International Journal of Scientific Research and Engineering Development-– Volume 7 Issue 5, Sep-Oct 2024
Available at www.ijsred.com

RESEARCH ARTICLE OPEN ACCESS

Microbial Cleanup Crew: Isolation and Characterization of Lipase-Producing Bacteria from Petrol Station Soil

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Abstract:

Lipases are enzymes with significant roles in everyday applications, ranging from the dairy industry to detergent manufacturing. In this study, we investigated the lipolytic activity of bacterial strains isolated from oil-contaminated soils collected from petrol stations, aiming to identify the strain with the highest lipase production potential. Out of 50 isolated bacterial strains, 10 were screened for lipase activity using a chromogenic medium, resulting in the identification of positive lipase producers, evident by clear zones on the media. Among these, three potent strains—*Bacillus* sp., *Pseudomonas* sp., and *Staphylococcus* sp.—were identified using Bergey's Manual. These strains were further analysed for qualitative and quantitative lipase production using various substrates. *Pseudomonas* sp. demonstrated the highest efficiency in oil stain removal from fabric, indicating its potential application in the detergent industry for effective oil stain removal.

Keywords — Lipase, Oil contaminated soil, Bacterial strains, Tributyrin agar, Lipase assay, 16s rRNA, detergent.

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1. INTRODUTION

Lipases have emerged as one of the leading biocatalysts with proven potential for contributing to the multibillion dollar underexploited lipid technology bio-industry and have been used in in situ lipid metabolism and ex situ multifaceted industrial applications (Verma N et al., 2012). The serine hydrolases are present in abundantly and which are contributed in wide range industries. Lipase synthesizes esters from glycerol and long-chain fatty acids in non-aqueous medium (Prem Chandra et al., 2020).

Lipases are produced by microorganisms (bacteria and fungi), plants and animals, However, microbial lipase especially from bacteria are more useful than their plant and animal origin. Since they known as lipase enzyme which belong to triacylglycerol ester hydrolase family. They can catalyze the hydrolysis (and synthesis) of long-chain triglycerides to fatty acids, diacylglycerol, monoacylglycerol and glycerol known as carboxylesterases. Besides hydrolysis activity they display interesterification, esterification, amino lysis and alcoholysis activity

have great variety of catalytic activities and microorganisms are easy to manipulate genetically and capable of rapid growth on inexpensive media. The microbial lipases are commercially most important mainly are secreted into the culture medium by many of microbial species are belong to bacteria, fungi, yeasts and actinomyces (Maytham Ayuob Alhamdani et al., 2016). Microbial lipases

have gained special industrial attention due to their ability towards extremes of temperature, pH, and organic solvents, and chemo-, region-, and enation-selectivity. Some important lipase-producing bacterial genera are *Bacillus, Pseudomonas* and *Burkholderia* and fungal genera include *Aspergillus, Penicillium, Rhizopus,* and *Candida.* Different species of yeasts belonging to seven different genera include *Zygosaccharomyces, Pichia, Lachancea, Kluyveromyces, Saccharomyces, Candida,* and *Torulaspor*a (Verma N et al., 2012).

The 3D structure of lipases from different microbial sources are not exactly alike, they exhibit high sequence diversity. As a result, these enzymes are unique and specific to the type of bioconversion processes they catalyze, finding relevance in a wide array of industrial processes. They are found to be useful in catalyzing various reactions synonymous to food, pharmaceuticals, medical and diagnosis, dairy, fatty acid, leather, cosmetic, detergent, beverage and paper industries (Hadiza Jamilu et al., 2022).

2. MATERIALS AND METHODS

2.1. Sample collection

For the present study oil-contaminant soil sample were collected from petrol bunk which was continuously exposed to petrol and diesel. The sample were collected from a depth of 5-10 cm using a sterile spatula and stored in sterile glass vials following collection, the sample ware immediately transferred to the laboratory for examination and subsequent analysis (Maytham Ayuob Alhamdani et al., 2016).

2.2. Isolation of lipase producing bacteria

The collected samples ware enriched by periodic sub-culturing of samples in nutrient broth (NB) medium composed from (5gm peptone and 3gm beef extract) per liter, pH of medium was adjusted to 7 with 0.1M NaoH.1gm of soil sample was dissolved in 10ml of sterile nutrient broth in 50ml Erlenmeyer flask and agitation at 120 rpm for 30min at 37℃ on a rotary shaker (Maytham Ayuob Alhamdani et al., 2016). The sample was serially diluted up to 10^{-6} dilution using 0.8% saline.100µl of each dilution was spread on Rhodamine B medium contained (0.8% w/v nutrient broth, 0.4% w/v NaCl, 500 µL of 0.01% Rhodamine B solution, 1% w/v agar and 7.5% v/v olive oil\glycerol). The pH of the medium was adjusted to 7.0 and plates were incubated at 37 °C for 24 h. After incubation, the plates were exposed to UV light (350 nm) for determination the lipase activity of the isolated strains (Mohammed Rabbani et al., 2013).

2.3. Morphological and biochemical characterization

Three colonies with fluorescence [B1, B2, and B3] were sub cultured on NA media and subjected to various tests to identify the bacterial strain. According to the Bergey's manual of systemic bacteriology, these tests were selected and were performed in triplicate (Claus and Berkeley, 2011). The tests included: Gram staining reaction, spore position and shape, and the biochemical test such as IMViC test, oxidase, catalase, starch test. The cell morphology was examined by light microscopy and biochemical characteristics were investigated at 37 °C (Mohammed Rabbani et al., 2013).

2.3.1. Gram staining

The bacterial smear was prepared on clean slide and heat fixed after drying and the entire slide could be moistened by a low volume of reagent that filled the cuvette in a specific order crystal violet (1-2 min), iodide solution (1 min), decolorant (70% ethanol for 15-20 sec), safranin (2 min), with water washing after each step (Hui Li et al., 2020). Last, the slides were air dried and observed under a bright field microscope using an immersion lens.

2.3.2. Endospore staining

The bacterial smear was prepared, heat fixed, and covered with malachite green solution (5%). Slides were passed through a flame for 5 min intermittently, making sure to not boil the dye. The slides were then washed with water and stained with safranin (2.5%) for 30 sec. Last, the slides were air dried and observed under a bright field microscope using an immersion lens (Paula de Camargo Bertuso et al., 2022).

2.3.3. Indole test

Tryptone broth (Tryptone 10g, NaCl 0.5g, $CaCl_3$ 0.03g in 1000ml water) was prepared, pH was adjusted to 7, inoculated with test organisms and incubated at 37°C for 24h. After incubation kovac's reagent (1ml) was added and observed for cherry red ring formation.

2.3.4. Methyl red test

MR-VP broth (buffered peptone 7g, glucose 5g, K_2HPO_4 5g in 1000ml water) was prepared, pH was adjusted to 7.5, inoculated with test organisms and incubated at 37°C for 24h. After incubation 2-3 drops of methylene red was added shaken well and left undisturbed for 18-20 min and observed for color change.

