Ann Microbiol (2015) 65:225–234
DOI 10.1007/s13213-014-0854-7

ORIGINAL ARTICLE

Degradation of polyaromatic hydrocarbons employing biosurfactant-producing *Bacillus pumilus* KS2

Kaustuvmani Patowary · Rashmi Rekha Saikia · Mohan Chandra Kalita · Suresh Deka

Received: 29 August 2013 / Accepted: 28 February 2014 / Published online: 21 March 2014

Abstract An efficient hydrocarbon-degrading native bacterial strain *Bacillus pumilus* KS2 (identified by partial 16S rDNA gene sequencing) was isolated from crude oil-contaminated soil collected from oil fields of Lakowa, Sivasagar district of Assam, India. Experiments were conducted under laboratory conditions to determine the efficiency of this biosurfactant-producing strain to degrade polycyclicaromatic hydrocarbons (PAHs). Quantification of the capacity of the biosurfactant to reduce the surface tension (ST) of the culture medium was used as a measure of biosurfactant production. In terms of total petroleum hydrocarbon (TPH) degradation, strain KS2 was able to degrade 80.44 % of the TPH by 4 weeks of incubation. It also demonstrated efficient degradation of PAHs, completely degrading nine of the 16 major PAHs present in the crude oil sample. Strain KS2 also produced biosurfactant which, based on biochemical and FTIR analyses, was glycolipid in nature. To our knowledge, this is the first report showing the potential of a native strain of the North-East region of India for efficient degradation of TPH and PAHs and, consequently, in the remediation of hydrocarbons from contaminated sites.

Keywords *Bacillus pumilus* KS2 · PAH degradation · Biosurfactant · Crude oil · Native strain

K. Patowary · S. Deka (✉)
Environmental Biotechnology Laboratory, Life Sciences Division, Institute of Advanced Study in Science and Technology, Guwahati, Assam, India
e-mail: sureshdeka@yahoo.com

R. R. Saikia
Department of Zoology, Jagannath Barooah College, Jorhat, Assam, India

M. C. Kalita
Department of Bioengineering and Technology, Gauhati University, Guwahati, Assam, India

Introduction

Oil pollution is a serious environmental problem worldwide. Various activities associated with oil exploration cause environmental pollution, including geophysical exploration, drilling of wells, pressure control and management of oil and natural gas gushing from the well, transportation and refining of crude oil. Crude oil is a homogeneous but complex mixture of hundreds of different hydrocarbons which vary widely in their characteristics. These compounds are toxic, persistent and have negative influence on living organisms, making it imperative to develop a technology for cleaning up polluted sites. Microorganisms capable of degrading hydrocarbons in various forms (Klug and Markovetz 1971) can be found in many environments depending on the specific compounds present in the environment (Atlas 1981). However, the rate of degradation of hydrocarbonaceous compounds in nature is limited due to their hydrophobic property which leads to their limited solubility in ground water and tendency to partition to the soil matrix. This partitioning can account for as much as 90–95 % or more of the total contaminant mass. As a consequence, hydrocarbon contaminants exhibit moderate to poor recovery by physicochemical treatments, limited bioavailability to microorganisms and limited availability to oxidative and reductive chemicals when subjected to in situ and/or ex situ applications (Pacwa-Plociniczak et al. 2011). A promising method that can improve the effectiveness of bioremediation of hydrocarbon-contaminated environment is the use of biosurfactants. Biosurfactants are surface-active amphipathic molecules produced by certain microorganisms. They have a wide structural diversity, ranging from glycolipids, lipopeptides and lipoproteins to fatty acids, neutral lipids, phospholipids and polymeric and particulate biosurfactants (Das et al. 2008). Biosurfactants reduce surface tension (ST) and critical micelle dilution in both aqueous solution and hydrocarbon mixtures, thereby facilitating the creation of

micro-emulsions with the formation of micelle in which hydrocarbons can solubilize in water or water in hydrocarbons (Banat 1995). Biosurfactants are often less toxic and more biodegradable than synthetic surfactants and thus are particularly suited for environmental applications such as hydrocarbon remediation (Oberbremer et al. 1990). Most biosurfactants, in comparison to chemical surfactants, have shorter persistence in the environment (Georgiou et al. 1992) and are more effective in enhancing the solubility and biodegradation of petroleum hydrocarbons, including polycyclic aromatic hydrocarbons (PAHs) (Wong et al. 2004; Hickey et al. 2007).

Crude oil contains more than 30 % PAHs. PAHs are important environmental contaminants because of their hydrophobic and recalcitrant nature (Toledo et al. 2006) and additionally are important health threats to human and animal life (Samanta et al. 2002). They occur in nature as a result of incomplete combustion of organic matter, as well as from many anthropogenic sources, including cigarette smoke and automobile exhaust (Jacques et al. 2007). A number of studies on degradation of multiple PAHs by biosurfactant-producing bacteria have been published. Ganeshalingam et al. (1994) suggested that applying surfactants as immobilizing agents might be one approach to enhance the solubility of PAHs. Biosurfactants are capable of increasing the bioavailability of poorly soluble PAHs (Gilewicz et al. 1997; Olivera et al. 2003). Nie et al. (2010) studied the biosurfactant-producing *Pseudomonas aeruginosa* NY3 as one of the components of PAH-degrading bacterial consortia tested for their efficacy in degrading a mixture containing equal amounts of fluorene, anthracene, phenanthrene, pyrene and fluoranthene. These authors reported that this bacterium could degrade all five of these PAHs at different degradation rates. The ability of members of the genus *Bacillus* to degrade PAHs has also been reported. However, experimental data on hydrocarbon degradation by biosurfactant-producing *Bacillus pumilus* are relatively scarce. Several bacterial strains, including *B. pumilus* JL, have demonstrated the capacity to degrade diesel and used engine oil effectively in liquid media (Mandri and Lin 2007; Singh and Lin 2008). In a PAH degradation study by bacterial consortia, Ma et al. (2010) observed that *B. pumilus* was one of the members of the consortia, but these authors did not determine whether the bacterium was responsible for degradation of any specific PAH. Khanna et al. (2011) reported that *B. pumilus* (PK- 12, MTCC 1002) could metabolically take up 64 % of pyrene from its growth medium. Yuliani et al. (2012) recently showed that *B. pumilus* C 15 has the capability to degrade the PAHs pyrene and phenanthrene because the strain possesses the dioxyrenese *nidA* and *nahAc* gene which are responsible for PAH uptake.

