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Screening and characterization of a novel solvent-activated esterase from *Lysinibacillus sphaericus*

Saminathan Muthusamy*, Muthukumaresan Kuppusamy Thirumalai, Deepa Arul Priya J, Divakar K and Gautam Pennathur*

Center for Biotechnology, Anna University, Chennai 600025, India
*Corresponding author: *samynathan@gmail.com/pgautam@annauniv.edu*

Abstract

We isolated a strain of bacteria from soil that can grow in the presence of water-miscible organic solvents, which can secrete a solvent-activated esterase. The isolated bacterium was identified as *Lysinibacillus sphaericus* based on morphological, physiological and phylogenetic analysis. The solvent-activated esterase hydrolyzes only short chain 4-nitrophenyl esters and hence it was identified as esterase. The purified enzyme showed high level of stability in water-miscible organic solvents such as methanol, ethanol and acetonitrile. The activity of the enzyme was enhanced 3-fold after incubating the enzyme with 80% acetonitrile and is also stable over a period of time in water-miscible organic solvents. The secreted enzyme undergoes temperature and time dependent aggregation. Ca^{2+} and Mg^{2+} ions enhanced and EDTA and PMSF inhibited the enzyme activity. The optimum pH and temperature for its activity was 8.0 and 40^{o}C respectively. The isolated strain's similarity to extremophiles could be the reason for stability in organic solvents. Stability and activation in the presence of water-miscible organic solvent makes it an attractive enzyme and provides advantage for stereospecific ester bond hydrolysis and synthesis.

Keywords: Solvent activated esterase; *Lysinibacillus sphaericus*, Enzyme aggregation.

1. Introduction

Esterases (E.C.3.1.1.1, carboxyl ester hydrolases) are enzymes that hydrolyze carboxylate esters and are widespread in animals, plants and microbes. Carboxyl esterases are defined as enzymes that catalyze the hydrolysis of acylglycerols with short acyl chains (<10 carbon atoms), while lipases catalyze the hydrolysis of acylglycerols with long acyl chains (≥10 carbon atoms) (Jaeger et al. 1999). These enzymes exhibit activity in organic solvents and find use in organic synthesis and industrial processes (Bornscheuer 2002). Esterases have been successfully used in transesterification reactions, the resolution of racemic mixtures and enantio- and regio-selective hydrolysis (Sebastian Torres 2005).

Over the last twenty years, biocatalysis in organic solvents has emerged as an area of systematic research and industrial development (Sebastian Torres 2004). The conditions for catalytic activity can be altered using enzymes in organic solvents. In organic solvents enzymes can catalyze the reactions that are not possible in water, become more stable and exhibit new properties. The most important aspect is the discovery that enzymatic selectivity, including substrate, stereo-, regio- and chemoselectivity, can be influenced in organic solvents. Synthesis of kilogram quantities of an azole antifungal agent has been reported, in a synthetic scheme where a pivotal stereoselective step is the acetylation of a symmetrical diol catalyzed by a yeast lipase in acetonitrile (Klibanov 2001). It has been reported that low-water environments can be used to stabilize enzyme conformations that exhibit novel catalytic properties (Sebastian Torres 2004). Replacement of some water molecules around the

enzyme by solvent molecules stabilizes the structure of enzyme making several hydrolytic enzymes stable in organic solvents (Ogino and Ishikawa 2001).

However, a high enzyme deactivation rate in organic solvents has been a major limitation (Sebastian Torres 2005). To overcome such a problem, novel esterases from extremophiles (Hotta et al. 2002) and meta-genome derived esterases (Rhee et al. 2005) have been explored.

Solvent-tolerant bacterial species that secretes organic solvent-stable lipolytic enzymes have been reported and their application in industrial processes has been described (Chin 2003; Li et al. 2013; Li et al. 2014; Ogino et al. 1994). A solvent tolerant *Bacillus licheniformis* esterase is stable and active in water-miscible organic solvents (Torres et al. 2009). Presence of an enantioselective esterase in the culture supernatant of *Bacillus sphaericus* was reported earlier but not studied in detail (Jackson 1995). Many novel esterase's and lipases have been characterized and described recently has shown industrial potential. In this context, ours is the first study of an organic solvent-activated esterase from *Lysinibacillus sphaericus*. Here, we report the isolation and identification of a novel solvent tolerant bacterium, purification and characterization of its solvent-activated enzyme. The secreted esterase shows similarities to archeal hydrolases in terms of solvent stability and sensitivity towards PMSF, a serine hydrolase inhibitor.

2. Materials and Methods

2.1. Materials

All reagents used in this work were of analytical or microbiological grade from Hi-Media (Mumbai, India), SRL (Mumbai, India), Sigma (St. Louis, Mo, USA), PALL Life Sciences (USA) and Merck (Darmstad, Germany).

