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*Full Length Research Paper*

# Optimization of lipase production by *Staphylococcus* sp. Lp12

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**A bacterial strain isolated from an oil contaminated soil, identified as *Staphylococcus* sp. Lp12 was screened for lipase activity on tributyrin agar and spirit blue agar medium. Maximum lipase production was observed at 48 h of growth (3.5 Eu/ml). Peptone was found to be as an ideal nitrogen source for production at a concentration of 1.0% (4.25 Eu/ml). Addition of any nitrogen source other than peptone to the medium resulted in a significant reduction of enzyme production. Lower lipase production was noted when an inorganic nitrogen source was used as the sole nitrogen source. Starch was used as a major carbon source for optimum production of lipase (4.25 Eu/ml) at a concentration of 1.5%. Of the natural oils, olive oil was able to induce more lipase (4.25 Eu/ml) rather than the oils like groundnut, coconut, castor oils. Basal medium containing tween 80 enhanced lipase production to a significant level. The pH 8 and temperature 45°C were found to be ideal pH and temperature for optimum production of lipase by this strain.**

**Key words:** *Staphylococcus* sp. Lp12, lipase, tributyrin, spirit blue agar.

## INTRODUCTION

Lipases or triacylglycerol acylester hydrolases are carboxylesterases (E.C. 3.1.1.3) that catalyze both hydrolysis and synthesis of esters formed from glycerol (Immanuel et al., 2008). Lipases are currently attracting an enormous attention because of their biotechnological applications. Lipases remain active in organic solvents, do not usually require a cofactor and display exquisite chemo, region and enantio selectivity. Hence they have become important biocatalysts in various industrial sectors, such as the agrochemical, pharmaceutical, detergent and food industries (Bouke et al., 2007).

In particular, lipases of microbial origin find immense applications in various fields as they can catalyze a variety of hydrolytic or synthetic reactions (Jaeger and Reetz, 1998). Bacterial lipases are glycoproteins, but some extracellular bacterial lipases are lipoproteins. Most of the bacterial lipases reported so far are constitutive and are 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 (Jaeger and Reetz, 1998), production of aromas, production of insecticides and synthesis of drugs such as naxopren and ibuprofen and as a biocatalyst of stereo selective transformations. The exponential increase in the application of lipases in various fields, in the past few years, necessitated both qualitative and quantitative improvement in enzyme production. The quantitative enhancement requires strain improvement and medium optimization for overproduction (Immanuel et al., 2008). Many bacterial lipases, particularly those from members of the genera *Pseudomonas*, *Bacillus*, *Staphylococcus*, *Achromobacter* have been cloned and characterized.

Bacterial lipases are mostly inducible enzymes and require some form of oil, fatty acid, fatty acid alcohol or fatty acid ester and surfactants for induction (Immanuel et al., 2008). Lipase biosynthesis by microorganisms under cultural conditions is influenced by factors such as medium composition, pH, temperature among others. Therefore, in the present investigations, a study was

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undertaken to optimize the lipase production by locally isolated *Staphylococcus* sp. Lp12 on relatively low cost media.

## MATERIALS AND METHODS

### Isolation and screening

The bacterial strain identified as *Staphylococcus* sp. designated as strain Lp12, was isolated from a soil contaminated with ground nut oil. The organism was tested for its lipolytic potential on tributyrin agar and spirit blue agar plates supplemented with tributyrin. After 24 h of incubation at 30°C, the lipolytic activity was confirmed by the formation of a clear zone around the colony. The strain was tested for lipase production and assessed first in 25 ml enrichment medium (peptone-10 g/l, beef extract-3 g/l, NaCl-5g/l, 1% olive oil and pH 7). After incubation for 24 h the preculture formed was inoculated into production medium (basal medium) of composition (g/l): starch 20, peptone 20, $NH_4Cl$ 3.8, $MgSO_4$ 1, $K_2HPO_4$ 5, olive oil 1%, pH 7.0. The culture was then incubated for 72 h in an orbital shaker at 100 rpm at 30°C.The cells were then harvested by centrifugation at 5000 rpm for 15 min and the supernatant was used for further assay at regular interval of 24, 48 and 72 h. 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. Uniform cultural conditions were maintained for all the optimization experiments and were carried out in triplicates. The production was also carried on formulation medium (g/l: starch 15, peptone 15, $NH_4Cl$ 2.5, $MgSO_4$ 0.7, $K_2HPO_4$ 2, olive oil 2%). During the optimization, the experiment was performed in a cumulative manner by incorporating previously optimized parameters.

