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Extraction and Characterization of Oil Degrading Bacteria

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Abstract: The present study was conducted to determine the isolation, identification and characterization of efficient oil degrading bacterial strains and to study the effect of different concentration of crude oil on the growth of bacterial strain. Two samples soil and water were collected from the crude oil contaminated areas for the isolation and characterization of efficient crude oil degrading strains. Sub culturing technique was employed to isolate 43 numerically dominant bacteria that had the ability to grow on 1.0% crude oil on nutrient agar plates. The isolates were then subjected to different concentrations of crude oil on nutrient agar, mineral salt agar media containing Phosphorous, Nitrogen and trace elements with glucose (PNTG) and without glucose (PER). These isolates showed rich growth on nutrient agar media along with crude oil. Out of 43 isolates 7 were able to grow up to 2.0% crude oil and were named as AA-1 to AA-7. These strains were also able to grow on mineral salt agar media with and without glucose but with different susceptibility to different concentrations of crude oil. Finally 3 prospective strains AA-1, AA-2 and AA-3 were selected for further studies. These strains exhibited good growth in PNTG containing 1.0% crude oil as evident by increase in Optical Density (OD) after every 24 h for five days. These isolated strains were identified by morphological and biochemical tests and were found to belong to genus *Bacillus*. These strains were subjected to shake flask transformation of crude oil in mineral salt media (PNTG) with glucose for 15 days. Marked change in crude oil colour was observed for these isolates, indicating their biodegradative ability. These isolated strains were able to use crude oil as the sole source of carbon and energy even under stressed environmental conditions. Thus these strains have bright potential for biodegradation of crude oil resulting in clean up of oil spills.

Key words: Biodegradation, optical density, morphological, *Bacillus*, nutrient agar

INTRODUCTION

Modern society continues to rely on the use of petroleum hydrocarbons for its energy needs. Despite recent technological advances, accidental spills of crude oil and its refined products occur on frequent basis during routine operations of extraction, transportation, storage, refining and distribution. It is estimated that between 1.7 to 8.8 million metric tons of oil is released into the world's water every year (NAS, 1985) of which more than 90% is directly related to human activities including deliberate waste disposal. Contrary to popular perception, only one eighth of the oil released into the aquatic environment is from tanker accidents. It is also estimated that about 30% of the spilled oil enters fresh water systems (Cooney, 1984). The data from last 20 years include oil spills of over 7 tons from tankers, combined carriers and barges. Although the data suggests a reduction in oil spills, the trend may only represent a temporarily downward fluctuation that is part of erratic cycling over the long term (Etkin and Welch, 1997). In the chemical and petroleum industries, considerable volumes of oily sludge are unavoidably generated. These inevitably result in the contamination of soil and ground water. Petroleum products contain many hazardous organic chemicals such as benzene, toluene, naphthalene and benzopyrene, some of which are recognized carcinogens Soriano *et al*., (1998).

Many microbial strains each capable of degrading a specific compound are available commercially for bioremediation (NCCLS, 1997). However oily sludge is a complex mixture of alkenes, aromatic, NSO (nitrogen, Sulphur, Oxygen) containing compounds and asphaltene fractions and a single bacterial species has only limited capacity to degrade all the fractions of hydrocarbon present (Loser *et al*., 1988).

Bioremediation is the act of adding materials to contaminated environments to cause an acceleration

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of the natural biodegradation processes (OTA, 1991). It has emerged as one of the most promising secondary treatment options for oil removal since its successful applications after the 1989 Exxon Valdez (Soriano *et al*., 1998; Prince *et al*., 1994). This technology is based on the premise that a large percentage of oil components are readily biodegradable in nature (Schülin *et al*., 1997; Atlas, 1981; Edine *et al*., 1997).

Petroleum contamination is dangerous and can play havoc with environment. In Pakistan Greek Ship MV Tasman spilled thousand of barrels of oil in the sea and jeopardize our sea environment, threaten marine life and posed serious health related problems. To cope up with such environmental and ecological issues only biotechnology would solve and minimize our environmental problem in effective and economical way (Bonnefoy *et al*., 1997).

