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ISOLATION, IDENTIFICATION AND MORPHOLOGICAL CHARACTERISATION OF CRUDE OIL DEGRADING BACTERIA OBTAINED FROM ONGC DRILLING SITE AT THIRUVARUR DISTRICT

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Abstract:

The initial population of bacteria and catabolic activities in a community are important factors in determining its efficacy for crude oil degradation. Optimization of the relative number of bacterial population may be almost impossible, but the strategy taken in this study, namely enrichment of bacteria by growth on residual crude oil may be one way to select the microbial population for better crude oil degradation. A combined approach of techniques like enrichment of culture, enumeration identification and screening of bacteria from crude oil were analyzed. In bacterial counts and degradation percentage of crude oil, the reduced substrate concentration and biomass concentration were measured for six weeks. All the bacterial isolates showed the ability to utilize crude oil, however the higher efficiency of utilization varied among strains with *Pseudomonas sp* (A) and *Pseudomonas sp* (B). In the development of the bacterial consortium six most efficient isolates were isolated and grown well in the medium with the crude oil, the highest growth rate of the bacterial consortium was obtained at the end of the 2nd week. The highest percentage of the crude oil degradation is 89.6%. Based on the results obtained from this research work, bioremediation could be considered as a key component in the clean-up strategy for crude oil and oily sludge contamination.

Keywords: Bioremediation, Crude oil, Consortium, Soil, *Pseudomonas* Sp., *Bacillus* Sp.

Introduction:

Bioremediation of oil spills requires the identification of microbes which have the ability to degrade hydrocarbons present in the soil or water, so that in case of a large spill these can be stimulated further in order to clean-up the area.

Identification of such strains can ensure better efficiency of remediation as these strains will be well adapted to grow in the respective environment. A number of microorganisms have been found to have the capability to degrade the hydrocarbons from the oil spills. These include strains of bacteria, fungi, yeast, algae etc. (Kazuya, 2001).

Bioremediation oil spills involve highly heterogeneous and complex processes. Its application can be limited by the composition of the oil spilled. The degradation of oil spills by microorganisms mainly depends on the type of microbial species, and its efficiency for the degradation under favorable environmental conditions. Concerns also arise about potential adverse effects associated with the application of bioremediation agents (Prince *et al*., 1994). To enhance the bioremediation process addition of nutrients (N and P) were very essential (Wang, 2011).

The world today is very much dependent on crude oil, either to fuel the vast majority of its mechanized transportation equipment or as the primary feedstock for many of the petrochemical industries. Crude oil is a naturally occurring complex mixture of hydrocarbon and non-hydrocarbon compounds which possesses a measurable toxicity towards living system.

Some microorganisms, though, cannot produce bio-surfactants but are still able to degrade oil substrates effectively via formation of extra cellular or cell membrane-bound bio-emulsifiers (such as exo-polysaccharides, EPS) (Hino, *et al*.,1997).

Crude oil is a complex mixture of hydrocarbons, composed of aliphatic, aromatic and asphaltene fractions along with nitrogen, sulfur and oxygen-containing compounds. The constituents of these hydrocarbon compounds are present in varied proportions resulting in high variability in crude oil from different sources. Biological cleaning approach is done by adding microbes which have the potential to consume hydrocarbons to use as a food source, giving out water and carbon dioxide as waste products. It occurs on the water surface, in the water column, in the sediments and at the shoreline. The biodegraded petroleum-derived aromatic hydrocarbons in marine sediments by *Arthrobacter, Burkholderia, Mycobacterium, Pseudomonas, Sphingomonas,* and *Rhodococcus* were found to be very effective [Jones *et al*., 1983].

Bacterial genera, namely, *Acinetobacter Sp., Gordonia, Brevibacterium, Aeromicrobium,* and *Mycobacterium* were isolated from petroleum contaminated soil in North East India. Among them *Acinetobacter Sp.,* was found to be capable of utilizing n-alkanes of chain length C10–C40 as a sole carbon source [Das and Mukherjee, 2007].

So the present work was aimed to enumerate isolate, screen and characterize the microbial flora (bacteria) from the hydrocarbon contaminated site of Thiruvarur ONGC drilling site and to standardize and optimize a suitable microbial consortia for lab and field evaluation.

Methodology:

Hard core (HC) samples for microbial analysis were collected from four locations at this site at Thiruvarur District of Tamil Nadu. The sampling locations were designated as HC_{1}, HC_{2}, HC_{3}, and HC_{4} (Vishvesh *et al.,* 2002) (Figure -1).

Enrichment of all soil microorganisms collected from the field was carried out in 500 ml Erlenmeyer flasks containing 100 ml of mineral salt medium (MSM) (Ijah and Upke, 1992) and it was sterilized by autoclaving at 121°C, 15 psi for 15 minutes. (Pokethitiyook *et al.,* 2003) Enumeration of bacteria from the Enrichment cultures was done by spread plate technique (Pokethitiyook *et al.,* 2003). Identification of bacterial isolates were characterized to generic level on the basis of colony morphology, Grams staining and biochemical tests .The characteristics of the isolates were compared with those of Known taxa described In Bergey's manual (Holt *et al*., 1994), and so identified to genus. Screening for crude oil degradation by bacteria was carried out by spread plate technique. Screening for individual aromatic hydrocarbon like Napthalene, Anthracene, phenanthrene, degradation by bacteria on agar plates (Kiyohara *et al.,* 1982) Determination of growth rate and percentage of crude oil degradation by individual bacterial isolates (Gogoi *et al*., 2003)

Residual oil recovery

After six weeks, The total petroleum hydrocarbon extract was obtained by adding 40 ml of DCM and hexane (1:1) to flasks, the aqueous and solvent phases were transferred to four 50 ml centrifuge tubes and flasks were rinsed three more times to bring the total volume of DCM hexane used to 100 ml. Tubes were centrifuged at 8000 rpm .The upper layer was discarded and the lower oil containing layer phase was filtered into a round bottom flask through sodium sulphate to remove residual water. The solvent was evaporated and the weight of the oil after degradation was taken.

