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Mineralization of Phenanthrene by *Paenibacillus* sp. PRNK-6: Effect of Synthetic Surfactants on its Mineralization

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Abstract

Paenibacillus sp. PRNK-6 isolated from a soil contaminated with polycyclic aromatic hydrocarbons (PAHs) was able to utilize 69.48% of phenanthrene (320 mg l^{-1}) in 48 h. The effect of three synthetic surfactants namely, the nonionic surfactant Tween-80, the anionic surfactant sodium dodecyl sulphate (SDS) and the cationic surfactant cetyltrymethyl ammonium bromide (CTAB) on the phenanthrene mineralization by Paenibacillus sp. PRNK-6 was studied. Results showed that the effect of each surfactant on phenanthrene mineralization by PRNK-6 and serve as growth substrate varied considerably. Tween-80, at the concentration of 1.5%, enhanced the growth and mineralization of phenanthrene from 69.48% to 90%; whereas SDS and CTAB, have a negative effect on the growth and mineralization of phenanthrene by Paenibacillus sp. PRNK-6.

Keywords: *Phenanthrene, Mineralization, Paenibacillus sp. PRNK-6, Synthetic surfactants*

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INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are organic contaminants of concern for human health and natural ecosystems due to their known carcinogenic and mutagenic properties [1]. Although poor aqueous solubility of PAHs poses a challenge for microbial degradation of these compounds, several microorganisms with PAHs degradation ability have been reported [2–4]. However, the rate and extent of degradation of PAHs are often restricted due to poor aqueous solubility and high hydrophobicity [5, 6].

Several studies have been conducted using surfactants to overcome bioavailability limitation in the biodegradation of PAHs. Surfactants are amphiphilic molecules that can enhance the apparent solubility of PAHs by forming micelles when present at a concentration above the critical micelle concentration (CMC). Some researchers have reported enhancement in biodegradation of PAHs in presence of surfactants [7–9]. However, sometimes non beneficial effects such as growth inhibition was observed [10–12]. Possible reasons for growth inhibition in surfactant-mediated degradation include surfactant toxicity to microorganisms due to permeabilization of cell membrane [13, 14], and decreased bioavailability of micelle solubilized PAHs [10].

In the present study, the effect of nonionic surfactant Tween-80, the anionic surfactant sodium dodecyl sulfate (SDS), and the cationic surfactant cetyltrimethyl ammonium bromide (CTAB), on the mineralization of phenanthrene by the isolated strain were investigated.

MATERIALS AND METHODS

Chemicals

Phenanthrene (97%) was purchased from Sigma-Aldrich (Steinheim, Germany). SDS, CTAB, anhydrous sodium sulphate and ethyl acetate were purchased from S D Fine-Chem Limited Mumbai, India. Surfactant Tween-80 was purchased from HiMedia, Mumbai, India. All other chemicals used in this study were of analytical grade.

Isolation, Media and Growth Conditions

The bacterium capable of utilizing phenanthrene as the sole carbon source was isolated from a PAHs contaminated soil collected from pharmaceutical companies

situated at Humnabad, Karnataka, India, by selective enrichment culture technique. About one gram of soil was suspended in 50 ml of mineral salt medium (MSM) supplemented with 160 mg l^{-1} phenanthrene in a 250 ml Erlenmeyer flask. Flasks were incubated in orbital shaker (180 rpm) at 30 °C for 15 days. Further enrichment was carried out by transferring 2% inoculum to fresh MSM during which phenanthrene concentration was gradually increased to 240 mg l^{-1}. After several such transfers, the culture was streaked on MSM agar plates spread with phenanthrene (dissolved in acetone). The purity of the culture was checked periodically by plating on Luria-Bertani (LB) agar plates.

