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ORIGINAL ARTICLE

Differential utilization of pyrene as the sole source of carbon by *Bacillus subtilis* and *Pseudomonas aeruginosa* strains: role of biosurfactants in enhancing bioavailability

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Keywords
Bacillus subtilis, biosurfactant, *Pseudomonas aeruginosa*, pyrene biodegradation.

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Abstract

Aims: Our goal is to compare the efficiency of utilization of pyrene as the sole source of carbon for growth and energy by two nonactinomycetous groups of bacteria viz., *Bacillus subtilis* DM-04 and *Pseudomonas aeruginosa* mucoid (M) and nonmucoid (NM) strains, isolated from a petroleum-contaminated soil sample of north-east India.

Methods and Results: *Bacillus subtilis* DM-04 and *P. aeruginosa* M and NM bacterial strains were capable of secreting biosurfactant in the culture medium while growing on pyrene and their pyrene utilizing efficiency was demonstrated by correlating the bacterial growth in the presence of pyrene as the sole source of carbon along with a concomitant decrease in pyrene content from the culture medium with respect to time. The biosurfactant secreted by the respective bacterial strains enhanced the apparent solubility of pyrene by factors of 5–7 and influenced the bacterial cell surface hydrophobicity resulting in higher uptake and utilization of pyrene by bacteria. The growth of *B. subtilis* DM-04 and *P. aeruginosa* M and NM strains at the expense of pyrene after 96 h showed an assimilation of about 48·0 ± 1·1% (mean ± SD) and 32·0 ± 0·6% (mean ± SD) of pyrene carbon, respectively, showing differences in metabolism of pyrene by these bacterial strains.

Conclusions: *Bacillus subtilis* DM-04 strain exhibited higher utilization and cellular assimilation of pyrene compared with *P. aeruginosa* M and NM strains. Further, the biosurfactants produced by the bacteria under study are capable of enhancing the solubility of pyrene in aqueous media and can influence the cell surface hydrophobicity of the biosurfactant-producing strains that results in a higher uptake of pyrene.

Significance and Impact of the Study: It may be suggested that the bacteria used in this study are suitable candidates for practical field application for effective *in situ* bioremediation of pyrene-contaminated sites.

Introduction

Polycyclic aromatic hydrocarbons (PAHs) produced as a consequence of fossil fuel utilization, combustion process and chemical manufacture (Evans *et al.* 1990; Christensen and Zhang 1993), represent an important class of environmental pollutants owing to their toxic nature and carcinogenic potential (International Association of Research Chemists 1983). Further, due to the hydrophobic nature of PAHs, most of these compounds in aquatic and terrestrial ecosystems bind to particulates in soil and sediments, rendering them available for biological uptake resulting in their accumulation in food chains that imposes a severe health threat to human populations (Morehead *et al.* 1986). Pyrene represents one of the most abundant high-molecular weight pericondensed PAHs,

possessing mutagenic and carcinogenic potential, and therefore its removal from the environment is a challenging task for scientists.

Degradation of pyrene or other PAHs in the environment occurs predominantly by microbial processes and therefore during recent years interest in the utilization of bacterial processes for the treatment of PAH-contaminated soil and ground waters has increased significantly (Gibson and Sayler 1992; Sims *et al.* 1993; Boonchan *et al.* 2000; Feitkenhauer *et al.* 2003). However, it has been shown that biodegradation of high-molecular weight compounds with four or more rings, such as pyrene, are more difficult than low-molecular weight compounds (Chen and Aitken 1999). This problem has been aggravated further due to the low bioavailability of pyrene or other PAHs to biodegrading microbes owing to their hydrophobic nature (Mihelcic *et al.* 1993). To overcome this problem, several researchers have investigated the addition of chemical surfactant or biosurfactant in culture medium to enhance the biodegradation of hydrophobic substrates (Zhang and Miller 1992; Miller and Zhang 1997; Barkay *et al.* 1999). This has stimulated research aimed at isolating and characterizing biosurfactant-producing microbial cultures able to degrade and/or utilize pyrene and other PAHs (Heitkamp *et al.* 1988; Foght and Westlake 1988; Feitkenhauer *et al.* 2003; Guo *et al.* 2005).

Although several reports are available on the degradation of pyrene by the actinomycetes group of bacteria (Kanaly and Harayama 2000; Vila *et al.* 2001; Guo *et al.* 2005), a survey of the literature shows that there is a dearth of knowledge on the biodegradation of pyrene by nonactinomycetes group of bacteria. Our preliminary studies have shown that three thermophilic strains of bacteria viz. *Bacillus subtilis* DM-04, and *Pseudomonas aeruginosa* mucoid (M) and nonmucoid (NM), isolated from a petroleum-contaminated soil sample from north-east India, are capable of utilizing hydrocarbons as the sole source of carbon and can secrete a large amount of biosurfactant while growing on a hydrocarbon-rich medium (Das and Mukherjee 2005; Mukherjee and Das 2005), a property that is suitable for field application for bioremediation of pyrene-contaminated sites. However, prior to advocating *in situ* field application, there is an urgent need to explore further the pyrene utilization capacity of these bacteria in a laboratory bench scale study.

