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Isolation and Characterization of Biosurfactant Producing Bacteria from Caspian Sea

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Abstract: Marine biosurfactants produced by some marine microorganisms have been paid more attention, particularly for the bioremediation of the sea polluted by crude oil. The goal of this study was isolation and characterization of biosurfactant producing bacteria from Caspian Sea water. Ten morphologically distinct microbial colonies were isolated and screened for biosurfactant production. From the results, tow strains isolated from Caspian Sea in Iran, which were able to grow on crud oil as sole carbon sources and to produce biosurfactants. Primary screening of biosurfactant-producing colonies was performed using the qualitative drop-collapse test, oil displacement test and hemolytic test. Among tow strains CpA1 completely emulsified crud oil in MSM medium within 48 h of cultivation. This strain exhibited the highest activity for oil displacement test toward crud oil (3.14 cm^2) and emulsification activity. In second screening, the surface tension of culture supernatants for CpA1 isolate grown under identical conditions was measured by Captive Drop Cell instrument. This strain reduced the growth medium surface tension below 40 mN m^{-1} and selected as a best biosurfactant producer in this study.

Key words: Marine bacteria, bioremediation, crude oil, drop shape, surface tension

INTRODUCTION

The annual release of crude oil in the oceans is estimated to be around 1.7 to 1.8 metric tons and the impact of this pollution can be severe environmental imbalance (Bicca *et al*., 1999). Crude oil is a highly hydrophobic material with most of its components having low water solubility (Kuyukina *et al*., 2005). When oil spread in the environment, most of the oil hydrocarbons remain on the water surface or adhered to soil particles due to their low solubility (Batista *et al*., 2006). The microbial biodegradation of hydrocarbons appears to be a promising tool to control such pollution (Bicca *et al*., 1999). To increase the bioavailability of hydrocarbon pollutants, surface-active agents (surfactant) may be used, allowing desorption and solubilization of petroleum hydrocarbons and thus facilitating their assimilation by microbial cell (Kuyukina *et al*., 2005; Batista *et al*., 2006). In recent years the interest in surface-active agents has increased (Queiroga *et al*., 2003). Microbial compounds, which exhibit pronounced surface activity, are classified as "Biosurfactant" (Maneerat, 2005). Biosurfactant are unique Amphipatic molecules with properties that have been explored for a variety of industrial and bioremediation applications (Bodour *et al*., 2003). Current worldwide surfactant markets are around $9.4 billion per annum and their demand is expected to increase at a rate of 35% toward the end of the century. Moreover, many commonly used synthetic surfactants are toxic (Desai and Banat, 1997; Batista *et al*., 2006) and poorly biodegradable; their application may lead to the accumulation of ecologically harmful compounds in soil (Kuyukina *et al*., 2005). Biosurfactants have several advantages over the chemical surfactants, such as lower toxicity; higher biodegradability, better environmental compatibility, higher foaming, high selectivity and specific activity at extreme temperatures, pH and salinity and the ability to be synthesized from renewable feedstock (Zajic *et al*., 1977; Kretschmer *et al*., 1982; Georgiou *et al*., 1992; Razafindralambo *et al*., 1996; Desai and Banat, 1997; Kuyukina *et al*., 2005). Also, these compounds can be produced in situ, at the contaminated site (Cha, 2000). These compounds can be used to enhance oil recovery from wells, reduce the heavy oil viscosity, clean oil storage tanks, increase flow though pipelines and stabilize fuel water-oil emulsions (Ghurye and Vipulanandan, 1994; Desai and Banat, 1997; Makkar and Cameotra, 1997; Bognolo, 1999; Banat *et al*., 2000; Batista *et al*., 2006). The most important surface-active properties evaluated in screening for microorganisms with potential industrial application are surface tension reduction (Batista *et al*., 2006). Surface tension is a parameter that is commonly used to describe the effectiveness of a surfactant (Bodour *et al*., 2003). The criterion used for selecting

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biosurfactant-producer is the ability to reduced the surface tension below 40 mN m^{-1} (Cooper *et al*., 1979; Bodour and Maier, 1998; Batista *et al*., 2006). Marine biosurfactants produced by some marine microorganisms have been paid more attention, particularly for the bioremediation of the sea polluted by crude oil. The use of biosurfactant protect the marine environment seems possible since a number of marine bacterial strains can produce biosurfactant during growth on hydrocarbons (Bodour *et al*., 2003). For the sake of the environment, the use of biosurfactant is preferable to those of synthetic surfactants. However, little information on either biosurfactant produced by marine microorganism or biosurfactants active in saline has been reported so far. The objective of this study was to isolate microorganisms with elevated potential for biosurfactant production and to characterize the surface-active properties of the metabolites produced.