2.3.5. Voges Proskauer test

MR-VP broth (Buffered peptone 7g, glucose 5g, K_2HPO_4 5g in 1000ml water) was prepared, pH was adjusted to 7.5, inoculated with test organisms and incubated at 37°C for 24h. After incubation 12 drops of VP reagent I and 2-3 drops of VP reagent II was added and observed for color change.

2.3.6. Citrate test

Simmon's citrate agar (sodium citrate 2g, NaCl 5g, $MgSO_4$ 0.2g, bromothymol blue 0.8g, $(NH_4)_2HPO_4$ 1g, K_2HPO_4 1g in 1000ml water) was prepared, pH was adjusted to 7, inoculated with test organisms and incubated at 37°C for 24h. After incubation observed for color change.

2.3.7. Oxidase test

The loop full of test organisms were smeared on the colorless oxidase disc on clean glass slide and observed for color change.

2.3.8. Catalase test

The loop full of test organisms were immersed in hydrogen peroxide and observed for bubbling (James G. Cappuccino et al.).

2.3.9. Starch test

Starch agar (Peptone 5g, Beef extract 3g, NaCl 5g, Starch 20%, Agar 15g in 1000ml water) was prepared, pH adjusted to 7, inoculated with test organisms and incubated at 37°C for 24h. After incubation plates were immersed with gram's iodine and observed for clear zone (Ajay Kumar Sahu et al., 2019).

3. QUALITATIVE ANALYSIS OF LIPASE ENZYME (plate assay)

Lipolytic organisms were screened by qualitative plate assay. Isolated organisms were grown on Tributyrin agar (peptone 0.5%, yeast extract 0.3%, ammonium nitrate 1%. K_2HPO_4 0.05%, KH_2PO_4 0.05%, glucose 0.1%, tributyrin/mustard oil 1%, agar 2% (Shubham Verma et al., 2014)) base plates and incubated at 37°C for 2 days. Zone of clearance was observed due to hydrolysis of tributyrin (E. Sirisha et al., 2010).

4. CHARACTERIZATION AND MOLECULAR IDENTIFICATION OF BACTERIA

The identification of 3 isolated lipolytic bacteria (B1, B2, and B3) was confirmed by using nucleotide sequence analysis of 16S rDNA genes. The genomic DNA of all 3 bacteria was isolated by the CTAB method. 16S rRNA gene of isolated strains was amplified from its genomic DNA using a bacterial primer for B1, 27F (5'-AGAGTTTGATCCTGGCTCAG - 3') as forward primer and 800R (5'-GGTTACCTTGTTACGACTT -3') as reverse primer, for B2, PA-SS-F (5'-GGGGGATCTTCGGACCTCA -3') as forward primer and PA-SS-R (5'-TCCTTAGAGTGCCCACCCG -3') as reverse primer, and for B3, arc up (5'-TTGATTCACCAGCGCGTATTGTC -3') as forward primer and arc dn (5'-AGGTATCTGCTTCAATCAGCG -3') as reverse primer respectively. The highly purified DNA was then amplified in a thermocycler at conditions: 95°C for 2min, 30 cycles of 95°C for 30 seconds, 50°C for 30 seconds and 72°C for 1 min and finally 72°C for 10 min. PCR products were visualized by agarose gel electrophoresis. DNA sequencing was performed in a highly automated gene sequencer. These sequences had been submitted to the GenBank database (BLASTN) and compared with the other sequences to analyze the bacterial classes and their phylogenies.

5. LIPASE PRODUCATION MEDIA

The production medium consist of (%w/v) Peptone 0.2, $NH_4H_2PO_4$ 0.1; NaCl 0.25; $MgSO_4.7H_2O$ 0.04; $CaCl_2.2H_2O$ 0.04; Olive oil/mustard oil 2.0 (v/v); pH 7.0; 1-2 drops of Tween 80 as emulsifier. (Mobarak- Qamsari et al., 2011). 24hrs old culture was used to prepare inoculum by transferring loopful of cells into production media and the flasks were incubated 24hr at 37°C (Sadia Faiz et al., 2020). Overnight cultures were inoculated into the 250ml Erlenmeyer flasks containing 100ml media and were kept in rotary shaker for 150rpm. Sample were collected after 24 hours and centrifuged at 10,000 rpm for 10mins at 4°C. The cell filtrate was used as a source of extracellular enzyme lipase (Aliyu et al., 2011).

6. LIPASE ASSAY (quantitative analysis)

Lipase activity was measured by titrimetric method with minor modifications and using Groundnut oil as a substrate .Oil substrates (1ml) and 1ml 0.05M phosphate buffer (pH 7.0) were added to a test tubes and mixed thoroughly; the mixture was incubated in a water bath at 37°C for 5 min, then 0.5ml crude lipase (supernatant of culture medium) was added to the reaction mixture and incubated at 37°C for 20 min (Qiongli Ma et al., 2010); After that, reaction was stopped by adding of 1ml of acetone and ethanol mixture in 1:1 ratio. The titration of released fatty acids was done with 0.05M NaOH using 1-2 drops of phenolphthalein as indicator until pink color appeared (Sadia Faiz et al., 2020).

7. CALCULATION OF LIPASE ACTIVITY

Lipase enzyme activity can be calculated as follows:

Enzyme assay (U\ml) = $\frac{(A-B)\times[NaOH]\times 1000}{20}$

Description: A = ml NaOH for the titration of the sample, B = ml NaOH for the blank titration, factor 1000 for conversion from mmol to µmol, and 20 = reaction time [20 min] (Meliynda Pomeistia 2017). One unit of lipase activity is defined as the amount of enzyme required to hydrolyze µmol of fatty acids from triglycerides (Hemkala 2017).

2. Analytical reagents: Alkaline copper reagent

a) 2% of sodium carbonate in 0.1N NaOH solution

b) 0.5% of Copper sulphate in 1% Sodium potassium tartrate.

Mix both a and b reagents in the ratio of 5:1.

8. OPTIMIZATION OF LIPASE ACTIVITY

Bacterial lipases are mostly extracellular and are greatly influenced by nutritional and physico-chemical factors, such as temperature, pH, nitrogen and carbon sources, presence of lipids, inorganic salts, agitation and dissolved oxygen concentration (Brune and Gotz, 1992; Jaeger et al., 1994).

8.1. Effect of temperature on lipase activity and stability

The temperature optimum for the enzyme was determined in the range 30 to 80°C, at pH 7.4, as above. The enzyme stability at different temperatures (20°C, 37°C, 50°C) was studied by using different substrates and enzyme was incubated in 0.05M phosphate buffer, pH 7.0 at different temperatures for 20min, followed by the activity estimation at 37°C (R.O. Fashogbon et al., 2021).

