Isolation, Screening and Molecular characterization of Lipase producing Bacteria and its application in detergents

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Abstract- **This study aims to investigate the commercial potential of lipases produced by bacteria. Four isolates were obtained from the lake water. A unique lipolytic medium with 1% (w/v) mustard oil was used to cultivate the isolated lipolytic bacteria. Egg yolk was used in the tests to measure primary lipase activity. The maximum lipolytic activity enzyme extracts from Isolate-2 and Isolate-4 were used for further use. The maximum displacement area was ascertained by separating the displaced oils (mustard oil and vegetable oil) before analyzing the oil displacement tests. The Isolate-2 was identified as *Pseudomonas aeruginosa* through 16S rRNA gene sequence analysis. The efficiency of employing crude enzyme extracts to remove oil stains from cotton fabric pieces was assessed. The lipase enzyme has oil stain removal properties and has proven effective as a detergent additive.**

Keywords: **Lipase producing bacteria, Detergent additive, *Pseudomonas aeruginosa*.**

I. INTRODUCTION

By a two-step process, lipases catalyze the hydrolysis of ester linkages in lipids. An acyl-enzyme intermediate is first formed when the catalytic serine residue in the active site is attacked by a nucleophile at the ester bond's carbonyl carbon. Water molecules interact with the intermediate as a result, going through hydrolysis to release glycerol and fatty acids. [1].

Numerous factors influence the catalytic activity of lipases, such as pH, temperature, and the existence of inhibitors or cofactors. The source organism and the physiological role of the enzyme determine the ideal pH range for lipase activity. The regulation of lipase activity is significantly influenced by temperature as well. For catalysis, each enzyme has a range of temperatures that works well. Enzyme reaction rates are also influenced by substrate concentration. Generally, the reaction rate increases with increasing substrate concentrations until saturation is attained. By modifying the catalytic efficiency, substrate binding affinity, or enzyme stability, cofactors and inhibitors can further regulate lipase [2].

An essential ingredient in enzymatic detergents that accelerate the removal of dirt and lipid stains from clothes and dishes is lipase. When it comes to cleaning, stain removal, and fabric care, enzyme detergents outperform conventional detergent formulas. Numerous stains, such as oils, grease, and food residues, can be successfully removed from fabrics, textiles, and hard surfaces with lipase. When it comes to lipid stains, enzyme stain removers function by hydrolyzing the ester bonds, dissolving the fat stain, and then eliminating it during the cleaning process. [3].

Lipases facilitate the biodegradation of lipid-based contaminants, including oils, greases, and fats, in the context of environmental remediation and wastewater treatment. reduces the environmental damage caused by lipid pollution by dissolving complicated lipid molecules into simpler substances that microbes can digest. A sustainable method of purifying soil and water is enzymatic bioremediation utilizing lipases, particularly in contaminated areas with a high lipid content. Ecosystems harmed by industrial spills or accidents can recover more quickly when lipid contaminants are broken down by lipase. [4].

It takes careful consideration of process parameters, throughput, and commercial viability to scale up lipase-mediated processes from laboratory to industrial production. The effective development and commercial integration of lipase biocatalysts depend on testing, optimization, and techno-economic analysis [5].The goal of the current study was to

determine how well bacteria's lipase enzyme removed greasy stains, demonstrating the enzyme's usefulness as a detergent ingredient.

II.MATERIAL AND METHODS

Sample Collection

In Chennai, Tamil Nadu, India, samples of water were taken from Porur Lake using plastic containers. As soon as possible, samples were sent to the lab for a variety of microbiological examinations.

Isolation of lipolytic bacteria

Make a lipolysis medium using the ingredients listed below: pH7.0, 0.3% yeast extract (w/v), 1% mustard oil, and 2.9% protein agar (w/v) are added. The culture media should be autoclaved and sterilized for 15 minutes at 121°C. Fill sterilized Petri dishes with the culture medium after sterilization. After the plates were solidified, they were layered, the spreader was gently rotated, and 10 µL of each dilution was equally distributed into the solidification media. The plates were then incubated for 48 hours in order to isolate the lipolytic bacteria. Bacterial cells were isolated using nutrient agar medium as well as lipolytic media. Identification of colony morphology and biochemical testing were carried out.

Egg-yolk test for primary lipolytic activity

The nutrient agar was melted, cooled to 55˚C, and then the egg-yolk suspension was added to create the egg-yolk test medium [6].Saturated copper II sulphate (CuSO4) solution was added to the plate after two days of incubation, and it was left to stand for 20 minutes. The plates were then dried in the incubator for a brief period of time after the extra solution was drained off. Lipolysis was verified by the greenish-blue color of copper soaps containing fatty acids.