MATERIALS AND METHODS

Microorganism isolation: Marine (sediment and water) samples were collected of Caspian Sea from Babolsar in Mazandaran province of Iran in 2007. We was applied a direct method for biosurfactant-producing bacteria isolation in this study. In this method, only bacterial population enrich that capable to use crude oil as a carbon source. 100 mL of each sample was placed into a 250 mL flask containing 100 mL of mineral salts medium (MSM) and 1 g crud oil as a sole carbon source and incubated at 30°C on a shaker at 150 rpm for 1 week. The MSM was a mixture of solution A and solution B. Solution A contained (per liter) 2.5 g of $NaNO_3$, 0.4 g of $MgSO_4$ _ $7H_2O$, 1.0 g of NaCl, 1.0 g of KCl, 0.05 g of $CaCl_2$ _ $2H_2O$ and 10 mL of concentrated phosphoric acid (85%). This solution was adjusted to pH 8.2 with KOH pellets. Solution B contained (per liter) 0.5 g of $FeSO_4$ _ $7H_2O$, 1.5 g of $ZnSO_4$ _ $7H_2O$, 1.5 g of $MnSO_4$ _ H_2O, 0.3 g of K_3BO_3, 0.15 g of $CuSO_4$ _ $5H_2O$ and 0.1 g of Na_2MoO_4 _ $2H_2O$. One milliliter of solution B was added to 1,000 mL of solution A to form the MSM (Bodour *et al*., 2003). In this method, only bacterial population enrich that capable to use crude oil as a carbon source. After observation the crud oil microemulsion, enrichment cultures were diluted in sterile 0.85% saline solution and plated on TS agar for microorganism isolation. Morphologically distinct colonies were reisolated by transfer to fresh agar plates at least three times to obtain pure culture. Pure cultures have been stored at -80°C in mixed with sterile glycerol at final concentration of 15%.

Qualitative screening of surface tension: Hemolytic activity: Isolated strains were screened for hemolytic activity on blood agar plates containing 5% (v/v) human blood and incubated at 37°C for 24 and 48 h (Bicca *et al*., 1999). Hemolytic activity was detected as the occurrence of a define clear zone around a colony. Positive and negative controls were also plated.

Drop-collapse tests: Qualitative drop-collapse tests were performed in the polystyrene lid of a 96 microwell 12.7 by 8.5 cm plate. (Bodour and Maier, 1998; Bodour *et al*., 2003). The lids have 96 circular wells (internal diameter, 8 mm). A tin coat of 10 W-40 oil was applied to each well. The coated wells were equilibrated for 24 h at 23°C and then a 5 μL aliquot of supernatant was delivered into the center of each well. If the drop remained beaded, the result was scored as negative. If the drop spread and collapsed, the result was scored as positive for the presence of biosurfactant. Cultures were tested in triplicate. The mineral salt medium alone had a negative drop-collapse test.

Oil displacement test: Fifteen microliter of crude oil were placed on the surface of distilled water (40 μL) in a Petridish (150 mm in diameter). Then, 10 μL of the culture supernatant were gently put on the center of the oil film. The diameter and area of clear halo visualized under visible light were measured and calculated after 30 sec (Morikawa *et al*., 1993; Maneerat and Phetrong, 2007).

Quantitative measurement of surface tension: Measurement of surface tension: The change in surface tension of cultures was evaluated by using a Captive Drop Cell instrument. Drop Shape Analysis is a powerful technique for the measurement of interfacial properties from the shape of drops/bubbles (Hoorfar and Neumann, 2006). This instrument is a novel system developed for the measurement of surface and interfacial tensions at elevated temperatures and/or pressures. All isolates that tested positive in the drop-collapse and hemolytic test, then tested quantitatively for biosurfactant production. A droplet or bubble is held captive against a specially coated hydrophilic surface, its image digitalized, its shape fit to a mathematical function and then its tension determined by a calculation procedure. Cell suspensions were centrifuged (10,000 g) and the cell-free supernatant was place into a clean transfer vessel. Drop image of supernatant was used to measurement the surface tension.

