtained indicated clearly that the enzyme is thermostable in nature and can tolerate higher temperatures above 50 °C. In fact temperature optimum of 45 °C and above has been reported for lipases isolated from many *Bacillus* species (Lee et al. 2001; Jinwal et al. 2003). Most of the industrial processes involving enzymes operate at temperatures above 50 °C, and consequently thermostable enzymes assume great significance (Sharma et al. 2002). Moreover it has been also suggested that it would be favorable for industrial or diagnostic use if *Bacillus* sp. lipase could function up to 60 °C (Sugihara et al. 1991). In this context the present findings strongly indicated the potential of *B. smithii* lipase for use in various biotechnological industries that require thermostable enzymes.

The lipase isolated from *B. smithii* was found to have substrate specificity for pNPB followed by pNPC and pNPA. This particular observation strongly suggests that the lipase has strong inclination for short to medium chain fatty acids than for the long chain fatty acids. A low K_m value represents a high affinity. For most industrially relevant enzymes, K_m values range between 10^{-1} and 10^{-5} M, although the K_m values of the enzyme generally vary widely (Fullbrook 1996). The reaction kinetics of the purified enzyme of *B. smithii* determined from Lineweaver–Burk plots with *p*NPB under standard assay conditions indicated that the enzyme had an apparent K_m value of 0.1 mM, and a V_{max} value of 100 U/ml for the hydrolysis of *p*NPB. Further, the Lineweaver–Burk plots also indicated that the hydrolysis of *p*-nitrophenyl butyrate by *B. smithii* lipase followed Michaelis–Menten kinetics.

A comparison of the chromatogram obtained for the hydrolytic products of triolein from thin layer chromatography (TLC) with that of BTID-A *Bacillus* lipase (Lee et al. 2001) indicated that 1,2- and 1,3-diolein and monoolein were released as the main products by *B. smithii*

lipase and the enzyme is likely to be positionally non-regiospecific. Further, this observation also indicated that this enzyme may probably act synergistically to hydrolyze extracellular lipids to free fatty acids and glycerols. In fact most of the bacterial lipases reported so far are constitutive and are non-specific in their substrate specificity (Macrae and Hammond 1985).

The majority of enzymes require the presence of metal ion activators to express their full catalytic activity, and thus metal ion activation of enzyme reactions is important in industrial applications for achieving maximal catalytic efficiency and a number of enzymes require the presence of metal ions, such as calcium ions, for the maintenance of their stable and active structures (Gray 1995). The study conducted on the effect of various cations on the activities of *B. smithii* lipase revealed that all ions tested were found to enhance lipase activity except Cu^{2+} which showed an inhibitory effect on the lipase activity. *B. stearothermophilus* MC 7 lipase was reported to be inhibited by divalent ions of heavy metals, entirely by Cu^{2+} and strongly by Fe^{2+} and Zn^{2+} (Kambourova et al. 2003) while the lipase activity of thermophillic *B. coagulans* BTS-3 isolate was reported to be enhanced by K^{+}, Fe^{3+}, and Mg^{2+} ions and inhibited by Co^{2+}, Mn^{2+}, and Zn^{2+} (Kumar et al. 2005). The enhanced catalytic activity of *B. smithii* lipase observed in the presence of Co^{2+} could be attributed to the probable formation of insoluble Co-salts of fatty acids released during the hydrolysis which could have prevented product inhibition.

In the present study *B. smithii* lipase was observed to be completely inhibited by SDS, PMSF and DEPC at all the concentrations studied. Inhibition by PMSF, suggests that the enzyme is a serine lipase having a serine residue at its active site. The results obtained for the experiment with β-mercaptoethanol showed that the enzyme activity was significantly influenced by β-mercaptoethanol indicating that thiol group was not present or not critical for the catalytic function. Enhanced activity also indicated the probable absence of disulfide bonds in *B smithii* lipase or disulfide bridge is not required for activity of this lipase. Similar results were observed with some lipases (Lotrakul and Dharmsthiti 1997; Dharmsthiti and Luchai 1999; Lee et al. 1999). EDTA is a potent inhibitor of metal-dependent enzymes, and is generally used as a commercial inhibitor of proteases, where it acts by chelating metals such as lead and zinc. EDTA treatment strongly inhibited the lipase activity with the increasing concentrations confirming that *B. smithii* lipase requires metal ions for their activity. Similar result was reported for *B. thermolevorans* ID-1 lipase (Lee et al. 2001). Unlike most other bacterial lipases, the *B. smithii* lipase was observed to be a metallo-enzyme inhibited by EDTA as well as PMSF. These observations indicate that *B. smithii* lipase may possess a triad of three amino acids at its catalytic site just like many other lipases (Winkler et al. 1990). A purified lipase from *B. coagulans* MTCC 6375 was also reported to be inhibited by EDTA, PMSF and total loss of activity in the presence of SDS (Nawani et al. 1998; Yu et al. 2007). The results observed with *B. smithi* lipase corroborates with earlier reports with respect to EDTA, PMSF and SDS.

