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## Investigation on Lipase Producing Actinomycete Strain LE-11, Isolated from Shrimp Pond

<sup>1</sup>B. Gunalakshmi, <sup>2</sup>Maloy Kumar Sahu, <sup>2</sup>K. Sivakumar,  
<sup>2</sup>T. Thangaradjou, <sup>1</sup>S. Sudha and <sup>3</sup>L. Kannan

<sup>1</sup>Department of Biotechnology,  
Dhanalakshmi Srinivasan College of Arts and Science for Women,  
Perambalur 621 212, Tamil Nadu, India

<sup>2</sup>Center of Advanced Study in Marine Biology, Annamalai University,  
Parangipettai 608 502, Tamil Nadu, India

<sup>3</sup>Thiruvalluvar University, Fort Campus, Vellore 632004, Tamil Nadu, India

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**Abstract:** Actinomycetes (20 strains), isolated from the sediments of shrimp pond were examined for their lipase activity. Strain LE-11, which was tentatively identified as *Streptomyces griseochromogenes*, showed higher lipase activity and it was taken for further study. Effects of various physical and chemical factors such as pH, temperatures, sodium chloride concentrations, carbon and nitrogen compounds on the lipase activity of *S. griseochromogenes* were studied. It was found that at pH 7, temperature 55°C, 0.05% NaCl concentration, carbon compound mannitol and nitrogen compound L-phenylethylamine, the enzyme activity was maximum. Protein content of the crude enzyme was 2.057 µl mL<sup>-1</sup>. The crude protein and partially purified protein were run in the SDS-PAGE and a band was found on equal position; however molecular weight of the protein was not determined. The study indicated that *S. griseochromogenes* can effectively be used in large scale production of lipase enzyme for commercial purposes, after ascertaining the strain's ability in large scale fermentations.

**Key words:** Shrimp pond, *Streptomyces griseochromogenes*, lipase enzyme

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### INTRODUCTION

Lipases possess the unique feature of acting at an interface between the aqueous and nonaqueous (i.e., organic) phases and this feature distinguishes them from esterases. Lipases are the most versatile biocatalysts and they bring about a range of bioconversion reactions such as hydrolysis, interesterification, esterification, alcoholysis, acidolysis and aminolysis. Lipases act under extremely mild conditions (Sharon *et al.*, 1999) and they can be used in a variety of organic solvents. Lipases are an excellent alternative to many classical organic techniques in the selective transformation of complex molecules. They possess many features that favour their use as excellent biocatalysts. They impart specificity to a reaction, in which a chemical process is typically more non-specific. In addition, the use of lipase enzyme can decrease side reactions and simplify post-reaction separation problems (Pandey *et al.*, 1999). Lipase catalyzed processes are reported to offer cost-effectiveness too, in comparison with traditional downstream processing in which energy consumption and toxic by-products might often pose problems (Jansen *et al.*, 1996). Many fatty food stains and human sebum contain triglycerides which are hydrolyzed by lipases to produce fatty acids, monoglycerides

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**Corresponding Author:** Dr. K. Sivakumar, Center of Advanced Study in Marine Biology, Annamalai University, Parangipettai 608502, Tamil Nadu, India  
Tel: +91-4144-252099 Fax: +91-4144-243999

and diglyceries, which are easier to remove than unhydrolyzed triglycerides (Fuji *et al.*, 1986). The lipases find application in various industries like food, chemical, pharmaceutical, cosmetic, leather and detergent industries.

From the existing literature it is evident that most of the lipase related study were undertaken earlier on terrestrial bacteria and only a few were on terrestrial fungi and actinomycetes (Sztajer *et al.*, 1988; Rapp and Backhaus, 1992) and there is no single report on marine actinomycetes. As marine actinomycetes are considered to be potential microorganisms, capable of producing novel secondary metabolites, the present investigation aims at to study the lipase activity of actinomycetes isolated from the sediments of shrimp pond, under different experimental conditions like pH, temperatures, sodium chloride concentrations, carbon and nitrogen compounds and to identify the potential lipase producing actinomycetes using chemotaxonomical and conventional methods.

