

Isolation, Characterization and Identification of Three Petroleum Tolerant and Degrading Bacteria (*Micrococcus*, *Staphylococcus* and *Pseudomonas* Spp.) From Petroleum Oil Contaminated Soil

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ABSTRACT: Three petroleum degrading bacteria, *Micrococcus*, *Staphylococcus* and *Pseudomonas* have been isolated from petroleum contaminated soil. The isolates were identified by using standard biochemical tests and morphological studies. After identification, the bacterial strains were optimized for maximum growth under different growth parameters such as temperature, pH, carbon and nitrogen sources. Bushnell Haas agar medium proved best for bacterial growth and enrichment. The optimum temperature recorded for growth was 37°C in case of *Micrococcus* and *Staphylococcus* spp. and 43°C in *Pseudomonas* spp. As carbon source, 15µl petrol was found optimum for all the three isolates. Calcium nitrate tetrahydrate was found to be a better source of nitrogen in case of *Micrococcus*, *Staphylococcus* and peptone for *Pseudomonas* spp. The UV mutagenesis study revealed that all the strains exhibit considerable improvement in their growth after mutagenesis. Among the three isolates, strain of *Pseudomonas* was found to be better choice for further studies like standardization, ability of producing various extracellular enzymes, surfactants and genetic profiling.

KEYWORDS: *Micrococcus*, *Staphylococcus*, *Pseudomonas*, petroleum tolerant bacteria, soil contamination, bioremediation

1. INTRODUCTION

Nature takes thousands of years to form the fossil fuel. All fossil fuels primarily consist of a complex mixture of molecules called hydrocarbons (Alexander, 1994). Petroleum is a complex mixture of many thousands of compounds mainly consisting of carbon and hydrogen. These can be divided into four major groups: alkanes, aromatics, resins, and asphaltenes. In general, an alkane fraction is the most biodegradable, whereas the polar fraction (i.e., resins and asphaltenes) is resistant to biological degradation. The aromatic compounds, especially the polycyclic aromatic hydrocarbons (PAHs), are of intermediate biodegradability, but these are of most concern owing to their toxicity and tendency to bioaccumulate (Wrenn *et al.*, 1995).

The utilization of crude petroleum all over the world serves as the sources for fuel and energy. Lubricants and other petrochemical products cause a lot of serious problems in oil contamination. These sources serve as the major contributor to the environmental pollution problems especially in the soil and water. Contamination of the environment by petroleum products is known to be toxic and hazardous to the environment.

As industrialization expands, petroleum hydrocarbons become a greater potential source of soil contamination. Soil contamination with hydrocarbons causes extensive damage of local ecosystem since accumulation of pollutants in animals and plant tissues may cause progeny's death or mutation. It has become one of the major environmental pollution that is becoming more stringent and to be given a lot of attention. The elevated loading of petroleum hydrocarbons in soil cause a significant decline soil quality and these soils have become unusable. To

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control the environmental risk caused by petroleum products, various new regulations have been introduced and, at the same time, research focusing on remediation (Alvarez *et al.*, 1991).

Physical, chemical and biological methods can be used for cleaning up the polluted sites. Biodegradation is most often the primary mechanism for contaminant destruction including petroleum contaminants. It has been proposed for cleanup of oil-spills as cost effective technology of removing contaminants (Lyman *et al.*, 1990). Microorganisms survive in contaminated habitat because they are metabolically capable of utilizing its resources and can occupy a suitable niche. Contaminants are often potential energy sources for microorganisms. It shows that microorganisms have broad range of enzymes that enable them to degrade many chemicals. To investigate the countermeasure to remediate soils contaminated with oils, bioremediation provide an effective and efficient strategy to speed up the clean-up process (Yavari *et al.*, 2015).

Hydrocarbons contaminated soil is classified as hazardous wastes and is toxic to microorganisms as well as to the plants (Atlas, 1981). Due to widespread use of petroleum products, the number of petroleum contaminated sites has abounded. Natural attenuation, which relies on in-situ biodegradation of pollutants, has received a large amount of attention, especially for petroleum contamination (Dadrasnja *et al.*, 2014). These strategies appear to be the most environmentally friendly method of removal of oil pollutants since other method such as surfactant washing and incineration lead to introduction of more toxic compounds to the environment (Atlas *et al.*, 1978). Researchers are being forced to investigate novel technologies for the degradation of petroleum compounds.