This has prompted us to investigate and compare the pyrene utilization efficiency of *B. subtilis* DM-04 and *P. aeruginosa* M and NM strains. There is hardly any information on pyrene biodegradation by a *B. subtilis* strain and the biodegradation potential of pyrene by *Pseudomonas* sp. isolated from Indian subcontinent has never explored. Further, major biosurfactant secretion by *B. subtilis* DM-04 is lipopeptide in nature containing higher amount of surfactins compared with iturins (Mukherjee and Das 2005), whereas biosurfactant secreted by *P. aeruginosa* M and NM is found to be a complex mixture of lipopeptides and glycoproteins (Das and Mukherjee 2005). Therefore, our second objective was to explore the role played by the different groups of biosurfactant in enhancing the apparent solubility and further utilization of pyrene by the biosurfactant-producing bacterial strain.

Materials and methods

Chemicals and media

Pyrene was purchased from Merck-Schuchardt, Germany. Acetone and hexane were purchased from Merck India Ltd, India. All other chemicals used were of analytical grade with high purity.

Micro-organisms

Isolation, characterization and taxonomic identification of biosurfactant-producing micro-organisms viz., *B. subtilis* DM-04, *P. aeruginosa* M and NM strains were done by following the standard biochemical and morphological tests, and by gas-chromatographic (GC) analysis of bacterial cellular fatty acid methyl esters (Das and Mukherjee 2005; Mukherjee and Das 2005). These bacteria, isolated from a crude petroleum oil-contaminated soil sample from north-east India, were capable of secreting biosurfactant at thermophilic growth conditions (Das and Mukherjee 2005; Mukherjee and Das 2005). They were subcultured on nutrient agar plates before use as inocula for pyrene degradation study.

Media and microbial growth in presence of pyrene

The composition of mineral-salts medium used in this study was described previously (Das and Mukherjee 2005). To measure the microbial growth at the expense of pyrene as the sole source of carbon, this compound dissolved in *N*′,*N*′-dimethylformamide (5% w/v) was added in excess to sterile mineral-salts medium to give a final concentration of 250 mg 100 ml^{-1} of culture. The flasks were shaken at 200 rev min^{-1} and 37°C before inoculation for the removal of volatile *N*′,*N*′-dimethylformamide. If glucose was used as the sole or co-carbon source, it was supplied to the culture medium at a final concentration of 100 mg l^{-1}.

Three replicate batch cultures were grown in 250-ml Erlenmeyer flasks containing 100 ml of mineral-salts medium supplemented with either pyrene or glucose, or pyrene and glucose as carbon source(s). Although these bacteria are capable of growing in a wide range of tem-

peratures (25–60°C), pyrene degradation study was performed at their optimum growth conditions, viz. at 45°C temperature and pH 7·0 for *P. aeruginosa* M and NM strains (Das and Mukherjee 2005) or at 55°C temperature and pH 8·0 for *B. subtilis* DM-04 strain (Mukherjee and Das 2005) with 200 rev min^{-1} rotary shaking. Uninoculated flasks and flasks without pyrene served as controls. Pyrene and protein concentrations were measured after 24, 48 and 96 h of microbial growth.

Determination of bacterial growth in presence of pyrene

Bacterial growth by utilizing pyrene was assessed by measuring the bacterial cell population, protein concentration (Lowry *et al.* 1951), dry biomass and the residual pyrene remained in the culture medium (see below). Bacterial dry biomass was determined by gravimetric method after extracting the residual pyrene from the culture medium followed by pelleting and drying the cells (Makkar and Cameotra 1998).

Determination of pyrene biodegradation

Pyrene biodegradation by bacteria was determined by quantifying the amount of pyrene remaining in the culture medium at different time intervals. The residual pyrene in the flasks was extracted with a mixture of chloroform and methanol (v/v = 20 : 10 ml) (Zhang *et al.* 2004). One millilitre of extracted samples was then filtered through a 0·45-μm pore size filter and 20 μl of the filtrate was then analysed for pyrene content by high-performance liquid chromatography (HPLC) on a Waters reverse-phase C_{18} Nova pak column (3·9 × 150 mm; Waters, Vienna, Austria) by using isocratic elution with acetonitrile–water (Pickard *et al.* 1999). Flow rate was adjusted to 1·0 ml min^{-1} and elution of pyrene was monitored at 273 nm. The amount of pyrene remaining in the experimental flasks was estimated by measuring and comparing the HPLC peak area of the eluted pyrene from the experimental and control flasks. Pyrene concentration was expressed as mean and standard deviations based on the results obtained with triplicate flasks. Quantity of pyrene remaining in the control (uninoculated) flasks at different time intervals was considered as 100% amount and other values were compared with that.

