SCREENING, CHARACTERIZATION AND OPTIMIZATION OF LIPASE PRODUCING Bacillus SP. ISOLATED FROM PETROLEUM HYDROCARBONS CONTAMINATED SITE

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ABSTRACT

The present study was aimed at isolating lipase producing bacteria from different soil and water samples which are rich in lipid content like diesel oil spillage. As oil contaminated samples are rich in lipid and fatty acid content it makes a very good source to find microorganisms incapable of degrading lipids byproducing lipase enzymes. In the present study, the isolates were characterized by morphologically and biochemically. Lipase activity was optimized by varying both physical and chemical parameters such as pH, temperature, incubation period and carbon, nitrogen and substrate sources as well lipid substrate. Tween 80 fructose and extract was found to be appropriate substrate for lipase production. Also lipase activity was optimum at 50°C with pH 8. Also lipase production from Bacillus sp. was found to maximum at incubation period of 48 hrs.

Key Word: Lipase, Bacillus sp., Biodiesel, Aromatic, transesterification

INTRODUCTION

Lipases (E.C. 3.1.1.3) have emerged as key product of rapidly growing biotechnology industry. Lipase enzyme has versatile applications by virtue of their unique properties. Lipases have been applied in a wide array of industrial applications, such as food technology, detergent, chemical industry and biomedical science, due to their shorter generation time, ease of bulk production, which is further improved with advancement in fermentation technologies and ease of manipulation, either genetically or environmentally. Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) catalyze the hydrolysis and the synthesis of esters produced from glycerol and long-chain fatty acid. Lipases occur extensively in nature, but only microbial lipases are commercially significant. Lipase producing microorganisms have been found in diverse habitats such as industrial wastes, vegetable oil processing factories, dairies, soil contaminated with oil, etc. Many microorganisms such as bacteria, yeast and fungi are known to secret lipases. Of all these, bacterial lipases are more economical and stable. Biodiesel, production through transesterification of fat or vegetable oils has emerged as an attractive substitute topetroleum fuel, since it is biodegradable, nontoxic and essentially sulphur free and non-aromatic. Existing industrial biodiesel production chiefly relies on base-catalyzed transesterification of animal fat or vegetable oils, which have the drawbacks of pollution during the removal of catalyst and the requirement of energy-intensive process, because it require high temperature and pressure. Therefore, many researchers consider the use of lipase to catalyze biodiesel production under mild condition. Whereas, nitrogen source and other media components regulate the growth of producer organism and thus the fermentation process. Gram-positive lipase producers are Staphylococcus (especially, S. aureus and S. hyicus), Streptomyces and Bacillus species. The most useful lipase producer genus used in industry is Bacillus among gram-positive bacteria. Most bacterial species studied for lipase production are non-pathogenic.

AIMS AND OBJECTIVES

Isolation, screening, characterization and optimization of lipase production from Bacillus sp.
MATERIAL AND METHODS

Sample collection and isolation of bacterial strains

For the present study, soil sample was aseptically collected from oil-spilled areas of automobiles industry (Hisar) in a sterile container for the isolation of lipase producing organisms under laboratory condition. The soil surface was characterized as black hard and there was no vegetation grown on soil. The bacterial species present in the collected soil sample were isolated using standard nutrient agar media.

Screening of isolated bacteria on basis of lipase production

To begin with, lipolytic microbes were isolated from the collected soil sample. For this, 1.0 g of soil was dissolved in 100 ml of distilled water. Then it was serially diluted (10^{-1} to 10^{-6}) and the diluted samples were plated on nutrient agar for total viable count. Then, the dominant organisms were isolated and individually streaked on tributyrin, Tween 80 and Rhodamine agar plates.

Tributyrin agar plate assay

Lipase producing micro-organisms produced a zone of clearance (hydrolysis) when their inappropriate dilutions were spread on the TBA medium containing per liter of peptone, 5g; beef extract, 3g; tributyrin, 10ml and agar-agar, 20g. The zone size was examined after 12, 24, 36 and 48 h of incubation at 37°C.

TWEEN 80 agar plate assay

The lipolitic activity of bacterial strains was observed on Tween 80 medium composed of (g/L): Peptone, 10; NaCl,5; CaCl_{2}.2H_{2}O, 0.1; agar-agar, 20; Tween 80, 10 ml (v/v). Zone of precipitation observed as an indication of lipase activity.