The present study was carried out with the following objectives:

- i. To explore the potential indigenous bacterial strains with degradative ability from petroleum contaminated soil samples.
- ii. To study the efficacy of bacterial isolates in degradation of petroleum compounds present in the soil samples.
- iii. To identify the bacterial isolates by microscopic and biochemical characterization.
- iv. To optimize the growth parameters such as temperature, pH, carbon source and nitrogen sources for the isolates.
- v. To study the optimized bacterial isolates mutationally to assess the enhanced capability in the degradation of petroleum.

II. MATERIALS AND METHODS

Collection of petroleum contaminated soil samples

100gm of three different petroleum contaminated soil samples were collected using sterile bags from three petrol bunks. And they were brought to the laboratory without further contamination. They were mixed together and used as a single sample for further studies. The samples are shown in Fig. no.1 (a-c).



(a)



(b)



(c)

Fig. 1. Soil samples collected from different locations of Daltonganj (a) Ratan Lal Petrol Pump (b) Chianki Petrol Pump and (c) Durga Petrol Pump

Isolation of indigenous bacteria from petroleum contaminated soil samples

Bushnell Haas broth, Mineral Salt broth and Basal Salt broth are used for isolating the bacteria from petroleum contaminated soil samples. 100 ml of each broth media were prepared in 250 ml Erlenmeyer flask and sterilized at 121° C for 15 lbs/sq.inch for 15 minutes. After sterilization, the broth media were incorporated with 0.1 ml of petrol as a

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carbon source and 1 gm of each soil samples were inoculated. They were incubated at 170 rpm at 30° C for a week. After a week of incubation, the turbidity of the broth cultures was observed by spectrophotometer at 600nm.

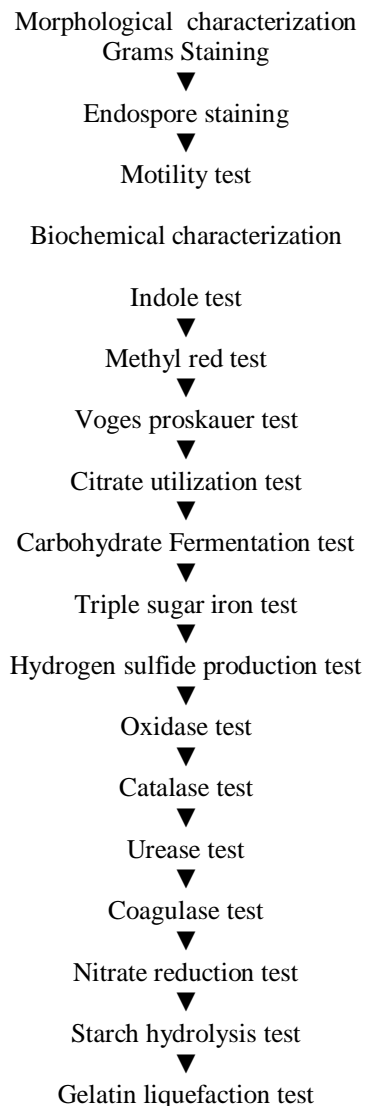
Enrichment of indigenous bacteria from petroleum contaminated soil samples

The similar protocol which was followed for the isolation of indigenous bacteria from petroleum contaminated soil samples was repeated twice to enrich the isolates using the same culture media. During enrichment, the growth of the isolates were estimated by turbidity method at regular intervals of 48 hours for 14 days. The maximum growth was identified by OD values in the three different culture media. Based on the OD values obtained, the best medium that shows maximum growth was selected for further studies.

