Isolation and optimization of lipase producing *Micrococcus* sp. from oil contaminated soil

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Abstract

Lipases are important hydrolytic enzymes that catalyze the hydrolysis of triglycerides to free fatty acids and glycerol having numerous applications and industrial potential. Studies on lipase enzyme production were carried out with a bacterial strain (Micrococcus sp. SJ4) isolated from oil contaminated soil sample. Due to the reason that oil contaminated soil sample are rich in lipid and fatty acid, it makes them a very good source to find microorganisms in capable of degrading lipids by producing lipase enzymes. The bacterial strain SJ4 was studied and identified based on morphological and biochemical characteristics and 16S rDNA sequencing.

Lipase activity was optimized by physical and chemical parameters such as pH, temperature, incubation time and carbon and nitrogen sources. A high level of lipase production was found at 35ºC with pH 8.0 and incubation time 48 hours by the lipase producing bacteria SJ4 Micrococcus sp. Maximum activity was observed in olive oil and peptone as carbon and nitrogen sources respectively. The results of the present study demonstrate that the Micrococcus sp. is ideal for extracellular lipase production at industrial level.

Keywords: Lipase, *Micrococcus* sp., Enzyme production, Lipase activity, 16S rDNA.

Introduction

The class of hydrolytic enzymes catalyzing the hydrolysis of acylglycerols to di-acyl glycerol, mono-acyl glycerol and glycerol at oil-water interface is lipases (EC 3.1.1.3)[19]. Lipases are serine hydrolases which act at lipid- water interface with Ser-Asp/Glu-His triad in its catalytic active site[5].The sequences of bacterial lipases show a conserved pentapeptide sequence of Gly-X-Ser-X-Gly with serine residue in its active site[9]. They have a wide source of origin and are ubiquitously found in bacteria, fungi, yeast, plants and animals[29]. Microbial lipases are found commercially more important due to their high versatility and high stability and moreover the advantage of being readily produced in high yields[22].

Due to its versatile role, it has become a necessity to isolate the enzyme through cost effective process. Many microbial lipases have been commercially available in free or immobilized form[23]. The diversity in properties of lipase is exhibited at molecular and biochemical level. Lipases find its use in pharmaceutical industry, dairy industry, agriculture industry, agro-chemical industries, paper industries, cosmetic and flavoring industry and biodiesel production[8,24].

Bacteria are the microbes of choice for the commercial production and isolation of lipases because of the ease of their growth and optimization of culture conditions for higher yield[2]. Bacterial lipases are mostly stable at extreme temperature and different pH, so this forms the basis of analyzing and optimization of lipase production from bacterial origin. Moreover, the substrate spectrum of bacterial lipase is also wide[6]. Lipase from bacteria *Acinetobacter venetianus* RAG-1 is used in biodiesel production by trans-esterification of waste cooking oil[26].

In present study, bacterial strains were isolated from oil contaminated soil of petrol pump and automobile garage and then best lipase producing bacterial isolates screened out by primary and secondary screening. The enzyme production was then optimized for higher yield at various pH, temperature, incubation time, different nutrient sources and varying salt concentrations.

Material and Methods

Chemicals and reagents: Glycerol tributyrate, *p*-Nitrophenyl palmitate (pNPP) (Sigma-Aldrich), sodium deoxycholate (Sigma-Aldrich) and olive oil were purchased from Angel scientific store (Patna, India).

Collection of samples: For the present study, samples were collected from oil contaminated soils from different areas of Patna viz. petrol pump and automobile garage. The samples were aseptically collected from a depth of 4-5cm with the help of sterile spatula in sterile polyethylene bags for the isolation of lipase producing organisms under laboratory condition and kept at 4 ºC for further investigation.

Isolation of lipase producing bacteria: The bacterial species present in the collected soil samples were isolated by using serial dilution up to 10^{-7} using 0.8 % saline and 100 µl of each dilution was spread on nutrient agar plate (NA) by spread plate method and incubated at 37ºC up to 72 hrs. Those microbial colonies which appeared on agar plates were isolated and screened for lipase activity.

Screening of lipase producing bacteria: The isolates having the ability of lipase production were further screened to examine the best possible lipase producing bacteria. The isolates were now plated on Tributyrin agar base media

containing beef extract 3.0 gm/L, peptone 5.0 gm/L, agar 15.0 gm/L and glycerol tributyrate 10 ml/L and then incubated at 37ºC for 24 hrs. Halos around the colonies on tributyrin agar plates are observed as positive colonies for lipase enzyme production. The isolates showing maximum zone of clearance are selected for further analysis.

