African Journal of Biotechnology Vol. 9(45), pp. 7724-7732, 8 November, 2010
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB10.369
ISSN 1684–5315 © 2010 Academic Journals

Full Length Research Paper

Purification and characterization of 60 kD lipase linked with chaperonin from *Pseudomonas aeruginosa* BN-1

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Accepted 21 October, 2010

An extracellular lipase from *Pseudomonas aeruginosa* BN-1 was purified to 42.99 fold. N-terminal sequence of purified enzyme was AAKEVKFGDS identical to sequence of a chaperonin and enzyme may be linked to it. It has an estimated molecular weight of 60 kD, while temperature and pH optima were 37°C and 8.0, respectively. The enzyme obtained has considerable thermostability retaining 70% of activity at 50°C for 1 h. The enzyme was stable at pH 9.5 for 1 h having 70% of the residual activity. Long acyl chains were preferred as substrate and highest hydrolytic activity was observed against C-16 and C-18 4-nitrophenyl esters. Mustard oil was found to be the preferred substrate as lipolytic activity was 2.75 fold higher when compared with the activity with olive oil as the substrate. The enzymatic activity declined in the presence of Al^{3+}, Hg^{2+}, Co^{2+} and Mn^{2+}, while Ca^{2+} and Ba^{2+} ions enhanced the activity. Non ionic detergents, Tween 80 and sodium deoxycholate, increased the activity by 1.2 and 2.5 fold, respectively. Ethylenediaminetetraacetic acid (EDTA), 2-mercaptoethanol and 1,4-dithio-DL-threitol (DTT) had no effect on lipolytic activity. Sodium dodecyl sulfate (SDS) and phenylmethylsulfonyl fluoride (PMSF) inhibited the enzyme activity by 90 and 98%, respectively. The lipase showed stability in organic solvents.

Key words: *Pseudomonas aeruginosa* BN-1, chaperonin, mustard oil, organic solvent, phenyl methyl sulfonyl fluoride, sodium deoxycholate, Swiss Prot Accession # P 30718.

INTRODUCTION

Lipases belong to the class hydrolase (EC 3.1.1.1). It hydrolyzes acylglycerides into free fatty acids and glycerol (Jaeger and Eggert, 2002) and also catalyzes a variety of organic transformations which are chemo-, regio-enantio selective beside esterification under reduced aqueous conditions (Rubin and Denis, 1997; Kazlauskas and Bornscheuer, 1998). Due to such multifaceted properties, it is one of the important industrial enzymes that has found usage in a wide array of industrial applications, such as food, detergent, and chemical industry and also in biomedical sciences (Pandey et al., 1999).

Lipases are serine hydrolases with catalytic triad composed of Ser-Asp/Glu-His. It usually contains consensus sequence (Gly-x- Ser-x-Gly) that is present around the active site serine. The three-dimensional structures reveal the presences of a characteristic α/β-hydrolase fold (Nardini and Dijkstra, 1999). Lipases are widely distributed in the earth's flora and fauna but are found more abundantly in microbial flora comprising bacteria, fungi and yeast (Pandey et al., 1999). For commercial bulk production, bacterial sources are used, as the enzyme is extracellular and easy to handle (Gupta et al., 2004). Lipase producing bacterial sources is enormous but only few wild or recombinant strains have been used for commercial production of lipases. The

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Abbreviations: CFS, Cell free supernatant; **BSA,** bovine serum albumin; **SDS-PAGE,** sodium dodecyl sulfate polyacrylamide gel electrophoresis; **DTT,** 1,4-dithio-DL-threitol; **EDTA,** ethylenediaminetetraacetic acid; **DMSO,** dimethyl sulfoxide; **DEAE, diethylaminoethyl.**

lipases of *Pseudomonas* are widely used for variety of biotechnological applications (Beisson et al., 2000). It has been reported that lipase from *Pseudomonas aeruginosa* retained activity in the presence of organic solvents and also has temperature and pH stability (Karadzic et al., 2006).