The present study comprising of isolation, identification and characterization of efficient oil degrading bacterial strains and to study effect of different concentrations of crude oil on the growth of bacterial strains was therefore, undertaken to optimize their growth under different concentrations of crude oil.

MATERIALS AND METHODS

This study was conducted from June to November 2005.

Growth and Maintenance of bacterial cultures: Nutrient broth and mineral salt media were used for growth and characterization of the bacterial strains. Crude oil was added after sterilization through the Millipore 0.2 filters directly in the liquid broth medium. For identification, selective media and different biochemical reagents were used. Stock bacterial cultures maintained by periodic sub-culturing on the nutrient agar plates were stored at 4°C. The composition of the nutrients used was as follows:

Peptone	5 g L^{-1}
Yeast Extract	3 g L^{-1}
Dist. Water	1000 mL
Agar	1.5% was used as solidifying agent.

The composition of mineral salt media (PNR) per litre of the distilled water is as under:

Composition of the mineral salt medium

PN (20×50 mL) used/per L		R salt used as 7 mL/per L	
KH_2PO_4	13.6% (W/V)	$MgSO_4$	8% (W/V)
$(NH_2)_2SO_4$	2.6% (W/V)	$FeSO_4.7H_2O$	0.2% (W/V)
NaOH	2.5% (W/V)	HCl (Conc.)	0.4% (V/V)

Bacterial growth was measured by taking absorbance at 600 nm. All spectrophotometer measurements were recorded on a Shimadzu UV 240 Spectrophotometer.

Collection of samples: Two oily sludge samples were collected, one from crude oil waste pit Tando Allah Yar (TAY) district Hyderabad i.e. TAY Well No. 1 and 2. Two water samples were also collected from same area. one from TAY oil well No. 2 and other from waste pit of the same area. These samples were tested for the presence of micro-organisms having the ability to grow and degrade crude oil.

Isolation of bacterial strains: Nutrient agar plates were prepared containing 0.5% crude oil. One gram of soil sample was then dissolved in 100 mL sterilized saline distilled water. The flask were then kept in shaker for 30 min at 30°C. Serial dilution of the two sludges was made separately. Six test tubes all containing 9 mL distilled water was sterilized. Ten fold serial dilution was made by mixing 1 g of soil sample in the first test tube and then transferring 1 mL of the dilution to the second one and so on. A sterile micropipette tip was used to dispense 0.1 mL from each dilution onto duplicate nutrient agar plates. Glass spreader, dipped in alcohol, flamed and cooled was used for spreading on the plates. The plates were incubated at 28°C temperatures for the growth of bacteria for 24 h and then the colonies were counted. The CFU (colony forming unit) was calculated as under

$$\text{CFU per mL} = \frac{\text{No. of colonies} \times \text{dilution factor}}{\text{Size of inoculum}}$$

For the isolation from the water samples directly 0.1 mL was spread on nutrient agar plates with crude oil.

Screening of crude oil utilizing micro-organisms: From the bacterial colonies, which appeared on the nutrient agar plates containing oil, 43 bacterial isolates were picked randomly, purified and streaked on nutrient agar plates containing 0.5% crude oil as sole source of carbon and energy for screening. The plates were incubated at 28°C for 24 h. Three types of media were used for this purpose:

- Nutrient agar + crude oil
- PNRG + crude oil
- PNR + crude oil

Those strains that showed best growth on these media at higher concentrations of crude oil were identified and used for further studies.

Growth behavior of selected strains in PNRG crude oil: After selection and adaptation on the crude oil in different media, growth of these strains were observed in PNRG medium for 5 days at 37°C and 100 rpm in shaking incubators. Identification and characterization of selected bacterial isolates was performed by morphology and culture characterization using colony morphology, Gram's staining and spore formation test. For biochemical characterization of the isolates, following tests were performed, Nitrate Reduction Test, Catalased Production Test, Hydrogen Sulphide Production Test, Tripple Sugar Iron Agar Test, Citrate Utilization Test, Oxidase Test, Gelatin Liquification Test, Starch Hydrolysis Test and Lactose Fermentation Test according to the Bergys Manual of Determination Bacteriology (9th Edition).