Growth studies were carried out with MSM containing (g l^{-1}): K_2HPO_4, 6.3; KH_2PO_4, 1.8; NH_4NO_3, 1.0; $MgSO_4.7H_2O$, 0.1; $CaCl_2$, 0.1; $FeSO_4.7H_2O$, 0.1; $MnSO_4.4H_2O$, 0.1; $Na_2MoO_4.2H_2O$, 0.006. The pH of the medium was adjusted to 7.0 using 2 N NaOH or 2 N HCl. The culture was inoculated in 250 ml flask containing 50 ml MSM supplemented with increasing concentrations of phenanthrene (240 to 400 mg l^{-1}) and incubated in an orbital shaker (LabTech, India) at 180 rpm and 30 °C. Simultaneously, culture flasks without phenanthrene as well as flasks containing phenanthrene without inoculum were served as controls. All experiments were performed in triplicate. Bacterial growth was determined spectrophotometrically (analytik jena-SPECORD 50) at 660 nm.

16S rDNA Sequencing

The genomic DNA of the strain PRNK-6 was extracted and was used for polymerase chain reaction (PCR) amplification. The PCR amplification of 16S rDNA gene was carried out using universal primers forward 27F (5'-AGA GTT TGA TCM TGG CTC AG -3') and reverse 1,492R (5'- TAC GGY TAC CTT GTT ACG ACT T -3'). All PCR amplifications were performed in the thermal cycler (BioRad, USA). The PCR programming for 16S rDNA consisted of a preheating step at 95 °C for 4 min followed by 35 cycles of denaturation at 95 °C for 40 sec, annealing at 50 °C for 60 sec, elongation at 72 °C for 60 sec. The PCR products were purified using a Montage PCR Clean up kit (Millipore). DNA sequencing analysis was performed using Big Dye terminator cycle sequencing kit (Applied Bio Systems, USA).

The 16S rDNA sequence of strain PRNK-6 was compared to sequences in public databases with the BLAST search program on the NCBI website (http://www.ncbi.nlm.nih.gov/) to find closely related bacterial 16S rDNA sequences. Phylogenetic analysis was carried out with MEGA 6.0 [15] using neighbor-joining test. The nucleotide sequence of 16S rDNA of isolate PRNK-6 have been deposited in GenBank under the accession number KT185017.

Effect of pH and Different Concentrations of Phenanthrene on the growth of PRNK-6

The effect of pH and different concentrations of phenanthrene on the growth of the strain PRNK-6 was studied. The increasing concentrations of phenanthrene (240–400 mg l^{-1}) were added and inoculated with exponential growth phase cells of PRNK-6. Similarly, the effect of pH of the medium (6.0–8.0) on the growth of the strain PRNK-6 was studied by culturing the bacterium in 50 ml of MSM containing 320 mg l^{-1} of phenanthrene with respective pH. All the flasks were incubated in shaking incubator at 180 rpm at 30 °C. The growth was determined by measuring the absorbance at 660 nm.

Phenanthrene Mineralization

To study the phenanthrene mineralization by the strain PRNK-6, the bacterium was grown in 50 ml of MSM supplemented with phenanthrene (320 mg l^{-1}). Once the cells reached the mid-exponential phase, they were harvested by centrifugation (10000 rpm for 15 min at 4 °C), washed twice with 50 mM phosphate buffer (pH 7.0) and resuspended in same volume of MSM. About 2 ml of this cell suspension served as inoculum. Phenanthrene degradation study was conducted using 100 ml of MSM in 250 ml flask containing 320 mg l^{-1} of phenanthrene and inoculated with PRNK-6 and incubated at 30 °C (180 rpm). Control flask without inoculum was also incubated under the same conditions. Samples were collected at different time intervals to determine the residual phenanthrene concentration by gas chromatography (GC).

Effect of Surfactants on Phenanthrene Mineralization

To study the effect of surfactants on growth and phenanthrene mineralization by bacterial strain PRNK-6, the bacterium was cultured in a 250 ml Erlenmeyer flasks containing 100 ml of autoclaved MSM supplemented with 320 mg l^{-1} phenanthrene along with Tween-80 (1.5%), SDS (100 mg l^{-1}) and CTAB (100 mg l^{-1}) individually. Cells in the mid-exponential phase (2%) were used as inoculum. The flasks were incubated on a rotary shaker at 30 °C (180 rpm), and culture flasks without surfactants served as controls. All experiments were performed in triplicate. Bacterial growth was determined spectrophotometrically at 660 nm. Residual phenanthrene concentration was determined using GC by withdrawing the spent medium at regular time intervals.

