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

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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 Y IVI. In electrophoretic separation many bands were detec. 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^{0}C and 80^{0}C respectively. The activity was found to be less at 20^{0}C. The results revealed that a lipase activity of 124% and 109% was observed in presence of metal ions $CaCl_2$ and NaCl respectively. Inhibition of lipase by LP5 was detected with EDTA, $ZnSO_4$, $HgCl_2$ and $CuSO_4$.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 carbonylesterases (E.C. 3.1.1.3) that catalyse both hydrolysis and synthesis of esters formed from glycerol. Lipases are produced by many microorganisms[1] and higher eukaryotes. Microbial lipases have drawn much attention because of their potential use in biotechnology, mainly due to their availability and stability[2,3]. 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[4], 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, agro-chemicals, and flavor compounds[5]. The chemo-regio-and enantio-specific behavior of these enzymes has generated interest among scientists and industrialists[6]. Many *Bacillus sp*. strains, which grew successfully under highly alkaline conditions, were found to be useful in leather processing[7]. The alkaline thermophilic lipases find application in detergent industry [8,9]. A variety of

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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

Alignment View	ID	Alignment Result	Description
	Consensus	0.96	Sample LP5 16S rDNA
	GQ475486.1	1.00	*Bacillus subtilis* strain Y-IVI 16S ribosomal RNA gene
	GQ421472.1	0.99	*Bacillus subtilis* strain L4 16S ribosomal RNA gene
	GQ402829.1	1.00	*Bacillus sp.* G3(2009) 16S ribosomal RNA gene
	GQ375227.1	0.99	*Bacillus subtilis subsp. subtilis* strain CICC 10076 16S ribosomal RNA gene
	GQ199597.1	0.99	*Bacillus subtilis* strain I527 16S ribosomal RNA gene
	GQ169813.1	1.00	*Bacillus subtilis* strain B107 16S ribosomal RNA gene
	FJ641016.1	1.00	*Bacillus subtilis* strain IMAUB1036 16S ribosomal RNA gene
	FJ641015.1	1.00	*Bacillus subtilis* strain IMAUB1035 16S ribosomal RNA gene
	FJ641014.1	1.00	*Bacillus subtilis* strain IMAUB1031 16S ribosomal RNA gene
	FJ641007.1	1.00	*Bacillus subtilis* strain IMAUB1018 16S ribosomal RNA gene

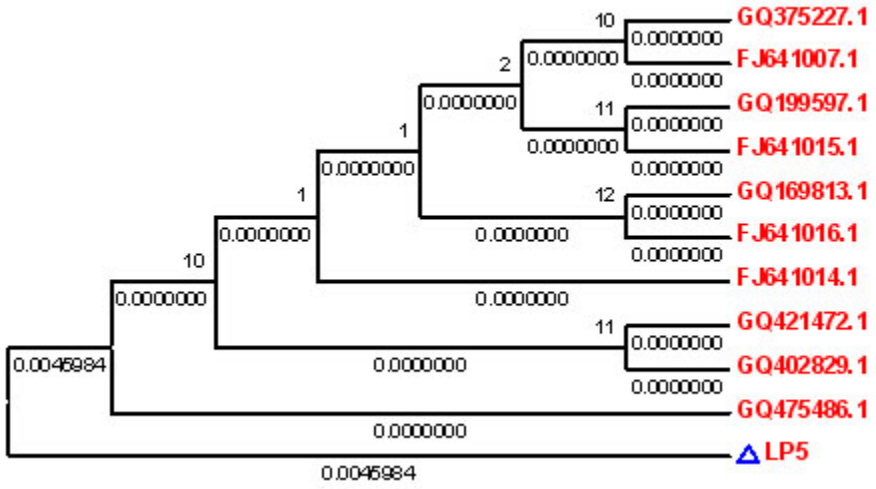

Fig. 1. Phylogenetic tree showing evloutionary relationship of Lp5 with 10 other related taxa