MATERIALS AND METHODS

Organism

Extracellular lipase producing *P. aeruginosa* BN-1 was isolated from the well-water samples of Karachi using the method of Noman et al. (2004).

Production of lipase

P. aeruginosa BN-1 was grown in mustard oil medium (1% tryptone, 0.05% $CaCl_2$, 0.05% $MgSO_4$, 0.001% K_2HPO_4, 1.0 % mustard oil) at 37°C for 48 h. The fermented broth was centrifuged (Sigma 3K30 centrifuge using 12150 rotor) at 30000 x g for 15 minutes at 4°C to remove cells. A clear cell-free supernatant was obtained containing crude lipase.

Purification of lipase

Concentration of crude enzyme through ultrafiltration

Clear cell free supernatant (CFS) was concentrated by ultrafiltration under nitrogen (Amicon 8400 stirred ultrafiltration cell) using cutoff membrane of 30 kD. Retentate was lyhpolized and stored at –80°C till further analyses. Retentate was reconstituted in 55 mM Tris-HCl buffer of pH 8.0 before use.

Size exclusion chromatography by Sephadex G-100

Retentate (2.0 ml) was applied to 2.5 x 30.0 cm sephadex G-100 column pre equilibrated 55 mM Tris-HCl buffer of pH 8.0. Elution was carried out with same buffer with a flow rate of 60 ml/h and 2.0 ml fractions were collected. Fractions having absorbance at 280 nm were assayed for lipase activity and the ones showing enzyme activities were pooled and lyhpolized.

Ion exchange chromatography by Diethylaminoethyl (DEAE) A-50

Active fractions were re-suspended in 20 mM Tris-HCl buffer of pH 8.0 and applied to pre 1.5 x 20.0 cm column of DEAE pre equilibrated with same buffer. Elution of proteins was done using a linear gradient of NaCl from 0.0-1.0 M in 20 mM Tris HCl buffer (pH 8.0). The fractions (1.0 ml) were collected and monitored at 280 nm. Protein fractions were assayed for lipase activity and those with enzyme activity were pooled, subjected to centrifugal filtration (Centricon using 10 kD cutoff) to remove salt and lyhpolized.

Protein estimation

Protein was estimated by the method described by Lowry et al. (1951) using bovine serum albumin (BSA) as standard.

Electrophoresis and molecular mass determination

Molecular weight of lipase was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) carried out according to the method described by Laemmli (1970).

Native electrophoresis and activity stain

Native PAGE gels for separation of non denatured proteins were carried out according to Hames & Rickwood (1981). The gels were subjected to lipase or esterase activity according to Takahashi et al. (1998).

Lipase assays

Colorimetric assay

Colorimetric assay was based on the hydrolysis of 4-nitrophenyl palmitate as described by Ruiz et al. (2004). In the reaction mixture, Tris-HCl buffer (55 mM pH 8.0) was used instead of phosphate buffer. One lipase unit “U” is defined as µmol of 4–nitrophenol released per minute under assay conditions.

Titrimetric assay

Titrimetric assay was carried out as described by (Watnabe et al., 1977). One Unit of lipase activity is defined as µmol of fatty acids released per minute under assay conditions.

Characterization of enzyme

Optimum temperature and thermal stability

Optimum temperature assays were performed by the titremetric assay at temperatures ranging from 4-70°C. Thermostability of the enzyme was determined by preparing enzyme solutions in Tris-HCl buffer (55 mM pH 8.0) and incubated for 1 h at temperatures ranging from 4-70°C. After incubation, enzyme activity was assayed by colorimetric method at the optimum temperature.

Optimum pH and pH stability

Optimum pH assays were performed by titremetric assay replacing Tris-HCl buffer in the reaction mixture by the following buffers (final concentration: 55 mM): citrate phosphate buffer (pH 4.0-7.0), phosphate buffer (pH 6.0-8.0), Tris–HCl buffer (pH 7.5-9.0) and glycine–NaOH buffer (pH 9.0-10.0). The enzyme activity was assayed at optimum temperature. For pH stability assay, enzyme solutions were prepared in the above mentioned buffers and incubated for 1 h.

