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Biodegradation of pyrene and phenanthrene by bacterial consortium and evaluation of role of surfactant

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Abstract

High molecular weight poly aromatic hydrocarbons (HMW PAHs) are well known for their hydrophobicity and they get strongly adsorbed onto the soil particles. Generally, surfactants facilitate the biodegradation of PAH by enhancing their solubility and desorption of hydrophobic compounds from soil particles. To investigate the role of synthetic surfactant in biodegradation of PAHs, two bacterial strains BP10 and P2 were incubated in soil spiked with pyrene and phenantherene (100 µg $g^{-1}$of soil each) in isolation and in combination with/without Tween 80. After 14 days of incubation, pyrene and phenantherene were degraded by a combination of BP10 and P2 to the extent of 98% and 99%, respectively. Addition of tween 80 reduced the degradation of pyrene and phenantherene by 35 and 10%, respectively. Biosurfactant produced by selected strains i.e. BP10 and P2 could enhance desorption of pyrene (100 µg $g^{-1}$of soil) by about 27% and 12%, respectively. However, desorption activity was relatively higher (32 and 29%, respectively) in case of phenanthrene (100 µg $g^{-1}$of soil) from the spiked soil. Present study showed that in spite of additional chemical surfactant, bioaugmentation of highly petroleum hydrocarbon degrading bacterial combination was very effective in boosting the bioremediation of PAHs- contaminated sites.

Key words: Biodegradation, Poly aromatic hydrocarbons, Bacterial consortium, Surfactant, Cell hydrophobicity.

Introduction

PAHs are ubiquitous contaminants present in the environment, mainly originating from anthropogenic activities like burning of fossil fuels, coal liquefaction and gasification process, oil seepage and accidental spillage of hydrocarbons and petroleum industries (1). Low Molecular weight PAHs are known to be highly toxic while HMW PAHs are genotoxic. Besides, many of the constituents of PAHs are not only carcinogenic and mutagenic, but also potent immunotoxicants (2). Pyrene and phenanthrene are two PAHs that are listed in 16 PAHs as priority pollutants by United State Environmental protection Agency (USEPA). They are highly recalcitrant under normal conditions because of their strong molecular bonds and hydrophobic nature.

In the past two decades, a number of bacteria have been identified as "PAH degraders". Among them, *Pseudomonas* sp. has been widely reported but least information is available on *Ochrobactrum* sp. Various authors supported the idea to use consortium than monoculture for achieving higher efficiency of biodegradation (3). The co-operation between the individual species in the consortium and complementary effects of microbes on each other may ultimately account for enhanced growth and survivability of consortium members (4) that directly or indirectly boost the rate of biodegradation.

The low water-solubility of petroleum hydrocarbon (PAH) increases sorption of compound to soil micelle and limits their availability to biodegrading microorganisms (5). Biodegradation of PAH is reportedly enhanced if the bioavailability to organisms could be increased (6). Surfactants are chemical agents that facilitate the mass-transfer of hydrophobic pollutants from a solid or non-aqueous liquid phase into the aqueous phase by decreasing the interfacial tension. Application of non-ionic surfactants (NISs) is usually preferred to boost the PAH biodegradation as they are less toxic to bacteria than the anionic and cationic surfactants (7).

In this study, degradation of two PAHs i.e. pyrene and phenanthrene was observed in the presence of two PAHs degrading bacterial strains i.e. *Pseudomonas stutzeri* BP10 and *Ochrobactrum intermedium* P2 in isolation and combination with /without surfactant to determine the role of consortium and surfactant on the degradation process. Simultaneously, bacterial biomass, enzyme induction and desorption of pyrene by bacterial biosurfactants were also observed.

Materials and methods

Bacterial Strains

Two bacterial strains *Pseudomonas stutzeri* BP10 and *Ochrobactrum intermedium* P2 selected for this study were capable of degrading high molecular weight PAH such as pyrene (8). These strains were isolated from the crude oil contaminated soil collected form Barauni Oil Refinery, Barauni, Bihar (India). These strains are registered in NCBI (National Center for Biotechnology Information) database having accession no. KC493413 and KC493414, respectively.