Lipase production

The lipolytic bacteria were inoculated with extra olive oil substrate (2.5%), or 2.5 ml in 100 ml of broth, to the salt basal medium (which included 12.8 g Na2HPO4.7H2O, 3 g KH2PO4, 0.5 g NaCl, 2 Mm MgSO4.7H2O, 1 g NH4Cl, 2 g Glucose, and 10 ml). For every strain, the media pH was later adjusted to 7.0 and 8.0, and it was then incubated for 24 to 48 hours at 37 to 42 degrees Celsius.

Preparation of crude lipase

To prepare the inoculum, a 24-hour-old culture was utilized. A loopful of microbial cells was transferred into the production media, and the flasks were maintained for 24 hours at 30°C in a shaker incubator at a speed of 100 rpm to facilitate the synthesis of lipase. The supernatant was produced after centrifuging the crude enzyme-containing medium at a speed of 12,000 rpm. Further studies of crude enzyme were carried out.

Lipase assay method

Due to its simplicity, accuracy, and repeatability, the titration method, which uses olive oil as a substrate based on hydrolysis, is a frequently used technique for measuring lipase activity [7]. After adding 1 ml of culture supernatant to the reaction mixture along with 1 ml of 0.1 M Tris HCl buffer, 3 ml of distilled water, and 3 ml of olive oil, the mixture was incubated for 24 hours at 37°C. After that, 3 milliliters of 95% ethanol were added to stop the process. Using 0.1% alcoholic phenolphthalein as an indicator, 0.1 M NaOH was used to titrate the released fatty acids until a pink color was visible.

Oil displacement test

A test for oil displacement was used to measure lipase activity [8].Each isolate received 100 ml of broth culture (0.3 g of peptone, 0.1 g of yeast extract, 0.05 g of sodium chloride, and 0.5 g of tween 80 per 100 ml) in order to extract the crude lipase enzyme extract. Sterilization was then performed for 15 minutes at 121ºC. Once the media had cooled, isolates were added and it was cultured for 72 hours at 30 degrees Celsius. After centrifuging the culture for 30 minutes at 7000 rpm, the crude enzyme extract, or supernatant, was separated. 40ml of distilled water was put to two separate petri dishes (diameter of 150 mm) together with 10ul of weathered hydrocarbons, such as vegetable or mustard oil. Subsequently, 10µl of each isolate's cell-free culture supernatant was positioned in the middle of each kind of oil film. After 30 seconds of incubation, the diameter of the clear halo zone was measured, and the results were recorded in triplicate.

Application of lipase for removal of oily stains from cotton cloth

Using technique, lipase enzyme extract was used as a detergent additive to cotton textile pieces (10 x 10 cm) stained with weathered hydrocarbons (mustard oil and olive oil)[9]. The effectiveness of removing greasy stains was investigated in three experimental groups:

Group 1: 100 ml flask filled with distilled water plus mustard and olive oil-stained cloth.

Group 2: 100 ml of distilled water in a flask, 1 ml of Surf Excel detergent (10 mg/ml), and a soiled cloth containing olive and mustard oils.

Group 3: 100 ml flask filled with distilled water, 1 ml of Surf Excel detergent (10 mg/ml), 2 ml of lipase enzyme extract (for I2 and I4), and a stained cloth (olive and mustard oil).

To assess how well oily stains were removed, each flask was incubated for 15 minutes at 60 degrees Celsius. Cloth pieces visually examined after treatment to assess stain removal efficacy.

Molecular identification of best lipase-producing isolate using 16S rRNA sequencing

16S rRNA gene sequencing was used to determine which lipase-producing isolates were the best, based on the strain removal results. From the previously obtained DNA, the 16S gene area was amplified by PCR using universal primers. Resolved on Agarose Gel, a single distinct 1000 bp PCR amplicon band was seen. To get rid of impurities, the PCR product was filtered. Using an ABI 3500 Genetic Analyzer and the BDT v3.1 Cycle sequencing kit, sequencing was carried out. Bioinformatics software is used to align and trim the sequencing data. Create a phylogenetic tree by applying maximum likelihood or neighbor-joining techniques. To identify the bacterial taxa, compare the acquired sequences with reference sequences found in open databases.

III.RESULTS AND DISCUSSION

Isolation and colony morphological examination of lipolytic bacteria

Colonies (Figure 1) were iden6tified in both lipolytic and nutrient media. Using lipolytic selective agar medium, four morphologically unique colonies were separated from the water samples and identified as I1, I2, I3, and I4 (I-Isolate). Table 1 lists the colony morphology tabulations. In Table 2, the results of the biochemical tests were tabulated.