Substrate range

The substrate range of the enzyme was determined by using 4-nitrophenyl derivatives (200 µM) with carbon chain length of 2-18: 4-nitrophenyl acetate (C2), 4-nitrophenyl butyrate (C4), 4-nitrophenyl valerate (C5), 4-nitrophenyl caproate (C6), 4-nitrophenyl caprylate (C8), 4-nitrophenyl caprate (C10), 4-nitrophenyl laurate (C12), 4-nitrophenyl palmitate (C16) and 4-nitrophenyl stearate (C18).

Activity against natural triglycerides

Mustard, castor, linseed, corn, soybean, sunflower, peanut, palm and coconut oil (1% in emulsion) were used as substrates to determine the lipolytic behavior of the enzyme with olive oil (1% in emulsion) taken as the control.

Effect of metal ions

Purified enzyme was mixed with solutions of metal salts (5 mM) and incubated for 30 min and assayed for residual activity.

Effect of surfactants, detergents & protein modifying agents

Solutions of surfactants, detergents and protein modifying agents were mixed with the enzyme, preincubated for 30 min and then assayed for residual activity.

Solvent stability

Stability of purified lipase in organic solvent was carried out according to Ogino et al. (2000). The enzyme activity at 0 h was taken as control (100%).

RESULTS AND DISCUSSION

Crude lipase obtained was first subjected to ultrafiltration followed by size exclusion chromatography on G-100 and 20.69 fold purification was achieved (Table 1). Similar strategy was adopted by Gaur et al. (2008) with 8.6 fold purification for lipase from *P. aeruginosa* PseA. Additional step of purification by ion exchange chromatography using DEAE A-50 increased it to 42.99 fold (Table 1). Using sephadex G-100 and DEAE–A50 chromatography simultaneously, Lee and Rhee (1993) reported 5.3 fold purification of lipase from *Pseudomonas putida* 3SK. The molecular weight of lipase on SDS-PAGE was found to be approximately 60.0 kD. A single non smearing band was observed on native PAGE confirmed by activity staining (Figure 1). It has been reported that the molecular weights of lipases from *P. aeruginosa* vary considerably. This was similar to the lipases isolated from *P. aeruginosa* S5 (Rehman et al., 2005) and *P. aeruginosa* Pse A (Gaur et al., 2008). Zymography in SDS- PAGE was not visible. This may be due to the inhibitory effect of SDS on enzyme under experimental conditions. A single band was observed in native electrophoresis, suggesting that enzyme did not form aggregation which is in contrast to the findings of Lessuise et al. (1993).

The optimum activity of the purified lipase was observed at 37°C (Figure 2a) and 70% of its activity was retained at 50°C and 30% at 60°C for at least 1 h (Figure 2b). The purified lipase had better thermostability at 60°C, compared with lipase from *P. aeruginosa* MB 5001 lipase which has been reported to retain only 10% of its activity (Chartrain et al., 1993). The purified lipase showed maximum activity at pH 8.0 (Figure 3). The stability of enzyme varies in different buffer compositions. In citrate buffer (pH 4.0-7.0), the stability of enzyme increased with rise in pH and 30% of residual activity was noted at pH 6.5. In phosphate buffer (pH 7.0-8.0), stability of the enzyme increased and at pH 7.5, residual activity was 70%. Best enzyme stability was observed with Tris-HCL buffer (pH 7.5-9.0) where 98% of its activity was retained at pH 8.0, 85% at pH 9.0 and 70% at pH 9.5. In glycine-NaOH buffer, enzyme stability declined to 50% at pH 9.0, while activity was completely lost at pH 10.0 (Figure 4).