Tagging of Bacterial Strains against Antibiotics

As soil autoclaving decreases the soil nutrition va-

lue and cannot be applicable in field, bacterial strains were tagged with antibiotics to know the survivability of inoculated bacterial strains in the soil (unsterilized) during experiment. These strains were tagged with vancomycin at a concentration of 250µg ml^{-1}whichdid not permit the growth of native bacteria in soil. Tagging of bacteria was performed by growing them separately on NA (Nutrient Agar) plate supplemented with different antibiotics with increasing concentration of vancomycin (up to 250µg ml^{-1}).

Screening of Non-ionic Surfactants

To select a surfactant for microcosmic study, four non-ionic surfactants i.e. Tergitol 7, Triton X-100, Tween 80 and Brij 35 were screened based on the emulsification index and bacterial sensitivity to these detergents before using them in microcosmic study. For this purpose, 5 ml of benzene or hexadecane was added to 5 ml of aqueous phase containing 500 mg l^{-1} of selected non-ionic surfactant and agitated vigorously for 5 min on a vortex. After 24 h, total height of both organic and aqueous phase (HT) and height of emulsified layer (HE) were recorded. Emulsification index was calculated as following:

$$E24\ (\%) = (HE/\ HT) \times 100 \qquad (1)$$

To check the toxic effect of selected surfactants, BP10 was inoculated in MSM (Minimal Salt Media) with/without these surfactants and incubated at 35°C for 6 days. Growth of bacterial strain was observed based on the absorbance of media at 600 nm at the interval of 24 hr.

Reduction of surface tension by different concentration of selected surfactant was determined to decide its concentration for microcosmic study. Surface tension of distilled water with or without surfactant was determined through drop method by using stalagmometer and r.d. bottle using relative density of distilled water as standard at room temperature 25°C. Surface tension was calculated as given below.

$$\gamma m = (nw \times dm \times \gamma w)/(nm \times dw) \qquad (2)$$

where γm and γw are surface tension of media and distilled water, dm and dw are weight of 25 ml of water with surfactant and distilled water and nw and nm are the no. of drops of water with surfactant and distilled water for a fixed volume through stalagmometer.

Experimental Setup

Garden soil, collected from CSIR-National Botanical Research Institute, Lucknow, U.P. (India) was air dried, sieved through 2 mm sieve. Soil was spiked with pyrene and phenantherene (each of 100 µg g^{-1} of soil) by adding pyrene and phenanthrene with acetone. The mixture was vigorously stirred and left in dark place for removal of acetone for 24 h. Hundred gram of spiked garden soil was uniformly spread over each petri dish (n=15). Two selected bacterial strains, BP10 and P2, were enriched in 250 ml nutrient broth enriched with 100 mg l^{-1} pyrene and phenanthrene in 500 ml Erlenmeyer flask in an orbital shaker for 24 h at 35°C and 150 rpm. Cells were harvested by centrifugation at 5000 x g for 10 min to get pellets of intact bacterial cells. Subsequently, cell pellets were washed with sterilized double distilled water (DDW) to remove nutrients and PAHs sticking to the cell surface. Cell pellets were resuspended in sterilized DDW and vertexed properly before they were inoculated in spiked soil in petri dishes. One set of three petri dishes was inoculated with P2 and the other set by BP10. The three petri dishes out of six petri dishes inoculated with P2+BP10 contained tween 80 (200 µg g^{-1} of soil) The bacterial inocula were maintained in the range of 6-7 x 10^5 CFU (Colony Forming Unit) g^{-1} of soil. Three petri plates without any bacterial inoculums were maintained to study natural degradation of pyrene in soil which served as control. All the petri plates were kept in culture room at 37°C for 28 days. 5 g soil samples were taken out from each set at zero day, 7th day and 14th day. Simultaneously, growth of bacteria in terms of CFU, protein content of soil and specific activity of dioxygenase enzymes (catechol 1,2dioxygenase and catechol 2, 3 dioxygenase) were also monitored.

