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Bioremediation of Petroleum-Polluted Soil Using Biosurfactant Producing Bacteria, *Pseudomonas* sp.

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Abstract: **The oil degradation capability of *Pseudomonas* sp. were tested using lipase enzyme producing bacterial strains from petroleum oil contaminated areas. The high level of lipase production was observed at temperature range from 30° to 37°C and a pH of 4 to 7. The optimum pH and temperature was 7 and 37°C for the strain *Pseudomonas* sp. respectively. The presence of sucrose and yeast extract in the medium enhanced enzyme production when compared with other carbon and nitrogen sources. *Pseudomonas* sp. can be effectively used to degrade petroleum oil contaminated soils and also considered as a key component in the cleanup strategy for bioremediation of petroleum oil.**

Index Term : **Bacterial strains, Bioremediation, Oil degradation, Petroleum oil, *Pseudomonas* sp.**

I. Introduction

Petroleum oil pollution is a great hazard to the environment. The soil contamination by petroleum oils particularly engine oil (diesel) are one of the major concerns. Approximately five million tons of petroleum oil enters the environment each year as a result of anthropogenic activities. Oil spill develops as a result of oil leakage from oil pipelines, mechanical workshops, accidents of oil tankers during transportation and anthropogenic activities (Urum et al., 2004). In addition to oil spill from oil pipelines, oily sludge contamination is also a main problem. The constituents of the petroleum oil are highly toxic (Saxena et al., 1999), carcinogenic and are poorly biodegradable. Oil contamination results in the loss of beneficial properties of soil such as fertility, water holding capacity, permeability and binding capacity. The oil contaminant persists in the soil and can cause groundwater contamination (Margesin and Schinner, 1998). The process of bioremediation is an advanced technique used to degrade the petroleum hydrocarbons using microorganisms efficiently (Alexander and Martin, 1994). The benefit of microorganism to degrade the petroleum hydrocarbon is effective and economic technique to treat oil pollution (Thomas et al., 1992). Microorganisms degrade the petroleum hydrocarbon compounds by producing enzymes like dehydrogenase, lipase and amylase. Bacterial lipases (lipolytic enzymes) demolish lipid components of petroleum hydrocarbons such as fats, oils and triglycerides which are water insoluble compounds. The synthesized bacterial lipases are of great demand due to their specificity of reaction and less energy consumption. (Margesin and Schinner, 1998). Bacterial lipases are significant in bioremedial process, as it is safer environmentally friendly way to process hazardous waste and used as a viable method for cleanup of oil spills and contaminants as an alternative to solve petroleum pollution. The development of lipase based technologies for bioremediation is rapidly expanding and is used in oil degradation and pollution control (Hasan et al., 2006).

The study aims to optimize the culture parameters for the maximum production of lipase for the degradation of petroleum oil contaminated soil resulting in the isolation of lipolytic potentials and production of lipase by the selected strain and used for the processing of bioremediation of petroleum hydrocarbons in oil polluted soil with *Pseudomonas* sp. owing to their enzymatic and economic efficiency.

II. Materials and Methods

A. Isolation, Identification and characterization of bacterial strains

Oil spilled soil samples were collected from a mechanical workshop in Vytilla (9.9682°N, 76.38182°E), Ernakulam,

Kerala. Serial dilution technique was used to isolate bacterial strains. Isolated bacterial strains were subjected to Grams staining for morphological and biochemical identification. Biochemical tests such as (a) Indole test, (b) Methyl Red (MR) and (c) Voges Proskauer test (VP), (d) Citrate test, (e) Triple Sugar Iron test (TSI), (f) Oxidase test, (g) Catalase test was performed. a) Indole test demonstrate the ability of isolated bacterial strain to metabolize the amino acid tryptophan. b) MR and c) VP test was performed to detect the fermentation pathways of the bacterial strain for acid and carbohydrate respectively. d) Citrate test was performed to screen the isolated microorganism that capable of utilizing citrate as sole source of carbon. e) TSI test was executed to distinguish the members of Enterobacteriaceae from other gram-negative bacteria and identify the production of hydrogen sulphide. f) Oxidase test was done to distinguish oxidase negative and positive bacteria. To identify the isolated bacterial strain belongs to aerobic or anaerobic habitat g) catalase test was performed. Bacterial isolates were identified by referring Bergey's Manual of Determinative Bacteriology (Holt et al.,1994).

