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# Molecular Identification of Lipase Producing Bacteria based on 16S rDNA Sequencing

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**A B S T R A C T**

*Lipases* or triacyl glycerol acylester hydrolases or carboxyl esterases (E.C 3.1.1.3) that catalyze both hydrolysis and synthesis of esters formed from glycerol. Lipases are currently attracting an enormous attention because of their biotechnological applications. In particular, lipases of microbial origin finding immense applications in various fields as they can catalyze a variety of hydrolytic or synthetic reactions. A bacterial strain isolated from an oil contaminated soil using Nutrient agar medium with 1% olive oil. The isolated strains were screened for lipolytic activity on tributyrin agar and the lipolytic potential was measured. The strains with lipolytic potential (R/r) >2 were selected and further screened for lipase production on ideal medium. The Lipase assay was carried out by measuring the growth using optical density at regular time intervals of 24hrs, 48hrs and 72hrs respectively. The selected bacterial strain with maximum lipase production was observed at 48hrs, 37oC (9.0 EU/ml). In our studies, the best producer of lipase was subjected to molecular identification based on 16S r DNA nucleiotide sequence homology and phylogenetic analysis, a newly isolated indigenous potential lipase producing strain (LP5) was identified as Bacillus subtilis strain Y-IVI.

## Introduction

*Lipases* (glycerol ester hydrolases, EC 3.1.1.3) are one of the most important classes of industrial enzymes that catalyze the hydrolysis of triglycerides to fatty acids and glycerol (Jager and Reets, 1998; Rajendran and Thangavelu, 2007). They are produced by many bacteria, fungi, plants, animals and are being employed in food, cosmetics, detergents and pharmaceutical industries (Vulfson, 1994; Suk- Jung *et al*., 2003). Recently, there have been attempts to use lipase for the deacetylation of cephalosporins (Lee *et al*., 2001). Lipases are known to have certain roles in human pathogenesis and their activity modulators have been suggested as potent pharmaceuticals for the treatment of obesity (Kanwamura *et al*., 1999; Nonaka *et al*., 1996; Park, 2001). Lipases perform essential roles in the digestion, transport and processing of dietary lipids (triglycerides, fats, oils) in most living organisms.

Although the existence of *lipolytic* bacteria is known for many years, our understanding of bacterial lipolysis stems from the fact that most of the studies are with crude enzyme systems. Only few studies have been made with partially purified lipases (Mencher *et al*.,

1967). In view of the importance of bacterial lipases, in the present investigations an attempt was made to isolate, screen and characterize efficient strains so that they can be employed for commercial production.

## Materials and Methods

### Isolation

The oil contaminated soil samples were collected aseptically and isolations were made by spread plate method using serial dilutions on nutrient agar medium amended with olive oil as substrate. (composition: peptone 5g/l; beef extract 3g/l; NaCl 5g/l; distilled water 1 liter; olive oil 1%) and the plates were incubated at 30oC for 48 hours. The bacterial colonies developed on the medium were isolated and were selected for screening.

The selected strains were maintained on nutrient agar medium amended with 1% olive oil medium. Isolations were also made from direct oil samples (ground nut oil, coconut oil, palm oil etc.,) by taking 0.1ml of oil sample and spreading it on to tributyrin agar plates.

### Screening

The isolated strains were screened for lipolytic activity and lipolytic potential (R/r), using tributyrin agar medium and spirit blue agar medium. The strains were spread on tributyrin agar (Collins, 1964; Collins and Lyne, 1980; Limpon *et al*., 2006) and spirit blue agar medium and incubated for 24hours at 30oC. Then, the bacterial colonies which formed clear zone around them on the plates were recorded and their lipolytic activity and lipolytic potential was calculated by the formula:

Lipolytic potential = hydrolytic zone diameter / colony diameter.

The strains which had exhibited high lipolytic potential were selected and screened further for efficient lipase production.

The strain was tested for lipase production and assessed first in 25ml of enrichment medium (peptone-10g/l, beef extract-3g/l, NaCl-5g/l, 1% olive oil and pH-7). After incubation for 24 hours the preculture formed was inoculated into production medium (basal medium) of composition (g/l): starch 20, peptone 20, NH4Cl 3.8, MgSO4 1, K2HPO4 5, olive oil 1%, pH 7.0. The culture was then incubated for 72 hours in an orbital shaker at 100 rpm at 30oC.The cells were then harvested by centrifugation at 5000 rpm for 15min and the supernatant was used for further assay at regular interval of 24 hours, 48 hours, and 72 hours. Bacterial growth was determined by measuring the absorbance at 550 nm (Sangiliyandi and Gunasekaran, 1996) and the final pH of the medium was also determined.

### Lipase assay

The lipase activity in the culture filterate was assayed by titrimetry (Venkateshwarlu and Reddy, 1993). The reaction mixture included 2ml of enzyme, 5ml of citrate phosphate buffer (pH 8.0), 2ml of triacetin and was incubated at 37oC 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 using phenolphthalein indicator. The activity of enzyme was expressed in terms of enzyme units. One unit of enzyme activity is 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 utilized as a template for the performance of PCR

amplification for 16S rDNA identification with a set of universal primers that are highly conserved among prokaryotes and could amplify 1,500bp. The universal primers used were as follows: forward and reverse DNA sequencing reaction of PCR amplicon was carried out with 8F and 1492R primers
8F: 5' AGA GTT TGA TCC TGG CTC AG 3'
1492R: 5' ACG GCT ACC TTG TTA CGA CTT 3' using 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). A phylogenetic tree was constructed using Tree Top phylogenetic Tree prediction software (http://www.genebee. msu.su).