Screening of petroleum degrading bacterial isolates

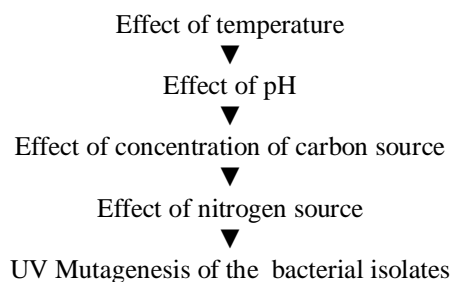
The enriched bacterial isolates were purified by subculturing. They were screened for the ability to degrade the petroleum compounds present in the soil samples collected as follows. 100ml of Bushnell Haas Agar medium was prepared and sterilized at 121°C for 15lbs/sq.inch for 15 minutes. After sterilization the medium and 1ml of each enriched broth cultures were mixed, poured into petriplates. After solidification, four wells were created within the plates. Then the wells were loaded with different concentrations of petrol sample (5µl, 10µl 15µl and 20µl) was incubated at 37°C for 2-4 days. After incubation colonies were obtained around the wells and they were used for further studies.

Identification of the bacterial isolates



Optimization of growth parameters on the growth of isolates

After identification, the bacterial strains were optimized under different growth parameters such as temperature, pH, carbon source and nitrogen sources.



III. RESULTS

1. Isolation of indigenous bacteria from petroleum contaminated soil samples

The primarily isolated broth cultures of Bushnell haas broth, Mineral salt broth and Basal salt broth were observed for the growth of bacteria by estimating optical density at 600nm which confirms the degradative ability of the isolates by utilizing petroleum compounds that are present in the soil samples collected (Table no. 1, Fig. no. 1).

Incubation period (in days)	OD values (at 600 nm)		
	Bushnell haas broth 1	Mineral salt broth 2	Basal salt broth 3
7	0.27	0.26	0.20

Table 1: Estimation of growth of bacterial isolates during isolation

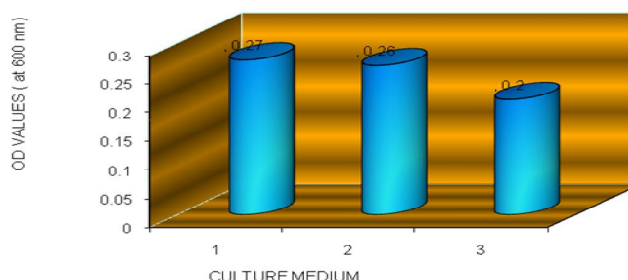


Fig. no. 2a: Graphic representation of growth of the three bacterial isolates in different culture media.



Fig. no. 2b: Broth cultures of petroleum degrading bacterial isolates in different media *i.e.* Bushnell haas broth, Mineral salt broth, basal salt broth.

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2. Enrichment of indigenous bacteria from petroleum contaminated soil samples

During enrichment the growth was estimated by reading optical density at 600nm. The experimental outcome proved that the isolates have the ability to degrade the petroleum compounds present in the soil sample incorporated in the medium. Bushnell haas agar medium exhibited better growth within a short period of incubation *i.e.* 5 days comparatively. Hence, it was selected for further studies on optimization (Table no. 2 and Figs 2a&b).

Incubation period (in days)	OD values (at 600 nm)		
	Bushnell haas broth	Mineral salt broth	Basal salt broth
1	0.46	0.45	0.34
3	0.50	0.51	0.40
5	0.67	0.63	0.45
7	0.61	0.65	0.47
9	0.46	0.41	0.63
11	0.23	0.25	0.45
13	0.18	0.21	0.38

Table no. 2. Estimation of growth of bacterial isolates during enrichment

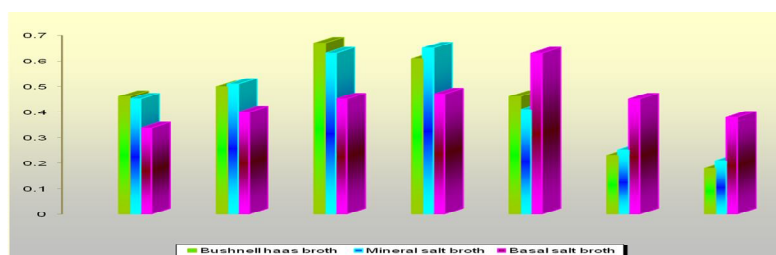


Fig. no. 3. Estimation of growth of bacterial isolates during enrichment

3. Screening of petroleum degrading bacterial isolates

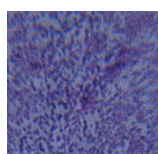
Pure colonies were obtained after enrichment on Bushnell Haas agar medium surrounding the wells. Based on varied colony morphology, 3 different types of isolates were obtained. They were designated by numbers as isolate no.1, 2 and 3(Fig. no. 4.1).