B. Screening for lipase producing bacterial strains

(i) Primary screening

(a) Tributyrin Agar Plate Assay (TBA)

The primary screening of isolated bacterial strains was made by the method suggested by Limpon (Limpon et al., 2006). The isolated strains were screened for their lipolytic activity using TBA assay (Ronald, 1946). The strains spread on the TBA with 1.0% olive oil (Kouker and Jaegar, 1987) and were incubated at 37° C for 24-48 hours. Detection of lipolytic activity was noticed by zone of hydrolysis/zone of clearance in TBA medium (Limpon et al., 2006) and (Blake et al., 1996).

(ii) Secondary screening

(a) Qualitative assay

The pure culture of *Pseudomonas* sp. plated on TBA showing high zone of clearance were inoculated to peptone medium to generate mass culture as referred in Manual of microbiological culture broth media, (Difco and BBL, 2009). Pure culture of *Pseudomonas* sp. was maintained in continuous agitation in 150 rpm for 24 hours in submerged fermentation method (Mobarak et al., 2011). After incubation, the culture was subjected to centrifugation and culture supernatant fluid was used for the quantitative determination of enzyme units (Lowry and Tinsley, 1976).

(b) Quantitative assay

1) Lipase assay

Lipase activity was estimated by using quantitative method to screen potential lipase producer. Lipase assay was performed. Lipase activity was measured by measuring the amount of free fatty acids (oleic acid) released and measures the absorbance at 715 nm using uv-vis Spectrophotometer (SL 159). One unit of lipase activity was defined as the amount of enzyme releasing one µm of fatty acid per min under standard assay conditions (Lowry and Tinsley, 1976).

C. Optimization of physico-chemical parameters and effect on Lipase enzyme production

The lipase enzyme production was tested in various physico-chemical environments. The standardization of enzyme production was considered. The *Pseudomonas* sp. obtained was maintained in a selected physico–chemical environment to understand the lipolytic activity. The efficiency of *Pseudomonas* sp. was assayed with different oil substrates (olive oil, sunflower oil, coconut oil and mustard oil), pH and temperature.

1) Effect of oil substrate

Studies were done to evaluate the effect of oil substrate on lipase activity (Bornscheur, 2002). Lipase production was assessed by using different oils, a) olive oil, b) coconut oil, c) sunflower oil and d) mustard oil. Different oils of 1% concentration were added to the medium separately and lipase activity was determined at 24 hours of incubation. The growth medium added with different oils separately was investigated for quantitative production of lipase enzyme was estimated using spectroscopy (SL 159).

2) Effect of pH

The *Pseudomonas* sp. was grown at varying pH ranges from 4 to 9 for studying their effects on lipase production (Larbidaoudi et al.,2015).

3) Effect of temperature

The *Pseudomonas* sp. was grown at varying temperature ranges from 28°C to 50°C for studying the effects on lipase production and all other parameters were left unchanged (Tembhurkar et al., 2012).

D. Screening for biosurfactant potential of pseudomonas sp. on oil degradation

Biosurfactant activity of bacteria *Pseudomonas* sp. was detected by a) Drop Collapsing Test (Youssef et al., 2004). b) Oil spreading test (Rodrigues et. al., 2006) and c) Emulsification test (E_{24}) and the height of the emulsion layer was measured (Cooper and Goldenberg, 1987).

III. Results

A. Isolation, Identification and characterization of bacterial strains

Total of six strains of naturally present lipolytic bacteria were isolated from the oil spilled soil sample. The bacterial isolates were identified with the help of morphological and biochemical studies. The isolated six bacterial strains were designated as *Sp*.1, *Sp*.2, *Sp*.3, *Sp*.4, *Sp*.5 and *Sp*.6. Four bacterial strains *(Sp*.2, *Sp*.3, *Sp*.5, and *Sp*.6*)* were gram-positive and two bacterial strains (*Sp*.1, *Sp*.4) were gram-negative. The isolated strains morphological appearance on the nutrient agar plates is shown in Fig.1.A and B. The Grams staining results of these bacterial isolates were shown in Table I.

