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Enrichment, isolation and Phylogenic identification of Polycyclic Aromatic hydrocarbon degrading rhizobacteria from grasses growing at hydrocarbon contaminated site in Cameroon and Phenanthrene Biodegradation potential

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ABSTRACT

A study was undertaken aimed at isolating indigenous rhizobacteria strains capable of utilizing petroleum hydrocarbons as energy source. Three bacteria strains capable of growing on a mixture of Phenanthrene, Napthalene and Anthren were isolated from vegetated aged oil contaminated rhizopheric soil by enrichment in liquid medium, screened and identified by 16S rDNA sequencing. The phenanthrene degradation potential of the three bacteria viz. *P. aeruginosa* strain N72, *P. aeruginosa* clone A11 and *Pantoea agglomerans* was evaluated in a specific medium in the presence and absence of glucose using GC-FID. On comparison it was found that, *P. aeruginosa* clone A11showed higher phenanthrene degradation (85 %) in the presence of glucose within 15 days, while *P. aeruginosa* strain N72 showed highest degradation (70 %), in the absence of glucose. Unlike the 1st day samples, FTIR-analysis of the samples collected on 15th day revealed absorption bands between 3500-3000cm^{-1}characteristic of alcohol and acidic groups produced during phenanthrene degradation. The isolates were assayed for biosurfactant production by oil displacement and emulsification activity using diesel oil in a liquid broth and confirmed by hallow formation on CTAB-methylene blue agar medium supplemented with phenanthrene. The phenanthrene degradation properties of the isolates based on these observations are discussed in this paper.

Key words: PAH; Ageing; Rhizosphere; Biosurfactant; Bioremediation; Phenanthrene.

INTRODUCTION

Petroleum pollution has been recognized as the most significant contamination problem on the continent because of their ubiquitous distribution, refractory nature, bioaccumulation potential, and its deleterious effects on health (Snape et al, 2001; Breedveld and Karlsen, 2000). Petroleum oil and petrochemicals include significant amount of polycyclic aromatic hydrocarbons (PAHs) (Wang, 2007). PAHs are the organic molecules basically known for their high persistence in the environment and affecting the ecosystems by bioaccumulation and biomagnification (Chang et al, 2000). Extensive use of fossil fuels, gas and coal tar manufacturing units, wood processing industries,

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oil spillage in water bodies and waste incineration industries have increased the concentration of PAHs in the environment. Phenanthrene has been identified as the major contaminant in the PAH polluted site in Cameroon (Philemon and Benoît, 2012). Various abiotic processes (such as ozonation, hydrogen peroxide oxidation, UV irradiation, chlorination, electrochemical oxidation and use of surfactants) and biotic process (bioremediation) have been developed for the degradation of PAHs (Henner et al, 1997). Bioremediation, the degradation of pollutant using microorganisms, is more advantageous over chemical approaches due to its hazardless, economically feasible and eco-friendly nature (Othman et al, 2011). Various species of bacteria viz. Pseudomonas putida, Comamonas testosteroni, Sphingomonas yanoikuyae, Mycobacterium sp, Escherichia coli, Alcaligenes sp. and Thiobacter subterraneus have been reported for their potential of PAHs degradation (Okere and Semple, 2012). Petroleum refinery soil and crude oil polluted soil are the natural habitat of these microorganisms as they adapt to this environment by various mechanisms which leads to developed resistance to PAH in bacteria (Simarro et al, 2013). The phytoremediation of PAHs is assisted by rhizoremediation which involves the breakdown of PAHs in soil by microbial activities in the rhizosphere (Gaskin et al, 2008). In turn, plant root exudates help to enhance rhizoremediation by stimulating the growth of microorganism in the rhizospheric soil, hence establishing a symbiotic relationship (Ukiwe et al, 2012). Hence, the microbial flora in the rhizosphere of such plants can be explored to understand phenanthrene biodegradation mechanism.

In the present investigation the bacteria isolated from the rhizosphere of the plants found in an oil contaminated soil in Cameroon were tested and compared for their potential for degradation of phenanthrene as well as emulsification activity through flask culture experiments. The effect of glucose on the phenanthrene degradation ability was also investigated. The results of these experiments are presented and discussed in this paper.

MATERIALS AND METHODS

Plant sampling and rhizospheric soil collection

Local grass species growing in petroleum waste contaminated soil at two industrial sites in Ngaoundere, Cameroon (Fig-1a-e) were chosen for screening of the rhizospheric bacteria. Rhizospheric soil samples of the selected plants were collected by carefully off-rooting the plants, loose soil was removed by gentle shaking off and stored at 4° C until future use.

Enrichment and isolation of PAH degrading bacteria

The Bushnell Haas minimal salt (B H) medium consisting of (per liter); 0.2g NH_4NO_3 ; 0.02g $CaCl_2.2H_2O$; 1g K_2HPO_4; 1 g KH_2PO_4; 1g $MgSO_4.7H_2O$; 0.05g $FeCl_2$ was used for enrichment and isolation of rhizospheric phenanthrene degrading bacteria (Atlas, 1994). The pH of media was adjusted to 7.2 using 0.1M NaOH and 0.1M HCl and sterilized by autoclaving (121ºC, 15 psi, 15 minutes) prior to the addition of phenanthrene. The media was supplemented with 0.2 g/L of phenanthrene as the sole carbon and energy source for growth bacteria. 0.5 g fine composite rhizospheric soil was added to the media and incubated at 37ºC in an orbital shaker at 200 rpm for 7 days. Following this period, an aliquot of 1 mL culture broth was transferred to fresh B H medium containing phenanthrene and re-incubated for further 7 days. After four successive enrichments the culture was serially diluted and spread on BH agar medium having a thin layer of hydrocarbon and incubated at 37º C for 72 hours. Individual bacterial colonies forming a clear zone around their colonies were picked and pure cultures were prepared by conventional streaking on BH agar media. All isolates were sub cultured after every 30 days and stored at 4 ºC to maintain the cultures.