8.2. Effect of ph. on lipase activity and stability

The pH of 4.0, 7.0, and 10.0 was selected to detect optimum pH where enzymes exhibit maximum activities. Citrate buffer (pH 4.0), phosphate buffer (pH 7.0), and sodium carbonate buffer (pH 10.0) was used to adjust the pH and followed the above procedure by keeping temperature constant [37°C] (Sadia Faiz et al., 2020).

8.3. Effect of oil substrate on lipase activity and stability

Different oil substrates were used to know the lipase activity by giving neutral pH (pH 7) and normal temperature. Different oil substrates diesel, ghee, coconut oil, sunflower oil and petrol was used and followed the above procedure (Rama T et al., 2020).

9. TOTAL PROTEIN ESTIMATION (lowry's method)

Reagents used for estimation of the protein content include;

1. BSA stock solution: 100mg of BSA was dissolved in 100ml of distilled water in a standard volumetric flask.

3. Folin Ciocalteau (FC) reagent: dilute the commercially available FC reagent with distilled water in the ratio of 1:3 on the day of use.

The phenolic group of tyrosine and tryptophan residues (amino acid) in a protein produces a blue purple color complex, with maximum absorption in the region of 660 nm wavelength, with Folin- Ciocalteau reagent which consists of sodium tungstate molybdate and phosphate. Thus, the intensity of color depends on the amount of the enzymes present and thus varies for different proteins. Most proteins estimation techniques use Bovine Serum Albumin (BSA) universally as a standard protein, because of its low cost, high purity and ready availability. The method is sensitive down to about 10 µg/ml and is probably the most widely used protein assay despite its being only a relative method, subject to interference from Tris buffer, EDTA, nonionic and cationic detergents, carbohydrate, lipids and some salts. The incubation time is very critical for a reproducible assay.

Different dilutions of BSA solutions ranging from 0.1-1ml was pipetted out into test tubes and the final volume in each of the test tubes was made up to 1ml using distilled water each has the concentration from 100-1000µg/ml and 1ml distilled water without BSA solution was used as blank. To these different dilutions, 5 ml of alkaline copper sulphate reagent (analytical reagent) was added and the solutions mixed thoroughly. The solution was incubated at room temperature for 10 mins, then added 0.5 ml of reagent Folin Ciocalteau solution (reagent solutions) to each tube and incubates for 30 min in dark. Zeroed colorimeter with blank, and the optical density (measured the absorbance) at 660 nm was taken. The absorbance against protein concentration was plotted to get a standard calibration curve (Hadiza Jamilu et al., 2022).

9.1. Unknown preparation

Unknown was prepared using mustard oil as a substrate. 1ml of mustard oil with 0.5ml of enzyme solution was incubated at room temperature for 5min and from that 1ml solution was used and followed the above procedure. The absorbance of unknown sample was used to determine the concentration of the unknown sample using the standard curve plotted.

10. OILY STAIN REMOVAL BY LIPASE ENZYME

Application of lipase enzyme extract (for B1, B2, and B3) as a detergent additive was carried out on cotton cloth pieces (10×10 cm) blemished with weathered hydrocarbons (sunflower oil). The experimental groups were set to study oily stain removal efficacy of lipase enzyme as follow:

1. Flask with distilled water (100 ml) + stained cloth (stained with sunflower oil).
2. Flask with distilled water (100 ml) + stained cloth (stained with sunflower oil) + 1 ml of detergent surf excel (10mg/ml).
3. Flask with distilled water (100 ml) + stained cloth (stained with sunflower oil) + 1 ml surf excel detergent (10mg/ml) + 2 ml of lipase enzyme extract.
4. Flask with distilled water (100 ml) + stained cloth (stained with sunflower oil) + 6 ml of lipase enzyme extract. All flasks were kept in incubator at 37°C for 5 minutes to check oily stain removal efficiency. Cloth pieces were visually examined after treatment (Sadia Faiz et al., 2020).

11. RESULTS AND DISCUSSION

1. Isolation of lipase producing bacteria

Isolation of lipase producing Bacteria in the present study from soil sample were collected from petrol bunk in Bangalore. The choice of areas was based on places with higher probability of lipase producing bacteria due to high level of fats accumulated by activities in that area.

A total of 50 x 10^{-5}CFU bacterial strains were isolated using Rhodamine B agar (fig.1) media by

using serial dilution technique which were screened for the production of lipase. 10 bacterial strains showed positive results in lipase production. The highest production of lipase by 3 potent bacterial strains named as B1, B2, and B3, amongst the 10 strains was confirmed based on the qualitative screening plate assay.

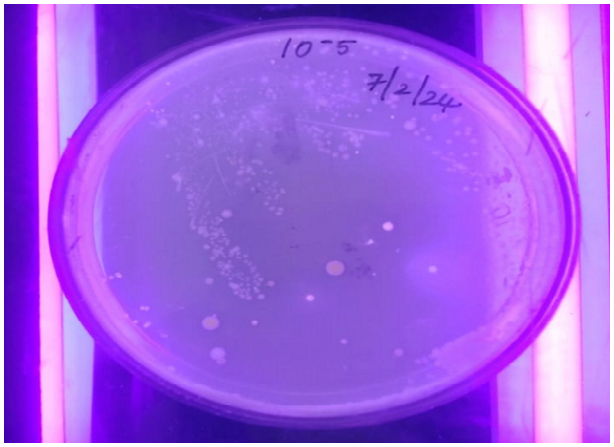


Fig.1. Isolation of lipase producing bacteria on Rhodamine B agar showing fluorescence around colonies after exposing to UV light.

2. Morphological and biochemical characterization

The morphological characters of isolated colonies were observed on NA media (table.1) and the biochemical characterization of the isolate identified the bacteria as a species of the genus *Bacillus.spp, Pseudomonas.spp, and Staphylococcus.spp* (table.2) by following the bergey's manual.

Table.1. Morphological characterization of the isolated colonies on NA media

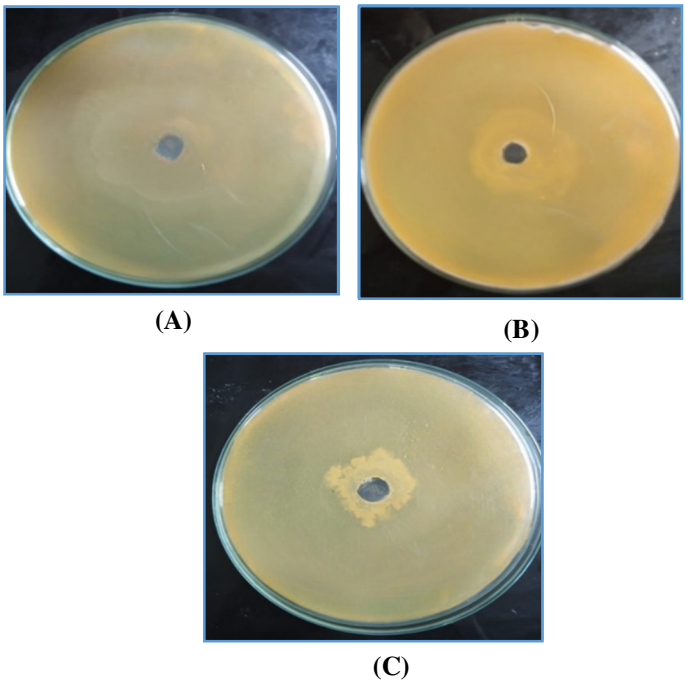
Characters	B1	B2	B3
Size	Moderate	Large	Large
Shape	Irregular	Irregular	Circular
Color	Creamish	Greenish	Golden yellow
Margin	Lobate	Irregular	Entire
Elevation	Raised	Flat	Convex
Texture	Rough	Smooth	Smooth
Appearance	Dull	Mucoid	Shiny
Optical property	Opaque	Opaque	Opaque

