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Research Article

SCREENING AND ISOLATION OF EXTRACELLULAR LIPASE PRODUCING BACTERIA FROM DIESEL CONTAMINATED SOILS

Sabika, A* Alekhya, M and Roopa, D

Department of Microbiology, A. Q. J Degree and Pg College, Anandapuram, Visakhapatnam, Andhra Pradesh

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ABSTRACT

Oil contamination of soil and water are causing serious problems in environment. Using lipolytic microorganisms, triglycerides and fatty acids can be oxidized which reduces the oil contaminants from all types of soils and waters. The present study focuses on isolation of lipolytic microbes from diesel contaminated soils and analysis of their ability to produce lipase in defined media under controlled conditions. Qualitative analysis and isolation includes illumination on ROA plates and tween hydrolysis on Tween 80 agar plates. Four different bacterial strains showing positive result were selected. These strains namely R1, R4, R6 and T3 were identified as gram positive bacilli. For quantitative analysis, production media (PM1, PM2) were used. Enzyme estimation was done by titrimetry method against 0.5 N NaOH. Four different substates viz. Tween 80, olive oil, diesel and petrol were used separately in different reaction mixtures. Of all the isolates, T3 strain has showed highest activity of 14µl/ml in PM2.

INTRODUCTION

Extensive utilization of diesel and petroleum products as a major source of energy is one of the main reasons of accidental oil spills leading to land and marine pollution in the environment. This hydrocarbon composition of this crude oil is considered as highly toxic to all living beings including human beings[1]. As per literature, about 30 % of the spilled oil is disposed into freshwater bodies[2]. It has been estimated that, every year about 1.7 to 8.8 million metric tons of oil are released into the water and soil worldwide of which 90% is contributed by human activities ((NAS) NAoS 1985). As per records, 10 largest countries were considered as emitters of oceanic pollution worldwide[3] which largely pass through the rivers and accounts for "90 percent of all the waste that reaches the world's oceans[4].

Decontamination of these soils and waters is the only remedy for the conservation of natural biodiversity. The primary mechanism for pollutant destruction is bioremediation/biodegradation. Bioremediation (Biological conversion) of oil spills includes the application of microbes that convert the highly toxic chemicals into medium to less toxic chemicals[5]. Microbial enzyme technology is one of the cost effective measure which causes partial or complete conversion of many hydrocarbon contaminants[6]. Microbial lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) are soluble biological catalysts which hydrolyze triacylglycerol to release diacylglycerols, monoacylglycerols, free fatty acids (FFA) and glycerol as end products[7,8]. Besides hydrolysis, they can also catalyze esterification and interesterification reactions (acidolysis, alcoholysis and transesterification) and aminolysis[9]. Microbial enzymes are considered to be more promising than animal and plant lipases due to their high productivity in short time duration, variety in catalytic activities, fast inexpensive cultivation and effortless gene handling[10].

Several applications of lipase have been identified in the field of biotechnology. Bacterial lipases have their wide applications in diary and food processing industry where hydrolysis of milk, fat milk, butter, margarine, alcoholic beverages, cheese ripening and manufacture of cheese and cream (lipolysis) can be done11. They can be further used to remove fats from lean fats10. Hydrolytic lipases had been used in the detergent as an additive for both domestic and commercial purposes12. The pulp and paper industry commonly faces the problem of sticky deposits in the pulp. The hydrophobic components of this pulp can be easily removed by the application of lipases13. Lipases are readily used in oil and fat industry to produce novel MUFA

Corresponding author:* **Sabika, A
Department of Microbiology, A. Q. J Degree and Pg College, Anandapuram, Visakhapatnam, Andhra Pradesh

and PUFA enriched plant and animal oils14. Processing of leather and removal of fats from animal skin can be effectively done by using lipases. Several other applications like production of cosmetics, synthesis of biodiesel, organic chemical synthesis15 and medicine synthesis in pharmaceutical industry16. In biodiesel production, lipase enzyme is used for less energy catalysis in mild reactions and easily recovered glycerol from biodiesel. These are produced using many types of oils such as palm oil, olive oil, sunflower oil and soybean oil17.