Identification of microbial consortia

All isolates were first identified on the basis of their morphological and colony characteristics. Further identification was carried using Gram staining and antibiotic sensitivity test using Kirby Bauer disc diffusion method

(Ben Said et al, 2008). This was followed by the amplification of the 16S rDNA gene from genomic DNA by PCR using 16S gene universal primers: 8F:5'AGAGTTTGATCCTGGCTCAG-3', 1492R: 5'ACGGCTACCTTGTTACGACTT 3' as previously described by Janbandhu and Fulekar (2011). The amplicons were electrophoresed in 1% agarose gel and visualized under UV light. Concentration of the amplicon was checked in a Nanodrop ND 2000. The amplicons were then purified using Nucleospin purification column (Macherey-Nagel). Sequencing of amplicon with forward and reverse primers was carried out in ABI 3730xl cycle sequencer. Forward and reverse sequences were assembled and contig was generated after trimming the low quality bases. The sequence analysis was carried out using bioinformatics tool BLAST of NCBI. Based on maximum identity score first few sequences were selected and aligned using multiple sequence alignment software MultAlin and the phylogenetic tree were constructed.

Biodegradation experiments

Biodegradation of Phenanthrene by isolated rhizo-bacteria in Liquid culture

Biodegradation experiments of phenanthrene was carried out in 100 mL autoclaved B H medium inoculated with 1 mL pure culture of bacteria (105 CFU/mL) in 250 ml Erlenmeyer flasks containing phenanthrene (0.2g/L) and incubated at 120 rpm and 37ºC. Culture broth without bacterial inoculums served as negative control while culture broth having 1 g/L D-glucose served as positive control. All experiments were carried out in duplicates using each of the isolated pure cultures individually. Incubation was carried out for a period of 15 days, while sampling was done for analysis of residual PAHs on day 3, 6, 9 and 15.

Extraction of phenanthrene from culture medium

Residual phenanthrene was extracted from the culture broth using Dichloromethane (DCM) (3:1) at different time intervals. The sample was mixed using a vortex shaker for 5 min. and refrigerated at 4ºC to separate organic phase and aqueous phase. The aqueous phase was discarded and the organic phase with residual phenanthrene was recovered in acid washed 5 ml tubes. The organic solvent was allowed to completely evaporate, leaving residual crystalline phenanthrene in the tubes. The crystalline phenanthrene layer was dissolved in 5 mL of methanol and filtered using a 0.22 µm syringe filter before analysis by GC-FID (Boonchan et al, 1998).

Analytical method

GC-FID analysis of residual phenanthrene

The residual PAH dissolved in methanol obtained from each experiment was analyzed by Gas Chromatography (GC; Claurus 500) with a Flame Ionization Detector (FID). A DB-5 column (Agilent technologies; 30meters length, 0.250mm internal diameter, film 0.25µm, and temperature limit from 60ºC to 325ºC) was used for the analysis. The GC-FID program used was; initial temperature 250ºC, then ramped to 300ºC at 7ºC /min and held for 2 min. The temperature of injector and detector was 300°C. Nitrogen was used as carrier gas at a flow rate of 0.8 mL/min while oxygen at 3 bar and hydrogen were used as the combustion gas. The total run time was set to 10 min. Residual concentration of phenanthrene was calculated in sample collected at different time intervals by height and area of phenanthrene peak. The percentage degradation (%D) of the hydrocarbon was then obtained using the following formulae

$$\%D = C0 - Ct / C0 \times 100 \quad \ldots\ldots (1)$$

where C0 is the initial concentration of the hydrocarbon, Ct is the concentration of the hydrocarbon at the given time interval (t) in days (3, 6, 9 and 15).

FTIR analysis

The products of biodegradation were analyzed by FTIR spectroscopy as it differentiates the compounds on the basis of absorption spectra of specific functional groups of compound present in sample. Sample was prepared by drying the extract of day 15 sample at 60ºC in oven. Dried extract was mixed with potassium bromide. Mixture was analyzed by using Bruker Vertex 70 FTIR spectrometer.

Biosurfactant production and extraction

To extract bisurfactnt, 1mL culture (105 CFU/mL) of each isolate was inoculated in 100 mL BH medium having 0.2g/L of phenanthrene and incubated at 37 °C at 150 rpm for 7 days The bacterial cells grown were removed by centrifugation at 9000 rpm at 4°C for 15 minutes and the supernatant was used to extract biosurfactant (Samadi et al, 2007). To precipitate biosurfactant Supernatant was acidified to pH 2 using 1M sulphuric acid.The precipitated biosurfactant mixed with a solution containing equal volume of chloroform-methanol (2:1) mixture. The organic phase containing biosurfactant was separated in a separating funnel and evaporated to concentrate the biosurfactant. The activity of the concentrated crude bio-surfactant was tested by oil displacement test, emulsification activity index and CTAB-methylene blue agar plate test.