Table 2 Biochemical characterization of islolated colonies

Tests	B1	B2	B3
Gram staining	Positive	Positive	Positive
Shape	Rods	Rods	Cocci in cluster
Endospore staining	Positive	Negative	Negative
Indole	Negative	Negative	Negative
Methyl red	Positive	Negative	Positive
Voges proskauer	Positive	Negative	Positive
Citrate	Positive	Positive	Positive
Oxidase	Positive	Positive	Negative
Catalase	Positive	Positive	Positive
Starch	Positive	Negative	Positive

3.Qualitative analysis by plate assay

The isolated 3 colonies, *Bacillus.sp, Pseduomonas.sp, Staphylococcus.sp,* showed maximum zone of clearance when plated on tributyrin agar base due to hydrolysis of tributyrin (fig.2).



(A) **(B)**

(C)

Fig 2 Isolated colonies showing zone of clearance on tributyrin agar after 42hrs of incubation; (A)-Bacillus.sp; (B)-psuedomonas.sp; (C)- Staphylococcus.sp

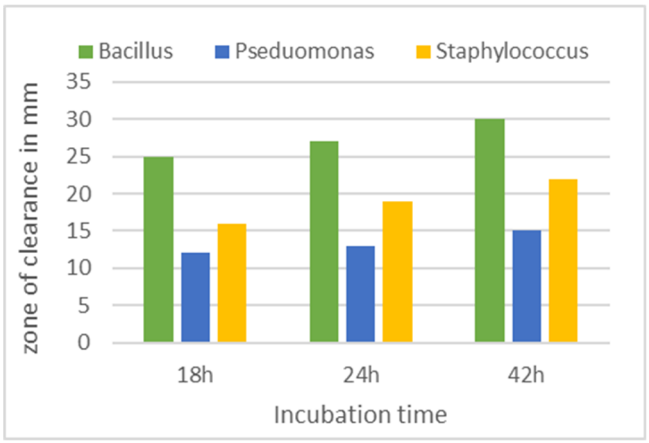


Fig.3 Graphical representation of zone of clearance on tributyrin agar plate

Table.3 Halo zone (mm) produced by lipase enzyme produced by the potent isolates, in plate assay after 18h, 24h, and 48h of incubation

Organisms	Halo zone in mm		
	18h	24h	48h
Bacillus.sp	25±0.5	27±0.5	30±0.5
Pseudomonas.sp	12±0.5	13±0.5	15±0.5
Staphylococcus.sp	16±0.5	19±0.5	22±0.5

In the Qualitative screening assay, all the isolated bacterial strains were screened for the production of lipase in plate assay which was followed by quantitative assessment of enzymes production. While screening for the efficient production of lipase enzyme, *Bacillus.sp* showed the highest zone of clearance (30mm), second highest was *Staphylococcus.sp* which showed moderate clearance zone (22mm), and the last was *Pseduomonas.sp* showed least clearance zone (15mm) compare to other two organisms (table.3). The maximum halo zone(mm) for lipase assay (30 ± 0.5 mm) was observed in Tributyrin Agar Plate at 48h of incubation and the graphical representation was noted (fig.3).

4. Molecular identification of bacterial strains

The highest lipase-producing bacterial isolates were identified based on the 16S rRNA sequence. They identified using BLAST as *Bacillus flexus strain TRS, Pseduomonas aeruginosa PA01* and, *Staphylococcus aureus SAL2* respectively.

From these studies it is very clear that *Bacillus.sp* is more predominant in the soil which contributes the production of lipase. Today most of the country relies on microbial enzymes for commercial exploitation. Lipase have diverse role in day to day life. For example, lipase is employed in various industries like detergent, dairy foods, beverages, health foods, fats, oils, paper, pharmaceuticals, bakery foods and cosmetics. These isolates can be used for further studies and the gene which is responsible for lipase production can be identified, isolated and cloned in expression vector and can get increased production of lipase.

5. Lipase assay

Quantitative analysis of lipase production in all the 3 isolates *Bacillus flexus, Psuedomonas aeruginosa, and Staphylococcus aureus* were determined by lipase assay (titrimetric method) by using groundnut oil as a substrate. Oil substrate with ph. 7 was incubated for 5min at 37°C and incubated with crude enzyme for 20min at 37°C. After incubation the mixture reaction was stopped by acetone and ethanol (1:1) and titrated against 0.05M NaOH using phenolphthalein as an indicator until pink color appears.

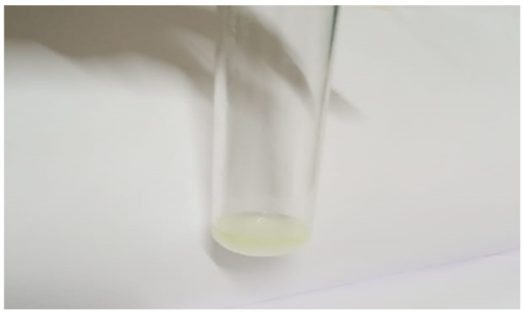
After titration, the enzyme activity was calculated by using formula;

$$\textbf{Enzyme assay (U\ml)} = \frac{(\mathbf{A-B})\times[\mathbf{NaOH}]\times\mathbf{1000}}{\mathbf{20}}$$

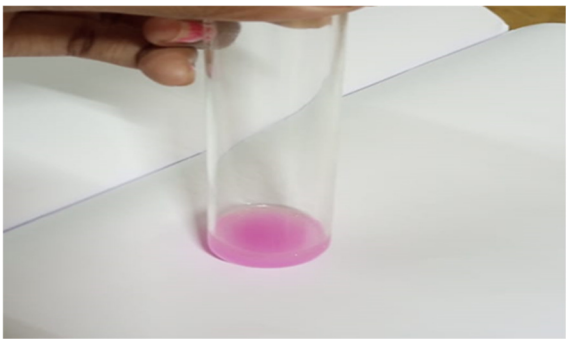
The calculated values were tabulated (table 4). One unit of lipase activity is defined as the amount of enzyme required to hydrolyze µmol of fatty acids from triglycerides.