The bacterial consortium that can degrade crude oil and oily sludge was developed. Out of 8 bacterial isolates 6 most efficient isolates were selected for further development of the consortium.(Mishra *et al.,* 2001)

Result and discussion

The use of microbial metabolic potential for eliminating environmental pollutants provide a safe and economic alternative to their disposal in waste dump sites and to commonly used Physico-chemical strategies. Thus a combined

approach was applied in this present research work. The hydrocarbon degrading bacteria were isolated, mass multiplied and applied in the field for better bioremediation.

Enumeration of Enrichment cultures of bacteria from (ONGC) Drilling site soil contaminated with crude oil, obtained from Thiruvarur District of Tamil Nadu.

Culturable heterotrophic bacteria in soil contaminated with crude oil were enumerated aerobically using standard heterotrophic plate count. The soil examined contained the highest amount of bacterial biomass ranging from 9×10^8 to 6.2×10^9 cells/g and active bacteria ranging from 4×10^8 to 2.8×10^9 cells/g soil (Vishvesh *et al.,* 2002).

In the present investigation the enrichment cultures enumerated from the crude oil contaminated soil contained the highest amount of active bacteria ranged from 2.3×10^5 to 1.4×10^5. The maximum range of active bacteria present in the samples collected on Nov 15th 2012. The bacteria comparatively with the sample collected on Feb 15th 2012 & Nov. 1st 2012 is given in Table -1.

In the period of north east monsoon November 2012 the active range of bacteria was above 1.9×10^5 to 1.4×10^5 where as in the period of southwest monsoon July 2012 the active range of bacteria was about 1.7×10^5 to 1.4×10^5 between the two monsoon season Feb.2012 the active range of bacteria was high and ranged from 2.3×10^5 to 2.0×10^5.Based on the above results, it has been observed that during both the monsoon season the growth rate of the bacteria was less when compared to bacterial load enumerated from the sample collected on February 2012. It may be due to the heavy rainfall that affected the growth rate of bacteria. Moreover in the sample collected on November 2012 the hard core sample (HC-1) showed the highest range of bacteria 1.9×10^5, whereas the hardcore sample HC-3 showed lowest range of bacteria 1.4×10^5.

Likewise in the sample collected from February 2004 the highest range of bacteria 2 .3 $\times 10^5$ was found is hardcore (HC-2) and the lowest range of bacteria 2.0×10^5 in HC-3. Furthermore in the sample collected on July 2012, the HC-l sample showed highest range of bacteria 1.7 x 10^5 ,where (HC- 4) has the lowest range of bacteria 1.4×10^5. This may be due to the variations in the distribution among the bacteria in the crude oil contaminated soil and due to the bio-availability of the contaminations.

Finally from the enrichment cultures enumerated from the crude oil contaminated soil, eight different bacteria were isolated with different colony morphology and cultural characteristics. (Table-1)

Identification of bacterial isolated obtained from enrichment cultures

The screened isolated were characterized to genetic level on the basis of colony morphology, gram staining and biochemical test. These characteristics of the isolates were compared with known taxa as described in Bergey's manual (Holt *et al.,* 1994) and so identified the genus. Eighteen bacterial strains were obtained by enrichment of oily sludge contaminated soil with the strains SV4, SV9, and SV17 are able to grow on a variety of hydrocarbons. These contrasted with other isolates which appeared to be restricted to a few hydrocarbon compounds. Comparing these characteristics with those known taxa. Isolate SV4 was identified as *Acinetobacter Sp.,* SV9 as *Bacillus Sp.,* and SV17 as *Pseudomonas Sp.*

Similarly in the present work, 8 different bacterial isolates were obtained from enrichment cultures and maintained individually on nutrient agar. These hydrocarbon degrading bacteria were characterized to genus level based on colony morphology. Gram staining and biochemical reactions given in Table 2. The bacteria were identified up to genus level. Two types of *Pseudomonas* species were identified, strain 1 was designated as *Pseudomonas Sp.,* (A), strain 2 as *Pseudomonas Sp.,*(B), strain 3 as *Bacillus Sp.,* strain 4 *Micrococcus Sp.,* (Figure-2) strain 5 *Flavobacterium Sp.,* strain 6 *Alcaligenes Sp.,* strain 7 *Aeromonas Sp.,* strain 8 was identified as *Corynebacterium Sp.,*(Figure- 2).

Screening for crude oil degradation by bacteria on agar plate.

Screening of crude oil degrading bacteria usually involves the growth on the medium that contains crude oil or refined petroleum product as the selective substrate because these complex substrate contain both aliphatic and aromatic compounds (Mulkins Philips and Stewart, 1974 and Haines *et al.,* 1996). Bacterial growth on pure hydrocarbons heavy oil was tested. The seven bacterial strains were able to grow well on aliphatic hydrocarbons such as tetradecane (C_{14}), hexadecane (C_{16}), octadecane(C_{18}),and eicodecane (C_{20}),as well as on heavy oil. Also they grew poorly on hexane (C_6), octane (C_8), octacosane (C_{28}), paraffin, pristine (branched C_{19}),and cyclohexane. However these bacterial strains did not appear to utilize aromatic hydrocarbons as carbon source, except that strains as grew poorly on phenanthrene and fluoranthene and strain A6 utilized various hydrocarbons. (Chaerun *et al.,* 2003). In this present investigation, crude oil was used as the selective substrate in which the isolated organisms in sterile discs are placed on the medium spreaded with crude oil. In this, both *Pseudomonas Sp.,* (A and B) shown very good growth and the maximum oil utilization zone of 25 and 22mm zone respectively.