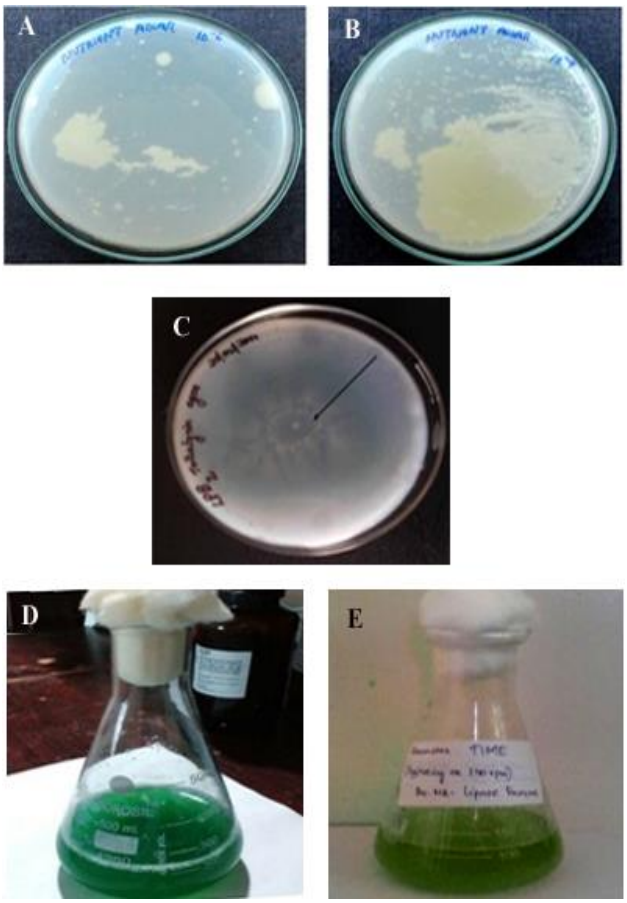

Fig.1.A: Isolated colonies on nutrient agar (spread plate method); B: Isolated bacterial colony growth on nutrient agar (spread plate method); C: Zone of clearance (pointed with arrow) around bacterial strain (*Pseudomonas* sp.); D: Production media after inoculating bacterial strain (*Pseudomonas* sp.); E: Production media after 48 hours of inoculation (color change shows enzyme production).

Table I: Bacterial strains and Grams staining characterization

Bacterial Strains	**Grams staining characterization**
Sp.1	-
Sp.2	+
Sp.3	+
Sp.4	-
Sp.5	+
Sp.6	+

a) Isolated bacterial strains (*Sp*.1, *Sp*.2, *Sp*.6) belongs to the family Enterobacteriaceae. b) Methyl Red test (MR) and c) Voges-Proskauer test (VP) determines glucose utilization efficiency of isolated strains. d) The bacterial strains (*Sp*.1, *Sp.2*, *Sp.4*, *Sp.5*) utilizes carbon as source of energy in Citrate test. e) Triple Sugar Iron test (TSI) differentiates Enterobacteriaceae family from other gram-negative bacteria and detect the production of hydrogen sulphide. The isolated bacterial strain (*Sp*.3) was TSI positive. f) Oxidase test was done to detect Pseudomadaceae family, thus the bacterial strains (*Sp*.3 and *Sp*.4) belongs to Pseudomadaceae family. g) Catalase test detect the release of oxygen with catalase enzyme in the isolated strains (*Sp*.2, *Sp*.3, *Sp*.4, *Sp*.6). The result of biochemical test results as shown in Table II. Bacterial isolates were identified and confirmed as *Pseudomonas* sp. by referring Bergey's Manual of Determinative Bacteriology.