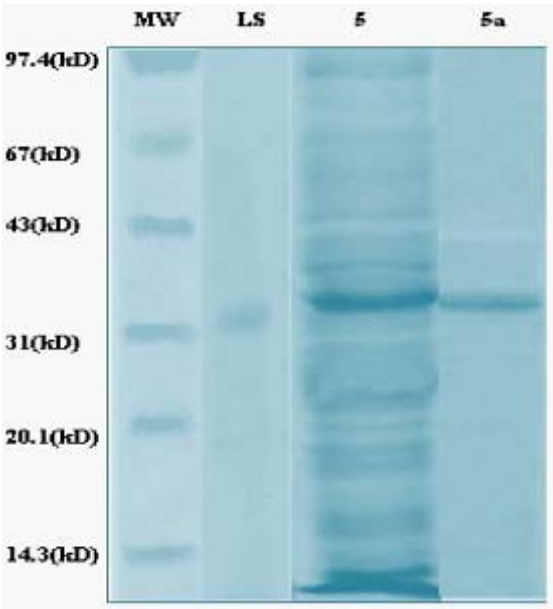

Fig. 2. Electrohoretic separation and size determination of the lipase protein
Lane 1: Protein Marker
Lane 2: Standard Lipase
Lane 3: LP5
Lane 4: LP5a

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 1µmol 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.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

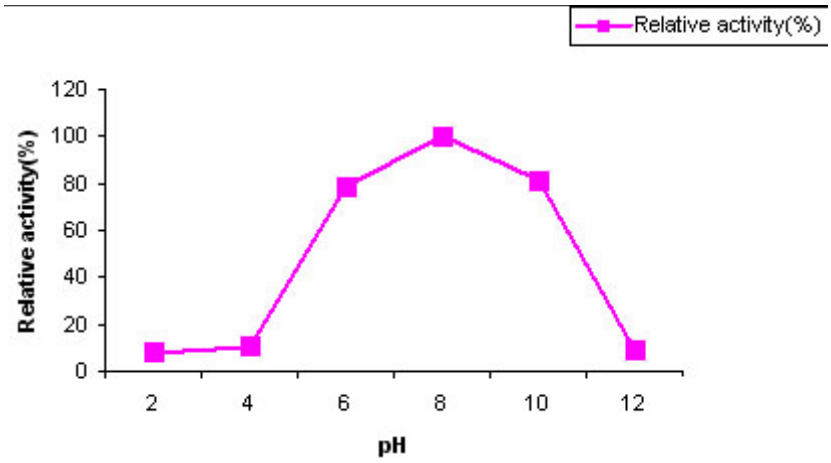

Fig. 3.Effect of pH on relative activity of lipase of LP5

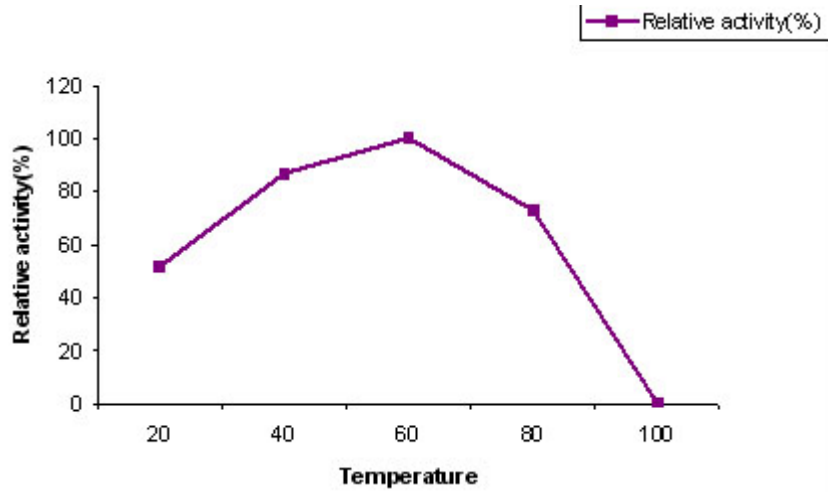

Fig. 4.Effect of temperature on relative activity of lipase of LP5

of 0.2M citrate buffer and dialyzed using the same buffer for 24 hours at 4°C to remove traces of $(NH4)_2SO_4$.

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 extra cellular protein was estimated by Folin- Lowry method[13] with crystalline bovine serum albumin (BSA) as the standard.