RESULTS

Ten morphologically distinct bacterial strains were isolated. Then these isolates tested qualitatively for biosurfactant-production with the drop collapse test,

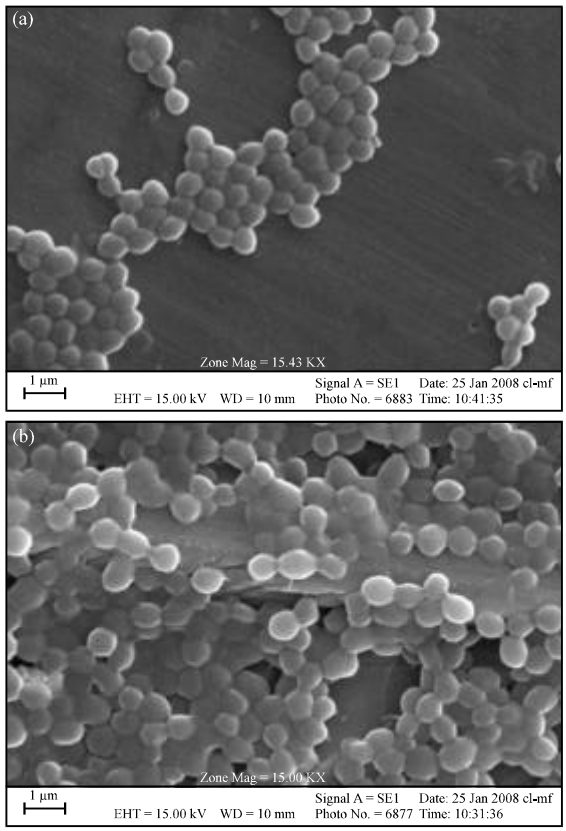

Fig. 1: Scanning electronic microscope image of CpA1 strain

Table 1: Oil displacement activity, hemolytic activity and drop collapsing test of culture supernatant from isolated strains

	Testing method		
Strains	Oil displacement test (cm^2)	Hemolytic activity	Drop collapsing test
CpA1	3.14	+	+
CpA2	2.55	+	+
CpB	-*	-	-
CpC	-	-	-
CpD	-	-	-
CpE	-	-	-
CpF	-	-	-
CpG	-	-	-
CpH	-	-	-
CpL	-	-	-

*Non detectable

hemolytic test and Oil displacement test. This study only strains exhibiting hemolytic activity and emulsifying activity showed the positive result with drop collapsing test and oil displacement test (Table 1). From the results, tow strains isolated from Caspian Sea in Iran, which were able to grow on crud oil as sole carbon sources and to produce biosurfactants. It is possible that other biosurfactant-producing populations were present but not

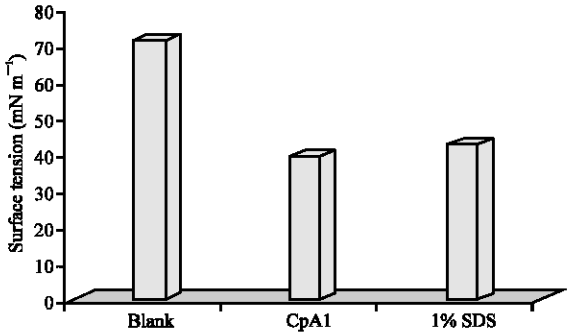

Fig. 2: CpA1 strain reduced the surface tension of culture medium below that of the positive control (1% SDS), a synthetic surfactant

enriched by the screening condition used for the samples tested in study. Two strains named CpA1 and CpA2. These strains exhibited clear haloes on blood agar plate. Among tow strains CpA1 completely emulsified crud oil in MSM medium within 48 h of cultivation, while strain CpA2 emulsified crud oil within 96 h. CpA1 strain exhibited the highest activity for oil displacement test toward crud oil (3.14 cm^2) and emulsification activity and was a good biosurfactant producer. Thus CpA1 selected as the best biosurfactant producer in this study. Scanning electronic microscope image of CpA1 strain showed (Fig. 1). The surface tension value obtained from Captive Drop Cell instrument for MSM medium is 71.23 (mN m^{-1}) which almost agrees well with the literature surface of water. CpA1 strain that tested positive for biosurfactant production in the qualitative tests, reduced the culture medium surface tension 39.4 mN m^{-1}. This strain reduced the surface tension below that of the positive control, 1% SDS, a synthetic surfactant (Fig. 2).