Extraction and Analysis of Pyrene and Phenantherene

Remaining amount of pyrene and phenantherene was extracted by benzene through solid: liquid (1: 10 w/v) extraction. Benzene extracted sample was collected after centrifugation at 10,000 x g for 10 min. Extracts were evaporated under a gentle nitrogen hood and dissolved in 2 ml acetone (2 ml). Samples were analyzed for pyrene by the gas chromatograph (Agilent GC model 7890A) with flame ionization detector using capillary BP-5 column (5% phenyl methyl polysiloxane column, 30 m x 0.32 mm x 0.25 µm). Both injection and detector temperatures were maintained at 280°C. The initial oven temperature was kept 80°C for 2 min and increased to 300°C with 15°C increase per min.

Bacterial Growth

Bacterial growth was assessed by CFU counting. CFU of BP10 and P2 per g of soil was determined on NA plates supplemented with vancomycin (250 µg m l^{-1}) after incubation for 48 h at 37°C in the culture cabinet.

Protein Extraction and Estimation

Soil protein was extracted in 1N NaOH solution by following the procedure of Rahman et al. (9). 1 g of soil was suspended in 10 ml of distilled water and mixed vigorously on vertex. After settling the soil particles, 1 ml of supernatant was centrifuged at 13000 rpm for 10 min. Thereafter, supernatant was discarded and the pellet was redissolved in 1 ml of 1N NaOH. It was kept for 3 min in water bath at boiling temperature for cell lysis. 1 ml of 1M H_3PO_4 was added after samples were cooled to room temperature (25°C). Sample, thus prepared, was used for the protein estimation and enzyme assay. Protein was estimated by following the method of Lowry et al. (10).

Dioxygenase Activity

Catechol 1, 2 dioxygenase (C12O, EC 1.13.11.1) was assayed by the method of Hegeman (11) using the colorimetric procedure to detect the product *cis, cis*-muconate (pH = 7.0, λ = 260 nm; ε = 25600 $molcm^{-1}$). Reaction mixture contained 1µmol of EDTA, 0.1 µM of

catechol, 8.7 µM of sodium phosphate buffer (pH=7.0) and protein sample (0.02-0.06 mg of protein) in a final volume of 1 ml. The increase in OD_{260} was used as a measure for accumulation of *cis, cis*-muconic acid.

Catechol 2, 3 dioxygenase (C23O, EC 1.13.11.2) was determined with an increase in OD_{375} concomitant with the formation of 2-hydroxymuconic semialdehyde (pH = 7.5, λ = 375 nm; ε = 33400 $mol^{-1}cm^{-1}$) using UV-VIS Spectrophotometer. Reaction mixture contained 48 µM of sodium phosphate buffer (pH = 7.5), 0.1 µM catechol and protein extract (0.02-0.06 mg of protein) in a final volume of 1 ml. Protein samples was heated for 10 min at 60° C before the enzyme activity was assayed following the method of Klecka and Gibson (12).

Cell Hydrophobicity of Bacterial Strains and Desorption of PAHs by their Biosurfactants

Bacterial surface activity of selected strains for the aromatic compound (benzene) was determined by using modified method of Rosenberg (13). Bacterial strains were enriched in 20 ml MSM supplemented with pyrene and phenanthrene (100 µg g^{-1} of soil each) for 7 days in an orbital shaker at 35°C and 150 rpm. Cells were harvested by centrifugation and resuspended in sterile MSM. OD_{600} of cell suspension (1.5 ml) was adjusted around 0.5. Benzene (200 µl) was added to cell suspension and vortexed for 3 min. Change in OD_{600} was recorded for cell suspension after allowing the benzene and aqueous phase to separate. Cell hydrophobicity was expressed as cell adherence (%) to the crude oil which was calculated as given below.

$$\text{Bacterial adherence (\%)} = (1-(A_c/A_o)) \times 100 \qquad (3)$$

Where A_c and A_o are absorbance of cell suspension after and before treatment with benzene, respectively.

Biosurfactant of selected bacterial strains were isolated by the following the method of Yakimov et al. (14). 100 ppm of isolated biosurfactant was prepared in 20 mM phosphate buffer (pH 7.0). 5 ml of this solution was added in 1 g of soil spiked with pyrene and phenanthrene (each100 µg g^{-1} of soil). After 5 min of vertexing, aqueous sample was separated by centrifugation (10,000 rpm at 4°C). Amount of pyrene and phenanthrene present in aqueous phase was extracted in benzene and detected by GC as given above.