Rhodamine agar plate assay

A sensitive and specific plate assay for revealing of lipase producing bacteria makes use of Rhodamine-olive oil-agar medium. The growth medium contained (g/L): nutrient broth, 8.0; NaCl, 4.0 and agar-agar 20. 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. It was then poured into petri plates under aseptic conditions and allowed to solidify. The bacterial culture was inoculated on the medium. 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 assay/lipase activity

For production of lipase : 5ml of seed culture inoculated into 50 ml of fermentation medium for 24 hrs. Fermentation liquid centrifuged at 6000 rpm for 15 minutes and clear supernatant containing lipase determined by using spectrophotometrically method using PNPP (P-nitrophenol palmitate) as substrate. One unit of lipase activity was defined as the amount of enzyme, which released one U/ml of Pnpp in 1 min. under assay condition (37°C, pH 8.0) Lipase yield of fermentation liquid expressed asumol/ml. Lipase activity calculated by using the formula :

\[ \text{Activity} \text{(U/ml)} = A \times 20 \times (\text{Dilution factor}) \]

Identification

The isolated dominant organisms were identified as Bacillus sp. based on morphological, biochemical and physiological characters according to Bergey’s manual of determinative bacteriology.

Biochemical characterization

Biochemical characterization of bacterial isolates was done by various biochemical tests like indole test, MR-VP test, simmons citrate, starch hydrolysis, H_{2}S production, catalase, oxidase, urease, nitrate reduction test and gelatin hydrolysis test.

Optimization of lipase activity of Bacillus sp.

Effect of incubation period

Lipase production was carried out for 96 hours and the samples were collected after every 24 hours to check the production of lipase and growth.

Effect of pH and temperature

Lipase production and growth of Bacillus sp. was observed on media with pH 5, 6, 7, 8 and 9 and temperature 30,37,45,50°C in shaking cum incubator at 120 rpm.
Effect of various carbon and nitrogen sources

Different carbon sources such as fructose, sucrose, lactose and dextrose (1% v/v) were added fermentation media at pH 8.0. Similarly, for optimization of nitrogen source substances such as beef extract, yeast extract, eptone and tryptone (1% v/v) were added to the media containing optimum carbon source.

Effect of lipids substrate on enzyme production

The production of lipase was carried out at different lipids (Tween 80, Tween 20, olive oil and coconut oil) (1% v/v) in the production media with pH 8 and 37°C.

RESULTS AND DISCUSSION

Enrichment culture technique enabled the isolation of strains with lipolytic activity in tributyrin, tween 80, Rhodamine agar media plates. In the plate assay method, bacterium forming the largest clear zone was further studied for lipase activity by employing the enzyme assay method found that Bacillus sp. gram positive, have potential of lipase production. In total 25 bacterial isolates were collected from different oil spilled soil sample. Among them 2 showed maximum lipase activity belong to Bacillus and Staphylococcus sp. The lipolytic microbe was further screened and characterized by its features and reactions and then identified as Gram positive, spore forming, motile organism (Table 1). Finally, the morphological and biochemical tests indicated that the suspected microorganism was Bacillus sp. and selected for further study.

Table 1 : Morphological identification and biochemical characterization of isolate

<table>
<thead>
<tr>
<th>Cultural characteristics</th>
<th>Colony morphology on agar plate</th>
<th>Colony morphology on agar plate</th>
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<tbody>
<tr>
<td>Microscopic characteristics</td>
<td>Gram staining, spore formation, motility</td>
<td>Gram positive, spore forming, motile</td>
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<tr>
<td>Biochemical characteristics</td>
<td>Indole test</td>
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<td></td>
<td>Methyl red</td>
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<td></td>
<td>Voges test</td>
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<td></td>
<td>Oxidase test</td>
<td>positive</td>
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<tr>
<td></td>
<td>Urease test</td>
<td>Negative</td>
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<tr>
<td></td>
<td>Nitrate reduction test</td>
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<td>Starch hydrolysis test</td>
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<td>Hydrogen sulphide test</td>
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<td></td>
<td>Glucose fermentation test</td>
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<td></td>
<td>Citrate utilization test</td>
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Fig. 1 to Fig. 3 showing zone of precipitation and clearance zone in Tween 80 and Trybutyrin agar plates indicating production of lipase. After incubation for 48 hrs at 37°C and photographed. Rhodamine agar plate showed orange halos on irradiation of ultraviolet radiation. The efficiency of lipolytic Bacillus sp. was assayed at different incubation period. The highest lipase activity was found to be 49.5 U/ml/min. at 48 hrs (Fig. 4). This
result is also supported by Tembhurkar, found similar result in Pseudomonas sp. while Staphylococcus spp. has maximum lipase production at 24 hrs.\textsuperscript{19,20}

