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Coal induced production of a rhamnolipid biosurfactant by *Pseudomonas stutzeri*, isolated from the formation water of Jharia coalbed

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HIGHLIGHTS

- ▶ *Pseudomonas stutzeri* was isolated from the formation water of an Indian coalbed.
- ▶ The *P. stutzeri* isolate produced biosurfactant in response to coal supplementation.
- ▶ The coal induced biosurfactant produced by *P. stutzeri* was a rhamnolipid.
- ▶ *P. stutzeri* produced more biosurfactant with lignite than bituminous or anthracite.

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ABSTRACT

A strain of *Pseudomonas stutzeri* was isolated form an enrichment of perchlorate reducing bacteria from the formation water collected from an Indian coalbed which solubilized coal and produced copious amount of biosurfactant when coal was added to the medium. It produced maximum biosurfactant with lignite coal followed by olive oil and soybean oil which was able to emulsify several aromatic hydrocarbons including kerosene oil, diesel oil, hexane, toluene etc. Haemolytic test, growth inhibition of *Bacillus subtilis* and FTIR analysis showed rhamnolipid nature of the biosurfactant. The stability of the coal induced biosurfactant in pH range of 4–8 and up to 25% NaCl concentration and 100 °C temperature suggests that due to its ability to produce biosurfactant and solubilize coal *P. stutzeri* may be useful in the coalbed for *in situ* biotransformation of coal into methane and in the bioremediation of PAHs from oil contaminated sites including marine environments.

1. Introduction

India is the 5th largest proven reservoir of coal in the world utilization of which as energy source results into atmospheric pollution including emission of green house gases (Hayward, 2010). *In situ* microbial conversion of coal into methane gas is considered an environment friendly alternative to produce the clean energy source. Thus, microbial biotransformation of coal into simpler, low molecular weight product is considered an economic and effective way of improving the utility of coal. A variety of microorganisms including fungi (Hatakka, 1994), bacteria (Standberg and Lewis, 1988) and actinomycetes (Quigley et al., 1989a) are capable of coal solubilization/degradation. Studies suggested the mechanism involved in coal biotransformation mainly comprises of (1) microbial production of alkaline substances (Quigley et al., 1989b), (2) biocatalysts especially produced by fungi (Hatakka, 1994), (3) metal ion chelators and surface active agents (Fakoussa, 1988; Polman et al., 1994). Surfactant decreases the coal surface tension and increases the solubility of coal in aqueous solutions (Fakoussa, 1988; Yuan et al., 2006). It is quite possible that both enzymatic and non enzymatic mechanisms may be involved in coal biotransformation process in the same microorganism. In a study it was shown that chemically synthesized surfactants and surfactant from *Bacillus licheniformis* and *Candida bombicoal* were able to solubilize different portion of coal (Breckenridge and Polman, 1994; Polman et al., 1994).

Biosurfactants can be used in coal dust control, enhancement of gas permeability of coal, removal of coal ash and bioremediation of soil and groundwater contaminated with hydrocarbon. Microorganisms producing biosurfactant can participate in oil degradation. Alternatively, they can function in a bacterial consortium, supplying the emulsifier for other bacteria that carry out the degradation of hydrocarbons (Ron and Rosenberg, 2002). In addition biosurfactant can be used in petroleum industry for transportation of crude oil, enhanced oil recovery by increasing the apparent solubility of petroleum components and effectively reducing the interfacial

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tensions of oil and water *in situ* (Singh et al., 2006). In this study we have described isolation and identification of a rhamnolipid producing *Pseudomonas stutzeri* strain from the formation water of an Indian coalbed, which produced maximum biosurfactant in presence of coal. We have shown here that the ability to produce biosurfactant is responsible for the coal solubilization ability of this bacterium.

2. Methods

2.1. Isolation and identification of perchlorate reducing bacteria

An enrichment culture was set up for isolation of perchlorate reducing bacteria with formation water collected from Jharia coalbed (Singh et al., 2012). The enrichment medium consisting of (g l^{-1}) KH_2PO_4, 1.2 g; $Na_2HPO_4{\cdot}12H_2O$, 10.8 g; $MgSO_4$ $7H_2O$, 0.04 g; $FeSO_4{\cdot}7H_2O$, 0.02 g; $MnCl_2{\cdot}4H_2O$, 0.02 g; NH_4C1, 3.0 g was prepared anaerobically and 10 mM sodium acetate and 10 mM sodium perchlorate was added from sterile anoxic stock as electron donor and acceptor, respectively. Finally, cysteine hydrochloride was added to a final concentration of 0.005% as reducing agent. Anoxic medium was inoculated with formation water and incubated at 37 °C for one week after which bacteria were isolated by standard serial dilution method on nutrient agar plates at 37 °C. Identification of the isolate based on 16S rRNA gene sequence (GenBank accession number JX429927) was carried out according to Singh and Tripathi (2011).