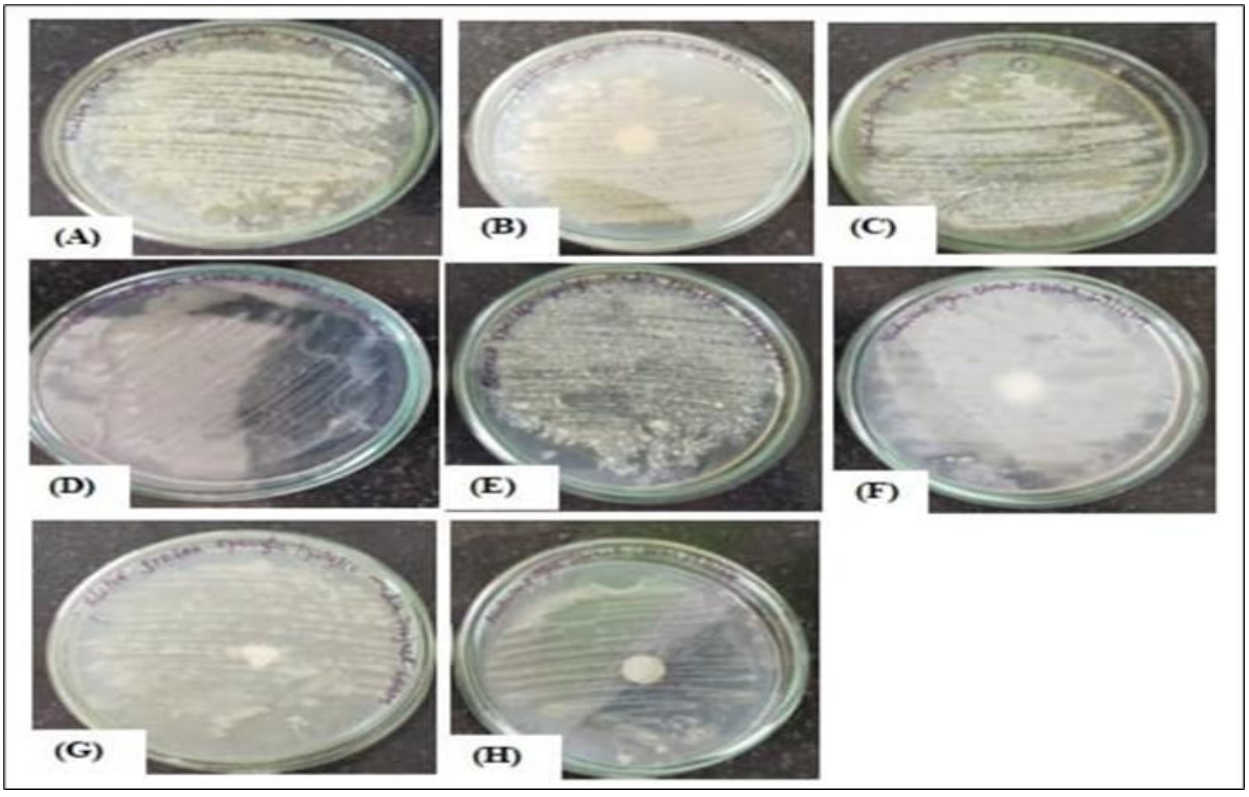

Figure1: Growth of isolated strains: (A) growth of Isolate-1 strain on selective media
(B) Isolate-1 strain on Nutrient agar media; (C) Isolate-2 strain on selective media
(D) Isolate-2 strain on Nutrient agar media; (E) Isolate-3 strain on selective media
(F) Isolate-3 strain on Nutrient agar media; (G) Isolate-4 strain on selective media
(H) Isolate-4 strain on Nutrient agar media.

Table 1. Morphological features of four isolates on both selective media and nutrient agar media

SM-Selective Media, NA-Nutrient Agar

Isolates	Size	Margin	Elevation	Surface texture	Optical Feature	Colour	Shape
I-1(SM)	8mm	Lobate	raised	creamy	Opaque	Off white	Irregular
I-1(NA)	5mm	Filiform	raised	Rubbery	Opaque	Off white	Irregular
I-2(SM)	7mm	Undulate	raised	Brittle	Opaque	Off white	Irregular
I-2(NA)	6mm	Fibril	raised	creamy	Translucent	Off white	lobate
I-3(SM)	9mm	undulate	raised	Brittle	Opaque	Off white	undulate
I-3(NA)	8mm	spread	flat	Buttery	Opaque	Off white	spreading
I-4(SM)	4mm	lobate	raised	brittle	Opaque	Off white	filliform
I-4(NA)	5mm	filliform	flat	Buttery	Translucent	Off white	lobate

Table 2. Biochemical characterization for four isolates bacteria
(I1-Isolate 1, I2-Isolate 2, I3-Isolate 3,I4-Isolate 4)

S.No	Tests	I1	I2	I3	I4
1	Gram Staining	_	+	_	+
2	Indole Test	+	+	+	+
3	Methyl red Test	+	+	+	+
4	VP Test	+	+	+	+
5	Citrate Utilization Test	_	_	+	_

Egg-Yolk Test for Primary Lipolytic Activity

The use of egg-yolk suspension allows for the detection of bacterial lipase activity. Lipolysis is characterized by the formation of a "confined" opalescence in solid media containing egg yolk suspension and a shimmering "pearly layer" enveloping the colonies [10]. When copper sulfate reacts with fatty acids, greenish-blue insoluble copper soaps are created that are evident as an opalescence in the medium as well as a sparkling layer. After the addition of copper II sulfate solution, lipase breaks down the lipids in the egg yolk, causing the colony to appear greenish blue in colour.In the egg yolk test, two of the four isolates, I2 and I4, exhibit strong lipolytic activity (Figure 2).

