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Improved bioavailability and biodegradation of a model polyaromatic hydrocarbon by a biosurfactant producing bacterium of marine origin

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ABSTRACT

Polyaromatic hydrocarbons (PAHs) are organic pollutants mostly derived from the processing and combustion of fossil fuels and cause human health hazards. In the present study a marine biosurfactant producing strain of *Bacillus circulans* was used to increase the bioavailability and consequent degradation of a model polyaromatic hydrocarbon, anthracene. Although the organism could not utilize anthracene as the sole carbon source, it showed better growth and biosurfactant production in an anthracene supplemented glycerol mineral salts medium (AGlyMSM) compared to a normal glycerol mineral salts medium (GlyMSM). The biosurfactant product showed high degree of emulsification of various hydrocarbons. Analysis by gas chromatography (GC), high performance thin layer chromatography (HPTLC) and Fourier transform infrared spectroscopy (FTIR) showed that the biosurfactant could effectively entrap and solubilize PAH. Thin layer chromatographic analysis showed that anthracene was utilized as a carbon substrate for the production of biosurfactant. Thus organic pollutant anthracene was metabolized and converted to biosurfactants facilitating its own bioremediation.

1. Introduction

Biosurfactants are surface-active amphipathic molecules produced by a plethora of microorganisms. They have a wide structural diversity, ranging from glycolipids, lipopeptides and lipoproteins to fatty acids, neutral lipids, phospholipids, polymeric and particulate biosurfactants. This endows them with their unique properties, including better environmental compatibility, greater foaming properties, higher selectivity and biodegradability (Desai and Banat, 1997; Mukherjee et al., 2006) in comparison to chemical surfactants. These molecules retain their activity at extremes of temperatures, pH and salinity conditions (Zajic et al., 1977; Kretschner et al., 1982; Banat, 1995). *Bacillus* species are one of the major producers of microbial surfactants. These include bioactive lipopeptide molecules such as surfactin, fengycin, lichenysin, iturin, pumilacidin and bacillomycin (Vater et al., 2002). Biosurfactants are less toxic and can be produced from cheap agro-based substrates (Mukherjee et al., 2006). Apart from the reported therapeutic potentials of these molecules (Rodrigues et al., 2006; Das et al., 2008), they facilitate the process of emulsification of hydrocarbons in aqueous phase by forming micelles thereby enhancing their availability for microbial uptake and degradation. Hence they have potential application in the field of bioremediation of persistent and recalcitrant organic pollutants (Noudeh et al., 2005). Polyaromatic hydrocarbons (PAHs) are the organic pollutants of major concern due to their persistence in the environment and their deleterious roles in human beings as mutagens and carcinogens. Anthracene, chosen as a model PAH in this study, is a solid polyaromatic hydrocarbon (PAH) with three benzene rings produced as a result of combustion of fossil fuels and anthropogenic activities. It is highly resistant to nucleophilic attack and hence, recalcitrant to biodegradation, due to very less solubility in water (Guieysse et al., 2001). In powdered form it causes irritation to the eyes, nose or lungs and is a probable inducer of tumors. Once anthracene enters the body, it appears to target the skin, stomach, intestines and the lymphatic system. It can cause burning, itching and edema.

Bioremediation is defined as the complete elimination or conversion of toxic recalcitrant compounds into non-toxic forms by a bacterium or a microbial consortium. It is a cost effective technology because a microbial culture is used in this process and also a performance effective technique, as the end products of this process are mostly not harmful to environment. PAH bioremediation approaches in soils include tillage, nitrogen and phosphorus addition and microbe inoculation (Abbondanzi et al., 2004). However, these approaches do not solve the problem of unavailability of these compounds for microbial degradation. Bioavailability is determined by the rate of substrate mass transfer into the microbial cells relative to their intrinsic catabolism and excretion (Johnsen et al., 2005). Though chemical surfactants could increase the bioavailability and consequent biodegradation (West and Harwell,

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1992; Volkering et al., 1998; Mulligan et al., 2001), they also have inhibitory effects on microbial growth and biodegradation at concentrations above their critical micelle concentrations (CMC). Thus bioremediation strategies with biosurfactants are drawing scientific attention and receiving a favorable publicity (King et al., 1998). Biosurfactant application in bioremediation is acceptable from environment point of view, because of their natural occurrence and has often been reported to enhance hydrocarbon biodegradation in liquid media, soil slurries and soil microcosms (Providenti et al., 1995; Ron and Rosenberg, 2002). Biosurfactant produced by a bacterium can improve remediation efficiency by solubilizing PAH for better utilization by the producer organism (Cameotra and Bollag, 2003).

The marine environment encompassing the vast majority of earth's surface is a repertoire of a large number of microorganisms. The environmental roles of the biosurfactants produced by many such marine microorganisms have been reported earlier (Poremba et al., 1991; Schulz et al., 1991; Abraham et al., 1998). Considering the structural diversity and consequent higher surface activities of the biosurfactants of marine origin, we had undertaken this study. The present study aims at assessing the potentiality of a marine bacterium to increase bioavailability and consequent biodegradation of anthracene, a model polyaromatic hydrocarbon. The role of biosurfactant in this process and the change in the level and quality of biosurfactant produced in presence of the polyaromatic hydrocarbon has also been assessed.