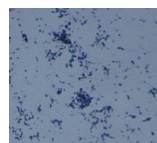
Fig. no.4.1. Pure colonies obtained on Bushnell haas agar medium

4.4. Identification of petroleum degrading bacterial isolates

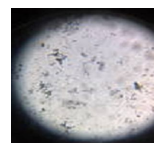
The three bacterial isolates were identified by microscopic examination and biochemical characterization. Among them, isolate no. 1 and 2 were gram positive and isolate no. 3 was gram negative. After biochemical characterization, based on the results of various tests performed they were preliminarily identified as strains of *Micrococcus sp.*, *Staphylococcus sp.* and *Pseudomonas sp.* (Fig. no. 4.2 a-c; Table no.4a).



(a)



(b)



(c)

Fig. no. 4.2 (a-c). Identified strains of (a) *Micrococcus sp.* (b) *Staphylococcus sp.* (c) *Pseudomonas sp.*

Sl. NO	TEST	Isolate No. 1 Micrococcus sp.,	Isolate No.2 Staphylococcus sp
1.	<u>Microscopic examination:</u>		
	a. Gram's staining	+	+
	b. Shape	Cocci	Cocci
	c. Motility	-	-
	d. Endospore staining	-	-
2.	<u>Biochemical tests:</u>	----	+
	a. Coagulase		
	b. Carbohydrate fermentation		
	(i) Glucose	+	+
	(ii) Lactose	-	+
	(iii) Mannose	-	+
	(iv) Sucrose	-	+

Note: + : Positive - : Negative

Table 4a. Gram positive isolates showing results of microscopic examination and biochemical tests.

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Sl. No.	Test	Isolate No. 3 <i>Pseudomonas sp</i>	
1	<u>Microscopic examination:</u>		
	a. Gram's staining	-	
	b. Shape		Bacilli
	c. Motility		-
	d. Endospore staining		-
2	<u>Biochemical tests:</u>		
	a. Indole Production		-
	b. Methyl Red	-	
	c. Voges -Proskeur		-
	d. Citrate Utilization		+
	e. Carbohydrate Fermentation:		
	(i) Glucose		+
	(ii) Lactose		+
	(iii) Mannose		+
	(iv) Sucrose		-
	f. TSI		
	g. H ₂ S Production		AK/A
	h. Catalase		+
	i. Oxidase		+
	j. Urease		+
	k. Coagulase	-	
	l. Nitrate reduction		-
	m. Starch hydrolysis		-
	n. Gelatin liquefaction	-	

Note:

+ : Positive
- : Negative

AK / A : Alkaline slant / Acid butt

Table 4b. Gram-ve isolate (*Pseudomonas sp.*) showing results of microscopic examination and biochemical tests.

5. Optimization of growth parameters on the growth of isolates

5.1. Effect of temperature

Effect of temperature on growth of the isolated strains of *Micrococcus sp.*, *Staphylococcus sp.*, *Pseudomonas sp.*, was studied after 24hrs of incubation period and the results are tabulated in Table no.5.1. It was found that better growth was obtained at 37° C for the strains of *Micrococcus sp.*, and *Staphylococcus sp.*, and 43°C was found to be the optimum for *Pseudomonas sp.*, (Fig. no.5.1).

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Sl. No.	Temp.	OD values (at 600 nm)		
		<i>Micrococcus sp.</i> ,	<i>Staphylococcus sp.</i> ,	<i>Pseudomonas sp.</i> ,
1.	4°C	0.07	0.08	0.21
2.	28°C	0.05	0.07	0.23
3.	37°C	0.10	0.15	0.23
4.	43°C	0.08	0.10	0.25

Table 5.1. Effect of varying temperature on growth of bacterial spp.