2.2. Isolation and identification of organic solvent tolerant strain

Esterase producing organisms were isolated and screened from soil samples. Serial dilution was performed and many pure cultures were isolated and screened on tributyrin agar plates. Colonies that gave the maximum zone of clearance were selected for solvent tolerance experiments. One strain, which was able to tolerate high levels of water-miscible organic solvent, was selected for further studies.

The isolated strain was identified according to the method described in "Bergey's Manual of Determinative Bacteriology (Holt and Krieg 1994) and also by 16S rRNA sequence analysis (Genbank accession number: AM493669). 16S rRNA sequences of twelve different *Bacillus* species obtained from NCBI database were compared. Sequences were analyzed using neighbor-joining method (Thompson et al. 1994).

2.3. Growth in the presence of water-miscible organic solvents

Microorganisms were grown with shaking at 30^{o}C in nutrient broth (w/v): peptone 0.5%, yeast extract 0.2%, beef extract 0.1%, NaCl 0.5%. Pre-culture was carried out in the absence of organic solvent, and 1% (v/v) of the pre-culture was inoculated into the fresh medium. When organic solvent was added to the medium, the cultivation vessel was plugged with a rubber stopper to prevent evaporation of the organic solvent. However, when organic solvent was not added, the culture flask was plugged with cotton. Nutrient broth without organic solvent was taken as control. Up to 4 % (v/v) of the solvents were tested. Growth of the microorganism as compared to control was measured. Relative growth indicates the tolerance of the organism to that particular solvent.

2.4. Effect of temperature on growth and enzyme production

Effect of temperature on bacterial growth and enzyme production studied in nutrient broth media without organic solvent for 40 h on a temperature controlled rotary shaker at 30^{o}C and 37^{o}C at 160 rpm. Esterase activity was determined at 4-h intervals. Growth rate was determined in both the temperature.

2.5. Culture conditions

Two different media (solid and liquid) were used for the growth of *L. sphaericus*. Nutrient broth agar was used as solid medium for maintaining the culture at 4^{o}C for a week. For long term storage the strain was stored at -80^{o}C after the addition of 6 volumes of glycerol to 4 volumes of a 10-h grown culture. The culture media was inoculated with 1% (v/v) of an exponentially growing pre-culture prepared in the same medium. The strain was grown in 250 ml shake flasks containing 50 ml of nutrient broth at 30^{o}C for 16 h on a refrigerated rotary shaker at 160 rpm. Growth monitored by measuring optical density at 600 nm (OD600).

2.5.1. Purification of the enzyme

The culture supernatant was collected after removing the cells in the culture by centrifugation at 12,000 g for 15 min at 4°C. Extracellular proteins in the supernatant were precipitated with 60% ammonium sulphate and the precipitated protein was pelleted at 15000 g for 20 min at 4°C. The pellet was dialyzed against 10 mM Tris-Cl (pH 8.0) for 24 h at 4°C. Dialyzed crude enzyme was used for further purification. All the buffers used were chilled at 4°C before use. A10 ml column was packed with fresh 2-3 ml of anion exchange support (DEAE Ceramic HyperD; PALL Life Sciences, USA) and the sorbent slurry was equilibrated with 25 mM Tris-Cl (pH 7.5) and then the column was equilibrated with three column volumes of 25 mM Tris-Cl (pH 7.5). The protein was loaded into the column at a flow rate of 15 ml/hour. The flow-through was loaded back to the column twice. The column was washed with five column volumes of the same buffer to remove unbound proteins. Proteins bound to the anion exchange gel was eluted by increasing the concentration of NaCl in 25 mM Tris-Cl buffer (pH 7.5) stepwise from 50 mM to 500 mM concentration of NaCl was used. The bound esterase was eluted with 100 mM NaCl. The enzyme solution was dialyzed overnight against 25 mM Tris-Cl (pH 7.5).The dialyzed protein was lyophilized and stored at -20°C.

2.6. Determination of protein concentrations

Protein concentrations in this study were determined by the method of Bradford (Bradford 1976) using bovine serum albumin as standard.

2.7. Enzyme assays

Esterase activity against 4-nitrophenyl esters was determined by measuring the amount of p-nitrophenol released by esterase-catalyzed hydrolysis. The production of p-nitrophenol was monitored at 400 nm in a spectrophotometer (HITACHI U-3210). Unless mentioned, in the standard assay, esterase activity was measured with 1 mM p-nitrophenyl valerate as a substrate in 50 mM Tris-Cl (pH 7.2) containing 2% acetonitrile at 37°C. The reaction mixture contained 10 µl of 50 mM p-nitrophenyl valerate (dissolved in pure acetonitrile), 25 µl of 1M Tris-Cl (pH 7.2), and 25 µl of enzyme solution and 440 µl of water. In every measurement the effect of non -enzymatic hydrolysis of substrates was taken into consideration and subtracted from the value obtained after the addition of enzyme. The extinction coefficients of p-nitrophenol were determined prior to the measurement under every condition. One unit of activity was defined as the amount of enzyme that liberated 1 µmol of 4-nitrophenol per min under the given assay conditions.

