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Anaerobic biodegradation of fluoranthene under methanogenic conditions in presence of surface-active compounds

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Abstract

Bacillus cereus isolated from municipal wastewater treatment plant was used as a model strain to assess the efficiency of two anionic surfactants, a chemical surfactant and a biosurfactant during fluoranthene biodegradation under anaerobic methanogenic conditions. The surfactants selected for the study were linear alkyl benzene sulphonates (LAS) and rhamnolipid-biosurfactant complex from *Pseudomonas* sp. PS-17. Biodegradation of fluoranthene was monitored by GC/MS for a period up to 12th day. No change in the fluoranthene concentration was registered after 7th day. The presence of LAS enhanced the cell growth as well as the fluoranthene biodegradation. The rhamnolipid-biosurfactant at both used concentrations inhibited the cell growth and had no effect on the biodegradation rate. It was shown that LAS did not affect the microbial cell permeability and its positive effect on fluoranthene biodegradation was most likely as a result of the increased fluoranthene solubility. The results indicate that LAS can be considered as a promising agent for facilitation of the process of anaerobic polycyclic aromatic hydrocarbons (PAH) biodegradation under methanogenic conditions.

Keywords: Anaerobic; Biodegradation; Fluoranthene; LAS; Biosurfactant

1. Introduction

Polycyclic aromatic hydrocarbons (PAH) are naturally occurring organic compounds, consisting of three or more fused aromatic rings. The increased number of aromatic rings and hydrophobicity makes PAH recalcitrant to biodegradation. As a result of anthropogenic activities as accidental oil leaks and spills, PAH concentration in the environment increased considerably. Higher concentrations are often found at old gas work stations or at sites formerly used for wood preservation. PAH are regarded as environmental pollutants by environmental and health agencies because they have toxic, mutagenic and carcinogenic effects on the living organisms [1].

Since the bioremediation techniques are cheaper than the other alternatives (soil washing, solidification and stabilization, incineration or thermal treatment) used for cleaning up of contaminated sites [2], implementation of the natural potential of microorganisms to utilize hydrocarbons is a promising approach to reduce PAH pollution. Although PAH were in the past considered to be recalcitrant to biodegradation in the absence of oxygen as electron acceptor, they have recently been proven to be biodegradable under a variety of anaerobic conditions [3–5]. There are many studies on anaerobic microbial degradation of aliphatic and monoaromatic hydrocarbons [6]. However, only a few studies are available on the anaerobic biodegradation of polyaromatic hydrocarbons [7].

In addition, the process of polyaromatic hydrocarbon utilization is very slow because of low water solubility of PAH resulting in a low bioavailability to the microorganisms. A promising approach to increase the solubility of such hydrophobic compounds is the use of surface active compounds (synthetic surfactants and biosurfactants, produced by microorganisms). The surfactants tend to increase PAH solubility by lowering the interfacial tension between the water phase and the hydrocarbons and actively desorbing molecules from hydrocarbon crystals. Biosurfactants have several advantages over synthetic surfactants such as lower toxicity, higher biodegradability and structural diversity [8]. So far, the studies are focused

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mainly on the effect of synthetic surfactants and biosurfactants on PAH biodegradation at aerobic conditions [1]. A little information is available about their effect on PAH degradation at anaerobic conditions [9,10]. One of the predominant compounds found in soils and sediments contaminated with polycyclic aromatic hydrocarbons is fluoranthene [11]. Fluoranthene degradation has already been demonstrated with *Bacillus cereus* under aerobic conditions [11,12] and with mixed anaerobic cultures under sulphate-reducing conditions [13]. To our knowledge till now no information is available about anaerobic fluoranthene biodegradation with this microorganism under methanogenic conditions although anaerobic growth of *B. cereus* has been previously shown under non-specified type of anaerobic electron acceptor conditions [14]. Other study showed that anaerobic growth of *B. cereus* was stimulated under nitrate-reducing conditions [15]. *B. cereus* was chosen as a model strain to study anaerobic fluoranthene biodegradation, as it was previously isolated in our laboratory and preliminary experiments showed its potential for fluoranthene biodegradation under methanogenic anaerobic conditions. This microorganism is wide spread in variety of soil habitats [16].

The aim of the present study was to estimate the effect of a synthetic surfactant and a biosurfactant on the biodegradation of fluoranthene (four-ringed PAH) by the *B. cereus* at strict anaerobic conditions. The selected surfactants were: a mixture of linear alkyl benzene sulphonates (LAS), and a rhamnolipid-biosurfactant complex.