The aim of this study was to isolate an efficient biosurfactant-producing/hydrocarbon-degrading native bacterial strain that can be used for the decontamination of the sites contaminated with petroleum hydrocarbons.

Materials and methods

Isolation of microorganism

Soil samples were collected from crude oil-contaminated sites of the Lakowa oil fields, situated in upper Assam, India, for isolation of biosurfactant-producing bacterial strains with hydrocarbon-degrading property. To this end, 1 g of collected soil sample was added to a 500-mL Erlenmeyer flask containing 100 mL of sterilized nutrient broth and mineral salt solution at 1:1 ratio (Francy et al. 1991) to which 2 %v/v crude oil was added as the sole carbon source. The flasks were incubated at 35 °C in a rotary shaker (model Orbitek LJEIL; Scigenics Biotech, Bangalore, India) at 150 rpm. After 3 days of incubation, 5 mL of culture broth was sampled from each flask and transferred into a second batch of flasks containing fresh medium; these flasks were then incubated under the same conditions to decrease the unwanted microbial load. This process was repeated three times, and each time 5 mL of culture broth was withdrawn from the "older" flasks and transfered into new ones. Serial dilutions of the culture broth from the last batch of flasks were inoculated onto diesel-containing (2 % v/v) mineral agar plates and the plates incubated at 35 °C for the development of bacterial colonies. The morphologically different bacterial colonies which developed on the plates were streaked on nutrient agar plates to obtain pure cultures of the isolates, and these pure cultures were maintained on nutrient agar slants and kept at 4 °C in the refrigerator.

Screening for biosurfactant-producing bacteria

The isolated bacterial strains grown in nutrient broth for 24 h at 35 °C with shaking at 150 rpm were used as mother inoculum. A 5-mL sample of each mother inoculum of each isolate was transferred to a 500-mL Erlenmeyer flask containing 100 mL sterilized mineral medium with 2 %w/v glucose as the carbon source and incubated at 35 °C with shaking at 150 rpm. The composition of the mineral medium used was (g/L): NH_4NO_3 (4.0), KCl (0.1), KH_2PO_4 (0.5), K_2HPO_4 (1.0), $CaCl_2$ (0.01), $MgSO_4{\cdot}7H_2O$ (0.5), $FeSO_4{\cdot}7H_2O$ (0.01), yeast extract (0.1) and 10 mL of trace element solution containing (g/L) 0.26 g H_3BO_3, 0.5 g $CuSO_4{\cdot}5H_2O$, 0.5 g $MnSO_4{\cdot}7H_2O$, 0.06 g $(NH_4)_6Mo_7O_2{\cdot}\ 4H_2O$ and 0.7 g $ZnSO_4{\cdot}\ 7H_2O$ (Saikia et al. 2012). The pH of the medium was adjusted to 7.0±0.2. The production of biosurfactant by the bacterial

isolates was assayed in terms of drop collapse assay and ST reduction of the culture medium.

Drop collapse assay

A single drop of crude oil was placed on a glass slide, following which a single drop of 48-h-grown culture broth was dropped onto the crude oil drop (Bodour and Miller-Maier 1998) and drop collapse activity was observed.

ST measurement

Surface tension reduction was measured at 24-h intervals up to the fifth day using a tensiometer (model K11; Kruss Optronic, Hamburg, Germany). The values reported are the mean of five measurements. The isolates which reduced the ST of the medium to <45 mN/m were selected as efficient isolates for further experiments.

Screening of the most efficient hydrocarbon-degrading bacterial isolate

The method of Rahman et al. (2002), with minor modifications, was used to screen the bacterial isolates for the most efficient hydrocarbon-degrading bacterium. As a first step, mother cultures were prepared from all of the biosurfactant-producing bacterial isolates in nutrient broth as per the procedure mentioned above. For the screening, a 5-mL sample of the mother culture of each bacterium was inoculated into a 500-mL Erlenmeyer flask containing 100 mL of sterilized mineral medium with crude oil 2 % (v/v) collected from the Noonmati refinery, Guwahati, India, and cultured in a shaking incubator at 35 °C and 150 rpm for 7 days. The bacterial growth in the medium of each flask was measured by determining the optical density (OD) at 600 nm using a UV–Vis spectrophotometer (model UV-1800; Shimadzu, Kyoto, Japan). The bacterial strain showing the maximum growth in the crude oil-containing medium was selected for further study.

Identification of the bacterial strain

The most efficient hydrocarbon-degrading/biosurfactant-producing bacterial isolate was identified by 16S rDNA sequencing and subsequent alignment of the sequence in the NCBI GenBank (performed by National Center for Cell Science, Pune, India). Following standard protocols, we extracted genomic DNA from a pure culture and amplified a 16S rDNA fragment of approximately 1.5 kb using Taq DNA polymerase. The fragment was bi-directionally sequenced using universal bacterial primers UFUL (GCCTAACACATGCAAG TCGA) and URUL (CGTATTACCGCGGCTGCTG) (Nilsson and Strom 2002). Sequence data were aligned and analyzed to identify the closest homolog of the isolated bacterial strain. A neighbor-joining phylogenetic tree (Fig. 1) was constructed with the aligned 16S rDNA gene sequences using 1,000 bootstrap replication with MEGA5 software (Tamura et al. 2011).

Study of total petroleum hydrocarbon degradation

Crude oil (2 g) was added to each of twenty 500-mL Erlenmeyer flasks, following which 100 mL of mineral media was added and the mixture mixed thoroughly. The mixture was then sterilized to kill the unwanted microorganisms present in the crude oil. Microbial growth was initiated in 15 of the 20 flasks by inoculating 5 mL of the strain KS2 mother culture into each flask (OD_{600} 1.8). The remaining five flasks were kept as abiotic controls and were not inoculated with the bacterial culture. The flasks were incubated in a rotary shaker at 35 °C to study the degradation of crude oil for a period of 1, 2, 3, 4 and 5 weeks (Kumari et al. 2012). Crude oil was extracted from three flasks containing culture media and from one from control flask at weekly intervals using a solvent extraction method with petroleum benzene (boiling range 40–60 °C). The solvent was evaporated in a rotary evaporator, and the hydrocarbon solution was captured in a clean, previously weighed beaker and kept for few days to obtain the constant weight. The amount of crude oil degraded was then determined gravimetrically (Mittal and Singh 2009).