Oil displacement test and emulsification activity measurement

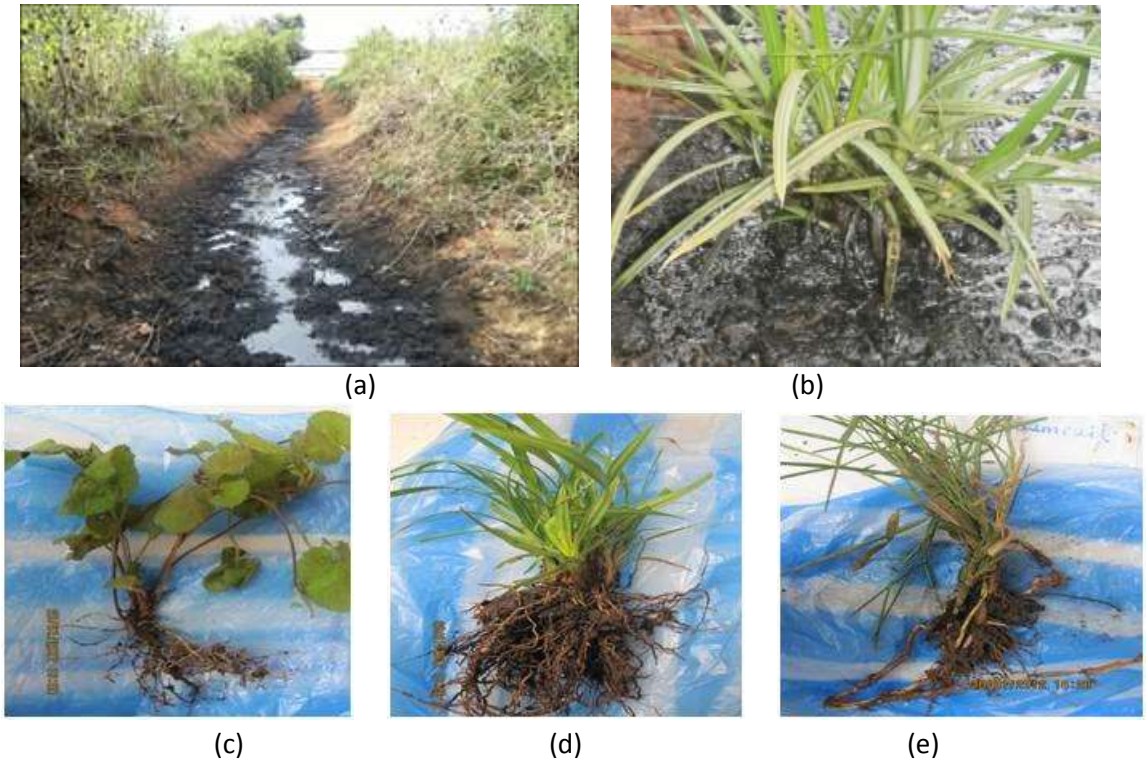
Biosurfactant activity was monitored by the oil displacement test described by Rodrigues et al (2007). Distilled water was taken large petri dishes (15cm diameter) and 20 µL of diesel oil mixed with Sudan red dye was added to the surface of water. Then 10µLof supernatant from the culture broths was added to the surface. The surface was observed for oil displacement made visible by the dye. The emulsification index (E24) was measured using a method described by Satpute et al (2008) to check the activity of the crude biosurfactant. 1 mL of diesel oil as added to 6 ml of the extract and vortexed at high speed for 5 minutes to promote hydrocarbon–water emulsification at the top of test tube. Tube left for 24 hours intact and height of the emulsified layer was measured. 1% (w/v) solution of the synthetic surfactant, Sodium Dodecyl Sulphate (SDS) in deionized water was used as a positive control. The emulsification index (E24 index) was calculated using the formulae shown below (Sarubbo et al, 2006):

Emulsification Index (E24) = Height of emulsion layer/height of liquid coloum× 100…….. (2)

CTAB-methylene blue agar plate test

To identify the nature of biosurfactant CTAB-methylene blue agar plate test was done. Pure bacterial culture was streak on CTAB-methylene blue agar plates supplemented with phenanthrene and incubated to detect hallow around the bacterial growth.

Figure-1. Petroleum waste contaminated soil at Cameroon (a), plant growing in the soil (b), plant samples collected from the contaminated soil (c-e).



RESULTS AND DISCUSSION

Isolation and identification of potential phenanthrene degrading bacteria

Restoration of soil polluted by PAHs is carried out by the microorganisms found in the rhizosphere of the plants growing in the soil contaminated with crude oil. Therefore, exploring rhizosphere microflora is an important area of research for bioremediation of polluted soil. In this endeavor, three potential bacterial cultures designated as NAP3EBD, P2EBD and A1EBD were isolated from the rhizosphere of plants growing on soil contaminated with petroleum oil as evident from Fig-1(a-e). The biochemical and physiological tests revealed that all the isolates were Gram negative and mostly occurred as single rods or chains (Table 1). Researchers have reported that Gram negative bacteria are the dominant bacterial species in the rhizosphere of plants found in PAHs polluted sites (Banks et al, 2003; Seo et al, 2007). The antibacterial sensitivity test against various antibiotics showed all the isolates were resistant to tetracycline, ampicillin and penicillin while sensitive to ciprofloxacin. The isolates P2EBD and A1EBD were found sensitive to vancomycin, while only isolate NAP3EBD was found resistant to vancomycin as shown in Table 1.

