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Optimization of Fermentation parameters for Enhanced production of Lipase from lipolytic *Pseudomonas spp.*

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Abstract

Lipases (Triacylglycerol acylhydrolases, EC 3.1.1.3) are enzymes which catalyze the enzymatic hydrolysis of triacylglycerol into diacylglycerol and fatty acids. Lipases are one of the most important enzymes that are used in various industries like Detergent, Food, Leather, Textiles, Pharmaceuticals, etc. Though various organisms used for the production and purification of lipase for industrial applications, Pseudomonas spp. remains considerably less explored. The present study thus explores the ability of Pseudomonas spp. isolated from oil contaminated soil samples for production of extracellular lipase enzyme. For the present study, 29 oil contaminated soil samples were collected from different regions of Pune, Solapur and Mumbai. Total 50 lipolytic Pseudomonas spp. were isolated by using Tributyrin agar medium containing Rhodamine B. Isolate showing maximum lipase activity (SP45) was then selected for further optimization studies. During optimization of fermentation parameters, SP45 showed optimum lipase activity of 50.4967 U/ml when Glucose was used as a carbon source, while lipase activity of 61.6567 U/ml was observed when Yeast extract was used as a nitrogen source. On optimizing the pH and incubation temperature of the fermentation medium, SP45 showed maximum lipase activity of 66.2567 U/ml in fermentation medium of pH 7at 37^{0}C.

Keywords: lipase, *pseudomonas spp.*, tributyrin, fermentation, optimized parameters.

Introduction

Enzymes play a vital role in our day to day life. Lipases (Triacylglycerol acylhydrolases, EC 3.1.1.3) catalyze hydrolysis of Mono-, Di- and Tri-acylglycerides into glycerol and free fatty acids in presence of water-lipid interface. Although lipase enzyme was isolated and purified from fungi, bacteria, yeast, animal and plant sources, but of all these bacterial lipases are considerably commercially important and physiologically significant. Some of the important industrial applications of lipase include its use in various industries like dairy industry for developing flavors in milk, cheese and butter while food industry uses lipase for shelf life prolongation in bakery items and also in beverages. In addition to this, lipase can also be used in leather industry for hydrolysis of leather products while detergent industry uses lipase for removal of oil stains from fabrics. Lipase acts as a key enzyme in cosmetics industry for production of emulsifiers, moisturizers while as far as textile industry is concerned, lipase can be used for increasing the fabric absorbency. In addition, lipase can be used as a catalyst for production of various products that are used in pulp and paper industry, in biodiesel synthesis and also in pharmaceutical industry[1-3].

Presently bacterial lipases are the enzymes with tremendous demand due to their potential industrial applications and stability. Various bacteria and fungi like *Bacillus spp., Staphylococcus spp., Streptococcus spp., Candida spp., Aspergillus spp.,* etc. are routinely used for the production and purification of lipases for industrial applications[4].

Of all these, the *Pseudomonas spp.* remain considerably less explored. The present study hence explores the potentials of these isolated *Pseudomonas* strains obtained from various oil contaminated soil samples in the production and purification of lipase enzyme with optimized fermentation parameters. For enhanced production of lipase enzyme, different fermentation parameters like pH of medium, temperature at which fermentation process was carried out along with carbon and nitrogen sources of fermentation medium have to be optimized. These fermentation parameters were studied in the present study for enhanced lipase production.

Material and Methods

Collection of soil samples: Soil samples used in the present study were collected from oil contaminated sites like petrol - diesel filling pumps, car-bike servicing centers, oil seeds bearing plants, bus stand-railway car shed, etc. situated in and around Pune, Solapur and Mumbai regions. Oil contaminated soil samples were collected randomly 4-5cm beneath the upper surface using spatula and packed in sterile container. Then these soil samples were transferred to the laboratory for further analysis[5].

Isolation of lipolytic bacteria: Oil contaminated soil samples were serially diluted from 10^{-1} to 10^{-5} dilutions and plated onto the Tributyrin Agar base (pH 7.0) containing Rhodamine B

plates by using spread plate method. Tributyrin Agar base containing Rhodamine B consists of peptic digest of animal tissue 0.5%(w/v), yeast extract 0.3%(w/v), agar 1.5%(w/v), tributyrin 1%(v/v) and Rhodamine B 0.0001%(w/v). Petriplates were then incubated at 37^0C for 24-48 hours in an incubator[6].