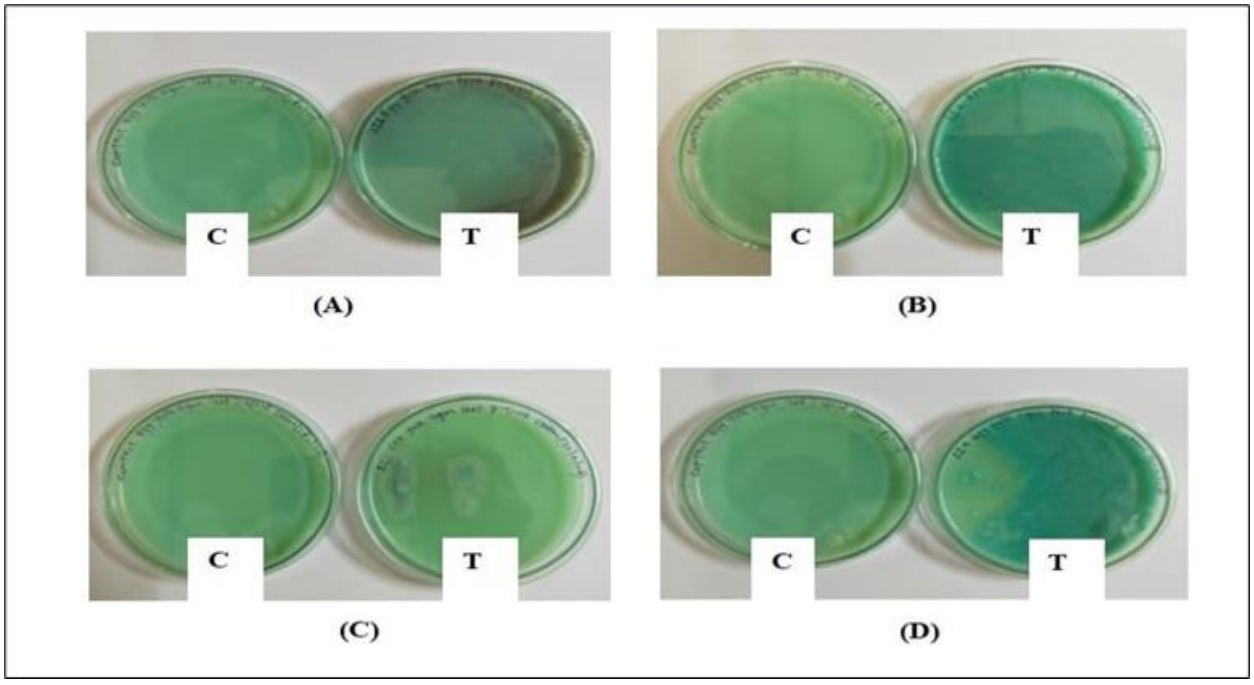

Figure 2: Egg yolk screening test for primary lipolytic activity
(A) Isolate1- negative; (B) Isolate2- positive; (C) Isolate3- negative; (D) Isolate4-positive

Oil displacement test

Two isolated strains, I2 and I4, had more lipolytic activity than I1, I3, according to an oil displacement test conducted with vegetable and mustard oils (Figure 3). I2 and I4 were then employed in additional studies to see if they might be used in real-world scenarios. To determine each isolate's lipolytic capability, the Zone of Hydrolysis was assessed (Table 3). All seven isolated isolates were found to be lipolytic by an oil displacement test, while SI1 and SI7 exhibited higher lipolytic activity [11].

