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*Full Length Research Paper*

# Thermostable, alkaline tolerant lipase from *Bacillus licheniformis* using peanut oil cake as a substrate

**N. Annamalai*, S. Elayaraja, S. Vijayalakshmi and T. Balasubramanian**

Marine Sciences, CAS in Marine Biology, Annamalai University, Parangipettai 608502, Tamil Nadu, India.

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**Thermostable alkaline tolerant lipase producing *Bacillus licheniformis* isolated from marine environment. Peanut oil cake was used as substrate for enzyme production and purified to 3.6 fold with a specific activity of 148.4 U/mg of protein with molecular weight of 35 kDa. The enzyme was 100% stable at 60°C, it retains more than 80% activity after 1 h incubation at 70°C and pH 9-11.5. Metals like $Ca^{2+}$, $Ba^{2+}$ and $Mg^{2+}$ enhanced whereas $Ni^{2+}$ and $Fe^{3+}$ inhibited the enzyme activity. No effect on enzyme activity found when treated with Tween 80 and sodium deoxycholate, but completely inhibited with SDS.**

**Key words:** *Bacillus licheniformis,* lipase, peanut oil cake, enzyme activity, thermostable, metals.

## INTRODUCTION

Lipases (EC 3.1.1.1) and esterases are act on the hydroxyl ester bonds present in triglycerols, liberating fatty acids and glycerols. Esterases act on water soluble substrates with a preference for short fatty acid chains but true lipases work at water-lipid interface and their major substrates are long chain triglycerols (Jaeger et al., 1994). Much attention is paid to lipases of microbial origin, such as *Candida* sp., *Pseudomonas* sp., *Bacillus* sp., and *Rhizopus* sp. for their unsurpassed roles in biotechnology. They function as biocatalyst, capable of catalyzing hydrolytic ester cleavage, transesterification, alcoholysis, acidolysis, and aminolysis and are thus widely used in the food, detergent, chemical and biomedical industries (Pandey et al., 1999).

Lipases have also been used as biocatalyst for the synthesis of chiral compounds, which offer tremendous potential in production of compounds of pharmaceutical interest. Since the last decade, lipases have been perceived by research scientists as one of the most important classes of industrial enzymes (Arbige and Pitcher, 1989) for instance lipases have been used extensively in the dairy industry, for household detergents (Falch, 1991), in the oleochemical industry (McNeil et al., 1991) and to produce structured triacylglycerols (Macrae, 1983).

*Corresponding author. E-mail: annabact@gmail.com.

A relatively smaller number of bacterial lipases have been well studied compared to plant and fungal lipases (Peterson and Drablos, 1994). In recent years there has been a great demand for thermostable enzymes in industrial fields. The importance of thermostable lipases for different applications has been growing rapidly (Shah et al., 2007; Chen et al., 2007). Thermostable enzymes from microbial sources are highly advantageous, for biotechnological applications, since they can be produced at low cost and exhibit improved stability (Handelsman and Shoham, 1994).

Ramakrishnan and Banerjee (1950) investigated that oil seed cakes for their lipolytic activity with a view to get a cheap and active lipase for fat hydrolysis. Among these, peanut oil cake is a cheaper as well as fat rich source. Hence the present study was carried out on production of thermostable lipase using peanut oil cake (*Arachis hypogea*) by alkalophilic thermophilic bacteria, purification and characterization such as effects of temperature, pH and various metal ions, detergents on enzyme activity and stability of lipase.

