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Role of nutrients in bacterial biosurfactant production and effect of biosurfactant production on petroleum hydrocarbon biodegradation

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ABSTRACT

Petroleum hydrocarbons' insolubility (due to hydrophobic nature) remains an important factor in microbial degradation of these compounds. The use of microbial biosurfactants significantly decreases the hydrophobicity and increases the rate of hydrocarbon biodegradation. Four bacterial strains, *Pseudomonas poae* BA1, *Acinetobacter bouvetii* BP18, *Bacillus thuringiensis* BG3, and *Stenotrophomonas rhizophila* BG32, isolated from petroleum hydrocarbon-contaminated soil, were used to test biosurfactant production capacities under different nutrient conditions. The hydrocarbon degradation by biosurfactant producing strains was compared with a non biosurfactant producing hydrocarbon degrading *Pseudomonas rhizosphaerae* BP3 strain. The percentage increase in biosurfactant production in nutrient rich medium, which was nutrient broth (NB), as compared to nutrient deprive medium, which was Bushnell-Haas broth (BHB), was BA1 = 20.48%, BP18 = 24.81%, BG3 = 16.71% and BG32 = 14.55%. The biosurfactant producing strains showed 16–28% increase in hydrocarbon degradation, as compared to non biosurfactant producing strain. The highest hydrocarbon degradation (96.07%) was exhibited by BA1, followed by BP18 (93.53%), BG3 (89.97%), BG32 (87.10%), and BP3 (74.60%). We concluded that biosurfactant production is influenced by the availability of nutrients. Cell hydrophobicity, surface tension and biosurfactant production influence hydrocarbon degradation, which can be enhanced with the use of biosurfactant producing bacteria.

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1. Introduction

Soil contamination resulting from petroleum hydrocarbons (diesel, gasoline and fuel oils) is critical, regarding health and environment (Kingston 2002). In soil these organics scarcely degrade, causing damage to human wellbeing, ruining environment, and imposing threats to animal and plant species (Khan et al., 2016). They are highly hydrophobic in nature and oil permeation into the soil makes the degradation process really difficult, as the hydrocarbons have less water solubility and get attached to soil particles, resulting in the decreased bioavailability to microorganisms, limiting mass transfer rate in the biodegradation (Iwabuchi et al., 2002). To enhance the bioavailability of the contaminants in soil, it is necessary to transfer the pollutant into bulk phase.

Many physical (disposal, land filling, incineration and electrokinesis), chemical (application of nanoparticles, addition of oxygen, and use of surfactant) and biological methods (use of plants, plant debris and microorganisms) are adopted for the treatment of noxious contaminates (Cristóvão et al., 2016). Biological treatment methods, due to their natural approach have gained appreciation in recent decades. They are not only applied as standalone remediation strategies but are also incorporated as a process of integrated treatment methods along with physicochemical method in the contaminant treatment scheme (Chrispim and Nolasco, 2016; Diplock et al., 2009). One of the very long practiced remediation strategy is the inoculation of hydrocarbon degraders to polluted sites and the importance of indigenous bacteria involved in oil degradation is well understood (Barathi and Vasudevan, 2001; Verma et al., 2006). However, due to insolubility of the hydrocarbon components the bioavailability of pollutant remains an important factor (Van Hamme et al., 2003). One of the very promising methods in

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the domain of bioremediation of hydrocarbons is the application of bacterial strains capable of producing biosurfactants (Pacwa-Płociniczak et al., 2011).

Biosurfactants were first discovered as extracellular amphiphilic compounds in hydrocarbon fermentation, in late 1960′s. Microbial surfactants or biosurfactants are the mimics of synthetic surfactants and include different groups of surface active amphiphilic particles produced by microbes including bacteria, fungi and yeasts that are present in nature (Khopade et al., 2012). These molecules comprise of fatty acids, glycolipids, lipopeptides and lipoproteins, phospholipids, polymeric and particulate biosurfactants (Souza et al., 2014). Bacterial biosurfactant synthesis is an important characteristic, particularly in various hydrocarbon contaminated sites, as the production enables microbes to grow efficiently on hydrophobic substrates (Bouchez-Naïtali and Vandecasteele, 2008). The role of microbial biosurfactants is to enhance the natural reduction process and intensify the extent of hydrocarbon biodegradation (Mulligan, 2005). In addition biosurfactants activate the bacteria to putrefy the contaminants, which favor soil pollutants' in-situ bioremediation, along with their solubilization and desorption.

Despite of having many attractive features, like less or no toxicity, renewability of resources, ease in production, high activity, and natural degradability (Thavasi et al., 2011; Shubina et al., 2016), when compared with the unnatural counterparts, the role of biosurfactants is commercially very insignificant. A number of parameters are important when designing a strategy for increased hydrocarbon degradation facilitated by biosurfactants (Mukherjee et al., 2006). The parameters that affect biosurfactants production and efficacy include limiting nutrients (N, Fe and Mg) (Amézcua-Vega et al., 2007; Wei et al., 2003), temperature (Zeraik and Nitschke, 2010), agitation speed of culture (Oliveira et al., 2009; Silva et al., 2010), pH and salinity (Abouseoud et al., 2010; Bai et al., 1998).