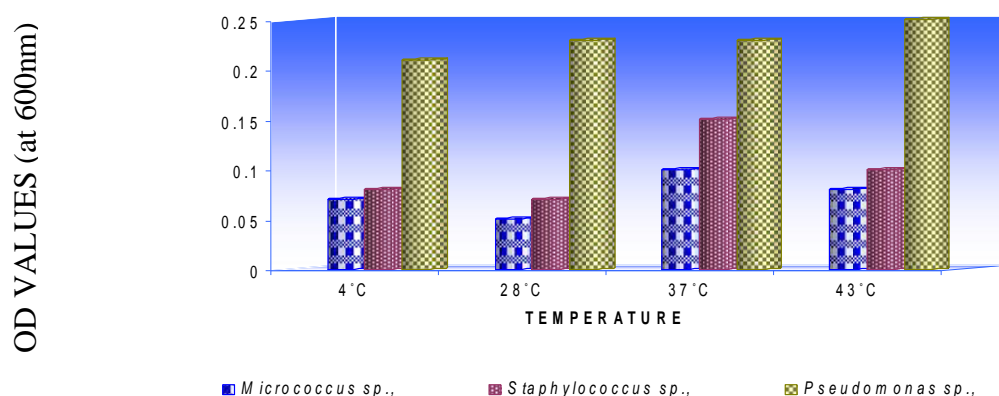


Fig. No. 5.1. Graphic representation of the effect of varying temperatures on the growth of three isolated bacterial spp.

2. Effect of pH

Effect of pH on growth of the isolated strains of *Micrococcus sp.*, *Staphylococcus sp.*, *Pseudomonas sp.*, was tested. The experimental results are tabulated in Table no.5.2. It was clearly evidenced that the peak growth was obtained at pH 7 for the strains of *Micrococcus sp.*, and *Staphylococcus sp.* and pH 8 was found to be the optimum for *Pseudomonas sp.* It exhibited a maximum growth at that pH while comparing with other pH (Fig. no 5.2).

Sl. No.	pH	OD values (at 600 nm)		
		<i>Micrococcus sp.</i> ,	<i>Staphylococcus sp.</i> ,	<i>Pseudomonas sp.</i> ,
1.	5	0.03	0.08	0.14
2.	6	0.06	0.10	0.16
3.	7	0.09	0.13	0.18
4.	8	0.08	0.09	0.54

Table no.5.2. Effect of pH on growth of the three bacterial spp.

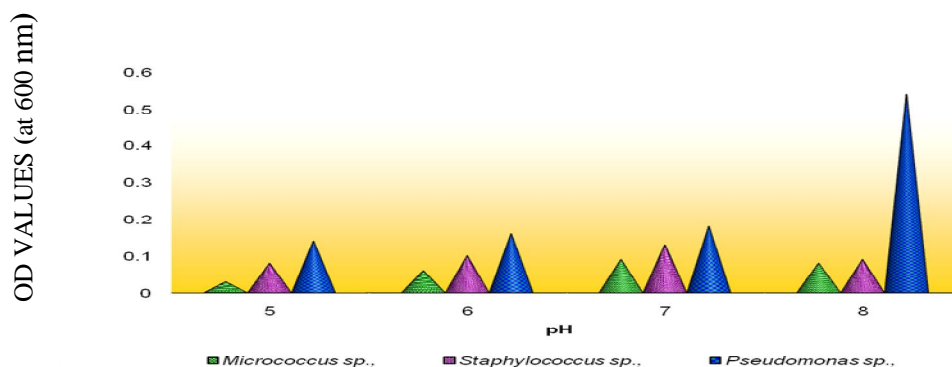


Fig. 5.2. Graphic representation of varying pH on the growth of the three bacterial spp.

5.3. Effect of different concentration of carbon source

Effect of concentration of carbon source on growth of the isolates was examined experimentally using various concentrations of petrol as carbon source and the results are tabulated in Table no.5.3.

It was evidently found that 15 µl of petrol as carbon source served as optimum which provided the maximum growth for all the isolates i.e. *Micrococcus sp.*, *Staphylococcus sp.*, and *Pseudomonas sp.* (Fig. no.5.3).