Study of degradation of PAHs

The degradation of PAHs was analyzed in the crude oil samples extracted from the flasks containing mineral media and crude oil inoculated with *B. pumilus* strain KS2 mother culture after 4 weeks of culture. Crude oil from the abiotic control was also extracted for analysis. Samples were analyzed by gas chromatography (GC) on a Master GC chromatograph (DANI, Cologno Monzese, Italy) equipped with a flame ionization detector and DN5 capillary column with Supelco (Sigma-Aldrich, St. Louis, MO) standards. The flow rate of carrier gas was 25 mL/min, and the fuel source was supplied at 40 mL/min. Air was supplied as oxidant at a rate of 280 mL/min. The initial oven temperature was 160 °C for 2 min.

Extraction of biosurfactant

To extract the biosurfactant from the culture media, we centrifuged 48-h-old culture broth at 10,000 rpm for 20 min at 4 °C to obtain the cell-free supernatant. The clear supernatant served as the source of crude biosurfactant. The pH was adjusted to pH 2 by adding 6 N HCl to the collected supernatant. The acidified supernatant was kept at 4 °C overnight, following which the biosurfactant was extracted continuously

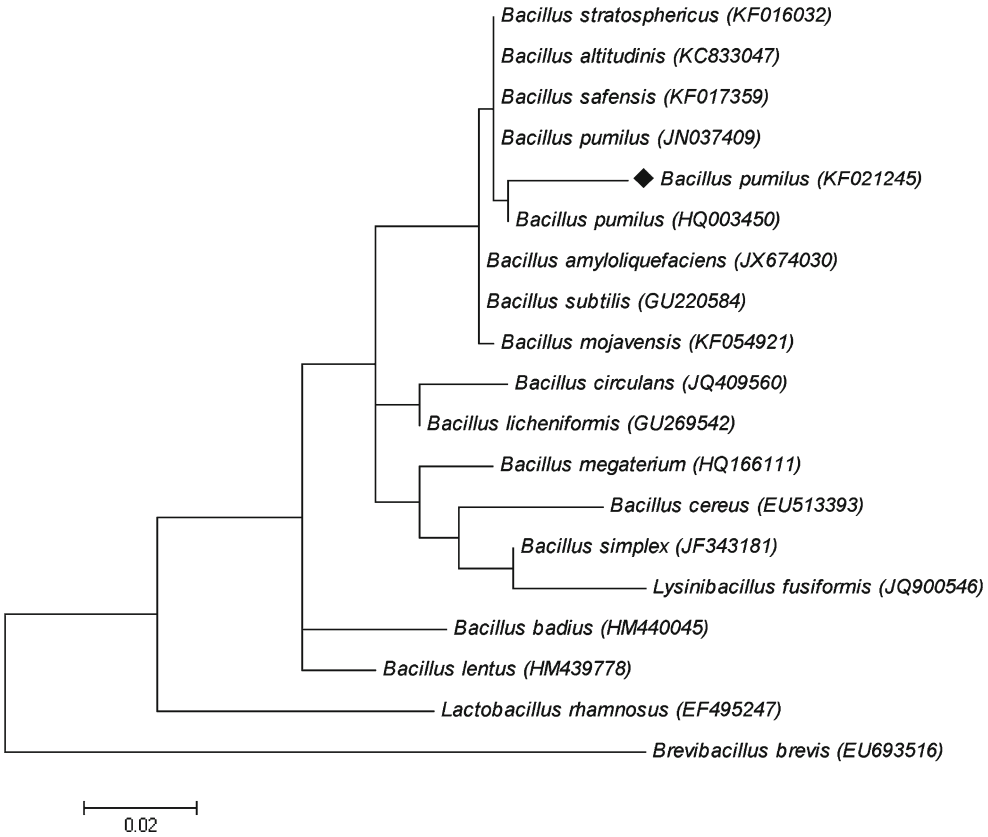

Fig. 1 Phylogenetic tree of *Bacillus pumilus* strain KS2 based on 16S rDNA sequencing and its closest relatives. *Bar* 0.02 nucleotide substitutions, *values in parenthesis* GenBank accession number

with vigorous shaking in a mixture of supernatant and ethyl acetate (ratio 1:1) at room temperature and then left static for phase separation. The organic phase was then transferred to a rotary evaporator and a viscous solid product was recovered (i.e. crude biosurfactant) after solvent evaporation at 40 °C under reduced pressure (George and Jayachandran 2009). The crude biosurfactant was subsequently dried and determined gravimetrically.

Characterization of biosurfactant

Biochemical

The presence of different bio-molecules in the extracted crude biosurfactant was determined by different biochemical assays according to standard procedures reported by Sawhney and Singh (2000). The ninhydrin test was carried out to determine the presence of amino acids and their polymer proteins, the anthrone test was performed to determine the presence of a carbohydrate moiety in the biosurfactant sample and the saponification test was done to estimate lipid content.

In the ninhydrin test, 5 drops of ninhydrin solution were added to 5 mL of cell-free supernatant and the mixture kept for 5 min in a boiling water bath. Color formation indicated the presence of amino acids and their polymer proteins. In the anthone test, equal amounts of culture supernatant and anthrone reagent (5 mL) were mixed, and a color change indicated the presence of a carbohydrate moiety. The saponification test was carried out by mixing 5 mL of cell-free supernatant with 2 mL of 2 % NaOH solution followed by vigorous shaking (i.e. saponification).

Spectral analysis

Spectral analyses were carried out to examine the type of biosurfactant produced by determining the functional groups present in the biosurfactant. For the analysis of functional groups, the extracted biosurfactant was analyzed in a Vector-22 Fourier transform infrared (FTIR) spectrometer (Bruker Corp., Fremont, CA). The spectral region used was 4,000–400 cm^{-1} at a resolution 4 cm^{-1} using a KBr palate of 0.26-mm thickness.