Results

Screening of non-ionic surfactant

As non-ionic surfactants are known as less toxic to microbes, four non ionic surfactants were screened on the basis of their emulsification capability with petroleum hydrocarbons. Benzene was taken as model compound for aromatic hydrocarbons. It was found that E_{24}% with benzene and hexadecane was highest (35.5% and 70.8%) in the presence of Tween 80.

When BP10 was grown in MSM with and without different non-ionic surfactants, it was observed that Tween 80 had minimum effect on the growth of bacteria and might be utilized by bacteria for its growth because the growth of BP10 in MSM supplemented with Tween 80 was comparatively higher than that in MSM alone (Fig.1). Therefore, this surfactant was selected for the microcosmic study to see the role of surfactant on PAHs degradation.

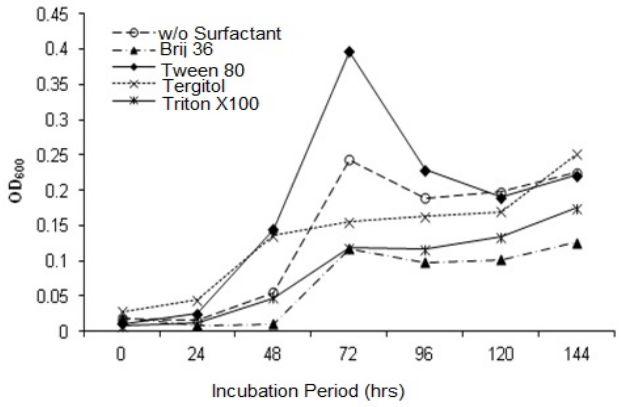

Figure 1. Growth of BP10 (OD_{600}) in MSM supplemented with / without non-ionic surfactants.

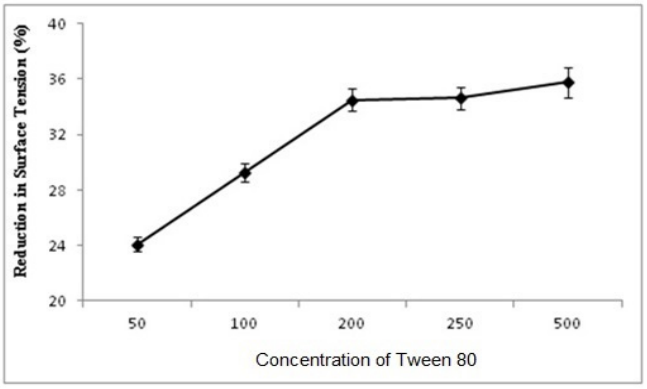

Figure 2. Surface tension reduction (%) of distilled water due to addition of Tween 80.

For determination of its concentration, reduction of surfactant tension of water was observed at different concentrations of Tween 80. After 200 ppm of Tween 80, reduction in surface tension became stable (Fig.2). Hence, it was assumed that this concentration represented the critical micelle concentration of this surfactant.

Degradation of Pyrene and Phenanthrene

When two bacterial strains were incubated in soil spiked with two PAHs (pyrene and phenanthrene, 100 ppm each) for 14 days, LMW PAH i.e. phenanthrene was degraded at higher rate than pyrene. Phenanthrene degradation was more than 80% by individual bacterial strains, where as their combination enhanced the degradation up to 93% within 7 days of incubation (Fig 3A). But addition of surfactant reduced the phenanthrene degradation by bacterial combination up to 64% after 7 day of incubation. After 14 days of incubation, bacterial consortium degraded 99% of phenanthrene in absence of surfactant, where as presence of surfactant reduced the efficiency of degradation by about 20%.