Table 4 Lipase activity of isolated bacteria in U\ml

Organisms	Enzyme activity (U\ml)
Bacillus sp	150
Pseudomonas sp	140
Staphylococcus sp	140



(A)



(B)

Fig.4. Lipase assay; (A) – before titration; (B) – after titration

Lipase activity is measured in units (U). One unit of lipzone activity is equivalent to 1 µmol of free fatty acid produced from the hydrolysis of the substrate catalyzed by lipase per minute. Lipase enzyme activity shows the quality of the enzyme, where if the activity is high then when the enzyme used will produce good quality and vice versa. The more substrates hydrolyzed by the enzyme the higher the activity, but at a certain point when the enzyme has been reacted so much any substrate concentration added enzyme activity will not increase. The activity of lipase is obtained by hydrolysis reaction, because this reaction is most easily observed and performed. The principle of activity test with this method is that when the oil in this case groundnut oil consisting of triglyceride hydrolysis reaction which is catalyzed by lipase enzyme, then triglyceride will decompose into glycerol and free fatty acid. The occurrence of this chemical reaction is where the substrate is bound to the active side of the enzyme.

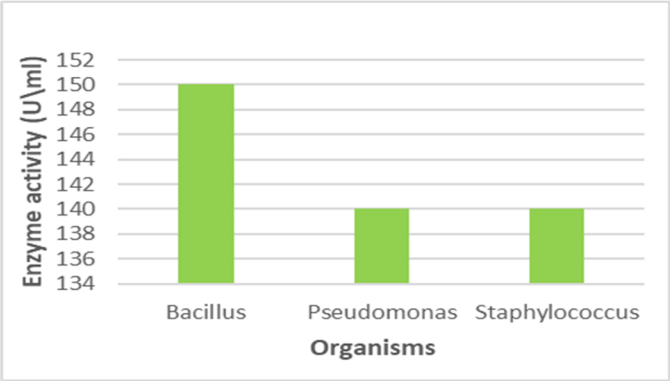


Fig.5. Graphical representation of enzyme activity

In this lipase assay, the groundnut oil was used as a substrate for estimation of lipase activity. Upon calculation, *Bacillus.sp* showed highest enzyme activity (150U/ml) compare to other 2 organisms. Whereas, *Pseudomonas.sp* and *Staphylococcus.sp* showed equal amount of enzyme activity (140U/l) which is represented (fig.5).

6. Optimization of lipase activity

Bacterial lipases are mostly extracellular and are greatly influenced by nutritional and physico-chemical factors, such as temperature, pH, nitrogen and carbon sources, presence of lipids, inorganic salts, agitation and dissolved oxygen concentration. pH and temperature are the most important effects which affects the activity of enzyme. To know the optimum pH and temperature of the enzyme, the different pH and temperature was used in the experiment.

6.1. Effect of temperature on lipase activity and stability

The effect of temperature on *Bacillus.sp, Pseudomonas.sp,* and *Staphylococcus.sp* lipase activity was done by incubating at different temperature 20°C, 37°C, and 50°C. The enzyme was optimally active at 37°C (100% relative activity). However, there was decrease in the lipase activity of all 3 enzymes as the temperature increases from 37°C to 50°C and as decrease from 37°C to 20°C. But *Bacillus.sp* enzyme was more active in all the 3 different temperatures compare to other organisms *Pseudomona.sp* and *Staphylococcus.sp* which is presented (table.5 and fig.6).

Table.5 Effect of temperature on lipase activity in U\ml

°C	*Bacillus sp*	*Pseudomonas sp*	*Staphylococcus sp*
20	130	115	110
37	150	140	140
50	130	115	125

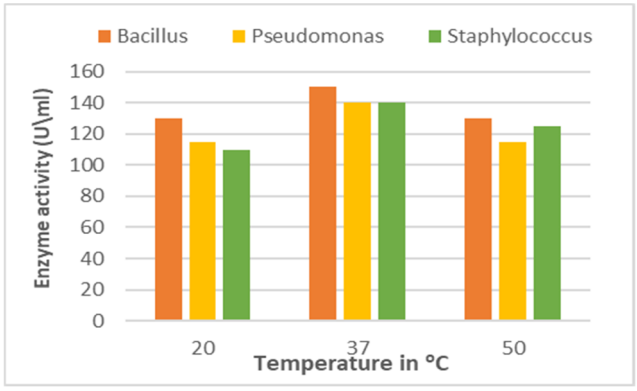


Fig.6. Graphical representation of temperature effect on enzyme activity

6.2. Effect of pH on lipase activity and stability

The activity of *Bacillus.sp, Pseudomonas.sp, and Staphylococcus.sp* lipase was observed in pH of 4.0, 7.0, & 10.0 and optimum pH was found to be 7.0 respectively. It was reported that lipase of *Bacillus sp*. exhibited maximum activity at pH7.0. But the enzyme activity was decreased to 10 folds as the ph. decreases to acidic condition (ph. 4.0) and as well as in the alkaline condition (ph. 10.0) also. All 3 isolates *Bacillus.sp, Pseudomonas.sp, and Staphylococcus.sp* exhibits maximum enzyme activity in neutral ph. (ph. 7.0). Enzyme activity of all 3 isolates were tabulated (table 6) and presented (fig.7).

Table.6 Effect of p H on lipase activity in U\ml

p H	*Bacillus sp*	*Pseudomonas sp*	*Staphylococcus sp*
4.0	35	40	45
7.0	150	140	140
10.0	25	25	25

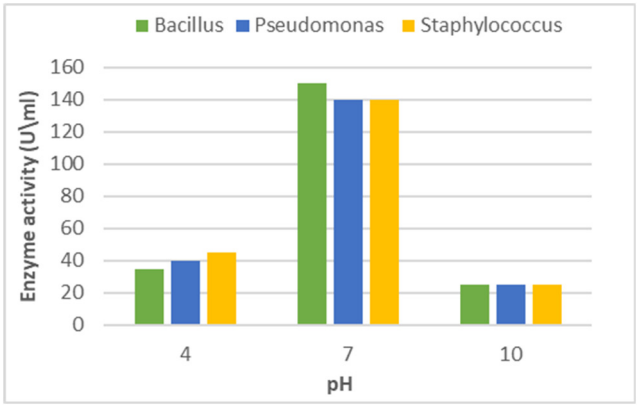


Fig.7. Graphical representation of pH effect on enzyme activity

6.3. Effect of oil substrate on lipase activity and stability

Oil substrates also have a great effect on lipase activity due to their molecular weight, viscosity. Using different oil substrates (diesel, ghee, coconut oil, sunflower oil, and petrol) to know the activity of *Bacillus.sp, Pseudomonas.sp and Staphylococcus.sp* lipase enzyme by giving neutral pH and normal temperature.