Sl. No.	Carbon source	OD values (at 600 nm)		
		<i>Micrococcus sp.</i> ,	<i>Staphylococcus sp.</i> ,	<i>Pseudomonas sp.</i> ,
1.	5 µl	0.01	0.01	0.19
2.	10 µl	0.02	0.02	0.18
3.	15 µl	0.04	0.03	0.21
4.	20 µl	0.01	0.01	0.19

Table no. 5.3. Effect of varying concentrations (5,10,15&20µl) of petrol as carbon source on growth of bacterial spp.

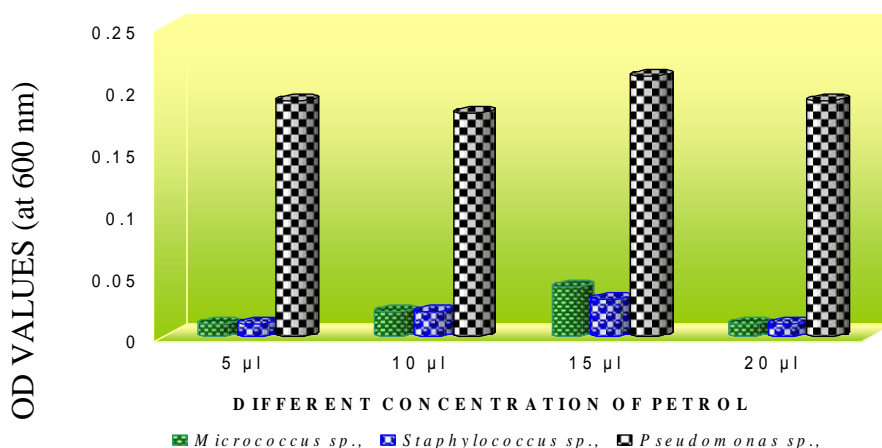


Fig. no. 5.3. Graphic representation of different concentration of petrol on growth of the three bacterial spp.

Effect of nitrogen source on growth of the isolates of *Micrococcus sp.*, *Staphylococcus sp.*, *Pseudomonas sp.*, was studied and the results are tabulated in Table no.5.4. The experimental outcome proved that calcium nitrate tetrahydrate is the better source for the strains of *Micrococcus sp.*, *Staphylococcus sp.*, which supported the maximum growth and peptone for *Pseudomonas sp.* (Fig. no.5.4).

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Sl. No.	Nitrogen source	OD values (at 600 nm)		
		<i>Micrococcus sp.</i> ,	<i>Staphylococcus sp.</i> ,	<i>Pseudomonas sp.</i> ,
1.	Ammonium nitrate	0.05	0.05	0.18
2.	Potassium nitrate	0.03	0.05	0.27
3.	Sodium nitrate	0.03	0.05	0.17
4.	Calcium nitrate	0.47	0.52	0.43
5.	Peptone tetrahydrate	0.03	0.06	0.33

Table no. 5.4. Effect of different nitrogen sources on growth of *Micrococcus sp.*, *Staphylococcus sp.*, and *Pseudomonas sp.*

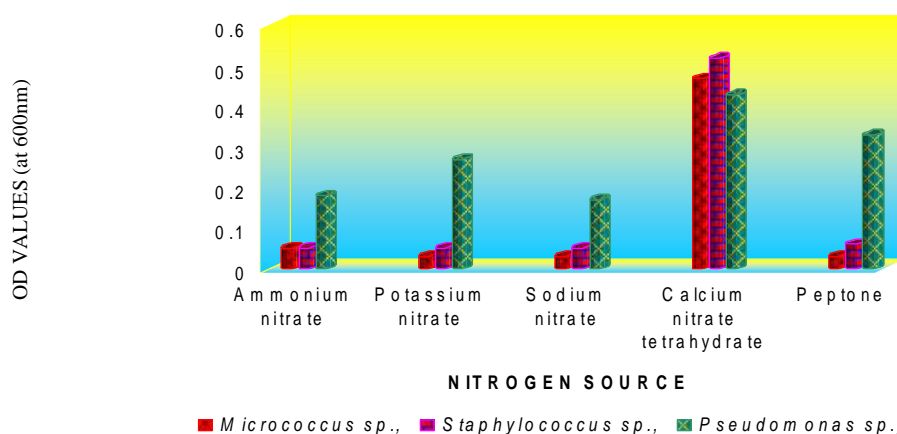


Fig. 5.4. Graphic representation of the effect of different nitrogen sources on growth of the three isolated bacterial spp.