The size of 16S rDNA amplicons amplified from the genome of all the isolates was 1.5 kb (Fig. 2). The phylogenetic analysis of 16S rDNA sequences and the sequences of other bacterial species previously stored in NCBI genbank database obtained by BLAST, identified the isolates NAP2EBD, P2EBD and A1EBD as Pseudomonas aeruginosa strain N72, Pseudomonas aeruginosa clone A11and Pantoea agglomerans respectively (Fig-3).

Figure-2. Gel electrophoresis of 16s rDNA amplicons of three bacteria isolated from the contaminated soil showing bands at1500bp

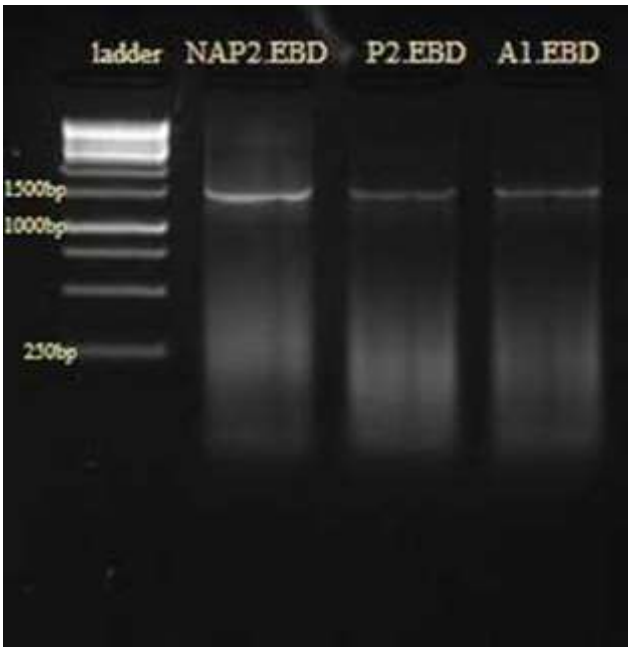


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Table-1. Characteristics of the microbial isolates

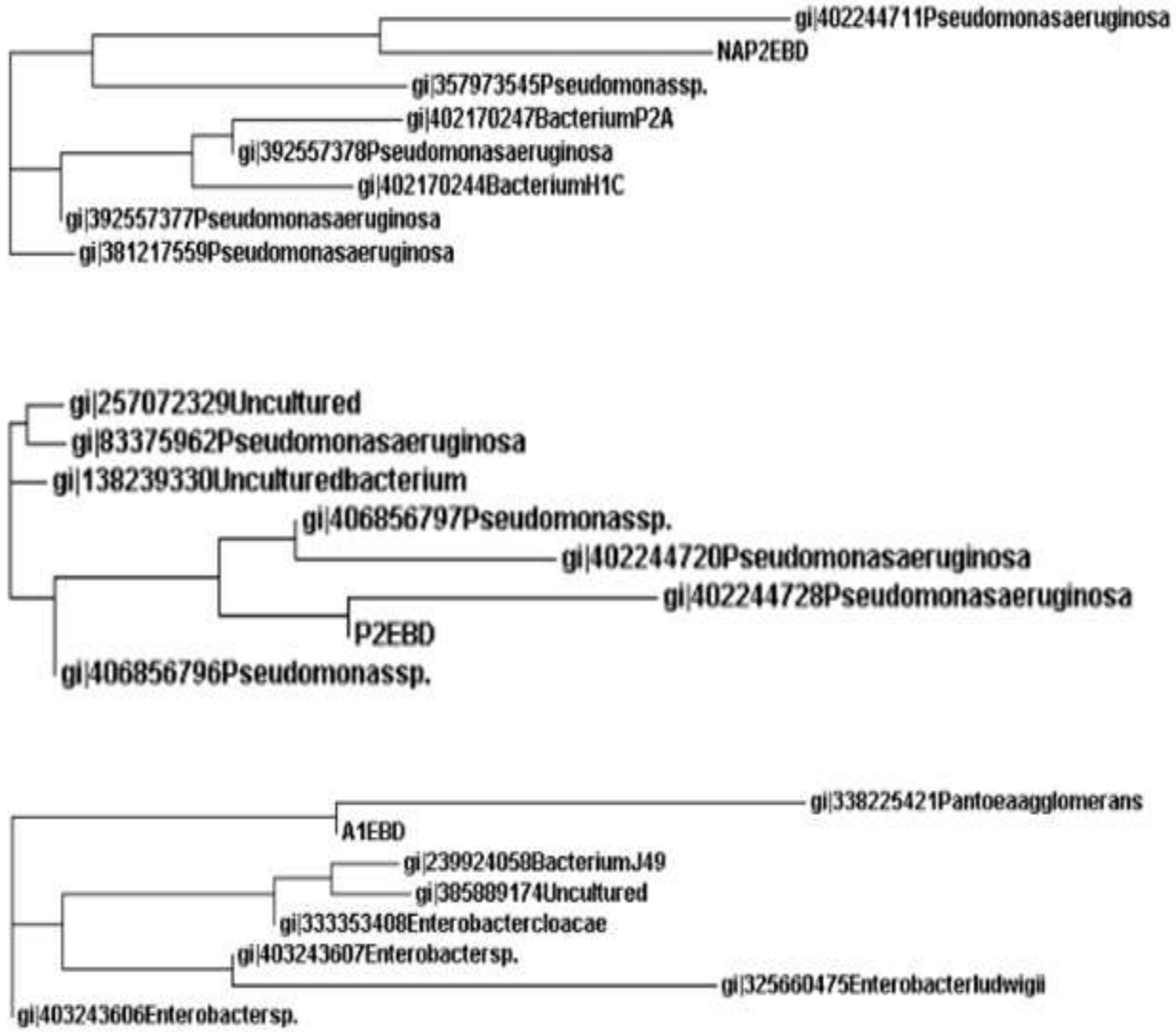
Isolate identity	Gram reaction	Shape	Antibiotics resistance				
			VA30[a]	P10[b]	CIP5[c]	T[d]	A[e]
NAP-3	Gram Negative	Rod shape	-	-	+++	-	-
P2	Gram Negative	Rod, occurring in chains	+	-	+++	+	-
A1	Gram Negative	Rods in chain	++	-	+++	-	-