Corynebacterium sp. shown 20mm zone of oil utilization *Bacillus Sp.,* and *Micrococcus Sp.,* shown 16 and 15mm zone respectively. *Aeromonas* Sp., and *Flavobacterium Sp.,* shown very less utilization zone 06 and 09 mm respectively, which is given in Table 3. This indicates that *Pseudomonas* strains (Figure -3) and *Corynebacterium Sp., Bacillus Sp.,* and *Micrococcus Sp.,* are efficient strains which can grow in the complex substrate (crude oil).

Screening for individual aromatic hydrocarbon degradation by bacteria on agar plates.

Polyaromatic hydrocarbon degraders have been selectively enumerated by spraying the surface of plates with solution of PAH in volatile solvent in which *Bacillus Sp., Pseudomonas Sp., Micrococcus Sp.,* shows the ability to grow on a variety of hydrocarbon groups tested by (Kiyohara *et al*., 1982). A fluoranthene metabolizing mixed culture was successfully isolated from soil contaminated with PAHs for several years using the enrichment procedure carried out in cyclone fermenters.

PAH contaminated soil or sediments have proved to be rich source of PAH degrading bacteria (Walter *et al.,* 1990; Weissenfels *et al.,* 1991; Iteitleamp and Cernigilia (1998). The isolated mixed culture grew and removed fluoranthene in media supplemented with glucose, yeast extract and peptone. Yeast extract supplementation has also been found to enhance transformation of PAHs in soil.

The presence of co-substrates has been shown to enhance PAH degradation in several studies (Bosert and Bartha 1986 ; Sanglard *et al.,* 1986; Shiaris and Cooney 1983; Mckenna and Health 1976). More than half of the fluoranthene metabolizing species isolated by Cooney and Shiaris (1982) used the substrates as sole carbon source while others utilized phenanathrene in the presence of yeast extract, peptone, glucose and benzoate.

The mixed culture was capable of degrading a range of PAHs including benzo (a) pyrene, pyrene, fluoranthene, anthracene, phenantherene, acenaphthene and fluorene. As in typically observed (Bossert and Bartha 1986; Wodzinski andcoyle 1974) low molecular weight compounds were degraded at faster rates than high molecular weight structures in time course studies. Four bacterial strains were isolated from the original mixed culture, three of which were identified as *P.pudita*, *Flavobacterium sp.,* and *P.aeruginosa*.

Pseudomonas and *Flavobacterium* species have been found to degrade different PAHs in numerous studies. (Muller *et al.,*1990) demonstrated the *Pseudomonas pavimobilis* EPA 505 degraded a range of aromatic compounds including napthalene, fluorene, anthracene, phenanthrene and fluoranthene.

Similarly result were obtained in the present research work the bacterial isolates are subjected to determine the efficiency of degrading the individual poly aromatic hydrocarbons like napthalene, anthracene, phenanthrene in mineral salts medium supplemented with yeast extract and peptone given in Table 3. As a result, the *Pseudomonas Sp.,* (A) and *Pseudomonas Sp.,*(B) have been found to utilize all the three hydrocarbons efficiently and grown well on the medium with zone of clearance around the growth of the organism which indicated the degradation has been occurred. *Bacillus Sp.,* and *Corynebacterium Sp.*, showed medium growth on the three hydrocarbons with the zone of clearance around the growth of the organism.

These hydrocarbons (Phenanthrene, Anthracene, Napthalene) served as a substrate by which the bacteria utilized these substrate as sole source of carbon and degraded. Whereas *Micrococcus* Sp. exhibited growth on the medium with phenanthrene only, remaining bacteria like, *Flavobacterium Sp., Alcaligens Sp.,* and *Aeromonas Sp.,* showed a negative result for all the three hydrocarbons.

Bacterial counts and degradation percentage of crude oil in broth.

There are large numbers of reports describing the properties of pure and mixed microbial cultures capable of degrading crude oil (Walker and Colwell, 1975; Atlas 1981; Leahy and Colwell 1990; Venkateswaran *et al*., 1991; 1993c, 1993d). Most of these studies concern the characterization of microorganisms growing on saturates, while bacteria that grow on the recalcitrant components of crude oil such as heavy molecular saturates, aromatics, resins and asphalteness have been neglected. It is possible to various microorganism that degrade different components of crude oil, but the combination of these strains did not provide efficient decomposition of crude oil and removed only 40% of saturates and 20% of aromatics present in crude oil. However the sequential enrichment technique allowed us to isolate an improved ability to degrade crude oil (Venkateswaran and Harayama 1995).

Seven different types of bacteria were obtained from oil contaminated soil in Bangkok area. The enrichment culture was inoculated in mineral salt medium with 0.5% Tapis crude oil as sole carbon source. Four types of crude oil degraders *Acinetobacter Sp., Pseudomonas Sp.,* MU01, MUO2 and MU03 could degrade crude oil well. The percentage of total hydrocarbon (THC) degradation was highest ranges from 80.76% to 62.22% (Pokethitiyook *et al.,* 2003).