2. Materials and methods

2.1. Chemicals

The rhamnolipid- biosurfactant-alginate complex used in this study was isolated from *Pseudomonas* sp. PS-17 in the Laboratory of Biotechnology, Ukrainian Academy of Sciences (Lviv town), and provided by Dr. E. Karpenko and Dr. A. Shulga. It is a unique natural complex that contains of two types rhamnolipids—RL1 and RL2 and a biopolymer-alginate with high surface and emulsifying activities [17]. Linear alkyl benzene sulphonates were provided from Huls, Germany, and represented chemically synthesized compounds with different length of the alkyl chain that are among the most broadly used surfactants [18]. Fluoranthene with 99% purity (CAS no. 206-44-0), fluoranthene-d10 (CAS no. 93951-69-0), naphthalene-d8 (CAS no. 1146-65-2) and 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) (CAS no. 28718-90-3) were purchased from Sigma–Aldrich.

2.2. Microorganism and media

A microbial culture of *B. cereus* utilizing fluoranthene at strict anaerobic methanogenic conditions was isolated from activated sludge received from a municipal wastewater treatment plant (Lundtofte, Denmark). The stock culture was maintained at 4 °C on agar slants containing 0.5% nutrient broth (MPB) through regular subculturing. A loopfull from an agar slant of this culture was used to prepare an inoculum in basic anaerobic (BA) medium [19] autoclaved at 115 °C for 20 min and supplemented aseptically with filter (0.45 μm) sterilized peptone and yeast extract to final concentration of 10 and 2 g/L, respectively. Prior to inoculation, the medium was dispensed under anaerobic conditions by flushing the headspace of the vessels with sterile gas mixture of N_2:CO_2 (80:20, v/v). After 3 days incubation at 37 °C cell suspensions were centrifuged and washed three times in sterile phosphate buffered saline (PBS) in order to remove nutrients. Washed cells diluted in PBS to $OD_{570} = 0.1$ were used as inoculum in the further experiments carried out in 26 mL anaerobic glass tubes (Bellco Biotechnology, USA).

2.3. Experimental set-up

Initially, all tubes were thoroughly washed with tap water and held at 220 °C for a minimum of 12 h to remove any trace organic residue prior to use. Tubes without bacteria prepared identically were used as controls.

Five-millilitre sterile BA medium was dispensed in the tubes and inoculated with 0.3 mL bacterial culture. A stock solution of fluoranthene dissolved in acetonitrile was amended to a final concentration of 120 mg/L. The headspace of the tubes was flushed with a sterile gas mixture of N_2:CO_2 (80:20, v/v). Immediately after flushing, the surfactant (LAS or biosurfactant-rhamnolipid-alginate) was added aseptically at final concentrations of 10 and 100 mg/L, respectively. After mixing, the tubes were closed with Teflon screw caps and were incubated at static conditions and 37 °C for 12 days.

2.4. PAH analysis

At the end of the experiment 2 mL of MiliQ water pH 12 (adjusted with 2 M NaOH) and 5 mL of extraction solvent (pentane:diethyl ether (15:85, v/v) containing 5 mg/L of fluoranthene-d10 as internal standard) were added to each tube. The tubes were capped with a Teflon lined stopper and shaken at 20 rpm for 4 h in darkness on a tube rotator. Thereafter 2 mL samples were withdrawn from each tube and centrifuge at 1.5 × *g* for 5 min. One millilitre of supernatant was transferred to 2 mL GC vials where 0.035 mL of injection standard (naphthalene-d8) was added to final concentration of 1.4 mg/L.

PAH analyses were performed by gas chromatography coupled to mass spectrometry (GC, Agilent 6890N; and MS, Agilent 5973). All the samples were injected split-less into the GC by an automatic sampler. The GC was equipped with a HP-5MS column. The injector and auxiliary temperature were respectively of 275 and 270 °C. Helium was used as carrier gas with a constant flow of 0.8 mL/min. The oven temperature was initially set at 60 °C for 1 min. Then an increasing temperature rate of 24 °C/min was applied up to 310 °C which was kept for 10 min and afterwards increased by 20 °C/min to 340 °C kept for 2 min. The MS was run in SIM mode. Fluoranthene was quantified using 202 *m/z* as quantifier ion and 101 *m/z* as qualifier ion.

