

Optimization of Lipase Production Medium for a Bacterial Isolate

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Abstract: Lipases are versatile biocatalysts that are used extensively in detergent and pharmaceutical formulations. Lipases have an immense potential for being employed as industrial catalysts for production of fine chemicals. Their superior value arises from specificity and efficacy as compared to chemical catalysts. However new lipases with properties amenable for application in specific industrial process are being sought by the researchers. In the present paper we have isolated a bacterial strain ISC 1 that showed high lipase production of 25 units/ml from soil obtained from a site of regular oil spill. The process optimization studies like media manipulation and optimization of environmental conditions resulted in further enhancement of enzyme activity to about 42 units/ml. Lipase production known to be induced when lipids such as vegetable oils are included in the medium as carbon source. The potential to induce lipase production is dependent on the type of oil used. In our study inclusion of mustard oil in medium resulted in highest lipase production. The oil in the medium is utilized effectively when it is emulsified with emulsifier like gum acacia.

Keywords: Lipase, *Bacillus*, Enzymes, lipolysis, ROA plate assay, carbon sources, media optimization.

INTRODUCTION

In recent years lipases have emerged as key enzymes in swiftly growing biotechnology, owing to their application in a wide array of industrial applications. The industries include detergent, baking, fine chemicals, pharmaceuticals and food. Lipases are ubiquitous in nature and are produced by various plants, animals, and microorganisms. Microbial enzymes by the virtue of their versatility and amenability to modification in properties are widely employed for industrial applications.

Lipases from several sources have been purified, and some of their properties investigated. Generally, they are acidic glycoproteins of molecular weights ranging from 20000 to 60000. Their biological function is to catalyze the hydrolysis of triacylglycerol to give free fatty acids diacylglycerol and monoacylglycerol & glycerol. Lipases isolated from different sources have different stabilities with respect to conditions like pH, temperature, presence of organic solvents. These also differ in their activity and specificity. Largest industrial market for lipases is in the detergent industry. The mere volume of market is a key driving factor for research on novel lipases that are better suited for their application in detergent formulations.

Different media have different stimulation effects on lipase production (1). Considering the enormous market demand we have attempted the isolation of novel lipase producing bacterial strain. Fungal lipases have lower temperature and pH optima. Present paper focuses on screening and isolation of lipase producing microorganisms and optimization of media and physical parameters for maximal enzyme activity.

MATERIAL AND METHODOLOGY

Material

All the chemicals used were of analytical grade, chemicals used in production of lipase are as follows: Sodium Chloride (Merck), Agar (HiMedia, India), Rhodamine (Lobachemie, India), Olive oil (Figaro), Peptone (HiMedia, India), Yeast (HiMedia, India), Disodium hydrogen phosphate (Merck, India), Potassium dihydrogen phosphate, Magnesium sulphate Septshydrate ($MgSO_4 \cdot 7H_2O$), Calcium chloride (Merck India), Ammonium sulphate (Merck, India).

Sample collection

Lipases are ubiquitous in nature and are produced by various plants, animals, and microorganisms. Lipase producing bacteria have been found in diverse habitat such as soil contaminated with oil dairy waste, industrial waste, oil seeds decaying food compost heaps coal tips & hot springs. Soil sample was collected from Shiv Mandir, Faridabad where soil is preconditioned with oil spills.

Screening for lipase producing bacteria

A plate assay given by (2) was used for the screening of lipase producing bacteria in the soil samples. In this highly specific and sensitive assay, Rhodamine B and olive oil are incorporated in the screening medium. Lipase production in the medium leads to lipolysis of olive oil resulting in formation of free fatty acids. Free fatty acids form a fluorescent complex with Rhodamine B that is incorporated in the medium. Thus the lipase-producing colonies give a fluorescent halo that is visible under UV light.

The Mineral growth medium (MGM) used for screening of lipase producing bacteria was prepared according to the composition given by Makula, and Finnerty, 1968 (3). MGM contained the following ingredients MGM were; Na_2HPO_4 12 g/l, KH_2PO_4 2 g/l, $MgSO_4 \cdot 7H_2O$ 0.3 g/l, $CaCl_2$ 0.25 g/l, Ammonium sulphate 2g/l. The only modification was addition of Olive oil 2% v/v since olive incorporation of oils induces lipase activity. The medium was adjusted to pH 7.0, autoclaved and cooled to about 60°C. Then, 31.25 ml of olive oil and 10 ml of Rhodamine B solution (1.0 mg/ml distilled water and sterilized by filtration) was added with vigorous stirring. The medium was then poured into petri-plates under aseptic conditions and allowed to solidify.