[a]Vancomycine, [b]Penicillin, [c]Ciprofloxacine, [d]Tetracycline, [e]Ampicilline
- not resistant, + slightly resistant, ++ resistant, +++ very resistant

database obtained by BLAST, identified the isolates NAP2EBD, P2EBD and A1EBD as Pseudomonas aeruginosa strain N72, Pseudomonas aeruginosa clone A11and Pantoea agglomerans respectively (Fig-3). The incidence in PAHs and oil contaminated soil (López-Fuentes et al, 2012).

Comparative evaluation of biodegradation of phenanthrene using isolates

Figure-3. Phylograms of isolated bacteria A1EBD, NAP2EBD and P2EBD



Pseudomonas species is well known for using wide range of carbon sources as it has plasmids for degrading various poly aromatic hydrocarbons viz. phenanthrene, naphthalene, anthracene and other xenobiotic compounds (Sudhakar-Babu et al, 1996). The plasmid encodes the enzyme which oxidizes the aromatic hydrocarbon ring and make the PAH easily available for the rhizobacteria as a carbon source (Obayori and Salam, 2010). Pantoea agglomerance has also been reported for its

The isolates were evaluated for their individual potential for the degradation of phenanthrene through flask culture experiments in a specific medium with and without supplementing glucose as a growth factor. The isolates P. aeruginosa strain N72, P. aeruginosa clone A11 and Pantoea agglomerans showed degradation of phenanthrene to a magnitude of 70%, 61% and 50% respectively in the absence of glucose by the end of the experiment (Fig-4a). In the presence of D-glucose (0.1 % w/v)

Figure- 4a Phenanthrene degradation (%) by the three isolates in the absence of glucose