## MATERIALS AND METHODS

### Isolation of Actinomycetes

The sediment sample was collected from a shrimp pond (Lat. 11° 28' 53.7" N and Long. 79° 45' 31.1" E) located opposite to the Vellar estuary, southeast coast of India, by inserting a sterilized polyvinyl corer (10 cm) into the sediments. The centre portion of the 2 cm sediment sample was taken out with the help of a sterile spatula. The collected sample was transferred to a sterile polythene bag and taken immediately to the laboratory. After arrival at the laboratory, the sample was air-dried aseptically for one week. The air-dried sediment sample was incubated at 55° C for 5min. (Sivakumar *et al.*, 2005). Then, 10 fold serial dilutions of the sediment samples were prepared, using filtered and sterilized 50% seawater. One of the serially diluted samples was plated in the Actinomycetes Isolation Agar medium (Hi-Media, Mumbai) in duplicate petriplates after suitable dilution. To minimize bacterial and fungal contaminations, all agar plates were supplemented with 20 and 100 mg L<sup>-1</sup> of nystatin and cycloheximide, respectively (Kathiresan *et al.*, 2005). The actinomycete colonies that appeared on the petriplates were counted from 5th day onwards, up to 28th day. All the colonies growing on the petriplates were separately streaked in petriplates, subcultured, ensured for their axenicity and maintained in slants.

### Assay of Lipase Activity

Lipase activity was assayed following method of Arima *et al.* (1972) on the basis of the extent of the enzyme diffusion zone after 24, 48, 72 and 96 h of incubation. The strains that showed the greatest diffusion areas were further examined by titration methods with tributyrin or Tween-80 as an enzyme substrate. The reaction mixture consisted of 5 mL tributyrin-polyvinyl alcohol (PVA) emulsion (10% tributyrin in 2% PVA, 4 mL of 0.05 M Tris-HCl buffer (pH-7) and 1 mL of culture supernatant. The mixture was incubated at 30°C for 30 min. with continuous shaking. Enzyme reaction was terminated by addition of 20 mL of acetone-ethanol mixture (1:1 v/v). Released acids were titrated with 0.05 M NaOH in the presence of thymolphthalein. One unit of lipase activity was defined as the amount that liberated 1 µmol of free fatty acid per minute under test conditions.

### Effects of pH, Temperature, Sodium Chloride, Carbon Compounds and Nitrogen Compounds on Lipase Activity of the Actinomycetes

Lipase activities of the actinomycetes were measured at different pH, temperatures, sodium chloride, carbon and nitrogen compounds. The experiments were conducted in 250 mL Erlenmeyer flasks containing the sterilized Lipase Production Medium (LPM) broth at 15 lbs pressure for 15 min. After sterilization of the broth by autoclaving, the flask was cooled and the strain was inoculated and incubated differently for different parameters as described in the following paragraphs by taking one parameter at one time. Appropriate controls were maintained in all the experiments.

#### **Effect of pH**

This was studied by varying the pH from 4 to 11. After inoculation of the strain, it was incubated for seven days at 55°C.

#### **Effect of Temperature**

After inoculation of the strain in LPM broth, it was incubated at various temperatures viz., 45, 50, 55, 60, 65 and 70°C for seven days.

#### **Effect of Sodium Chloride Concentrations**

To study the tolerance towards sodium chloride, the LPM broth prepared with distilled water was added with sodium chloride of known concentrations viz. 0.05, 0.5, 1, 2, 3 and 4%. After the inoculation of the strain, inoculation was done at 55°C for seven days. Simultaneously, a control was maintained without sodium chloride.

#### **Effect of Various Carbon Compounds**

The LPM Broth was used for studying the effect of various carbon compounds viz., mannitol, sucrose and glucose. The broth was distributed into different flasks and 1% of each carbon source was then added before inoculation of the strain and incubated for seven days at 55°C.

#### **Effect of Various Nitrogen Compounds**

The APM broth was used for studying the influence of L-asparagine, L-phenylalanine, L-histidine and L-hydroxyproline. The broth was distributed into various flasks and 0.8 mL<sup>-1</sup> of each nitrogen compound was then added and incubated for seven days at 55°C.

At the end of the incubation period, the lipase activity was determined by the procedure as described earlier.