2. Materials and methods

2.1. Microorganism, production medium and cultivation conditions

Marine water samples were collected from Andaman and Nicobar Islands, India. The samples were serial diluted and plated on Zobell marine agar (HiMedia, Mumbai, India) plates for obtaining individual bacterial colonies. Those colonies were grown in a glycerol mineral salts medium having the following composition in % (w/v): glycerol (2%), NH_4NO_3 (0.3%), K_2HPO_4 (0.22%), KH_2PO_4 (0.014%), NaCl (0.001%), $MgSO_4$ (0.06%), $CaCl_2$ (0.004%), $FeSO_4$ (0.002%) and 0.5 ml l^{-1} of trace elements solution containing (l^{-1}): 2.32 g $ZnSO_4 \cdot 7H_2O$, 1.78 g $MnSO_4 \cdot 4H_2O$, 0.56 g H_3BO_3, 1.0 g $CuSO_4 \cdot 5H_2O$, 0.39 g $Na_2MoO_4 \cdot 2H_2O$, 0.42 g $CoCl_2 \cdot 6H_2O$, 1.0 g EDTA, 0.004 g $NiCl_2 \cdot 6H_2O$ and 0.66 g KI. Biosurfactant producers were screened by monitoring change in the surface tension of the medium with respect to the uninoculated production medium. The colonies which significantly reduced the surface tension of the production medium were selected as those of biosurfactant producers. The biosurfactant producer used in this study was isolated from one such sample and was then characterized as *Bacillus circulans* by standard biochemical microbial identification methods (data not shown). The medium used for biosurfactant production was glycerol mineral salts medium (GlyMSM) as described earlier. The culture was incubated at 37 °C with shaking at 180 rpm for about 60 h. The microbial growth was estimated by measuring the optical density of the culture broth at 600 nm.

2.2. Isolation of surface-active molecules

The culture broth was centrifuged after fermentation and concentrated hydrochloric acid (HCl) was added to the cell free supernatant to lower its pH to 2. The acidified supernatant was then kept at 4 °C overnight for complete precipitation of the biosurfactant (Yakimov et al., 1996). The precipitate was centrifuged at 10000 g for 20 min to get the crude biosurfactants as a pellet. This pellet was then re-suspended in water; pH was raised to 7.5 and lyophilized.

2.3. Emulsification index

The ability of the biosurfactant to emulsify some liquid hydrocarbons, such as petrol, kerosene, diesel, hexadecane and benzene was determined. Emulsification index was measured using a previously reported protocol (Cooper and Goldenberg, 1987). Briefly equal volumes of the hydrocarbons and aqueous biosurfactant solution (5 mg ml^{-1}) were mixed and vortexed at high speed for 5 min. The resulting mixture was incubated at 25 °C for 24 h and then the emulsification index value (E_{24}) was calculated using the formula

$$E_{24} = (\text{Height of emulsion layer}/\text{Height of the total mixture}) \times 100$$

2.4. PAH solubilization assay

PAH (polyaromatic hydrocarbon) solubilization assay was performed as described below. Stock solution of anthracene (0.6 mg ml^{-1}) was prepared in hexane. 1 μl from this was added in separate glass tubes so as to yield 0.6 μg anthracene in each tube (Barkay et al., 1999). These tubes were then kept in an open chemical fume hood to remove the solvent. Subsequently 3 ml of assay buffer (20 mM Tris–HCl, pH 7) and the biosurfactant in increasing concentrations (100–500 μg ml^{-1}) were added to these tubes. A tube containing the assay buffer with PAH but no biosurfactant served as the positive control in this experiment. Assay buffer containing the biosurfactant, but no PAH, was used as blank. Tubes were capped with plastic closures and incubated in a vertical position overnight at 30 °C with shaking (150 rpm) in the dark. Samples were filtered through 1.2 μm pore-size filter (Whatman, Springfield Mill, United Kingdom) and 2 ml of this filtrate was extracted with equal volume of hexane. Aqueous and hexane phases were separated by centrifugation at 10000 g for 2 min. Concentration of anthracene in the hexane extracts was measured spectrophotometrically at 250 nm (Barkay et al., 1999). All experiments were performed in triplicate.

2.5. Production of biosurfactant in presence of anthracene and PAH solubilization

The organism's potential to utilize anthracene as a sole source for carbon was evaluated in a mineral salts medium with glycerol being replaced by 0.2% (w/v) anthracene. The growth and biosurfactant production was also evaluated in a 2% (w/v) GlyMSM supplemented with 0.2% (w/v) anthracene (AGlyMSM). The batch fermentation was carried out for a week. Broth samples were collected at an interval of 24 h for further analysis using HPTLC and GC. The samples were also tested for their potential to emulsify petrol, kerosene, diesel, hexadecane and benzene.

2.6. Analytical methods

2.6.1. Surface tension measurements

The surface tension (ST) of the culture supernatants was measured with a digital surface tensiometer (DCAT, DataPhysics Instruments GmbH, Filderstadt, Germany) working on the principles of Wilhelmy plate method (Fernandes et al., 2007; Kumar et al., 2007; Vasileva-Tonkova and Gesheva, 2007). The validity of the surface tension readings was checked with pure water (70.78 ± 0.02 mN m^{-1}) before each reading. All surface tension readings were taken in triplicate.

2.6.2. Gas chromatography

The cell free broth samples were extracted with equal volumes of dichloromethane for estimation of amount of anthracene left in