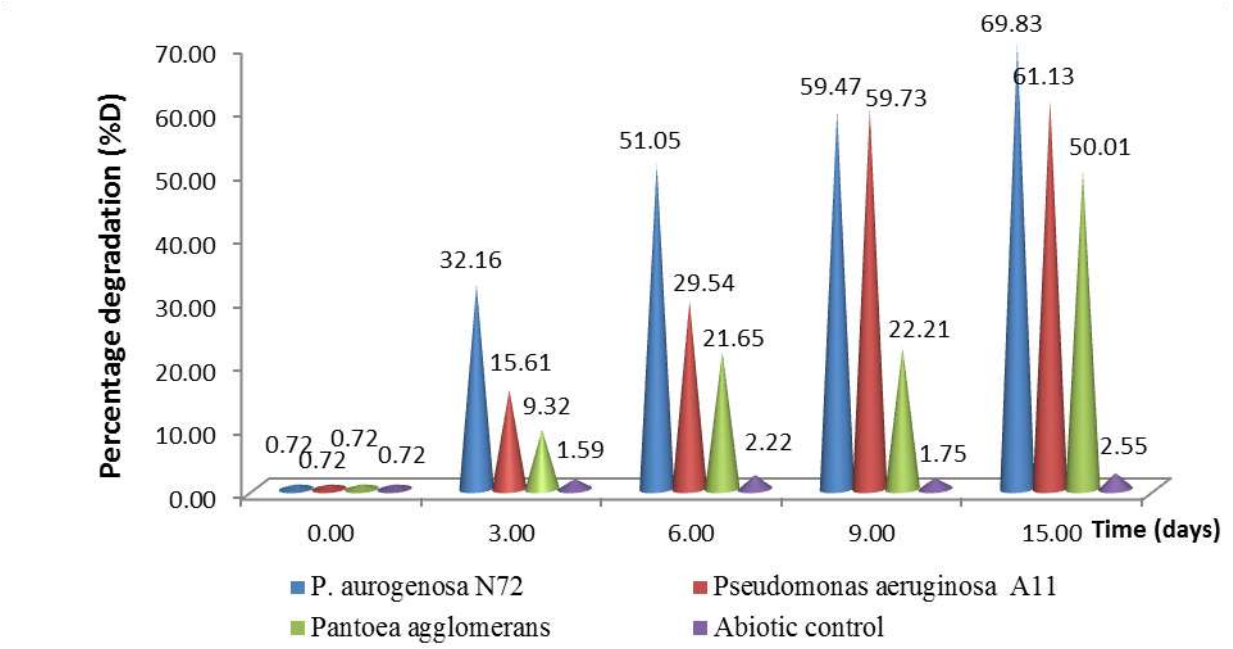
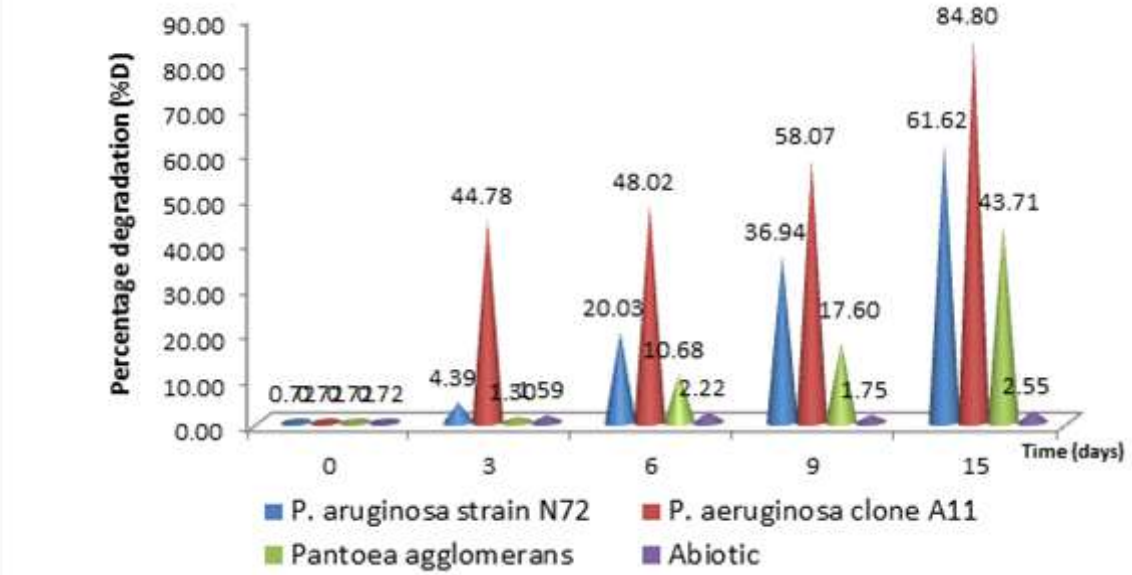


Figure-4b. Phenanthrene degradation (%) by the three isolates in the presence of glucose