Detergent industry consumes the largest share of the microbial lipase produced. The addition of lipases to detergent formulations has been investigated in the context of removal of fat stains and removal of oil or fatty deposits by lipase is attractive owing to its suitability under washing conditions. To be a suitable additive in detergents, lipases should be both thermostable and alkaline besides demonstrating catalytic activity in the presence of various components of washing powder formulations. In this context the results of the study showed that the *B. smithii* lipase could demonstrate high stability in all the detergents tested by retaining more than 90 % of enzyme activity, confirming the detergent compatibility of this lipase. In fact it was observed that the enzyme was highly compatible with Surf Excel, followed by Sunlight, Ariel, Ujala, and Henko. It must be noted here that the pH value of all detergent solutions tested had an alkaline pH and the *B. smithii* lipase recorded significant activity at alkaline pH in the presence of the detergents. Probably the alkaline conditions in the detergent could have attributed to the compatibility of the enzyme to the detergents. The observation adds further evidence to suggest that this alkaline lipase has potential for use as an additive in detergent formulations. Furthermore the results obtained for the wash performance study in removing oily stains from cotton cloths also testify the scope for use of this lipase as a detergent additive.

Enzymes in organic synthesis, using organic solvents as catalytic media, have been realized as a potential technological revolution towards obtaining highly enhanced enzyme activity, when compared with other natural aqueous reaction media, since they exhibit the phenomenon of "molecular memory." Enzyme-catalyzed reactions in organic solvents, and even in supercritical fluids and the gas phase, have found numerous potential applications, some of which have already been commercialized. Various organic solvents were tested for their effect on *B. smithii* lipase activity. A marked stimulation of the enzyme activity was observed upon adding organic solvents to the assay mixture. An increased residual lipase activity was observed with the increasing percentage of concentration of all the organic solvents tested namely methanol, ethanol, propanol, and hexane up to 5.0 % (v/v). These features of the enzyme indicate potential for probable application of this enzyme in the synthesis of chiral compounds in non-aqueous solvents (Zaks and Klibanov 1988).

Besides applications as additives in detergents, lipases are used in the esterification and transesterification reactions due

to their regiospecificity and steriospecificity. During evaluation of lipase for use in transesterification and synthesis of esters, the reactions have been allowed to occur in media containing water immiscible organic solvents and a small amount of water because the enzymes are less susceptible to denaturation in such systems (Sugihara et al. 1991). The marked stimulating effect of *B. smithii* lipase in the solvents tested may be attributed to the probable sheath of water molecule tightly bound to the enzyme which protects the enzyme's hydrophilic surfaces and allow retention of the native conformation even in the presence of apolar hydrophobic solvents (Klibanov 1986). Based on the observation that, the *B. smithii* lipase is stable in organic solvents methanol, propanol, ethanol, and n-hexane, it is suggested that the enzyme holds potential for application in transesterification and ester synthesis. Further it was observed that *B. smithii* lipase could effectively catalyze methyl–ester synthesis between fatty acids of varying carbon chain lengths and methanol and prefer medium-chain fatty acids to long chain fatty acids (C8: 0 to C18: 0) which were etherified at higher conversion rates (70 %) indicating scope for exploitation in synthesis of medium-long-medium-chain (MLM) triglycerides (Kazlauskas and Bornscheuer 1998). It must be noted that methyl and ethyl esters of long-chain fatty acids have been used to enrich diesel fuels (Vulfson 1994) and these esters have extensive uses as emulsifiers, antioxidants and flavour compounds in the food, pharmaceutical, detergent and oleochemical industries (Saxena et al. 1999). In this context the present observations on potential of marine lipase for use in ester synthesis deserve attention.

Considering the overall properties of different alkaline lipases of microbial origin and the alkaline lipase of marine *B. smithii* BTMS 11 it is inferred that marine *Bacillus* lipase has more desirable properties with regard to pH and temperature stability, detergent compatibility and wash performance for a potential application in the detergent industry and enzyme mediated organic synthesis. In a nutshell *B. smithii* stands out as a potential marine alkaline lipase producing bacterium which can be exploited for varied industrial applications especially in detergent industry and ester synthesis. Further the present study also endorses the faith that marine microorganisms are unique in terms of their characteristics and holds potentials for varied applications in industries and environmental protection.

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