#### **Partial Purification of the Lipase Enzyme**

The strain LE-11, which showed maximum enzymatic activity among the cultures screened for the production of the lipase enzyme, was used for partial purification. The crude sample of the enzyme was subjected to centrifugation at 1,00,000 rpm for 60 min in the refrigerated centrifuge. The supernatant and the pellet suspended in a mixture of volume of buffer were used for the enzyme assays. The crude extract was treated with protamine sulphate (1 mg protamine sulphate to 10 mg of protein) and centrifuged at 27,000 rpm for 10 min to remove the nucleic acids and the supernatant was collected. This supernatant was brought to 45% saturation by mixing ammonium sulphate (pH 8.5) slowly with gentle agitation and allowed to stand for 24 h at 4°C in a cold room. After the equilibration, the precipitate was removed by centrifugation (10,000 rpm at 4°C for 20 min) and the supernatant was once again brought to 95% saturation with ammonium sulphate and allowed to stand for 30 min at 4°C. This precipitate was again centrifuged at 10,000 rpm at 4°C for 20 min and the precipitate obtained was dissolved in 10 mL of 0.5 M Tris-HCl buffer (8.5) and the protein content was estimated (Lowry *et al.*, 1951). Then, 10 cm pretreated dialysis bag (Hi-Media, Mumbai) was taken and activated by rinsing in doubled distilled water. One end of the dialysis bag was tightly tied and the precipitate recovered was taken inside the bag. The other end of the dialysis bag was tightly tied to prevent the leakage. After that, the dialysis bag was suspended in a beaker containing 0.5 M Tris-HCl buffer (pH 8.5) for 24 h; then it was transferred to 5% sucrose solution so that the excess water was removed and got absorbed by sucrose solution. After the dialysis, the volume was measured and analyzed for proteins and stored in deep freezer. The crude protein and partially purified protein were run in the SDS-PAGE.

### Taxonomic Investigation

The genus level identification was made for the strain LE-11 using cell wall composition analysis and micromorphological studies (Lechevalier and Lechevalier, 1970). Characterization of the strain LE-11 was made by following the methods described by Shirling and Gottlieb (1966) using the standard yeast extract-malt extract agar (ISP-2). The species level identification of the strain was based on the keys of Nonomura (1974) and Bergey's Manual of Determinative Bacteriology (Buchanan and Gibbons, 1974).

## RESULTS AND DISCUSSION

### Enzyme Assay

Among 20 strains of actinomycetes, only 3 strains showed lipase activity. Out of these 3 strains, one strain LE-11 exhibited maximum enzymatic activity ( $1.53 \mu\text{mol fatty acid mL}^{-1} \text{h}^{-1}$ ) and it was selected for further studies. Umamaheswary *et al.* (2005) isolated 40 strains of *Streptomyces* from the fish, *Mugil cephalus* and out of them, only the strain LG-33 showed good L-glutaminase activity. Sivakumar *et al.* (2006) reported only one strain of *Streptomyces* (LG-10) isolated from the fish, *Chanos chanos* with good L-glutaminase activity. Sahu *et al.* (2007) isolated 40 strains of *Streptomyces* from different parts of three estuarine fishes viz., *M. cephalus*, *C. chanos* and *Etroplus suratensis* and tested them for L-asparaginase. Out of the 40 strains, 6 showed significant L-asparaginase activity. From the present findings and also from other studies, it could be inferred that though the marine environs are harboring a good number of actinomycete strains, only a few of them are capable of producing novel enzymes which have very good industrial and commercial applications.

### Effects of pH, Temperatures, Sodium Chloride, Carbon Compounds and Nitrogen Compounds on Lipase Activity

The selected isolate LE-11 was allowed to grow in different pH, temperatures, salinities, carbon and nitrogen sources. The enzymatic activity was optimum at pH 8 (Fig. 1). Kulkarni (2002) observed the maximum lipase activity at pH 7 while Sekhon *et al.* (2006) reported maximum lipase activity at pH 7.5. In the present study, maximum activity was recorded at in the pH 8, which indicates that the enzyme produced by the actinomycetes could be in alkaline condition.

The strain LE-11 showed lipase activity at different temperatures and it was most active in the temperature between 50 and 60°C, with the maximum at 55°C (Fig. 2). The activity dropped rapidly above 60°C. Sztajer *et al.* (1991) have reported a temperature optimum for oil hydrolysis between

