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Original Research Paper

Isolation and Identification of Gram Positive Biosurfactants Producing Bacteria from Mighan Wetland in Iran

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ABSTRACT

Biosurfactants are surface active compounds which are produced by bacteria, fungi and yeasts. Most of them have different structures including: lipopeptides, glycolipids, polysaccharides, protein complexes, fatty acids and phospholipids. Nowadays, due to their useful properties, they have attracted attention of many. Therefore, the present study was conducted to isolate Gram positive bacteria capable of producing biosurfactants from Mighan Wetland in Iran. Accordingly, the isolated microorganisms were evaluated using oil spreading technique with different types of oil and haemolysis tests. The selected microorganisms were detected by their ability to produce surfactants using TLC. The results indicated that out of seven different isolated genera two were Gram positive, and they were characterized as *Bacillus firmus* and *Staphylococcus* sp. On the other hand, oil spreading technique indicated that organisms are able to produce biosurfactants. In addition, extracted biosurfactant on TLC plates and applying ninhydrin reagent indicated the lipopeptide structure of the biosurfactant by producing red spot. Hence, the present study illustrated that this area of investigation could be a suitable place for isolation of microorganism with capability to produce biosurfactants and it could be used for further study and applications.

INTRODUCTION

Biosurfactants are surface active agents produced by bacteria, fungi and yeasts (Gupta & Debnath 2011). These amphiphilic substances could be released into the extracellular medium or located on the cell surface of the organisms (Ward 2010). Biosurfactants belong to different groups including: glycolipids, lipopeptides, fatty acids, phospholipids, neutral lipids and lipopolysaccharides (Rosenberg 1986). In fact low or non toxicity, biodegradability, excellent surface activity, high specificity and effectiveness under extreme temperature and pH conditions are the advantages of biosurfactants (Urum & Pekdemir 2004, Rodrigues et al. 2006).

These specific substances could increase the surface area and bioavailability of hydrophobic water-soluble substrates, heavy metal binding (Kavamura & Esposito 2010, Hoffiman et al. 2010), bacterial pathogenesis and biofilm formation (Fiechter 1992, Gautam & Tiagi 2006, Healy et al. 1996, Simoes et al. 2010). On the other hand, they are used for different applications such as food and cosmetic industries, medicine, environmental protection and crude oil recovery (Fiechter 1992, Desai & Banat 1997, Gudiana et al. 2010). In addition, scientists have shown the antibacterial, antifungal (Joshi et al. 2008) and antiviral properties of biosurfactants which have been extracted from different types of microorganisms (Mulligan 2005, Rodrigues et al. 2004, 2006a, Singh & Comeotra 2004). Hence, the present study was undertaken for isolation and characterization of Gram positive microorganisms which have the ability to produce biosur-factants from Mighan Wetland in Iran.

MATERIALS AND METHODS

Sampling area: For isolation of biosurfactant producing bacteria, samples were collected from soil of Mighan Wetland, which is located at 34.16° N and 49.46°E, and is located at 15 km away from Arak, Iran.

Sample collection: Totally, 1020 g soil sample was collected from the Mighan Wetland. The sample was taken in sterile polythene bags. Out of all, 1000g of the soil was sent to the soil laboratory for identification of soil properties.

Isolation and characterization of biosurfactant producing bacteria: For isolation of biosurfactant producing bacteria, 5 g of the soil sample was inoculated in 50 mL of R2B broth and the flask incubated at room temperature for 48 hr. Then serial dilution technique using R2A agar was performed to isolate bacteria from the soil, following by characterization of different morphological colonies using biochemical tests, and some were selected for further analysis (Anandaraj & Thivakaran 2010).

Screening of biosurfactant producing microorganisms: Two selected methods for primary isolation of biosurfactant producing bacteria have been selected *viz.*, oil spreading

technique and blood haemolysis test (Rodrigues et al. 2006, Banat 1995).

Oil spreading technique: For evaluation of oil spreading test, 30mL distilled water was poured in Petri dishes followed by 1 mL of coconut oil, olive oil, paraffin and petroleum was separately added to the centre of each plate. Subsequently, 20µL of the supernatant from microbial isolates was added to the centre of the plates and the diameter of clear zones of triplicate assays from the same sample were determined and recorded.

Blood haemolysis test: Each single colony from the isolated cultures was taken and streaked on blood agar plates. The plates were then incubated at 37°C for 48-72 hours. The bacterial colonies were examined for the presence of clear zone around the colonies. The clear zone indicates the existence of biosurfactant producing organisms.

Extraction and characterization of biosurfactants: For extraction of biosurfactants, the selected microorganisms were separately inoculated in 50 mL of R2B broth with 5 mL of petrol. The flasks were incubated at room temperature for 7 days under shaking condition (120 rpm). The bacterial cells were removed by centrifugation at 9000rpm for 20 minutes, subsequently the pH of supernatant was adjusted to 2 and equal volume of chloroform : methanol (2:1) was added to the mixture. The mixture was left at overnight for evaporation and white colour sediments were evaluated as biosurfactants.