Statistical analysis

All of the experiments were carried out three times and studied in triplicate. Results represent the mean ± standard deviation. One-way analysis of variance (ANOVA) with the least significant difference (LSD) test was conducted to determine the significant differences in hydrocarbon degradation efficacy of the bacterial strain at different time periods. SPSS ver. 17

Table 1 Surface tension measurements[a] of the bacterial isolate on glucose-containing mineral medium at different time intervals

Bacterial isolates	ST at 0 h	ST at 24 h of culture	ST at 48 h of culture	ST at 72 h of culture	ST at 96 h of culture	ST at 120 h of culture
C[b]	71.1±0.30	71.0±0.24	71.0±0.40	69.9±0.30	69.9±0.23	69.8±0.27
KS1	67.9±0.40	69.4±0.44	66.5±0.43	65.0±0.31	65.0±0.33	65.2±0.23
KS2[c]	59.1±0.30	48.5±0.0.25	38.5±0.30	40.2±0.25	41.7±0.15	43.7±0.25
KS3[c]	60.8±0.30	45.8±0.44	31.4±0.22	34.5±0.23	36.9±0.24	41.8±0.28
KS4	69.8±0.12	68.5±0.17	60.1±0.24	60.0±0.43	62.7±0.41	62.8±0.31
KS5	60.8±0.15	57.7±0.21	54.4±0.16	57.8±0.23	57.0±0.16	57.3±0.26
KS6	69.4±0.16	59.2±0.24	58.6±0.22	57.1±0.35	56.2±0.40	56.1±0.30
KS7[c]	62.9±0.40	47.4±0.21	33.4±0.41	37.3±0.12	41.2±0.31	44.3±0.22
KS8	68.8±0.12	60.5±0.17	54.1±0.24	57.8±0.43	60.7±0.41	62.8±0.31
KS9[c]	59.4±0.30	44.5±0.25	32.5±0.30	38.3±0.25	42.8±0.15	44.3±0.25
KS10	63.8±0.15	58.4±0.21	50.2±0.16	52.7±0.23	56.1±0.16	57.3±0.26
KS11	68.3±0.14	62.2±0.24	58.6±0.22	57.1±0.35	59.2±0.40	61.7±0.30
KS12[c]	60.9±0.41	45.4±0.23	3.3±0.27	38.4±0.32	42.4±0.41	47.1±0.12

[a] Surface tension (ST) measurements (in mN/m) are given as the mean ± standard deviation (SD) of five measurements

[b] Abiotic control

[c] Denotes the biosurfactant-producing strains

software (SPSS, Chicago, IL) was used to carry out the statistical analysis.

Results and discussion

Screening for biosurfactant-producing bacteria

A total of 12 morphologically different bacterial colonies were isolated from the collected soil samples. These 12 isolates were screened for biosurfactant production.

Drop collapse assay

The culture broths of five of the 12 bacterial isolates were able to collapse the drop of crude oil, indicating the presence of biosurfactant in the respective culture broth medium. Drop collapse, when observed, occurred almost immediately but always within 1 min of the addition of culture broth. The remaining bacterial culture broths were unable to collapse the drop of crude oil even after 1 min.

ST measurement

All five bacterial isolates able to collapse the drop of crude oil (positive drop collapse assay result) were able to reduce the ST to <45 mN/m (Table 1). According to Viramontes-Ramos et al. (2010), , isolates able to reduce the ST of the medium to ≤45 mN/M can be considered to be biosurfactant-producing microbes. Based on these results, we studied these five bacterial isolates for degradation of hydrocarbon.

Screening for the most efficient hydrocarbon-degrading bacterial strain

Among the five biosurfactant-producing bacterial isolates, bacterial strain KS2 showed the highest growth (OD_{600}) after 1 week of incubation in mineral media containing 2 % (w/v) crude oil (Fig. 2). In contrast, of all five biosurfactant-producing bacterial isolates, strain KS2 produced the lowest amount of biosurfactant when cultured in medium containing 2 % (w/v) glucose (Fig. 3). Strain KS2 showed maximum growth on culture day 7. This result indicates that the petroleum hydrocarbon was best utilized by the KS2 strain through degradation and establishes that not all efficient biosurfactant producers are good degraders

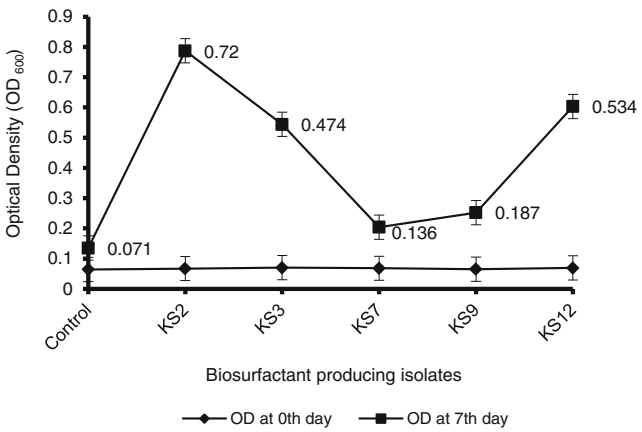

Fig. 2 Growth characterization of biosurfactant-producing isolates in mineral medium containing crude oil as the carbon source. *Bars* Standard error (SE) of three determinations

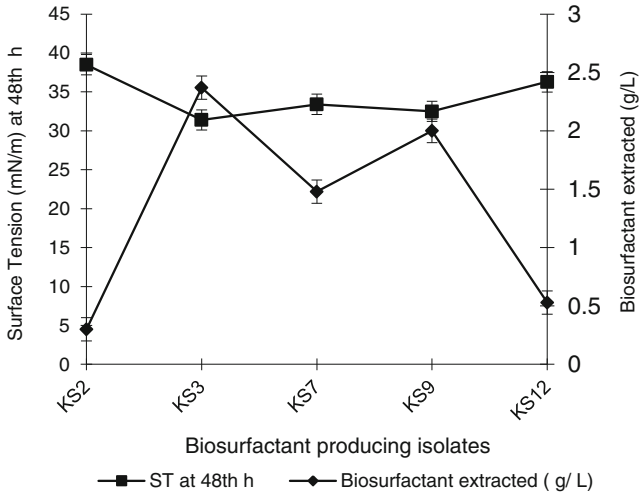

Fig. 3 Surface tension (*ST*; mN/m) reduction and biosurfactant extraction (g/L) at 48 h of culture of biosurfactant-producing isolates in mineral medium with 2 % (w/v) glucose. *Bars* SE of three determinations

of petroleum hydrocarbons—i.e. biosurfactant production/hydrocarbon degradation is dependent upon the nature and properties of the respective strain. At 48 h of culture, bacterial strain KS3 produced the highest amount of biosurfactant (2.37 g/L), but bacterial growth in crude oil-containing media on culture day 7 was 0.474 (OD_{600}). In comparison, bacterial strain KS2 produced the lowest amount of biosurfactant (0.3 g/L) at 48 h of incubation, but showed its maximum growth in crude oil-containing media at culture day 7 was 0.720 (OD_{600}) (Figs. 2, 3). Based on these results, we selected strain KS2 for further degradation studies.