16S rDNA analysis of lipase production: 16S rDNA was used for the identification of the bacterial strains. The 16S rDNA gene fragment was amplified through the PCR amplification system. A single discrete PCR amplicon band was observed when resolved on Agarose gel. The PCR amplicon was then purified by column purification to remove contaminants. DNA sequencing reaction of the purified PCR amplicon was carried out with 8F and 1492R 16S rDNA primers using BDT v3.1 Cycle sequencing kit on ABI 3500xl Genetic Analyzer. The 16S rDNA sequence was used to carry out BLAST with the database of NCBI Genbank database. Based on maximum identity score, first ten sequences were selected and aligned using multiple alignment software programs. A phylogenetic tree was constructed using the Neighbor-Joining method of Clustal Omega program.

Lipase production medium: Screened positive bacterial isolate PP35 was inoculated in lipase producing media for enzyme production. Lipase production media consist of yeast extract (3%), sucrose (3%), $CaSO_4$ (0.1g/l), KH_2PO_4 (0.5g/l), $MgSO_4.7H_2O$ (0.1g/l), olive oil (1%) and distilled water (100ml) in 250 ml conical flask. Inoculated flasks were incubated at 37ºC on a rotatory shaker (120 rpm). Samples were collected every 24hrs and centrifuged at 10,000 rpm for 20 min at 4ºC. The cell free culture supernatant was used as a source of lipase.

Lipase activity assay: Lipase activity in the supernatant was assayed quantitatively by calorimetric method using para-nitro phenyl palmitate (*p*NPP) as the substrate[30]. The reaction mixture contained 700 µl of *p*NPP solution and 300 µl of lipase solution. The *p*NPP solution was prepared by adding solution A into solution B with stirring until it was dissolved. Solution A contains 0.001 g *p*NPP in one ml isopropanol and solution B contains 0.01 g gum arabic, 0.02 g sodium deoxycholate, 50µl triton X-100 and 9 ml of 50 mM tris-HCl buffer, pH 8. The absorbance was measured at 410 nm for the first 2 min of reaction[13,20]. One unit of lipase activity was defined as the amount of enzyme that liberated 1µmol of *p*NP per minute under the assay conditions.

Optimization of lipase activity on various parameters

Effect of temperatures and pH: Effect of temperatures was determined by growing the isolate in production media of about 50 ml in 250 ml Erlenmeyer flask at varied temperatures (20 ºC - 50 ºC). All the flasks were incubated on a rotatory shaker (120rpm) at different temperatures for 48 hrs. The resulting cultures were then centrifuged at 10,000 rpm for 20 min at 4 ºC. Then the lipase activity was assayed.

The effect of pH was determined by growing the isolate in production media of different pH ranging from 5 - 11 and then the flasks were incubated on a rotatory shaker (120rpm) at 37 ºC for 48 hrs. The resulting cultures were then centrifuged at 10,000 rpm for 20 min at 4 ºC. Then the lipase activity was assayed.

Effect of incubation time: Effect of incubation time on lipase production was determined by incubating the flasks on rotatory shaker (120rpm) at 37 ºC for 24 hrs to 120 hrs. Lipase activity was assayed after different incubation time from 24 hrs to 120 hrs.

Effect of carbon sources: Effect of carbon sources on lipase production was determined by replacing the olive oil with different oils like sunflower oil, flaxseed oil, mustard oil, soybean oil, coconut oil and sesame oil at a concentration of 1%w/v added into the production medium in 250ml Erlenmeyer's flasks containing 50ml of liquid medium on a rotatory shaker (120rpm) and incubated at 37 ºC for 24 hrs. The resulting cultures were then centrifuged at 10,000 rpm for 20 min at 4 ºC. Finally, the lipase activity was assayed.

Effect of nitrogen sources: Effect of nitrogen sources on lipase production was determined by adding different nitrogen sources like yeast extract, tryptone, peptone, beef extract, soya bean meal and NH_4NO_3 at a concentration of 1%w/v into the production medium in Erlenmeyer flasks containing 50ml of liquid medium on a rotatory shaker (120rpm) and incubated at 37 ºC for 24 hrs . The resulting cultures were then centrifuged at 10,000 rpm for 20 min at 4 ºC. Finally, the lipase activity was assayed.

Results and Discussion

Screening and morphological identification of lipase producing bacterial strains: Oil spilled areas are potential sites which contain lipolytic bacteria. The oily environment is a good source for lipolytic microorganisms to grow. In this study, the samples were collected from oil spilled areas. The microbial cultures were screened for lipase production on tributyrin agar medium. A total of 59 bacterial colonies were observed from different areas of Patna district. Among these 7 were screened for lipase activity since it shows the maximum activity on tributyrin agar base medium. Out of these 7 isolates, PP35 (Figure 1) was used for further analysis. Morphological and biochemical analysis were done on this isolate (Table 1). After morphological and biochemical test, PP35 was characterized and identified as *Micrococcus luteus* using 16S rDNA sequence analysis.

