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Isolation, screening, and characterization of surface-active agent-producing, oil-degrading marine bacteria of Mumbai Harbor

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ABSTRACT

Diverse marine bacterial species predominantly found in oil-polluted seawater produce diverse surface-active agents. Surface-active agents produced by bacteria are classified into two groups based on their molecular weights, namely biosurfactants and bioemulsifiers. In this study, surface-active agent-producing, oil-degrading marine bacteria were isolated using a modified Bushnell–Haas medium with high-speed diesel as a carbon source from three oil-polluted sites of Mumbai Harbor. Surface-active agent-producing bacterial strains were screened using nine widely used methods. The nineteen bacterial strains showed positive results for more than four surface-active agent screening methods; further, these strains were characterized using biochemical and nucleic acid sequencing methods. Based on the results, the organisms belonged to the genera *Acinetobacter*, *Alcanivorax*, *Bacillus*, *Comamonas*, *Chryseomicrobium*, *Halomonas*, *Marinobacter*, *Nesterenkonia*, *Pseudomonas*, and *Serratia*. The present study confirmed the prevalence of surface-active agent-producing bacteria in the oil-polluted waters of Mumbai Harbor.

1. Introduction

Surface-active agents (SAAs) are biological compounds produced by bacteria. Oil-degrading marine bacteria produce SAAs when grown on an immiscible carbon source such as diesel, crude oil, or lubricating oil. These compounds are divided into two groups based on their molecular weights: low molecular weight compounds as biosurfactants (BSs) and high molecular compounds as bioemulsifiers (BEs). These compounds are amphiphilic in nature. Due to their dual nature, SAA tend to partition into the oil–water interface to reduce the surface and interfacial tension and stabilize the new created interfaces (Mnif and Ghribi, 2015a). BSs show good surface tension (ST)-reducing activity. Rhamnolipids and surfactin produced by *Pseudomonas aeruginosa* and *Bacillus subtilis*, respectively, are the best-known examples of BSs. BEs are well known for their high emulsifying ability, for example, emulsan and alasan produced by *Acinetobacter venetianus* recombination-activating gene 1 (RAG1) and *Acinetobacter radioresistens*, respectively (Uzoigwe et al., 2015). Based on their diverse chemical structures, BSs/BEs are classified as glycolipids, lipopeptides, phospholipids, fatty acids, neutral lipids, and polysaccharide–protein complexes (Pacwa-Plociniczak et al., 2011).

The important feature of BSs/BEs is their hydrophilic and lipophilic balance (HLB), which indicates the hydrophobic and hydrophilic constituents in surface-active compounds. These molecules act as effective chemical surfactants because of their biodegradability, low toxicity and high efficacy at extreme temperatures, salinity, and pH values. BSs/BEs can also be produced from waste oil generated by the automobile, petroleum, and shipping industries. The marine environment is known to produce harsh living conditions due to its varying surface water temperature, pH, salinity, currents, and wind patterns. As a result, marine bacteria have evolved special physiological and metabolic mechanisms to survive under these adverse conditions by producing various secondary metabolites such as antibiotics, hydrolytic enzymes, and SAAs (BS/BE) (Dash et al., 2013). SAA-producing bacterial species have been isolated from different ecological niches such as soil, seawater, marine sediments, petroleum sludge, rhizospheres and phyllospheres of plants, homogenates of marine invertebrates, and even extreme cold and hot environments such as the Antarctic sea and hot springs (Yakimov et al., 1998; Cappello et al., 2012; Djeridi et al., 2013; Rizzo et al., 2013.).

The diverse SAA-producing bacterial species reported to date belong to different genera such as *Alcanivorax*, *Acinetobacter*, *Aeromonas*, *Alcaligenes*, *Arthrobacter*, *Azotobacter*, *Bacillus*, *Corynebacterium*, *Dietzia*, *Gordonia*, *Halomonas*, *Inquilinus*, *Flavobacterium*, *Planococcus*, *Pseudomonas*, *Nocardia*, *Rahnella*, *Rhodococcus*, *Serratia*, *Streptomyces*, *Marinobacter*, *Microbacterium*, *Micrococcus*, *Mycobacterium*, *Myroides*, *Nocardia*, *Ochrobactrum*, and *Oleomonas* (Thavasi et al., 2009; Satpute et al., 2010, Silva et al., 2015; Smulek et al., 2015; Mnif and Ghribi, 2015b).