Table.7 Effect of oil substrate on lipase activity in U\ml

Substrate	*Bacillus sp*	*Pseudomonas sp*	*Staphylococcus sp*
Diesel	115	115	115
Ghee	130	120	130
Coconut oil	135	125	105
Sunflower oil	125	135	145
Petrol	115	105	115

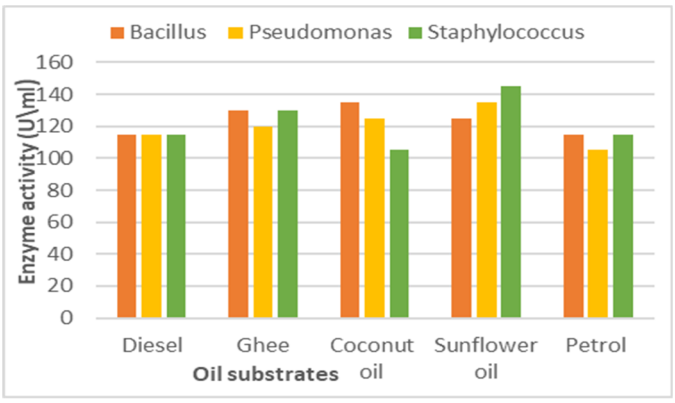


Fig.8. Graphical representation of oil substrate effect on enzyme activity

After calculation, *Bacillus.sp* shows maximum activity compare to *Pseudomonas* and *Staphylococcus.sp.* In diesel, all the 3 enzymes showed equal enzyme activity (115U\ml). In ghee and petrol, both *Bacillus.sp* and *Staphylococcus.sp* showed same amount of enzyme activity (130U\ml and 115U\ml) and *Pseudomonas.sp* showed last activity compare to those 2 organisms (120U\ml and 105U\ml). In coconut oil, *Bacillus.sp* showed highest activity (135U\ml), *Pseudomonas.sp* second highest activity (125U\ml) and *Staphylococuus.sp* showed least activity compare to those 2 organisms (105U\ml) but in sunflower oil, *Staphylococcus.sp* showed highest activity (145U\ml) and the second highest activity showed in *Pseudomonas.sp*

(135U\ml) and least activity was showed in *Bacillus.sp* (125U\ml). At neutral ph. and 37°C *Bacillus.sp* showed highest activity in ghee, coconut oil and petrol and *Staphylococcus.sp* showed highest activity in sunflower oil but *Pseudomonas.sp* showed moderate activity in all 5 substrates. The graphical representation of enzyme activity on different substrates were presented (table 7 & fig.8).

By subjecting different oil substrates to different pH (4.0 & 10.0) and temperature (20°C & 50°C), the activity of *Bacillus.sp, Pseudomonas.sp, and Staphylococcus.sp* lipase was measure. The neutral ph. and normal temp effect was mentioned above (table 7).

Table.8 Effect of pH 4.0 on lipase activity (U\ml) using different oil substrates

Substrate	*Bacillus sp*	*Pseudomonas sp*	*Staphylococcus sp*
Diesel	30	25	25
Ghee	20	25	25
Coconut oil	20	30	25
Sunflower oil	20	25	25
Petrol	20	30	20

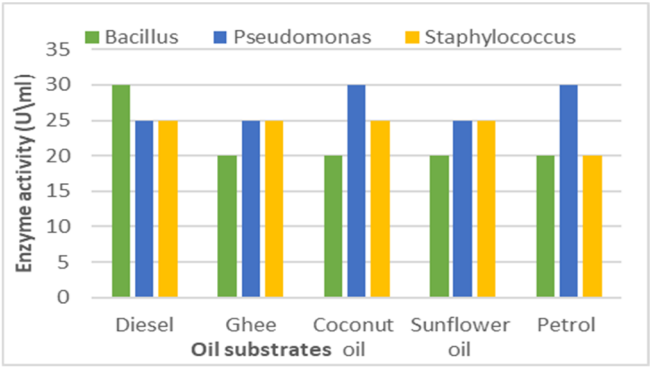


Fig.9 Graphical representation of ph. 4.0 effect on enzyme activity using different substrates

Table.9 Effect of ph. 10.0 on lipase activity (U\ml) using different substrates

Substrates	*Bacillus sp*	*Pseudomonas sp*	*Staphylococcus sp*
Diesel	5	10	25
Ghee	25	25	20
Coconut oil	10	10	10
Sunflower oil	5	5	10
Petrol	5	5	5

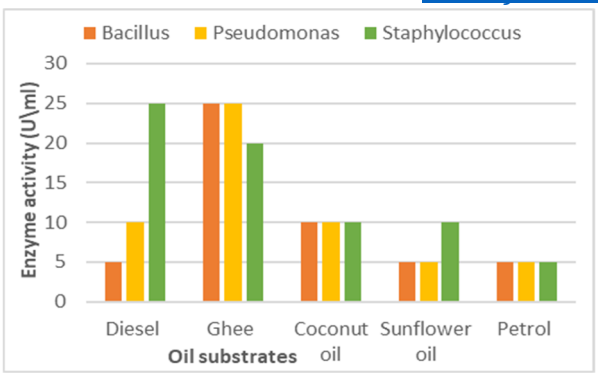


Fig.10 Graphical representation of ph 10.0 effect on enzyme activity using different substrates

Enzyme activity of all the 3 isolates are drastically decreases in both ph 4.0 and 10.0 even by using different oil substrates. Compare to acidic condition, the enzyme activity dropped to 5-25 U\ml in alkaline condition was presented (table 8, 9 and fig.9, 10) and effect of temperature (20°C & 50°C) of enzyme activity using different substrates was measured which shows decreases in activity in all 3 enzymes compare to normal temperature (37°C) was presented (table 10, 11 & fig. 11 & 13).

Table.10 Effect of temperature 20°C on lipase activity using different oil substrates

Substrate	*Bacillus sp*	*Pseudomonas sp*	*Staphylococcus sp*
Diesel	100	95	85
Ghee	115	100	95
Coconut oil	100	85	90
Sunflower oil	100	90	80
Petrol	100	80	85

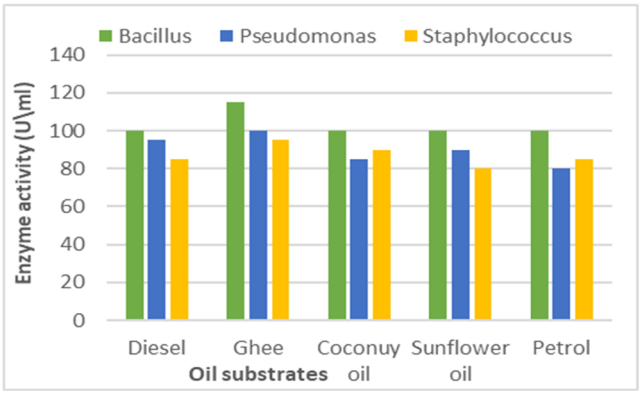


Fig.11 Graphical representation of 20°C effect on enzyme activity using different substrates