The samples were diluted and the dilutions were plated on Rhodamine Olive Oil Agar. Lipase producing strains were identified on spread plates after incubation for 48 h at 37 °C. The hydrolysis of substrate causes the formation of orange fluorescent halos around bacterial colonies visible upon UV irradiation.

Lipase production in submerged culture

The culture was grown in 100-mL Erlenmeyer flasks containing 20ml of mineral medium. The contents were sterilized by autoclaving at 121°C for 15 min. After cooling, the sterilized medium was inoculated with one loop-full of culture from the plates. The flasks were incubated at 37°C in a rotating shaker at 150 rpm for 8 days. The clarified supernatant was used as a source of extracellular enzyme.

Maintenance of Culture

The isolated cultures are maintained in Nutrient agar slants at 37°C. These cultures are revived after every 15 days.

Lipase Assay

The samples collected from the culture broth were centrifuged for 15 min at the speed of 5000 RPM and the supernatant was assayed for extracellular lipase activity. Lipase was determined titrimetrically on the basis of

olive oil hydrolysis by modified method of Jensen, 1983 (4). One ml of culture supernatant was added to assay substrate containing 10 ml of 10% (w/v) homogenized olive oil in 10 % (w/v) gum acacia, 2ml of 0.6% (w/v) CaCl₂ solution 5 ml of phosphate buffer (pH 7). The enzyme substrate was incubated on rotator shaker with 150 RPM at 30°C for 1 hour. To stop the reaction 20ml of acetone: alcohol (1:1) mixture was added to reaction mixture. The liberated fatty acid were titrated with 0.1 NaOH using phenolphthalein as an indicator.

One unit of lipase activity was defined as the amount of enzyme that liberated 1milli mol fatty acid per min at 37°C and at pH 7 under the assay conditions.

1 unit of Lipase activity = Volume of NaOH consumed (ml) × molarity of NaOH / volume of lipase × reaction time.

RESULTS AND DISCUSSION

Isolation of lipolytic bacteria

Lipolytic bacteria inhabit the natural environments rich in lipids. Considering this we selected the site of regular oil spill in Shiv Mandir premises, Faridabad. The samples were diluted and the dilutions were plated on Rhodamine Olive Oil Agar (ROA) plates and incubated at 37°C for 48h. The plates were observed under UV light. The lipase producing strains could be easily identified by the formation of orange fluorescent halos around the colonies (Figure 1). The isolated colonies were purified by repeated plating in the nutrient agar plates. Pure cultures of different isolates were obtained and were designated as ISC1 and IST1.

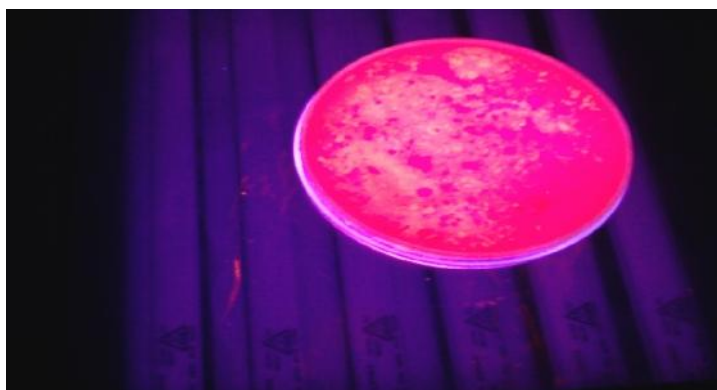


Figure 1: Lipase positive colonies on ROA under UV light.

Three different lipase producing organisms were purified from the soil sample (Table 1). The isolated strains were subjected to biochemical tests according to the Bergey's manual of bacteriology for identification (5). The study results were recorded in Table 1.

Table 1.Colony characteristics and nature (of Isolate 1 and 3)

| S.N | Colony characteristics | ISC1 | IST1 |
|-----|------------------------|-------------------|-----------------------------|
| 1 | Shape | Circular in shape | Round in cluster and tetrad |
| 2 | Colour | white | white |
| 3 | Margins | undulated | entire |
| 4 | Size | 1mm | 1µm |
| 5 | Morphology | Rods | Ellipsoid |
| 6 | Gram nature | Gram-positive | Gram negative |
| 7 | Motility | motile | Non motile |
| 8 | Gram stain | Gram +ve | Gram +ve |

Lipase producing bacterial strains ISC1 and IST1 were cultured for 7 days in MGM growth medium at pH 7 and 37°C. The culture was incubated for 7 days and the growth was harvested by centrifugation and the lipase activity in the supernatant was determined.