## MATERIALS AND METHODS

### Microorganism

Lipase producing bacteria was isolated from marine sediments of parangipettai coast (Lat; 11°46' Long; 79°46'), Tamilnadu, east coast of India using an enrichment procedure. The enriched culture

broth was serially diluted and spreaded on Tween 80 agar plates (pH-9). Inoculated plates were incubated at 55°C for 48 h. The isolated colonies were screened for lipase production on Tributyrine agar plates, colonies with highest clear zone on the plates were selected as potential lipase producing strain and identified through morphological, physiological and biochemical characteristics (Buchanan et al., 1974) and it was confirmed by 16S rDNA sequencing. The DNA was isolated by phenol chloroform method (Marmur, 1961). The primer sequences were chosen from the conserved regions previously reported for the bacterial 16S rDNA gene (Marchesi et al., 1998). Sequencing was done using forward primer (5'-CAGGCCTAACACATGCAAGTC-3') and reverse primer (5'-GGGCGGTGTGTACAAGGC-3'). PCR reactions were performed with the following program for the 16S rDNA gene: 30 cycles consisting of 95°C for 1 min, 55°C for 1 min and 72°C for 1.5 min, followed by a final extension step of 5 min at 72°C. The 16S rDNA sequence was analyzed by an automated DNA sequencer (Megabace, GE). The 16S rDNA gene sequence obtained was compared with other bacterial sequences by using NCBI BLAST for their pair wise identities. Multiple alignments of these sequences were carried out by ClustalX 2.0 version and the phylogenetic trees were constructed in MEGA (4.2 version) using neighbor joining (NJ) algorithms.

**Lipase production using peanut oil cake**

For the production of lipase, the bacterial cells were inoculated in 1000 ml erlenmeyer flasks containing 300 ml production media containing (g/L) soybean 20, peanut oil cake 20, soluble starch 10, $K_2HPO_4$ 5, $Na_2CO_3$ 10, NaCl 5 and pH of the medium was adjusted to 9.0 using 6 N NaOH (Sodium carbonate was sterilized separately and added to the medium) were incubated at 55°C on shaker incubator (125 rpm). The growth was measured at every 6 h intervals in terms of optical density at 660 nm (Systronics, Visiscan 167) and cell free supernatant was used as crude enzyme.

**Lipase assay**

Estimation of lipase activity was carried out by titrating the fatty acids liberated from olive oil with an alkali (Kunio et al., 1994). The liberated fatty acids were titrated with 1 mol/L NaOH solution. Simultaneously, a blank titration was also performed. One unit (U) was defined as the amount of lipase capable of releasing 1 $\mu$mol of titratable fatty acid per min under the assay conditions used.

**Production and partial purification of enzyme lipase**

The bacterial cells were removed by centrifugation at 3000 *xg* for 30 min at 4°C and enzyme from the cell free supernatant was precipitated by ammonium sulphate at 80% saturation at 4°C. The precipitate was collected by centrifugation at 3,000 *xg* for 30 min at 4°C and dissolved in 100 mM Tris-HCl buffer of pH 9.0. This aliquot was dialyzed against the same buffer overnight under refrigerated conditions. Dialyzed enzyme solution was loaded onto a DEAE-cellulose column (2.0 x 25 cm) equilibrated with 100 mM Tris-HCl buffer, pH-9.0. The enzyme was eluted with linear gradient of NaCl (0-1 M in 100 mM Tris-HCl buffer) at a flow rate of 25 ml/h. The eluted fractions were pooled together and assayed for enzyme activity. The resultant enzyme solution was loaded onto a Sephadex G-50 gel filtration column (2.5×120 cm), which had been equilibrated with 100 mM Tris-HCl buffer (pH 9.0), then eluted with the same buffer. The peaks exhibiting lipase activity were pooled together and used as a purified enzyme. This partially purified enzyme solution was used for investigating the effects of temperature, pH and various metal ions on enzyme activity.

Protein content in the crude and purified enzyme was determined according to the method of Lowry et al. (1951) using bovine serum albumin (Hi-Media chemical laboratories, India) as a standard.

**Molecular weight determination**

The molecular weight of the purified protein was estimated using SDS-PAGE (Laemmli, 1970). The electrophoresis was performed in Genei electrophoretic unit and stained with Coomassie Brilliant Blue R-250. Molecular weight was determined by comparing the mobility of standard molecular weight markers for 29, 43, 66, 97 and 205 kDa (Genei, Bangalore, India).