MATERIALS AND METHODS

Collection of Effluent Sample

Soils contaminated with oils like diesel and palm oil was collected and after 7 days the soil was taken as source for isolation of lipolytic microbes. These organisms present in the soil increased in number by lipolysis of available oils.

Isolation of Bacteria

The oil contaminated soils were serially diluted from 10^{-1} to 10^{5} dilution and 0.1ml of each dilution was spread on Rhodamine- B olive oil Agar (ROA) medium[4] and Tween 80 agar plates for identification of lipolysis[18]. The inoculated plates were incubated at 37°C for 24 - 48 hours. All the chemicals used in this project were obtained from Merck company, Mumbai where as culture media were obtained from Hiimedia, Mumbai.

Morphological and Biochemical Characterization

Morphological characteristics of isolates viz. shape, size, elevation, surface form, margins and surface texture and colour were observed on culture medium. The isolates were observed microscopically by performing gram staining and motility tests. The selected isolates were examined individually for biochemical characteristics and enzymatic activities (IMViC tests, urease test, gelatin hydrolysis test, H_2S production test, catalase – coagulase test and oxidase test) following the procedures mentioned in Cappuccino, J. and Welsh, C. (2014) manual[19].

Qualitative Analysis

Rodamine Olive oil Agar Medium (ROA) Assay

A sensitive and specific plate assay for detection of lipase producing bacteria makes use of rhodamine-olive oil-agar (ROA) medium. Nutrient agar medium (pH 7.0) was taken, autoclaved and cooled to about 60ºC. 10 ml/L of olive oil[20] and 10 ml of rhodamine B solution (1.0 mg/ml distilled water and sterilized by filtration) was added with vigorous stirring to the respective media. Further the plates were inoculated with soil dilutions by spread plate method and incubated for 48 h at 37 °C. Formation of orange fluorescent halos (reaction between rhodamine and released fatty acids) around bacterial colonies visible by UV irradiation was accepted as lipase producing organisms[7,21]. The positive colonies showing fluorescence were grown separately in 10 ml of Tween -80 broth and preserved in 30% (w/v) sterile glycerol solution at −80°C[22]. Standard inoculum was prepared of each organism was prepared from glycerol stocks. Cultures were cultivated individually on nutrient agar medium followed by incubation for 24 h at 37°C; young cultures were collected and suspended in sterile water to an OD of 0.3 at 600 nm. This solution served as inoculums[23].

Tween 80 Hydrolysis Assay

Agar plates were prepared with a medium composed of (g/L): peptone - 10; NaCl - 5; $CaCl_2.2H_2O$ - 0.1; agar-agar, 20; Tween 80 - 10 ml (v/v)[7]. After solidification wells are made in the agar using well borer. Stock solutions containing different strains were added to the separate wells and allowed to incubate at 37°C for 48 h. White colour precipitate around the wells were accepted as positive result[24].

Quantitative analysis – Submerged Fermentation

To study the effect of production media and incubation period, the lipase positive bacterial isolates were subjected to submerged fermentation by shake flask method in two different production media (PM1, PM2) for 48 h. The composition of these production media is given in Table 1. Inoculum size, pH of the production medium and incubation period selected for fermentation were kept optimum of that of bacterial growth. As per reports, lipase production was stimulated in the presence of Ca^{+2} solely[25] or in combination with others like Mg^{+2}, and Fe^{+3} ions[26]. As per some researchers, emulsifiers such as Tween and gum arabic may also increase lipase production[27]. Hence, based on availability, $MgSo_4$, $CaCl_2$, Tween 80 and gum acacia had been incorporated in the production media.

