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Isolation and characterization of biosurfactant producing bacteria from Persian Gulf (Bushehr provenance)

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ABSTRACT

Biosurfactants are surface active materials that are produced by some microorganisms. These molecules increase biodegradation of insoluble pollutants. In this study sediments and seawater samples were collected from the coastline of Bushehr provenance in the Persian Gulf and their biosurfactant producing bacteria were isolated. Biosurfactant producing bacteria were isolated by using an enrichment method in Bushnell-Hass medium with diesel oil as the sole carbon source. Five screening tests were used for selection of Biosurfactant producing bacteria: hemolysis in blood agar, oil spreading, drop collapse, emulsification activity and Bacterial Adhesion to Hydrocarbon test (BATH). These bacteria were identified using biochemical and molecular methods. Eighty different colonies were isolated from the collected samples. The most biosurfactant producing isolates related to petrochemical plants of Khark Island. Fourteen biosurfactant producing bacteria were selected between these isolates and 7 isolates were screened as these were predominant producers that belong to *Shewanella alga*, *Shewanella upenei*, *Vibrio furnissii*, *Gallaecimonas pentaromativorans*, *Brevibacterium epidermidis*, *Psychrobacter namhaensis* and *Pseudomonas fluorescens*. The largest clear zone diameters in oil spreading were observed for *G. pentaromativorans* strain O15. Also, this strain has the best emulsification activity and reduction of surface tension, suggesting it is the best of thee isolated strains. The results of this study confirmed that there is high diversity of biosurfactant producing bacteria in marine ecosystem of Iran and by application of these bacteria in petrochemical waste water environmental problems can be assisted.

1. Introduction

Microbial compounds which exhibit pronounced surface activity are classified as biosurfactants (Cappello et al., 2012b; Bodour and Maier 2002). They decrease surface tension at the air–water interface and between immiscible liquids, or at the solid–liquid interface. These molecules chemically belong to various categories such as glycolipids, lipopeptides, polysaccharide–protein complexes, phospholipids, fatty acids and neutral lipids (Cappello et al., 2012a,c). Excellent detergency, emulsification, dispersing traits, penetrating, thickening, microbial growth enhancement, metal sequestering and resource recovering (oil) are important characteristics of biosurfactants which make them suitable to replace with chemical surfactants. Therefore, they have wide applications in cosmetics, oil recovery and bioremediation (Emtiazi et al., 2005; Ghanavati et al., 2008).

The marine environment represents the major component of the Earth's biosphere. It covers a majority (70%) part of earth's surface and makes 90% of the volume of its crust. Oceans represent a vast and exhaustive source of natural products in the globe, harboring the most diverse groups of flora and fauna (Hasanshahian and Emtiazi, 2008). Marine microorganisms have developed unique metabolic and physiological capabilities to thrive in extreme habitats and produce novel metabolites which are not often present in the microbes of terrestrial origin (Fenical, 1993; Hassanshahian et al., 2013). Therefore, this rich marine habitat provides a magnificent opportunity to discover newer compounds such as antibiotics, enzymes, vitamins, drugs, biosurfactant (BS), bioemulsifier (BE) and other valuable compounds of commercial importance (Jensen and Fenical, 1994; Austin, 1989; Romanenko et al., 2001; Lang and Wagner, 1993).

Biosurfactant producing microorganisms are ubiquitous, inhabiting both water (sea, fresh water, and groundwater) and land (soil, sediment, and sludge) as well as extreme environments (e.g. hypersaline sites, oil reservoirs), and thriving at a wide range of temperatures, pH values and salinity. Microorganisms produce BS/BE to mediate solubilisation of hydrophobic compounds in their environment to be able to utilize them as substrates, however, this fact may not be always true. Few microbes produce BS/BE on water

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soluble substrates. It has been suggested that the presence of surface active molecules on the microbial cell surface increases the hydrophobicity of the cell and helps it to survive in hydrophobic environment (Hassanshahian et al., 2014b; Das et al., 2009).

Despite the fact that marine environment represents a wealthy basin of diverse microorganisms, it is important to know that at the same time they are suffering from anthropogenic pollution with domestic and industrial wastes (Hassan et al., 1996; Hassanshahian et al., 2014c).

Large quantities of crude oil, hydrocarbons, petroleum oil products and halogenated compounds finds their way into the marine ecosystem through accidental spillage (Satpute et al., 2005). To treat, emulsify or simply overcome such spills, on the one hand, many petroleum based synthetic chemical surfactants often get used (Hassanshahian et al., 2014a). Such synthetic compounds on the other hand, however often have detrimental ecological effects (Hassanshahian et al., 2012a). The use of biosurfactants and bioemulsifiers therefore may represent a better alternative to overcome the toxicity of synthetic compounds. The environmental roles of the biosurfactants produced by marine microorganisms have been reported earlier (Poremba et al., 1991; Schulz et al., 1991; Abraham et al., 1998).