## Results and Discussion

In the present investigations, a large number of bacterial strains were isolated from different oil mill soils. Out of them, four bacterial strains were selected for further screening for their extra cellular enzymatic activity. The lipolytic potential and hydrolytic zone diameter were calculated on tributyrin agar (Table 1). It is evident from the data presented in the table that the highest lipolytic activity was shown by Lp5and its lipolytic potential is 2.6.

Data presented in table 2 and 3, reveals that all the four strains of bacteria produced lipase in one or other medium. Lp5 produced maximum lipase (9.0Eu/ml) in medium, and had highest growth of OD 0.651 at 48 hours of incubation. All the other strains produced lipase at optimum level at an incubation of 48 hours using the medium. The continuous increasing in production was recorded till 48 hours and later the lipase production decreased gradually so, for the above isolates of bacterial strains lipase production was optimum at 48 hours incubation on medium.

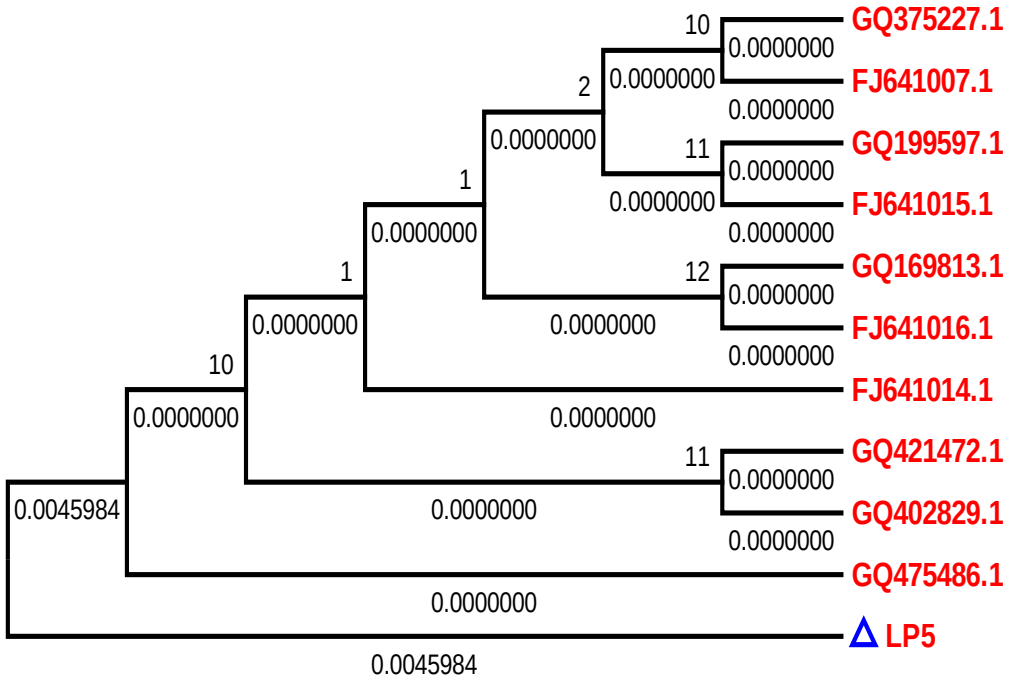
**Table.1** Evaluation of lipolytic potential of selected strains

| Isolate | Colony diameter(r)mm | Zone diameter® mm | Lipolytic potential(R/r) |
|---|---|---|---|
| Lp1 | 4.0 | 6.0 | 1.5 |
| Lp3 | 3.0 | 8.0 | 2.6 |
| Lp4 | 4.0 | 8.0 | 2.0 |
| Lp5 | 3.0 | 8.0 | 2.6 |

**Table.2:** Bacterial growth on Ideal medium

| Isolate | Growth(OD) | | |
|---|---|---|---|
| | 24hr | 48hr | 72hr |
| Lp1 | 0.193 | 0.527 | 0.682 |
| Lp3 | 0.429 | 0.651 | 0.506 |
| Lp4 | 0.249 | 0.563 | 0.663 |
| Lp5 | 0.375 | 0.603 | 0.712 |

**Fig.1** Phylogenetic tree showing evolutionary relationships of 11 taxa




**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 |

**Table.3:** Production of lipase on ideal medium

| Isolate | Eu/ml | | |
|---|---|---|---|
| | 24hr | 48hr | 72hr |
| Lp1 | 2.0 | 6.0 | 0.7 |
| Lp3 | 1.6 | 6.4 | 1.2 |
| Lp4 | 3.2 | 5.0 | 1.7 |
| Lp5 | 5.5 | 9.0 | 0.7 |

## 16S rDNA identification and phylogenetic tree analysis

Strain Lp5 was identified as Bacillus subtilis strain Y-IVI sp. 16S ribosomal RNA was employed for identification of the Lp5 strain. The 16S rDNA nucleotide sequence obtained for Lp5. 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.

The phylogenetic tree analysis of strain Lp5 was compared with 10 other bacillus sp. Sequences. It evidenced a high degree of homology with Bacillus subtilis strain Y-IVI. The phylogenetic relationship of closely related Bacillus sp. Is depicted in fig. On the basis of its morphological, cultural, biochemical characteristics, 16S rDNA strain Lp5 was identified as Bacillus subtilis strain Y-IVI.

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