Table 1 Chemical composition of two production media used for submerged production of lipase enzyme

Composition of Medium	Tween 80 liquid Media (PM1)	Diesel liquid Media (PM2)
Lipid	1% Tween-80	1% used diesel
Peptone	0.5%	0.5%
Yeast extract	0.5%	0.5%
Glucose	1%	1%
Gum acacia	1%	1%
$Na_2HPO_4.7H_2O$	1.2%	1.2%
KH_2PO_4	0.3%	0.3%
NH_4Cl	0.5%	0.5%
$MnSO_4.7H_2O$	0.1%	0.1%
$MgCl_2$	0.2%	0.2%
NaCl	0.5%	0.5%

This PM1 and PM2 media were incubated in a rotary shaker (120 rpm) and then assayed for lipolytic activity. The culture filtrates used in the enzyme determination were obtained by subjecting the fermentation medium to centrifugation at 10,000 rpm at 4℃ for 10 minutes.

Lipase assay – Titrimetry Method

Lipase assay was performed by simple titration method using Tween 80, olive oil, diesel and petrol as substrates. Assay mixture containing 1 ml of substrate in1 ml of 1% gum acacia, 1ml of tris HCl buffer and 1ml crude enzyme in 50 ml conical flask was taken, vortex and incubated at 37ºC for 30 min. After incubation the reaction is stopped by addition of ethanolic acetone solution.

The fatty acid released during the incubation was determined by titration with 0.5 N NaOH (alkali) using Phenolphthalein indicator (2 drops). Similarly a blank was run for each substrate and lipase activity was calculated[28,29].

One unit of lipase activity was defined as the amount of enzyme releasing in one mole of free fatty acids in one minute under standard assay condition.

$$\text{Lipase activity} = \frac{\text{vol of NaOH} \times \text{Normality of NaOH} \times 1000}{\text{Time of incubation} \times \text{Volume of enzyme solution}}$$

Where N is the normality of the NaOH titrant used (0.05) and 5 ml is volume of reaction mixture used.

Statistical analysis: All the experiments were carried out in triplicates. Means were calculated from the triplicates and ($p<0.05$). Standard deviations ($p<0.05$) for each of the experimental results were calculated using Microsoft Excel software.

RESULTS AND DISCUSSION

Lipase producing microorganisms are generally found in industrial water, soil contaminated with oil, oilseeds, coal crest, vegetable oil processing factories, compost blend, decaying food and dairy products[9,30]. Hence soils contaminated with oils like diesel and palm oil were selected as source of lipolytic organisms based on availability. These soils were serially diluted to obtain individual colonies which were further cultivated on selective medium for qualitative analysis of lipolysis.

Qualitative Screening of Lipase Activity

The lipase activity of 13 bacterial isolates/strains was assessed qualitatively using Rhodamine olive oil agar (ROA) plate assay and Tween 80 agar plate assay after 48 h of incubation. The observations were envisaged as follows (Table 2). The absence of colonies can be attributed to the inability of the microorganisms to grow on ROA and/or Tween 80 agar medium due to incapable of utilizing the lipid as carbon source as reported in several works[31]. 85% of the obtained bacterial isolates showed lipolysis on ROA medium whereas 30% of them showed their lipolysis on Tween 80 agar medium.

Table 2 Chart showing qualitative screening of bacterial isolates for lipolysis on Rhodamine olive oil agar (ROA) medium and Tween 80 agar medium

S no	Bacterial isolate	ROA plate	Tween 80 agar plate
1	R1	+ve	+ve
2	R2	+ve	-ve
3	R3	+ve	-ve
4	R4	+ve	+ve
5	R5	+ve	-ve
6	R6	+ve	+ve
7	R7	+ve	-ve
8	R8	+ve	-ve
9	T1	-ve	-ve
10	T2	-ve	-ve
11	T3	+ve	+ve
12	T4	+ve	-ve
13	T5	+ve	-ve

Prominent orange fluorescent halos were observed around the colonies on ROA medium. This fluorescence indicates the lipolysis of olive oil and release of free fatty acids during growth on ROA plates (fig 1). These free fatty acids combine with Rhodamine B and forms fluorescent complex when observed under UV irradiation[27]. Hence all the fluorescent colonies were considered as lipolysis positive isolates.