**Effect of Temperature, pH on enzyme activity and stability**

The effect of temperature on enzyme activity was tested by preincubating the reaction mixture (substrate + crude lipase solution and 1 ml 100 mM Tris HCl buffer, pH 9.0) at temperatures ranging from 30 to 90°C. To check the thermostability, activity of the enzyme incubated at different temperature for 1 h was checked at different time intervals (0 to 60 min). The effect of pH on the enzyme was determined by preincubating with four different buffers (100 mM): citric acid-$Na_2HPO_4$ (pH 4 to 6), phosphate buffer (pH 6 to 8), boric acid-NaOH buffer (pH 8 to 9.5) and phosphate-NaOH buffer (pH 9.5 to 12), hydroxide chloride buffer (12 to 13) for 2 h and the residual activity were determined by the standard assay.

**Effect of EDTA detergent and various metals on lipase activity**

Effect of detergents on lipase activity was analyzed by incubating enzyme for 1 h at 45°C in 100 mM Tris HCl buffer (pH 9.0) containing 0.1 or 1% (w/v) detergent. To determine the effects of various metal ions on the lipase activity, enzyme assay was performed for 1 h in the reaction mixture as described previously, with EDTA, SDS and different metal ions such as $MnSo_4$, $FeSo_4$, $CoCl_2$, $MgSO_4$, $CuSO_4$, $CoSO_4$, $MgCl_2$, $CaCl_2$, $FeCl_3$, $BaSO_4$, $HgCl_2$ and $NiSO_4$ at a final concentration of 1 mM.

## RESULTS

The thermostable alkaline lipase producing bacteria is a Gram positive, rod shaped, spore forming, motile, positive for citrate utilization, indole production, nitrate reduction, arginine, and acid production from glucose. Based on the morphological, physiological and biochemical characteristics, the isolate was tentatively identified as *Bacillus licheniformis* and it was confirmed by the 16 S rDNA sequencing (Gene bank accession number-HM370569). The phylogenetic tree was constructed and represented in Figure 1.

Lipase production by *Bacillus licheniformis* was highly correlated with the cell growth (Figure 2). Maximum lipase production (730 U/ml) was recorded in the stationary phase. The culture supernatant was used as a starting material for the purification of the lipase. A three-step procedure yielded a 3.6-fold enrichment of the protein with a 19% recovery. The summary of the purification depicted in Table 1 and molecular weight was estimated as 35 kDa (Figure 3).