Table II: Bacterial strains and biochemical characterization

Tests	**Observations**					
	***Sp*.1**	***Sp*.2**	***Sp*.3**	***Sp*.4**	***Sp*.5**	***Sp*.6**
Indole	+	-	-	-	+	+
MR	+	-	-	-	+	-
VP	-	-	-	-	+	-
Citrate	+	+	-	+	+	-
TSI	-	-	+	-	-	-
Oxidase	-	+	+	+	-	-
Catalase	-	+	+	+	-	+

(MR: Methyl red test, VP: Voges-proskauer test, TSI: Triple Sugar Iron test), + : positive, - : negative
Sp.1:*Proteus*, *Sp*.2: *Corynebacterium*, *Sp*.3: *Rhodococcus*, *Sp*.4: *Pseudomonas*, *Sp*.5: *Streptococcus*, *Sp*.6: *Bacillus*

B. Screening For Lipase Producing Bacterial Strains

1) Primary Screening

(a)Tributyrin Agar Plate Assay (TBA)

Screened bacterial strains shows zone of clearance (*Sp*.4 and *Sp*.6) on the TBA plate. Out of the two bacterial strains (*Sp*.4 and *Sp*.6), elevated zone of clearance was observed around bacterial strain *Pseudomonas* sp. (*Sp*.4) indicating lipase production on TBA (Fig.1.C). Through the biochemical test results, the isolated bacterial strains are belonging to the genera of *Proteus* sp. (*Sp*.1), *Corynebacterium* sp. (*Sp*.2), *Rhodococcus* sp. (*Sp*.3), *Pseudomonas* sp. (*Sp*.4), *streptococcus* sp. (*Sp*.5) and *Bacillus* sp. (*Sp*.6). The isolated bacterial strains on the TBA plate, four bacterial strains were identified as lipase producers (*Sp*.1, *Sp*.2, *Sp*.4, *Sp*.6). Among the cultured six bacterial strains, *Sp*.4 and *Sp*.6 showed large zones of clearance in TBA and *Sp*.1 and *Sp*.2 showed moderate zones of clearance and *Sp*.3 and *Sp*.5 strains did not show any zone around the colonies in TBA. The two strains considered for further analysis were *Sp*.4 and *Sp*.6 because it shows high zone clearance that denotes they are producing lipase enzymes. Gram staining results shows that *Sp*.4 is a gram-negative and *Sp*.6 is gram-positive bacteria. Considered only *Sp*.4 (*Pseudomonas* sp.) for further studies.

Morphological Characters of Pseudomonas sp.

The morphological and biochemical characteristics of the isolate were investigated and it indicated that the isolate (*Sp*.4) belonged to the genus *Pseudomonas* sp. as Gram negative, rod shaped, motile bacteria that are capable of hydrolysis of tributyrin.

ii) Secondary screening

(a) Qualitative assay

The *Pseudomonas* sp. (*Sp*.4) with high zone of clearance subjected to peptone medium shake flask culture shows enzyme production (Fig.1.D) and incubated at room temperature on a rotary shaker for 24-48 hours. The growth of bacterial strains in the production media was indicated as a colour change in the medium (Fig.1.E). After that it was subjected to centrifugation. The culture supernatant fluid contains the crude extracellular lipase enzyme.

(b) Quantitative assay

(1) Lipase assay

Enzyme assay for gram-positive, bacterial isolate *Bacillus sp*. (LPB1) and gram-negative isolate *Pseudomonas* sp. (LPB2) was performed (Fig.2.A). The OD value (Optical density) of the supernatant cultures was measured. The measured the OD values of *Pseudomonas* sp.(0.6) and *Bacillus* sp.(0.28). After performing the Lipase assay, zone of clearance produced by *Pseudomonas* sp. shows the maximum lipolytic activity (Fig.2.B). The Lipase activity of *Pseudomonas* sp. was observed as 0.23mg/ml/min.

C. Optimization of physico-chemical parameters and effect on Lipase enzyme production.

1) Effect of oil substrate

The *Pseudomonas* sp. cultured in the presence of 1% of different oil substrates such as a) olive oil, b) coconut oil, c) sunflower oil and d) mustard oil as shown in Fig.2.C. The screening of oil substrates showed maximum lipase activity in olive oil followed by coconut oil, sunflower oil and mustard oil. The activity of enzyme production by *Pseudomonas* sp. is summarized in Table III.