As per literature reports, Kouker G and Jaeger KE (1987) has analysed bacterial isolates for lipase production in ROA medium containing trioleoylglycerol as substrate. Bacteria like *P.aeruginosa* PAC 1R, *P. aeruginosa* ATCC 9027, *P. aeruginosa* PAO, *Serratia marcescens, Staphylococcus aureus* and *B. subtilis* exhibited orange fluorescent halos around the colonies after 48 hrs of incubation. Hence these colonies were accepted as lipase producing bacterial strains. However, *E. coli* did not show positive result, hence it was declared as non lipolytic bacterium[21].

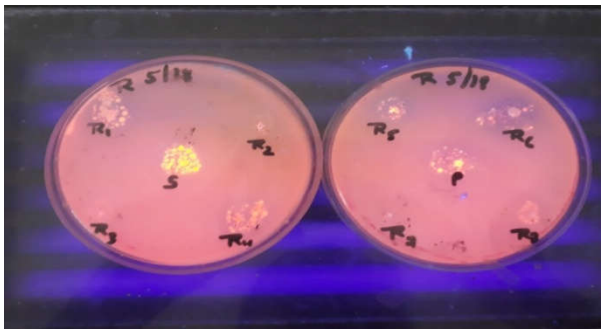


Fig 1 ROA plates showing +ve results of bacterial isolates

Tween 80 (fatty acid esters of polyoxyethylene sorbitan) contains esters of higher chain fatty acids like oleic acid. Hydrolysis of fatty acids is achieved by the microbial lipases. Liberated fatty acids combine with the calcium forming calcium – fatty acid complex. This calcium complex being insoluble crystals is visualized as precipitation around the colonies[7]. The zone of precipitation around the colonies on Tween 80 agar plates were confirmed as positive (Fig.2). Larger the ratio, higher is the lipase activity[18].

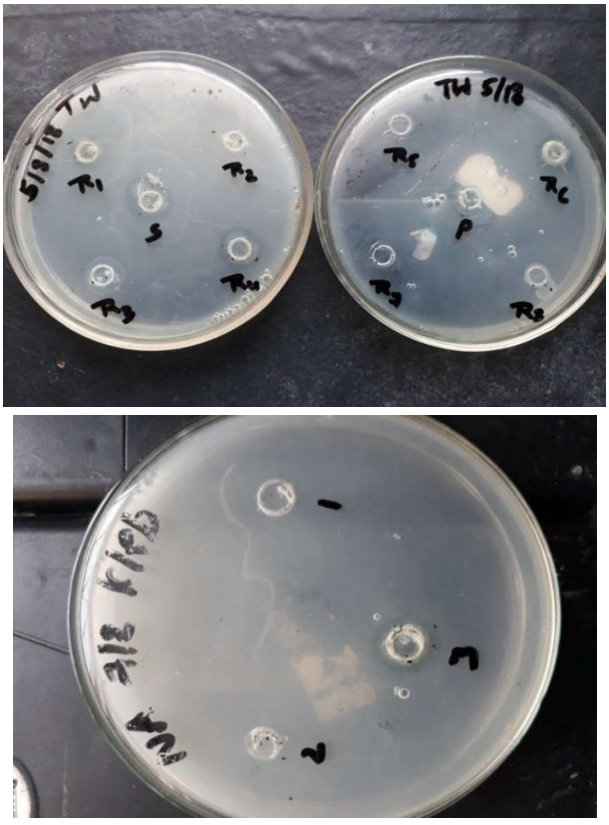


Fig 2 Tween - 80 plates showing +ve results for isolates R1, R4, R6, T3, T4, T5

In the present study, bacterial isolates R1, R4, R6 and T3 were selected as lipase positive based on ROA fluorescence and Tween 80 precipitation. Hence these cultures were subcultured

and utilized for further analyses. The negative isolates were discarded from further studies. Intracellular enzyme production may be one of the reasons for the absence of zone of clearance. Quantitative estimation of enzyme activity confirmed the above observations[31].

Quantitative Analysis

Submerged Fermentation

Presence of carbon is the criterion for the expression of lipase enzyme, since lipases are inducible enzymes[32].Thus the presence of a lipid source such as oil or any other hydrocarbon, such as triacylglycerols, hydrolysable esters, tweens, bile salts and glycerol has been practiced.