Measurement of pyrene uptake by bacterial cells

Pyrene uptake by resting bacterial cells was measured by spectrophotometric rate assay as described previously (Stringfellow and Aitken 1995). Briefly, $1·0 \times 10^7$ bacterial cells (final volume of 3·0 ml in 20 mmol l^{-1} phosphate buffer containing 150 mmol l^{-1} NaCl, pH 7·0) were placed in a 3·5-ml quartz cuvette in a U-2001 UV/Vis Spectrophotometer (Hitachi, San Jose, CA, USA) and 60 ng of pyrene (in 10 μl of acetone) was injected into the cuvette. A decrease in A_{273} was measured from 0 s to 30 min postaddition of pyrene. A control experiment was run in parallel where 10 μl of acetone (without pyrene) was added. Pyrene uptake was also measured in the presence of a suspension of killed cells. From a standard curve of pyrene, decrease in pyrene content was calculated and results were expressed as nanograms of pyrene uptake by $1·0 \times 10^7$ bacterial cells.

To study the bacterial uptake of biosurfactant-solubilized pyrene, stock solution of pyrene (in acetone) was incubated with biosurfactants (final concentration 0·5 mg ml^{-1}) from the respective bacterial strains overnight at 30°C (see *Pyrene solubilization assay*) and then 60 ng of biosurfactant-solubilized pyrene was injected into the cuvette containing $1·0 \times 10^7$ bacterial cells (final volume of 3·0 ml). Pyrene uptake measurement was done in the same manner as described above.

Pyrene solubilization assay

Pyrene solubilization assays were done as described by Barkay *et al.* (1999). Briefly, 60 μg of pyrene (from 6 mg ml^{-1} stock in hexane) was distributed into glass test tubes (10 × 170 mm) and kept open inside an operating chemical fume hood to remove the solvent. Then, 3·0 ml of assay buffer (20 mmol l^{-1} Tris–HCl, pH 7·0) and 1·0 ml of biosurfactant solution (0·5 mg ml^{-1}) were added. Tubes were capped with plastic closures and incubated in a vertical position overnight at 30°C with shaking (200 rev min^{-1}) in the dark. Samples were filtered through 1·2-μm filters (Whatman, Clifton, NJ, USA), 2·0 ml was removed in a clean test tube to which 2·0 ml of hexane was added prior to extraction by vortexing for 2 min. This emulsion was centrifuged at 9450 ***g*** for 10 min to separate the aqueous and hexane phases. Pyrene in the hexane extracts was measured spectrophotometrically at 273 nm and from a calibration curve of pyrene in hexane, the concentration of pyrene was determined. Control experiments were also run in parallel where no biosurfactant was added to pyrene before extraction with hexane.

Isolation of biosurfactant and determination of surface active properties

Biosurfactant from *B. subtilis* DM-04 strain was isolated by acid precipitation of cell-free fermentative broth followed by its extraction with dichloromethane or methanol (Mukherjee and Das 2005). Biosurfactant from *P. aeruginosa* M and NM strains was isolated by acetone precipitation followed by acidification by 6·0 N HCl (Das

and Mukherjee 2005). The biosurfactants thus obtained were dried in vacuum, weighed and expressed in grams per litre of the cell free culture broth. Surface tension of the culture medium was determined using a Du-Nouy Tensiometer (Kruss 9KT Tensiometer, Kruss, Germany) at room temperature (25°C) using the ring correction mode of the instrument (Das and Mukherjee 2005).

Cell surface hydrophobicity test

Affinity of bacterial cells towards pyrene was measured by determining the changes in cell surface hydrophobicity during growth in mineral-salts medium with pyrene as a substrate (Mukherjee and Das 2005). The cell surface hydrophobicity was expressed in terms of percentage of cells transferred to the pyrene phase.

Statistical analysis

Statistical analysis was done by determining the level of significance (1–5% level) between two sets of data by Student's *t*-distribution (*P*-value) analysis. Both the confidence coefficient and degree of freedom were taken into account when using the table of the *t*-distribution (Daniel 2000).