GC Conditions

GC system (Agilent 7820A) equipped with FID detector and HP-5 column (length 30 m, inner diameter 0.25 mm, 0.25 µm film) was used. Nitrogen was used as a carrier gas at a flow rate of 1 ml min^{-1}. The oven temperature was maintained at 80 °C for 2 min and increased to 280 °C with 8 °C rise min^{-1}, at which it was held for 4 min. Both detector and injector temperatures were maintained at 270 °C. The injection volume was 1µl.

RESULTS

Isolation, Characterization and Identification of Strain PRNK-6

By using selective enrichment culture technique, six bacterial strains were isolated from the contaminated soil using phenanthrene as the sole source of carbon and energy. Among these, the potential strain designated as PRNK-6 was selected for further studies. Various morphological, physiological and biochemical characteristics of the strain PRNK-6 are summarized in Table 1. The strain is Gram-negative, motile and rod-shaped. On phenanthrene MSM agar medium, the strain PRNK-6 formed pink colour round colonies with smooth edges. This strain exhibited positive tests for catalase, methyl red (MR), lipase, casein and starch hydrolysis. However, it tested negative for H_2S production, Voges-Proskauer (VP), indole production, gelatin liquefaction, citrate utilization, and urease activity.

16S rDNA Sequencing and Phylogenetic Tree Construction

Sequencing of 16S rDNA was used as an identification tool. The 16S rDNA sequence of strain PRNK-6 was compared against the NCBI public database. The 16S rDNA sequences from the type strains and the strain PRNK-6 were aligned and phylogenetic tree was constructed (Figure 1). A comparison showed that the isolated strain belongs to genus *Bacillus* and showed 100% similarity with *Paenibacillus lactis* MB 1871. Hence, the strain PRNK-6 was identified and named as *Paenibacillus* sp. strain PRNK-6. The 16S rDNA sequence of the strain PRNK-6 was deposited and is available in the Genbank database with accession number KT185017.

Optimization of Growth Conditions for Strain PRNK-6

Effect of pH and different concentrations of phenanthrene on growth of *Paenibacillus* sp. PRNK-6 was investigated. The maximum growth of PRNK-6 was observed at 320 mg l^{-1} of phenanthrene, 30 °C and pH 7.0. At optimum temperature (30 °C) and pH (7.0) conditions, strain PRNK-6 could grow faster at 320 mg l^{-1} of phenanthrene and enters the stationary phase by 72 h (Figure 2).

Table 1: *Morphological, physiological and biochemical characteristics of strain PRNK-6.*

Characteristics	Result
Cell morphology and grouping	Rod shaped, motile and individual
pH range for growth	6.0 to 8.0
Relation to oxygen	Facultative anaerobic
Gram-stain, H_2S production, Voges-Proskauer (VP), indole production, gelatin liquefaction, citrate utilization, urease activity and carbohydrate fermentation (glucose, lactose, sucrose).	Negative
Catalase, methyl red (MR), lipase, casein and starch hydrolysis	Positive