In the present research work an eight bacterial culture were subjected for crude oil degradation. The reduced substrate concentration (Crude oil % of degradation) and biomass concentration (CFU/ml) were measured for six weeks. The

Table 4 and Figure 4.1 to 4.8 represent the growth of individual's bacteria on crude oil and percentage of degradation. All the bacteria isolates showed the ability to utilize crude oil however the efficiency of utilization varied among strains with *pseudomonas Sp.,(*A) *pseudomonas Sp., (*B) being the best of (81.7% and 80.6% respectively removed in six weeks) with the growth rate ranges from $4.0x10^6$ for *pseudomonas Sp.,(*A) & $3.6x10^6$ to $2.2x10^6$ for *pseudomonas Sp.,(*B) given in Table 4.5, Figure 4.1 and 4.2 respectively. *Bacillus Sp.,* was able to degrade 78% of crude oil with growth rate 4.6×10^6 to 3.0×10^6 given in the Table 4. And Figure 4.3.Whereas *Micrococcus Sp.,* could degrade about 72% with the growth rate of $2.9x10^5$ to $1.7x10^5$ given the Table 4. and figure 4.4. In the case of C*orynebacterium Sp.,* it was able to degrade 68% with the growth rate of $2.8x10^6$ to $1.7x10^6$ given in the Table 4 and Figure 4.5. *Alcaligenes Sp.,* was able to degrade about 58% with the growth rate of $1.9x10^4$ to $1.0x10^4$ give in the Table 4 and Figure 4.6.

The result on the assessment for the degradation capacity of *Flavobacterium Sp.,* and *Aeromonas Sp.,* was less when compared to rest of the bacteria.

A small degree of growth and degradation was observed in cases of strains *Flavobacterium Sp.,* and *Aeromonas Sp.,* crude oil was utilized to the extent of 44% for *Flavobacterium Sp.,*with growth rate of $1.8x10^4$ to$1.0x10^4$and 39% for *Aeromonas Sp.,* with growth rate of $1.3x10^4$ to $0.7x10^4$ given in Table 4 and Figure 4.7 and 4.8 respectively. This result suggested that *Pseudomonas Sp., (A) & Pseudomonas Sp.,(B*) was the most efficient among the isolates.

Survival of bacteria for crude oil after their inoculation is a key deciding factor in the rate of biodegradation of hydrocarbons either in soil or in liquid phase (Ramos *et al*., 1991). Since all the bacteria in the present study were isolated from a petroleum contaminated soil sample, the survived and adapted the oil contaminated liquid environment very easily reported by authors (Rahman *et al*., 2002).This was the evident from the significant increase in the bacterial population, after inoculation. However out of 8 species of bacteria higher growth rate of *pseudomonas* strains and *Bacillus Sp.,* might be related to higher breakdown and, utilization of petroleum hydrocarbon in crude oil.

TPH biodegradation potential of *Pseudomonas* strains, *Bacillus Sp., Micrococcus Sp.,* and *Corynebacterium Sp.,* were for higher than the other strains like *Flavobacterium Sp., Aeromonas Sp.*

Crude oil degradation using consortium of bacterium

The components of the culture attacks different components of the crude oil at different ratio and the mixed culture of a whole degrade the oil by strong synergistic effect. The degradative capacity of any microbial consortium is not

necessarily the result of merely adding together of the capabilities of the individual strains forming the association. Many research groups observed consortial biodegradation. Komukai- Nakamura and Co-workers (1996) reported the sequential degradation of Arabian light crude oil by two different genera. *Acinetobacter Sp.,* T_4 biodegraded alkanes and other hydrocarbons producing the accumulation of metabolites. Following that *Pseudomonas putida* PB4 began to grow on the metabolites and finally degraded aromatic compounds in the crude oil. The advantages of employing mixed cultures as opposed to pure cultures in bioremediation have also been widely demonstrated. It could be attributed to the effects of synergistic interactions among members of the association. The mechanism in which petroleum degraders benefit from synergistic interactions may be complex. It is possible that one species preceding it. It is also possible that second species are able to do only partially (Alexander,1999). In this present research work, also based on their capabilities to grown on crude oil and individual hydrocarbons as their sole carbon source, six bacterial isolates were used in the construction of the bacterial mixtures or consortia namely (a) *Pseudomonas Sp.,*(A), (b) *Pseudomonas Sp.,*(B), (c) *Bacillus Sp.,* (d) *corynebacterium Sp.,* (e) Micrococcus *Sp.,* and (f) *Alcaligenes Sp.,*

The biodegradation of crude oil was studied using this consortium. The consortia grown well in the medium with crude oil, the growth rate ranges from 3.9 X 10^7 to 2.6 X 10^7 CFU/ml and degraded the crude oil upto 89.6% in six weeks (Tables 4; Figure 3A, B). The highest growth rate of the bacterial consortium was obtained at the end of the second week, with 3.9X 10^7 for which the degradation was about 45.2%.The highest percentage of crude oil degradation (89.6), obtained by using bacterial consortium. This may be due to that, the mixed bacterial population attacks different components of the crude oil by a strong synergistic effect and as a result the effective biodegradation was noticed.

Table :1 Soil Sample Collected on November 1 – 2012/Feb15 2013/ July 1-2013.

S.no	Sample collection Site ONGC Drilling site	Sampling Location	TVC CFU/ml of Enrichment culture collected on Nov.1-2012	TVC CFU/ml of Enrichment culture Collected on Feb.15 - 2013	TVC CFU/ml of Enrichment culture Collected on July 1 - 2013
1.	ONGC Drilling site	HC_1	1.9×10^5	2.2×10^5	1.7×10^5
2.	ONGC Drilling site	HC_2	1.8×10^5	2.3×10^5	1.5×10^5
3.	ONGC Drilling site	HC_3	1.4×10^5	2.0×10^5	1.5×10^5

4.	ONGC Drilling site	HC_4	1.5×10^5	2.1×10^5	1.4×10^5

Table 2 Identification of bacterial isolates obtained from enrichment cultures.

