Characterization of a Lipase Produced by *Bacillus* species Strain Lp5

P. Pallavi, S. Jeevan Chandra and S. Ram Reddy*

Department of Microbiology, Kakatiya University, Warangal - 506 009, India.

(Received: 22 October 2011; accepted: 08 December 2011)

In view of several biotechnological applications of microbial lipases, an attempt was made to characterize a lipase produced by *Bacillus* sp Lp5, a strain isolated from oil contaminated soil. Based on 16S rDNA analysis, it was identified as *Bacillus* strain YIVI. In electrophoretic separation many bands were detected. The molecular weight of this protein was calculated to be about 35KD. The partially purified lipase of LP5 exhibited activity in a wide range of pH (4-10). Maximal activity (100%) was observed at pH 8.0. The relative activity was found to be 10.02%, 78.49%, 81.25%, and 8.18% at pH 4.0, 6.0, 10.0 and 12.0 respectively. This lipase retained 86.90% and 73.16% of its activity at 40°C and 80°C respectively. The activity was found to be less at 20°C. The results revealed that a lipase activity of 124% and 109% was observed in presence of metal ions CaCl₂ and NaCl respectively. Inhibition of lipase by LP5 was detected with EDTA, ZnSO₄, HgCl₂ and CuSO₄. The Enzyme activity was 95.24%, 82.16%, 91.18%, and 62.15% with tributyrin, tween-20, tween-80, olive oil respectively. Inhibition of enzyme activity was observed when triton-x was employed as substrate.

**Key Words:** *Bacillus* species, Lipases, pH, Temperature, Metal ions.

Lipases or triacylglycerol acylester hydrolases are carbonyl esterases (E.C. 3.1.1.3) that catalyse both hydrolysis and synthesis of esters formed from glycerol. Lipases are produced by many microorganisms and higher eukaryotes. Microbial lipases have drawn much attention because of their potential use in biotechnology, mainly due to their availability and stability. Bacterial lipases are glycoproteins, but some extracellular bacterial lipases are lipoproteins. Most of the bacterial lipases reported so far are constitutive and non-specific in their substrate specificity and a few bacterial lipases are thermostable. Due to such attributes lipases are used in detergents, manufacture of food ingredients, pitch control in pulp and paper industry, production of aromas, production of insecticides, synthesis of drugs such as naxopren and ibuprofen and as biocatalysts of stereo selective transformations. Further more, novel biotechnological applications have been successfully established using lipases for the synthesis of biopolymers and bio-diesel, the production of enantiopure pharmaceuticals, agrochemicals, and flavor compounds. The chemoregio-and enantio-specific behavior of these enzymes has generated interest among scientists and industrialists. Many *Bacillus* sp. strains, which grew successfully under highly alkaline conditions, were found to be useful in leather processing. The alkaline thermophilic lipases find application in detergent industry. A variety of
pesticides (insecticides, herbicides, fungicides or their precursors) made with the applications of lipases are currently in use. The most important application of lipases has been in the organic synthesis of pesticides for the production of optically active compound 10.

Due to unlimited potentials of lipases in different areas, a continuous search is being conducted to discover new lipases that meet specific requirements of the industry. In the present investigations, an attempt was made to characterize a potential lipase produced by native Bacillus species.

MATERIAL AND METHODS

Samples collection
Isolation of lipolytic bacteria was made from three types of soil samples: i. normal soils, ii. oil contaminated soils and iii. stored oil samples. For the first two types soils, samples from a depth of 5cm were collected aseptically from different localities of Warangal district. The third type, oil samples were collected from oil mills, merchants, stores and households.

Isolation
Isolations of lipase producing bacteria were made by spread plate method using serial dilutions on nutrient agar medium supplemented with olive oil as substrate (Nutrient agar with 1% olive oil).

Screening
The primary screening of isolated bacterial strains was made by the method suggested by Limpon et al. 11 The isolated strains were screened for lipolytic activity and lipolytic potential (R/r), using tributyrin agar medium and spirit blue agar medium.