Identification of the most efficient isolate

The sequence of bacterial strain KS2 was submitted to the NCBI GenBank database under accession no. gb|KF021245| and a BLAST search was conducted to compare this sequence with existing sequences. The results revealed that strain KS2 has a maximum similarity with *Bacillus pumilus* strain NBGD45 (accession no. gb|HQ003450|) and *Bacillus pumilus* strain DSX6 (accession no. gb| JN037409|).

Degradation of total petroleum hydrocarbons

The degradation of total petroleum hydrocarbon (TPH) by bacterial strain KS2 at different time intervals is presented graphically in Fig. 4. There was a trend towards increasing TPH degradation with increasing length of culture up to the fourth week of culture, with maximum TPH degradation (80.45 %) at the fourth week of incubation and minimum TPH degradation (51.95 %) at first week of incubation. The differences in TPH degradation values at different time period was found to be statistically significant, although there was no significant increase in the value after fourth week of incubation (ANOVA LSD test, $p<0.05$).

Biosurfactants can enhance the degradation of hydrocarbons by two mechanisms, namely by increasing substrate bioavailability for the microorganisms and by interacting with the surface of the bacterial cell to increase the hydrophobicity of the surface, thereby allowing hydrophobic substrates to associate more easily with bacterial cells (Mulligan and Gibbs 2004). By reducing surface and interfacial tensions, biosurfactants increase the surface areas of insoluble compounds, leading to an increased mobility and bioavailability of hydrocarbons. Taken together, biosurfactants ultimately enhance the biodegradation and removal of hydrocarbons. Therefore, the addition of biosurfactant-producing bacteria to a culture system can be expected to enhance hydrocarbon biodegradation by mobilization, solubilization or emulsification (Nguyen et al. 2008; Déziel et al. 1996; Nievas et al.

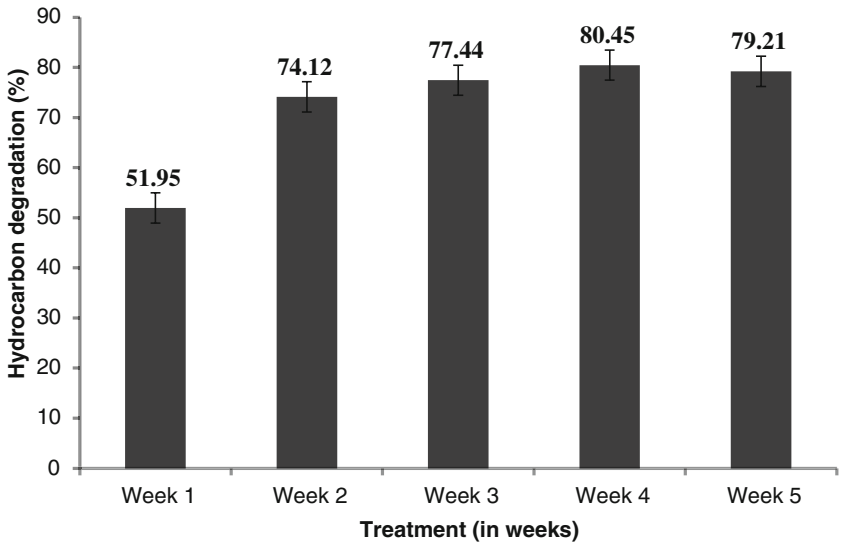

Fig. 4 Percentage degradation of total petroleum hydrocarbons by bacterial strain KS2 at different time points during the incubation period. *Bars* Standard deviation (SD) of three determinations

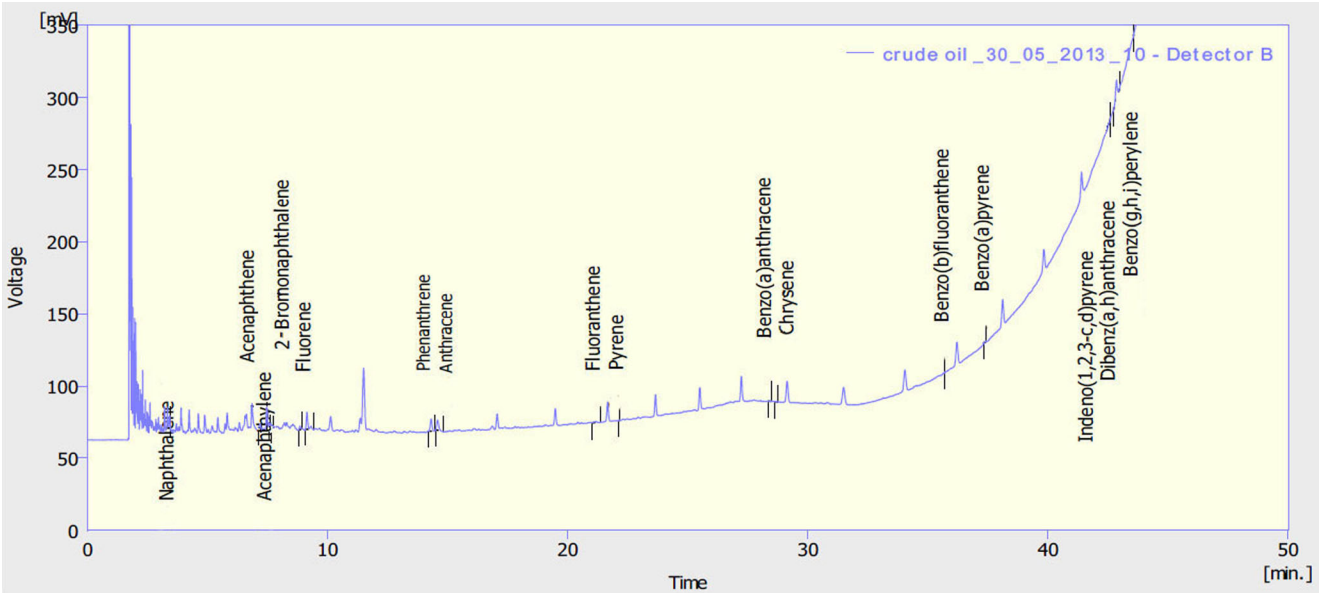

Fig. 5 Gas chromatograph of abiotic control at culture week 4

2008). Kumari et al. (2012) reported that under their respective optimal culture conditions biosurfactant-producing strains *Pseudomonas* sp. BP10 and *Rhodococcus* sp. NJ2 degraded 60.6 and 49.5 % of TPH, respectively, after 30 days of incubation in minimal salt media containing 2 % of crude oil. Based on the results of their comparative study on the biosurfactant activity of crude oil-degrading bacteria and its correlation to TPH degradation, Phan et al. (2013) reported that *Rhodococcus* sp. UKMP-7T, *Rhodococcus* sp. UKMP-5T, *Pseudomonas aeruginosa* UKMP-14T and *Acinetobacter baumanii* UKMP-12T degraded 93.3±1.0, 62.4±2.6, 75.2±0.6 and 62.8±0.5 % of TPH, respectively.