Results

Growth characteristics of microbes at the expense of pyrene

All the microbes in the present study were able to utilize pyrene as the sole source of carbon and energy as was evident from the decrease in pyrene content from microbial cultures containing pyrene (Fig. 1) with a concomitant increase in (i) the absorbance of the culture medium at 600 nm; (ii) bacterial dry biomass and (iii) protein content with respect to time (Table 1). Bacterial growth (biomass, protein content and bacterial cell density) was negligible when grown in absence of pyrene when compared with the growth of bacteria on pyrene medium, demonstrating further the utilization of pyrene as the sole source of carbon by bacteria (Table 1). However, the growth of *B. subtilis* DM-04 strain in pyrene medium was significantly higher ($P < 0{\cdot}01$) compared with the ability of *P. aeruginosa* M and NM strains to grow in pyrene-rich medium (Table 1). Further, the addition of 100 mg l^{-1} of glucose in the growth medium resulted in an enhancement of pyrene utilization and growth of bacteria under study (Table 1).

After 96 h of incubation with *B. subtilis* DM-04 strain, the concentration of pyrene decreased from 2·5 mg ml^{-1} (initial concentration) to 1·36 mg ml^{-1}, with a corresponding increase in *B. subtilis* DM-04 biomass from 0·3 mg l^{-1} at time of inoculation to a maximum of 1300 mg l^{-1} (Table 1). Given that carbon accounts for approximately 50% of cell dry weight (Vila *et al.* 2001) and considering that growth of *B. subtilis* DM-04 in absence of pyrene and glucose was negligible (Table 1), it indicates a conversion of approximately 48·0 ± 1·1% (mean ± SD) of pyrene as cellular carbon in *B. subtilis* DM-04 strain. Similar calculation shows cellular assimilation of pyrene in *P. aeruginosa* M and NM strains with an efficiency of 32·0 ± 0·6% (mean ± SD) post 96 h of growth.

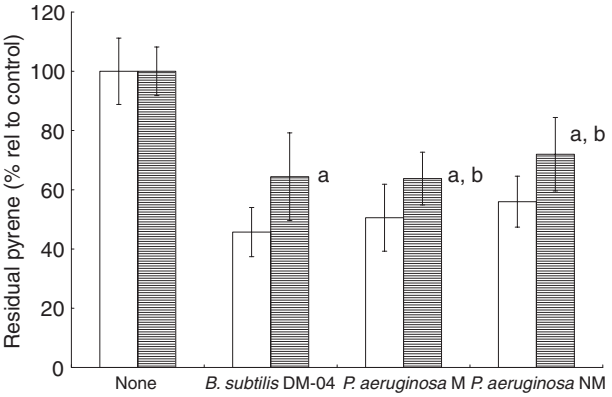

Figure 1 Comparison of pyrene biodegradation by *Bacillus subtilis* and *Pseudomonas aeruginosa* strains. Residual pyrene was extracted from the culture supernatants after 96 h of bacterial growth and quantified by reverse phase (RP)-high-performance liquid chromatography. Results represent the mean ± SD of three independent experiments. [a]Significance of difference with respect to pyrene utilization in presence of glucose ($P < 0{\cdot}01$). [b]Significance of difference with respect to pyrene utilization by *B. subtilis* DM-04 strain ($P < 0{\cdot}05$). (□) Glucose added, (▤) glucose depleted.

Biosurfactant production during microbial growth on pyrene

Observation of both surface-active properties and emulsifying activities supported that all the strains used in this study produced biosurfactant in pyrene-mineral salts medium (Table 2). The biosurfactant produced by *B. subtilis* DM-04, and *P. aeruginosa* M and NM strains reduced the surface tension of growth medium from 67·4 ± 0·5 mN m^{-1} (mean ± SD, $n = 3$) to 30·0 ± 0·2 mN m^{-1} (mean ± SD, $n = 3$), 45·9 ± 0·3 mN m^{-1} (mean ± SD, $n = 3$) and 45·0 ± 0·7 mN m^{-1} (mean ± SD, $n = 3$), respectively, post 48 h of growth in pyrene medium, demonstrating biosurfactant secreted by *B. subtilis* DM-04 was more efficient ($P < 0{\cdot}01$) than *P. aeruginosa* M and NM strains in reducing the surface tension of growth medium. The secretion of biosurfactant started after 24 h of growth and continued up to 96 h. Interestingly, as oppose to

Table 1 Bacterial growth on different carbon source(s) at different time intervals