Lipase assay
Lipase activity in the culture filtrate was assayed by titrimetry 12. The reaction mixture included 2 ml of enzyme, 5ml of citrate phosphate buffer (pH 8.0), 2 ml of triacetin and was incubated at 37°C for 3hours. At the end of incubation, the reaction was terminated by adding 10ml of ethanol and the mixture was titrated against 0.05M NaOH.

Table 1. BLAST DATA: Alignment view using combination of NCBI GenBank and RDP databases

<table>
<thead>
<tr>
<th>Alignment View</th>
<th>ID</th>
<th>Alignment Result</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Consensus</td>
<td>0.96</td>
<td>Sample LP5 16S rDNA</td>
</tr>
<tr>
<td>GQ475480.1</td>
<td>1.00</td>
<td>Bacillus subtilis strain Y-IVI 16S ribosomal RNA gene</td>
<td></td>
</tr>
<tr>
<td>GQ421472.1</td>
<td>0.99</td>
<td>Bacillus subtilis strain L4 16S ribosomal RNA gene</td>
<td></td>
</tr>
<tr>
<td>GQ402892.1</td>
<td>1.00</td>
<td>Bacillus sp. G3(2009) 16S ribosomal RNA gene</td>
<td></td>
</tr>
<tr>
<td>GQ375227.1</td>
<td>0.99</td>
<td>Bacillus subtilis subsp. subtilis strain CICC 10076 16S ribosomal RNA gene</td>
<td></td>
</tr>
<tr>
<td>GQ199597.1</td>
<td>0.99</td>
<td>Bacillus subtilis strain I527 16S ribosomal RNA gene</td>
<td></td>
</tr>
<tr>
<td>GQ169813.1</td>
<td>1.00</td>
<td>Bacillus subtilis strain B107 16S ribosomal RNA gene</td>
<td></td>
</tr>
<tr>
<td>FJ641016.1</td>
<td>1.00</td>
<td>Bacillus subtilis strain IMAUB1036 16S ribosomal RNA gene</td>
<td></td>
</tr>
<tr>
<td>FJ641015.1</td>
<td>1.00</td>
<td>Bacillus subtilis strain IMAUB1035 16S ribosomal RNA gene</td>
<td></td>
</tr>
<tr>
<td>FJ641014.1</td>
<td>1.00</td>
<td>Bacillus subtilis strain IMAUB1031 16S ribosomal RNA gene</td>
<td></td>
</tr>
<tr>
<td>FJ641007.1</td>
<td>1.00</td>
<td>Bacillus subtilis strain IMAUB1018 16S ribosomal RNA gene</td>
<td></td>
</tr>
</tbody>
</table>
using phenolphthalein indicator. The activity of enzyme was expressed in terms of enzyme units. One unit of enzyme activity was defined as the amount of enzyme required to liberate 1 µmol of equivalent fatty acid (ml/min) under the standard assay conditions.

**16S rDNA sequence identification and phylogenetic tree analysis**

Genomic DNA extraction was used as a template for the performance of PCR amplification for 16S rDNA. Amplification was done with a set of universal primers that are highly conserved.
among prokaryotes and could amplify a segment of 1,500bp. The universal primers used for amplification are:

8F: 5’ AGA GTT TGA TCC TGG CTC AG 3’; forward, 1492R: 5’ ACG GCT ACC TTG TTA CGA CTT 3’; reverse

DNA sequencing of PCR amplicon was carried out using a BDT v3.1 cycle sequencing kit on ABI 3730xl genetic analyzer. A DNA homology search was conducted using the Genbank database(http://WWW.ncbi.nlm.nih.gov) and a phylogenetic tree was constructed using Tree Top phylogenetic Tree prediction software (http://WWW.genebee.msu.su).

**Partial purification with (NH4)_2SO_4**

After incubation, cultures were centrifuged at 8000 rpm for 20 min (25°C) and supernatants were used as source of crude enzymes. The crude enzymes were precipitated from the supernatant using (NH4)_2SO_4, which is equivalent to 80% saturation. The precipitation was performed at room temperature (25°C) and later kept at 4°C for 24 hours. The precipitates were obtained after centrifugation and then resuspended in 30ml

---

**Fig. 3.** Effect of pH on relative activity of lipase of LP5

**Fig. 4.** Effect of temperature on relative activity of lipase of LP5
of 0.2M citrate buffer and dialyzed using the same buffer for 24 hours at 4°C to remove traces of (NH₄)₂SO₄.