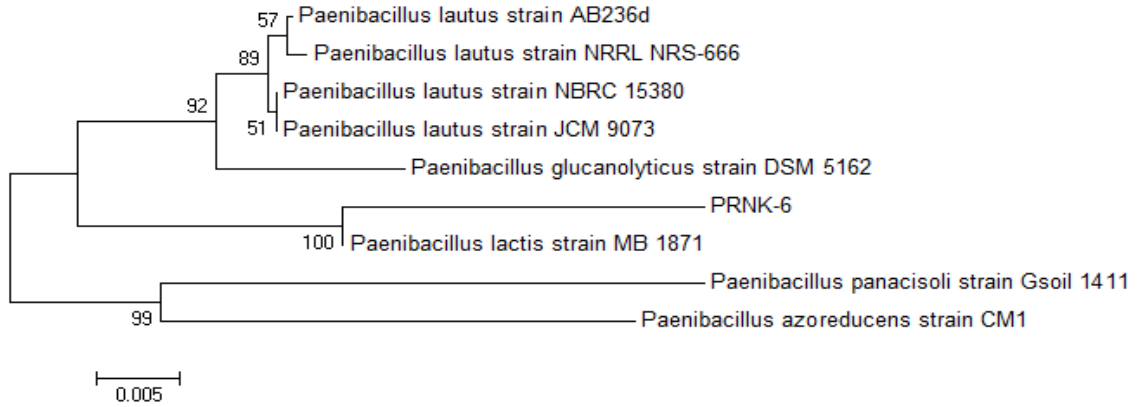

Fig. 1: *Phylogenetic tree showing the position of isolate PRNK-6 with reference to related strains. All 16S rDNA sequences of related strains have been retrieved from the NCBI database; 0.005 denotes the genetic distance.*

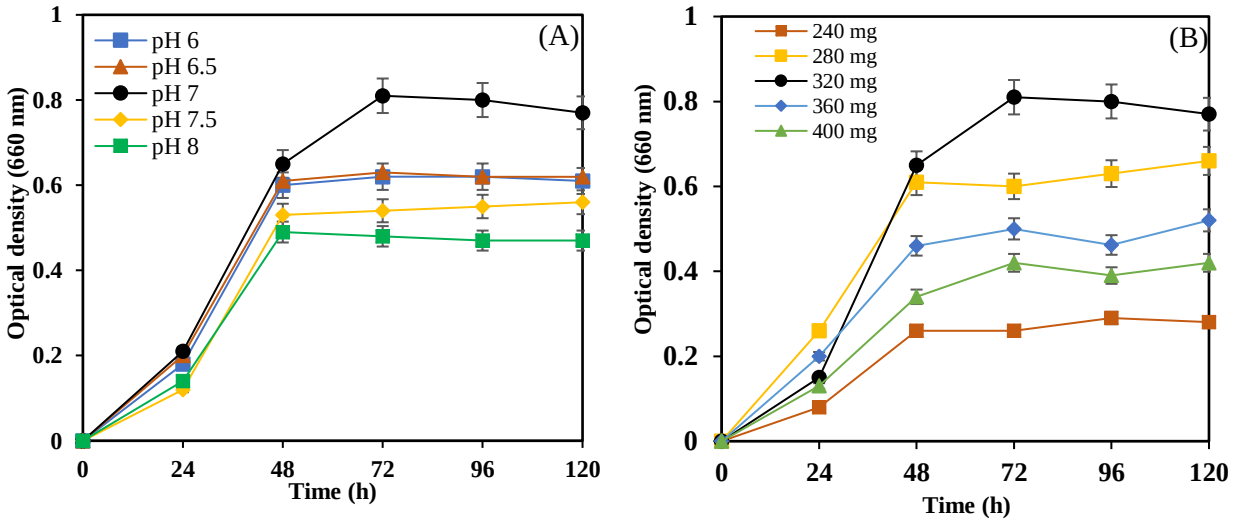

Fig. 2: *Optimization of growth conditions of Paenibacillus sp. PRNK-6 under different conditions. (A) Growth at different pH (6–8) at 30 °C and 320 mg l^{-1} of phenanthrene, (B) Growth at different concentrations of phenanthrene (240–400 mg l^{-1}).*

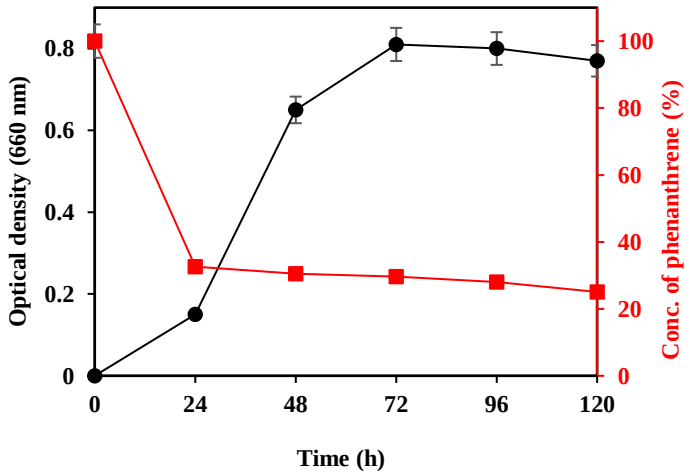

Fig. 3: *Growth of Paenibacillus sp. PRNK-6 and utilization of phenanthrene. Growth (•) and concentration of phenanthrene (■).*

Phenanthrene Mineralization by *Paenibacillus* sp. PRNK-6

Maximum growth was observed at 72 h of incubation when *Paenibacillus* sp. PRNK-6 was grown in MSM with phenanthrene as the sole carbon and energy source. The strain showed 69.48% utilization of phenanthrene (320 mg l^{-1}) (Figure 3) within 48 h. After 72 h, phenanthrene degradation slowed down as the cells entered the stationary phase.