Optimum activity of enzymes is pH dependent and varies from one enzyme to another. The pH of the environment influences the growth of organisms to a greater extent. The results on the effect of medium pH on the tested organism indicated that the lipase production were maximum (49.5 U/ml/min) at pH 8. (Fig. 5) and found to be less at pH 5,6,9 respectively. Similar result from Bacillus subtilis and Psuedomonas sp. for lipase activity revealed by Prasad and Tembhurkar \textsuperscript{5,19} Another finding also revealed lipase activity was maximum in Pseudomonas aerogionosa and Bacillus licheniformis between pH 7 to 10.\textsuperscript{21,22} Temperature is a critical parameter that has to be controlled, it varies from organism to organism and it influences secretion of extra cellular enzymes. The lipase activity of was high 48.6 U/ml/min. at 50 °C (Fig. 6) from Bacillus sp. when grown at the medium temperature of at the optimum pH of 8.0, but it have been found less at both incubation temperature 30 and 37 °C. Similar to lipases from other microorganisms, such as Bacillus licheniformis was found to be maximum at 55°C.\textsuperscript{23} it was reported that Pseudomonas aerinoussa showed enhanced lipase activity at incubation temperature 35 to 40°C. The optimum temperature for lipase activity was found to be at 55°C in B. licheniformis.\textsuperscript{24} Enhanced lipase production have been found in Psuedomonas sp. at 50°C.\textsuperscript{19} Also nitrogen and carbon sources have significant effects on lipase activity. Generally, microorganisms provide high yields of lipase when organic nitrogen sources are used, such as peptone and yeast extract. Lipase production is influenced by the type and concentration of carbon and nitrogen sources. Among 4 different organic nitrogen sources yeast extract 44.01U/ml (Fig.7) showed enhanced lipase activity of Bacillus strain. Similar, finding have been observed in various thermophilic Bacillus licheniformis and Pseudomonas.\textsuperscript{25} This result is also supported with the finding of Sirisa et al, according to their studies lipase production by Staphylococcus was better when peptone was used in place of yeast extract and tryptone as nitrogen source.\textsuperscript{25} Major content of lipase production media is carbon source which also act as inducer for lipase production. Since microbial lipases are often inducible enzymes.\textsuperscript{26} In our present study, isolated Bacillus sp. has maximum lipase yield in fructose (45.65 U/ml) (Fig.8) among various carbon sources but it was surprising to note that control has enhanced lipase production.According to another report mannose was found to be best carbon source for lipase production in Bacillus sp.\textsuperscript{22} In present investigation, it has been reported that lipid substrate have their significant effect. In our present investigation, Tween 80 with lipase activity (38.6 U/ml/min.) (Fig.9) appeared to be potential inducer for lipase production.\textsuperscript{27} This results also supported in case of Bacillus sp.\textsuperscript{28-33} In another report, it has been found that olive oil support best lipase production.\textsuperscript{34-39}

![Figure 1: Tween 80 agar plate](image1)
![Figure 2: Trybutyrin agar plate](image2)
![Figure 3: Rhodamine agar plate](image3)
Fig. 4: Optimization of incubation time for lipase production

Fig. 5: Optimization of medium pH for lipase production

Fig. 6: Optimization of incubation temperature for lipase production
Fig. 7: Effect of additional nitrogen sources on lipase production by *Bacillus* sp.

Fig. 8: Effect of sugars as additional carbon source on lipase production by *Bacillus* sp.

Fig. 9: Effect of various lipid substrates on lipase production by *Bacillus* sp.
CONCLUSION

Maximum production of lipase was obtained from batches isolated from hydrocarbon contaminated soil which was found to be of Bacillus sp. According to the result, cultivation at 120 rpm shaking, Bacillus sp. produced the highest amount of lipase after 48 h. Growth of Bacillus sp. with different nutritional and other physicochemical parameters revealed maximum lipase activity with fructose as carbon source, yeast extract as a nitrogen source with pH 8. Results also showed that the optimal temperature for lipase production was found to be 50ºC. Among carbon sources fructose showed highest lipase activity on the other hand yeast extract as an additional nitrogen source showed maximum lipase activity of Bacillus sp. On the other hand tween 80 showed maximum lipase production among tested lipid substrates. The results are promising because the strain produced more lipase activity after optimization as compared to the non-optimized growth conditions.

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