

Identification, production, and purification of a novel lipase from *Bacillus safensis*

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ABSTRACT

Lipase is an extracellular enzyme produced by various sources such as plants, animals, and microorganisms. Microorganisms are the best source of extracellular lipase and are used in various industrial processes for their potential application. In this research study, lipase producers were isolated in oil-contaminated soil collected from oil refineries of Aravalli district situated in Gujarat, India. It was screened on a selective medium like tributyrin agar. The isolate was identified using the 16S rRNA method and lipase enzyme activity was quantitatively assayed. Submerged fermentation was used for the production of lipase using olive oil as a substrate. Maximum lipase activity was found from *Bacillus safensis*. Lipase from *B. safensis* was purified using $(\text{NH}_4)_2\text{SO}_4$ precipitation and ion exchange chromatography and characterized by sodium dodecyl sulphate-polyacrylamide gel electrophoresis analysis. *B. safensis* exhibited specific activity of 13.71 U/mg with a yield of 16.16% and the molecular weight of lipase is 55kDa was obtained.

1. INTRODUCTION

In ancient times, various types of enzymes are being used for making various food and dairy products, leather, textiles, biodiesel production, pharmaceuticals, and other valuable products for human beings [1]. A new technique (submerged culture, solid state fermentation, and immobilized cell culture) was then introduced to produce enzymes in the purified form at a commercial level from microbial sources [2]. In the world, the global enzyme market was developed a few million dollars since the 1960s. From those days, the global market has been continuously developed miraculously. Today, different types of 200 enzymes are used at the commercial level. The demand for the enzyme market is increased by study enzyme biochemistry and found new methods of fermentation process and product recovery methods. There are six classes of enzymes, of which lipases belong to the EC 3, hydrolases. Lipase hydrolyses fats and lipids (triglycerides) into monoacylglycerols and free fatty acids [3]. Some important bacterial species (*Bacillus*, *Pseudomonas*, *Burkholderia*, *Serratia*, etc.) produced extracellular lipase by submerged fermentation and lipase production is increased by adding different lipid substrate [1,4]. The versatility of microbial

lipase to choose promises application in the manufacture of paper, pharmaceutical products, cosmetics, detergents, textiles, dairy products, synthesis of fine chemicals, processing of fat and oil, and commercially [1,4,5].

2. MATERIALS AND METHODS

2.1. Soil Sample Collection

Samples of oil-contaminated were taken from different Industrial areas of Aravalli situated in Gujarat, India. Soil samples were collected in airtight sterile bags and stored in the laboratory at 4°C until further use.

2.2. Screening of Lipolytic Bacteria

For isolation of lipolytic bacteria, 1 g of soil samples was taken in pure 100 ml distilled water. Serial dilution was made from this suspension from 10^{-1} to 10^{-6} . Take 1 ml suspension from each dilution and inoculated on tributyrin agar medium (Composition g%: Peptone-0.5, Yeast extract- 0.3, NaCl-0.1, Tributyrin-1.0, and Agar- 2.0) by spread plate method [6]. The plates were then incubated (37°C for 48 h). All plates were studied after 48 h of incubation for lipase production in an agar plate. Observed halo zone of clearance (lipid hydrolysis) surrounded bacterial colonies. All isolates were further screened as for maximum lipase producer were inoculated on tributyrin agar medium. The best lipase producer was selected based on the maximum zone of clearance for further studies.

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2.3. Identification of Lipolytic Bacteria

The selected bacterial isolate (KKSC12) was identified morphologically by Gram staining and genetically by 16S rRNA sequencing. The molecular identification (16S rRNA sequencing) was performed at Biokart PVT Ltd. Bangalore.