S. No.	Bacterial strain number	Gram's reaction and morphology	Motility	Spore	Growth on Maconkey	Indole	Methyl red	Voges proskauer	Cirtate	Catalase	Oxidase	Nitrate reduction	Glycerol	Starch hydrolysis	Casein hydrolysis	ONPG	Urease	Gelatinase	Fluorescent pigment	H2S	DNAase
1.	Strain 1	- Rods	+	-	+	-	+	-	+	+	+	-	+	-	-	-	-	-	-	-	-
2.	Strain 2	- Rods	+	-	+	-	+	-	+	+	+	-	+	-	+	-	-	+	+	-	-
3.	Strain 3	+ Rods	-	+	-	-	-	+	+	+	-	-	-	+	+	+	+	-	-	-	-
4.	Strain 4	+ Cocci	-	-	-	-	+	-	+	+	+	+	+	-	-	-	+	-	-	-	-
5.	Strain 5	- Rods	-	-	+	+	-	+	-	+	+	-	+	+	+	-	+	+	-	-	+
6.	Strain 6	- Rods	+	-	+	-	+	-	+	+	+	-	-	-	-	-	-	-	-	-	-
7.	Strain 7	- Rods	+	-	+	+	+	-	+	+	+	+	+	+	+	+	-	+	-	-	+
8.	Strain 8	+ Rods	-	-	-	NA	NA	NA	NA	+	NA	+	NA	NA	NA	NA	NA	NA	NA	NA	NA

(Bergey's Manuel of Determinative Bacteriology) NA = Not Applicable

Table :3 Screening for crude oil degradation by bacteria on agar plate and degradation of PAH by bacterial isolates.

Organism	Zone of clearance in mm	Naphthalene	Anthrancene	Phenathrene
Pseudomonas sp.(A)	25	+++	+++	+++
Pseudomonas sp.(B)	22	+++	+++	+++

Bacillus sp.	16	++	++	++
Corneybacterium sp.	20	++	++	++
Flavobacterium sp.	09	-	-	-
Alcaligenes sp.	12	-	-	-
Micrococcus sp.	15	-	-	+
Aeromonas sp.	06	-	-	-

(+++) Good growth; (++) Medium growth; (+) Slow growth

Table: 4 Bacterial counts and degradation percentage of crude oil in broth.

***Pseudomonas* sp. (A)**	**No. of weeks**	I	II	III	IV	V	VI
	% of hydrocarbon degradation by solvent extraction with hexane & DCM (1:1)	23.5 ± 1.0	40.5 ± 1.6	52.3 ± 1.5	69.0 ± 2.1	74.8 ± 2.0	81.7 ± 1.7
	TPH degrading bacteria (cfu /ml)	3.6 X 10^6	4.0 X 10^6	3.6 X 10^6	3.7 X 10^6	3.4 X 10^6	2.8 X 10^6
***Pseudomonas* sp. (B)**	**% of hydrocarbon degradation by solvent extraction with hexane & DCM (1:1)**	22.3 ± 0.8	42.5 ± 1.3	55.0 ± 2.2	65.8 ± 1.6	73.0 ± 1.1	80.6 ± 2.3
	TPH degrading bacteria (cfu /ml)	3.0 X 10^6	3.6 X 10^6	3.4 X 10^6	3.2 X 10^6	2.6 X 10^6	2.2 X 10^6
***Bacillus* sp.**	**% of hydrocarbon degradation by solvent extraction with hexane & DCM (1:1)**	17.2 ± 1.2	35.9 ± 1.0	50.4 ± 2.1	61.7 ± 1.4	70.0 ± 3.1	78.0 ± 2.4
	TPH degrading bacteria (cfu /ml)	4.0 X 10^6	4.6 X 10^6	4.3 X 10^6	3.9 X 10^6	3.4 X 10^6	3.0 X 10^6
***Micrococcus* sp.**	**% of hydrocarbon degradation by solvent extraction with hexane & DCM (1:1)**	15.8 ± 0.5	31.7 ± 1.1	42.4 ± 1.6	50.9 ± 1.2	61.2 ± 2.0	72.0 ± 1.8

	TPH degrading bacteria (cfu /ml)	2.2 X 10^5	2.9 X 10^5	2.6 X 10^5	2.4 X 10^5	2.1 X 10^5	1.7 X 10^5
***Corneybacterium* Sp.**	**% of hydrocarbon degradation by solvent extraction with hexane & DCM (1:1)**	12.4 ± 0.6	29.8 ± 1.0	36.2 ± 0.8	43.4 ± 1.6	57.4 ± 1.2	68.0 ± 1.7
	TPH degrading bacteria (cfu /ml)	2.0 X 10^6	2.8 X 10^6	2.6 X 10^6	2.3 X 10^6	1.9 X 10^6	1.7 X 10^6
Alcaligenes sp.	**% of hydrocarbon degradation by solvent extraction with hexane & DCM (1:1)**	11.6 ± 0.9	22.4 ± 1.2	30.1 ± 0.5	39.1 ± 0.7	47.9 ± 1.9	58.0 ± 1.6
	TPH degrading bacteria (cfu /ml)	1.4 X 10^4	1.9 X 10^4	1.6 X 10^4	1.5 X 10^4	1.3 X 10^4	1.0 X 10^4
***Flavobacterium* sp. (B)**	**% of hydrocarbon degradation by solvent extraction with hexane & DCM (1:1)**	7.0 ± 0.6	16.2 ± 0.93	25.4 ± 1.0	32.2 ± 1.3	42.0 ± 1.1	44.0 ± 1.3
	TPH degrading bacteria (cfu /ml)	1.0 X 10^4	1.8 X 10^4	1.4 X 10^4	1.3 X 10^4	1.0 X 10^4	0.9 X 10^4
***Aeromonas* sp.**	**% of hydrocarbon degradation by solvent extraction with hexane & DCM (1:1)**	6.2 ± 0.91	14.1 ± 0.56	20.4 ± 1.0	26.5 ± 1.2	31.2 ± 1.6	39.0 ± 1.7
	TPH degrading bacteria (cfu /ml)	1.0 X 10^4	1.3 X 10^4	1.1 X 10^4	0.9 X 10^4	0.7 X 10^4	0.5 X 10^4
***Consortium* Sp.**	**% of hydrocarbon degradation by solvent extraction with hexane & DCM (1:1)**	28.4 ± 1.3	45.2 ± 1.9	58.2 ± 1.2	71.3 ± 1.7	82.4 ± 1.2	89.6 ± 2.1
	TPH degrading bacteria (cfu /ml)	3.0 X 10^7	3.9 X 10^7	3.7 X 10^7	3.4 X 10^7	3.0 X 10^7	2.6 X 10^7