The 16S rDNA sequence was used to carry out BLAST with the database of NCBI Genbank database. Based on maximum identity score, first ten sequences were selected and aligned using multiple alignment software programs. A phylogenetic tree was constructed using the Neighbor-Joining method of Clustal Omega program (Figure 2). The sequence was submitted in NCBI BLASTn and analysed for the bacterial class and species with the other sequences.

Then it is submitted and accession number (MK240570) *Micrococcus* sp. strain SJ4 has been allotted to the PP35 isolate.

Effect of temperatures and pH: Temperature and pH are the two important parameters which influences the lipase production. The lipase activity was determined under different temperatures under assay conditions. Temperature is critical parameter that has to be controlled and it varies from organism to organism. Temperature influences the secretion of extracellular enzymes. The lipase enzyme exhibited activity maximum at 35 ºC which was 64.57±0.801 U/ml (mean± SEM) and beyond 35 ºC the activity decreased uniformly (Figure 3A). The data was significant with $p \leq 0.001$. This result is also supported by the finding of Rashid et al.[21] According to their studies, the optimal temperature for lipase production by pseudomonas sp. was 35 ºC. It was reported that the lipase activity of *Pseudomonas flouroscens* was maximum at 36 ºC[10].

The effect of pH on lipase activity was assayed between 5.0 – 11.0 for the organism PP35. PP35 showed maximum lipase activity at pH 8.0 (70.35±1.270 U/ml) and then declined at pH 9 (37.56±1.239) and above (Figure 3B). The lowest activity was observed at pH 11 (21.54±0.784). The data was significant with $p \leq 0.001$. Nisha et al[18] reported that *Micrococcus flavus* has maximum lipase activity at pH 8. Similar results were reported for *Bacillus* sps.[1, 12, 14-17]. Low activities were seen at low pH. Hasan et al[7] reported that lipase active under this condition was preferred because in detergent industries, laundering is generally conducted at alkaline pH.

Effect of incubation time: It was noted that highest lipase activity of 64.83±3.992 U/ml was recorded after 48hrs of incubation. Then a declining trend of the lipase activity was observed with further increase in incubation time and a minimum activity (15.31±2.589 U/ml) was observed after 120 hours of incubation (Figure 4).

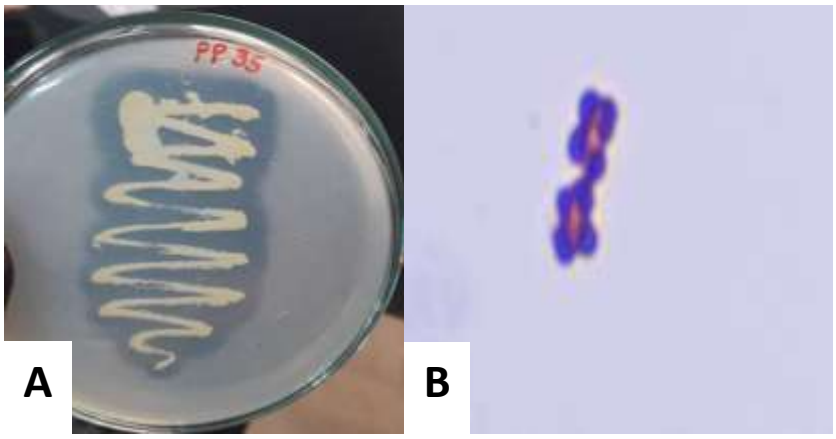

Figure 1: (A) Zone of hydrolysis formed by lipase strain SJ4 (PP35). (B) Bacterial strain +ve *Micrococcus* species