The collected dialyzate is separated by centrifugation and to a volume of 100ml of enzyme taken individually 500 ml of chilled acetone was added. The mixture was allowed to precipitate for an hour and then centrifuged at 500 rpm for 15 mins and the centrifugate collected was dissolved in minimum amount of 20ml Tris/Hcl buffer (pH-8). The sample was collected in a sterile container and stored at 4°C for further analysis. The activity of the enzyme was determined as described earlier.

**Estimation of protein**

Total extracellular protein was estimated by Folin-Lowry method with crystalline bovine serum albumin (BSA) as the standard.

**SDS-PAGE**

To determine the homogeneity and molecular weight, the enzyme preparations and

![Image](image1.png)

**Fig. 5.** Effect of different metal ions on the relative activity of lipase of LP5

![Image](image2.png)

**Fig. 6.** Effect of different substrates on the relative activity of lipase of LP5
known molecular weight markers were subjected to electrophoresis. Characterization of partial purified lipase
Effect of pH
The effect of pH on lipase activity was studied by carrying out the enzyme activity at different pHs in the range of 4.0 – 12.0 using different buffers at 0.2M concentrations and the reaction mixture was incubated for 30mins. Different buffers used for observing effect of pH on the enzyme activity were: citrate phosphate buffer (pH 4.0 – 6.0); sodium phosphate buffer (pH 7.0); Tris – HCl buffer (pH 8.0) and glycine NaOH buffer (pH 9.0 – 12.0). For the determination of pH stability of the enzyme, it was pre-treated in buffers at different pH at 50°C for 30 mins and assay was carried out using standard procedures described earlier.
Effect of temperature
To determine the effect of temperature on lipases, enzyme activities were estimated in a temperature range of 30°C – 100°C at pH 8.0 in 0.2M phosphate buffer. Thermostability of the partially purified lipases were tested by pre-incubating aliquots of enzyme in 0.2M phosphate buffer (pH 8.0) at varying temperatures ranging from 30°C – 100°C for 30min. The samples were then rapidly brought to room temperature and the residual enzyme activity was then assayed using standard procedures mentioned earlier.
Effect of metal ions
To determine the effect of different metal ions on lipase activity, the enzyme (10µg) was incubated with respective metal ions at pH8 and 50°C and the final concentration was 5mM for each metal ion. Aliquot (500µl) was taken after 30mins of incubation for the determination of lipase activity under standard assay conditions.
Substrate specificity
The lipase activity on different substrates such as triacetin, tributyrin, olive oil, tween-20, tween-80, triton-X was studied.

RESULTS AND DISCUSSION

16S rDNA identification and phylogenetic tree analysis
Strain Lp5 isolated from oil contaminated soil on the basis 16s rDNA sequence was identified as Bacillus subtilis strain Y-IVI(Accession number: GQ 475486.1). The 16S rDNA nucleotide sequence was obtained. The phylogenetic tree analysis of Lp5 strain was constructed on the basis of comparison of the 16S rDNA sequence of this strain with other Bacillus sp. strains available in the NCBI Genebank database. It evidenced a high degree of homology with Bacillus subtilis strain Y-IVI (Table 1). The phylogenetic relationship of closely related Bacillus sp. is depicted in fig.-1. On the basis of its cultural, morphological, biochemical characteristics and 16S rDNA sequence analysis strain Lp5 was identified as Bacillus subtilis strain Y-IVI.