2.4. Production of Lipase Enzyme

Lipase was produced from a selected bacterial isolate (KKSC12) by submerged fermentation. Production medium (Composition g/L; Peptone [2.0 g], $\text{NH}_4\text{H}_2\text{PO}_4$ [1.0 g], NaCl [2.5 g], $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ [0.4 g], $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ [0.4 g], Olive oil [2%(v/v)], Tween 80 (1–2) drop, and D/W (1000 ml); pH 7.0) [7]. The activated culture of the isolate was inoculated into the production medium (100 ml) contained in Erlenmeyer flasks (500 ml) after sterilized in the autoclave at 121°C for 15 min. The flask was placed on a metabolic shaker (150 rpm) at 37°C for 48–72 h. After incubation, the crude enzyme extract was obtained at 10,000 rpm (centrifugation) for 20 min, and for analysis of lipase enzyme quantitatively, the supernatant was used.

2.5. Spectrophotometric Assay of Lipase Activity

The lipase activity in the supernatant was examined by the spectrophotometric method using phenyl acetate using as a substrate [8].

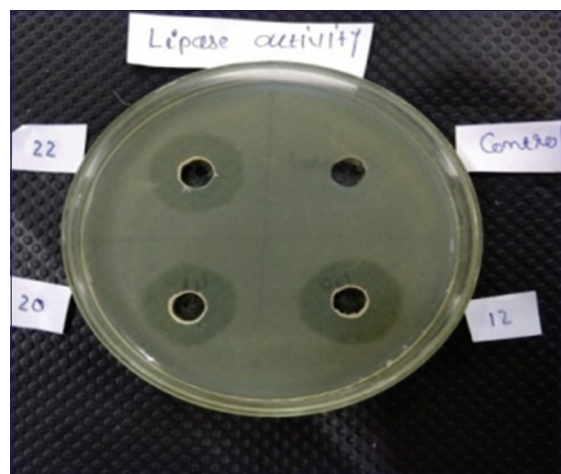


Figure 1: Zone of clearance of KKSC12.

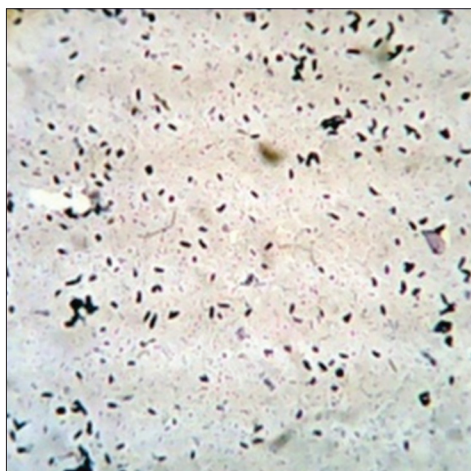


Figure 2: Morphological Identification of KKSC12.

2.6. Lipase Enzyme Purification

$(\text{NH}_4)_2\text{SO}_4$ precipitation and Diethylaminoethyl (DEAE) cellulose anion exchange chromatography were used for purification of extracellular lipase from bacterial isolate (KKSC12) [9]. The supernatant was precipitated at different ammonium sulfate saturation. Precipitation of protein was done by addition of ammonium sulfate at 4°C with stirring for 2 h to obtain fractions, that is, 40% and 60%. At the end of each fraction, precipitates were collected and dissolved in phosphate buffer pH 7.2. The collected ammonium sulfate precipitate was dissolved in 50mM Tris-HCl buffer, pH 8.0, and applied onto the DEAE-cellulose anion chromatography for further clarification.

2.7. Molecular Mass Determination of Lipase Enzyme

According to the procedure of Laemmli [10], the Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) electrophoresis method was used to the determined molecular mass of purified bacterial lipase. For gel electrophoresis, 4% stacking gel was used. Electrophoresis was performed at 200v for 2 h. After gel electrophoresis, the gel was removed from plates and stained with 0.025% Coomassie brilliant blue dye. Then molecular mass was determined.