The lipase from *Bacillus licheniformis* exhibited good

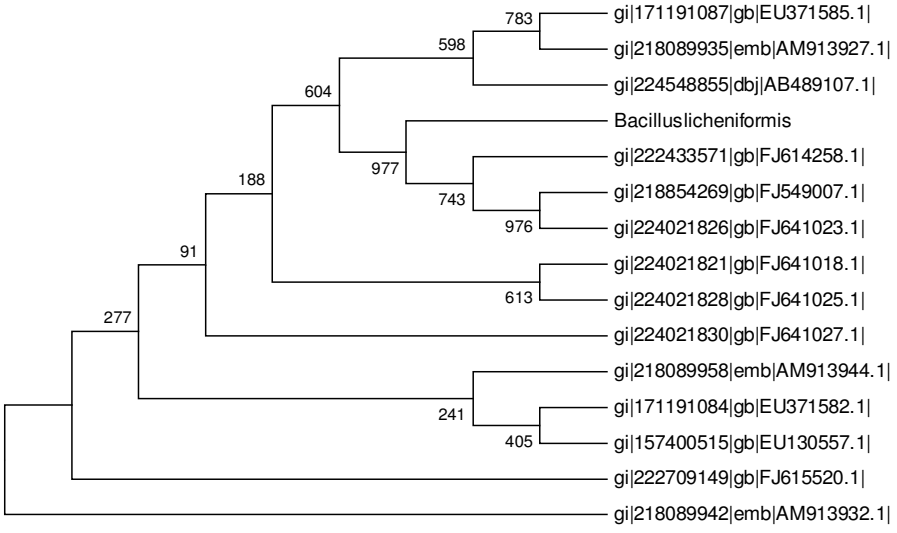


**Figure 1.** Phylogenetic tree showing the taxonomic position of the *Bacillus licheniformis.*

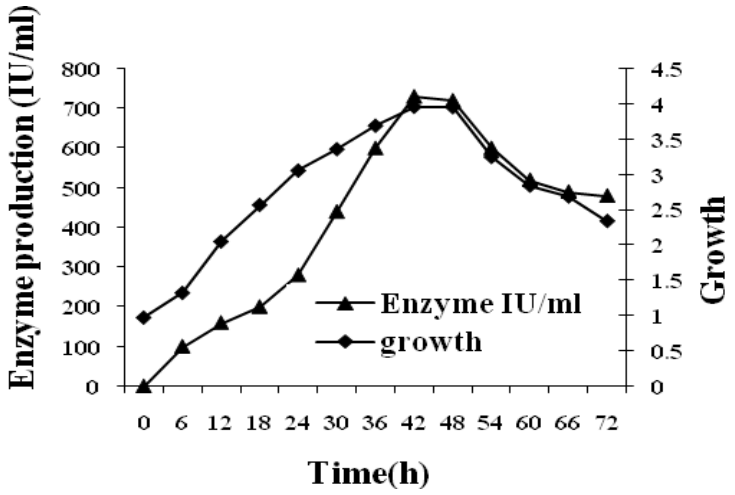


**Figure 2.** Kinetics of growth and lipase production.

**Table 1.** Summary of the purification of lipase from *Bacillus licheniformis*.

| Fraction (mg) | Total protein (U) | Total activity (U/mg) | Specific activity (Fold) | Purification (%) | Yield |
|---|---|---|---|---|---|
| Culture broth | 416 | 17000 | 40.8 | 1.0 | 100 |
| $(NH_4)_2SO_4$ Precipitate | 83 | 6238 | 75.1 | 1.8 | 36.6 |
| DEAE- Cellulose | 34 | 4250 | 125 | 3.06 | 25 |
| Gel filtration | 22 | 3265 | 148.4 | 3.6 | 19 |

enzyme activity between the temperature of 37 to 60°C and optimum being at 45°C (Figure 4). Regarding stability, enzyme was 100% stable after 1 h incubation at 60°C. It retained 82% original activity when heated at 70°C for 60 min and enzyme was 65% stable up to 30 min at 80°C (Figure 5).

The effect of pH on enzyme activity and stability were investigated by using different buffer solutions. The