2.8. Electrophoresis and Zymography

Native polyacrylamide gel electrophoresis was performed using the discontinuous gel system of Davis (Davis 1964). The stacking and separating gels were prepared with 4% (w/v) and 10% (w/v) acrylamide respectively. Gel was loaded in duplicate with 100 µg of ammonium sulphate precipitated protein. The gel was run at 20°C and 60 V. The gel was run for a period of about 4 h until the dye front reached the end of the gel.

One half of the gel was used for activity staining while the other half was stained for locating the corresponding enzyme bands. For activity staining the gel was first equilibrated in 25 mM Tris-Cl (pH 7.5), for 30 min. Subsequently, the gel was sandwiched between substrate-agar emulsions for the detection of esterase activity. The substrate-agar emulsion was prepared using 1.3% (w/v) agar, 1% (v/v) tributyrin, 25 mM Tris-Cl (pH 7.5), 10 mM CaCl2 and 0.1% (v/v) Triton X-100. The plate was incubated at room temperature until a clear zone was observed (Saminathan et al. 2008).

2.9. Effect of pH and temperature

The effect of pH on esterase activity was studied in a pH range of 3.0 to 9.0. The buffers used were 50 mM sodium citrate (pH 3.0 to 4.0), 50 mM sodium acetate (pH 4.0 to 5.5), 50 mM sodium phosphate buffer (pH 6.0), 50 mM Tris-Cl (pH 7.0 to 8.0) and 50 mM Bicine (pH 8.0 to 9.0). Production of 4-nitrophenolate and 4-nitrophenol was monitored at 348 nm, the pH independent isosbestic wavelength of 4-nitrophenolate and 4-nitrophenol (Hotta et al. 2002).

Thermostability was measured by incubating the purified esterase (200 µg/ml) in 20 mM phosphate buffer (pH 7.2) at 20 to 80°C range for various intervals of time. At each time interval, aliquots were taken from these mixtures and added as the enzyme sample in the standard assay, in order to measure the residual activities. For determining the optimal temperature; standard assay mixture was incubated at different temperatures.

2.10. Effect of metal ions, inhibitors and substrate specificity

To determine the effect of the salts $CaCl_2$, $ZnCl_2$, $MgCl_2$, and KCl and of EDTA as well as phenylmethylsulfonylfluoride (PMSF) on enzyme activity, different concentrations of up to 10 mM were added directly to the standard p-NPV assay mixture. The substrate specificity and chain length selectivity were determined spectrophotometrically using a variety of *4*-NP esters.

2.11. Effect of organic solvents on the stability of esterase

Stability in organic solvents was measured by incubating the purified esterase (100 µg/ml) in 50 mM Tris-Cl (pH 7.2) containing 0, 50, or 80% organic solvent (vol/vol) at 37^{o}C for 60 min. Aliquots were taken from these mixtures and added as the enzyme sample in the standard assay, in order to measure the residual activities. Enzyme incubated with buffer alone was used as control to compare the effect of solvents on enzyme activity. The final concentration of the solvent in the assay solution is very negligible and does not affect the extinction coefficient.

2.12. Statistical Analysis

Determinations were made in triplicate and results shown are the average of three independent experiments. Data are represented as mean ± standard deviation. Analysis of variance (ANOVA) was performed on data sets using a significance of p values lower than 0.05.

3. Results

3.1. Strain characterization

The strain grew in the presence of 4% acetonitrile, methanol and ethanol. The optimum temperature for growth was 37^{o}C (range 16-45^{o}C) and the optimum pH was 7.0-8.0 (range pH 5.5-10.5). It hydrolyzed only tributyrin and not olive oil (long chain fatty acid ester), which showed that the enzyme was an esterase. Some properties of the isolate are listed in Table 1.

Table1. Phenotypic properties of the isolated strain *L. sphaericus*

Characteristic	Results
Morphological	
Shape	Rods
Gram stain	+
Spore position	Terminal
Motile	+
Biochemical	
Denitrification	+
Citrate utilization	+
Catalase	+
Oxidase	-
Hydrolysis of:	
Tributyrin	+
Triolein	-
Gelatin	+
Starch	-

Comparison of the 16S rRNA gene sequence with the sequences from other bacteria revealed that very high similarity (98%) with *Lysinibacillus sphaericus*, therefore the isolated strain was established as *Lysinibacillus sphaericus*. It was recently proposed that *B. fusiformis* and *B. sphaericus* be reclassified into the genus of Bacillus, with the renaming of *B. sphaericus* as *Lysinibacillus sphaericus* [20]. The rooted phylogenetic tree (Fig. 1) based on the 16S rRNA sequence revealed the isolated strain's similarity to extremophiles such as *Bacillus alcalophilus, Bacillus halodurans, Bacillus thermaleovorans, Bacillus methanolicus* and *Bacillus sporothermodurans*. It also exhibits close similarity with *B. sphaericus* DSM 396, *B. sphaericus C3-41*, *B. sphaericus* 205y *and Bacillus* sp strain NRRL B-14905.