Characterization of partially purified lipase
SDS-PAGE
Molecular weight of the partially purified lipases of LP5 was determined by SDS-PAGE. The gel revealed many bands in crude sample of LP5 and a single band in purified sample during electrophoresis. The molecular weight of this protein was calculated to be about 35kDa respectively by comparing with the standard marker proteins and standard lipase marker (Fig. 2).
Chartrain et al. reported that Pseudomonas fluorescens lipase to have different molecular weights like 20.9 kDa, 33kDa, 48kDa and 45 kDa. Costa et al. reported that lipases are monomeric proteins having molecular weights in the range of 16-670 kDa. Among Gram-positive bacteria, the Bacillus lipases were classified into two subfamilies. The first subfamily included lipases with low molecular mass in the range of 19-20 kDa from Bacillus licheniformis, Bacillus subtilis, and Bacillus pumilus. The lipases from Bacillus thermocatenulatus, Bacillus thermoleovorans, and B. stearothermophilus were included in the second sub family and they had molecular mass around 43 kDa.
Effect of pH
The protein nature of enzymes implies that pH will affect the ionization state of the amino acids which dictate the primary and secondary structure of the enzyme and hence, its overall activity. A change in pH will have a progressive effect on the structure of the protein and the enzyme activity. The pH dependent activity and stability of partially purified lipase of LP5 is presented in fig.-3. Results presented in fig.-3 indicate that the partially purified lipase of Lp5 exhibited activity in a very wide range of pH (4-10). However maximal activity (100%) was observed at...
pH 8.0 for Lp5. The enzyme activity was less in acidic and highly alkaline pH range. For Lp5, the relative activity was found to be 10.02%, 78.49%, 81.25% and 8.18% at pH 4.0, 6.0, 10 and 12.0 respectively.

Among Gram-positive bacteria, a Bacillus subtilis lipase was shown to have a very alkaline pH optimum between 10 and 11.5. Pseudomonas lipases were reported to have optimum pH in acidic as well as alkaline environment. Lipases of Pseudomonas and Bacillus were reported to be more stable in alkaline pH range.

**Effect of temperature**

The effect of temperature and thermal stability of partially purified lipase of Lp5 was studied and the results are presented in fig-4. The partially purified lipase of Lp5 exhibited maximum activity at 60°C. The lipase of Lp5 retained 86.90% and 73.16% of its activity at 40°C and 80°C respectively. The activity dropped rapidly above 60°C. The activity was also found to be less at 20°C and no activity was detected at 100°C.

Sztajer et al. have reported a temperature optimum for oil hydrolysis between 50 and 55°C for a lipase from Pseudomonas fluorescence. The temperature optima of lipases from mesophilic microorganisms were found to be normally in the range of 45-60°C. Many thermophilic Bacillus strains have been reported to produce lipases that are active at temperatures between 60 and 75°C. The lipases from thermophilic bacilli are relatively more stable at higher temperatures (above 60°C), than those from mesophilic organisms. The lipases from mesophilic Pseudomonas sp. were stable till 50°C; on the other hand there are a few reports of thermostable lipases from Pseudomonas sp.

**Effect of metal ions**

The results pertaining to the effect of various metal ions on lipase activity by Lp5 are presented in fig-5. The results reveal that an enhanced activity of 124% was observed in presence of CaCl₂. Similarly an enhanced activity of 109% was observed in presence of NaCl. Inhibition of lipase was recorded by EDTA, ZnSO₄, HgCl₂, and CuSO₄.

Metal ions are reported to show both stimulatory as well as inhibitory effects. Metal ions like Hg²⁺, Zn²⁺ and Cu²⁺ are reported to have inhibitory effect on Pseudomonas lipases by several workers. Barium was shown to have marginal stimulatory effect in case of Pseudomonas sp. lipase. Several workers have found that calcium ions are able to stimulate lipase activity. In accordance with the present observations EDTA was reported to inhibit activity of a few lipases.

**Substrate specificity**

Enzymes exhibit substrate specificity and they show maximum activity on preferred substrate. The results pertaining to substrate specificity are precisely in fig-6. A critical perusal of fig reveals the substrate specificity of lipases. It is evident that the lipase activity of Lp5 was maximum when triacetin was employed as the substrate. The lipase activities were 95.24%, 82.16%, 91.18% and 62.15% with tributyrin, tween-20, tween-80, olive oil respectively. The inhibitory activity was exhibited when triton-x was employed as a substrate.

**ACKNOWLEDGEMENTS**

Thanks are due to Head, Department of Microbiology, Kakatiya University for encouragement and providing the necessary laboratory facilities.

**REFERENCES**

9. Jaeger, K.E., Ransac, S., Dijkstra, B., Colson,


J PURE APPL MICROBIO, 6(2), JUNE 2012.