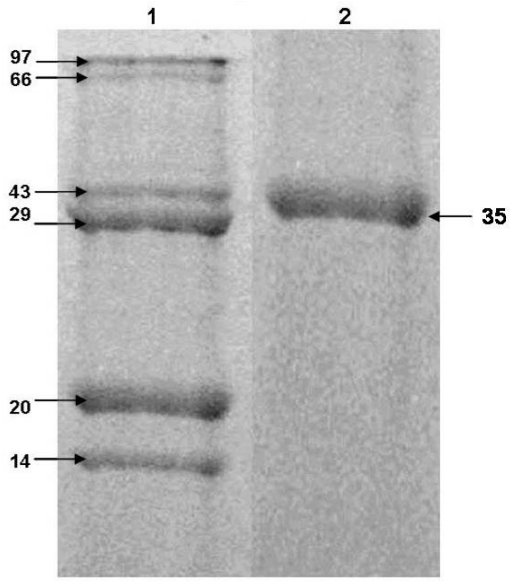


**Figure 3.** SDS-PAGE of lipase from *Bacillus licheniformis*. Lane. 1. Molecular markers, Lane 2. Purified protein.

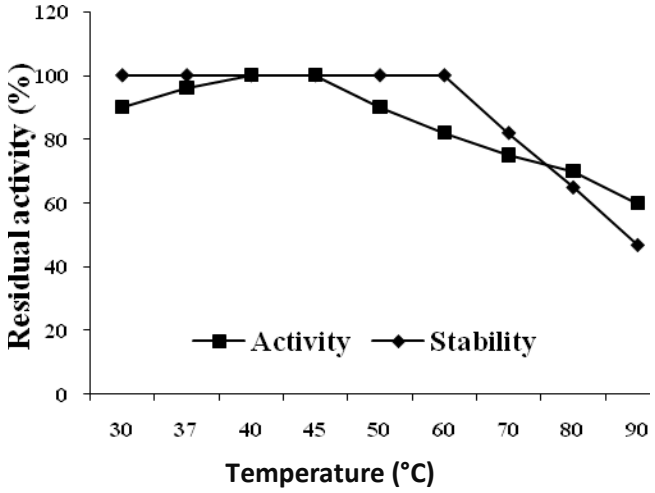


**Figure 4.** Effect of temperature on the activity and stability of the lipase enzyme. (Activity is expressed in enzyme units and stability is expressed in % of retained activity).

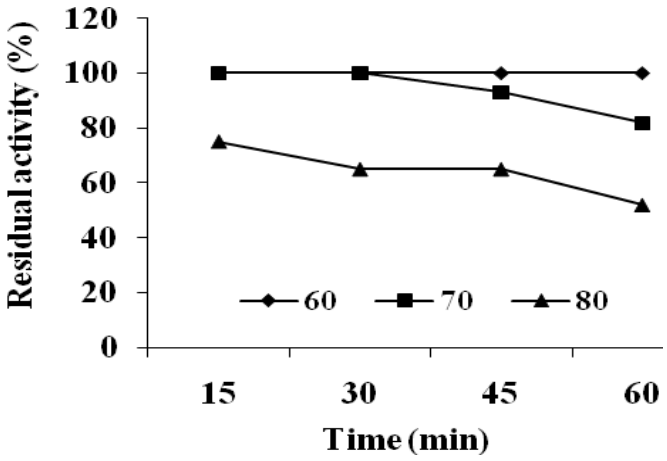


**Figure 5.** Stability of the lipase at different time in different temperature levels.

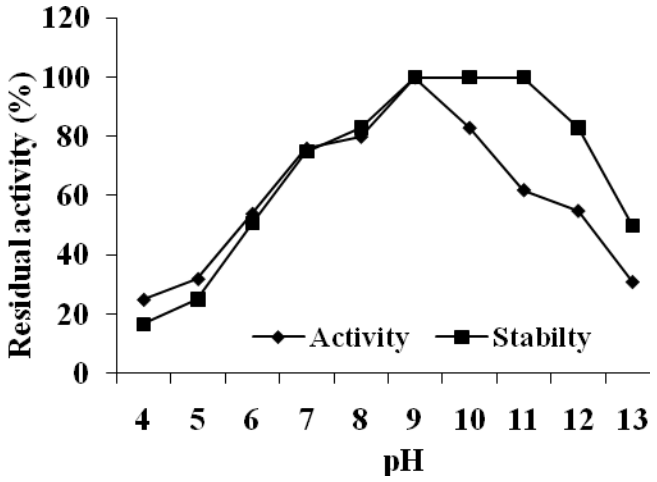


**Figure 6.** Effect of pH on the activity and stability of the lipase enzyme. (Activity is expressed in enzyme units and stability is expressed in % of retained activity.

enzyme was active between pH range of 6 (53%) and 11 (58%) optimum at pH 9.0 (Figure 6). The enzyme was most stable between at pH 9.0 to 12 (83%).

Among metal ions, $Ni^{2+}$, $Mn^{2+}$, $Hg^{2+}$, $Fe^{2+}$, $Fe^{3+}$ and $Co^{2+}$ were slightly inhibited enzyme activity whereas $Ca^{2+}$, $Mg^{2+}$ and $Ba^{2+}$ were enhanced the enzyme activity (Table 2) and no effect was found with $Cu^{2+}$. Only 25% inhibition was found at 1 mM concentration of SDS and EDTA, but completely inhibited at 10 mM. Tween 80 and sodium deoxycholate had no effect on enzyme activity at 10 mM and it exhibits 110% and 72 activity at 1mM concentration respectively (Table 3). Triton X 100 enhanced (126%) at 10 mM concentration.