2.2. Utilization and solubilization of coal

Ability of the isolate to utilize and solubilize coal was detected in mineral salt medium as described previously by Singh and Tripathi (2011). Further, coal solubilization ability of the isolate was detected in Yeast extract mannitol broth (YMB; Himedia). Flasks containing YMB with and without coal (1% w/v) were inoculated with equal number of overnight-grown culture and incubated at 37 °C with shaking at 200 rpm for 7 days. Culture from each flask was centrifuged at 10,000 rpm for 5 min to pellet the cells and the cell free culture supernatant was used for spectrophotometric monitoring of coal solubilization at 450 nm as described elsewhere (Willmann and Fakoussa, 1997).

2.3. Screening of biosurfactant activity of culture supernatant

For screening of the biosurfactant activity, the culture supernatant was taken from the flasks containing YMB supplemented with and without coal. Cells were separated by centrifugation at 12,000 rpm for 10 min and cell free culture supernatant was used for tests. Reduction in the surface tension of medium was measured by du Nouy ring type tentiometer (Krauss, Germany) (Chandran and Das, 2010). Triton X-100 was used as positive control for the measuring reduction in the surface tension of water. Emulsifying ability of the culture supernatant was measured by vortexing equal volume of cell free culture supernatant and kerosene oil for 1–3 min and determining the percentage of volume occupied by the emulsion. The mixture was allowed to settle for 24 h and the height of the emulsion was measured to determine the emulsification index (E_{24} values) which is described as ratio of the height of emulsified zone (EZ) to total height (TH); [E_{24} = (EZ/TH) × 100] (Bonilla et al., 2005; Sriram et al., 2011a). Chemical surfactants such as Tween 80, Triton-X 100 (Himedia) and 10% sodium dodecyl sulfate (SDS; Merck) were used as positive test control (Chandran and Das, 2010). Further, the ability of cell free supernatant to emulsify different hydrophobic substrates was tested by mixing equal volume of kerosene, diesel, olive oil, soyabean oil, hexane, benzene, toluene, xylene, carbon tertrachloride, and chloroform with cell free culture supernatant. The samples were vortexed for 1–3 min and E_{24} value was determined as described above. All the measurements were repeated in twice for two times and an average value was obtained.

2.4. Lipase production

Plate assay for lipase activity was determined using tributyrin agar plates (Lakshmipathy et al., 2010). The cultures were streaked on the tributyrin agar plates (Himedia) and incubated at 37 °C for 48 h. The plates were then examined for clear zone formation around the colonies.

2.5. Structural characterization of biosurfactant

The carbohydrate group of the biosurfactant was assayed by rhamnose test (Dubois et al., 1956). Briefly, 500 μl of culture supernatant was mixed with 500 μl of 5% phenol solution and 2.5 ml of concentrated sulfuric acid, and incubated for 15 min before measuring absorbance at 490 nm using the UV–Vis spectrophotometer GeneSys (Thermo Scientific, USA). The amount of rhamnose was determined with a reference standard constructed with L-rhamnose.

The chemical nature of the biosurfactant was determined by haemolytic assay (Hazra et al., 2010) and growth inhibition of *Bacillus subtilis* (Ito et al., 1971). Sterile disk soaked in cell free culture supernatant of YMB with and without coal was placed on the agar plate containing 5% blood, and on the nutrient agar containing a lawn of *B. subtilis* cells. Blood agar plates were incubated at room temperature for 2 days and nutrient agar plates of *B. subtilis* at 37 °C overnight to observe zone for haemolysis and growth inhibition, respectively.

For FTIR analysis the pH of the cell free supernatant was adjusted to 2.0 and incubated at 4 °C overnight for precipitation of the biosurfactant, which was then extracted with equal volume of $CHCl_3$:CH_3OH (2:1) and evaporated on a rotary evaporator to yield a viscous honey colored pellet (Hazra et al., 2010). The peaks were recorded in the 4000 to 400 cm^{-1} spectral region at a resolution 2 cm^{-1}, using 0.23 mm KBr liquid cells with Perkin Elmer Spectrum Version 10.03.05.

2.6. Production of biosurfactant with different hydrocarbons

Production of biosurfactant with different hydrocarbon was tested in YMB containing different hydrocarbons (1% w/v and v/v) *viz* H_2O_2 treated coal (oxidized coal), raw coal, kerosene oil, diesel oil, olive oil, soyabean oil, naphthalene, anthracene and phenanthrene. Flasks were inoculated with equal number of cells from an overnight grown culture. Inoculated flasks were incubated at 37 °C at 200 rpm for 7 days. The concentration of rhamnolipid was determined by measuring the amount of rhamonse (Dubois et al. (1956) by the colorimetric phenol sulfuric acid method at 490 nm using the UV–Vis spectrophotometer GeneSys (Thermo Scientific, USA). Protein content was measured as described by Bradford method of protein estimation (Bradford, 1976). The E_{24} values of the cell free culture supernatant were measured after equalization of the amount of rhamnolipid from each treatment.

2.7. Production of biosurfactant with different types of coal

Production of biosurfactant with different types of coal was evaluated as described above. YMB supplemented with different types of coal *viz* Lignite, Bituminous and Anthracite (1% w/v) were inoculated with equal numbers of cells from overnight grown