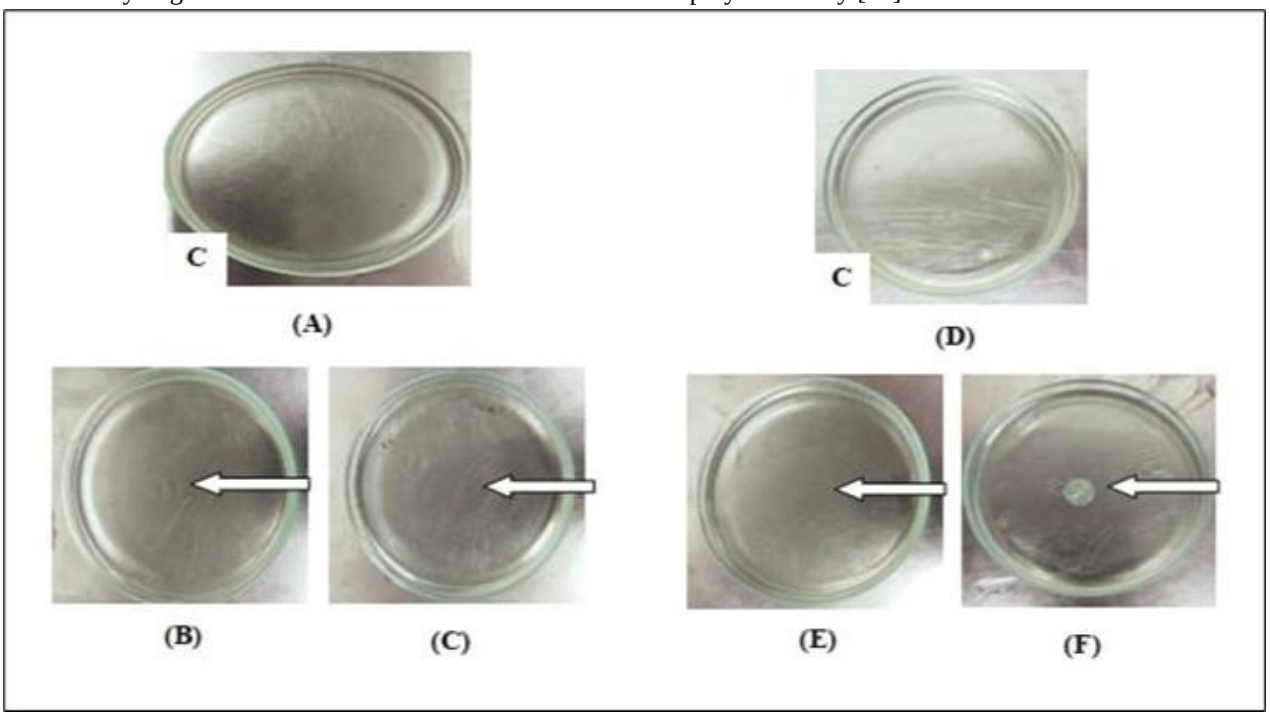

Figure 3:Shows the results for oil displacement test
(A) Vegetable oil control, (B) Arrow indicates the clear zone formed by Isolate 2 in vegetable oil, (C) Arrow indicates the clear zone formed by Isolate 4 in vegetable oil.
(D) Mustard oil control, (E) Arrow indicates the clear zone formed by Isolate 2 in Mustard oil, (F) Arrow indicates the clear zone formed by Isolate 4 in Mustard oil.

Table 3. Diameters (in cm) of clear zone formed by I2 ,I4 isolates in oil displacement test

Isolates	Vegetable oil	Mustard oil
I2	4.60±0.63	6.55±0.48
I4	5.20±0.30	5.8±0.28

Data represents Mean ± Standard error of mean

Application of lipase for removal of oily stains from cotton cloth.

It was demonstrated that the oily stains on clothing could be removed by lipase enzymes derived from I2 and I4. When lipase enzyme extract was applied to clothes to remove stains, stains were almost fully removed, in contrast to incomplete stain removal that was shown when clothes were washed with just water or water mixed with detergent. Figures 4 and 5 depicted this. To ascertain the effectiveness of the enzyme to enhance detergent wash performance, researchers examined lipases' capacity as detergent additives. Given that *Bacillus methylotrophicus* PS3 lipase was shown to be effective at removing grease, butter, vegetable oil, and olive oil stains in less than 30 minutes, these enzymes could be considered significant candidates for laundry detergent additive [12].