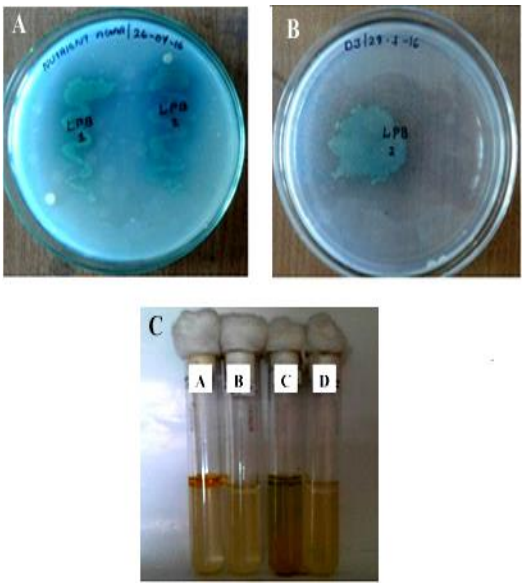

Fig.2.A: Lipase producing bacterial strains LPB1 (*Bacillus* sp.) & (*Pseudomonas* sp.) on TBA plate; B: Zone of clearance around bacterial strain LPB2 (*Pseudomonas* sp.) on TBA plate; C: Screening of lipase producing bacterial strain (*Pseudomonas* sp.) on different substrate A: mustard oil, B: coconut oil, C: olive oil, D: sunflower oil.

Table III: Effect of different oil substrates on Lipase activity

	Substrate	**Lipase activity(µg/ml)**
a	Olive oil	0.610
b	Coconut oil	0.288
c	Sunflower oil	0.233
d	Mustard oil	0.122

2) Effect of pH

The initial pH of the growth medium (lipase broth) was optimized for maximum lipase production by the *Pseudomonas* sp. ranges from 4 to 9. The isolated bacterial strain *Pseudomonas* sp. showed maximum lipase production at pH 7. It was noted that the lipase production reduces with increase in pH from 7 to 9 (Fig.3.A).

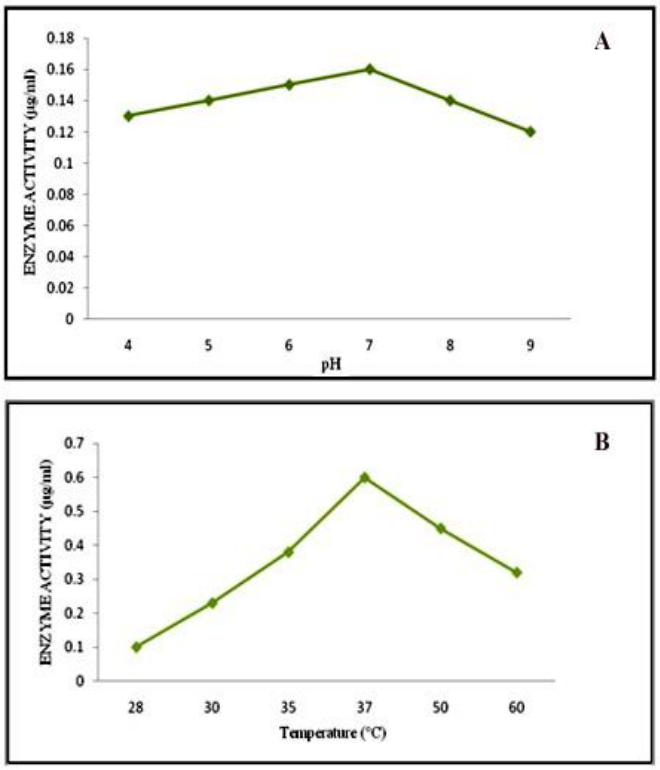

Fig.3.A: Graph showing effect of pH and enzyme activity; B: Graph showing effect of temperature and enzyme activity

3) Effect of temperature

The temperature of the growth medium (lipase broth) was optimized for maximum lipase production by growing the *Pseudomonas* sp. at different temperature. The optimization of temperature shows that the bacterial isolate produce lipase in wide range of temperature from 28° to 60°C. It was noted that the optimum temperature for lipase enzyme production was at 37°C. The enzyme production was decreased by increasing the temperature above 37°C (Fig.3.B).

