

Screening and Production of Lipase Producing Bacteria from Vegetable Oil Industry

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ABSTRACT

Lipases are industrially important enzymes having applications in numerous industries. The present study aims to isolate different bacteria and screen them for lipase production. Oil spilled soil samples were collected from vegetable oil industry of Harur, Dharmapuri district. A total of thirteen morphologically distinct bacteria isolated were screened for lipase production in Tween 80 agar plate. Lipolytic activity was confirmed by zone of precipitation around the colony. Among thirteen isolates, six isolates exhibited lipolytic activity in plate assay. The bacterial isolate HLB7 produced high amount of lipase 2.6U/ml in lipase production medium by submerged fermentation was identified as *Pseudomonas* sp. The optimum incubation time, inoculum size, temperature and pH for lipase production were determined. The lipase production was maximum at incubation time 48 hr (3.26U/ml), Inoculum size 4% (4.37U/ml), temperature 40°C (4.65U/ml) and pH 7 (4.95U/ml) by the *Pseudomonas* sp.

Keywords : Oil Spilled Soil, Lipase, Fermentation, *Pseudomonas*, Tween80 Agar

I. INTRODUCTION

India is amongst the largest producer and consumer of vegetable oils in the World. Vegetable oils are generally obtained through extraction and refining processes of oils and fats from vegetable and animal sources [1,2]. Environmental pollution due to oil processing industry is one of the major problems faced by the industrialized world today. Vegetable oil processing activities generate significant quantities of organic solid waste and by-products [3]. Oil product spills are possible in all stages of oil extraction, transportation or usage which strongly affect the ecosystem [4,5,6].

Thus, there has been constant research on bioremediation of lipid-rich waste, either aerobically or anaerobically. The conventional treatment methods are discouraged due to their disadvantages [7]. In order to restore the biological properties of soil, the biological treatment by enzymatic hydrolysis is preferred due to the advantages of high selectivity and specificity [8].

Lipases (triacylglycerol acylhydrolases; EC 3.1.1.3), which catalyze both hydrolysis and ester synthesis, are the most important class of hydrolytic enzymes [9]. Lipase-producing microorganisms have been found in diverse habitats such as industrial wastes, fats and oil processing factories, dairies, oil contaminated soils, oil seeds and decaying foods, compost heaps, coal tips and hot springs [10,11].

Microbial lipases have applications in fats and oils processing, food processing, synthesis of fine chemicals, cosmetics, pharmaceuticals, detergents and biosurfactants [12,13,14].

A variety of lipases were isolated from many different bacteria such as including *Bacillus* [15,16], *Pseudomonas* [17,18,19], *Staphylococcus* [20,21], *Acinetobacter* [22], *Enterococcus* [23], *Geobacillus* sp [24].

The industrial demand in view of the diversity in applications for lipolytic enzymes continues to stimulate the search for new enzyme source. Hence the present

study has been carried out to isolate a novel lipase producing bacteria from oil spilled soil of vegetable oil processing industry and optimize its growth conditions, including incubation time, inoculum size, temperature and pH for lipase production.

II. METHODS AND MATERIAL

Isolation of lipolytic bacteria

Soil samples were collected from different vegetable oil extracting units, Harur, Dharmapuri districts of Tamilnadu State, India in a sterile container by adapting aseptic technique from a depth of 5-10 cm. Lipolytic bacteria were isolated by enrichment culture method. 1gm of soil sample was aseptically transferred to the 100ml of tributyrin broth and incubated in a rotary shaker at 37°C for upto 72 hrs. After incubation 1ml from each enrichment culture was spreaded over the tributyrin agar medium plate and incubated at 37°C for upto 48 hrs.

Screening of the bacterial isolate for lipolytic activity

The lipolytic activity of isolated colonies were observed by spot inoculation on Tween 80 agar medium plates and incubated at 37°C for 48 hrs. Lipase production is indicated by the formation of precipitation around the bacterial isolate. The isolates which had exhibited high lipolytic potential were selected and screened further for efficient lipase production.

Lipase enzyme production by submerged fermentation

Six bacterial isolates which shows lipolytic activity in the Tween 80 screening medium were selected for the lipase production by submerged fermentation. The composition of lipase enzyme production medium used in the study was according to veerapagu et al. [25]. 2ml of the each isolate was inoculated into a 500 ml Erlenmeyer flasks containing 100 ml of lipase enzyme production medium on a rotary shaker (120 rpm) and incubated at 37°C upto 48 hrs.

