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Production and structural characterization of biosurfactant produced by newly isolated *staphylococcus xylosus* STF1 from petroleum contaminated soil

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ABSTRACT

Petroleum-contaminated soil was used to isolate and characterize biosurfactant producing bacteria. The strain could produce higher amount of biosurfactant in medium supplemented with motor oil as sole source of carbon and energy. A new biosurfactant producing bacterium, designated as *Staphylococcus xylosus* STF1 based on morphological, physiological, biochemical tests and 16S rRNA gene sequencing. The isolated bacterium was first screened for the ability to produce biosurfactant. Partial sequence of STF1 strain of 16S rDNA gene was highly similar to those of various members of the family Staphylococcaceae. Biochemical characterizations including FT-IR, Raman spectroscopy and Mass spectroscopy studies suggested the biosurfactant to be lipopeptide. Study also confirmed that the cell free supernatant exhibited high emulsifying activity against the different hydrocarbons. Moreover, the partially purified biosurfactant exhibited antimicrobial activity by inhibiting the growth of several bacterial species. The strain could be a potential candidate for the production of polypeptide biosurfactant which could be useful in a variety of biotechnological and industrial processes, particularly in the food and oil industry.

1. Introduction

Surfactants are amphipathic molecules capable of reducing surface and interfacial tensions between liquids, solids and gases (Desai and Banat, 1997; Banat et al., 2000; Nitschke and Costa, 2007). All surfactants have two ends, one of which is hydrophobic and the other hydrophilic (Desai and Banat, 1997). A hydrocarbon part usually comprises the hydrophobic end, which is less soluble in water, whereas the water-soluble hydrophilic end may be a carbohydrate, amino acid, cyclic peptide, phosphate, carboxylic acid or alcohol (Rahman, 2008).

The disposal of oil residues from storage, processing and transportation facilities has always been a major issue faced by the petroleum industry. As a result of increasing environmental awareness and the emphasis placed on a sustainable society in harmony with the global environment, natural surfactants of microbial origin have recently been recommended to replace chemically synthesized surface active agents (Das and Mukherjee, 2007; Gusmão et al., 2010). These natural surfactants offer a number of advantages over chemical surfactants, such as adequate inherent biodegradability, low toxicity and ecological acceptability. These substances may also be used at extremes of acidity, temperature and salt concentration and can be produced from inexpensive, renewable substrates (Banat et al., 2010; Santos et al., 2013).

Biosurfactants are structurally diverse surface active agents produced mainly by hydrocarbon-utilizing bacteria, yeasts and filamentous fungi (Desai and Banat, 1997; Banat et al., 2000; Makkar and Cameotra, 2002; Rahman, 2008). Biosurfactants are utilized in commercial applications in various industries (Desai and Banat, 1997; Kosaric, 1992; Banat et al., 2000) and can be classified by their molecular weight as low-molecular-mass and high-molecular-mass polymers. Low-molecular-mass biosurfactants include glycolipids, lipopeptides and phospholipids, while high-molecular-mass biosurfactants includepolymeric and particulate surfactants and may act as emulsion stabilizing agents (Nitschke and Costa,

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2007). Some of the most common biosurfactants are glycolipids, rhamnolipids, sophorolipids, trehalolipids, lipoproteins and lipopeptides, fatty acids, phospholipids and polymeric structures such as emulsan and liposan (Chrzanowski et al., 2012; Banat et al., 2010).

A great number of microorganisms are capable of secreting various types of biosurfactants. Among these, genera most commonly utilized for biosurfactant production are *Pseudomonas* sp., *Bacillus* sp., *Rhodococcus* sp., *Candida* sp., *Lactobacillus* sp., *Arthobacter* sp. and *Acinetobacter* sp. (Rosenberg and Ron, 1999; Banat et al., 2000; Rufino et al., 2013). While chemically synthesized surfactants are highly effective, such chemicals also tend to be toxic, hazardous to environment and difficult to degrade (Ławniczak et al., 2013). Biosurfactants are biodegradable and less toxic compared to conventional surfactants and as such do not threaten the environment to the same extent as the latter (Vasileva-Tonkova et al., 2011). As such, biosurfactants offer a more environmentally friendly alternative to conventional surfactants (Jacques et al., 2005; Boonchan et al., 1998; Miguel et al., 2009).

Further, biosurfactants are biocompatible, easily degraded in the environment and specific in their action due to the presence of specific functional groups (Bharali et al., 2011). In addition, biosurfactants have better foaming properties, are stable in extreme pH, temperature and salinity ranges (Banat et al., 2000) and can be produced by processing of as industrial wastes or by-products as a nutrient source to biosurfactant-producing microorganisms (Kosaric, 1992).