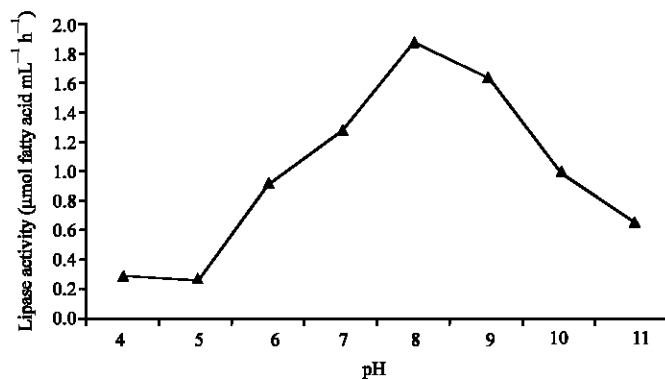


Fig. 1: Effect of pH on lipase activity of the strain LE-11

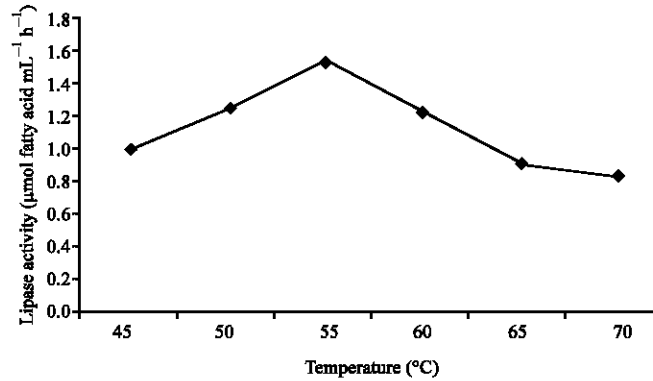


Fig. 2: Effect of temperature on lipase activity of the strain LE-11

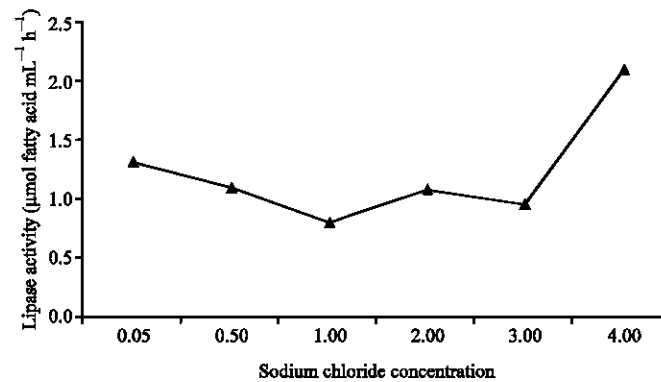


Fig. 3: Effect of sodium chloride concentration on lipase activity of the strain LE-11

50 and 55°C for a lipase produced by *Pseudomonas fluorescens*. The temperature optima around 55-65°C have also been reported for lipases of *Pseudomonas* sp. by several other workers (Yamamoto and Fujiwara, 1988; Iizumi *et al.*, 1990; Kojima *et al.*, 1994; Castellar *et al.*, 1997; Kulkarni and Gadre, 1999).

In the present study, since the cultures were isolated from the shrimp pond, it was felt necessary to understand the influence of various concentrations of sodium chloride on the enzymatic activity of LE-11. The maximum activity was recorded at 4% and minimum activity was recorded at 1% sodium chloride concentration, respectively (Fig. 3). This could indicate that the microbes prefer higher alkaline conditions for enzyme production. This might be one of the reasons for the strain to prefer the alkaline pH condition for its maximum production.

Among the different carbon sources used, the enzyme activity was maximum in sucrose and minimum in mannitol (Fig. 4). Contradictory to this Sekhon *et al.* (2006) found that the highest lipase activity was in the crude enzyme of the isolate *Bacillus megaterium* in mannitol and the enzyme was produced in lower amounts when glucose, galactose, xylose and sucrose were used as carbon sources. However, Nahas (1988) suggested that xylose, glucose and mannitol can be used as good carbon sources for lipase production by *Rhizopus oligosporus*.

Among the different nitrogen sources used, the enzyme activity was maximum in L-phenylalanine and minimum in L-asparagine (Fig. 5). But Sekhon *et al.* (2006) used different nitrogen sources for testing lipase activity in *B. megaterium* and observed highest activity in peptone while Kulkarni (2002)