Due to high molecular weight, pyrene was found to be more recalcitrant than phenanthrene. Only combination of bacterial strain could degrade more than 50% of pyrene after 7 days of incubation (Fig. 3B). For same incubation period, bacterial combination boosted the degradation rate of pyrene by 55% and 75% as compared to degradation by individual bacterial strains i. e. BP10 and P2, respectively. But application of exogenous surfactant reduced the degradation rate by about 80%.After

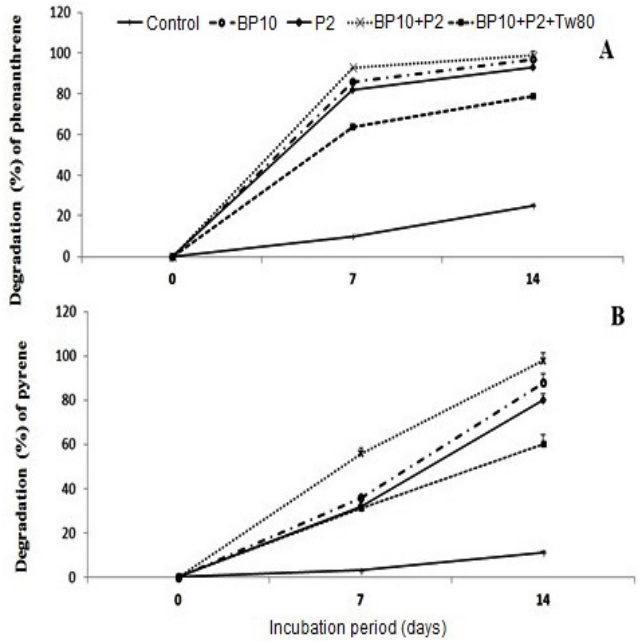

Figure 3. Degradation of PAHs, spiked in soil, by different treatments.

14 days of incubation, about 87% degradation of pyrene and 74% degradation of phenanthrene was contributed by this combination after substraction of natural degradation of pyrene (11%) and phenanthrene (25%) as observed in control.

Bacterial biomass

As selected bacterial strains were already tagged with the antibiotic, only colonies of bioaugmented bacterial strains were appeared on nutrient agar plates supplemented with vancomycin (250 µg ml^{-1}) after 48 h of incubation. Highest bacterial count (4.15 x 10^7 CFU g^{-1} of soil) was observed in soil inoculated with bacterial consortium, followed by bacterial count of BP10 (3.75 x 10^7 CFU g^{-1} of soil) and P2 (3.27 x 10^7 CFU g^{-1} of soil) in soil after 14 days of incubation (Fig.4). The bacterial count in soil without surfactant (4.15 x 10^7 CFU g^{-1} of soil) was higher than that supplemented with surfactant (2.18 x 10^7 CFU g^{-1} of soil).

Soil protein

Induction of protein by soil microbes and bioaugmented bacterial strains in soil increased with increasing the incubation period (Fig.5). Induction of protein correspond the bacterial growth and degradation of PAHs. The highest protein induction (6.14 mg g^{-1} of soil) was observed in soil inoculated with bacterial consortium, followed by individual member BP10 (4.95 mg g^{-1} of soil) and P2 (4.57 mg g^{-1} of soil). The protein content inpresence of Tween 80 (3.23 mg g^{-1} of soil) was almost reduced to half the value of recorded for consortium (6.14 mg g^{-1} of soil).

Dioxygenase activity

Polyaromatic hydrocarbons are degraded by both mono-oxygenase and di-oxygenase enzymes however, dioxygenase activity is more predominant during degradation. Bioaugmentation of selected bacterial strains enhanced the dioxygenase activity in soil as compared to very low dioxygenase activity in control (no bacterial augmentation). Activity of catechol 1,2 dioxygenase

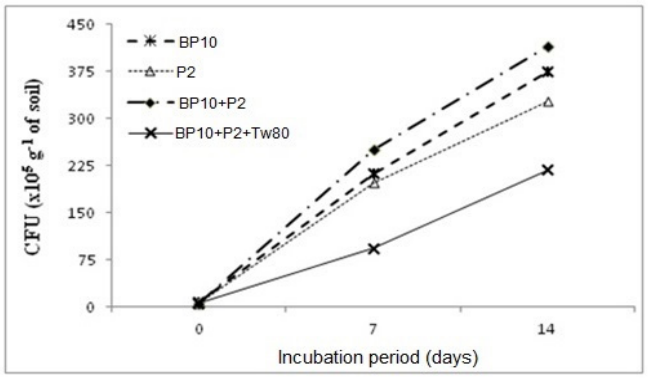

Figure 4. Bacterial count of bioaugmented strains in soil.