As per the literature reports, lipid carbon sources (especially natural oils) stimulate lipase production[33] in microbes. Thermophilic *Bacillus significantly produced high* lipase activity in the presence of olive oil as carbon source in the medium[34, 35]. However catabolic repression had been observed with the presence of glucose in the production medium[36].

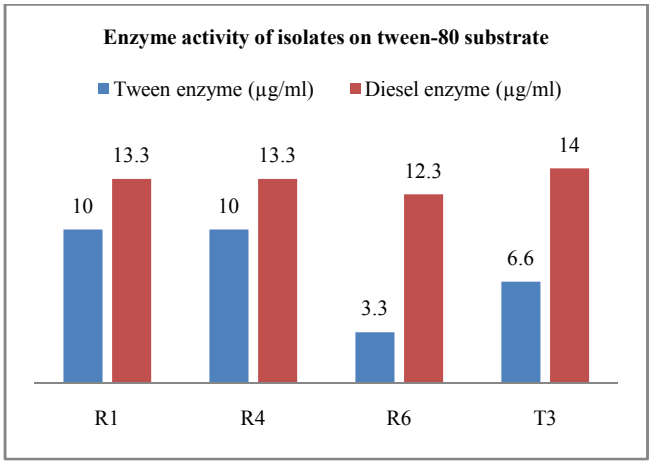
The lipase productivity of the four selected bacterial strains was studied by submerged fermentation using the liquid media supplemented with diesel and Tween 80 individually (Table.1) as carbon source in different media.

Enzyme Activity of Isolates on Tween 80 Substrate

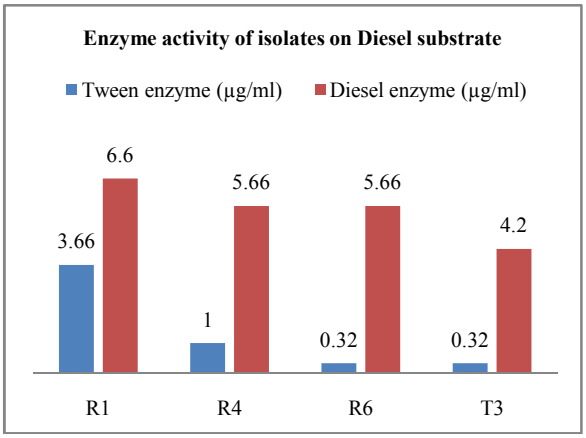
The culture free extracts of the two production media were used as lipase enzyme source using Tween 80 substrate for estimation of enzyme activity by titration with 0.5N NaOH. All the culture filtrates obtained from diesel production medium exhibited maximum enzyme activity compared to Tween medium (Graph.1).

With diesel medium culture filtrates, maximum enzyme activity of 14µg/ml was observed in T3 isolate. Whereas R1 and R4 isolates exhibited 13.3 µg/ml enzyme activities. The lowest enzyme activity was recorded in the case of R6 isolate which expressed 12.3 µg/ml enzyme activity.

Contrary to the above readings, R1 and R4 isolates expressed higher enzyme activities of 10µg/ml. whereas T3 and R6 showed 6.6 µg/ml and 3.3 µg/ml enzyme activities respectively. The poor production of enzyme in diesel medium may be due to incapability of gene expression under applied conditions.



Graph 1 Enzyme activities of 4 bacterial isolates on Tween 80 substrate from PM1 & PM2



Graph 2 Enzyme activities of 4 bacterial isolates on diesel substrate from PM1& PM2

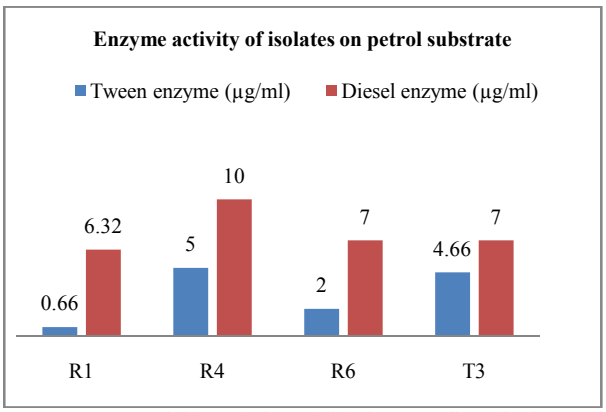
Enzyme Activity of Isolates on Diesel Substrate