DISCUSSION

From the results, CpA1 exhibited the highest activity for oil displacement test toward crud oil (3.14 cm^2) and emulsification activity and was a good biosurfactant producer. It was noted that the strain with higher emulsifying activity toward crud oil showed the greater oil displacement activity and emulsification activity. These results are in accordance with the study of Batista *et al.* (2006) and Maneerat and Phetrong (2007). On the other hand, we used of the qualitative drop-collapse test and hemolytic activity in primary screening. Although Bodour and Maier (1998) proposed use of the qualitative drop-collapse test for screening biosurfactant-producing organisms But Batista *et al.* (2006) indicated that this test actually screens for production of bioemulsifier and not biosurfactants. In the previous studies, hemolytic activity

has been used for the isolation of Lipopeptide biosurfactants (Mulligan *et al*., 1984; Lin *et al*., 1998) and Rhamnolipids (Iqbal *et al*., 1995). The hydrophilic part of biosurfactant (the cationic part) is proposed to initiate electrostatic interaction with the negatively charged components of the membrane of microbes; the hydrophobic portion is supposed to permit the peptides to insert into and permeate the membrane (Pag *et al*., 2004). Biosurfactant producing capacity in liquid medium was found to be associated with hemolytic activity (Carrillo *et al*., 1996; Fiebig *et al*., 1997). Hemolytic activity therefore appears to be a good screening criterion for surfactant producing strains (Carrillo *et al*., 1996). In another study it has been reported that only 13.5% of the hemolytic Strains lowered the surface tension below 40 mN m^{-1} and not all biosurfactants had a hemolytic activity (Youssef *et al*., 2004). In addition, other microbial products such as virulence factors lyses blood agar and biosurfactants that are poorly diffusible may not lyses blood cells (Iqbal *et al*., 1995; Lin *et al*., 1998). Thus, it is not clear whether blood agar lyses should be used to screen for biosurfactant production. However, such screening can be used as a rapid method, in which samples with the positive result are subsequently subjected to biosurfactant-activity tests in liquid media. Present results are in agreement with observation in previous study that most bacteria isolated from sites with a history of contamination by oil or its byproducts are Gram-negative and this may be a characteristic that contributes to survival of these populations in such harsh environments (Bicca *et al*., 1999). Surface tension is a parameter that is commonly used to describe the effectiveness of a surfactant (Bodour *et al*., 2003). Drop shape methods have been developed to determine the liquid-vapor or liquid-liquid interfacial tensions and the contact angle from the shape of a sessile drop, pendant drop or captive bubble. The advantages of drop shape methods are numerous. In comparison with a method such as the Wilhelm plate technique, only small amounts of the liquid are required. Drop shape methods are easy to handle. They can be used in many difficult experimental conditions such as studies of temperature or pressure dependence of liquid-fluid interfacial tensions. Also, they do not depend on adjustable parameters to determine interfacial tensions and contact angles. Drop shape methods have been applied to materials ranging from organic liquids to molten metals and from pure solvents to concentrated solutions. Also, since the profile of the drop may be recorded as digital images, it is possible to study interfacial tensions in dynamic systems, where the properties are time dependant (Hoorfar and Neumann, 2006). Isolates that liberate biosurfactants into the culture medium are interesting from an industrial point of view, because the product recovery process can be simplified (Fiebig *et al*., 1997; Cameotra and Makkar, 1998; Kuyukina *et al*., 2001; Batista *et al*., 2006). Despite several reports of biosurfactant producing bacteria, present study was first one that isolated these microorganisms from Caspian Sea and used of drop shape technique for measurement of surface tension in this geographical region. CpA1 strain was a good biosurfactant producer and was able to grow on hydrocarbon sources, suggesting its possible exploitation in future biotechnological processes, either directly as a field-released microorganism, or as a biosurfactant producer under controlled conditions. Biosurfactant have been proven as the promising agents for bioremediation of hydrocarbons, particularly oil polluted in marine environment. Nevertheless, metabolites from bioremediation process show the toxicity to some marine organisms. Therefore, novel microorganisms should be intensively screened for bioremediation without causing adverse effects. Further studies are under way to scale up growth conditions and biosurfactant production in bioreactors and to identify strains involved in the synthesis of these bimolecules.

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