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Petroleum hydrocarbons are used as a primary source of energy for power generation, industries, and transportation in daily life. The growth of the human population and advances in science and technology have increased the consumption of petroleum products. In turn, many developing countries import crude oil from the oil fields located in the Persian Gulf and Arabian countries primarily by sea. Due to frequent accidents involving oil-carrying cargo ships, large amounts of oil are released into the marine environment every year. Cleaning of oil tankers also contributes significantly to marine oil pollution. Rules to prevent marine hydrocarbon pollution are often disregarded, thus causing oil spill and damage to beaches, marine organisms, and the coastline (Harayama et al., 1999; Dash et al., 2009).

Marine oil spills are a pressing environmental issue. Bioremediation is a green cleanup alternative that uses oil-degrading bacteria, SAAs, and essential inorganic nutrients such as phosphate and nitrogen to enhance the growth of natural bacterial communities that specifically degrade hydrocarbons. Recently, many hydrocarbonoclastic bacteria (HCB) such as *Alcanivorax*, *Marinobacter*, *Dietzia*, and *Oceanobacter* have been reported to produce BS with crude oil as the carbon source (Yakimov et al., 2007; Bonin et al., 2015; Wang et al., 2014). The primary role of BS in bioremediation is to increase the solubility and bioavailability of hydrocarbons, for easy uptake and metabolization by natural oil-degrading bacteria (Ron and Rosenberg, 2001).

The Mumbai Harbor is one of the important commercial harbors located on the west coast of India. This harbor faces a high risk of oil spill accident because of its heavy shipping traffic. On 7 August 2010, the Panama-flagged *MV MSC Chitra* and *MV Khalijia 3* collided at the mouth of the Mumbai Harbor (18.5199° N 72.4903° E), resulting in a major oil spill. Thus, the waters at harbors and ports are known to be an optimal source for the isolation of BS-producing bacterial strains that specifically biodegrade a variety of hydrocarbons.

The aim of this study was to isolate, screen, and characterize oil-degrading marine bacteria present in the oil-polluted waters of the Mumbai Harbor. In addition, different SAA screening assays were performed to select potential SAA-producing bacterial strains.

2. Materials and methods

2.1. Sampling sites

Seawater samples were collected from three stations in the Arabian Sea near Mumbai: (1) Gateway of India (GI; 18.921836° N 72.834705° E), (2) Karanja Reef (KR; 18.9051° N 72.8760° E), and (3) Prongs Reef (PR) off Colaba (18°5419.6° N 72.4812.2 °E). Seawater samples were collected at a depth of 15 cm in sterile 100-ml bottles and transported within 4 h on ice to the Department of Marine Biotechnology laboratory, Naval Materials Research Laboratory, Ambernath, to isolate the SAA-producing bacteria.

2.2. Isolation of SAA-producing, oil-degrading marine bacteria by enrichment culture method

A modified Bushnell–Haas broth (MBHB) was used to isolate BS-producing bacteria. MBHB ($g\ l^{-1}$) is composed of 1 g of KH_2PO_4, 0.2 g of K_2HPO_4, 0.2 g of $MgSO_4 \cdot 7H_2O$, 0.02 g of $CaCl_2$, 1 g of NH_4NO_3, two droplets of 60% $FeCl_3$, and 30 g of NaCl. The pH was adjusted to 8.2 and autoclaved at 121 °C for 15 min. After the MBHM was cooled to 40 °C, it was supplemented with 1% (*V*/V) high-speed diesel (HSD) (Bharat Petroleum Corporation, India) as the sole carbon and energy source. To prepare a solid modified Bushnell–Haas agar (MBHA) medium, agar (Himedia, Mumbai, India) ($20\ g/l^{-1}$) was added to the solution (Hassanshahian and Emitazi, 2008).