After selecting and growth in PNRG crude oil media, shake flask transformation was carried out at 37°C on 100 rpm for 15 days to check degradation of crude oil in PNRG media. Results were obtained on the basis of visual change in crude oil colour, produced by bacterial strains. One hundred milliliter of PNRG media was autoclaved and after sterilization selected strains were inoculated in 250 mL flask with 1% crude oil at 100 rpm and 37°C for 15 days. After 15 days change in colour in the crude oil for each strain was observed.

RESULTS

Isolation and selection of crude oil degrading bacteria: Two soil samples and two water samples were taken from crude oil contaminated sites and were subjected to viable cell count for soil and water samples (Table 1 and 2). There was different number of bacterial colonies for soil and water samples. Pure colonies were isolated by replica plating on the basis of their morphology. Initially 43 isolates were taken and checked for their growth on crude oil (Table 3 and 4).

Table 1: Viable cell count on nutrient agar with 0.5% crude oil from soil

Sample No.	Dilution factor	No. of colonies	Viable cell counts/g of soil
1	10^{-3}	76	76×10^{4}
2	10^{-3}	83	83×10^{4}

Table 2: Viable Cell Count on Nutrient Agar with 0.5% Crude oil from water

Sample No.	No. of Colonies	Viable Cell Counts/mL of water
1	88	9 cells mL
2	92	9 cells mL

Table 3: Colony morphology of bacterial isolates.

Strain	Size	Colour	Form	Margin	Elevation
AA-1	Large	White	Circular	Wavy	Convex
AA-2	Medium	Pure White	Circular	Entire	Convex
AA-3	Large	Opaque (Shiny)	Circular	Entire	Flat

Nutrient agar and PNRG agar media were used. Isolates showed different level of growth with different concentration of crude oil. Majority showed rich growth on nutrient agar from 0.5 to 1% crude oil. Some showed growth on PNRG and very little showed growth on PNR media. Seven best strain were selected and named AA-1, AA-2, AA-3, AA-4, AA-5, AA-6 and AA-7 for adaptation on the higher concentration of crude oil with and with out glucose. Crude oil when supplied as sole source of carbon and energy, growth was observed by all strains but with different abilities on higher concentrations. The growth in case of AA-1, AA-2, AA-3, AA-4, AA-5, AA-6 and AA-7 on nutrient agar was up to 2% crude oil while on PNRG media growth of all strains was up to 0.5% crude oil except AA-4, AA-5, AA-6 and AA-7 did not show rich growth. In case of PNR agar only AA-1, AA-2 and AA-3 showed growth on 0.5% crude oil and showed that these three isolates were able to utilize crude oil as sole source of carbon and energy (Table 5-7). The comparative growth of these isolates in PNRG containing 1% crude oil was also checked. The results showed gradual increase in optical densities after every 24 h for 5 days. *Bacillus* strain AA3 showed higher level of growth as compared to *Bacillus* strain AA-2 and AA-3. The strains AA-1, AA-2 and AA-3 showed better results for the adaptation on crude oil.

Table 4: Biochemical Characterization of Bacterial Isolates

Strain No	Gram'sStaining	Spore Form	TSI	Cit	OX
AA-1	Rods +	SF	+	+	-
AA-2	Rods +	SF	+	-	-
AA-3	Rods +	SF	+	-	-

Cat	H_2S	Nit	Gel	St.Hydro.	Lac.Ferm.	Species
+	-	-	-	+	-	*Bacillus* Sp.
+	-	-	-	+	-	*Bacillus* Sp.
+	-	-	+	+	-	*Bacillus* Sp.