Diesel is considered to be a major contaminant in environment. Bioremediation of diesel, especially in the case of oil spills[37], is a struggling point in research. Usage of lipase in degradation of diesel has been used in several cases. The enzyme extract of PM2 (diesel production medium) from all the four isolates has shown more enzyme activity than that from PM1 (Tween medium) while using diesel as substrate (Graph.2). Among all the isolates, R1 isolate showed highest enzyme activity of 6.6µg/ml obtained from PM2 while all the culture filtrates showed very low enzyme activity.

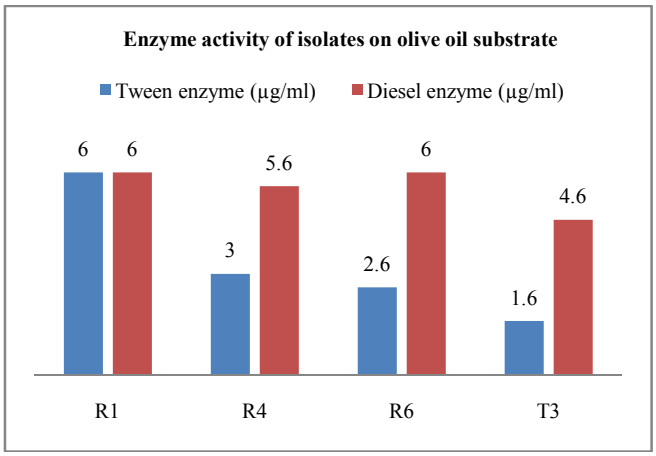
Enzyme Activity of Isolates on Olive oil Substrate

Olive oil was used as substrate for estimation of enzyme activity in the second case where the culture free extracts of the two production media (PM1, PM2) were used as lipase enzyme source. The estimation was done by titration with 0.5N NaOH. All the culture filtrates obtained from diesel production medium exhibited maximum enzyme activity compared to Tween medium (Graph.3).

With diesel medium culture filtrates, maximum enzyme activity of 6µg/ml was observed in both R1 and R6 isolates. Whereas T3 and R4 isolates exhibited 4.6 µg/ml and 5.6 µg/ml enzyme activities respectively. The lowest enzyme activity was recorded in the case of R6 isolate which expressed 12.3µg/ml.
In contrast to the above readings, Tween production medium culture filtrate of R1 isolate expressed higher enzyme activities of 6µg/ml. whereas R4, R6 and T3 showed 3µg/ml, 2.6µg/ml and 1.6µg/ml enzyme activities respectively.



Graph 3 Enzyme activities of 4 bacterial isolates on olive oil substrate from PM1& PM2



Graph 4 Enzyme activities of 4 bacterial isolates on petrol substrate from PM1 & PM2

Enzyme Activity of Isolates on Petrol Substrate

Petroleum pollution is considered to be a catastrophe globally and especially a major phenomenon in the industrial and oil rich areas. Oil spill clearance has been a critical point and extensively explored over twenty years. Several researchers had used plants and microorganisms to clear these oil contaminants[38]. Petrol was used as substrate for estimating the lipase enzyme activity obtained from the two production media. The reaction was further analysed by titration with 0.5N NaOH.

A maximum of 10 µg/ml enzyme activity was recorded by R4 isolate whereas R1, R6 and T3 isolates expressed 6.32 µg/ml, 7 µg/ml each respectively. These results were obtained from the culture filtrates taken from PM2 (diesel production medium). Whereas the results attained from the culture filtrates of PM1 (Tween production medium) showed very poor enzyme activity on petrol substrate. A maximum of 5 µg/ml enzyme activity was recorded by R4. This indicates the incapability of the isolates in degradation of petrol.Of the two production media tested, only PM2 (diesel medium) could produce lipase effectively. Bacterial isolate T3 proved its potential in lipase productivity. The results of production have been concisely shown in Table.3 in µg/ml concentration. From all the four different substrates tested, Tween 80 substrate only showed considerable enzyme activity of 14±0.02 µg/ml produced from diesel medium.