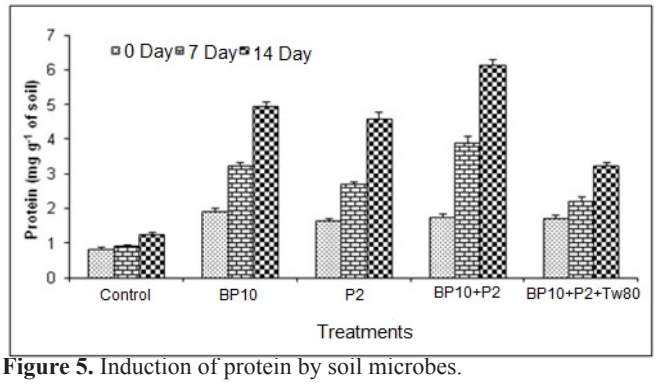

Figure 5. Induction of protein by soil microbes.

was found to be always higher than the catechol 2,3 dioxygenase. The results indicated that ortho cleavage pathway was more prominent in the degradation of poly aromatic hydrocarbons.

Catechol 1, 2 dioxygenase activity was higher on 7th day of incubation but decreased on 14th day in case of bioaugmented bacterial strains used either individually or in the combination without Tween 80 (Table 1). The highest activity of catechol 1, 2 dioxygenase (653.5 µ mol mg^{-1} of protein) was recorded in consortium, followed by BP10 (453.2 µ mol mg^{-1} of protein) and P2 (379.3 µ mol mg^{-1} of protein) on 7th day of incubation. The enzyme activity was reduced by about 62% in the presence of Tween 80.

Catechol 2, 3 dioxygenase activity increased with increasing the incubation day except in the case of BP10. Its activity was recorded highest again in consortium (57.8 µ mol mg^{-1} of protein) followed by BP10 (44.2 µ mol mg^{-1} of protein) and least in the case of bacterial combination with Tween 80 (31.2 µ mol mg^{-1} of protein). Similar to the activity of catechol 1, 2 dioxygenase, the activity of catechol 2, 3 dioxygenase activity was also reduced (85%) due to supplementation of chemical surfactant.

Cell hydrophobicity and desorption of pyrene and phe-

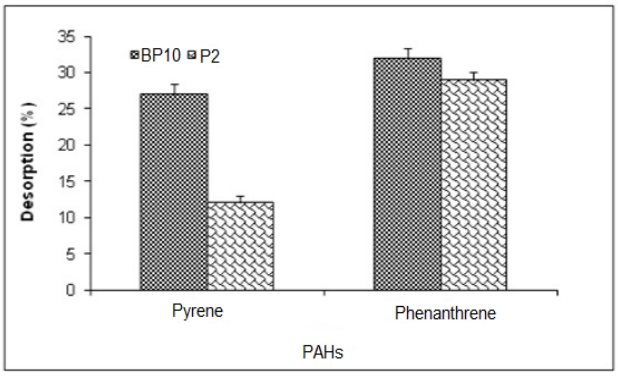

Figure 6. Desorption of pyrene and phenantherene by biosurfactant of selected strains.

Table1. Catechol dioxygenase activities (μ mol mg^{-1} of protein) in different treatments.

Treatments	Incubation period					
	0 day		7th day		14th day	
	C12O	C23O	C12O	C23O	C12O	C23O
Control	18.2 ± 1.4	10.2 ± 0.8	110.1 ± 5.8	23.6 ± 0.7	135.1 ± 3.4	32.7 ± 2.5
BP10	25.4 ± 0.5	9.8 ± 0.6	453.2 ± 2.3	47.2 ± 2.3	372.7 ± 2.8	44.2 ± 1.9
P2	21.2 ± 1.1	11.6 ± 0.7	379.3 ±11.2	29.8 ± 1.7	323.6 ± 4.2	32.6 ± 0.5
BP10+P2	25.4 ± 1.3	12.3 ± 0.6	653.5 ± 6.7	37.5 ± 2.9	530.4 ± 9.8	57.8 ± 0.8
BP10 +P2+Tw80	25.1 ± 0.9	11.9 ± 0.4	250.2 ± 4.7	21.6 ± 1.6	270.6 ± 19.4	31.2 ± 1.1