Characterization of the biosurfactants was done using thin layer chromatography. A spot of crude biosurfactants was placed on the silica gel (F254) plate and the biosurfactants were separated on the plate using chloroform : methanol : water (20:10:0.5). Ninhydrin reagent was sprayed to detect lipopeptide biosurfactant as red spots and anthrone reagent for detection of glycolipid biosurfactant as yellow spots.

RESULTS

Characterization of soil samples: According to the percentage of sand, silt, clay and TNV, the soil texture belong to clay loam with pH 7.65 (Table 1). On the other hand, as the EC is 81.3 mS, it could be concluded that the amount of salt in this area is relatively high and this situation may be able to help the microorganisms to produce biosurfactants.

Table 1: Macro and micro parameters of soil samples.

Macro-parameters	Micro-parameters	Soil texture
EC (mS) - 81.3	Fe (ppm) - 11	Sand - 23.8%
pH - 7.65	Zn (ppm) - 11	Silt - 37.6%
SP (%) - 86	Cu (ppm) - 29.2	Clay - 38.6%
K (ppm) - 814	Mn (ppm)- 1.06	TNV - 31 %
P (ppm) - 9.4		
OC (%) - 1.11		
N (%) - 0.11		

Isolation and characterization of biosurfactant producing bacteria: In this study, seven different types of colonies were isolated and identified. The characterized organisms belong to: *Enterobacter* sp., *Salmonella* sp., *Aeromonas, Pasteurella, Arizonae, Bacillus firmus and Staphylococcus* sp.

Oil spreading technique: The Gram positive bacteria were separately inoculated with the R2B for 48 hrs at room temperature. The samples were then centrifuged and added to the different types of oils. As seen in Table 2, both Gram positive microorganisms were able to displace the petroleum while only *Staphylococcus* was able to dislocate paraffin.

Blood haemolysis test: After evaluating the oil spreading technique the isolated and characterized colonies were streaked on the blood agar medium. The results indicated that *Bacillus firums* shown β haemolysis (Fig. 1), while the *Staphylococcus* sp. showed γ haemolytic activity on blood agar plate.

Extraction and characterization of biosurfactants: The extracted biosurfactants were characterized using TLC plates. The sediments were placed in different TLC plates and the plates were separately sprayed with anthrone as well as ninhydrin reagents for observation of yellow or red color. As shown in Fig. 2, the results indicated that the isolated and characterized organisms show red color indicating the presence of lipopeptide biosurfactants in the organism.

DISCUSSION

Biosurfactants are categorized mainly based on their chemical composition and microbial origin. Generally, their structure include a hydrophobic and hydrophilic moiety. Consequently, the main classes of the biosurfactants include glycolipids, lipopeptides and lipoproteins, phospholipids and fatty acids, polymeric surfactants, and particulate surfactants

Table 2: Oil displacement (mm).

Bacterial isolates	Petroleum	Olive oil	Paraffin	Coconut oil
Bacillus firmus	40	-	-	-
Staphylococcus sp.	55	-	70	-

Fig. 1: β haemolysis by biosurfactant producing bacterium on blood agar plate.

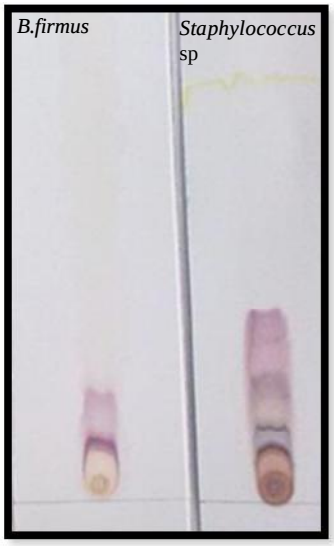

Fig. 2: Characterization of biosurfactants by TLC technique.

(Desa & Banat 1997).

Among the reports published by many scientists various aspects such as their biomedical and therapeutic properties (Cameotra & Makkar 2004, Rodrigues 2006), natural roles (Ron & Rosenberg 2001), production on cheap alternative substrates (Makkar & Cameotra 2002, Maneerat 2005) for the biosurfactants have been suggested. The biosurfactant-producing microbes are distributed among a wide variety of genera, for example, surfactant with lipopeptide nature have been reported by several scientists, which were characterized as *Bacillus* strains (Sheppard & Cooper 1991, Ohno et al. 1995). In addition, Thavasi et al. (2010) have isolated three types of bacterial strains with potential of biosurfactant production. They identified and characterized the organisms as *Bacillus megaterium, Corynebacterium kutscheri* and *Pseudomonas aeruginosa*. In the present study although seven different genera of microorganisms have been detected and characterized from soil of Mighan Wetland in Iran, two of them were Gram positive. Then, these types of organisms were screened for biosurfactants production using petroleum, olive oil, paraffin and coconut oil by oil spreading technique and they were confirmed by TLC. The results indicated that petroleum could be a main source for the microorganisms to produce biosurfactant while other researchers believed that diesel oil was the best carbon source for biosurfactant production (Priya & Usharani 2009). On the other hand, although researchers have reported that glycolipid is the most known biosurfactant which is produced by microorganisms (Desa & Banat 1997), in this study the type of biosurfactant characterized was lipopeptide. Therefore, the present study illustrated that Mighan Wetland in Iran could be an area of investigation for isolation of microorganisms which are capable of producing biosurfactants and could be used for further study and application.

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