Extraction of Enzyme

After the desired period of incubation the extracellular lipase enzyme was extracted by centrifugation at 10000 x g for 30 min in a refrigerated centrifuge. The resulting supernatant contained crude extracellular lipase was used as source of enzyme for enzyme assay. The pellet was retained for estimation of total cell biomass. Biomass concentration was estimated by measuring

optical density at 600 nm using a UV-Vis spectrophotometer (Systronics, India) and a standard curve previously determined (Biomass g l⁻¹ = 0.270 X OD600 nm) [26].

Lipase enzyme assay

The lipase activity of crude extracellular lipase enzyme extracted by centrifugation was assayed by titrimetric method using olive oil emulsion [27]. The reaction mixture containing 5ml of olive oil emulsion, 2 ml of 0.03 % Triton X-100, 2 ml of 3 M NaCl, 1 ml of 0.075 % CaCl₂ and 5 ml of phosphate buffer and 1 ml of crude enzyme extract was incubated at 37°C for 15 mins. The reaction was terminated by adding 20ml of ethanol: acetone (1:1, v/v) to the mixture. The free fatty acids liberated during the reaction was titrated against 0.02 N NaOH using phenolphthalein as indicator. The control containing the reaction mixture and ethanol:acetone solution without the addition of enzyme was titrated against 0.02 N NaOH similarly. One unit of lipase enzyme was defined as the amount of enzyme that released 1 μmol of fatty acid per minute under assay conditions.

Characterization of the bacterial isolate

The isolate which produces maximum lipase production by submerged fermentation was selected as a novel lipase producing bacteria and identified by phenotypic characterization based on morphological, biochemical and physiological characters [28].

Selection of Bioprocess variables

Influence of Incubation Time

The bacterial isolate HLB7 which shows maximum lipase production among the six isolate was selected for further optimization studies. The effect of incubation period on growth and lipase production was monitored in the lipase production medium used previously for screening for 72 hrs. Samples were harvested at every 8hr intervals for both bacterial growth and lipase production.

Statistical analysis

All experiments were carried out in triplicates and the results are presented as the mean of three independent observations. Standard deviation for each experimental result was calculated using Graph Pad Prism 6.0.

Optimization of Inoculum concentration

The effect of inoculum concentration for production of lipase by bacteria isolate HLB7 was studied by varied inoculums concentration (1% to 10 %) to the enzyme production medium on a rotary shaker (150 rpm) and incubated at 37°C upto 48 hrs.

Optimization of Incubation Temperature and medium pH

The effect of incubation temperature and pH on lipase production was studied by varying temperatures from 20°C to 50 °C and medium pH from 4 to 10 while other parameters are remain unaltered.

III. RESULTS AND DISCUSSION

Lipase-producing microorganisms have been found in diverse habitats such as industrial wastes, vegetable oil processing factories, dairies, soil contaminated with oil, oilseeds, and decaying food , compost heaps, coal tips, and hot springs [29]. Soil is a reservoir of a large and diverse microbial population. Lipase producers have been isolated mainly from soil that contains vegetable oil [30]. Bioremediation, the use of microorganisms or microbial process to detoxify and degrade the oil effluents is among the innovative technologies. In the present study oil spilled soil samples from vegetable oil processing units from Harur, Dharmapuri district, Tamilnadu were collected to isolate a novel lipolytic bacteria. After the enrichment of soil samples thirteen morphologically different bacteria were isolated by spread plate method in tributyrin agar medium plate.

All the thirteen isolates were screened for the lipolytic activity by Tween 80 agar qualitative plate assay. Among the thirteen bacterial isolates six isolates showed zone of precipitation. Screening using tween agar plates shows precipitation around the lipase producing micro-organisms [31]. Evaluation of the lipase producing efficiency, based on the clear precipitation zone around colony, indicated that six bacterial isolates could produce lipase enzyme. The isolate HLB7 showed high lipolytic activity 15mm (Table 1).

In order to select a best lipase producing bacteria six bacterial isolates were investigated for the production of lipase by submerged fermentation. The bacterial isolate HLB7 produced 2.6U/ml was selected for further study

while others produced less than 1.2U/ml (figure 1). The isolate HLB7 which produced high amount of lipase than the other was preliminarily examined for morphological characteristics by gram staining, shape, motility and for the presence of endospore. The results of the morphological and biochemical characteristics of the lipase HLB7 was presented in table 2. From the results it is inferred that the selected isolate HLB7 belongs to *Pseudomonas* sp.