Screening of the lipolytic isolates: After 24-48 hours of incubation, Gram negative, rod shaped, motile bacteria which are lipolytic in nature were selected for further studies. The isolated lipolytic bacteria were screened microbiologically by streaking on Tributyrin Agar plates containing Rhodamine B by four quadrant method. These plates were then incubated at 37^0C for 24-48 hours. After incubation, zone of clearance was observed around the bacterial colonies due to the hydrolysis of tributyrin. These isolated bacteria were preserved in the form of glycerol stock preparation for further use[6].

Biochemical characterization of the isolates: Morphological and biochemical characteristics of the isolates were studied for the identification of the isolated bacteria. Morphological characteristics involved bacterial colony characters, Gram's nature and motility of the organism while biochemical characteristics involved sugar fermentation along with different tests like oxidase Test, nitrate reduction test, catalase test and indole test. In this way, isolated lipolytic bacteria were characterized up to genus level with the help of Bergey's manual of Systematic Bacteriology. Species level identification of the selected strain was performed by 16SrRNA sequence analysis[7].

Lipase assay: Activity of extracellular lipase enzyme was measured with the help of polyoxyethylene sorbitan ester (Tween 80) as substrates by the method described by Tirunarayanan and Lundbeck with slight modifications. Tween 80 is the ester of oleic acid. Briefly, the reaction mixture contains 0.1ml of 10% Tween 80 in 50mM Tris hydrochloride buffer (pH 7.6), 0.1ml of 1M $CaCl_2$ in Tris buffer, 0.5ml of concentrated culture supernatant as a source of enzyme, and 2.3ml of Tris buffer (pH 7.6). Reaction mixture with 0.5ml of deionized water instead of supernatant was treated as a blank. Enzyme assay for each isolate was performed in triplicates. Then the reaction mixtures were incubated at 37°C in an incubator for 2 hours. In this assay, Tween was cleaved and a fatty acid and an alcohol were produced. Due to the presence of calcium in the reaction mixture, an insoluble fatty acid salt was formed, giving a precipitate which can be measured spectrophotometrically at 400nm. One unit of lipase activity was defined as that amount of enzyme resulted in an increase of optical density at 400nm (OD400) of 0.01 after 2 hours under the assay conditions[8]. Lipase activity was calculated according to the equation 1 as follows:

$\text{Lipase activity}\,(\text{U/ml}) = \Delta A/0.01$, Where ΔA is the difference between the absorbance@400nm (A_{2hour}-A_{0hour})

Isolate showing maximum enzymatic activity was selected for optimization of fermentation parameters study.

Optimization of fermentation media parameters for enhanced lipase production: Effect of different culture conditions such as pH of medium on the activity of lipase enzyme and temperature at which the culture was incubated were evaluated. The effects of medium components like carbon sources and nitrogen sources on lipase activity were also investigated in the present study. The final amount of SP45 inoculated in the fermentation medium was 1 % (v/v) equivalent to 0.5 McFarland's standard. Then the inoculated medium was kept in an incubator shaker at agitation speed of 110 rpm and 37^0C for 24-48 hours of fermentation. After 24 and 48 hours of fermentation, 10ml medium was taken out for centrifugation at 4000rpm for 15min to get the culture supernatant which can be used in the spectrophotometric lipase enzyme assay[9].

Effect of different carbon sources on lipase activity: The effects of different carbon sources on the activity of lipase were determined by using different components like Glucose, Sucrose, Lactose, Maltose, and Mannitol at a final concentration of 1 % (w/v). After 24 hours and 48 hours of fermentation, spectrophotometric lipase assay was performed and the carbon source showing optimum lipase activity was selected for further studies[6].

Effect of different nitrogen sources on lipase activity: Different nitrogen sources like Peptone, Tryptone, Tryptose, Beef Extract, Yeast Extract and Soyabean Meal at a final concentration of 0.5%(w/v) were studied for determining the effects of nitrogen sources on the lipase enzyme activity. After 24 hours and 48 hours of fermentation, spectrophotometric lipase assay was performed and the nitrogen source showing optimum lipase activity was selected for further studies[6].

Effect of pH of the medium on lipase activity: The influence of initial culture pH on the lipase activity was determined in various media of pH ranging from pH 5.0 to 10.0. The optimum pH for lipase activity was studied in the fermentation medium by keeping remaining parameters unaltered. After 24 hours and 48 hours of fermentation, spectrophotometric lipase assay was performed and pH of fermentation medium showing optimum lipase activity was selected for further studies[6].

Effect of incubation temperature on lipase activity: The temperature at which fermentation was carried out plays a vital role on the activity of lipase enzyme. The fermentation of SP45 was carried out at different incubation temperature ranging from 30^0C-50^0C for determining the effect of incubation temperature on activity of lipase by keeping remaining parameters same. After 24 hours and 48 hours of fermentation, spectrophotometric lipase assay was performed and the fermentation temperature showing optimum lipase activity was selected for production of lipase enzyme[10].