Carbon source(s)	After 24 h			After 48 h			After 96 h		
	Dry biomass (mg l^{-1})	Cell density (cells ml^{-1})	Protein content (mg ml^{-1})	Dry biomass (mg l^{-1})	Cell density (cells ml^{-1})	Protein content (mg ml^{-1})	Dry biomass (mg l^{-1})	Cell density (cells ml^{-1})	Protein content (mg ml^{-1})
Bacillus subtilis DM-04									
None (control)*	0·3 ± 0·1	$(9·9 \pm 0·6) \times 10^5$	0·003 ± 0·001	0·3 ± 0·1	$(9·9 \pm 0·4) \times 10^5$	0·0035 ± 0·0015	0·48 ± 0·15	$(1·5 \pm 0·2) \times 10^6$	0·004 ± 0·001
Pyrene†	500 ± 50	$(1·65 \pm 0·4) \times 10^9$	0·5 ± 0·05	1000 ± 110	$(3·3 \pm 0·7) \times 10^9$	0·83 ± 0·1	1300 ± 150	$(4·29 \pm 0·2) \times 10^9$	1·07 ± 0·5
Glucose	108 ± 10	$(5·9 \pm 0·1) \times 10^8$	0·3 ± 0·04	220 ± 25	$(8·2 \pm 0·3) \times 10^8$	0·45 ± 0·08	270 ± 25	$(9·9 \pm 0·3 \times 10^8$	0·65 ± 0·1
Pyrene + glucose‡	800 ± 100	$(2·64 \pm 0·4) \times 10^9$	0·6 ± 0·08	1200 ± 130	$(3·96 \pm 0·1) \times 10^9$	1·0 ± 0·15	1500 ± 20	$(5·28 \pm 0·12) \times 10^9$	1·33 ± 0·17
Pseudomonas aeruginosa mucoid									
None (control)*	0·31 ± 0·1	$(1·0 \pm 0·2) \times 10^6$	0·003 ± 0·001	0·31 ± 0·1	$(1·0 \pm 0·1) \times 10^6$	0·0055 ± 0·0025	0·38 ± 0·1	$(1·2 \pm 0·3) \times 10^6$	0·007 ± 0·003
Pyrene	380 ± 35	$(1·25 \pm 0·15) \times 10^9$	0·38 ± 0·09	700 ± 75	$(1·3 \pm 0·2) \times 10^9$	0·65 ± 0·09	800 ± 90	$(2·64 \pm 0·11) \times 10^9$	0·75 ± 0·15
Glucose	100 ± 10	$(3·3 \pm 0·1) \times 10^8$	0·2 ± 0·05	130 ± 15	$(4·9 \pm 0·1) \times 10^8$	0·34 ± 0·06	200 ± 25	$(8·2 \pm 0·18) \times 10^8$	0·58 ± 0·12
Pyrene + glucose‡	409 ± 45	$(1·48 \pm 0·1) \times 10^9$	0·45 ± 0·05	800 ± 90	$(2·31 \pm 0·2) \times 10^9$	0·75 ± 0·1	1000 ± 150	$(3·96 \pm 0·24) \times 10^9$	1·13 ± 0·5
Pseudomonas aeruginosa nonmucoid									
None (control)*	0·3 ± 0·1	$(9·9 \pm 0·1) \times 10^5$	0·002 ± 0·001	0·3 ± 0·1	$(1·0 \pm 0·1) \times 10^5$	0·005 ± 0·0015	0·36 ± 0·1	$(1·1 \pm 0·1) \times 10^6$	0·005 ± 0·002
Pyrene	400 ± 43	$(1·3 \pm 0·1) \times 10^9$	0·35 ± 0·1	600 ± 65	$(1·3 \pm 0·1) \times 10^9$	0·6 ± 0·1	700 ± 80	$(2·31 \pm 0·1) \times 10^9$	0·7 ± 0·1
Glucose	100 ± 11	$(3·3 \pm 0·2) \times 10^9$	0·18 ± 0·02	130 ± 10	$(4·2 \pm 0·2) \times 10^8$	0·25 ± 0·05	230 ± 20	$(7·5 \pm 0·5) \times 10^8$	0·55 ± 0·15
Pyrene + glucose‡	500 ± 55	$(1·65 \pm 0·15) \times 10^9$	0·42 ± 0·13	800 ± 92	$(2·3 \pm 0·2) \times 10^9$	0·8 ± 0·1	1100 ± 100	$(3·63 \pm 0·4) \times 10^9$	1·1 ± 0·35

Values represent the mean ± SD of three individual experiments.

*Growth of all bacteria on control medium was significantly lower ($P < 0·01$) compared with growth on pyrene, glucose, or pyrene and glucose medium.

†Growth of *B. subtilis* DM-04 on pyrene was significantly higher ($P < 0·01$) compared with growth of *P. aeruginosa* M and NM strains on the same medium.

‡Growth of bacteria on pyrene and glucose medium was significantly higher ($P < 0·01$) compared with growth on individual (pyrene or glucose) carbon source.