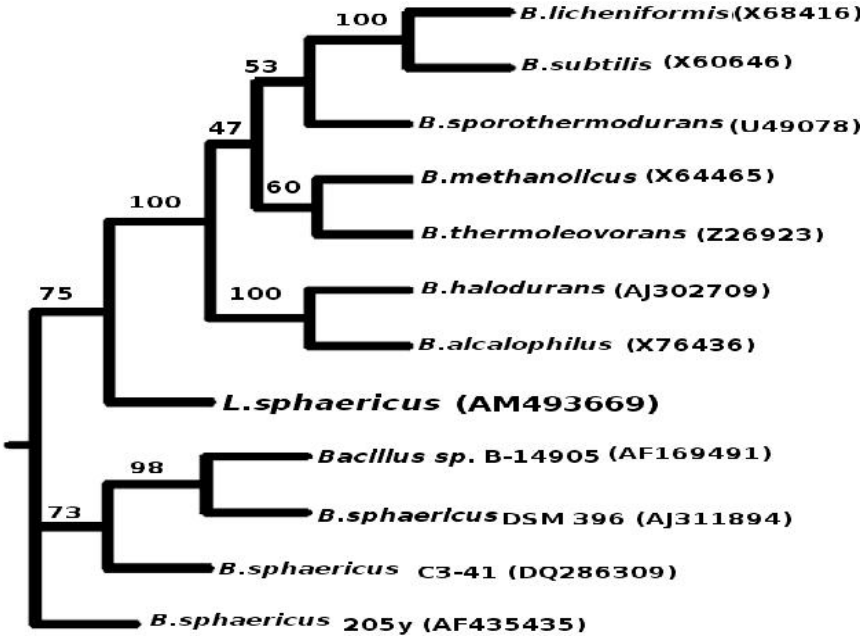

Fig.1. Rooted phylogenetic tree based on 16S rRNA sequence, showing the relationship of *Lysinibacillus sphaericus* to other *Bacillus* species. Values shown in the parentheses are GeneBank accession number.

3.2. Effect of water-miscible organic solvents on bacterial growth

Lysinibacillus sphaericus can grow up to 4% (v/v) of methanol, ethanol and acetonitrile at 30°C. It showed tolerance to methanol, ethanol and acetonitrile, as revealed by 70% growth even at the concentration of 4% (v/v). Butanol was toxic to the cells at the concentration of 2% (v/v) since only 20% of the growth was observed and at 4% (v/v) concentration there was no growth (Fig. 2).

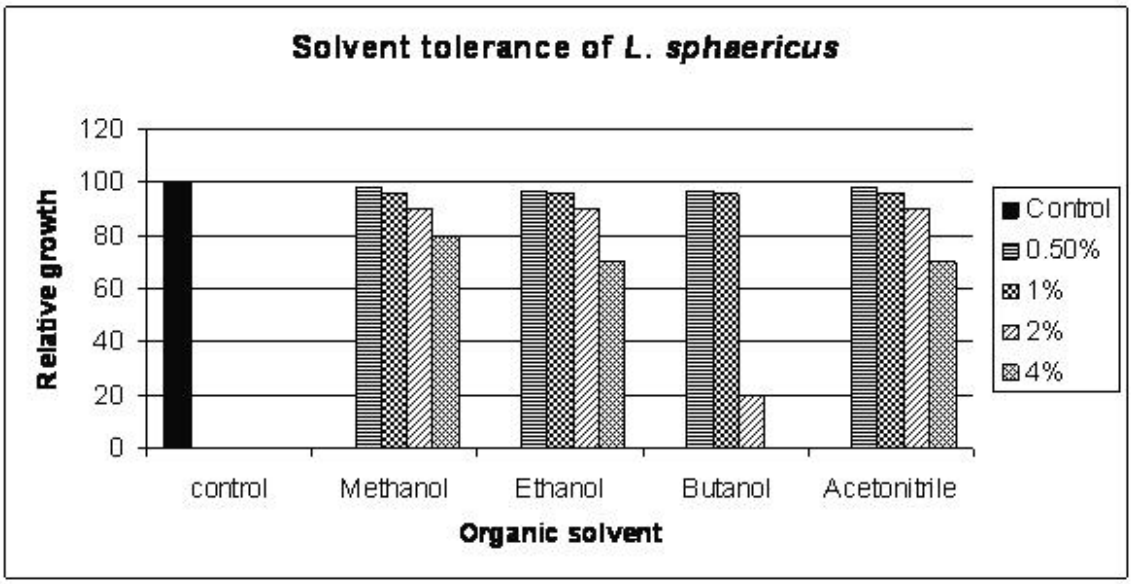

Fig. 2. *L.sphaericus* growth in the presence of organic solvents.