Results and Discussion

Screening, Isolation and Identification of the isolates: Total 50 (SP01-SP50) lipolytic *Pseudomonas spp.* were isolated from 29 oil contaminated soil samples from different areas of Pune, Solapur and Mumbai. After performing the lipase assay of these isolates, it was found that SP45 showed the maximum enzymatic activity among them as shown in figure-1. Hence SP45 was selected for the further study. Morphological and biochemical studies of SP45 were carried out as described in Table 1. According to the Bergey's manual of Systematic Bacteriology and 16SrRNA sequencing analysis, the isolate SP45 was identified and confirmed as *Pseudomonas aeruginosa.*

Effect of carbon sources on lipase activity: On studying the effect of different carbon sources on lipase activity, Glucose shows the optimum enzymatic activity of 50.4967 U/ml when used at a final concentration of 1% (w/v) in the fermentation medium as a carbon source as shown in figure-2.

Effect of nitrogen sources on lipase activity: While studying the effect of various nitrogen sources on lipase activity, Yeast extract shows the maximum enzymatic activity of 61.6567 U/ml when used at a final concentration of 0.5%(w/v) in the fermentation medium as a nitrogen source as indicated in figure-3.

Effect of pH and temperature of the medium on lipase activity: The pH of the fermentation medium and the temperature at which fermentation process was carried out has a major influence on the enzymatic activity of the enzyme of interest. The present study indicates that SP45 showed the optimum lipase activity of 66.2567 U/ml at pH 7.0 as shown in figure-4. While as far as temperature parameter was considered, SP45 exhibits maximum lipase activity of 66.2567 U/ml at 37^0C as indicated in figure-5.

Table-1
Morphological and Biochemical Characterization of SP45

SP45 Colony Characters	Details	Biochemical Tests	Results
Shape	Circular	Gram Staining	Gram Negative Bacilli
Size	1-2 mm	Motility	Motile
Color	Creamy	Indole	Negative
Margin	Smooth	Catalase	Positive
Opacity	Transluscent	Nitrate Reduction	Positive
Elevation	Slight	Oxidase	Positive
Consistency	Sticky	Sugar Fermentation	Glucose: Negative Sucrose: Negative Lactose: Negative Maltose: Negative Mannitol: Negative
Pigment	Green		

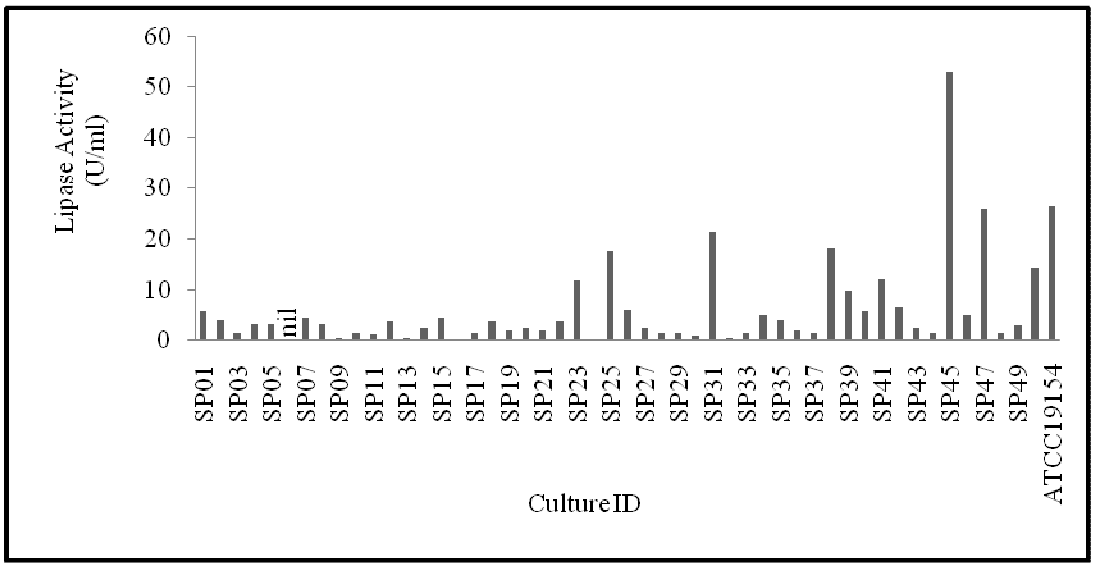

Figure-1
Lipase activity of lipolytic *Pseudomonas spp.* by spectrophotometric assay