Rods+Gram Positive Rods, H_2S Hydrogen Sulfide, SF = Spore Formation, Nit = Nitrate Reduction, TSI = Tripple Iron Sugar, Gel = Gelatin Liquefaction, Cit = Citrate Utilization, St. = Hydro Starch Hydrolysis, Ox = Oxidase, Lac. = Ferm Lactose Fermentation

Table 5: Growth of different bacterial strains on nutrient agar and crude oil

Strain	0.5%	1.0%	1.5%	2.0%
AA-1	+++	+++	+++	+++
AA-2	+++	+++	+++	+++
AA-3	+++	+++	+++	+++
AA-4	+++	+++	++	++
AA-5	+++	+++	+++	++
AA-6	+++	+++	+++	+++
AA-7	+++	+++	+++	+++

+++ Rich Growth, ++ Good Growth, + Growth, - No Growth

Table 6: Growth of different bacterial strains on PNRG and crude oil

Strain	0.5%	1%	1.5%
AA-1	+++	++	-
AA-2	+++	++	+
AA-3	+++	++	+
AA-4	++	-	-
AA-5	++	+	-
AA-6	+	-	-
AA-7	+	-	-

+++ Rich Growth, ++ Good Growth, + Growth, - No Growth

Table 7: Growth of different bacterial strains on PNR and crude oil

Strain	0.5%	1%
AA-1	++	-
AA-2	++	-
AA-3	++	+
AA-4	-	-
AA-5	+	-
AA-6	-	-
AA-7	+	-

+++ Rich Growth, ++ Good Growth, + Growth, - No Growth

DISCUSSION

Microbial degradation process in nature results from the combined enzyme, cell and community based activity of microorganism whose regulation and kinetics are likely to be highly individualized and variable in space and time. A bacterial community in contaminated sites tends to dominate by the strains that can survive toxicity and are able to utilize the contaminant itself for growth. As a response to biodegradation treatment, these populations may begin to actively degrade the pollutants and detoxify the environment, allowing other starving populations to increase their numbers, leading to an increase of the bacterial community in the soil. Such a process can result in a quantitative increase in number of the cells and their activities, implying a succession of leading bacterial groups and change of the observable diversity. Samples of soil and water harbour oil degrading bacteria, as indicated by CFU values 76×10^4, 83×10^4 and 9 cells mL^{-1} in tested soil and water, respectively.

Adaptation of microbial communities to hydrocarbons i.e., increase in rates of transformation of hydrocarbons associated with oil contaminated environments has been reported in many studies (Boswell *et al*., 1998). Sediment microbial population within the Athabasca oil sands were more capable of oxidizing hydrocarbons than were population from controlled sites, based on respiration rates of radiolabelled hexadecane and naphthalene. Many microbes are endowed with metabolic properties enabling them to degrade these compounds. These include the presence of mono and *di oxygenase* enzyme for oxidation of organic compounds. Communities of microorganisms may have increased degradative potential as they embody the complementary metabolic capacity through rapid alteration and the exchange of genetic material. During bioremediation treatment, bacterial diversity change is accompanied in the increase in the quantity of gene involved in contamination degradation pathway.

There potential strains were selected due to their resistance on higher concentrations of the crude oil belonging to *Bacillus* species identified by biochemical tests. The screening results showed that *Bacillus* species were the predominated hydrocarbon degrader in the soil and water. This finding is in agreement with many reports (Jamjian *et al*., 1997), which indicate the wide spread existence of *Pseudomonas* and *Bacillus* species at the hydrocarbon contaminated areas. Among the catabolic versatility of these genera include tolerance to high concentrations of solvents and ability to metabolize complex mixture of crude oil and engine oil. Our isolates *Bacillus* strains AA-1, AA-2 and AA-3 showed significant growth on PNRG crude oil agar plates which can use the crude oil as sole source of carbon and energy. In PNRG crude oil gradual increase in growth was observed by increase in Optical Densities (OD) and turbidity during five days of incubation for these isolates. Shake flask transformation of crude oil in PNRG showed that *Bacillus* strain AA-3 produced better change in crude oil colour as compared to *Bacillus* strain AA-1 and AA-2.

CONCLUSIONS

It is inferred from this study that the three isolates were belonging to *Bacillus* group according to biochemical characterization and were gram-positive rods. *Bacillus* strain AA-3 was the most active strain and produced more effective visual colour change in crude oil than the other two strains and can be used for biodegradation of the oil spills clean up.

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