3. RESULTS AND DISCUSSION

3.1. Screening of Lipase Producing Bacteria

The best bacterial isolate (KKSC12) was chosen from 46 bacterial based on the production of the maximum zone of clearance as detected by hydrolysis of lipid on tributyrin agar medium due to the extracellular lipase production. This bacterial isolate (KKSC12) was produced highest zone of clearance, 25 mm diameter on tributyrin agar medium [Figure 1] among the isolates used in the study. Olawaseya *et al.* [11] isolated *Pseudomonas aeruginosa* from soil and *Bacillus* sp. from water using tributyrin agar, rhodamine olive

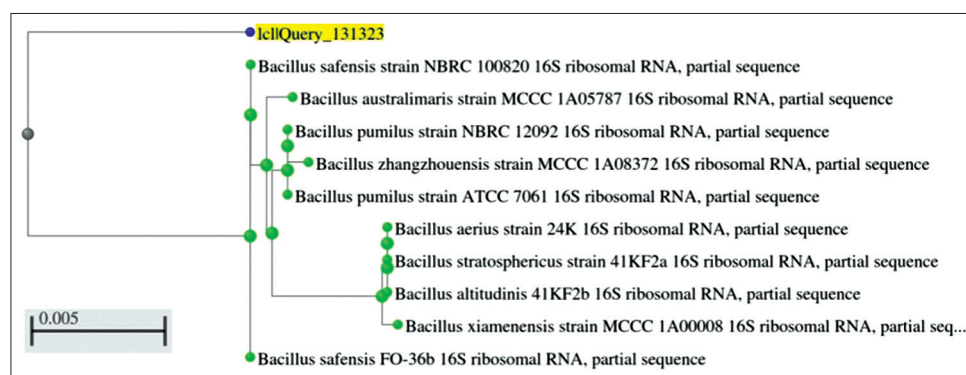
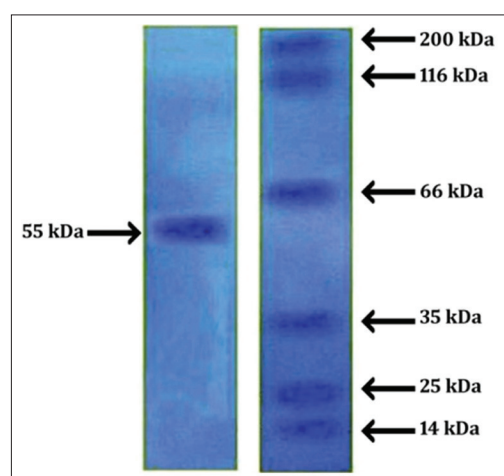
Table 1: Blast analysis report of the bacterial isolate KKSC12 (*Bacillus safensis*).

Sl. No.	Organism Name	Accession No.	Percentage Match
1	<i>Bacillus safensis</i> strain NBRC 100820 16S ribosomal RNA	NR_113945.1	98.04%
2	<i>Bacillus safensis</i> FO-36b 16S ribosomal RNA	NR_041794.1	98.04%
3	<i>Bacillus australimaris</i> strain MCCC 1A05787 16S ribosomal RNA	NR_148787.1	97.89%
4	<i>Bacillus pumilus</i> strain NBRC 12092 16S ribosomal RNA	NR_112637.1	97.89%
5	<i>Bacillus pumilus</i> strain ATCC 7061 16S ribosomal RNA	NR_043242.1	97.89%
6	<i>Bacillus zhangzhouensis</i> strain MCCC 1A08372 16S ribosomal RNA	NR_148786.1	97.81%
7	<i>Bacillus aerius</i> strain 24K 16S ribosomal RNA	NR_118439.1	97.57%
8	<i>Bacillus stratosphericus</i> strain 41KF2a 16S ribosomal RNA	NR_042336.1	97.57%
9	<i>Bacillus altitudinis</i> 41KF2b 16S ribosomal RNA	NR_042337.1	97.57%
10	<i>Bacillus xiamenensis</i> strain MCCC 1A00008 16S ribosomal RNA	NR_148244.1	97.50%

Table 2: Purification steps of lipase from *Bacillus safensis*.