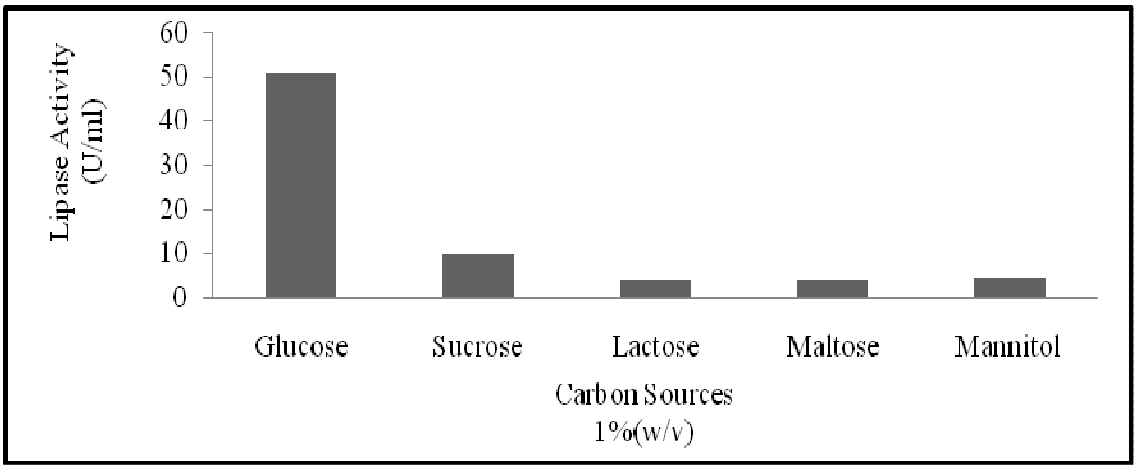

Figure-2
Effect of different carbon sources on lipase activity

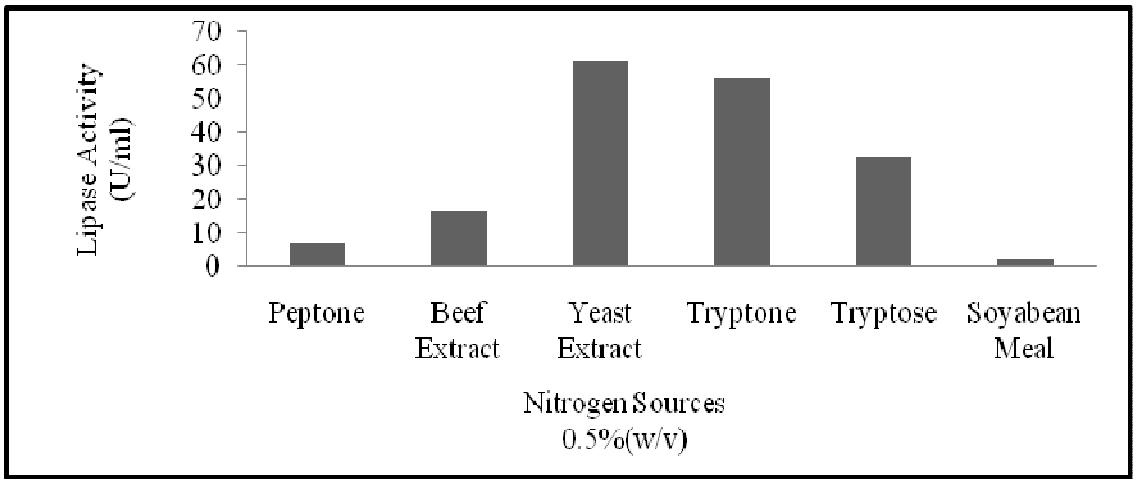

Figure-3
Effect of different nitrogen sources on lipase activity

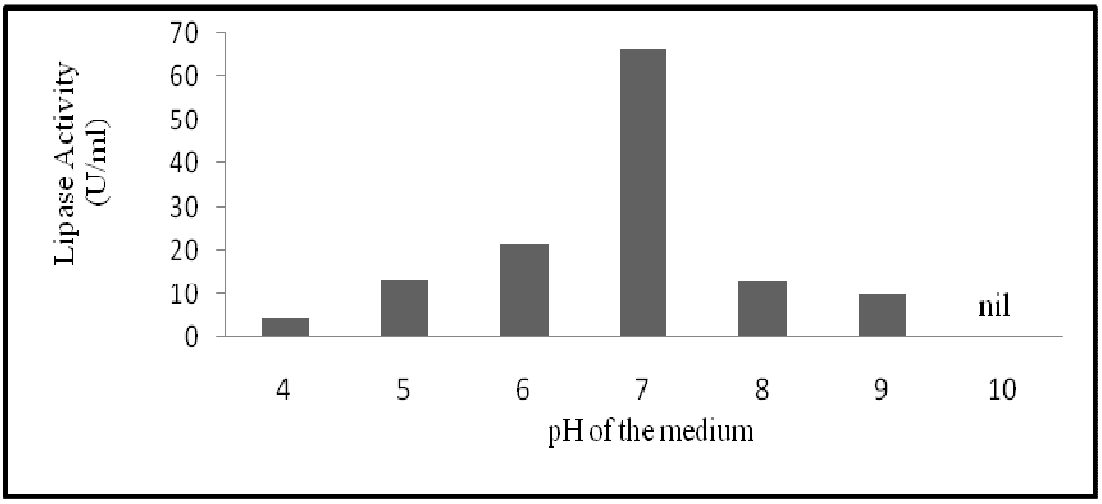

Figure-4
Effect of different pH on lipase activity

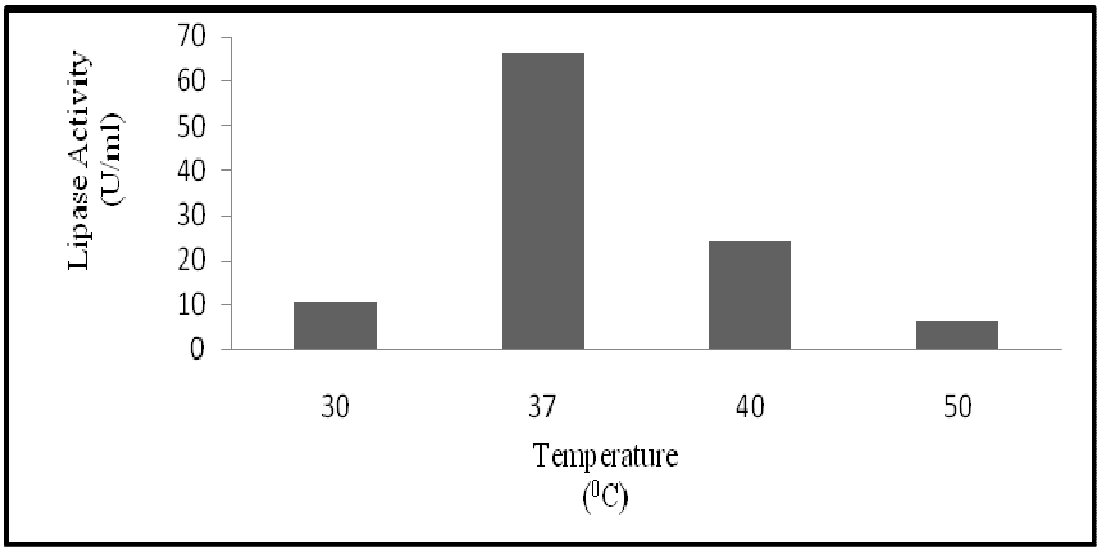

Figure-5
Effect of different temperatures on lipase activity

Lipase enzyme produced from microbial origin has tremendous applications in multiple industries. The present study helps us to understand the role of the optimized fermentation parameters for enhanced production of lipase enzyme. Isolated *Pseudomonas spp.* from oil contaminated soil samples shows the ability of production of extracellular lipase.

Out of 50 isolates, SP45 displayed maximum enzymatic activity. With the help of Bergey's manual of systemic Bacteriology and 16SrRNA sequence analysis, SP45 was confirmed as *Pseudomonas aeruginosa.*

This study also reveals the fact that on altering the different media components, there was a considerable increase in the lipase activity of SP45. Glucose and Yeast Extract were found as an optimum carbon and nitrogen sources respectively when the fermentation was carried out at 37^0C in a fermentation medium of pH 7. Optimum lipase activity of 66.2567 U/ml was obtained after optimization of different fermentation parameters.

Conclusion

In conclusion, a total of 50 lipolytic *Pseudomonas spp.* were isolated on screening 29 oil contaminated soil samples. Amongst them, as SP45 showed optimum lipase activity, hence SP45 was selected for further optimization studies. On performing the optimization of fermentation parameters, we have found that Glucose was considered as optimum carbon source while Yeast extract was found to be the optimum nitrogen source in a fermentation medium of pH 7 at temperature of 37^0C. With these optimized fermentation conditions, extracellular lipase produced from fermentation can be partially purified by ammonium sulphate precipitation followed by dialysis.

Acknowledgement

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