The lipase from *P. aeruginosa* BN-1 showed alkliophilic character as it retained more than 70% of its activity for 1 h 37°C at pH 9.5. However, it is lower when compared to that reported for *Pseudomonas* sp. PK-12 CS lipase (Jinwal et al., 2003). Broad lipolytic activity was observed against natural triacylglycerols of plants sources (Table 2). Mustard oil was the best of all substrates as the relative lipolytic activity was 2.75 fold higher than the activity in the presence of olive oil as substrate taken as the control. It was reported that relative lipolytic activity of lipase from *Pseudomonas* sp was 3.75 fold in the presence of mustard oil as substrate (Rathi et al., 2000). The relative lipolytic activity was higher in the presence of soybean, castor, corn and sunflower oil, while in the presence of palm, peanut and coconut oil, the relative activity was similar to that of the control. Relative activity of the lipase from *Pseudomonas pseudoalcaligenes* F-111 was reported to be lower in the presence of sunflower oil as substrate (Lin et al., 1996), while coconut oil has been reported to be the best substrate for lipase from *P. aeruginosa* LST-03 (Ogino et al., 2000). The purified lipase from *P. aeruginosa* S5 has been reported to have highest relative activity against palm oil (Rahman et al., 2005). The difference in the relative activity may be due to the difference in the type of fatty acyl chains present in the glycerides.

It had been proposed that lipolytic activity has dependence on appropriate surface tension and viscosity (Gargouri et al., 1989), which ultimately affect micelle formation. Kordel et al. (1991) attributed low lipolytic activity against short chain glycerides to low micelle formation due to low hydrophobicity and viscosity. As the acyl chain length increases, there is an increase in the hydrophobicity and viscosity that results in an increase in the hydrolysis of glycerides. The purified lipase hydrolyzes 4-nitrophenyl esters of various acyl chains with preference for higher fatty acyl chains (Figure 5). The lipase from *Burkholderia* sp. HY-10 has been reported to have broad substrate range from C4 to C18 4-nitrophenyl esters, with maximum hydrolytic activity against 4-nitrophenyl caproate (C6) (Park et al., 2007).

Ca^{2+} and Ba^{2+} increased the lipolytic activity while in the presence of Na^{+}, K^{+} and Mg^{2+} metal ions, the activity was similar to that of the control. Fe^{3+}, Al^{3+}, Co^{2+}, Hg^{2+}, Mn^{2+} and Ni^{2+} on the other hand, markedly decreased the enzyme activity (Figure 6). Ca^{2+} ions have been known to

Table 1. Purification of lipase from *Pseudomonas aeruginosa* BN-1.

Parameter	Volume (ml)	Total protein (mg)	Total Lipase Activith	Specific activity (U/mg)	Purification fold
Cell free supernatant	500	1200	5310	4.43	1
Ultrafiltration	50	272.72	4731	17.35	3.92
Sephadex G 100	10	35	3209	91.69	20.69
DEAE Sephadex A50	10	13	2476	190.46	42.99

Table 2. Relative enzymatic activity towards various substrate.

Substrate	Relative activity (%)
Olive oil (Taken as Reference)	100±2.1
Mustard oil	275±2.4
Soybean oil	139±2.0
Castor oil	136±2.0
Corn oil	110±2.0
Sunflower oil	105±2.1
Linseed oil	100±2.0
Peanut oil	98±1.9
Palm oil	98±1.1
Coconut oil	95±1.7
Groundnut oil	94±1.5

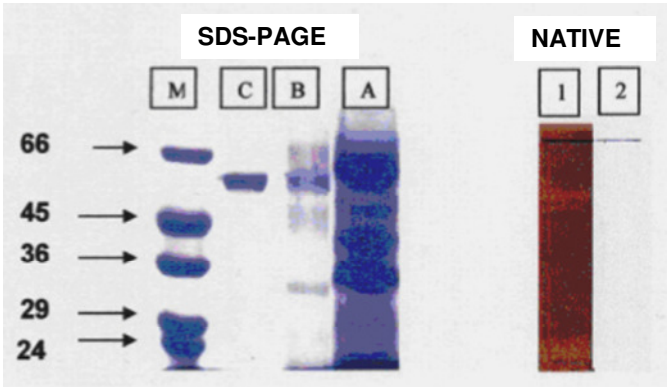

Figure 1. SDS-PAGE/native electrophoresis and zymography of lipase from *Pseudomonas aeruginosa* BN-1. A, Concentrated cell free filtrate after ultrafiltration; B, fraction from size exclusion chromatography using sephadex G-100; C, fraction from ion exchange chromatography using DEAE Sephadex A-50; M, molecular weight marker; 1 , zymography; 2, purified lipase.