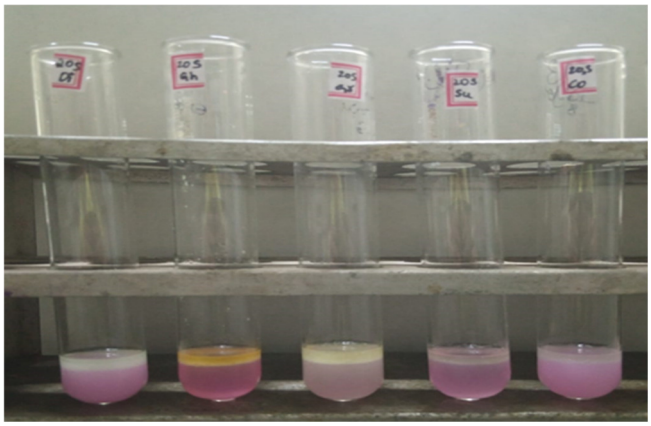


Fig.12 Lipase assay of different oil substrates after titration

Table.11 Effect of temperature 50°C on lipase activity using different oil substrates

Substrate	*Bacillus sp*	*Pseudomonas sp*	*Staphylococcus sp*
Diesel	95	90	95
Ghee	110	105	100
Coconut oil	100	95	95
Sunflower oil	95	100	90
Petrol	85	90	90

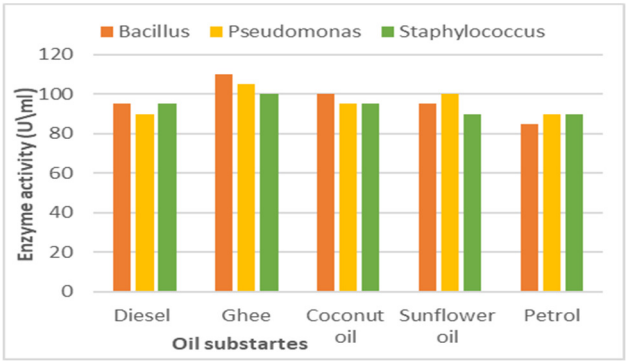


Fig.13 Graphical representation of 50°C effect on enzyme activity using different substrates

7.Total protein estimation by Lowry's method

The total protein content was determined by Lowry's method using BSA as a standard. Protein content of all 3 lipases was shown Maximum protein content was observed in *Staphylococcus aureus SAL2* (874.81µg/ml) followed by *Bacillus flexus strain TRS* (541.90µg/ml) and *Pseudomonas aeruginosa PA01* (605.92µg/ml). It shows that *Staphylococcus.sp* has high lipase content compare to other 2 organisms. In previous all test, *Bacillcus.sp* lipase showed maximum activity but has low lipase content than other 2 organisms. The optical density of standard was measured at 660nm by using blank was tabulated (table 12).

The unknown sample was prepared by incubating 1ml mustard oil with 0.5ml enzyme for 5min at room temperature and 1ml of above mixture taken to follow the Lowry's method without adding water. The optical density of unknown sample was measured at 660nm using same blank was tabulated (table 13). The concertation of protein present in unknown sample was determined by using standard curve (fig 15).

Table.12 Optical density of standard curve

Vol of BSA (ml)	Vol of water (ml)	Concentration of protein (µg/ml)	OD at 660nm
Blank	1.0	00	0.00
0.1	0.9	100	0.21
0.2	0.8	200	0.24
0.3	0.7	300	0.33
0.4	0.6	400	0.38
0.5	0.5	500	0.54
0.6	0.4	600	0.6
0.7	0.3	700	0.62
0.8	0.2	800	0.65
0.9	0.1	900	0.74
1.0	0.0	1000	0.83

Table.13 Optical density of unknown samples

Samples	OD at 660nm	Concentration of protein (µg/ml)
Bacillus sp	0.5	541.90
Pseudomonas sp	0.55	605.92
Staphylococcus sp	0.76	874.81

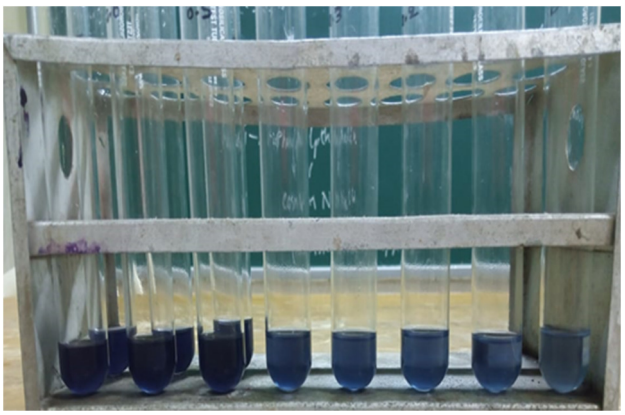


Fig.14 Color development after incubation (Lowry's method)

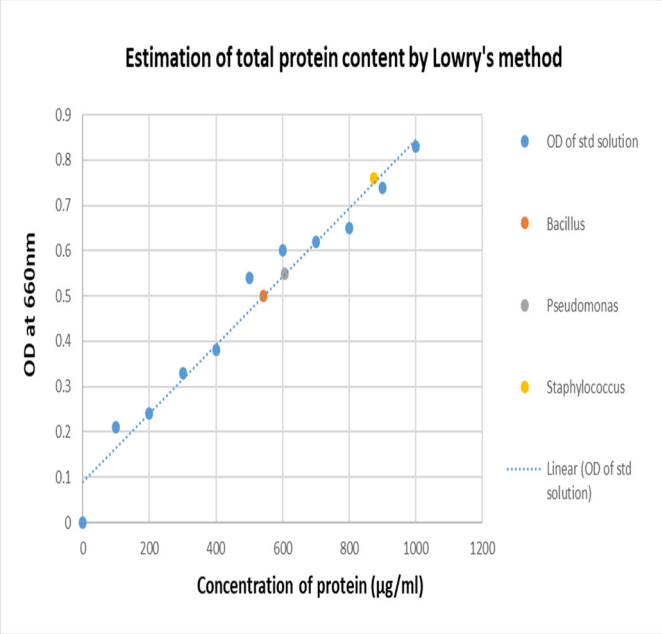


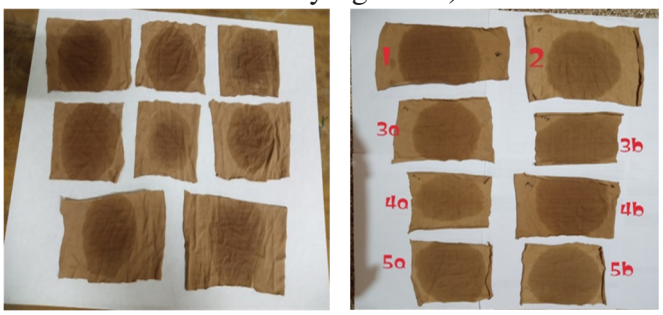
Fig.15 Standard curve of protein estimation by Lowry's method

8. Oily stain removal by lipase enzyme

The detergent additives capability of lipases was studied to determine the efficiency of the enzyme to improve wash performance of detergent. The addition of lipase in detergent had significant impact on improvement of the oil stains removal within period of 5-10 minutes. The oily stain was simply removed from cloth by incubating them in enzyme extract. It was proved that lipase enzymes obtained from microbial species were capable of removing the oily stains from clothes. Incomplete stains removal was observed when clothes were washed only with the water and with water containing detergent, whereas as stains were almost completely removed when lipase enzyme extract was added to remove stains from clothes. The detergents containing enzymes as detergent additives also have advantage of fabric quality improvement and no change in color brightness of clothes.