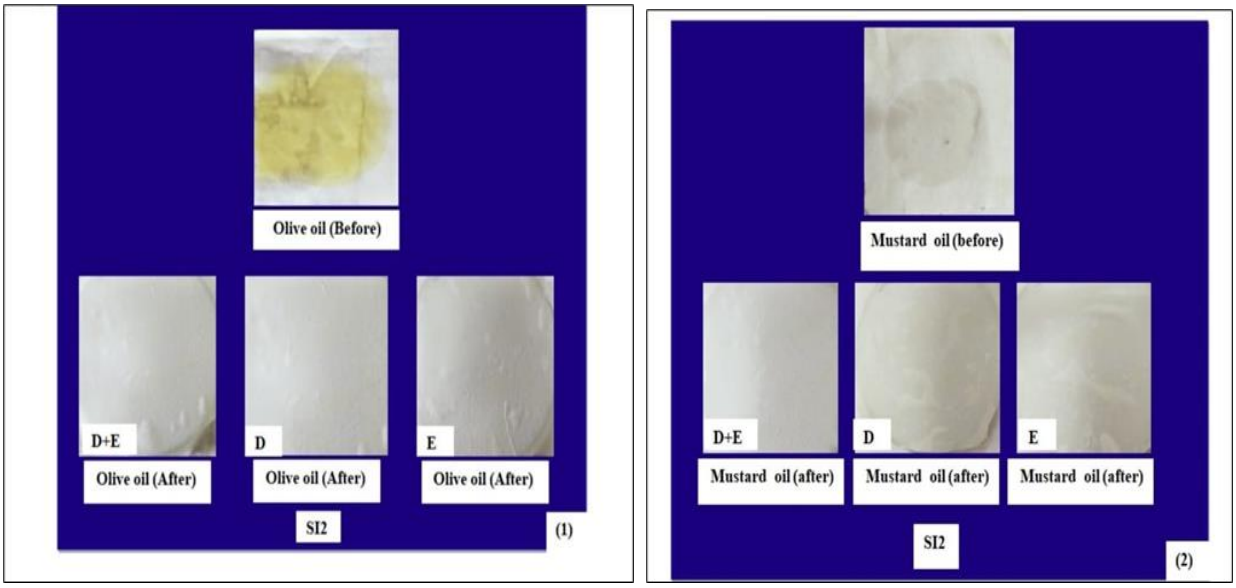

Figure 4:Washing performance of Isolate 2 lipase enzymes (D-Detergent, E-Isolate 2 lipase)
(1) olive oil stained cloth (2) mustard oil stained cloth

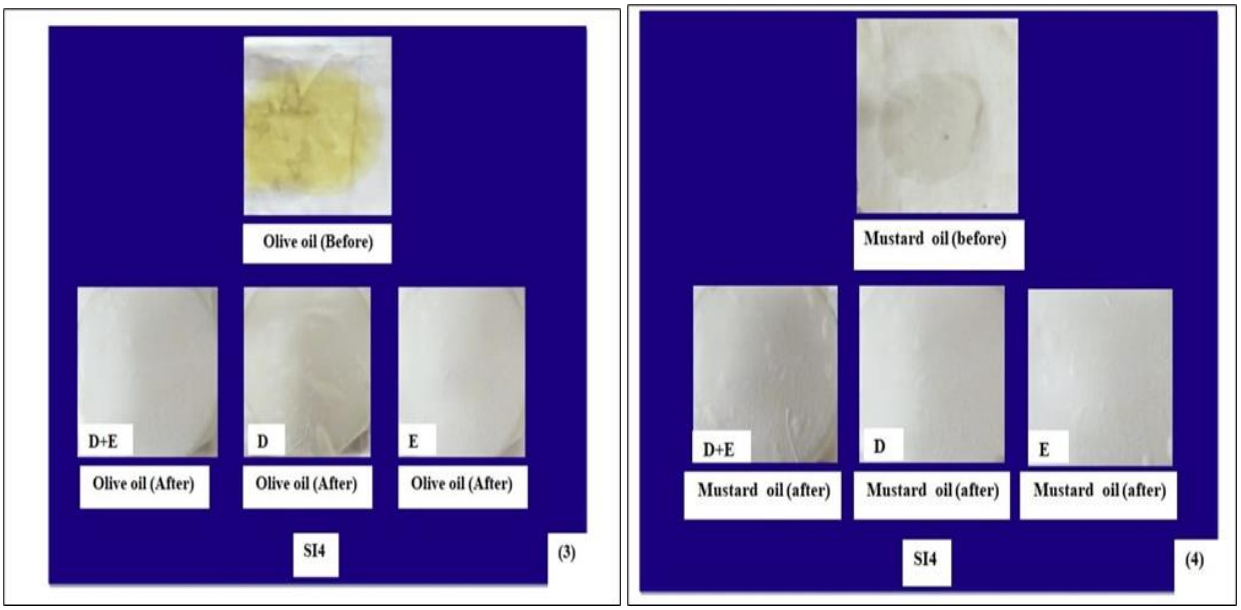

Figure 5:Washing performance of Isolate 4 lipase enzymes (D-Detergent, E-Isolate 2 lipase)
(3) olive oil stained cloth (4) mustard oil stained cloth

Molecular identification of the Isolate 2 (I2) using 16S rRNA sequencing

Based on the 16S rRNA sequence, the highest lipase-producing bacterial isolates, I2, were found. From the extracted DNA, the 16S gene area was amplified by PCR using universal primers. Resolved on Agarose Gel, a single distinct 1000 bp PCR amplicon band was seen. To get rid of impurities, the PCR product was filtered. Lane 2: DNA Sample; Lane 1: 250 Step up ladder. Using the Universal forward and reverse primers, the purified PCR amplicon was sequenced. Using an ABI 3500 Genetic Analyzer and the BDT v3.1 Cycle sequencing kit, sequencing was carried out.