Table 3 lipase enzyme activity levels on different substrates obtained from PM2 (diesel production medium) of four isolates

Isolates Substrates	R1	R4	R6	T3
Tween 80	13.3±0.03	13.3±0.04	12.3±0.06	14.0±0.05
Olive oil	6.00±0.03	5.60±0.02	6.00±0.04	4.60±0.02
Petrol	6.32±0.02	10.0±0.04	7.00±0.02	7.00±0.07
Diesel	6.60±0.05	5.66±0.06	5.66±0.02	4.20±0.03

Characterization of Bacterial Isolates

Characterization of the isolates was done for the identification of the isolates at genus and species level by performing microscopic, cultural and biochemical examinations.

Staining & Cultural Characteristics

The morphological characterization of the four selected isolates was done by cultivating them on nutrient agar medium individually. The results of morphological characters revealed had been displayed in Table 4. All the four isolates belong to gram positive bacilli. However all the isolates were different as the colonies developed showed a lot variations among them.

Table 4 Cultural characterization of four isolates showing good lipase enzyme production

S no	Test	R1	R4	R6	T3
1	Colony	Transparent, irregular	Transparent, irregular	Transparent, round	Transparent, irregular
2	Shape	Rods	Rods	Rods	Short Rods
3	Gram's staining	Gram +ve	Gram +ve	Gram +ve	Gram +ve
4	Colour	White	Creamish white	White	Yellowish white
5	Texture	Mucoid	Normal	Sticky	Normal to dry
6	Margin	Regular	Regular	Irregular	Regular
7	Motility	Motile	Motile	Slightly motile	Motile

Biochemical Characterization

All the four strains were subjected to 10 different biochemical tests which may determine the species of the isolate. The isolates were cultivated in the respective biochemical media individually and incubated. Later the media were tested for the presence of product. The results of all the tests have been specified in Table 5.

Table 5 Biochemical characterization of four isolates showing good lipase enzyme production

S no	Test	R1	R4	R6	T3
1	Indole test	-ve	-ve	-ve	-ve
2	MR test	-ve	+ve	-ve	-ve
3	VP test	+ve	-ve	+ve	-ve
4	Citrate test	+ve	+ve	+ve	+ve
5	Urease test	+ve	+ve	-ve	-ve
6	H_2S production test	+ve	+ve	+ve	+ve
7	Catalase test	+ve	+ve	+ve	+ve
8	Coagulase test	+ve	-ve	+ve	+ve
9	Oxidase test	+ve	+ve	+ve	+ve
10	Gelatinase test	+ve	+ve	-ve	-ve

Basing on the "Bergey's Manual of Determinative Bacteriology".[39] the above mentioned results indicate that the isolated microorganisms named T3 was found to be *Bacillus cereus,* R1 is found to be *Bacillus subtilis,* R4 is *Bacillus megaterium* and R6 may be *Enterobacter* species.

CONCLUSION

The aim of present study was to isolate identify and characterize lipolytic microorganism from various oil contaminated soil samples. Based on the growth on lipid containing selective medium and zone of hydrolysis four bacterial isolates were selected. Quantitative estimation was done to identify highest lipase producer of the four isolates in two production media viz PM1 and PM2. Culture free filtrates were treated with four different substrates viz Tween 80, olive oil, petrol and diesel to determine the lipase activity. All the strains were characterized biochemically by employing "Bergey's Manual of Determinative Bacteriology" and was identified as *Bacillus cereus (T3), Bacillus subtilis (R1),*

Bacillus megaterium (R4) and Enterobacter spp (R6). Of all the isolates T3 has produced lipase effectively in PM2 and showed its activity in Tween 80 substrate. Hence T3 is termed as a good lipolytic strain under optimum laboratory conditions.

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