D. Screening for biosurfactant potential of pseudomonas sp. on oil degradation

Biosurfactant activity of *Pseudomonas* sp. was detected by a) Drop Collapsing Test, b) oil spreading test and c) oil emulsification test as described in materials and methods.

a) Drop collapsing test

In the drop collapse test the shape of the drop of oil on the surface was collapsed. The culture supernatant makes the drop collapse indicates the biosurfactant activity. This clearly indicated that the bacterial strain *Pseudomonas* sp. produced biosurfactant activity.

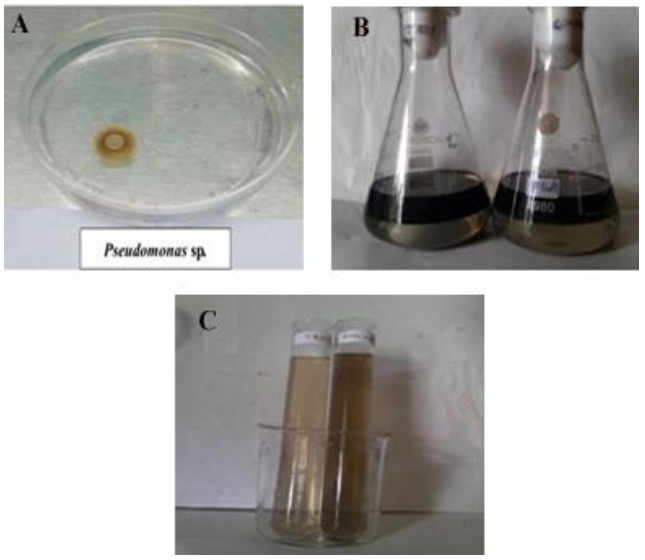

Fig.4.A: Clear zone around oil drop (oil degradation); B: Oil degradation activity of *Pseudomonas* sp. on initial stage of inoculation; C: Oil degradation activity of *Pseudomonas* sp after a month of inoculation.

(b) Oil spreading test

In oil spreading test the bacterial strain *Pseudomonas* sp. introduced into the water surface coated with petroleum oil (diesel). The occurrence of a clear zone indicates the bio-surfactant activity (Fig.4.A).

(c) Oil Emulsification test

In oil emulsification test, moderate Emulsification index (E_{24}) value was observed in *Pseudomonas* sp. The bio-surfactant and oil degradation (Fig.4.B) activity obtained was about 50 %. The oil degradation by the bacterial strain *Pseudomonas* sp. after a month of inoculation shows as in Fig.4.C.

IV. Discussion

Cleaning up of petroleum hydrocarbons in the environment is a real world problem. A better understanding of the mechanism of biodegradation by microorganism has high ecological significance. The petroleum hydrocarbon compounds are susceptible to microbial degradation (Geetha et al., 2014). Microorganism degrades hydrocarbons through a natural mechanism i.e. bioremediation by bio-surfactant activity of enzymes (Batista et al., 2006). *Pseudomonas* sp. flourishes on oil contaminated soil utilizing the hydrocarbons as a nutrient source for its growth and development and extracellular lipase enzyme obtained from bacteria used for bioremediation (Thomas et al., 1992). Hence they play a significant role in oil degradation (Vitolo et al., 1998). The present study helps us to explore the potential application of *Pseudomonas* sp., optimization of lipase production and its application as a natural remedy for oil pollution. This study suggested that the oil contaminated environment provides bacterial strains a good environment for lipase activity. The sample collected from the study area was