Table 2. Lipase production by different isolates.

| Isolate | Lipase activity (milli-units/ml) |
|---------|----------------------------------|
| ISC1 | 19 |
| IST1 | 15 |

The maximum activity was observed in the case of isolate 1 (Table 2). So all the activities and optimization was carried out in the presence of isolate 1 (ISC1). The culture was now subjected to biochemical test that aid in identification of the bacteria according to Bergey's manual of determinative bacteriology (5). The results of the tests were tabulated in Table 3:

Table 3: Biochemical tests for primary identification of ISC1

| SN | Test | Result |
|----|---------------|----------|
| 1 | Endospore | Positive |
| 2 | Catalase test | Positive |
| 3 | Amylase test | Positive |

The rod like, motile, Capsule forming bacteria were isolated from temple soil. A pure culture of this isolate was obtained and was referred to as ISC1. It can be inferred from the morphological and biochemical study that they could be considered to be a strain of *Bacillus*.

Medium optimization

Production of lipases has been found to be majorly inducible by presence of lipase substrates. Lipase production by various *Bacillus* sp. was stimulated in the presence of Ca^{2+} alone (6, 7) or in combination with other ions such as Mg^{2+} , and Fe^{2+} (8). Therefore calcium chloride, magnesium sulphate and olive oil were incorporated in the basal medium used for initial studies. Some researchers have proposed that addition of emulsifiers such as Tween and gum arabic increase lipase production (9). The inductive effect of oils differs with type of oil used (10). We have therefore studied the effect of gum acacia as an emulsifier and different oils on lipase production by the isolate ISC1.

Effect Of Emulsifier

It has been reported by different researchers that non-metabolizable polysaccharides such as gum arabic are able to augment the lipase production. To investigate the effect of emulsifier on lipase production, MGM medium containing 2% v/v olive oil was emulsified with 5% w/v gum acacia was inoculated with Isolate ISC1 and incubated at 37°C in a rotating shaker at 150 rpm for 7 days. The lipase activity obtained after 7 days of culture time was compared with the lipase activity obtained in un-emulsified medium (Figure 2). In the presence of emulsifier the lipase production increases by 15%. Therefore further studies were performed with a lipase production media emulsified with 5% w/v Gum acacia. The increase in production is credited to increase in the interfacial area between oil and water owing to the emulsification of culture media (11).

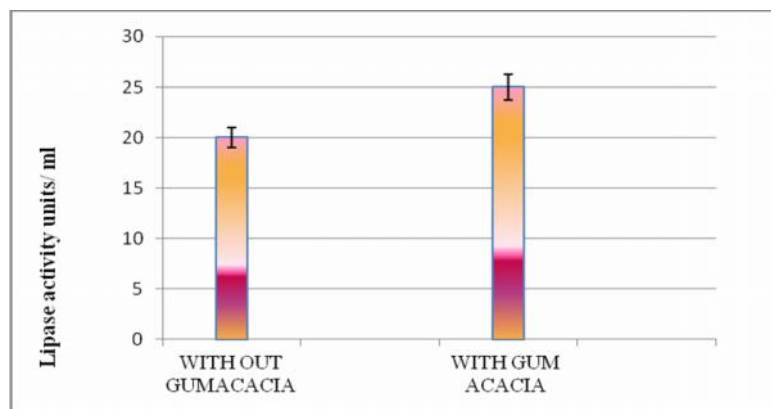


Figure 2: Effect of gum acacia on the production

Effect of different oils on the lipase production

The oil included in the lipase production media is utilized by the lipase producers as the carbon source and also it induces lipase production (12). We used different types of oils as the sole carbon source in the production media. Previous studies have shown that maximum lipase production occurs with olive oil. We used olive oil, mustard oil, soya oil, and coconut oil as carbon sources and lipase production was monitored.

ISC1 was inoculated in MGM (pH 7.0) containing 10% v/v of different oils (Mustard oil, soyabean oil, olive oil, coconut oil). And the flasks were incubated at 37°C, lipase production was monitored every 12h (Figure 3). Maximum activity (u/ml) was obtained in medium containing Mustard oil at 48h. Therefore we have used mustard oil supplemented MGM in all our further studies.