Selection of physical parameters for lipase production

The effect of incubation time on growth and lipase production revealed growth starts at 8 hr of incubation but lipase production does not occurred and it starts after 16hr of incubation time. Both growth (3.76g^l⁻¹) and lipase production (3.26U/ml) is maximum at 48hr of incubation time (Figure 2) and further increase in incubation period did not lead to an increase in lipase production, rather a slow decrease in lipase yield was observed. The lipase activity appeared to couple with growth .The enzyme production was found to be maximum when the cell population entered into the stationary phase suggesting that enzyme secretion is growth associated. These results are in agreement with earlier results [32, 33] where maximum lipase activities were observed at the onset of the stationary phase of bacterial growth. It was reported that maximum lipase production was obtained at 48hrs for *Pseudomonas putida* [34], staphylococcus [35] *Trichoderma viride* [36] and 72 hrs for *Pseudomonas* sp [37] and *Bacillus coagulans* [38].

Table 1: Lipolytic activity of bacterial isolates in Tween 80 agar plates.

Bacterial Isolate	Zone of precipitation diameter mm	Bacterial Isolate	Zone of precipitation diameter mm
HLB1	-	HLB8	4
HLB2	10	HLB9	-
HLB3	-	HLB10	-
HLB4	2	HLB11	7
HLB5	-		6
HLB6	-	HLB13	-
HLB7	15		

Table 2: Characterization of the bacterial isolate HLB7

Test	Result
Gram's Staining	Gram Negative
Shape	Rod
Motility	Positive
Spore Staining	Negative
Oxidase Test	Positive
Citrate Test	Positive
O/FGlucose test	Oxidative
King's B medium	Positive
King's A medium	Negative
Nitrate Reduction test	Positive
Levan Production Test	Positive
Indole Test	Negative
Methyl Red Test	Negative
Voges-Proskauer test	Negative

The inoculum concentration was studied to obtain optimum size of inoculum and results indicated that lipase production increased with an increase in inoculum size (Fig. 3). Maximum lipase production (4.37 Uml⁻¹) was obtained at 4% (v/v) inoculum size, however, further increase in inoculum size resulted in sharp decrease in lipase production, especially when 8% (v/v) inoculum size was used. YU et al [39] reported that highest lipase was produced when the inoculums concentration was 6% for *Pseudomonas* Lip35.

The Influence of temperature on growth and lipase production of *Pseudomonas* was investigated by different temperatures ranges from 20°C to 50°C at an interval of 5°C. The growth was maximum (4.13 gl⁻¹) at 40 °C and lipase production was found to be maximum (4.37 Uml⁻¹) at 35°C (Figure 4). The result showed that the growth of the *Pseudomonas* sp was not affected at temperature above 35 °C but the production of lipase was decreased. This implies that the increase in temperature immediately affect the metabolic activity of a bacteria i.e enzyme synthesis rather growth. Temperature influences secretion of extra cellular enzymes by changing the physical properties of the cell membrane. It was reported that the maximum lipase production was at 37°C by *Pseudomonas xinjiangensis* [40]. Temperature is a critical parameter that has to be controlled and it varies from organism to organism [41].

The initial pH of the culture medium was found to be one of the most important critical environmental parameter which affects the growth and enzyme production by *Pseudomonas* sp. Maximum biomass (5.34 gl⁻¹) and lipase production (4.95 Uml⁻¹) was

obtained at an initial medium pH of 7.0 (Figure 5). Lipase production and biomass decreased significantly at pH of 8.0 and above. The similar optimal pH 7 for maximum lipase production was also reported for *Pseudomonas aeruginosa* [42,43,44], *Pseudomonas* sp.[45], pH 7.5 *Pseudomonas* sp [46] and *Bacillus subtilis* [47]. In general bacterial lipases are produced over a wide range of pH from acidic, neutral and alkali [48,49,50,51].

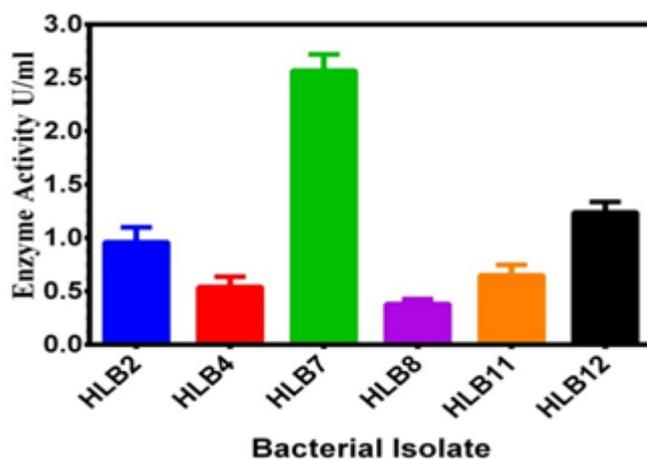


Figure 1: Production of lipase by the selected bacterial isolates. The values are mean ± SD of three replicates