dominated by *Pseudomonas* sp. The isolated strains of bacteria Lipolytic bacterial strain, *Pseudomonas* sp. identified and characterize as common bacterial strain from oil contaminated soil (Sirisha et al., 2010) and were capable of producing the extracellular lipase enzyme (Gupta et al., 2004). A variety of lipases are produced from both gram-positive and gram-negative bacteria (Miral et al., 2016). In this study it was observed that bacterial strains isolated from oil contaminated area includes both positive and gram-negative bacterial strains but greater part of bacterial lipases comes from gram-negative bacterial strains and the bacterial genus among the isolated strains is *Pseudomonas* sp., which is capable of hydrolysis of tributyrin agar (TBA). The zone of hydrolysis produced by the enzyme is a function of concentration of enzyme activity (Shelley et al., 1987). Bacterial strains *Pseudomonas* sp. showed high intensity of clear zone in TBA, signifies bacterial strain *Pseudomonas* sp. as a lipase producer.

The extracellular lipolytic enzyme productions by the bacterial strains are greatly influenced by physico-chemical parameters, such as pH, temperature, nitrogen and carbon sources (Jaeger et al.,1994). Here the bacterial strain *Pseudomonas* sp. the growth medium was experimented with different oils, olive oil shows maximum lipase activity and is referred as the best substrate for lipase production (Bornscheur, 2002). The lipase production was noticed at both acidic and alkaline pH range 3 to 12, enzyme was most active at pH 6 to 9 (Bayoumi et al., 2007). *Pseudomonas* sp. also shows maximum enzyme activity at temperature 37°C and pH 7. The microbial treatments are capable of oil remediation in temperate and tropical regions (Mercer and Trevors, 2011). The temperature specific enzymatic activity of lipase is maximum at relatively high 37°C ensures the activity of this enzyme as a bioremediate to restore oil contaminated sites of equatorial and temperate regions. Lipolytic activity showing bacterial strain *Bacillus* sp. shows maximum lipase production after 24 to 36 hours of incubation (Davender et al., 2012), whereas the lipase producing bacterial strain *Pseudomonas* sp. shows maximum enzyme activity after 48 hours of incubation and the quantitative analysis of lipase enzyme done by lipase assay showed maximum activity of about 0.23 mg/ml/min.

The bacterial strain *Pseudomonas* sp. shows positive results to oil spreading test, drop collapsing test and oil emulsification index test clearly indicates the bio-surfactant activity of the bacterial strain *Pseudomonas* sp. confirmed its applicability against different petroleum hydrocarbon pollution (Thavasi et al., 2010). Bio-surfactant activity of *Pseudomonas* sp. shows its ability to utilize petroleum oil as a carbon source and has been widely used for hydrocarbon degradation (Rodrigues et al., 2009).

Two approaches are suggested for bioremediation are bioaugmentation and biostimulation (Chandrakant et al., 2011). In the former one oil contaminated areas are inoculated with hydrocarbonoclastic (degrade hydrocarbon) microorganisms and later depends on enhancing already existing microorganisms by adding nutrients, surfactants and other materials. The nutrients stimulate the microbial community and the isolated bacterial enzyme used for the transformation of contaminants into non-toxic compounds. This approach helps to restore different petroleum contaminated sites. The *Pseudomonas* sp. can be effectively used to degrade petroleum oil contaminated soils and also considered as a key component in the cleanup strategy for bioremediation of petroleum oil.

V. Conclusion

The isolated bacterial strain *Pseudomonas* sp. from oil contaminated soil exhibits significant potential for extracellular lipase production. Among different lipid substrates, olive oil showed best results and the addition of 1% olive oil to peptone broth production media showed a remarkable increase in lipase production. The isolated bacterial strain *Pseudomonas sp*. showed maximum enzyme activity after 48 hours of incubation at a pH 7 and temperature 37°C. *Pseudomonas* sp. can be effectively used for the production of extracellular lipase enzymes in tropic and temperate regions with optimum pH and temperature. So it is applicable for degradation of oil contaminated regions to control oil pollution. Thus the future prospect of the study can involve in-situ and ex-situ bioremediation. The bioremediation is an eco friendly process which helps to cleanup environment utilizing the bacterial strain *Pseudomonas* sp. and the bio-surfactant activity of lipase enzyme tested using different oil substrate also prove the petroleum hydrocarbon degradation by the bacterial strain *Pseudomonas* sp.

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