### Lipase assay

The lipase activity in the culture filterate was assayed by titrimetry (Venkateshwarlu and Reddy, 1993). The reaction mixture included 2 ml of enzyme, 5 ml of citrate phosphate buffer (pH 8.0), 2 ml of triacetin and was incubated at 37°C for 3 h. At the end of incubation, the reaction was terminated by adding 10 ml of ethanol and the mixture was titrated against 0.05 M 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 1 µmol of equivalent fatty acid (ml /min) under the standard assay conditions.

### Optimization of medium components for lipase production

The optimization of medium components was carried out at the optimum pH 7 and temperature 30°C by substituting components present in the production medium.

### Carbon sources

The effect of carbon sources on lipase production was investigated by using different carbon sources namely glucose, sucrose, maltose, lactose. They were tested individually by replacing the starch present in the basal formulated production medium at the concentration 15 g/l. Later, the maximum enzyme inducing carbon source was further optimized by varying its concentration (1, 1.5 and 2%).

### Nitrogen sources

To test the effect of nitrogen sources on lipase production four different organic nitrogen sources viz, beef extract, yeast extract, proteose peptone, tryptone and two inorganic nitrogen sources namely ammonium sulphate, potassium nitrate were used. They were individually tested by replacing the peptone present in the basal formulated production medium at the concentration of 1.0%.

### Oil source and surfactants

The lipase production was accelerated by incorporation of different lipid sources namely olive oil, coconut oil, groundnut oil, triacetin, tributyrin and surfactants namely Tween 20, Tween 40, Triton -X in the optimized medium. They were tested individually at the fraction of 2% in the medium. The oil source and surfactants producing maximum lipase were optimized by varying their fraction (1, 1.5, 2 and 2.5%).

### pH and temperature

The effect of pH and temperature on lipase production was determined by adjusting the pH with a buffer (2, 4, 6, 8 and 10) and the influence of temperature was assessed by incubating the flasks at 25, 35, 45, 55 and 65°C.

### Statistical analysis

The results obtained in the present study were subjected to relevant statistical analysis using Statistical Package for the Social Sciences (SPSS) 12.0 software version.

## RESULTS AND DISCUSSION

The bacterial isolate under investigation *Staphylococcus* sp. Lp12 showed lipolytic with a potential of 3.33 and the diameter of hydrolytic zone was 10 mm after 24 h of incubation at 30°C on tributyrin agar plate.

### Time course studies on bacterial growth and lipase production

Time course studies were conducted in order to determine the growth and lipase production characteristics of *Staphylococcus* sp. Lp12. Figure 1 shows the different parameters determined, including bacterial growth (biomass) lipase production and pH at regular intervals of time 24, 48 and 72 h on the reported medium. It is evident from the figure that lipase production was detected in late logarithmic phase (after 24 h) and maximum production was achieved after 48 h of incubation. Gradually the production decreased after 72 h. The optical density values of growth reveal that OD was higher at 72 h but production was maximum at 48 h. The increase in OD may be due to increase in turbidity because of byproducts released during decline phase

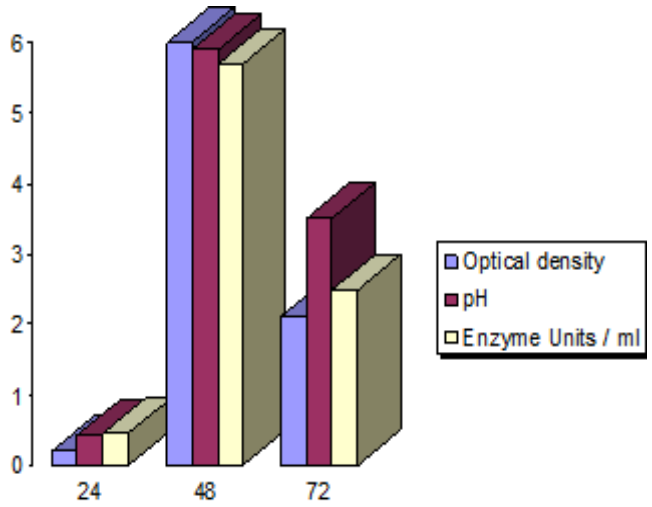


**Figure 1.** Production of lipase on reported media by *Staphylococcus* sp. Lp12.

(Rahman et al., 2006). This suggests that the lipase production effected by *Staphylococcus* sp. Lp12 occurred in a growth dependent fashion.

The pH of the culture dropped from the initial neutral pH to pH 6 during the first 24 h of incubation. This drop in pH continued till the 72 h. This may be attributed to the production of acids during growth. The production of lipase was found to be optimum at 48 h and hence all the experiments were carried out at 48 h incubation as a standard incubation period. The lipase production increased gradually from 24 (2.1 Eu/ml) to 48 h (3.5 Eu/ml) and after 72 h the production decreased to 2.5 Eu/ml. The production was found to be optimum (4.25 Eu/ml) in formulated medium in 48 h.