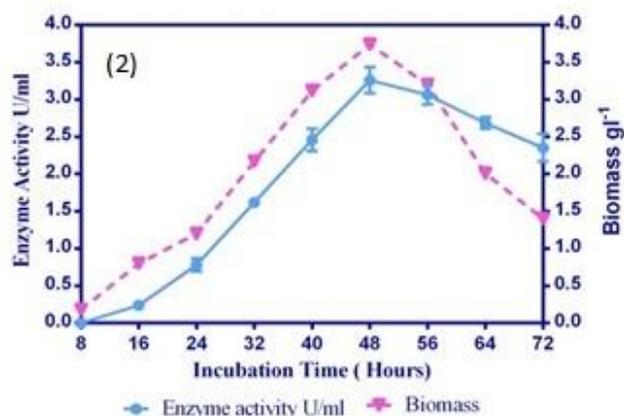


Figure 2: Effect on incubation time on growth and lipase production.

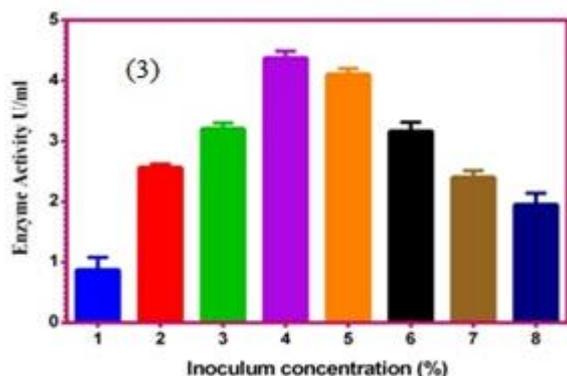


Figure 3: Effect of inoculum concentration on lipase production.

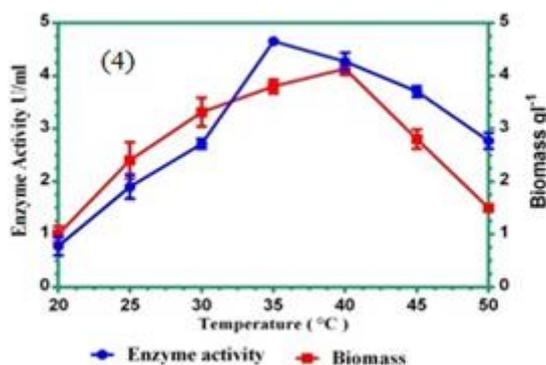


Figure 4: Effect of incubation temperature on growth and lipase production.

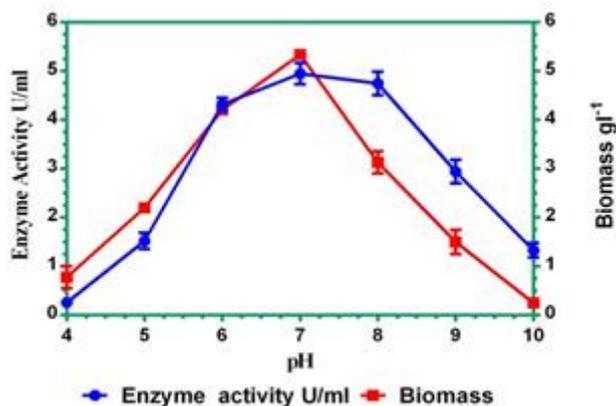


Figure 5: Effect of medium pH on growth and lipase production. The values are mean \pm SD of three replicate.

IV. CONCLUSION

In the present study an attempt was made to isolate lipolytic bacteria from the oil spilled soil. The isolate HLB7 was identified as *Pseudomonas* sp. and the bioprocess variables for lipase production were optimized. *Pseudomonas* exhibit the ability to biotransform a wide range of organic compounds and are able to degrade various chemical pollutants such as

simple hydrocarbons, aromatic hydrocarbons, nitroaromatics, chlorinated polycyclic aromatics etc. It was concluded that *Pseudomonas* sp could be used as a new bacterial source of lipase which can be useful for the bioremediation of oil contaminated soil.

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