3.3. Time course and temperature studies on bacterial growth and esterase production

A time and temperature course study was conducted in order to determine the growth and esterase production (Fig. 3) characteristics of *L. sphaericus*. Different parameters were determined for optimal esterase secretion. At 37°C maximum growth was observed after 8 h, but the maximal enzyme secretion was at 16 h. The cell reaches the stationary phase within 8 h but maximal esterase was secreted into the media only after the cell reaches the stationary phase. On native

polyacrylamide gel electrophoresis the secreted enzyme failed to enter into separating gel and aggregated (Fig. 4a). At 30°C maximum growth was observed after 10 h and the maximal enzyme secretion was at 18 h. The amount of secreted esterase was low at 30°C as compared to 37°C. Up to 16 h, the esterase secreted at 30°C did not aggregate and readily entered into the separating gel (Fig. 4b). Even at 30°C, after prolonged incubation, the enzyme aggregated and failed to enter the gel.

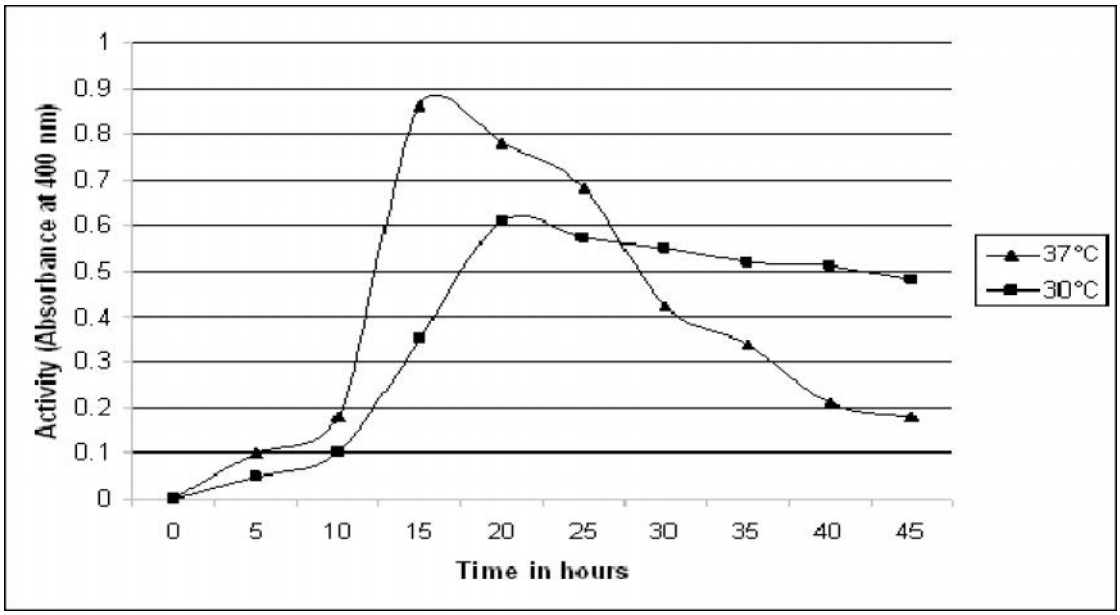

Fig. 3. *L. sphaericus* esterase secretion at 30 and 37°C. Esterase activity against p-nitrophenyl valerate was determined by measuring the amount of p-nitrophenol released at 400 nm. Culture was sampled at different time points