6. UV Mutagenesis of the bacterial isolates

The mutational study revealed that the strains exhibit considerable improvement after mutagenesis. It was evidenced by their growth as well as degradative ability (Table 6a&b; Figs. 6&7)

Sl. No.	Isolate	OD value (at 600 nm)	
		Mutant	Wild type
1.	<i>Micrococcus sp.</i>	0.95	0.87
2.	<i>Staphylococcus sp.</i>	0.97	0.90
3.	<i>Pseudomonas sp.</i>	0.99	0.96

Table 6a. The isolated bacterial spp. showing improved growth after UV mutagenesis

Concentration Of petrol		No. of. Colonies		
		10 µl	20 µl	30 µl
<i>Micrococcus sp.</i>	Mutant	3	7	12
	Wild type	7	4	8
<i>Staphylococcus sp.</i>	Mutant	2	6	21
	Wild type	4	8	4
<i>Pseudomonas sp.</i>	Mutant	5	7	25
	Wild type	7	5	10

Table 6b. The bacterial spp. show increased ability of degrading petrol after strain improvement

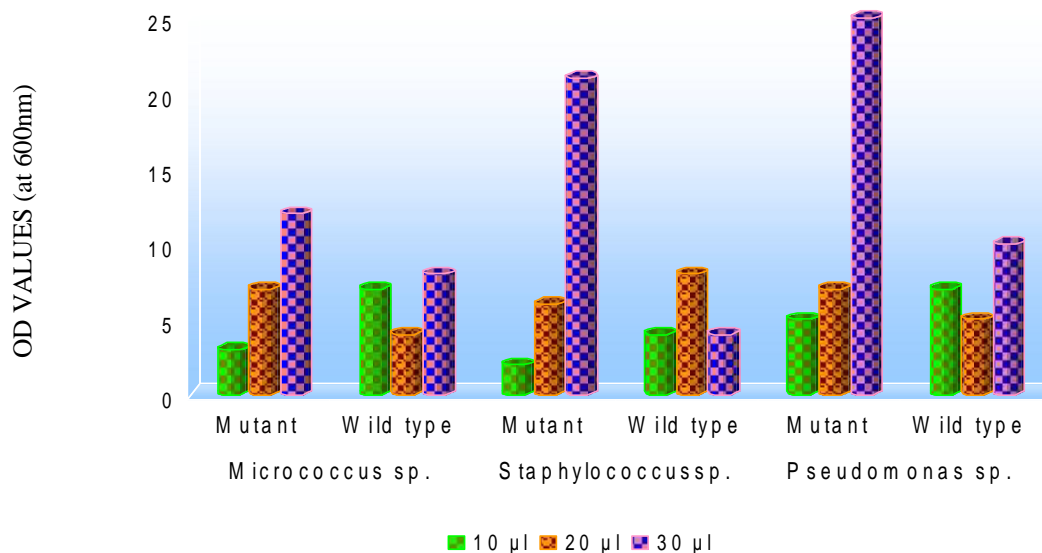
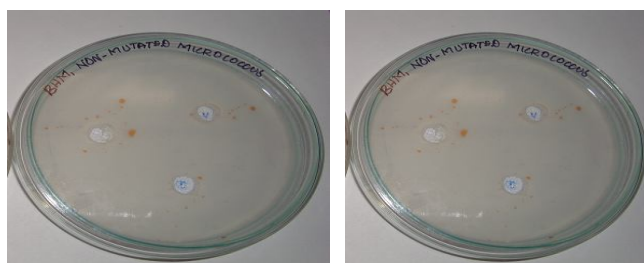


Fig. no. 6. Graphic representation of the degradative ability of petrol by the three bacterial *spp.*, where all the three isolates show marked improvement in their action after strain improvement.