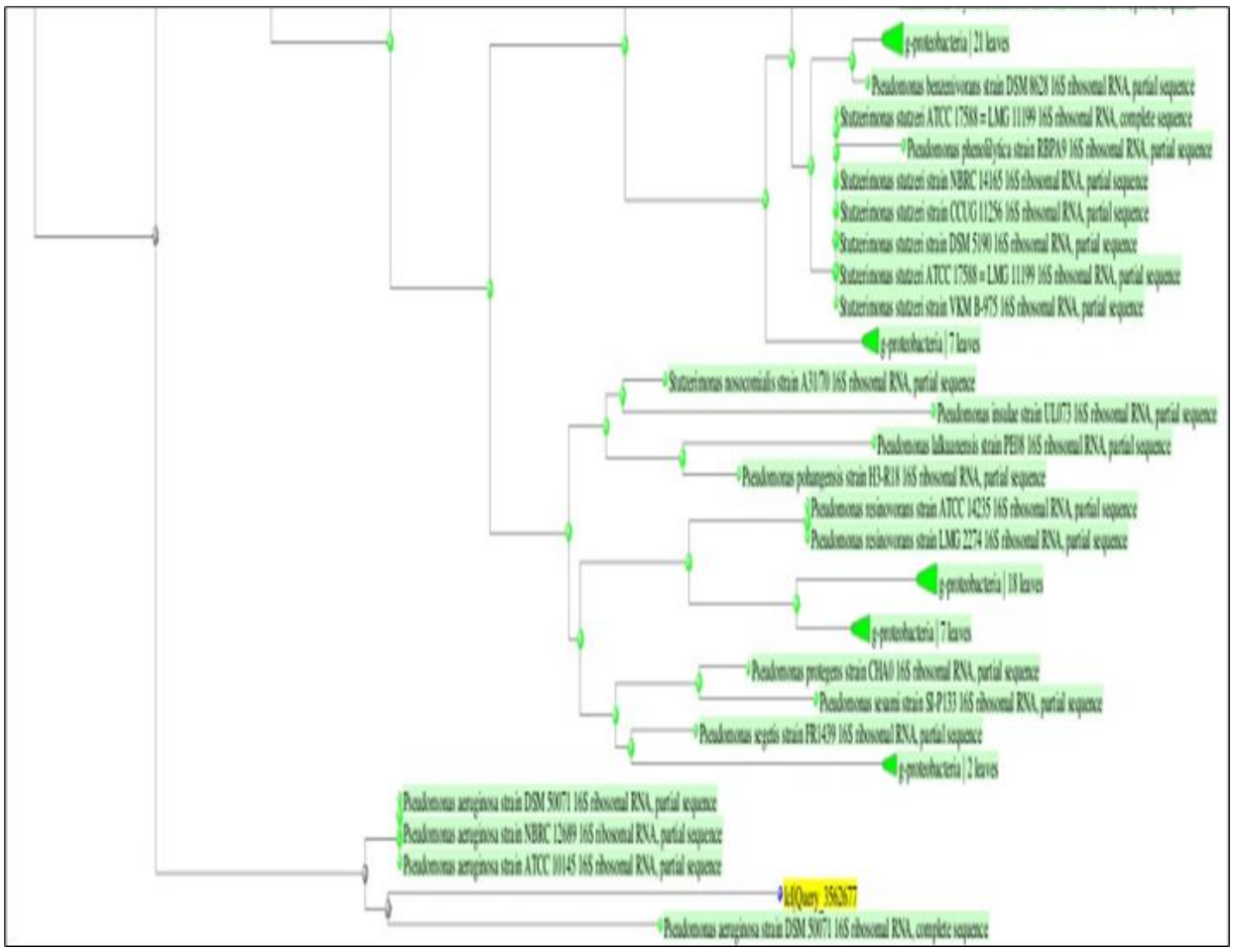

Figure 6: Representing the phylogenetic tree based on the sequence of the 16S rRNA gene shows the evolutionary relationships of *Pseudomonas aeruginosa* strain DSM 50071

Most bacterial strains that have been identified to generate lipases include those belonging to the genera *Bacillus, Geobacillus, Burkholderia, Enterococcus, and Staphylococcus* [13]. A potent method for prokaryote identification and classification is 16S rRNA gene sequencing [14]. The most effective isolates for generating lipase were found to be *Brevibacillus sp*. strains HC1 and HS5 [15]. *Pseudomonas aeruginosa* strain DSM 50071 16S ribosomal RNA, partial sequence; GenBank Accession Number NR_117678.1; was determined by sequence homology and phylogenetic analysis, culture. The strain was shown to be the best lipase-producing isolate (Figure6). *Pseudomonas aeruginosa strain* DSM 50071 produced lipase enzymes with high activity, and the lipase enzyme obtained from the *Pseudomonas aeruginosa strain* DSM 50071 capable of eliminating the oily stains from cotton garments, according to the performed lipase activity assay. Since the oil stains significantly improved after the lipase was added to the detergent. It was mentioned that *Pseudomonas aeruginosa strain* DSM 50071 lipase was appropriate for use as a detergent additive.