Effect of Surfactants on Phenanthrene Mineralization

The phenanthrene degradation by PRNK-6 was performed in MSM with and without Tween-80 (1.5%), SDS (100 mg l^{-1}) and CTAB (100 mg l^{-1}) individually. The growth and residual concentration of phenanthrene is shown in Figure 4. Tween-80 had shown positive effect on phenanthrene degradation as well as on growth of PRNK-6, whereas adding SDS and CTAB in MSM along with phenanthrene shows toxic effect on the growth of PRNK-6. Phenanthrene (320 mg l^{-1}) degradation was enhanced from 69.48% to 90% in presence of Tween-80 at 48 h. However, SDS and CTAB surfactants has shown inhibitory effect on growth of PRNK-6.

DISCUSSION

Numerous phenanthrene degrading bacterial strains documented (Table 2) so far degrades very low concentrations of phenanthrene (10–250 mg l^{-1}) either as a sole carbon source or co-metabolically [16]. *Paenibacillus* sp. strain PRNK-6 a soil bacterium efficiently degrades high concentration of phenanthrene (320 mg l^{-1}).

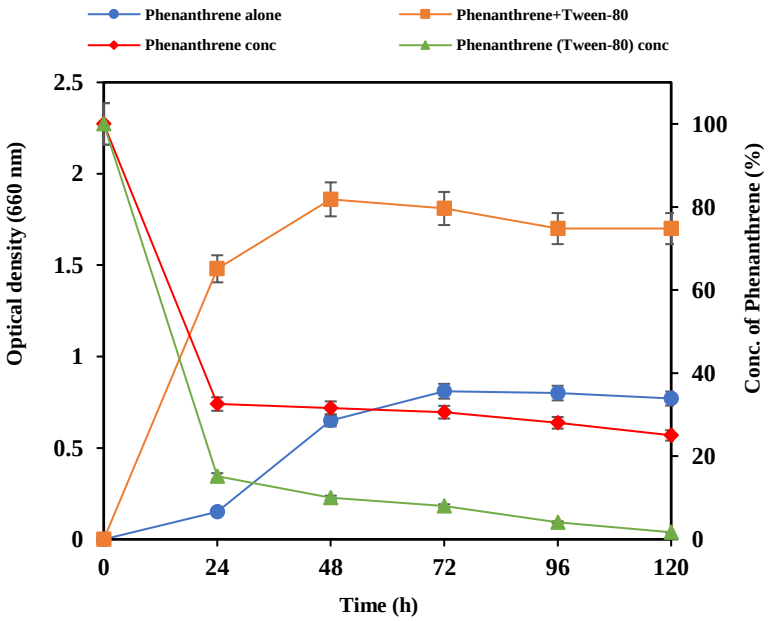

Fig. 4: *Growth of Paenibacillus sp. PRNK-6 and utilization of phenanthrene. Growth on phenanthrene (●), phenanthrene along with Tween-80 (■), concentration of phenanthrene (◆) and concentration of phenanthrene along with Tween-80 (▲).*

Table 2: *Comparison of phenanthrene degradation period and percent degradation in the present study with literatures.*

Phenanthrene concentration (mg l^{-1})	Degradation period (days)	% Degradation	Organism	References
100	30	99.5	*Rhodotorula glutini*	[17]
10	14	58	Mixed culture	[18]
250	14	56.9	*Bacillus cereus*	[19]
320	2	90	*Paenibacillus* sp. PRNK-6	This study

Increased utilization of phenanthrene occurred in presence of Tween-80, showing 90% of phenanthrene utilization (320 mg l^{-1}) in just 48 h (Figure 3) as compared to unamended. Bacterial uptake of solubilized compounds is hypothesized to be influenced by Tween-80, as the uptake of surfactant-solubilized molecules has been found to be faster than the uptake of dissolved molecules [20]. Surfactants linearly increased the utilization of phenanthrene as noticed in Figure 3. This indicates that a greater amount of phenanthrene degradation could be achieved through amendments with surfactants, when compared to the amount degraded without Tween-80 addition.