Biosurfactants have several industrial applications (Cameotra and Bollag, 2003; Soudmand-asali et al., 2007; Ferhat et al., 2011). In addition to their extensive utilization for enhanced oil recovery and bioremediation of pollutants, (Chrzanowski et al., 2012; Szulc et al., 2014; Xiao et al., 2012) surface tension-reducing biological agents have potential application areas in agriculture, detergents, cosmetics, personal care products, pharmaceuticals, textile manufacturing, laundry supplies, metal treatment and processing, pulp and paper processing and paint industries. Further, biosurfactants can be used in food industry as emulsifiers, solubilizers, foaming, wetting, anti-adhesive and antimicrobial agents (Singh and Cameotra, 2004).

In the light of the literatures, present study is the first study about the production of a biosurfactant from the bacteria *Staphylococcus xylosus* which isolated from petroleum contaminated soil. The properties of the biosurfactant produced with different oils and the effect of environmental factors on emulsification activity and stability are reported.

2. Materials and methods

2.1. Isolation, identification and culture conditions of microorganism

The biosurfactant producing bacteria were isolated from the petroleum contaminated soil samples of Ankara, Turkey (39.8679°N, 32.7489°E). Soil samples were collected from the surface layer (5–15 cm) and transported aseptically in a sterile plastic container to the laboratory; they were air dried and stored at 4 °C until use. Cultures were grown in Mineral Salt Medium (MSM) with 15 W-40 motor oil as the sole source of carbon and energy and incubated at 125 rpm and 30 °C. Cultures were stored at 4 °C to maintain viability. For long-term storage, cultures were maintained at −80 °C in 20% glycerol.

Identification of biosurfactant producing bacterium was done by Scanning Electron Microscopy (SEM), 16S rDNA technology and cultural characterization. Partial 16S rDNA gene sequencing was done at ABI 3130xl analyzer based on Sanger's dideoxy termination method at the REFGEN, Ankara, Turkey. The 16S rDNA sequence is available under the GenBank accession number: KP162129. The sequences obtained were analyzed using BLAST. The strain was kept at the public culture collection of the Sustainable Energy Laboratory (SUELab) of Gazi University, (Ankara, Turkey). Cell morphologies were examined under a scanning electron microscope (SEM; JSM-6390, JEOL, Japan) with 20,000 V accelerating voltage.

2.2. Preparation of the bacterial inocula

To screen the isolated bacteria for biosurfactant production, a single colony of each isolate was inoculated into 5 ml of MSM in tubes and incubated overnight until an OD_{580} of 1.5 was reached. These cultures were used as inocula for the screening cultures. In all screening assays, uninoculated growth medium was used as a negative control.

2.3. Screening for biosurfactant production

2.3.1. Hemolytic activity

The bacterium was tested for the ability to produce biosurfactant by using hemolytic assay as the method described by Youssef et al. (2009) with slight differences. Overnight culture of *S. xylosus* STF1 grown on LB medium was inoculated as 10 μl spots on 5% human blood agar plates and incubated at 30 °C for 24 h and then observed for the zone of hemolysis around the spot.

2.3.2. Drop-collapse oil assay

Biosurfactant production was screened using the qualitative drop-collapse oil test described by Bodour et al. (1998) with slight modifications. 2 μL of 15 W-40 motor oil was dropped at the center of each well on a 96-well micro-titer plate and allowed to stand for 24 h for equilibration. 5 μL aliquot of sample was dropped on the oil-coated wells and then the drop size was observed after 1 min with the help of a magnifying glass. The beaded drop was recorded as negative, indicating that the microorganism tested does not produce biosurfactant. Collapsed drops were taken as indicative of biosurfactant production.

2.3.3. Atomized oil assay

In order to determine the levels of biosurfactant production, the atomized oil assay was performed as described in Burch et al. (2010). Bacteria were spotted onto LB agar plates and, following growth, incubated with open lids for drying. A fine mist of mineral oil was applied onto the plates by an airbrush with an air pressure of 15 – 30 psi. Pressure settings were optimized in order to apply a uniform and controlled stream of oil droplets. Biosurfactant halos were observed immediately following oil application by an indirect bright light source, halo diameters were measured and recorded.

2.3.4. Emulsification index measurement

Emulsification index measurement was adapted from the method described by Youssef et al. (2009). Hydrocarbons such as waste motor lubricant oil, crude oil, diesel, kerosene, naphthalene, anthracene, xylene, and peanut oil were tested for emulsification activity. Cell free supernatants were mixed vigorously with the tested oils in a 1:1 ratio. Tubes containing the mixture were then left undisturbed for 24 h. The emulsification index (E24) was calculated as the percentage of height of the emulsion layer divided by the total height.

2.3.5. Antimicrobial test

The bacterial species *Bacillus subtilis* (ATCC 6633), *Staphylococcus aureus* (RSHM 25923), *Klebsiella pneumonia, Pseudomonas aeruginosa* and *Escherichia coli* DH5 were obtained from the