## DISCUSSION

In the present study, thermostable lipase produced by *Bacillus licheniformis* using peanut oil cake as cheaper source, was purified and characterized. Deive et al. (2003) and Ota et al. (2000) were found trybutyrin induced the lipase production compared with other carbon sources by *Kluyvermyces marxianus* and *Geotrichum* sp. F0401B respectively. In large scale

**Table 2.** Effect of EDTA and metal ions on the enzyme activity.

| Metals | Residual activity (%) (5 mM concentration) |
|---|---|
| Control | 100 |
| $MnSO_4$ | 75 |
| $FeSO_4$ | 69 |
| $CoCl_2$ | 95 |
| $MgSO_4$ | 150 |
| $CuSO_4$ | 100 |
| $MgCl_2$ | 127 |
| $CaCl_2$ | 225 |
| $FeCl_3$ | 50 |
| $BaSO_4$ | 175 |
| EDTA | 75 |
| $HgCl_2$ | 83 |
| $NiSO_4$ | 43 |

**Table 3.** Effect of detergents on stability of the enzyme.

| Detergents | Residual activity (%) | |
|---|---|---|
| | 1 mM | 10 mM |
| Control | 100 | 100 |
| Triton X 100 | 80 | 126 |
| Tween 80 | 110 | 100 |
| SDS | 25 | 0 |
| Sodium deoxycholate | 72 | 100 |

production of lipase, the addition of other inorganic carbon sources can be costly and a burden in downstream processing (Seghal et al., 2008). Hence, peanut oil cake is the potential cheaper substrate lipase production.

As described for most extracellular lipases, maximum enzyme production appeared when bacterial cells reached the late logarithmic phase (Jaeger et al., 1994). Purification yielded 3.6-fold with a 19% recovery and molecular weight was estimated as 35 kDa. In general many of the *Bacillus* lipases have low molecular mass about 20 kDa (Sugihara et al., 1991).

The optimum temperature for enzyme activity was 45°C and it is comparatively high with lipase of *Rhodotorula glutinis* (Dimitris et al., 1992) and *Pseudomonas* sp. (Sugihara et al., 1991). Lipase from *Bacillus licheniformis* shown to have good thermostability, it retains 100% activity at 60°C and 65% activity even at 80°C. The thermostability of the lipase was somewhat higher to other reported lipases from *Bacillus* J 33 (Nawani et al., 1998) and *Pseudomonas fluorescens* NS2 W (Kulkarni and Gadre, 2002). Many lipases with moderate thermostability have been found in different strains of fungi *Penicillium wortmanii*, stable at 40°C (Costa and Peralta, 1999) and lipase from *R. glutinis* stable only at 35°C (Papaparaskevas et al., 1992).

The lipase from *Bacillus licheniformis* was 100% stable at pH 9 to 11.5 and 83% of activity retains even at pH 12.0. But the purified lipase from *S. grimisii* (Abdou, 2003), *Pseudomonas* sp. (Fox and Stepaniak, 1983) were stable only between the pH ranges of 8.0 to 9.0.

The activity of the enzyme was inhibited by metal ions such as $Mn^{2+}$, $Hg^{2+}$, $Fe^{2+}$, $Fe^{3+}$ and EDTA and enhanced by $Ca^{2+}$, $Mg^{2+}$ and $Ba^{2+}$. Several lipases had enhanced lipolytic activity in the presence of $Ca^{2+}$ and hence it is called as $Ca^{2+}$ dependent metalloenzyme. Similar results have been reported for *Bacillus* sp. Strain 42 (Eltaweel et al., 2005) and *Bjerkandera adusta* R59 lipase (Bancerz and Ginalska, 2007). In contrast, Triton X-100 inhibited activity of the lipase from *Mucor* sp. (Abbas et al., 2002) and *Rhizopus* sp. (Sroka, 1994).

In the present study, the lipase produced by *Bacillus licheniformis* isolated from mangrove sediments was cost effective because of cheaper source as peanut oil cake, stable up to 70°C and pH 12.0. Hence it can be useful for industrial application because many of the processes performed at temperatures around 50°C and alkaline pH.

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