The addition of SDS inhibit the growth of PRNK-6. According to Neu *et al.* [21], SDS inhibits hydrophobic interactions. Experiments performed with *Streptococcus* revealed that hydrophobic bond-disrupting agents including SDS inhibited adhesion of the bacteria to hydroxylapatite [22]. Thus, if adhesion of cells to substrate is an important mechanism to PAHs uptake, SDS may have a negative effect if adhesion is mainly due to hydrophobic interactions. The inhibitory effect of SDS was reported by Deschenes *et al.* [23] during anthracene degradation.

In the presence of CTAB (100 mg l^{-1}), the growth of strain PRNK-6 was inhibited. Similar inhibitory effect of CTAB during fluoranthene and anthracene degradation was reported by Rodrigues *et al.* [24], suggesting that the inhibitory effect of CTAB, at a concentration of 100 mg l^{-1}, is probably related to the uptake mechanism of these PAHs. As previously mentioned, CTAB binds by chemisorption to the bacterial cell surfaces, due to the electrostatic attraction, preventing adhesion of the cells to the substrate [21].

CONCLUSIONS

Three surfactants were used to evaluate their effects on phenanthrene mineralization. It was found that except Tween-80, SDS and CTAB showed toxicity to strain PRNK-6. The biodegradation experiment showed that, in the presence of Tween-80 about 90% of phenanthrene was degraded in 48 h. For the PAHs contaminated sites, Tween-80 is the best choice among the surfactants studied. As two other surfactants inhibit the growth of PRNK-6 and phenanthrene degradation.

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REFERENCES

1. Enzminger JD, Ahlert RC. Environmental fate of polynuclear aromatic hydrocarbons in coal tar. *Environ Technol Lett*. 1987; 8: 269–78p. doi:10.1080/09593338709384486.
2. Margesin R, Moertelmaier C, Mair J. Low-temperature biodegradation of petroleum hydrocarbons (n-alkanes, phenol, anthracene, pyrene) by four actinobacterial strains. *Int Biodeterior Biodegrad*. 201: 185–91p. doi:10.1016 /j.ibiod.2012.05.004.
3. Obayori OS, Ilori MO, Amund OO. Degradation of spiked pyrene and non-pyrene hydrocarbons in soil microcosms by *Pseudomonas* species isolated from petroleum polluted soils. *Pet Sci Technol.* 2013; 31: 1674–80p. doi:10.1080/10916466.2010.551240.
4. Lu J, Guo C, Zhang M, *et al.* Biodegradation of single pyrene and mixtures of pyrene by a fusant bacterial strain F14. *Int Biodeterior Biodegrad*. 2014; 87: 75–80p. doi:10.1016/j.ibiod.2013.11.004.
5. Shuttleworth KL, Cerniglia E. Environmental aspects of PAH biodegradation. *Appl Biochem Biotechnol.* 1995; 54: 291–302p. doi:10.1007/BF02787927.
6. Seo JS, Keum YS, Li QX. Bacterial degradation of aromatic compounds. *Int J Environ Res Public Health*. 2009; 6(1): 278–309p. doi:10.3390/ijerph6010278.
7. Prak DJL, Pritchard PH. Solubilization of polycyclic aromatic hydrocarbon mixtures in micellar nonionic surfactant solutions.

Water Res. 2002; 36: 3463–72p. doi:10.1016/S0043-1354(02)00070-2.