stimulate the lipase activity in varying concentrations. It has been reported that in the presence of Ca^{2+}, lipase activity from *Bacillus licheniformis* strain H1 increased up to 120% (Khyami-Horani, 1996) while the activity of lipase from a *Pseudomonas* sp has been reported to be increased by 250% (Dong et al., 1999). Ba^{2+} are also known to enhance lipase activity of lipase isolated from *Burkholderia* sp (Rathi et al., 2001). Metal ions like Hg^{2+}, Zn^{2+} and Cu^{2+} have been reported to have inhibitory effect on *Pseudomonas* lipases (Iizumi et al., 1990; Kumura et al., 1993). *Pseudomonas* sp lipase has also been reported to be inhibited in the presence of Al^{3+}, Mn^{2+}, Ni^{2+} and Fe^{3+} (Dong et al., 1999).

Na-deoxycholate and Tween 80 increased the lipolytic activity as the presence of surfactants enhanced the micelle formation of hydrophobic substrate, facilitating more access to the enzyme enhancing the lipolytic activity. The enzyme activity in the presence of 1,4-dithio-DL-threitol (DTT) is similar to that of the control (Figure 7). *Burkholderia cepacia* ATCC 25416 lipase remained unaffected in the presence of DTT alone but marked inhibition was observed in conjunction with SDS (Wang et al., 2009). No effect on enzyme activity was observed in the presence of 2-mercaptoethanol. This can be explained as lipases contain very few sulfhydryl groups, important for lipase activity (Gupta et al., 2004). No effect of 2-mercaptoethanol were observed in case of lipases from *Chrysanthemum viscosum* (Sugiura et al., 1974) and *Staphylococcus aureus* 226 (Muraoka et al., 1982). It was also noted that lipase was not