IV. DISCUSSION

Contamination of the environment by petroleum products is known to be toxic and hazardous to the environment. The production, transportation, refining and ultimate disposal of petrochemicals are increasing day by day. Biodegradation by natural populations of microorganisms, or in situ attenuation, is a primary mechanism by which petroleum hydrocarbons could be eliminated from contaminated sites (Bartha, 1996). Microorganisms can survive in contaminated habitat because they are endowed with metabolic capability to utilize such xenobiotics as their sole carbon source. Hydrocarbon degrading microorganisms are widely distributed in marine, freshwater and soil ecosystems. Nearly all soils and sediments have populations of bacteria capable of degrading petroleum hydrocarbons. Rates of biodegradation vary with different microbial population, hydrocarbons, geochemical and hydrological conditions of the niche.

The microbial degradation of oil pollutants is a complex process. But with an understanding and studying this process in the environment, it is possible to develop strategies for utilizing microbial hydrocarbon degradation activities for the removal of hydrocarbons from contaminated areas. The objectives of this study are the isolation, identification, optimization of indigenous bacteria from petroleum contaminated soils and their mutagenesis to assess the enhanced degradative ability.

A preliminary study was carried out to isolate the indigenous bacteria from petroleum contaminated soil sample using Bushnell Haas broth, Mineral salt broth and Basal salt broth. An increase in turbidity during the growth

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demonstrates the ability of utilizing of petroleum present in the soil during enrichment. Bushnell Haas agar medium exhibited better growth within a short period of incubation i.e. 5 days comparatively. Hence, it was found to be the best medium and selected for further studies of optimization. Three different isolated strains were identified by using standard procedures. The experimental outcome of morphological and biochemical characterization proved that both gram positive and gram negative bacteria i.e. *Micrococcus sp.*, *Staphylococcus sp.*, *Pseudomonas sp.*, are able to survive in such a harsher niche. *Pseudomonas sp.*, is the most common bacterial hydrocarbon degraders reported in the literature, which is widespread in nature and can degrade a wide range of xenobiotics (Rusansky *et al.*, 1987; Kiyohara *et al.*, 1992; Barathi and Vasudevan, 2001; Bhattacharya *et al.*, 2002; Van Hamme *et al.*, 2003; Esin Erdogan *et al.*, 2011; Adeyemo *et al.*, 2013). The present study proved it and *Pseudomonas sp.*, was isolated as one of the isolates. Similar strains were isolated by Sabeen Survery *et al.*, 2004, Mandri and Lin, 2007. Studies on optimization of various growth parameters such as temperature, pH, different concentration of petrol as a carbon source and nitrogen sources revealed that the strains of *Micrococcus sp.*, and *Staphylococcus sp.*, showed better growth at 37° C, pH 7 and these parameters were found to be the optimum. Whereas, 43°C and pH 8 were found to be the optimum for *Pseudomonas sp.* A related work was carried out with the strains of *Flavobacterium sp.*, *Acinetobacterium Calcoaceticum* and *Pseudomonas aeruginosa* by Mandri and Lin, 2007 where similar results were obtained. It was evidently found that 15 µl of petrol as carbon source for all the strains and calcium nitrate tetrahydrate as nitrogen source were utilized more efficiently. They served as optimum which provided the maximum growth for the strains of *Micrococcus sp.*, and *Staphylococcus sp.* But the strain of *Pseudomonas sp.*, was found to be metabolically different and exhibited better growth in the medium incorporated with peptone as nitrogen source. This shows that the strain of *Pseudomonas sp.*, is able to show better degradation even by using a very simple and basic nitrogen source. The optimized isolates were utilized for strain improvement by mutagenesis. The mutational study revealed that the mutants exhibit considerable improvement in their growth. It was evidenced by increased optical density as well as the degradative ability.

The present study confirmed that among all isolates, the strain of *Pseudomonas sp.* was found to be the better choice for further studies like standardization, ability of producing various extracellular enzymes as well as surfactants and genetic profiling.

V. ACKNOWLEDGEMENT

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