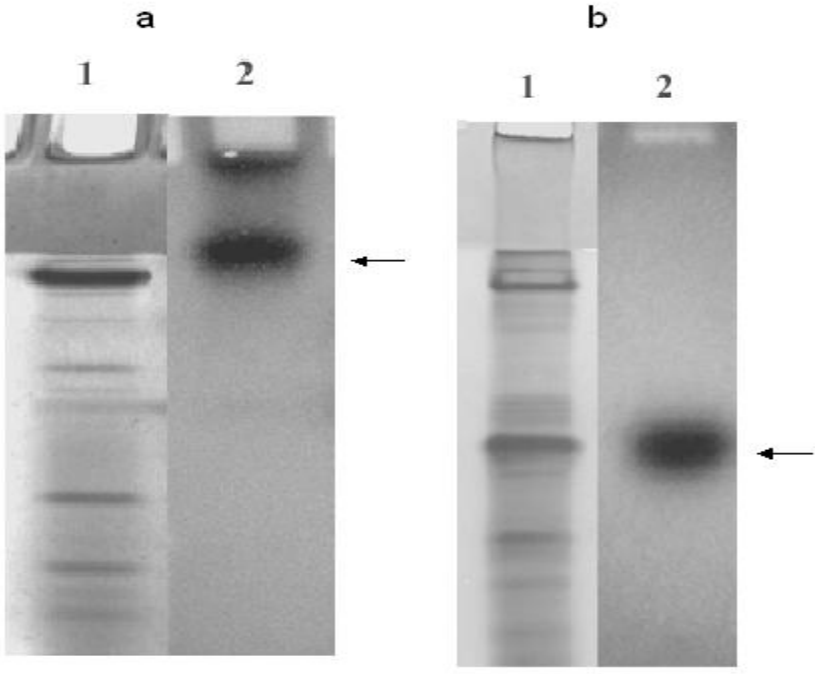

Fig. 4. Activity staining. (a) Native PAGE enzyme aggregation observed in 37°C. (b) Native PAGE non-aggregation of esterase in 30°C. Lanes: (1) Coomassie stained extracellular *L. sphaericus* protein sample, (2) Activity stained extracellular *L. sphaericus* protein sample. Arrows indicate the hydrolysis of tributyrin by *L. sphaericus* esterase.

3.4. Effect of pH and temperature on esterase activity

To determine the optimal pH for the esterase, we measured the activity of the crude *L. sphaericus* esterase protein at various pH values (pH 3.0 to 9.5), using 4-nitrophenyl valerate as a substrate, at 37°C. The amount of released 4-nitrophenol for each reaction was measured at 348 nm, the isosbestic point of 4-nitrophenol and 4-nitrophenolate (Hotta et al. 2002). *L. sphaericus* esterase exhibited >85% maximal activity in the pH range of 6.5 to 8.5, with the highest activity at approximately pH8.0. The esterase showed good stability after 30 min at pH 7.0-9.0; however the activity was reduced drastically at acidic pH. The enzyme retained 90% and 75% activity in pH 8.0 and 7.0, respectively, and the enzyme lost 80% of activity in pH 6.0 (Fig. 5a).

Effect of temperature on esterase activity and stability was investigated using p-nitrophenyl valerate as substrate. The activity was measured in the range of 20 to 80°C. The enzyme showed an optimal temperature of 40°C (Fig. 5b). The enzyme lost all of the original activity at 55°C after 2 hours incubation (Fig. 5b). The enzyme retained 100% of its maximal activity at 20°C after 4 h incubation.