nanthrene by bacterial surfactant

Among the two bacterial strains, cell surface activity of BP10 was found to be highest i.e. 82 ± 0.8% and 75 ± 1.1% for benzene. Desorption of phenantherene and pyrene was enhanced 32% and 27%, respectively, by the biosurfactant produced by BP10 (Fig.6).

Discussion

Use of autochthonous microbes are always preferred for bioremediation as they are expected to be more adapted to particular soil environment than exotic species (15). In this study, BP10 and P2, isolated from petroleum hydrocarbon contaminated soil showed their high efficiency to degrade PAHs compare to native microbes of soil. Combination of these strains boosted the degradation process as compared to bioaugmentation of individual bacterial strains. From environmental perspective, using a microbial consortium always provides the metabolic diversity and robustness needed for the stability of ecosystem (16).

Presence of LMW PAH like phenantherene is reported to enhance the degradation of HMW PAH like pyrene, fluoranthene and benzo(α)pyrene (17, 18).Whereas Bouchez et al. (19) reported that the presence of fluoranthene as a second PAH resulted into reduced degradation of phenanthrene by *Pseudomonas* sp. strain S PheNaL. Similar to previous work (8), the present study revealed that the presence of phenanthrene as a second PAH substrate could reduce the degradation of pyrene from 96% to 88% in case of BP10, but there was negligible effect of phenanthrene in case of P2 bacterial strain.

Surfactants are able to facilitate the mass-transfer of hydrophobic pollutants from solid or non-aqueous liquid phase into aqueous phase by decreasing the interfacial tension and by accumulating the hydrophobic compounds in the micelles (20). When surfactant concentration is above the critical micelle concentration (CMC), micelle aggregates provide an additional hydrophobic area in the central region of micelles enhancing the aqueous solubility of PAHs (21).

Besides the positive role played by the surfactant (22), sometimes, negative effects of these surfactants have also been observed by various researchers (23). In present study, addition of Tween 80 reduced the degradation rate of PAHs in soil. Biodegradation of certain poorly soluble petroleum hydrocarbons may be inhibited by surfactants as a result of (i) toxicity at high concentration of surfactant, (ii) preferential metabolism of the surfactant itself, (iii) interference with the membrane uptake process, or (iv) reduced bioavailability of micelle hydrocarbons (24, 25). Shin et al. (26) performed the toxicity test that indicated the presence of solubilized phenanthrene due to presence of surfactant increased the toxicity by 100 fold. Feng et al. (27) found that addition of tergitol enhanced the surface activity of *Pseudomonas putida* 852, but reduced the same activity in case of *Rhodococcus erythropolis* 3586. Thus, biosurfactants have found more attention than synthetic surfactant due to low toxicity, low CMC, biodegradability, ecological acceptability, high selective and specific activity at extreme temperature, pH and salinity (28).

Both selected strains in this study were able to produce biosurfactants (8). Biosurfactant produced by BP10 has high efficiency to mass transfer of pyrene and phenantherene from soil surface to aqueous solution. Simultaneously, cell hydrophobicity to aromatic compound (benzene) and enzyme induction were also found to be the highest in BP10 than P2 strains. A higher rate of degradation of PAHs in BP10 than P2strain, might be contributed by cell inherent characteristics like cell hydrophobicity, biosurfactant production and enzyme activity as they are directly correlated with the degradation of petroleum hydrocarbons.

Based on the foregoing observations, it was informed that a suitable combination of potent hydrocarbon degrading and biosurfactant producing microbes might be able to induce degradative enzymes which directly contribute to the remediation of petroleum hydrocarbon contaminated sites. Application of individual strain with or without exogenous surfactants limit the process of PAH degradation.

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Other articles in this theme issue include references (29-44).

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