## Effect of carbon source on lipase production

Carbon source is an important substrate for energy production in microorganisms. In order to investigate the effect of carbon source on lipase production by *Staphylococcus* sp. Lp12, a range of carbon sources, mainly carbohydrates were screened for their efficiency to support lipase production. Glucose, sucrose, maltose, lactose, starch were replaced with the basal carbon source in basal medium. However, for comparison purpose, lipase production was also investigated with olive oil which is a substrate to induce the lipase. Results pertaining to this aspect revealed that the medium containing starch is more suitable for maximum lipase (4.25 Eu/ml) production than other carbon sources. Next to starch, glucose (1.5 Eu/ml) and sucrose (1.0 Eu/ml) were found to be ideal carbon sources for lipase production. Maltose and lactose did not induce any lipase production and their incorporation was found to be as good as without carbon source. This showed that maltose and lactose were not utilized as carbon sources by *Staphylococcus* sp. Lp12 for production of lipase. Similar results were previously reported by Immanuel et al. (2008), while investigating the lipase production by S*erratia rubidaea*. Similarly, Rahman et al. (2006) demonstrated that lipase production in *Pseudomonas aeruginosa* Ys-7 was quite low when glucose and glycerol were employed as the sole carbon sources. The starch containing medium also had higher growth of OD 1.154 and the pH of the medium was 5.47. The influence of different carbon sources on the production of lipase was found to be statistically significant ($p < 0.05$) by the one-way ANOVA.

## Effect of nitrogen source

In the present studies, higher degrees of lipase production (4.25 Eu/ml) was observed when peptone was added to the basal medium, followed by yeast extract (3.25 Eu/ml), proteose peptone (2.5 Eu/ml), beef extract (2.0 Eu/ml), tryptone (0.5 Eu/ml). Peptone was found to be the best organic nitrogen source for lipase which had OD 1.154 for growth and pH of medium as 5.4 from neutral pH. Similar to present studies, peptone was also reported to increase lipase production for some microorganisms (Chander et al., 1980). The effect of inorganic nitrogen sources were determined by replacing the organic nitrogen source (peptone) from basal medium. Potassium nitrate (2.25Eu/ml) increased the lipase production than ammonium sulphate (0.25 Eu/ml) which showed less growth of OD 0.01 and pH of the medium drifted to 7.3 from neutral pH. The statistical analysis by one-way ANOVA showed that the influence of nitrogen sources on total lipase production was statistically significant ($p < 0.05$).

## Effect of lipid sources on lipase production

Lipid induced lipase production by *Staphylococcus* sp. Lp12 was investigated by the addition of lipids to the culture medium. The lipase production in lipid supplemented medium gave better production than the control medium. The effect on lipase production by different groups of triglycerides was investigated. In general, *Staphylococcus* sp. Lp12 lipase preferred natural triglycerides, compared to synthetic triglycerides. It hydrolysed all tested triglycerides with the highest degree of affinity to coconut oil (8.0 Eu/ml) (Table 1). With regard to the group of natural triglycerides, lower lipase production was observed when castor oil (1.0 Eu/ml) was used. Its OD was 0.121 of growth and pH 6.7. With olive oil, the corresponding figures were 4.25 Eu/ml of enzyme production, 1.154 OD for growth and 5.4 pH. These results revealed that this strain was more selective towards

**Table 1.** Effect of different oil sources on the growth and production of lipase by *Staphylococcus* sp. Lp12.

| Oil source | Optical density | pH (initial = 7.0) | EU/ml |
|---|---|---|---|
| Groundnut oil | 0.537 | 7.8 | 1.5 |
| Coconut oil | 0.120 | 8.3 | 8.0 |
| Castor oil | 0.121 | 6.7 | 1.0 |
| Olive oil | 1.154 | 5.4 | 4.25 |
| Triacetin | 0.00 | 7.0 | Nil |
| Tributyrin | 0.063 | 6.2 | 1.0 |