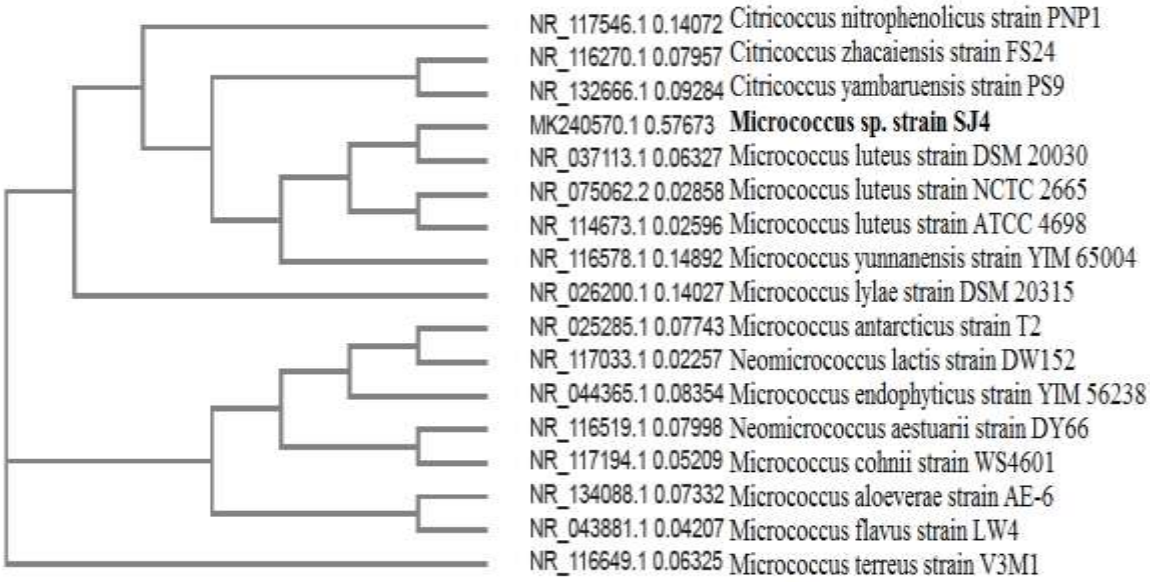

Figure 2: Phylogenetic tree of *Micrococcus* sp. strain SJ4 (PP35) based on 16S rDNA gene sequence based on neighbor-joining method.

The value is expressed as mean ± SEM and is statistically significant ($p \leq 0.001$). Nisha et al[18] reported that *Micrococcus flavus* showed maximum activity after 48hrs of incubation. Similar results from *Bacillus* sp. and *Pseudomonas* sp. were also reported[1,28,29].

Effect of carbon sources: Lipases are inducible enzymes, hence major factor which is responsible for the expression of lipase activity has always been carbon source. The effect of different carbon sources on lipase activity was examined. Among the various carbon sources, olive oil acted as best supplement for lipase activity by PP35 organism (99.68±3.635 U/ml) (Figure 5A). Minimum lipase activity was seen in flaxseed oil that is 12.48±0.745 U/ml. The statistical significance was observed with $p \leq 0.001$. Various thermophilic *Bacillus* sp. showed maximum lipase production in presence of olive oil as carbon source in the culture medium[4,11]. *Pseudomnas aeruginosa* also showed maximum lipase activity in presence of olive oil as carbon source[20].

Effect of nitrogen sources: The effect of different nitrogen supplements on lipase activity was examined. Among the various organic and inorganic nitrogen sources, peptone acted as best nitrogen supplement for lipase activity by PP35 organism (67.50±1.967 U/ml) while tryptone showed minimum activity of 36.31±3.411 U/ml (Figure 5B). The statistical significance was observed with $p \leq 0.001$. Beef extract and yeast extract were good nitrogen sources for lipase activity by *Micrococcus* sp.

Table 1
Biochemical characterization of SJ4 (PP35)

TESTS	SJ4 (PP35)
Gram test	Positive
Shape	Cocci
Motility	Negative
Indole	Negative
Citrate utilization	Negative
MR	Positive
VP	Positive
Catalase	Positive
Gelatin hydrolysis	Negative
Oxidase	Positive
Casein hydrolysis	Negative
Starch hydrolysis	Negative
Glucose	Negative
Fructose	Negative
Lactose	Negative
Sucrose	Negative
Urea	Negative
H_2S Production	Positive
Nitrate reduction	Positive

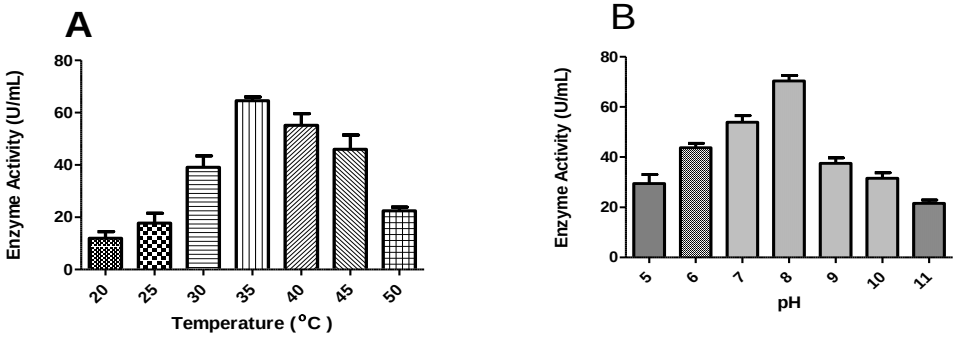

Figure 3: Effect of different (A) temperature and (B) pH on lipase production. The graph depicts the enzyme activity of triplicate experiments (Mean ± SE) with $p \leq 0.001$ statistically significant.