Persian Gulf is a marine environment that was polluted with crude oil during the 1991 Gulf war. The pollution impact of this episode has been evaluated in several studies, all indicated that crude oil accumulated and remained for long time in coastal area (Hassanshahian et al., 2010; Emtiazi et al., 2009). The oil pollution problem is particularly acute in an oil producing area of this marine environment such as Bushehr provenance.

The aims of this study was to understanding the diversity of biosurfactant producing bacteria in Persian Gulf and especially at Bushehr provenance, also isolation and characterization of these important bacteria is another purpose of this research.

2. Materials and methods

2.1. Sampling

Sediments and seawater samples were collected from five stations in the Persian Gulf at Bushehr provenance (Khark Island (KI), Ganaveh Port (GP), Siraf Port (SP), Halileh Shoreline (HS) and Asalooye Shoreline (AS) 26°15′N; 54°15′E). From each station five sediments and seawater samples were taken. These stations located near to petrochemical plants and oil refinery.

Sediment samples were taken from 1–12 cm below the surface of coastal using a sterile knife. Seawater samples were collected from a depth of 15 cm in sterile 100 ml bottles and transported on ice to the laboratory for isolation.

2.2. Isolation and selection of biosurfactant producing bacteria

ONR7a medium was used for isolation of biosurfactant producing bacteria. ONR7a contained (per liter of distilled water) 22.79 g of NaCl, 11.18 g of $MgCl_2{\cdot}6H_2O$, 3.98 g of Na_2SO_4, 1.46 g of $CaC1_2{\cdot}2H_2O$, 1.3 g of TAPS0 {3-[N-tris(hydroxymethyl)methylamino]-2-hydroxypropanesulfonica cid}, 0.72 g of KCl, 0.27 g of NH_4Cl, 89 mg of $Na_2HP0_4{\cdot}7H_2O$, 83 mg of NaBr, 31 mg of $NaHCO_3$, 27 mg of H_3BO_3, 24 mg of $SrCl_2{\cdot}6H_2O$, 2.6 mg of NaF, and 2 mg of $FeCl_2{\cdot}4H_2O$. For solid media, Bacto Agar (Difco) (15 g/l) was added to the solution (Dyksterhouse et al., 1995).

ONR7a medium were supplemented with 1% (v/v) diesel oil as sole carbon source and energy. Portion of sediment (10 g) or condensed seawater (10 ml) were added to Erlenmeyer flasks containing 100 ml of medium and the flasks were incubated for 10 days at 30 °C on rotary shaker (180 rpm, INFORS AG). Then 5 ml aliquots were removed to fresh medium. After a series of four further subcultures, inoculums from the flask were streaked out and phenotypically different colonies purified on ONR7a agar medium. The procedure was repeated and isolates only exhibiting pronounced growth on diesel oil were stored in stock media with glycerol at −20 °C for further characterization (Chaillan et al., 2004; Tebyanian et al., 2013).

2.3. Screening of biosurfactant producing bacteria

Three tests were used for screening and selection of prevailing biosurfactant producing bacteria. These tests were described below.

2.3.1. Hemolytic test

Hemolytic activity was carried out as described by Carrillo et al. (1996). Isolated strains were screened on blood agar plates containing 5% (v/v) blood and incubated at 30 °C for 24–48 h. Hemolytic activity was detected as the presence of a clear zone around a colony.

2.3.2. Drop collapse method

The drop-collapse technique was carried out in the polystyrene lid of a 96-microwell plate (Biolog, Harward, CA, USA) as described by Jain et al. (1991) and 100 μl culture supernatant was added to wells of a 96-well microtiter plate lid, and then 5 μl of crude oil was added to the surface of the culture supernatant. Biosurfactant-producing culture gave flat drops. Aliquots from a culture of each strain were analyzed on two separate microtiter plates.

2.3.3. Oil spreading method

Oil spread technique was carried out according to Morikova et al. (2000) and Youssef et al. (2004). 50 ml of distilled water was added to Petri dishes followed by addition of 100 μl of crude oil to the surface of the water. Then, 10 μl of the culture filtrates was put on the crude oil surface. The diameter of the clear zone on the oil surface was measured.

2.4. Liquid surface tension

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). The validity of the surface tension readings was checked with pure water (70.78 ± 0.02 mN/m) before each reading. All surface tension readings were taken in triplicate.

2.5. Emulsification activity and Bacterial Adhesion To hydrocarbon (BATH test)

The emulsification activity (E_{24}) was determined by the addition of hexadecane, to the same volume of cell free culture broth, mixed with a vortex for 2 min and left to stand for 24 h. The emulsification activity was determined as the percentage of height of the emulsified layer (mm) divided by the total height of the liquid column (mm) (Emtiazi et al., 2009). Bacterial adhesion to hydrocarbon was carried out according to Pruthi and Cameotra, 1997.

2.6. Identification of the isolates

2.6.1. Biochemical identification

In order to identify and characterize the bacteria isolates, some biochemical tests was carried out such as: Gram staining, oxidation/fermentation, production of acid from carbohydrates,