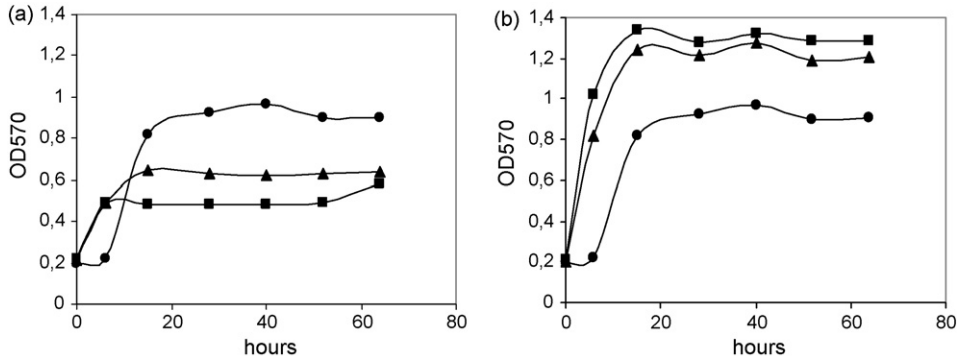

Fig. 1. Cell growth on fluoranthene in presence of (a) biosurfactant-rhamnolipid and (b) LAS. *Symbols*: (●) Without surfactant; (▲) with surfactant at the low concentrations (10 mg/L); (■) with surfactant at the high concentrations (100 mg/L). Error bars represent standard deviations from triplicate analyses.

2.5. Cell growth and cell permeability

The cell growth was followed by measuring the optical density at 570 nm (OD_{570}). Concentration of the extracellular protein was chosen as an easy to measure parameter directly reflecting cell permeability. Extracellular protein was measured using the method of Bradford [20].

2.6. Microscopy

DAPI (0.33 μg/mL in MiliQ water for 5 min) was used for total cell identification at the 7th day. An upright Axioplan epi-fluorescence microscope equipped with digital camera was used for microscopic observations.

3. Results and discussion

3.1. Microbial growth

Synthetic surfactant and the biosurfactant used in this study were tested at two concentrations: 10 and 100 mg/L, respectively. As clear inhibitive effect of LAS on anaerobic biodegradation processes was previously observed with concentrations above 100 mg/L [21] both surfactants were not tested above 100 mg/L final concentration.

The results obtained showed that no change in the microbial growth was observed after 60th hour (Fig. 1). Presence of LAS at both studied concentrations stimulated the cell growth (Figs. 1b and 2c). In contrast, the presence of biosurfactant suppressed cell growth of the studied culture on fluoranthene as a sole carbon source (Figs. 1a and 2b). The inhibitory effect of the biosurfactant was better expressed at the higher concentration of 100 mg/L (Fig. 1a). Although biosurfactants are known to have mild effect on the microbial cells, some reports demonstrate that rhamnolipid-biosurfactants produced by *P. aeruginosa* have toxic effect against *Bacillus subtilis* and other Gram-positive strains growing at aerobic conditions [22,23]. The toxic effect of the biosurfactant used in this study could be due to the presence of rhamnolipid as a main component in the biosurfactant complex. At higher complex concentrations where rhamnolipid concentration was increased, the effect on the cell growth of the studied strain was more suppressive. On the other hand our results showed that LAS do not possess such negative effect on the cell growth even at the high concentrations of 100 mg/L.

In order to understand in which way the synthetic surfactant affects the bacterial growth, the effect of LAS on the microbial cell permeability was studied. The results obtained for the extracellular protein release showed that there were no significant differences between the control without surfactant and the samples with LAS (Table 1). The inhibitory effect of LAS was suspended in presence of fluoranthene due to possible changes in the physicochemical properties of the surfactant. It is well known that processes such as sorption and biodegradation affect the surfactant mixture composition and thus the physicochemical properties of the mixture such as the critical micelle concentration and ability to solubilize [24]. It could be assumed that LAS affected mostly the process of fluoranthene solubilisation than the microbial cell permeability and proved the mild effect of LAS on the microbial cells.

3.2. Biodegradation of fluoranthene

The results for fluoranthene biodegradation under strict anaerobic methanogenic conditions showed that no change in

Table 1
Extracellular protein release (mg/ml) during anaerobic cell cultivation in the absence and in the presence of LAS at two concentrations

	Days		
	3rd (mean ± S.D.[a])	5th (mean ± S.D.)	7th (mean ± S.D.)
Control (no LAS)	0.020 ± 0.001	0.054 ± 0.002	0.050 ± 0.002
10 mg LAS/L	0.032 ± 0.001	0.050 ± 0.001	0.054 ± 0.002
100 mg LAS/L	0.024 ± 0.001	0.044 ± 0.002	0.075 ± 0.003

[a] Standard deviations (S.D.) were based on triplicate analysis.