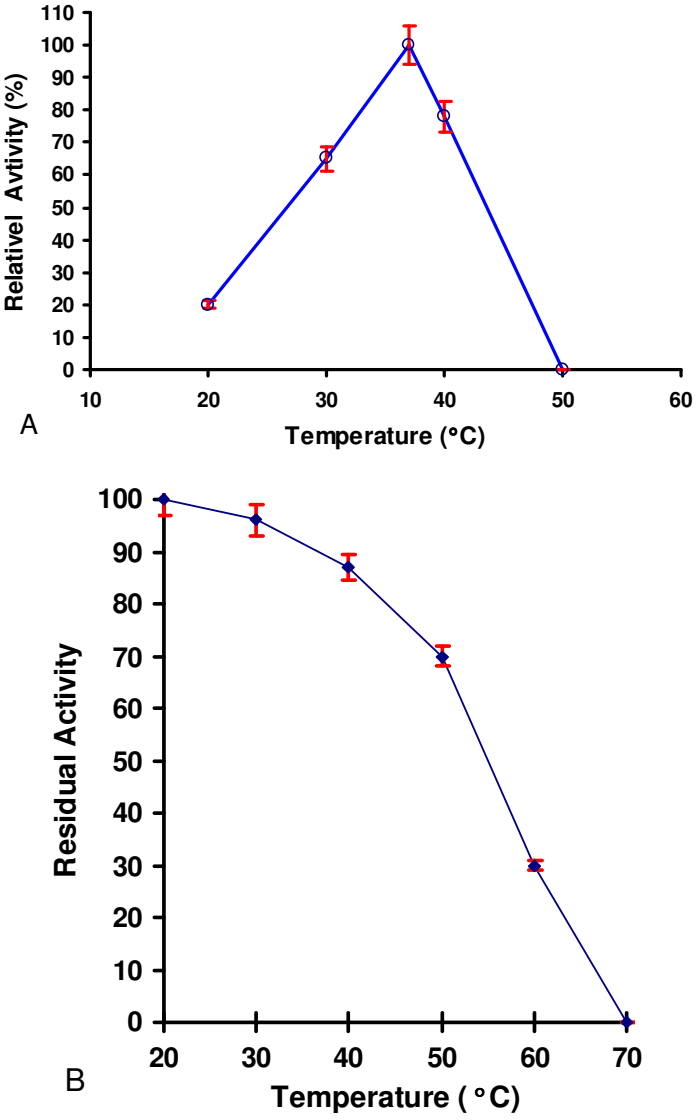

Figure 2. A. Optimum temperature and **B.** thermostability of lipase.

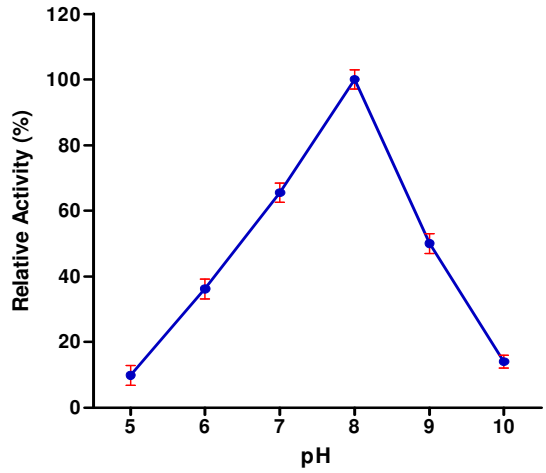

Figure 3. Effect of pH on purified lipase activity.

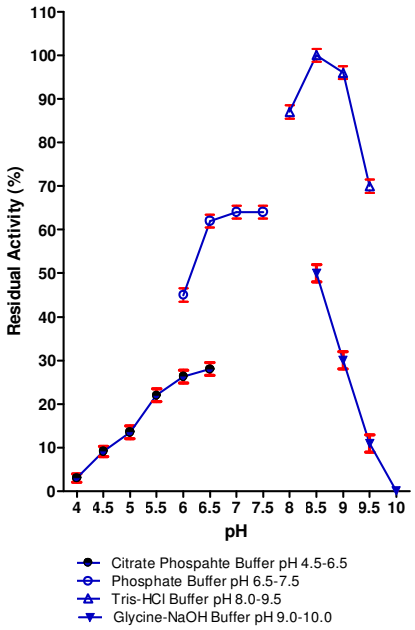

Figure 4. Stability of purified lipase at various pH.

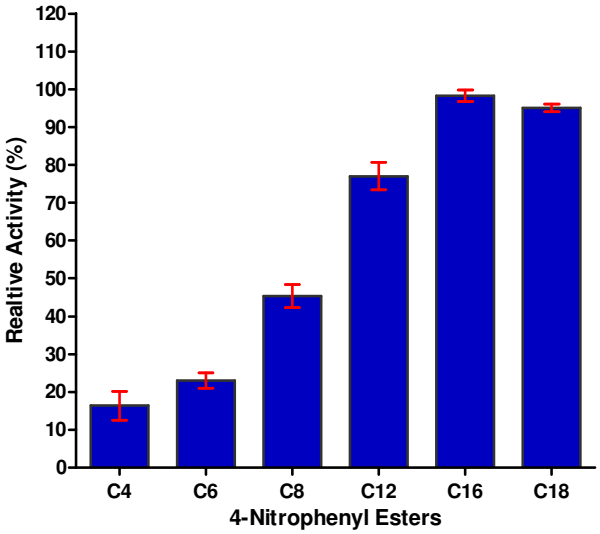

Figure 5. Hydrolytic activity of purified lipase against various 4-nitrophenyl esters. C_4, 4-Nitrophenyl butyrate; C_6, 4-nitrophenyl caproate; C_8, 4-nitrophenyl caprylate; C_{12}, 4-nitrophenyl laurate; C_{16}, 4-nitrophenyl palmitate; C_{18}, 4-nitrophenyl stearate.

sensitive to ethylenediaminetetraacetic acid (EDTA). This character is contrary to the lipase from *Pseudomonas* sp KB 700A (Rashid et al., 2001).