Purification steps	Enzyme volume (ml)	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Fold
Supernatant	100	81.49	24.96	3.26	100%	1
Ammonium sulfate (40%)	4	50.28	6.8	7.39	61.7	2.2
Ammonium sulfate (60%)	4	8.91	1.84	4.84	10.9	1.48
DEAE cellulose	2	13.17	0.96	13.71	16.1	4.2

DEAE: Diethylaminoethyl

**Figure 3:** Phylogeny of KKSC12 (*Bacillus safensis*).**Figure 4:** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of *Bacillus safensis*

oil agar, and phenol red agar medium. Leelatulasi *et al.* [12] reported that cold-adapted *Pseudomonas* sp. LSK25 containing lipase activities was isolated on tributyrin, triolein, and rhodamine olive oil agar from Antarctica.

3.2. Identification of Lipolytic Bacteria

The selected potential isolate was morphologically identified by performing Gram staining. The isolated bacteria (KKSC12) were observed gram-positive, big rods [Figure 2]. Using the 16S rRNA gene sequencing method isolated bacterial KKSC12 was identified as *Bacillus safensis* [Figure 3] as 98.4% of sequence similarity was matched [Table 1].

3.3. Production of Lipase

Extracellular lipase enzyme was produced by submerged fermentation. The production medium containing olive oil as a lipid substrate and fermentation was carried out at pH 7.0, at 37°C temperature, for 48–72 h in shaking condition at 150 rpm. The crude enzyme was assayed by the spectrophotometric method using phenyl acetate as a substrate from production medium. The selected *B. safensis* was produced the highest lipase activity such as 113.33U/ml after 48 hours. Pushpinder S. [4] reported using submerged fermentation to produce *Bacillus methylotrophicus* PS3 lipase using lipidic substrate (tributyrin) and the maximum activity was 320.01U at 40°C and pH-7 with 1% tributyrin. Venkata *et al.* [13] reported maximum lipase produced from *Stenotrophomonas maltophilia* using tributyrin as a substrate. Naif *et al.* [14] shown that the highest lipase activity from *Streptomyces* sp. was found 253U/ml at pH-11.0 and 35°C temperature with olive oil. Amira *et al.* [15] also reported that extracellular lipase production from *Aspergillus niger* using tween 80 broth by submerged fermentation.

3.4. Partial Purification and SDS-PAGE Gel Electrophoresis

The ammonium sulfate fractionation method was used for partial purification of lipase enzyme using crude extract of *B. safensis*. Crude enzyme filtrate was subjected to different fractions of ammonium sulfate precipitation at 40% and 60% saturation. The fractions obtained at 40% and 60% followed by DEAE-cellulose chromatography showed that maximum activity, specific activity, and yield were detected [Table 2]. The molecular mass of the purified lipase from *B. safensis* was 55kDa using different marker proteins as confirmed by the appearance of a single band on SDS- PAGE [Figure 4]. Gulhan *et al.* [16] studied that the 17.8 fold obtained from *Bacillus subtilis* LP2 was partially purified by the Ammonium fractionation method.

Iqbal and Rehman [17] reported that *B. subtilis* I-4 was purified by thin-layer chromatography, and molecular weight was determined to be 53kDa by SDS-PAGE gel electrophoresis. Loreni *et al.* [18] reported that lipase from *Pseudomonas helmanticensis* was purified resulting in 18.78 fold purification, 5.58% yield and high specific activity of 3368 U/mg. Anil *et al.* [19] reported that extracellular lipase from Psychrotrophic bacterium *Chryseobacterium polytrichastri* was purified by ammonium sulfate precipitation, size exclusion, and hydrophobic interaction chromatography method.

4. CONCLUSION

Lipase production by lipolytic bacteria is worth exploiting for detailed investigations and further applications at the industrial level. Lipase enzyme from bacteria is suggested for commercial production due to its constitutive nature and easy purification. Limited research reports are available on lipase production from bacteria and this would be one of them in this direction.

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6. AUTHORS' CONTRIBUTIONS

All authors made intellectual contribution to the completion of this research work. They also took part in an experimental design, analyzed the research data, and played a role in composing the describing result in an article.

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8. CONFLICTS OF INTEREST

The authors report no conflicts of interest.

9. ETHICAL APPROVALS

This study does not involve experiments on animals or human subject.

10. DATA AVAILABILITY

Data is available online as well as mentioned in references in bibliographic notes.

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