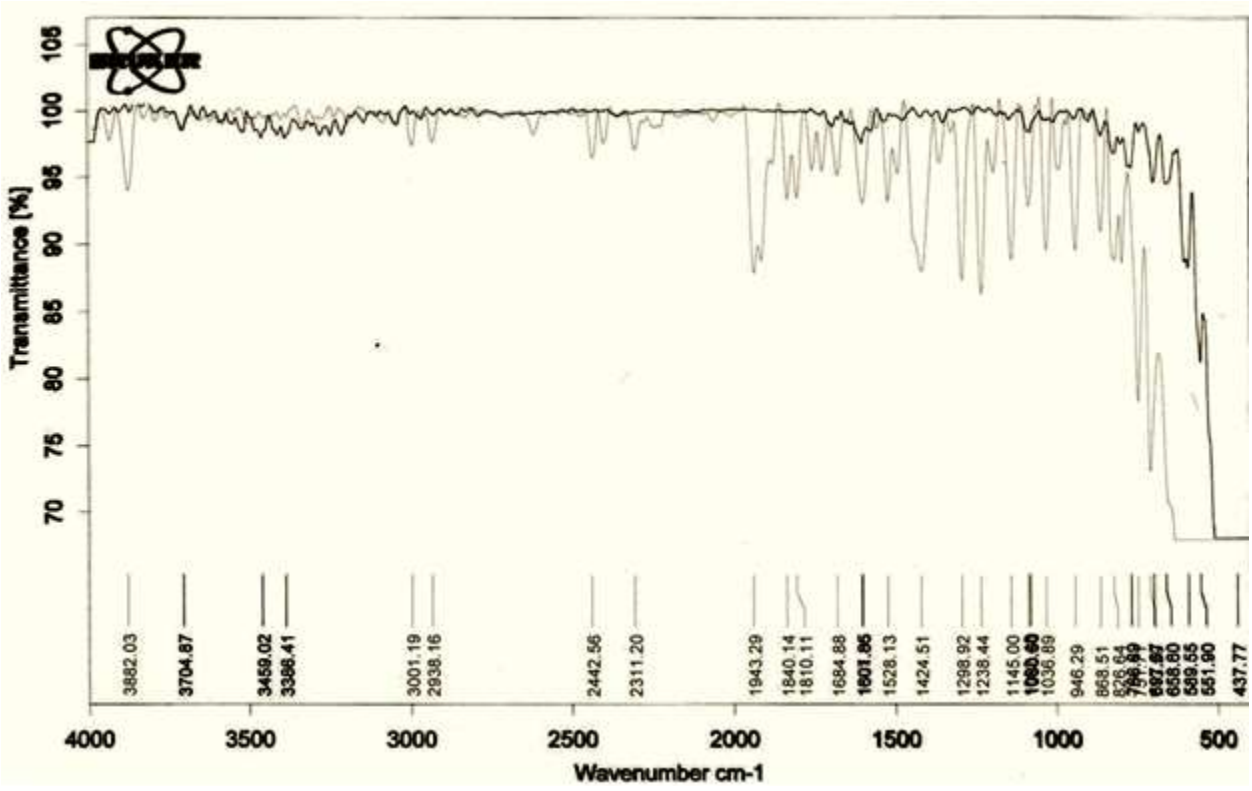


phenanthrene degradation increased to 85% by P. aeruginosa clone A11, while P. aeruginosa strain N72 and Pantoea agglomerans showed 61.62% and 43.71% phenanthrene degradation respectively (Fig-4b). The increased phenanthrene degradation by P. aeruginosa clone A11 in the presence of D-glucose may be attributed to increase in cell growth rate. Hence, P. aeruginosa clone A11 possessed phenanthrene degradation ability even in the presence of D-glucose, indicating that this bacterium was better acclimatized for phenanthrene degradation. On the other hand, P. aeruginosa strain N72 and Pantoea agglomerans showed lesser phenanthrene degradation in the presence of D-glucose during the first week of incubation as

Table 2 E_{24} using 0.2g/L mixture of Phenanthrene, Naphthalene and Anthracene with/without glucose

Isolate	Emulsification Index-E_{24} (%)	
	without Glucose	with Glucose
Pseudomonas aeruginosa strain N72	82.0	41.03
Pseudomonas aeruginosa clone A11	48.72	58.97
Pantoea agglomerans	33.33	30.77
Positive control (1% SDS)	69.23	69.23
Negative control (media broth)	09.38	09.35

Figure-5. FTIR spectra of the sample extracted from the media on Day 1 shown by light line. FTIR spectra of the sample extracted from the media on Day 15 shown by dark line.



shown in Fig. 4b. This could be because of higher glucose utilization rate of these bacteria as compared to phenanthrene as the primary carbon source. However, the phenanthrene degradation increased after that, indicating that glucose concentration in the media was depleted. These results showed that P. aeruginosa clone A11 can cometabolize glucose and phenanthrene, while P. aeruginosa strain N72 and Pantoea agglomerans prefer D-glucose over phenanthrene for their metabolic activity.

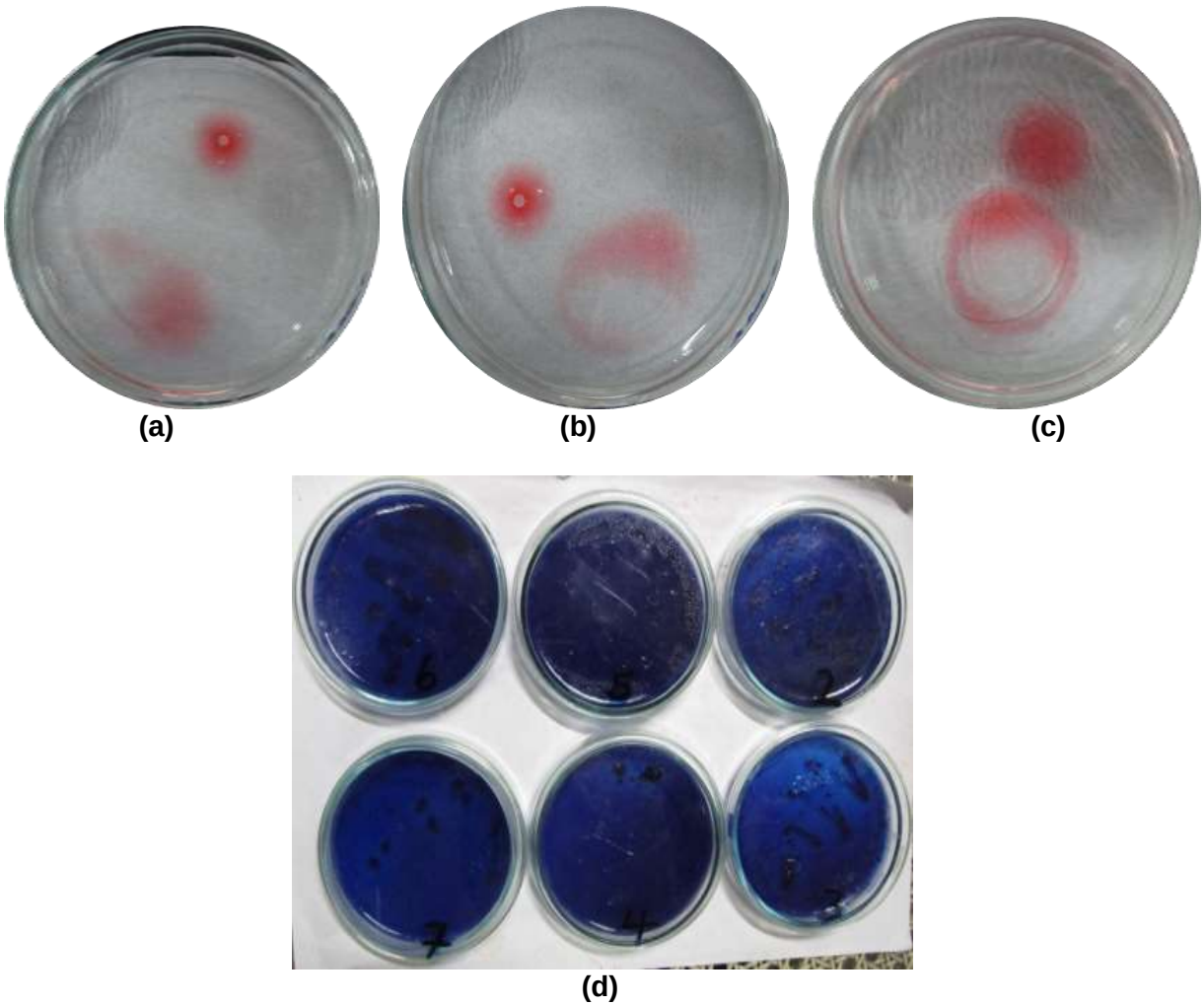
The samples extracted from the media before and after incubation for 15 days were analyzed by FTIR spectrometer and the result is shown in Fig. 5. As compared to the first day sample (shown by lighter line), the spectra of the 15th