IV.CONCLUSION

The goal of the current investigation was to isolate and identify lipolytic bacteria from a water sample. A zone of hydrolysis was seen and four bacterial isolates were obtained on a medium with lipid as the only carbon source. Isolates I2 and I4 with the highest lipolytic activity were chosen after screening and given more study. It was demonstrated that cotton clothing could be cleaned of oily stains using lipase enzymes derived from I2 and I4. The lipolytic bacteria I2 were identified using 16s rRNA sequencing. *Pseudomonas aeruginosa strain* DSM 50071 is the most promising

bacterial isolate that can be good candidates for the production of lipase enzymes to be used in detergent additive and other different applications.

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REFERENCE

[1] Lee K., Eisterhold M.L., Rindi F., Palanisami S. and Nam P.K. 2014. Isolation and screening of microalgae from natural habitats in the mid western United States of America for biomass and biodiesel sources. *Journal of natural science, biology and medicine*,5(2): 333–339.

[2] Antczak M.S., Kubiak A., Antczak T., Bielecki S. 2009. Enzymatic biodiesel synthesis – Key factors affecting efficiency of the process. Renewable Energy, 34(5): 1185–1194.

[3] Chauhan M, Chauhan RS & Garlapati VK (2013). Evaluation of a New Lipase from *Staphylococcus sp*. for Detergent Additive Capability. *J Biomed Biotechnol* 2013:1-6

[4] Azhdarpoor A, Mortazavi B & Moussavi G (2014). Oily wastewaters treatment using *Pseudomonas sp.* Isolated from the compost fertilizer. *J Environ Health Sci Eng* 12:77.

[5] Kalapatapu VP & Reddy IB (2015). A Study of Thermostable And UV Resistant Lipase Producing *Bacillus Species*. *Int J Adv Res* 3(3):1-19.

[6] Kandala H, Roy D, Sathiavelu M, Arunachalam S & Loganathan K (2010). Isolation And Characterization of Lipase Producing Bacteria From Vellore Soil. *Pharmacology online* 2: 356-361.

[7] Wai KP, OWWN & Shwe MT (2016). Screening on Lipase-producing Bacteria Isolated from Oil Mill Soil. 5th International Conference on Food, Agricultural and Biological Sciences (ICFABS-2016) Dec. 25-26, 2016 Bangkok (Thailand).

[8] Abbas H, Wsain SM, AL-Quraishi DA & Fartoos SK (2015). Using of biosurfactant and bacterial enzymes for treatment of the oily polluted soils. *Int J Adv Res* 3(5): 741-748.

[9] Kanmani R, Dhivya S, Jayalakshmi & Vijayabaskar P (2011). Studies on detergent additives of protease enzyme from an estuarine bacterium *Bacillus cereus. Int Res J Biotechnol* 2 (7): 157-163.

[10] Sadia Faiz, Zahida Nasreen, Aneela Sharif and Saira Naz. Isolation, screening and characterization of lipase from bacterial isolates and its application in detergents and oily waste water degradation. *Pure and Applied Biology*. 10(1)U:209-224.

[11] Sharma R, Chisti Y & Banerjee UC (2001). Production, purification, characterization and applications of lipases. *Biotechnol Adv* 19: 627–662.

[12] Sharma P, Sharma N, Pathania S & Hande S (2017).Purification and characterization of lipase from *Bacillus methylotrophicus* PS3 under submerged fermentation and its application in detergent industry. *J Genet Eng Biotechnol* 15:369-377.

[13] Javed, S.; Azeem, F.; Hussain, S.; Rasul, I.; Siddique, M.H.; Riaz, M.; Afzal, M.; Kouser, A. and Nadeem, H. (2018): Bacterial lipases: A review on purification and characterization. *Progress in Biophysics and Molecular Biology*, 132: 23-34.

[14] Hassan, S.W.; Abd El Latif, H.H. and Ali, S.M. (2018): Production of cold-active lipase by free and immobilized marine Bacillus cereus HSS: application in wastewater treatment. *Front Microbiol*, 9: 2377.

[15] Hayam S. Thabet ; Ismail Seddik ; Wegdan A. Mohamed ;Ahmed M. Sayed And Shaymaa Yusuf U(2003)U: Isolation, Screening, And Molecular Identification Of Lipase-Producing Bacteria From Different Bacterial Sources. *Assiut Veterinary Medical Journal, 69(178) : 134-144.*