Table 2 Production of biosurfactant by bacteria on different carbon source(s)

Carbon source(s)	Yield of biosurfactant (mg l^{-1})	
	After 48 h	After 96 h
Bacillus subtilis DM-04		
None (control)	ND	ND
Pyrene	200 ± 21	300 ± 29*,**
Glucose	150 ± 11	240 ± 12*,**
Pyrene + glucose	300 ± 34	600 ± 39*,***
Pseudomonas aeruginosa M		
None (control)	ND	ND
Pyrene	100 ± 12	200 ± 14*
Glucose	108 ± 11	100 ± 13
Pyrene + glucose	200 ± 19	500 ± 33*
Pseudomonas aeruginosa NM		
None (control)	ND	ND
Pyrene	100 ± 12	200 ± 12*
Glucose	110 ± 13	99 ± 10
Pyrene + glucose	198 ± 18	500 ± 29*

Values represent the mean ± SD of three individual experiments. Significance of difference with respect to biosurfactant production post 48 h of growth (*$P < 0{\cdot}01$). Significance of difference with respect to biosurfactant production by *P. aeruginosa* M and NM strains (**$P < 0{\cdot}01$, ***$P < 0{\cdot}05$).
ND, not determined (concentration was below the detectable limit).

B. subtilis DM-04 strain, biosurfactant production by *P. aeruginosa* M and NM strains did not increase ($P > 0{\cdot}05$) post 48 h of growth in glucose medium.

Pyrene solubilization by biosurfactant

The effect of biosurfactant on the apparent aqueous solubility of pyrene was determined by test tube solubilization assay in the presence of biosurfactant (500 μg ml^{-1}) produced by respective bacterial strains. Solubility of pyrene in presence of biosurfactants from *B. subtilis* DM-04, *P. aeruginosa* M and NM strains was significantly higher ($P < 0{\cdot}001$) than its solubility in the absence of biosurfactant (Fig. 2). Moreover, the crude biosurfactant secreted by *P. aeruginosa* NM strain showed a higher pyrene solubilization effect ($P < 0{\cdot}05$) compared with pyrene solubilization by crude biosurfactant from *P. aeruginosa* M and *B. subtilis* DM-04 strains (Fig. 2).

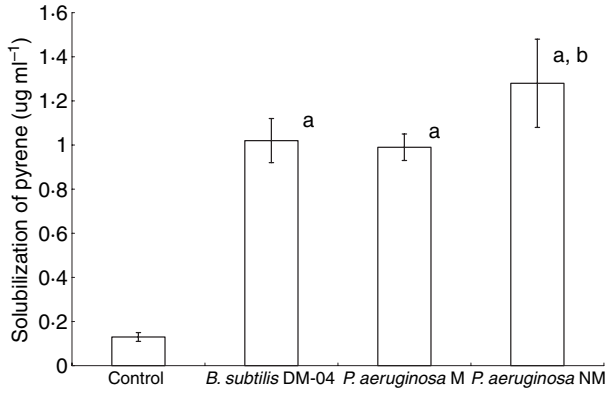

Figure 2 *In vitro* pyrene solubilization effect of crude biosurfactant (500 μg) from *Bacillus subtilis* DM-04, *Pseudomonas aeruginosa* mucoid (M) and nonmucoid (NM) strains. Results represent the mean ± SD of three independent experiments. In the control experiment, pyrene was not incubated with biosurfactant. Significance of difference with respect to pyrene solubilization in absence of biosurfactants (control) ($^aP < 0{\cdot}001$). Significance of difference with respect to pyrene solubilization by biosurfactants from *B. subtilis* DM-04 and *P. aeruginosa* M strain ($^bP < 0{\cdot}05$).

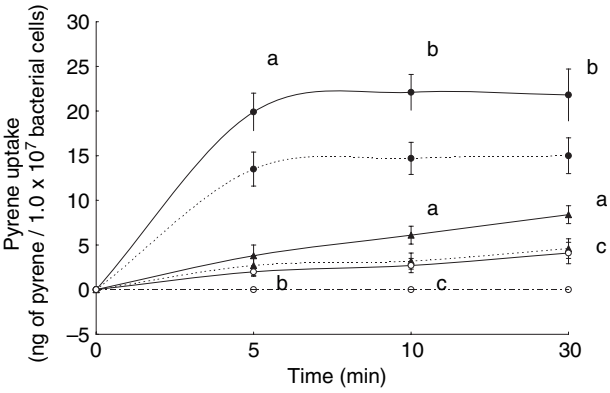

Figure 3 A comparison of uptake of unsolubilized and biosurfactant-solubilized pyrene by *Bacillus subtilis* DM-04 and *Pseudomonas aeruginosa* mucoid (M) and nonmucoid (NM) strains at different time intervals. The solid line (—) and broken line (- - - - -) indicate the incubation of bacteria with biosurfactant-solubilized or unsolubilized pyrene respectively. Results represent the mean ± SD of three individual experiments. Symbols: *B. subtilis* (●); *P. aeruginosa* M (▲); *P. aeruginosa* NM (○). Significance of difference with respect to uptake of unsolubilized pyrene. $^aP < 0{\cdot}05$, $^bP < 0{\cdot}01$ and $^cP < 0{\cdot}001$.