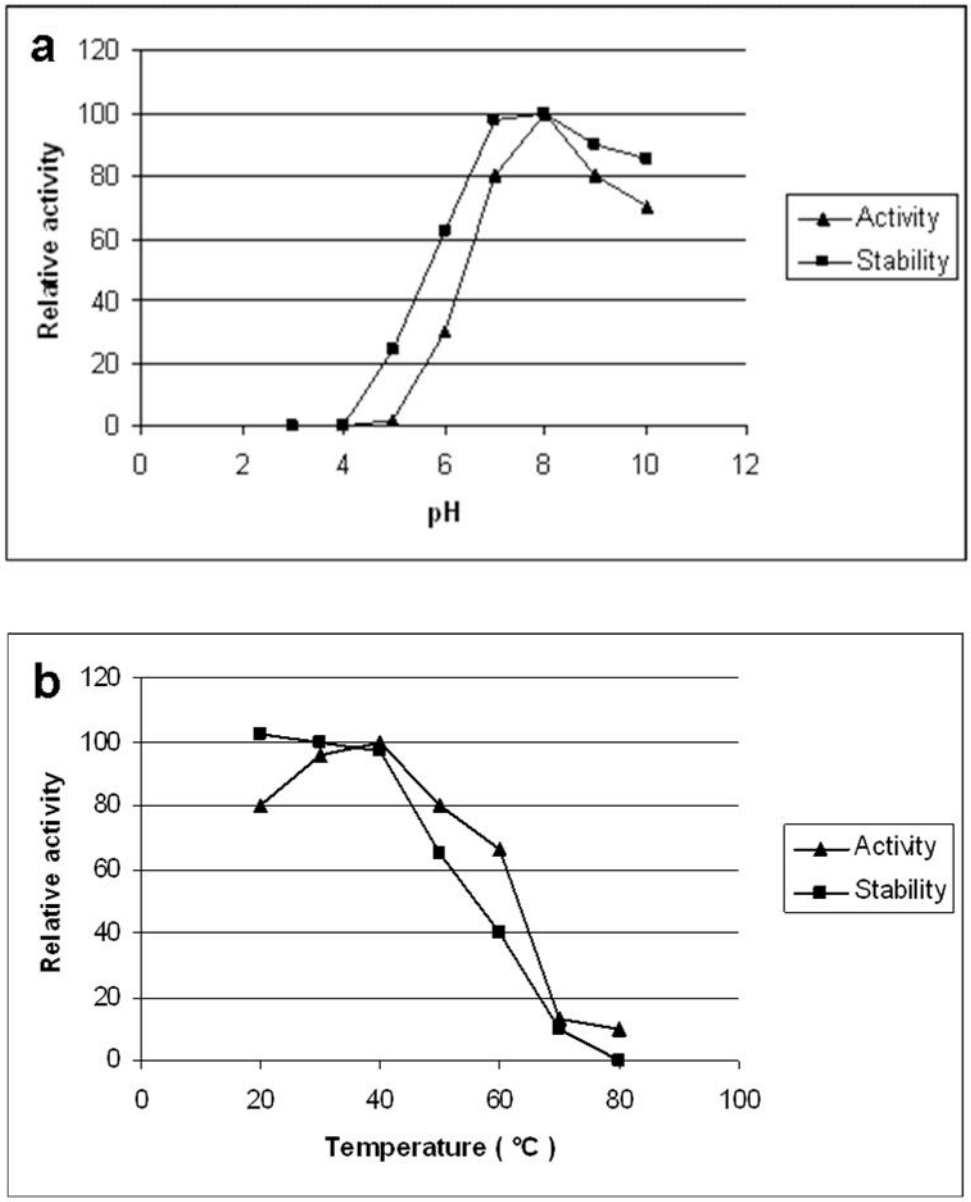

Fig. 5. (a) Effect of pH on esterase activity and stability. (b) Effect of temperature on esterase activity and stability.

3.5. Effect of metal ions, inhibitors and substrate specificity

Divalent metal ions such as $Ca^{2+,}$ Zn^{2+} and Mg^{2+} used for studies were found to activate the enzyme. On the contrary EDTA at 10 mM concentration inhibited the esterase activity. PMSF (Phenylmethyl sulfonylfluoride) completely inhibited the enzyme activity at the concentration of 1 mM (Table 2) and therefore the esterase was a serine-type enzyme with a metal ion co-factor. The enzyme showed maximum affinity for 4-nitrophenyl butyrate and 4-nitrophenyl valerate and less so for 4-nitrophenyl caproate and did not hydrolyze palmitate and stearate and therefore the enzyme was confirmed as an esterase (Table 3).

Table 2 Effect of various reagents on esterase activity

Reagent	Relative activity	
	1 mM	10 Mm
None	100	100
$CaCl_2$	210	300
$ZnCl_2$	190	270
$MgCl_2$	127	145
KCl	120	167
EDTA	10	05
PMSF	0	0

Table 3 Substrate specificity of *L. sphaericus* esterase

p-nitrophenyl substrate	Relative activity (%)
Acetate	96
Butyrate	137
Valerate	100
Caproate	13
Palmitate	0
Stearate	0

3.6. Effect of organic solvents on esterase activity

The stability of the purified (Fig. 6) *L. sphaericus* esterase in seven organic solvents with different log P values, defined as the logarithm of its partition coefficient in standard n-octane/water two-phase systems (Laane et al. 1987) was studied. Residual activity was increased considerably in methanol, ethanol and 2- propanol, while in acetonitrile it increased to 3.5-fold. n-hexane, 1-butanol and n-undecane slightly reduced the enzyme activity. Activity increased initially up to 20% concentration of water miscible organic solvents; however at 50% solvent concentration enzyme activity was reduced considerably when compared to control, because activity measured in terms of hydrolysis. As the solvent concentration increased hydrolysis was not favored (Table 4).

Table 4. Stability and activity in organic solvents and log P values of organic solvents used in this study.

Organic solvent	Log P	Residual activity after1 h incubation (%)		Activity in 50% solvent
		50 %	80 %	
None	-	100	100	100
Methanol	-0.764	127	120	19
Acetonitrile	-0.394	357	349	25
Ethanol	-0.235	129	121	10
2-Propanol	0.074	117	109	05
1-Butanol	0.8	90	87	10
n-Hexane	3.5	77	80	33
Undecane	6.1	93	86	35

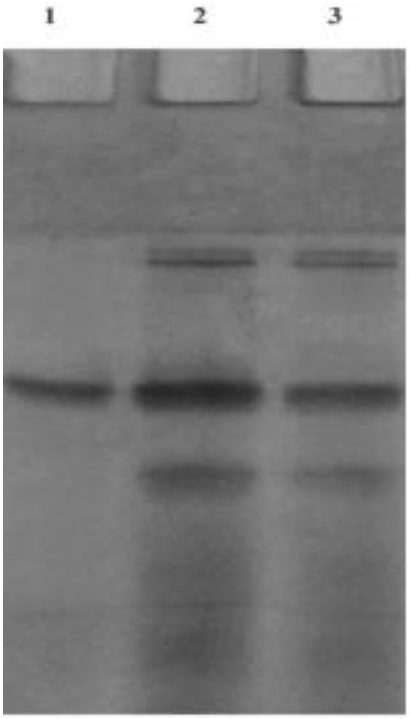

Fig. 6. Esterase purification. (a) Lanes: (1) 100 mM NaCl elution, (2) 200 mM NaCl elution, and (3) 300 mM NaCl elution.