Enrichment cultures were performed in 250-ml Erlenmeyer flasks containing 50 ml of MBHB and 1 ml of seawater samples. The flasks were incubated for 3 days at 30 °C in a rotary shaker (Orbitek Shaker, Scigenics Pvt. Ltd., Chennai, India) operating at 180 rpm. Then, 1 ml of the inoculum was transferred to fresh MBHB for subsequent subcultures. After a series of four further subcultures, 100-μl inocula from the flasks were streaked on MBHA with 1% (*V*/V) HSD as the carbon source in solid medium, and phenotypically different colonies were isolated. The isolates with significant growth on HSD were stored in glycerol stock at −80 °C for further characterization.

2.3. Screening of SAA-producing bacterial strains

The bacterial strains were screened for their SAA-producing ability with nine different methods. The bacterial strains were grown in 500-ml Erlenmeyer flasks with 100 ml of MBHB containing 1% (*V*/V) HSD as the carbon source. Flasks containing sterilized MBHB with 1% (*V*/V) HSD were inoculated with a loopful of bacterial culture grown in HSD-containing marine agar plates, and the culture flasks were incubated in a rotary shaker for 3 days at 180 rpm and 30 °C. After 3 days of incubation, the culture broth from each flask was centrifuged at 6000 rpm and 4 °C for 15 min, and the supernatant was passed through a membrane filter paper of 0.22-μm pore size (Millipore). This cell-free culture broth was used to perform the drop-collapse assay, oil spreading assay, microplate assay, penetration assay, stable emulsification assay, and ST measurement. The bacterial cells were used to perform the blue agar method, hemolytic assay, and bacterial adhesion to hydrocarbon (BATH) assay. All screening experiments were performed in triplicate, and the mean values were used as results.

2.3.1. Blue agar plate method

The blue agar method was performed as described by Satpute et al. (2008). MBHA medium supplemented with 20 $g\ l^{-1}$ of hexadecane or diesel as the carbon source, 0.2 $g\ l^{-1}$ of cetyltrimethylammonium bromide, and 0.005 $g\ l^{-1}$ of methylene blue was prepared. Then, 50 μl of the bacterial cultures was grown in MBHB for 3 days at 180 rpm and 30 °C, spot-inoculated at the center of the blue agar plate, and incubated at 30 °C for 24 h. A dark halo around the colonies indicated BS production. With this method, anionic BSs such as rhamnolipids can be detected.

2.3.2. Hemolytic assay

The hemolytic assay was performed as described by Sharma et al. (2015). Isolated colonies were inoculated on Zobell marine agar containing 5% (*v*/v) blood and incubated at 30 °C for 24–48 h. The hemolytic activity of the bacterial culture was detected by the clear halo zone around the colonies.

2.3.3. Drop-collapse assay

The drop-collapse assay was performed in the polystyrene lid of a 96-well microplate (Nunc, USA), as described by Bodour and Miller-Maier (1998) with slight modification. The culture supernatant (100 μl) was mixed with 5 μl of methylene blue and added to the center of wells of a 96-well microtiter plate lid. Then, 5 μl of diesel was added to the surface of the culture supernatant. The BS-producing culture produced flat drops. To visualize and photograph the drop collapse clearly, 5 μl of methylene blue was added, which did not alter the shape of the droplets. The tests were conducted in triplicate in three separate microtiter plate lids.

2.3.4. Oil spreading assay

The oil spreading assay was performed as described by Youssef et al. (2004). In 100-mm glass petri dishes (Borosil, India), 40 ml of distilled water was added, followed by 100 μl of crude oil on the surface of the water. Then 10 μl of the cell-free culture filtrates obtained by filtering through a membrane filter paper (Millipore) of 0.22-μm pore size was added to the center of the crude oil surface. The diameter of the clear zone formed by the oil displacement was measured.