Role of biosurfactants in pyrene uptake

Figure 3 shows that in all three groups of bacteria, uptake of biosurfactant-solubilized pyrene was much higher when compared with the unsolubilized pyrene. Uptake of pyrene did not occur in a suspension of killed cells (control), and even if a trace quantity of biosurfactant-solubilized pyrene was adsorbed on the surface of bacterial cells, this was taken into consideration for calculating the actual pyrene uptake by bacterial cells. Interestingly, *P. aeruginosa* NM strain did not demonstrate pyrene uptake up to 30 min of incubation (Fig. 3) and 12 ng of pyrene uptake was observed 60 min postincubation with unsolubilized pyrene (data not shown). However, the addition of biosurfactant-solubilized pyrene resulted in an

increase in the rate of pyrene uptake by *P. aeruginosa* NM strain.

Bacterial cell surface hydrophobicity

The cell surface hydrophobicity exhibited by *B. subtilis* DM-04 (6·0 ± 0·3%; mean ± SD, $n = 3$) in pyrene phase was significantly higher ($P < 0·01$) compared with the same property displayed by *P. aeruginosa* M (4·0 ± 0·5%; mean ± SD, $n = 3$) and NM (3·5 ± 0·5%; mean ± SD, $n = 3$) strains showing that the former bacteria possess much more affinity towards pyrene.

Discussion

There are many reports on bacterial degradation of pyrene, mainly by actinomycetes groups of bacteria such as *Mycobacterium* and *Rhodococcus* (Kanaly and Harayama 2000; Vila *et al.* 2001). Besides, a variety of nonactinomyces bacteria such as *P. aeruginosa*, *Pseudomonas pudita* and *Flavobacterium* sp. were reported to utilize pyrene, when supplemented with other forms of organic carbon (Trzesicka- Mlynarz and Ward 1995). Moreover, soil *Pseudomonas* sp. was capable of degrading PAHs, but failed to utilize them as the sole source of carbon and energy (Foght and Westlake 1988). In contrast, we demonstrated that utilization of pyrene as the sole source of carbon and energy by *B. subtilis* DM-04 and *P. aeruginosa* strains is possible, perhaps owing to their ability to produce biosurfactants in the culture medium. We have provided further evidence of utilization of pyrene by *Bacillus* and *Pseudomonas* bacteria as the sole source of carbon by demonstrating the correlation between increase in bacterial growth (dry biomass, protein concentration and bacterial cell density) and a concomitant decrease (biodegradation) in pyrene content from the culture medium with respect to time.

Survival of micro-organisms in pyrene medium after their inoculation is a key deciding factor in the rate of biodegradation of this compound either in soil or in liquid phase. As all the bacteria in the present study were isolated from a crude petroleum oil-contaminated (hydrocarbons rich) soil sample, they survived and adopted to the pyrene-rich liquid environment very easily, which was evident from the significant increase ($P < 0·01$) in the population of *B. subtilis* and *P. aeruginosa* strains in pyrene medium when compared with control. Further, the optimum growth temperature range of these bacteria is well suited for *in situ* bioremediation application in the soil of the tropical countries like India, where the outdoor temperature may reach up to 50°C.

The growth (dry biomass yield) of *B. subtilis* DM-04 and *P. aeruginosa* M and NM strains at the expense of pyrene after 96 h of inoculation indicated a greater ($P < 0·01$) assimilation of pyrene carbon in *B. subtilis* DM-04 compared with other two bacteria under study, showing differences in utilization of pyrene by these bacterial strains, isolated from the same soil. Therefore, we agree with the finding of Vila *et al.* (2001) that all pyrene lost from the growth medium cannot be considered assimilated because some of it must have been respired. Moreover, our result is also in good agreement with the findings of many other researchers, who observed that microbial growth on pyrene did not result in complete removal of the substrate (Mulder *et al.* 1998; Vila *et al.* 2001). Observed attenuation in pyrene degradation occurred presumable by biofilm formation on pyrene crystals and consequent prevention of pyrene dissolution, while previously accumulated intermediates would allow further cell growth (Vila *et al.* 2001).