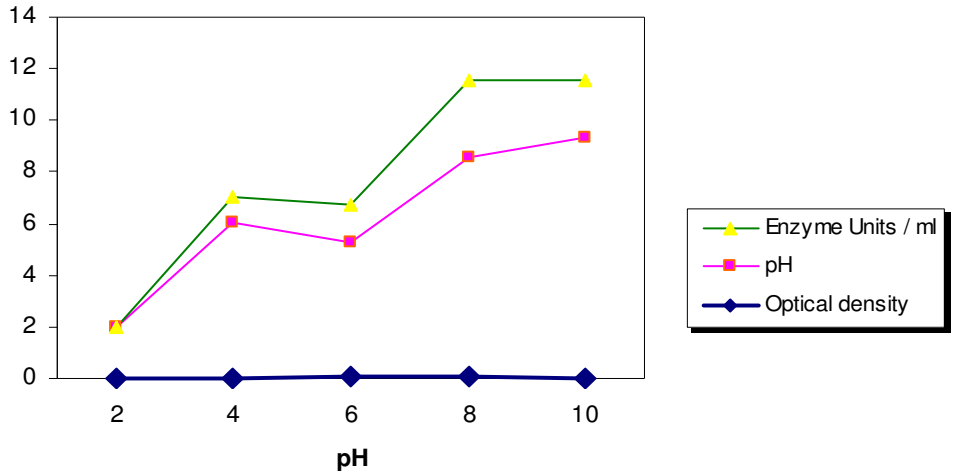


**Figure 2.** Effect of pH on the growth and production of lipase by *Staphylococcus* sp. Lp12.

long carbon chain natural oils (coconut oil, ground nut oil and olive oil). In the synthetic triglycerides, tributyrin showed better production than triacetin. At the same time, triacetin retarded the growth of the strain. Similar phenomenon was reported earlier by Rahman et al. (2006). One-way ANOVA showed that the influence of various lipid sources on total lipase production was statistically significant ($p < 0.05$).

## Effect of surfactant on lipase production

In order to determine the effect of surfactants on lipase production, three different types of surfactants were tested. Tween 80 (4.0 Eu/ml) was shown to enhance lipase production after 48 h of incubation. The lipase production rapidly decreased when Tween 20 and Triton-X were used, (Rahman et al., 2006). According to Wu and Tsai (2004), higher levels of lipase production were observed when the substrate formed an emulsion, thereby presenting an interfacial area to the enzyme. One-way ANOVA test for lipase production showed that the use of different surfactants was found to be statistically significant ($p < 0.05$).

## Effect of pH on lipase production

The effect of the pH of the medium on lipase production indicated a linear increase from 1.0 to 3.0 Eu/ml corresponding to the increase of pH from 4 to 8 (Figure 2). At pH 10, the lipase production decreased drastically. Evidently, *Serratia marcescens* preferred slightly acidic pH for lipase production (Gao et al., 2004). On the other hand *P. aeruginosa* MB preferred neutral pH (Marcin et al., 1993). The present study revealed that the lipase production by *Staphylococcus* sp. Lp12 requires alkaline pH. One-way ANOVA test conducted to obtain the data on lipase production as a function of medium pH revealed a highly significant variation ($p < 0.05$).

## Effect of temperature on lipase production

Experiments on effect of temperature indicated that the lipase production was maximum (5.0 Eu/ml) at the optimum temperature of 45°C. But low temperature (25 to 35°C) as well as high above 55°C the lipase production recorded was low (Figure 3). Lipase production by *S. marcescens* was higher at temperature of 25°C as

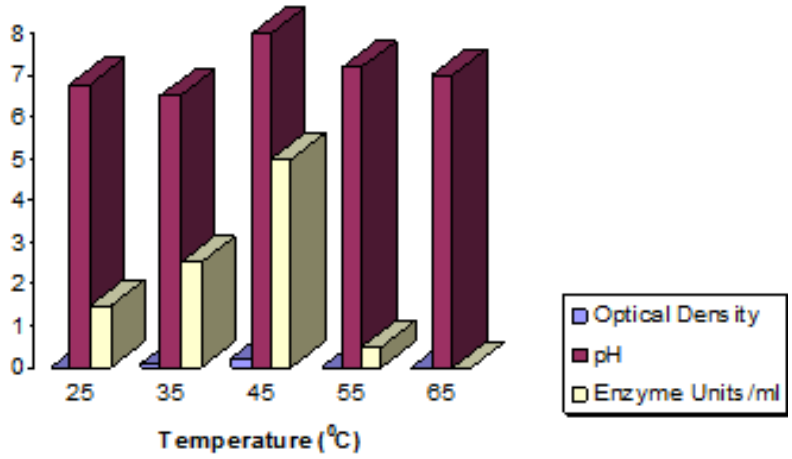


**Figure 3.** Effect of temperature on growth and production of lipase by *Staphylococcus* sp. Lp12.

compared to 30 and 35°C (Gao et al., 2004). Similarly, lipase production observed in *P. aeruginosa* MB was higher at 30°C (Marcin et al., 1993). But in the present study, lipase activity showed gradual increase with the increase of temperature from 25 to 45°C and further increase of temperature, beyond 45°C the production decreased. Such types of results were also reported by Immanuel et al. (2008). Statistical analysis by one-way ANOVA test for the data on lipase activity as a function of medium temperature change indicated a highly significant variation ($p < 0.05$).

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