Figure:1 Sample collection sites.

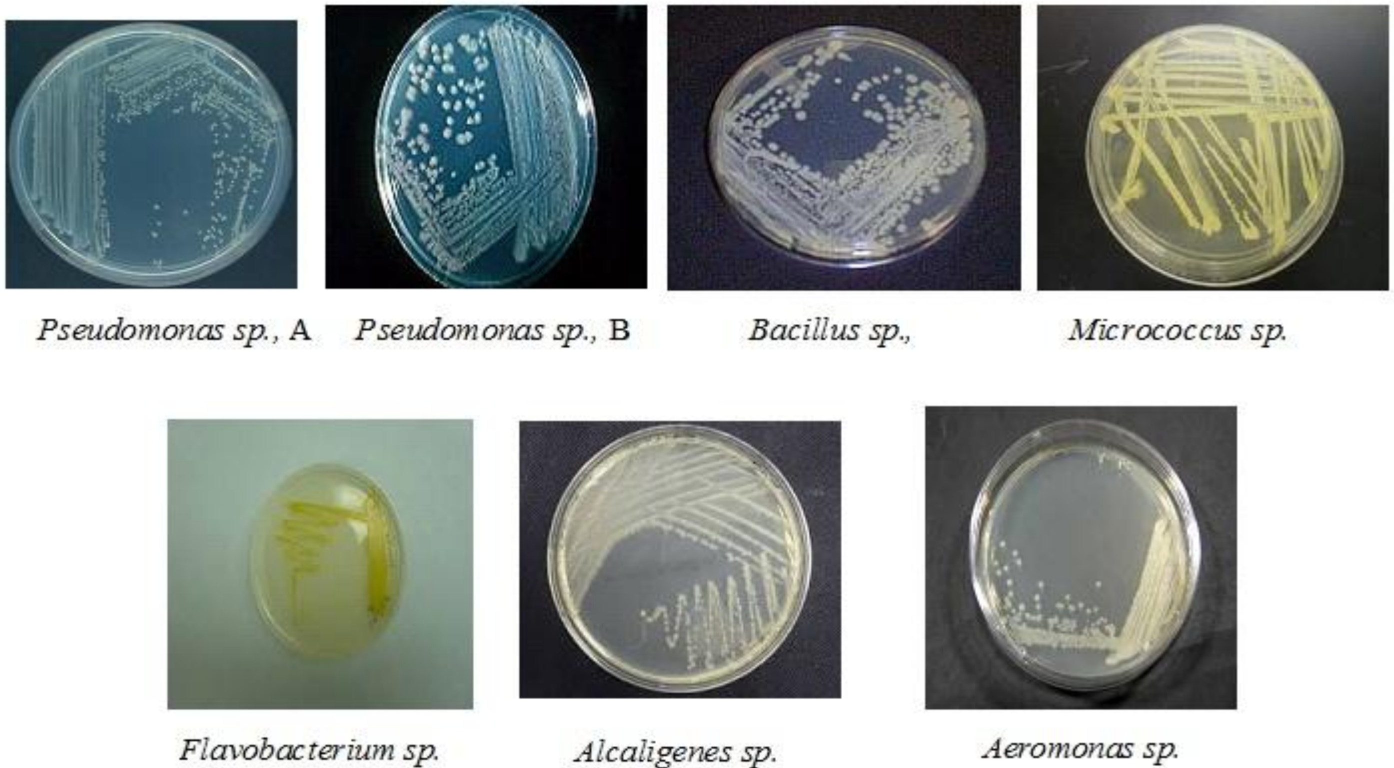


Figure 2: Isolation of cultures from enrichment cultures.