Purified lipase was markedly inhibited by phenylmethylsulfonyl fluoride (PMSF) because lipase belongs to the class of serine hydrolases. Extracellular lipase from *Bacillus subtilis* 168 has been reported to be strongly inhibited with 0.1 mM PMSF when preincubated only 10 min at 20°C (Lesuisse et al., 1993). SDS, an anionic detergent, strongly inhibited the enzyme. The complete loss of activity was observed in the case of *Burkholderia* sp. HY-10 lipase in the presence of SDS

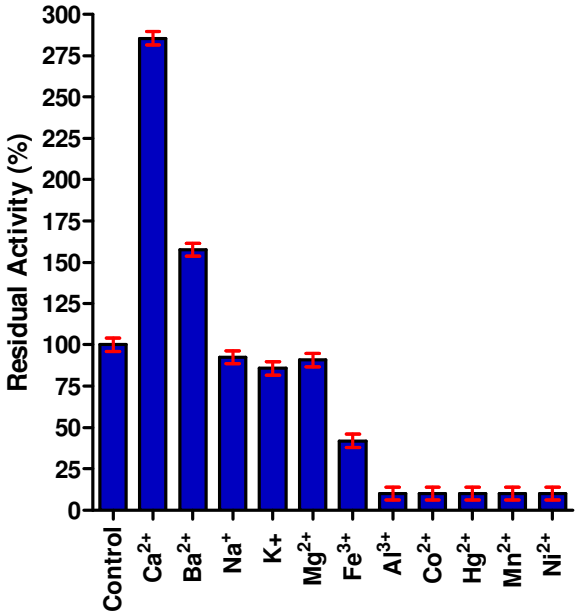

Figure 6. Effect of metal/non-metal ions on purified lipase activity.

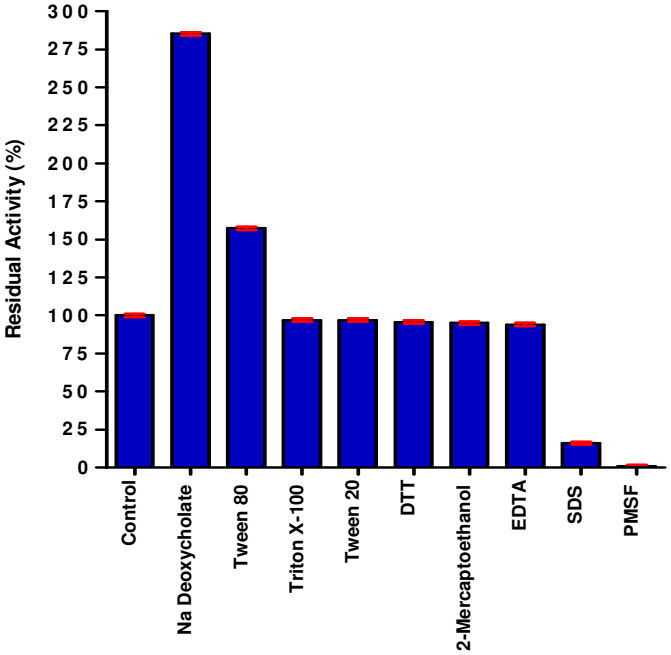

Figure 7. Effect of various detergents/surfactants/chelating and denaturating agent on purified lipase.

(Park et al., 2007). Extracellular lipase (Lip 2) from *Yarrowia lipolytica* also completely lost the activity in the presence of SDS, but activity was enhanced in the presence of 0.1% Tween 80 (Yu et al., 2007).

Lipase from *P. aeruginosa* BN 1 retained more than 90% of activity at 37°C for 24 h in dimethyl sulfoxide (DMSO), *n*-octane, *n*-decane, *n*-heptane, *n*-hexane. Residual activity of 80% or greater was recorded in cyclohexane, ethanol and methanol. In the presence of butanol, enzyme retained more than 60% of activity, while 50 and 40% of activity was retained in the presence of isopropanol and acetone, respectively. Very low

Table 3. N-terminal amino acid sequence of purified lipase.