4. Discussion

In this study we have isolated an organic solvent-tolerant novel *Lysinibacillus sphaericus* strain that produces acetonitrile-activated esterase and the secreted esterase was purified and biochemically characterized. Effects of non-polar organic solvents in Gram-negative bacteria have been reported in great detail (Sardessai and Bhosle 2004). However solvent tolerance studies with polar organic solvents like ethanol, methanol, and acetonitrile in Gram-positive organisms are limited. Reports have shown that organic solvent tolerant organisms are able to produce enzymes with high stability in the presence of organic solvents (Ogino et al. 1994). Strains of Gram-positive bacteria like *Rhodococcus*, *Arthrobacter* and *Bacillus* show tolerance to highly toxic organic solvents and most of them had originated from marine environment (Sardessai and Bhosle 2004). A strain of *Bacillus* (SB1) that tolerates butanol up to 2% has been reported. It has the lowest index value reported for any organic solvent tolerant bacterium (Sardessai and Bhosle 2004). However, we have isolated a novel strain *Lysinibacillus sphaericus* that can grow in the presence of 4% water miscible organic solvents that have much lower index value than butanol. This high level of tolerance of this bacterium to water-miscible organic solvents can be satisfactorily explained on the basis of its similarity to extremophiles.

Stability of few oligomeric enzymes (Mukhopadhyay et al. 2003) been increased by the process of multimerization of protomeric enzymes. The hydrophobic interaction at the subunit interface of the enzyme, interaction of hydrophobic surface amino acid residue of the enzyme and modification of surface loops of the enzyme (Mukhopadhyay et al. 2003) were all reported to improve stability of the enzyme. Aggregation was observed in lipases and esterases and in general, these enzymes are hydrophobic as a result, of which they tend to aggregate. *L. sphaericus* esterase undergoes temperature and time dependent aggregation. The aggregated esterase showed better stability. Amount of enzyme produced at 37^{o}C was more when compared to 30^{o}C. The enzyme may undergo aggregation when the concentration of the secreted esterase exceeds the threshold concentration or under conditions of physico-chemical stress. Aggregation may be the oligomerization of the monomeric enzyme or it could be protein-protein interaction as well. It is interesting to note that protein-protein interaction of extracellular enzymes was found to improve the rigidity as well as catalytic activity of the participating enzymes (Mukhopadhyay et al. 2003).

Stability of the enzyme vis-à-vis polarity of solvent does give varied results as reported in the literature. Solvents with high log P values (hydrophobic solvents) have been observed to cause less inactivation of biocatalysts than solvents with lower log P values (Laane et al. 1987). Interestingly, some enzymes produced by extremophiles have remarkable properties: a protease from ThermusstrainRt4A2 is extremely stable in 90% of acetone, methanol, ethanol and propan-2-ol (Freeman et al. 1993), whereas an esterase from the hyperthermophilic archaeon *Pyrobaculum calidifontis*VA1is stable for 1 hour in 80% of DMSO, DMF, acetonitrile, methanol, ethanol and propan-2-ol (Hotta et al. 2002).

Our data demonstrate that hydrophilic solvents like methanol, propanol, and ethanol did not inactivate the *L. sphaericus* esterase. Interestingly residual activity of the enzyme was increased 3.5-fold after incubating with 80% acetonitrile promises industrial potential. Its stability in acetonitrile is better than that of *Pyrobaculum calidifontis* VA1 esterase, whereas lipases from *Pseudomonas* species B11-1 (Choo et al. 1998), *Fusarium heterosporum* (Shimada 1993) and *Bacillus sphaericus* 205Y (Chin 2003) were drastically inactivated after incubation with acetonitrile. This is in a different perspective to the present work where we found the *L. sphaericus* esterase did not denature during incubation with organic solvent.

5. Conclusion

We isolated a new organic solvent tolerant *L. sphaericus* strain that is capable of growing in the presence of water-miscible organic solvents. To our knowledge, this is the first report of a mesophilic bacterium, which is tolerant to high concentrations of methanol, ethanol and acetonitrile isolated till date. We also provide novel evidence that *L. sphaericus* has some special features similar to bacteria that can grow in extreme environments. Taken together our data suggest that kinship to extremophiles as well as marine bacteria could be the reason for stability of the *L. sphaericus* esterase in the presence of water miscible organic solvents. Stability in organic solvent is important when using enzymes for synthesis of esters. The present enzyme was not only stable but also got activated by acetonitrile. Therefore it can be of potential use for reactions in media containing organic solvents, making it attractive for application in industry.

Acknowledgments

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