Degradation of PAHs

All 16 PAHs tested were detected in the chromatogram of the contol crude oil sample (Fig. 5) which was compared with the chromatogram of the bacterial treated experimental sample. Following treatment with the KS2 strain, nine of the PAHs present in the control had been degraded, including naphthalene, acenaphthene, acenaphthylene, 2-bromonaphthalene, fluorene, benzo[a]pyrene, indeno[1, 2, 3-c, d]pyrene, dibenz[a, h]anthracene and benzo[g, h, i]perylene (Fig. 6).

A large number of microorganisms are reported to use two- and three-ringed PAHs as the source of carbon and

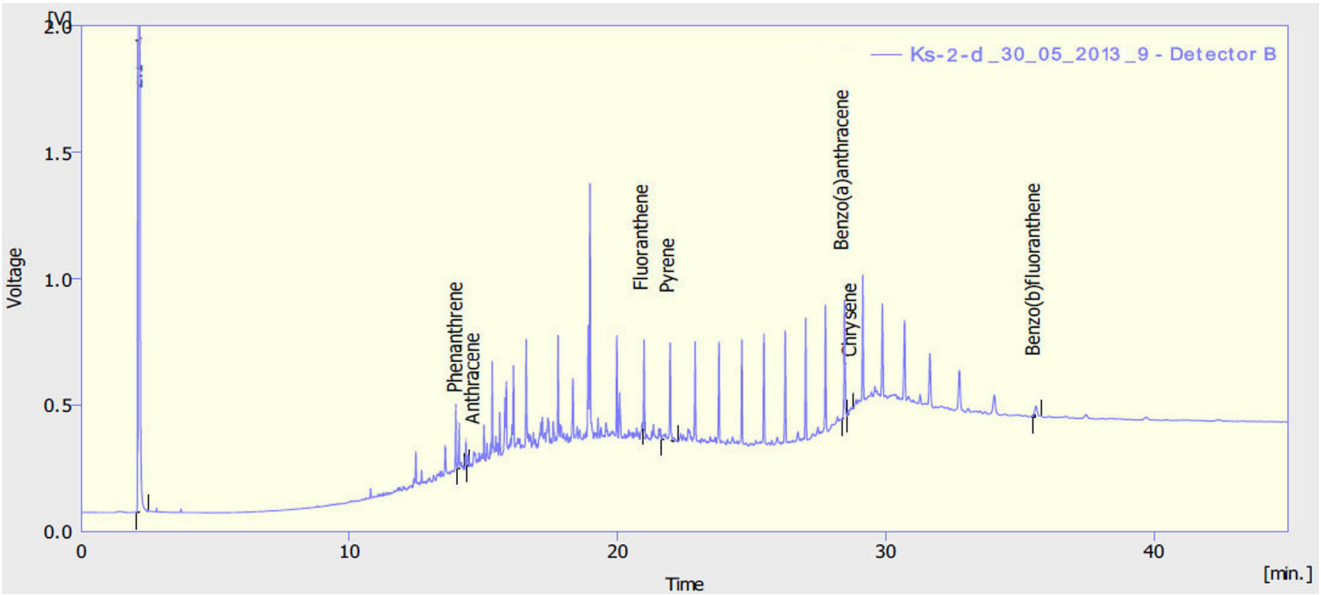

Fig. 6 Gas chromatograph of crude oil treated with strain KS2 at culture week 4

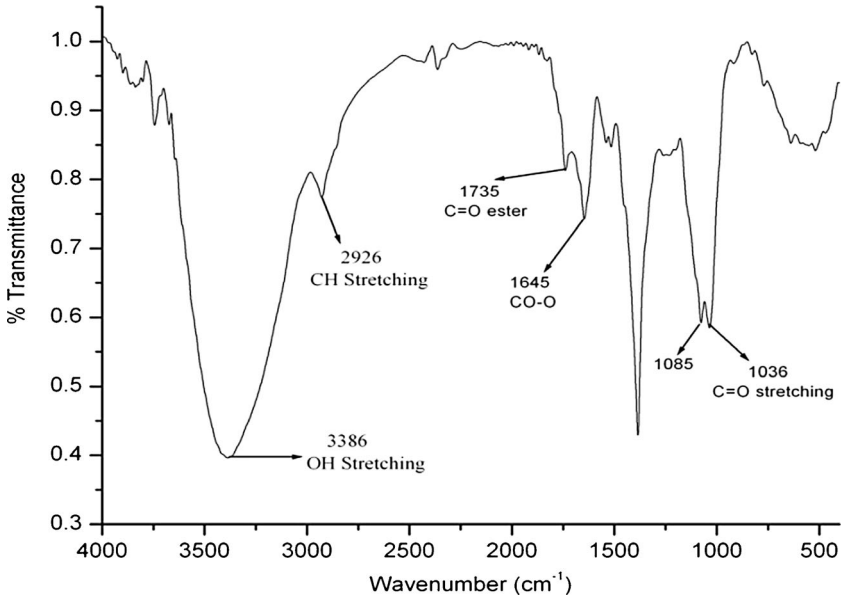

Fig. 7 Fourier transform infrared spectroscopy spectra of the crude biosurfactant obtained from strain KS2

energy (Bamforth and Singleton 2005). Only limited numbers of microorganisms are capable of degrading PAHs with four or more fused aromatic rings (Harayama 1997). Kelley and Cernigilia (1995) reported the degradation of benzo[a]pyrene in a mixture of PAHs by *Mycobacterium* sp., and Ye et al. (1996) reported that a *Sphingomonas paucimobilis* strain can degrade the five-ring PAH dibenz[a,h]anthracene and benzo[b]fluoranthene. In our study, the experimental strain *Bacillus pumilus* KS2 showed a wide range of PAH degrading ability, ranging from two-ringed structures to six-ringed fused structures, i.e., it was able to degrade four PAHs with a two-ringed structure (naphthalene, acenaphthene, acenaphthylene, 2-bromonaphthalene), one PAH with a three-ringed structure (fluorine), two PAHs with a five-ringed structure (benzo[a]pyrene and dibenz[a,h]anthracene) and two PAHs with a six-ringed structure (indeno[1,2,3-c,d]pyrene and benzo[g,h,i]perylene).