Figure: 3 –A:Screening for crude oil degradation by bacteria on agar plate

B: Crude oil degradation using consortium of bacterium

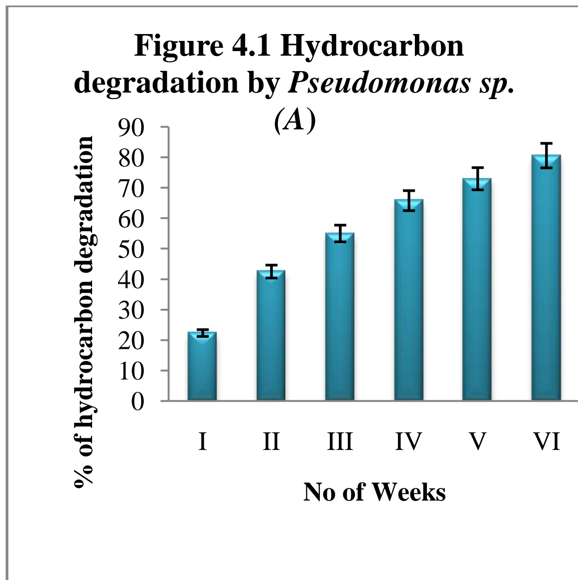


Figure 4.1 Hydrocarbon degradation by *Pseudomonas sp. (A)*

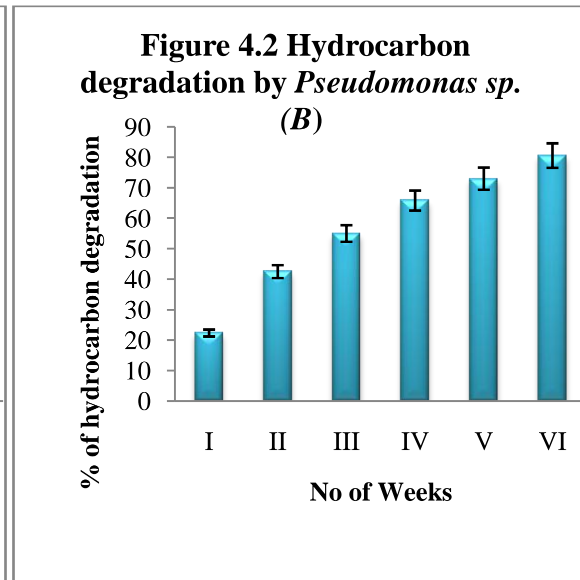


Figure 4.2 Hydrocarbon degradation by *Pseudomonas sp. (B)*

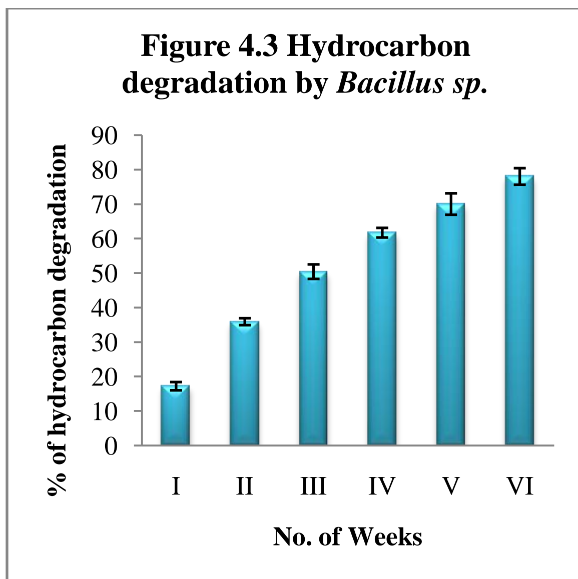


Figure 4.3 Hydrocarbon degradation by *Bacillus sp.*

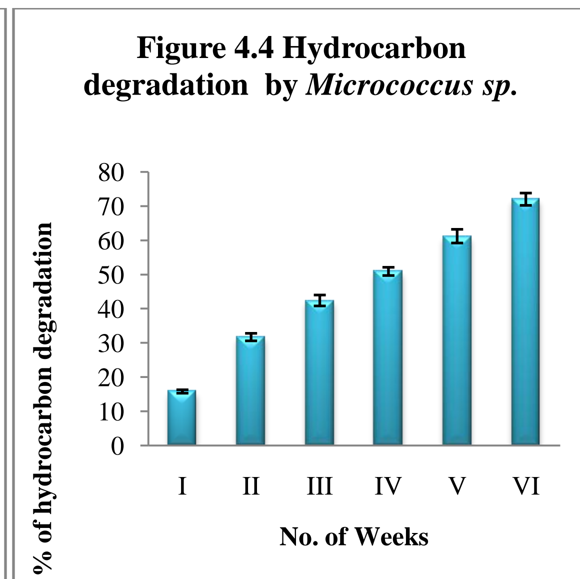


Figure 4.4 Hydrocarbon degradation by *Micrococcus sp.*

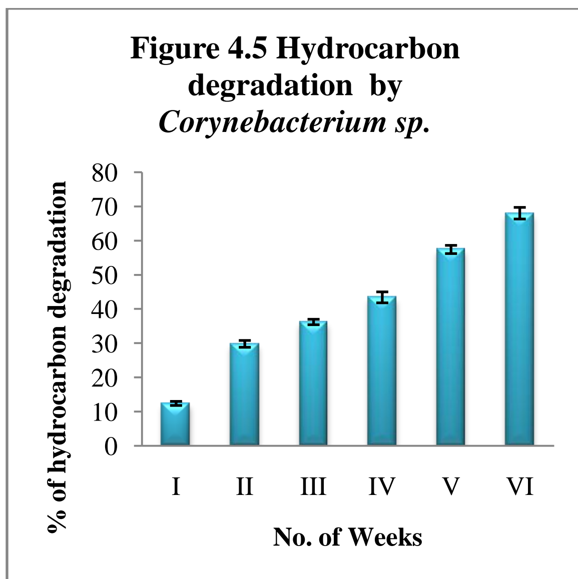


Figure 4.5 Hydrocarbon degradation by *Corynebacterium sp.*

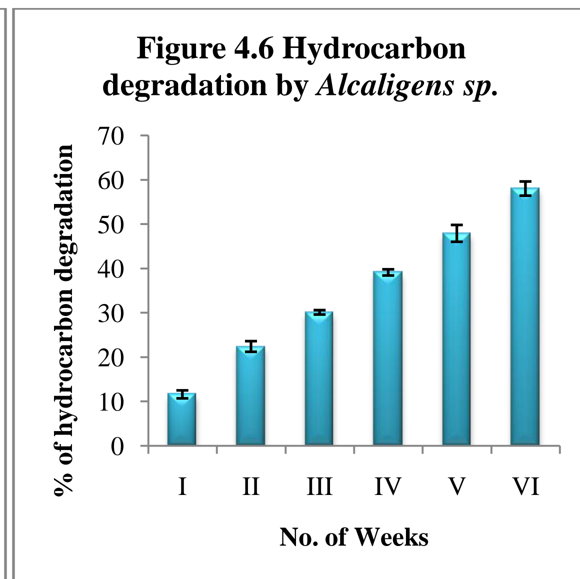


Figure 4.6 Hydrocarbon degradation by *Alcaligens sp.*

Figure 4.7 Hydrocarbon degradation by *Flavobacterium sp.* (B)