SDS- PAGE

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

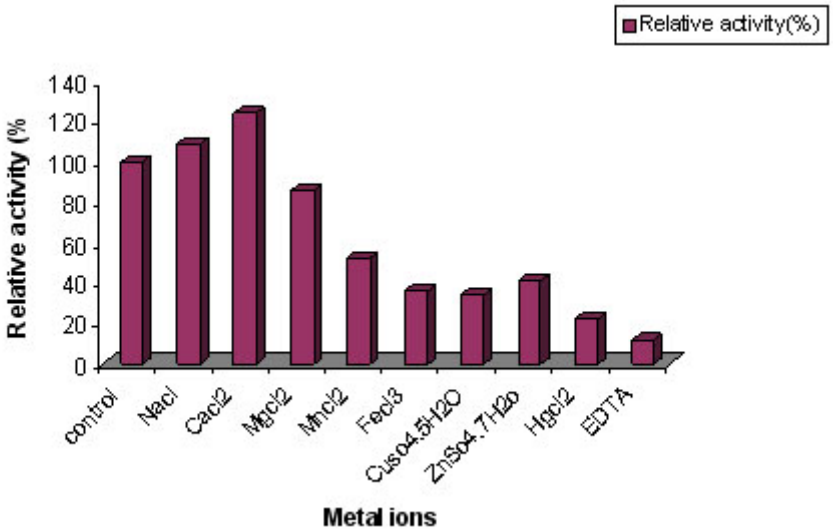

Fig. 5. Effect of different metal ions on the relative activity of lipase of LP5

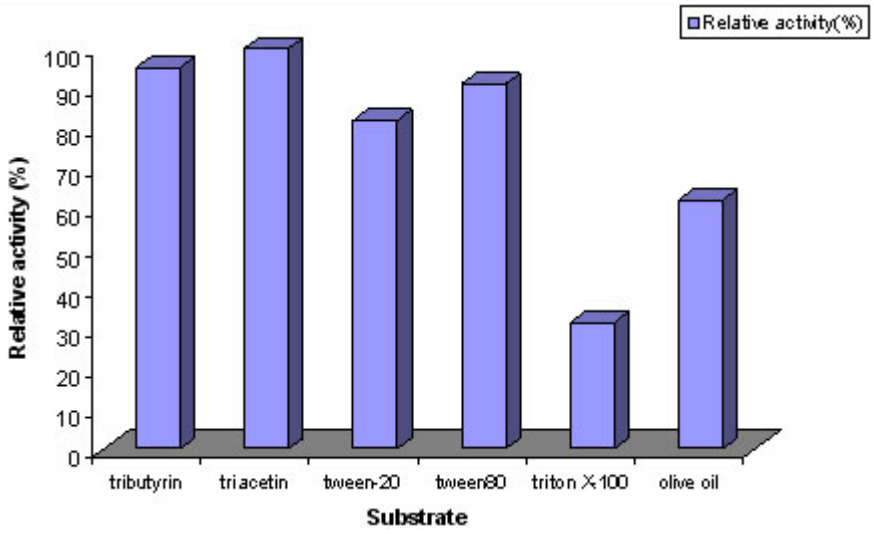

Fig. 6. Effect of different substrates on the relative activity of lipase of LP5

known molecular weight markers were subjected to electrophoresis [14].

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 50^0C 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 30^0C – 100^0C 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 ^{0}C – 100^0C 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 50^0C 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 35KD respectively by comparing with the standard marker proteins and standard lipase marker (Fig. 2).

Chartrain *et al.*[15] reported that *Pseudomonas fluorescens* lipase to have different molecular weights like 20.9 kDa, 33kDa, 48kDa and 45 kDa. Costa *et al.*[16] 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[17]. 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, it's overall activity. A change in pH will have a progressive effect on the structure of the protein and the enzyme activity[18]. 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[17]. Pseudomonas lipases were reported to have optimum pH in acidic as well as alkaline environment[19,20]. 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 ^{0}C. The lipase of Lp5 retained 86.90% and 73.16% of its activity at 40 ^{0}C and 80 ^{0}C respectively. The activity dropped rapidly above 60 ^{0}C. The activity was also found to be less at 20 ^{0}C and no activity was detected at 100 ^{0}C.

Sztajer *et al.*[21] have reported a temperature optimum for oil hydrolysis between 50 and 55^{0}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.[19, 22].

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 $CaCl_2$ $2H_20$. Similarly an enhanced activity of 109% was observed in presence of NaCl. Inhibition of lipase was recorded by EDTA, $ZnSO_4$, $HgCl_2$ and $CuSO_4$.

Metal ions are reported to show both stimulatory as well as inhibitory effects. Metal ions like Hg^{2+}, Zn^{2+} and Cu^{2+} are reported to have inhibitory effect on *Pseudomonas* lipases by several workers[15,19,23]. Barium was shown to have marginal stimulatory effect in case of *Pseudomonas* sp. lipase[24]. Several workers have found that calcium ions are able to stimulate lipase activity[15,25]. In accordance with the present observations EDTA was reported to inhibit activity of a few lipases [26].

Substrate specificity

Enzymes exhibit substrate specificity and they show maximum activity on preferred substrate. The results pertaining to substrate specificity are précised 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.

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