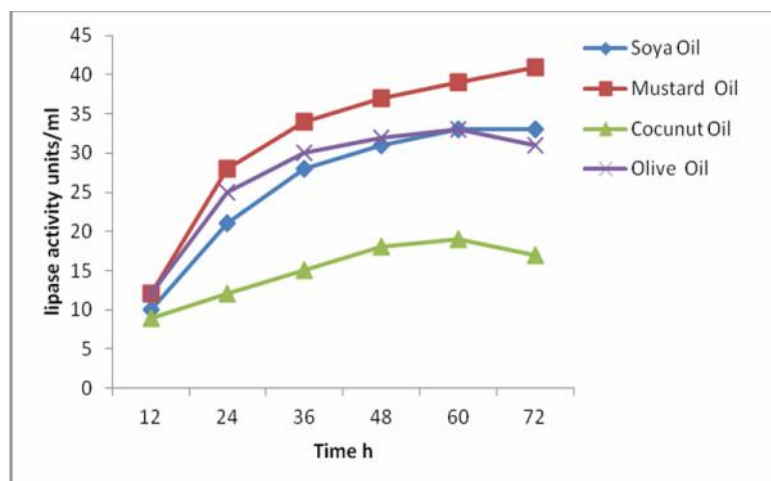


Figure 3: Effect of different oils incorporated in MGM on lipase activity

Effect of media pH

The ISC1 was inoculated in MGM formulated with 5% w/v gum acacia and 10% v/v mustard oil. The Ph of the medium adjusted to different Ph (at 4,5,6,7,8, and 9) and incubated at 37°C and 150 RPM. The samples were withdrawn after 48 h and the lipase activity obtained was determined. The maximum activity of 42 units/ml was found to be expressed at pH 7 (Figure 4). Therefore media pH of 7 was used for further investigations.

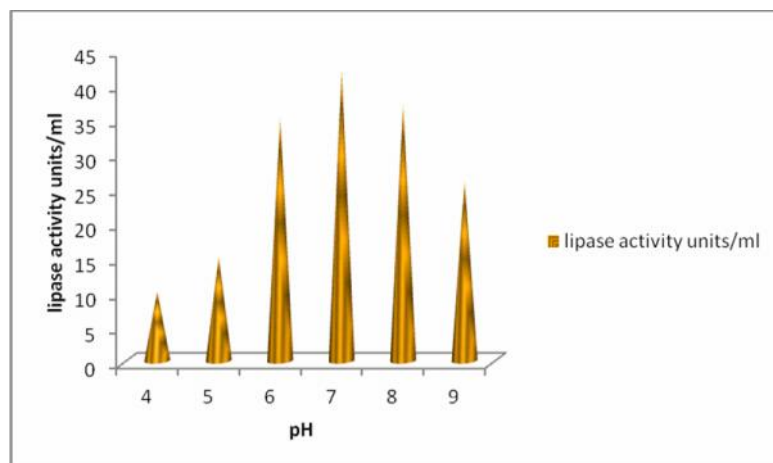


Figure 4: Effect of medium pH on Lipase production

Time course of lipase production

Isolate 1 was inoculated in MGM pH 7 (having mustard oil and gum acacia) the flasks were incubated at 37°C. The lipase activity was determined after different intervals of time. The maximum activity was observed at 48h. Figure 5 represents time course of growth of isolate 1.

The detectable lipase activity was obtained only after 16 h of culture period. Beyond these incubation periods, prolonged incubation didn't help any further increase in the lipase yield which might be related to the other components in the medium or change in pH of the medium.

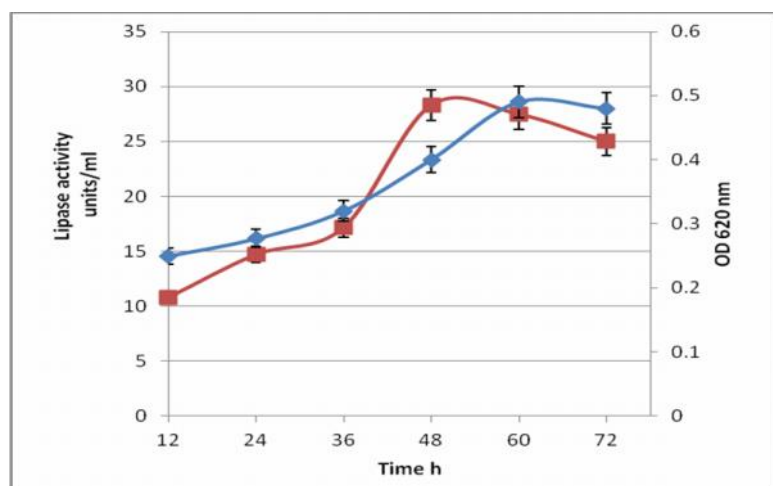


Figure 5: Time course of growth and lipase production by ISC1

CONCLUSION

The isolate ISC1 tentatively identified as *Bacillus* showed high lipase production amongst the different isolated. Inclusion of gum acacia as emulsifier increases the lipase production by 15%. Mustard oil incorporated as carbon source led to highest lipase production as compared to olive oil, coconut oil and soya oil. The maximum lipase activity of 41 u/ml was obtained at temperature 37°C, pH 7.0 after 48 h of culture.

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