In this study, we investigated the potential of four bacterial strains (*Pseudomonas poae* BA1, *Acinetobacter bouvetii* BP18, *Bacillus thuringiensis* BG3, and *Stenotrophomonas rhizophila* BG32) for biosurfactant production, and hydrocarbon degradation in comparison to a non-biosurfactant producing (NBP), hydrocarbon degrading *Pseudomonas rhizosphaerae* BP3 strain. Studies focused on comparing hydrocarbon degradation between biosurfactant producing and non biosurfactant producing bacteria were not conducted to the finest of our information. The biosurfactant production by bacterial strains in nutrient rich (Nutrient broth, NB) and nutrient deprived (Bushnell-Haas broth, BHB) conditions was also compared, and correlation between biosurfactant production and hydrocarbon degradation was explored. NB is considered nutrient rich as it contains yeast extract in its composition, and BHB is considered nutrient deprived, as it provides minimal salts and is deprived of other complex nutrients i.e. nitrogenous compounds, carbon, sulfur, trace nutrients, vitamin B complex and other important growth factors, to support bacterial growth when it is amended with a specific hydrocarbon, as a carbon source (Wang and Koch, 1978).

2. Materials and methods

2.1. Screening of bacteria for hydrocarbon utilization

Six pre-isolated bacterial strains; BA1 *Pseudomonas poae* KT758715, BP18 *Acinetobacter bouvetii* KT758716, BG31 *Pseudomonas proteolytica* KT758717, BG32 *Stenotrophomonas rhizophila* KT758718, BP3 *Pseudomonas rhizosphaerae* KT758719, and BG3 *Bacillus thuringiensis* KT758720 were used in this study (Khan et al., 2016). Soil samples were collected from historically petroleum contaminated site of Hattar industrial estate, Haripur, Pakistan. Bacterial strains from 2 mm sieved soil samples were isolated on nutrient agar (NA) plates amended with 1% filter sterilized diesel oil and fluconazole (100 μg ml^{-1}). Morphologically different isolates were pure cultured, and screened for hydrocarbon utilization. Bacterial strains growing on BHA were subjected to a 15 days preliminary screening experiment. The strains were inoculated in Bushnell Haas broth (BHB) in static conditions at 30 °C. Composition of BHB was (g l^{-1}) NH_4NO_3, 1; $FeCl_3$, 0.05; KH_2PO_4, 1; K2HPO4, 1; MgSO4, 0.2; $CaCl_2$, 0.02; pH 7. Diesel at 10 ml l^{-1} was added as the carbon source in BHB. The experiment was conducted in triplicates. After incubation period the optical density of strains was monitored, the strains showing maximum growth were selected for different carbon sources utilization experiments.

For this purpose, Bushnell-Haas broth (BHB) media, amended with 1% filter sterilized one hydrocarbon representative as a C source was used (Ilori et al., 2005). Three different carbon sources (i.e. diesel, crude, and engine spent oil) were used to evaluate the growth of each strain after 13 days of incubation at 30 °C. As a carbon source, diesel represents a mixture of cycloparaffin, paraffin and olefinic hydrocarbons (Khan et al., 2016), crude oil is predominated by alphatic, alicyclic and aromatic petroleum carboxylic acids, and polar heteroatoms (like sulfur, nitrogen, and oxygen) (Rowland et al., 2014), while engine spent oil contains high concentrations of polyaromatic hydrocarbons (PAHs) and additives, in comparison with diesel and crude oil (Jia et al., 2014).

2.2. Bacterial screening for biosurfactant production

2.2.1. Drop collapse test

All bacterial strains, selected on the basis of their potential to utilize different carbon sources as substrate were used for biosurfactant production screening, using drop collapse method (Tugrul and Cansunar, 2005). In this assessment, 7 μl of diesel oil was added in each well of a 96-well microtiter plate. The plate was then equilibrated for 60 min at 30 °C. After equilibration, 20 μl cultivated culture of each strain was added to the oil surface. The drop collapsed indicated the presence of extra cellular biosurfactant whilst if the drops remained, it was considered negative.

2.2.2. Emulsification index (E_{24})

Cell free supernatants (CFS) of Nutrient broth (NB) and BHB, were used for the estimation of emulsification index (E_{24}) for selected four biosurfactant producing bacterial strains, by adopting method defined by Kumari et al. (2012). Briefly, 3 ml diesel oil was added to equal volume of CFS, homogenized at a high speed with a vortex for 2 min, left at room temperature for 24 h and the stability of emulsion was observed for one month. The CFSs were prepared through centrifugation of incubated cultures at 7000*g* for 20 min. After 24 h, E_{24} was calculated using the height of the emulsion level and the total height of the liquid layer in the following equation (Petrikov et al., 2013);

$$E_{24}\% = 100 * \left(\frac{\text{Total height of the emulsified layer}}{\text{Total height of the liquid layer}} \right)$$

2.2.3. Surface tension measurement

Each of the selected bacterial strains was enriched in 20 ml BHB media supplemented with diesel oil (1% w/v) for 7 days. Before and after 7 days of enrichment incubation, cell free supernatants of each strain were prepared and statistical triplicate for each sample's surface tension at 30±2 °C was measured using du Noüy ring tensiometer (Kumari et al., 2012). Supernatants were prepared following the same method as done for CFSs in emulsification index.

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