8. Aryal M, Liakopoulou-Kyriakides M. Biodegradation and kinetics of phenanthrene and pyrene in the presence of nonionic surfactants by *Arthrobacter* strain Sphe3. *Water Air Soil Pollut*. 2013; 224: 1426. doi:10.1007/s11270-012-1426-8.
9. Chen K, Zhu Q, Qian Y, *et al.* Microcalorimetric investigation of the effect of non-ionic surfactant on biodegradation of pyrene by PAH-degrading bacteria *Burkholderia cepacia*. *Ecotoxicol Environ Saf*. 2013; 98: 361–7p. doi:10.1016/j.ecoenv.2013.08.012.
10. Laha S, Luthy RG. Inhibition of phenanthrene mineralization by nonionic surfactants in soil-water systems. *Environ Sci Technol.* 1991; 25: 1920–30p. doi:10.1021/es00023a013.
11. Margesin R, Schinner F. Biodegradation and bioremediation of hydrocarbons in extreme environments. *Appl Microbiol Biotechnol.* 2001; 56: 650–63p. doi:10.1007/s002530100701.
12. Mesbaiah FZ, Mansour F, Eddouaouda K, *et al.* Surfactant effects on biodegradation of polycyclic aromatic hydrocarbons. *Desalin Water Treat.* 2014; 57: 37–41p. doi:10.1080/19443994.2014.988651.
13. Willumsen PA, Karlson U. Effect of calcium on the surfactant tolerance of a fluoranthene degrading bacterium. *Biodegradation.* 1998; 9: 369–79p. doi:10.1023/A:1008357904624.
14. Avramova T, Sotirova A, Galabova D, *et al.* Effect of Triton X-100 and rhamnolipid PS-17 on the mineralization of phenanthrene by *Pseudomonas* sp. cells. *Int Biodeterior Biodegrad.* 2008; 62: 415–20p. doi:10.1016/j.ibiod.2008.03.008.
15. Tamura K, Stecher G, Peterson D, *et al.* MEGA6: Molecular evolutionary genetics analysis version 6.0. *Mol Biol Evol.* 2013; 30: 2725–9p. doi:10.1093/molbev/mst197.
16. Sponza DT, Oztekin R. Removals of PAHs and acute toxicity via sonication in a petrochemical industry wastewater. *Chem Eng J.* 2010; 162: 142–50p. doi:10.1016/j.cej.2010.05.014.
17. Romero MC, Cazau MC, Giorgieri S, *et al.* Phenanthrene degradation by microorganisms isolated from a contaminated stream. *Environ Pollut.* 1998; 101: 355–9p. doi:10.1016/S0269-7491(98)00056-6.
18. Tam NFY, Guo CL, Yau WY, *et al.* Preliminary st. udy on biodegradation of phenanthrene by bacteria isolated from mangrove sediments in Hong Kong. *Mar Pollut Bull*. 2002; 45: 316–24p. doi:10.1016/S0025-326X(02)00108-X.
19. Janbandhu A, Fulekar MH. Biodegradation of phenanthrene using adapted microbial consortium isolated from petrochemical contaminated environment. *J Hazard Mater*. 2011; 187: 333–40p. doi:10.1016/j.jhazmat.2011.01.034.
20. Johnsen AR, Wick LY, Harms H. Principles of microbial PAH-degradation in soil. *Environ Pollut*. 2005; 133: 71–84p. doi:10.1016/j.envpol.2004.04.015.
21. Thomas RN. Significance of Bacterial Surface-Active Compounds in Interaction of Bacteria with Interfaces. *Am Soc Microbiol.* 1996; 60: 151–66p.
22. Nesbitt WE, Doyle RJ, Taylor KG. Hydrophobic interactions and the adherence of *Streptococcus sanguis* to hydroxylapatite. *Infect Immun*. 1982; 38: 637–44p.
23. Taylor D, Samson P. The effect of an anionic surfactant on the mobilization and biodegradation of PAHs in a cresote-contaminated soil. *Colloid Polym Sci*. 1996; 274: 1061–71p. doi:10.1007/BF00658371.
24. Rodrigues A, Nogueira R, Melo LF, *et al.* Effect of low concentrations of synthetic surfactants on polycyclic aromatic hydrocarbons (PAH) biodegradation. *Int Biodeterior Biodegradation.* 2013; 83: 48–55p. doi:10.1016/j.ibiod.2013.04.001.

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