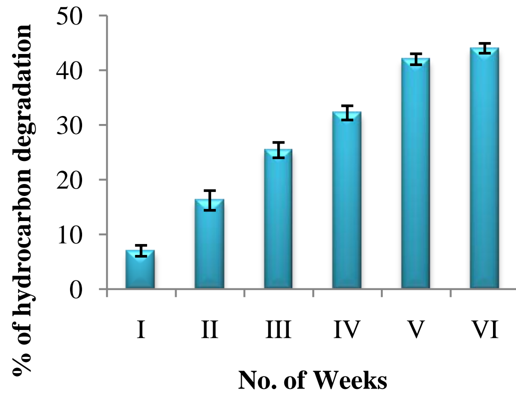


Figure 4. 8 Hydrocarbon degradation by *Aeromonas sp*

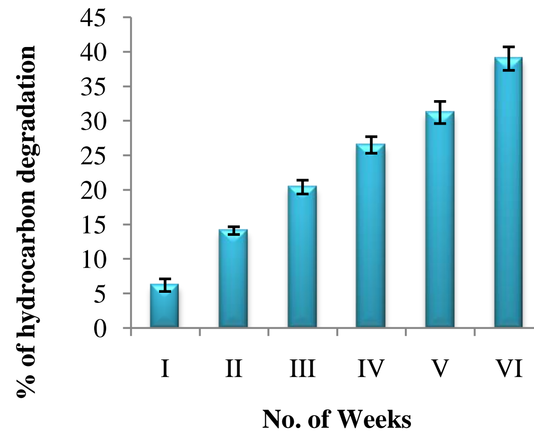
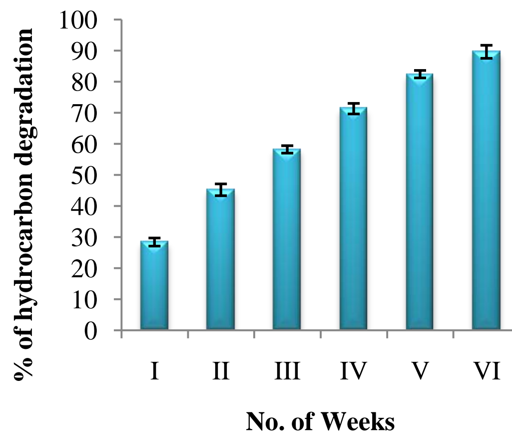


Figure 4.9 Consortium Sp hydrocarbon degradation



6. Conclusion

Rahman *et al.,* 2002 isolated 130 oil degrading bacterial cultures isolated from oil contaminated soil samples, *Micrococcus* Sp.,GS2-22 *Corynebacterium*Sp.,GS5-66, *Flavobacterium* Sp., DS5-73, Bacillus sp. Ds6-86 and *Pseudomonas* sp.Ds10-129 were selected for the study based on the efficiency of crude oil utilization. At 1% crude oil concentration, the mixed bacterial consortium degraded a maximum of 78% of BH crude oil. This was followed by 66% by *Pseudomonas Sp.,*DS10-129, 59% by Bacillus sp.DS6-86, 49% by *Micrococcus* sp.GS2-22, 43% by *CorynebacteriumSp.,*GS5-66 and 41% by *FlavobacteriumSp.,*DS5-73. The percentage of degradation by the mixed bacterial consortium decreased from 78% to 52% as the concentration of crude oil was increased from 1% to 10%. Temperature of 30°C and pH 7.5 were found to be optima for maximum biodegradation.

Based on the evident that not even one species of microorganism is capable of degrading all the components of crude oil. The metabolic pathway and gene regulation is not been common to all the strains. For e.g.*Pseudomonas Sp.*

The discovery of petroleum brought a lot of relief to the word's energy requirement because of sourcing and conversion. The ease of production, refining and distribution has also brought with it an ever increasing problem of environmental pollution. One of the ways through which petroleum pollutants can be removed is bioremediation which was practically experienced during the period of this research work. In the present research work, the bacterium for effective bioremediation of hydrocarbons was isolated from crude oil contaminated soil sample by selective enrichment. Eight different bacteria were isolated, identified and screened for hydrocarbon degradation. *Pseudomonas* strains showed the highest percentage of degradation of crude oil and growth rate. *Pseudomonas* strains degraded the crude oil upto 81% and 80% apart from *Pseudomonas Sp.,Bacillus Sp.* ,showed effective degradation upto 78%. The *Corynebacterium Sp.,* and *Micrococcus Sp.,* also degraded the crude oil to some extent.

Based on the ability to degrade the crude oil and individual hydrocarbons bacterial consortium was formed. The consortium consists of *PseudomonasSp.,*(A), *PseudomonasSp.,*(B),*Bacillus Sp.,Corynebacterium Sp.,Micrococcus Sp.,*and *Alcaligenes Sp.,*This consortium degraded the crude oil upto 89% in 6 weeks (in lab) and the oily sludge upto 86% in 120 days in field. The result and discussion presented above are expected to provide a meaningful insight into the biodegradability of the hydrocarbons in crude oil and oil sludge. The experimental results of this study can be utilized to assess the role of varies factors which controls the success of bioremediation. These factors include the availability of bacteria that can metabolize the contaminant and utilizing it as a sole carbon source under favorable conditions.

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