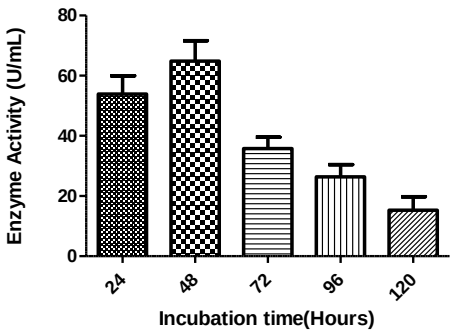

Figure 4: Effect of different incubation periods on production of lipase. The graph depicts the enzyme activity of triplicate experiments (Mean ± SE) with $p \leq 0.001$ statistically significant.

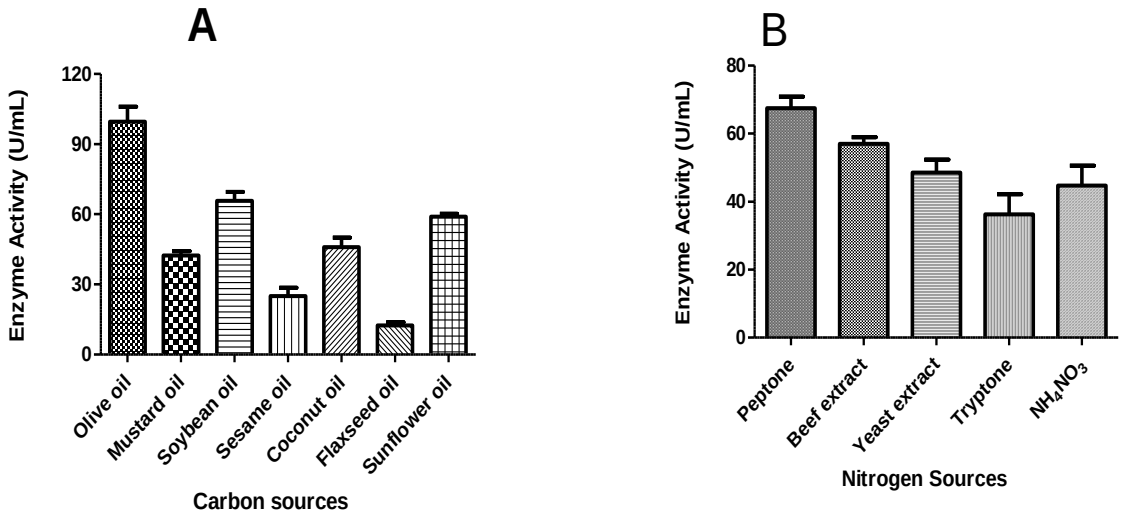

Figure 5: Effect of different (A) carbon and (B) nitrogen sources on the production of lipase. The graph depicts the enzyme activity of triplicate experiments (Mean ±SE) with a statistical significance of $P \leq 0.001$.

This result is in concordance with Sirisha et al[25] where lipase activity was found maximum when peptone was used as nitrogen source in *Staphylococcus* sp. Similar results were also reported in *Bacillus* sp.[3,13,27]. *Pseudomnas aeruginosa* also showed maximum lipase activity in presence of peptone as nitrogen source[20].

Conclusion

With an increase in world oil demand, there is a simultaneous increase in waste production as well. This poses a serious threat to the environment if discharged untreated into the nature. Microbes are an efficient waste removal organism. Thus, there is a need to develop cost effective technology which exploits the waste degrading capability of microbes. Lipase is one such enzyme produced by an array of microbes that catalyzes the hydrolysis of triglycerides to fatty acids and glycerol at oil water interface and also catalyzes the reverse reactions like esterification, transesterification and interesterification in non-aqueous solvent systems.

Therefore, lipases have tremendous applications in many industries like food, dairy, detergent, leather, pharmaceutical and agrochemical. In present study, the production of lipase from Micrococcus sp. SJ4 was optimized. The optimum conditions for growth and enzyme production were 48hr, 35ºC, pH 8.0 with olive oil as carbon source and peptone as nitrogen source. The result of the present study indicates that the *Micrococcus* sp. SJ4 is a potential lipase producing strain that can be efficiently utilized in many industries.

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