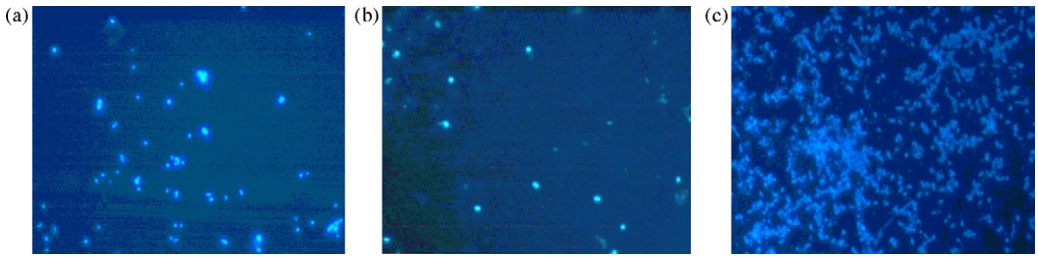

Fig. 2. Fluorescent pictures of microbial cells growing on fluoranthene. (a) Without surfactants; (b) in presence of 100 mg biosurfactant/L; (c) in presence of 100 mg LAS/L.

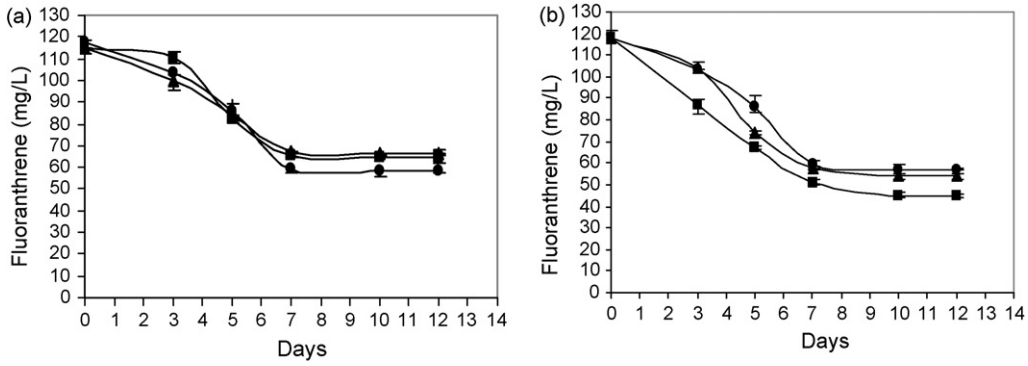

Fig. 3. Biodegradation of fluoranthene in presence of (a) biosurfactant-rhamnolipid and (b) LAS. Symbols as in Fig. 1. Error bars represent standard deviations from triplicate analyses.

the fluoranthene concentration was registered after 7th day. Biodegradation rate was almost not influenced by the presence of rhamnolipid-biosurfactant at both studied concentrations (Fig. 3a). In the presence of LAS, however, accelerated fluoranthene biodegradation was observed (Fig. 3b). The positive effect was more pronounced at the higher concentration of 100 mg/L. After 7th day, the fluoranthene concentration (42 mg/L) in presence of 100 mg LAS/L was about 30% lower compared to the control without surfactant (60 mg/L). Results obtained showed that *B. cereus* mediated biodegradation of fluoranthene was surfactant-dependent. This finding is also supported by other studies demonstrating that effect of surfactant on the biodegradation depends on the target compound, the bacterial specie and surfactant used [25–27]. Our study clearly indicated that although the presence of biosurfactant complex considerably inhibits the cell growth, the biodegradation rate of fluoranthene was not affected. This has never been reported in the literature. The effect of LAS on the fluoranthene biodegradabily could be explained by the enhanced fluoranthene bioavailability due to the increased fluoranthene solubility or the improved direct mutual interaction between the microbial cells and the fluoranthene crystals [28].

4. Conclusions

Anaerobic biodegradation of PAH is a very slow process that could be enhanced by the use of solubilizing agents as surfactants. Our results showed that rhamnolipid-biosurfactant complex suppressed the anaerobic cell growth of *B. cereus* and its presence did not affect the fluoranthene biodegradation rate. The observed positive effect of LAS on fluoranthene biodegradation was probably due to the improved bioavailability of fluoranthene for the microbial cells. To our knowledge there is no information available for use of LAS as solubilizing agent under strict anaerobic conditions. Our results indicate that LAS can be considered as a promising agent for facilitation of the process of anaerobic PAH biodegradation under methanogenic conditions.

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