The biochemical pathway for the biodegradation of pyrene by microbes has been proposed (Cerniglia 1992; Vila *et al.* 2001), and some of the metabolites of pyrene metabolism are detected (Heitkamp *et al.* 1988; Vila *et al.* 2001). The identification of metabolites accumulating during the growth of *Mycobacterium* sp. strain AP1 in pyrene suggested that this strain initiates its attack on pyrene by either mono-oxygenation or dioxygenation at its C-4, C-5 positions to give *trans*- or *cis*-4,5-dihydropyrene, respectively (Vila *et al.* 2001). Dehydrogenation of the *cis*-dihydroxypyrene to the corresponding diol, followed by *ortho* cleavage of the oxidation ring to produce phenanthrene 4,5-dicarboxylic acid in *Mycobacterium* sp. was initially proposed by Cerniglia (1992). However, the pathway(s) for pyrene degradation and assimilation in *B. subtilis* DM-04 and *P. aeruginosa* M and NM strains are yet to be discovered. Furthermore, it might be reasonable to assume that several key factors, such as optimum growth condition of bacteria (pH, temperature), presence of a specific and or higher amount of inducible enzyme(s), substrate specificity of PAHs degrading enzymes (Gibson and Subramanian 1984; Sharanagouda and Karegoudar 2001), nature of biosurfactant secreted by bacteria and bacterial cell surface hydrophobicity (Mukherjee and Das 2005) are responsible for differential uptake and higher metabolism (consumption) of pyrene by *B. subtilis* DM-04 strain compared with *P. aeruginosa* M and NM strains. In the present study bacteria consumed a significantly higher proportion of pyrene than that reported for the *Mycobacterium* sp. strain AP1 that is capable of cellular assimilation of pyrene with an efficiency of about 10% (Vila *et al.* 2001).

Phylogenetic analysis revealed that great diversity exists among biosurfactant-producing microbes suggesting that biosurfactant production is an important survival tool for the biosurfactant-producing microbes (Maier 2003). Production of biosurfactant is related to the utilization of

available hydrophobic substrates by the biosurfactant-producing microbes from their natural habitat, presumably by increasing the surface area of the substrates and enhancing their apparent solubility and also influencing the cell-surface hydrophobicity of bacterial cells towards hydrophobic substrates like pyrene (Ron and Rosenberg 2001; Mukherjee and Das 2005). In the present study, all the three bacteria were found to be efficient biosurfactant producers on pyrene medium based on (i) surface tension measurement of culture supernatants obtained post 48 h of bacterial growth and (ii) by determining the yield of biosurfactant. The production of biosurfactant by all the three bacterial strains increased significantly when the medium was supplied with glucose (as a co-substrate) along with pyrene. Glucose, which is a versatile precursor for many biomolecules, acts as an inducer and co-source of carbon, energy and reducing power for microbes that leads to substantial increase in bacterial biomass with a corresponding increase in biosurfactant production. Moreover, greater reduction of surface tension of growth medium by biosurfactant produced by *B. subtilis* DM-04 strain compared with *P. aeruginosa* M and NM strains may be related to production of surfactin-like lipopeptides by former bacteria (Mukherjee and Das 2005).

The pyrene uptake rates in all groups of bacteria appear to be highly nonlinear, with much higher uptake in the first 10 min than in the subsequent time interval. However, we think that the argument of possible interference in the absorbance of pyrene in the present experiment due to release of pyrene metabolites is unlikely, because no pyrene metabolite was detected by HPLC analysis post 60 min incubation of bacteria with pyrene (Das, K and Mukherjee, A.K., unpublished observation). The literature contains little information on pyrene uptake by bacteria and its link to biosurfactant production. In this study, we present the persuasive data showing that biosurfactants secreted by *B. subtilis* DM-04, *P. aeruginosa* M and NM strains at a concentration of 0·5 mg ml^{-1} enhanced the apparent solubility of pyrene by factors 5–7 resulting in its higher uptake and metabolism by bacteria. The difference in pyrene solubilization effect of biosurfactants from different bacterial strains in this study may be related to the chemical nature as well as surface properties of biosurfactants (Das and Mukherjee 2005; Mukherjee and Das 2005). For example, low-molecular weight biosurfactant-like lipopeptides secreted by *Bacillus* sp. have low critical micelle concentrations (CMCs) that increase the apparent solubility of hydrocarbons by incorporating them into the hydrophobic cavities of micelles (Wick *et al.* 2001) and alasan, a high-molecular weight bioemulsifier complex produced by *Acinetobacter radioresistens* KA 53 enhances the aqueous solubility of PAHs by a physical interaction, most likely of a hydrophobic nature and increases the biodegradation rate of PAHs (Barkay *et al.* 1999). Moreover, the significantly higher ($P < 0·05$) pyrene solubilization effect of biosurfactant from *P. aeruginosa* NM strain compared with *P. aeruginosa* M strain reinforces the hypothesis that a minor variation in biosurfactant isoforms between these two strains may result in a large variation of the emulsification property and specificity of biosurfactants (Das and Mukherjee 2005). It may be concluded that higher pyrene solubilization effect of biosurfactant from *P. aeruginosa* NM strain enhanced the uptake and subsequent utilization of pyrene and thus sustained the growth of this bacteria in pyrene, otherwise it might not be able to grow on pyrene. Further studies to identify the metabolites in the degradation of pyrene by *B. subtilis* DM-04 and *P. aeruginosa* M and NM are in progress.

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