Extraction of crude biosurfactant

The yield of biosurfactant from strain KS2 was 0.3 g/L. The color of the crude biosurfactant was light honey.

Characterization of biosurfactant

Biochemical

Ruhemann's purple complex formation was absent in the ninhydrin test, indicating the absence of amino acids or proteins in the biosurfactant. In the anthrone test for carbohydrates, however, the formation of a blue-green color was observed, indicating the presence of carbohydrates in the sample. In the saponification test, NaOH saponified the lipids present in the biosurfactants, indicating the presence of lipids. These results indicate that the crude biosurfactant produced by bacterial strain KS2 contains sugar and lipid molecules but not protein molecules.

FTIR analysis

In the spectrum for crude biosurfactant from strain KS2 (Fig. 7), strong absorption band was observed at 3,386 cm^{-1} and at 2,926 cm^{-1}. Carbonyl stretching band was found at 1,735 cm^{-1}. Absorption bands at 1,645 cm^{-1} and 1,036 cm^{-1} were also observed in the spectrum. Comparison of FTIR spectrum with Bordoloi and Konwar (2009) and Thenmozhi et al. (2011) revealed the presence of different functional groups in the biosurfactant sample. Strong and broad band of the hydroxyl group (−OH) free stretch was observed at 3,386 cm^{-1} which was due to the presence of hydrogen bonding. The absorption band observed at 2,926 cm^{-1} confirmed the presence of C-H stretching vibrations of hydrocarbon chain of alkyl (CH_2– CH_3) groups. Carbonyl stretching band found at 1,735 cm^{-1} was characteristic for ester compounds. The absorption at 1,645 cm^{-1} was because of stretching of COO^- group. The spectrum also showed absorption band at 1,036 cm^{-1} which corresponded to stretching vibration of –C-O–. The pattern of absorption bands observed in FTIR analysis indicates that some polysaccharide or polysaccharide-like substances are present in the biosurfactant. So, from the above discussion it can be concluded that the biosurfactant is of glycolipid in nature.

Conclusion

We report here the isolation of a new biosurfactant-producing/hydrocarbon-degrading bacterial strain (*Bacillus pumilus* KS2) from crude oil-contaminated soil of the Lakowa oil field, Upper Assam, India. The biosurfactant produced was glycolipid in nature. Although the KS2 strain produced relatively less biosurfactant than the other biosurfactant-producing isolates, it showed maximum growth on crude oil, indicating maximum degradation of crude oil and PAHs. Therefore, we conclude that the degradation capacity of this bacterium is strain specific but does not depend on the biosurfactant production. Strain KS2 showed excellent degradability against a number of very complex PAHs present in crude oil, which has not been reported previously. Therefore, this strain could be used for the decontamination of the sites contaminated with toxic pollutants of PAHs. Further studies are underway to scale up growth conditions for better degradation. However, field trials are necessary to ascertain the laboratory-scale findings which indicate that this bacterial strain has a potential use in cleaning up hydrocarbon-contaminated sites.

Acknowledgments The authors would like to thank the Director, Institute of Advanced Study in Science and Technology (IASST), Guwahati, India for providing laboratory facilities and encouraging the research. Kaustuvmani Patowary is also grateful to the Department of Science and Technology, Govt. of India for providing assistance as a Junior Research Fellow (JRF) to carry out the research work. We also thank Dr. N. Sen Sarma, Associate Professor, and Dr. A. Devi, Assistant Professor, IASST, for their assistance with the FTIR and GC analysis, respectively, at IASST, Guwahati. We expression our appreciation to Dr. Hemen Deka, RA, IASST, for the statistical analysis and Rajeev K. Brahma, Tezpur University, for the phylogenetic analysis.

References

Atlas RM (1981) Microbial degradation of petroleum hydrocarbons: an environmental perspective. Microbiol Rev 45(1):180–209

Bamforth SM, Singleton I (2005) Bioremediation of polycyclic aromatic hydrocarbons: current knowledge and future directions. J Chem Technol Biotechnol 80:723–736

Banat IM (1995) Biosurfactant production and possible uses in microbial enhanced oil recovery and oil pollution remediation: review. Bioresour Technol 55:1–12

Bodour AA, Miller-Maier RM (1998) Application of a modified drop-collapse technique for surfactant quantitation and screening of biosurfactant-producing microorganisms. J Microbiol Methods 32: 273–280

Bordoloi NK, Konwar BK (2009) Bacterial biosurfactant in enhancing solubility and metabolism of petroleum hydrocarbons. J Hazard Mater 170:495–505

Das P, Mukherjee S, Sen R (2008) Improved bioavailability and biodegradation of a model polyaromatic hydrocarbon by a biosurfactant producing bacterium of marine origin. Chemosphere 72:1229–1234

Déziel E, Paquette G, Villemur R, Lepine F, Bisaillon J (1996) Biosurfactant production by a soil *Pseudomonas* strain growing on polycyclic aromatic hydrocarbons. Appl Environ Microbiol 62:1908–1912

Francy DS, Thomas JM, Raymond RL, Ward CH (1991) Emulsification of hydrocarbons by subsurface bacteria. J Ind Microbiol 8:237–246

Ganeshalingam S, Legge RL, Anderson WA (1994) Surfactant enhanced leaching of poly aromatic hydrocarbons from soil. Trans Inst Chem Eng 72:247–251

George S, Jayachandran K (2009) Analysis of Rhamnolipid biosurfactants produced through submerged fermentation using orange fruit peelings as sole carbon source. Appl Biochem Biotechnol 158:694–705

Georgiou G, Lin S, Sharma MM (1992) Surface-active compounds from microorganisms. Biotechnology 10:60–65

Gilewicz M, Ni'matuzahroh T, Nadalig H, Budzinski H, Doumenq P, Michotey V, Bertrand JC (1997) Isolation and characterization of a marine bacterium capable of utilizing 2-methylphenanthrene. Appl Microbiol Biotechnol 48:528–533