The 0.5%-wheel detergent along with supplementation of lipase resulted in maximum oil removal (62%) from the oiled cloth. The enzyme lipase was applied effectively and safely on the archeological textiles to remove the oily dirt which is difficult to be removed by traditional methods.

In this experiment, lipase enzyme from all 3 isolates *Bacillus.sp, Pseudomonas.sp, and Staphylococcus.sp* were checked for stain removal property using cloth stained with sunflower oil. The stained cloth was washed with water, detergent (wheel), and detergent with enzyme and only with enzyme after incubating for 5min in all the above maintained reagents. After washing and dried, stained cloth was observed to check the efficiency of all 3 enzymes in removal of oil stain. The difference in stained cloth which is washed with *Pseudomonas.sp* was seen after drying. It proves that lipase enzyme of *Pseudomonas.sp* has the capacity to remove the stain on cloths in 5min (fig.16 4a). And the stained cloth which was washed with lipase enzyme of *Staphylococcus.sp* along with detergent was also has the capacity to remove the oil stain on cloth in 5min but not fully fig.16 5b).



(A) **(B)**

Fig.16 Oily stain removal by lipase enzyme; (A) – Before washing; (B) – After washing; (1)- washed with water; (2)- washed with detergent; (3a)- washed with Bacillus.sp lipase; (3b)- washed with Bacillus.sp lipase along with detergent; (4a)- washed with Pseudomonas.sp lipase; (4b)- washed with Pseudomonas.sp lipase along with detergent; (5a)- washed with Staphylococcus.sp lipase; (5b)- washed with Staphylococcus.sp lipase along with detergent.

9. Applications of lipase enzyme

Microbial lipases have assumed a great deal of importance as industrial enzymes in view of their potential for use in various biotechnological purposes. Lipases are being exploited due to their low cost of extraction, thermal and pH stability, substrate specificity and activity in organic solvents. Lipases are the most widely used enzymes in organic synthesis and more than 20% bio-transformations are performed with lipases (Gitleson et al., 1997). The field of industrial enzymes is now experiencing major R and D initiatives, resulting in both the

development of a number of new products and in improvement in the process and performance of several existing products. Today nearly 4000 enzymes are known and out of these, about 200 are commercially used.

Lipases found promising application in chemical processing, dairy industry, agrochemical industry, paper industry, oleo chemical industry, cosmetics, pharmaceuticals, and synthesis of surfactants, detergent industry, polymer synthesis and personal care products (Sharma et al., 2011).

9.1. Lipases in food industry

Lipases have become an integral part of the modern food industry. It is desirable for the production of flavors in cheese and for interesterification of fats and oils. It also accelerates the ripening of cheese and lipolysis of butter, fats and cream. Addition of lipases to such products primarily releases short-chain (C12 and C14) fatty acids leads to formation of a smooth taste. Lipases are also commonly employed in dairy industry for the hydrolysis of milk fat. Improvement of flavor in cheeses, the acceleration of cheese ripening, the production of cheese like products and the lipolysis of butterfat and cream is achieved by the help of lipase enzymes (Sharma et al., 2001).

9.2. Lipases in the detergents

The addition of lipases to detergent formulations has been investigated in the context of removal of fat stains (Schmid and R. Verger, 1998). Ever since the discovery of lipases it became part and parcel of the detergent industry along with proteases, amylases and cellulases. Lipases, protease, amylase and celluloses were used as they can split fats, proteins, starch and cotton-fluff, respectively.

In addition to their application in laundry, lipases are also included in dish washing, contact lens cleaning, degradation of organic wastes on the surface of exhaust pipes and toilet bowls etc. (Hasan et al., 2006).

9.3. Lipases in pulp and paper industry

Wood is the main source of paper and pulp industry and the presence of the hydrophobic components (mainly triglycerides and waxes), also name pitch, in wood causes serious problems in the production of paper and pulp. Lipases are used to remove the pitch from pulp produced for paper making. The enzymatic pitch control method using lipase was put into practice in a large-scale paper-making process as a routine operation in the early 1990s and was the first case in the world in which an enzyme was successfully applied in the actual paper-making process (Bajpai, 1999).

9.4. Lipases in cosmetics and perfumery

Lipases have potential application in cosmetics and perfumeries because it shows activities in surfactants and in aroma production (Metzger and Bornscheuer, 2006). Monoacyl glycerol and diacyl glycerol are produced by esterification of glycerol and are used as a surfactant in cosmetics and perfume industries.

9.5. Lipases in leather industry

The main process in leather industry is the removal of subcutaneous fat and detailing. Conventional methods including organic solvents and surfactants to remove fat from animal skins can be harmful to environment due to the production of dangerous final products like volatile organic compound (VOC) emissions (Hasan et al., 2006).

Usage of lipases in association with other hydrolytic enzymes like proteases is a new approach in leather processing. Since the process in carried out at alkaline pH, alkalophilic lipases are used in combination with alkaline or neutral proteases and other necessary hydrolytic enzymes (Pandey et al., 1999).

9.6. Lipases in bioremediation

Employment of lipases in bioremediation process is a new aspect in lipase biotechnology. Oil spills in refinery, shore sand and processing factories could be handled by the use of lipases from different origins (Nakamura et al., 1994). It has been also used for the degradation of wastewater contaminants such as olive oil from oil mills. Another important application has been reported for the degradation of polyester waste and removal of biofilm deposits from cooling water systems (Miral Patel et al., 2016).

10. CONCLUSION

The soil is a reservoir of a huge and diverse microbial population, which is considered a rich source of many types of microbial strains which can afford a particular group of microbial strains necessary for the degradation of different contaminants thrown into the soil. Hence the soil samples may be used to isolate the novel strains that can be used as a part of the microbial collection for the production of lipase at research labs and industries.

All the bacterial isolates as *Bacillus flexus strain TRS, Pseudomonas aerignosa PA01*, and *Staphylococcus aureus SAL2* were isolated during the survey from oil-contaminant soil, and all the isolates were exhibited the lipase producing activity. The isolates *Bacillus flexus* showed the highest lipase production activity. The use of Rhodamine B-plate agars as a good media to study lipase producing activity. So the using of 16SrDNA sequence database provides excellent identification at the species and subspecies levels and it can lead to the recognition of novel species of bacteria.

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