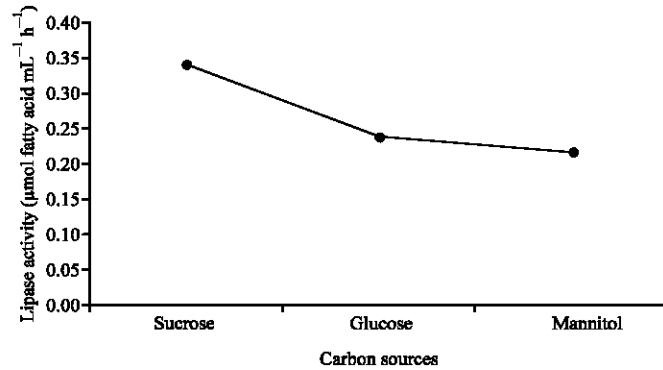


Fig. 4: Effect of carbon compounds on lipase activity of the strain LE-11

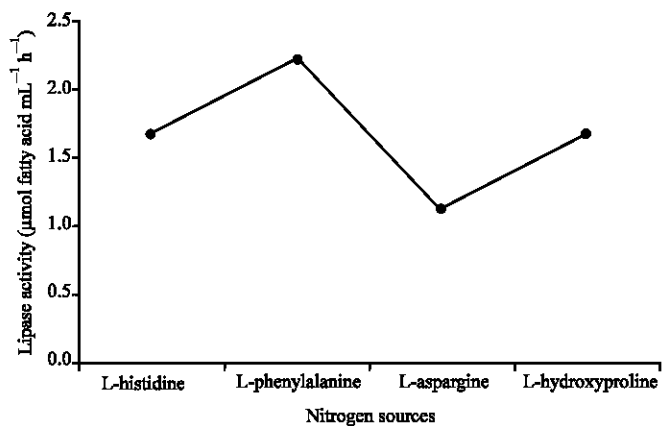


Fig. 5: Effect of nitrogen compounds on lipase activity of the strain LE-11

reported maximum enzymatic activity in the yeast extract obtained from *Pseudomonas* sp. Likewise, Ficker *et al.* (2004) reported that tryptone was found to be the best nitrogen source for lipase production by *Yarrowia lipolytica*.

Protein content of the crude enzyme of the strain LE-11 was quantified by means of Lowry's method and it was 2.057 µl mL<sup>-1</sup>. Then the crude protein and partially purified protein were run in the SDS-PAGE and a band was found on equal position; however molecular weight of the protein was not determined in this experiment. But, the available literature indicates that the molecular weight of the lipase enzyme produced by the microbial sources was found to be between 30 and 33 kDa (Baral and Fox, 1997; Kulkarni, 2002).

### Taxonomic Investigation

The strain LE-11 possesses LL-Diaminopimelic (LL-DAP) and the strain rested contains glycine in its cell wall. Presence of LL-DAP along with glycine indicates the cell wall chemotype-I. The strain with chemotype-I does not have characteristics pattern of sugars (Lechevalier and Lechevalier, 1970). The species belonging to the wall type I are *Streptomyces*, *Streptovorticillium*, *Chainia*, *Actinopycnidium*, *Actinosporangium*, *Elyptrosporangium*, *Microellbosporia*, *Sporichthya* and *Intrasporangium* (Lechevalier and Lechevalier, 1970). The micromorphological observations of the

Table 1: Comparison between the strain LE-11 and *S. griseochromogenes*

Characters studied	Strain LE-11	<i>S. griseochromogenes</i>
Aerial mycelium	Grey	Grey
Melanoid pigment	-	+
Reverse side pigment	+	-
Soluble pigment	-	-
Spore chain	Rectiflexibiles	Rectiflexibiles
<b>Utilization of sole carbon sources</b>		
Arabinose	+	+
Xylose	+	+
Inositol	+	+
Manitol	+	+
Fructose	+	+
Rhamnose	+	-
Sucrose	-	+
Raffinose	+	+
<b>Utilization of sole nitrogen sources</b>		
L-asparagine	+	+
L-hydroxyproline	-	-
L-histidine	+	+
L-phenylalanine	+	+
<b>Biochemical properties</b>		
Cellulose degradation	-	-
Hydrogen sulphide production	+	+
Melanin production	-	-
Nitrate reduction	-	-
Starch hydrolysis	+	+
Milk coagulation	+	+

+: Denotes positive; -: Denotes negative

strain LE-11 reveal that it belongs to the genus *Streptomyces*. The morphological, physiological and biochemical characteristics of the amylase producing strain LE-11, tested in the present study, are given in Table 1. The comparison showed that the present strain differed with the reverse side and soluble pigmentations. The test strain utilizes rhamnose as carbon source but the reference strain *S. griseochromogenes* has failed to utilize the rhamnose as carbon source. Likewise, the strain LE-11 is not able to utilize sucrose but reference strain utilized the same. Except these, all the other characters are exactly similar to those of *S. griseochromogenes*. Hence, the strain LE-11 has been tentatively identified as *S. griseochromogenes*.

## CONCLUSION

Sediments of shrimp ponds are a good source material for the isolation of potential actinomycetes. Tentatively identified species, *S. aureofasciculus* isolated from the sediments of a shrimp pond possesses good lipase activity. The study has also standardized the growth parameters of the actinomycetes for the maximum enzyme production, which can be effectively used in the large scale production of lipase enzyme for commercial purposes.

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