Harayama S (1997) Polycyclic aromatic hydrocarbon bioremediation design. Curr Opin Biotechnol 8:268–273

Hickey AM, Gordon L, Dobson AD, Kelly CT, Doyle EM (2007) Effect of surfactants on fluoranthene degradation by *Pseudomonas alcaligenes* PA-10. Appl Microbiol Biotechnol 74(4):851–856

Jacques RJS, Okeke BC, Bento FM, Peralba MCR, Camargo FAO (2007) Characterization of a polycyclic aromatic hydrocarbon-degrading microbial consortium from a petrochemical sludge land farming site. Biorem J 11:1–11

Kelley I, Cernigilia CE (1995) Degradation of a mixture of high-molecular-weight polycyclic aromatic hydro-carbons by a *Mycobacterium* strain PYR-1. J Soil Contam 4:77–91

Khanna P, Goyal D, Khanna S (2011) Pyrene degradation by *Bacillus pumilus* isolated from crude oil contaminated soil. Polycycl Aromat Compd 31(1):1–15

Klug MJ, Markovetz AJ (1971) Utilization of aliphatic hydrocarbons by microorganism. Adv Microb Physiol 5:1–4

Kumari B, Singh SN, Singh DP (2012) Characterization of two biosurfactant producing strains in crude oil degradation. Process Biochem 47:2463–2471

Ma B, Chen H, He Y, Xu JM (2010) Isolations and consortia of PAH-degrading bacteria from the rhizosphere of four crops in PAH contaminated field. In: 19th World Congress of Soil Science, Soil Solutions for a Changing World. Brisbane, pp 63–66 (published on DVD)

Mandri T, Lin J (2007) Isolation and characterization of engine oil degrading indigenous microorganisms in Kwazulu-Natal, South Africa. Afr J Biotechnol 6(1):23–27

Mittal A, Singh P (2009) Isolation of hydrocarbon degrading bacteria from soils contaminated with crude oil spills. Indian J Exp Biol 47:760–765

Mulligan CN, Gibbs BF (2004) Types, production and applications of biosurfactants. Proc Indian Natl Sci Acad 70:31–55

Nguyen TT, Youssef NH, Mclnerney MJ, Sabatini DA (2008) Rhamnolipid biosurfactant mixtures for environmental remediation. Water Res 42:1735–1743

Nie M, Yin X, Ren C, Wang Y, Xu F, Shen Q (2010) Novel rhamnolipid biosurfactants produced by a polycyclic aromatic hydrocarbon degrading bacterium *Pseudomonas aeruginosa* strain NY3. Biotechnol Adv 28(5):635–643

Nievas ML, Commendatore MG, Estevas JL, Bucalá V (2008) Biodegradation pattern of hydrocarbons from a fuel oil-type complex residue by an emulsifier producing microbial consortium. J Hazard Mater 154:96–104

Nilsson WB, Strom MS (2002) Detection and identification of bacterial pathogens of fish in kidney tissue using terminal restriction length polymorphism (T-RFLP) analysis of 16S rRNA genes. Dis Aquat Org 48:175–185

Oberbremer A, Müller-Hurtig R, Wagner F (1990) Effect of the addition of microbial surfactants on hydrocarbon degradation in a soil population in a stirred reactor. Appl Microbiol Biotechnol 32(4):485–489

Olivera NL, Commendatore MG, Delgado O, Esteves JL (2003) Microbial characterization and hydrocarbon biodegradation

potential of natural bilge waste microflora. J Ind Microbiol Biotechnol 30(9):542–548

Pacwa-Plociniczak M, Plaza GA, Piotrowska-Seget Z, Cameotra SS (2011) Environmental applications of biosurfactants: recent advances. J Mol Sci 12:633–654

Phan CW, Bakar NFA, Hamzah A (2013) A comparative study on biosurfactant activity of crude oil-degrading bacteria and its correlation to total petroleum hydrocarbon degradation. Biorem J 17(4): 240–251

Rahman KSM, Banat IM, Thahira J, Thayumanavan T, Lakshmanaperumalsamy P (2002) Bioremediation of gasoline contaminated soil by a bacterial consortium amended with poultry litter, coir pith and rhamnolipid biosurfactant. Bioresour Technol 81:25–32

Saikia RR, Deka S, Deka M, Banat IM (2012) Isolation of biosurfactant-producing *Pseudomonas aeruginosa* RS29 from oil-contaminated soil and evaluation of different nitrogen sources in biosurfactant production. Ann Microbiol 62:753–763

Samanta SK, Singh OV, Jain RK (2002) Polycyclic aromatic hydrocarbons: environmental pollution and bioremediation. Trends Biotechnol 20(6):243–248

Sawhney SK, Singh R (2000) Introductory practical biochemistry. Narosa Publishing House, New Delhi, pp 16–17

Singh C, Lin J (2008) Isolation and characterization of diesel oil degrading indigenous microorganisms in Kwazulu-Natal, South Africa. Afr J Biotechnol 7(12):1927–1932

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 28(10):2731–2739

Thenmozhi R, Sornalaksmi AN, Nagasathya A, Praveenkumar D, Thajuddin N (2011) Characterisation of biosurfactant produced by bacterial isolates from engine oil contaminated soil. Adv Environ Biol 5(8):2402–2408

Toledo FL, Calvo C, Rodelas B, Lez-Lopez GJ (2006) Selection and identification of bacteria isolated from waste crude oil with polycyclic aromatic hydrocarbons removal capacities. Syst Appl Microbiol 29:244–252

Viramontes-Ramos S, Portillo-Ruiz MC, Ballinas-Casarrubias ML, Torres-Muñoz JV, Rivera-Chavira BE, Nevárez-Moorillón GV (2010) Selection of biosurfactant/bioemulsifier-producing bacteria from hydrocarbon contaminated soil. Braz J Microbiol 41:668–675

Wong JWC, Fang M, Zhao Z, Xing B (2004) Effect of surfactants on solubilization and degradation of phenanthrene under thermophilic conditions. J Environ Qual 33:2015–2025

Ye D, Siddiqi MA, Maccubbin AE, Kumar S, Sikka HC (1996) Degradation of polynuclear aromatic hydro-carbons by *Sphingomonas paucimobilis*. Environ Sci Technol 30:136–142

Yuliani H, Sahlan M, Hermansyah H, Wijanarko A (2012) Selection and identification of polyaromatic hydrocarbon degrading bacteria. World Appl Sci J 20(8):1133–1138