Parameter	N-Terminal Sequence											Reference
P. aeruginosa BN-1		G	V	Y	D	Y	**K**	N	F	G	D	Kojima and Shimizu, 2003
P. flourescens HU 380	M	G	I	F	D	Y	**K**	N	L	G	T	Tan and Miller, 1992
P. flourescens B 52	M	G	V	F	D	Y	**K**	N	L	G	T	Chung *et al* , 1991
P. flourescens SIK WI		**A**	D	T	Y	A	A	T	R	Y	P	Batenberg *et al* ,1991
P. glumae		**A**	D	T	Y	A	A	T	R	Y	P	Park *et al,* 2007
Burkholderia sp HY-10		**A**	A	G	Y	A	A	T	R	Y	P	Jorgensen *et al*, 1991
P. cepacia		G	V	Y	D	Y	**K**	N	F	G	D	Kojima and Shimizu, 2003

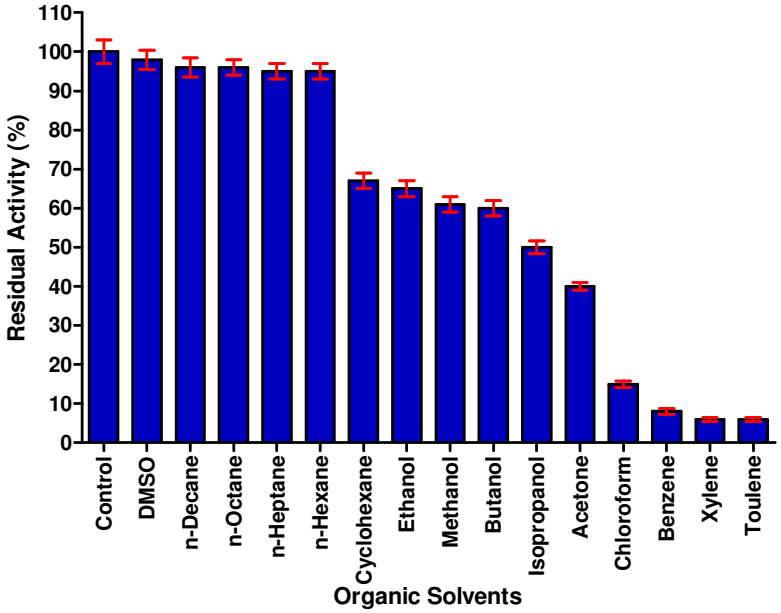

Figure 8. Stability of purified lipase in organic solvent.

tolerance was observed towards chloroform, benzene, toluene and xylene (Figure 8). Lipases showed variable tolerance towards organic solvents. Lipase from *P. aeruginosa* PseA (Gaur et al., 2008) and *P. aeruginosa* LST-03 (Ogino et al., 2000) showed similar relative stability in the presence of DMSO, *n*- hexane, *n*-decane and *n*-octane. Klibanov (1986) explained the effect of organic solvents with variable hydrophobicity on the enzyme. Polar solvents remove the essential water molecules from active sites of enzyme required for conformational flexibility, that cause denaturation.

N-terminal sequence for first 10 residues of the purified lipase revealed the presence of hydrophobic, positively and negatively charged amino acid (Table 3). No homology was observed when the sequence obtained was matched with known sequences of lipases (Table 3). However, it was similar to the N-terminal amino acids of a chaperonin. There are reports that lipase and chaperone are coded together and it is essential for proper lipase activity in some organisms (Pauwels et al., 2006; Sullivan et al., 1999). *Pseudomonas* sp lipases need a chaperone whose gene is located down stream of the lipase gene for efficient secretion and folding of active lipase (Hobson et al., 1993). This suggests that lipase from *P. aeruginosa* BN-1 may also contain a chaperone at its N-terminal for proper folding, secretion and lipolytic activity.

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