day sample (shown by darker line) showed disappearance of the strong absorption bands between 2000 cm-1 – 1000 cm-1 corresponding to the C=C bands (Sindhu, 2006). This reveals the breaking of double bonds between two carbon moieties present in the aromatic ring. New peaks between 3,000cm-1- 2,500cm-1 corresponding to C-H stretch and between 3,500cm-1 – 3,000cm-1 corresponding to O-H and N-H stretches (Lin et al, 2007) were observed in the 15th day sample which was absent in the FTIR spectra of first day sample as shown in Fig-5. These peaks are the characteristic of alcohol and acid functional groups which indicated the presence of acidic and alcoholic degradation byproducts. All these results confirmed that phenanthrene was degraded by the bacteria during incubation, since the isolated microorganisms have had a long exposure of crude oil in the rhizosphere, therefore have acquired potential for degradation of phenanthrene.

Comparative evaluation of isolates for emulsification activity

Hydrophobic nature of phenanthrene makes its high persistence in the environment and also causes non bioavailability for its biotransformation. Hydrocarbon degrading bacteria have a significant ability to emulsify hydrocarbons by producing extracellular surface-active agents called biosurfactants, which reduce the surface tension between hydrocarbons and water thereby making the hydrocarbons

Figure-6. Oil displacement shown by the culture broths of P2EBD, NAP2EBD and A1EBD respectively (a-c) Hallow formation around dark blue colonies on CTAB-methylene blue agar media



(a) (b) (c)

(d)

available for degradation to the bacteria (Desai and Banat, 1997; Salihu et al 2009). Many rhizobacterial species have been reported for the production of biosurfactant (Sachdev and Cameotra, 2013). In this study, microbial isolates were screened positive for tensio-active and emulsifying activities as observed by the oil spread technique, emulsification activity (E24) and the production of hallow on CTAB-methylene blue agar media (Pinzon and Ju, 2009). The oil displacement test revealed the presence of biosurfactant in the culture broth which decrease surface tension of oil. This resulted in spreading of oil drop over water surface as shown in Fig. 6 (a-c); this observation could not be made with oil drop containing dye alone. Further emulsification activity of biosurfactant produced by all the isolates with different magnitude was observed by determining the E24 value. The highest E24 of 82.05% was observed with P. aeruginosa strain N72 in the absence of D-glucose than in its presence (41.03%) (Table-2). This is further supported by the literature report as mention by Sudhaker et al (1996), while P. aeruginosa clone A11 gave a higher E24 of 58.97% in the presence of D-glucose than in its absence (48.72%). There was no significant difference in the case of Pantoea agglomerans which had an E24 of 33.33% and 30.77% in the absence and presence of D-glucose respectively. All these isolates also produced dark blue colonies on CTAB-methylene blue agar media which indicated that the biosurfactant produced by the bacteria have glycolipid content which have the tendency to precipitate methylene blue molecules and this resulted in the formation of dark blue colonies surrounded by a hallow (Fig. 6d). Similar observation have also been reported by Burd and Ward (1996), who reported that Pseudomonas marginalis, growing on PAHs produced an extracellular surface-active factor of high molecular weight, composed of protein and lipopolysaccharide.

CONCLUSION

- Potential phenanthrene degrading rhizobacteria P. aeruginosa strain N72, P. aeruginosa clone A11 and Pantoea agglomerans were isolated from crude oil polluted soil
- P. aeruginosa strain N72, P. aeruginosa clone A11 and Pantoea agglomerans degraded phenanthrene to the tune of 70%, 61% and 50% respectively in the absence of glucose. Supplementing glucose in the medium increased the degradation capability of P. aeruginosa clone A11, while it was suppressed in case of other two isolates.
- Emulsification activity was found to be highest for P. aeruginosa strain N72 in the absence of glucose and for P. aeruginosa clone A11 in the presence of D-glucose.

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CONFLICT OF INTERESTS

The authors declare that there is no conflict of interests regarding the publication of this paper.

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