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Evaluation of Biosurfactant/Bioemulsifier Production by a Marine Bacterium

Anita Suresh Kumar · Kalpana Mody · Bhavanath Jha

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Abstract *Planococcus maitriensis* Anita I (NCBI GenBank Accession number EF467308) was tested for its biosurfactant/bioemulsifying efficacy. The crude extracellular polymeric substance (EPS) produced by this bacterium contained carbohydrate (12.06%), protein (24.44%), uronic acid (11%) and sulfate (3.03%). The oil spreading potential of this EPS was comparable to Triton X100 and Tween 80. This exopolymer emulsified xylene more efficiently as compared to few standard gums. It also formed stable emulsions ($E_{1,080}$=100) with jatropha, paraffin and silicone oils. The cell free supernatant of this bacterium successfully reduced the surface tension (from 72 to 46.07 mN m^{-1}). It also decreased interfacial tension of hexane and xylene. Based on the emulsifying and tensiometric properties, this bacterium or its exopolymer could be used for bioremediation, enhanced oil recovery and in cosmetics.

Keywords Bioemulsifier · Biosurfactant · Exopolymer · *Planococcus maitriensis* Anita I.

Oil spills are often treated using synthetic surfactants to disperse oil and accelerate its mineralization (Iqbal et al. 1995). Desorption and solubilization are the rate-limiting factors for mineralization of hydrophobic petroleum hydrocarbon in soil and water (Bognolo 1998). Surfactants are molecules consisting of a polar head and a non-polar tail. In an aqueous solution they reduce the surface tension and facilitate the formation of emulsion between liquids of different polarities (Frazer 2000). Surfactants increase the surface area of hydrophobic contaminants in soil or water and thus increase their aqueous solubility and consequently their microbial degradation (Karanth et al. 1999).

Biosurfactants are a structurally diverse group of surface-active molecules that are synthesized by microorganisms (bacteria, fungi and yeast). They include glycolipids, lipopeptides, phospholipids, fatty acids, and polymeric compounds. Biosurfactants are totally or partially extracellular, with an amphipathic structure, which allows them to form micelles that accumulate at the interface between liquids of different polarities such as water and oil. This process is based upon the ability of biosurfactants to reduce surface tension, blocking the formation of hydrogen bridges and certain hydrophilic and hydrophobic interactions. Growth of microorganisms on hydrocarbons accompanied with production of biosurfactants or bioemulsifiers help to disperse the oil by emulsifying the oil, thus increasing the surface area for growth (Ron and Rosenberg 2002). Bushnell and Haas (1941) were among the first to demonstrate bacterial production of biosurfactants. Biosurfactants also exhibit natural physiological roles in increasing bioavailability of hydrophobic molecules and can complex with heavy metals, and some also possess antimicrobial activity (Van Hamme et al. 2006).

Recently diverse functions have been demonstrated for biosurfactants, including emulsification, foaming, antiviral and antimycoplasmic (Dehghan-Noude et al. 2005). Biosurfactants with proven potential for remediation of contaminated sites include surfactin, produced by *Bacillus subtilis* and the rhamnolipid from *Pseudomonas aeruginosa* (Mulligan et al. 2001). There are a number of approaches that measure the surface activity of

A. Suresh Kumar · K. Mody (✉) · B. Jha
Discipline of Marine Biotechnology and Ecology, Central Salt and Marine Chemicals Research Institute, Bhavnagar 364002, Gujarat, India
e-mail: khmody@csmcri.org

biosurfactants. These include surface and/or interfacial tension measurement, axisymmetric drop shape analysis profile (ADSA-P), glass-slide test, drop collapse method and the oil spreading technique. Reduction in surface tension has traditionally been used to detect biosurfactant production (Youssef et al. 2004). Surface tension measurement and emulsifying index are usually used to quantify biosurfactant production (Bicca et al. 1999). Batista et al. (2006) screened biosurfactant/bioemulsifier producing bacteria from terrestrial and marine using the qualitative drop-collapse test, wherein he reported eight isolates that efficiently reduced the surface tension of the growth medium below 40 mN m^{-1}.

The present investigation evaluates the potential of a marine bacterium, *Planococcus maitriensis* Anita I to produce biosurfactant/bioemulsifier.

Materials and Methods

An extracellular polymeric substance producing bacterium was isolated from seawater collected from coastal area of Bhavnagar district, Gujarat, India (Latitude: 21°18′17″N Longitude: 72°05′52″E). Based on the morphological characteristics and 16S ribosomal RNA gene sequence (http://www.ncbi.nlm.nih.gov), this bacterium showed 98% homology with *Planococcus maitriensis* (NCBI GenBank Accession number AJ 544622) also described as *Planococcus maitrii*. The bacterium was maintained on Zobell Marine Agar (Hi Media, India).

The salt tolerance (0.5%–12%) of this bacterium was tested by growing it on solid media viz. Nutrient agar (Hi Media, India, containing 0.5% salt), Modified Nutrient agar (containing 1% and 2% sodium chloride), Zobell Marine agar (Hi Media, India containing 3.5% salts) and Modified Zobell agar (containing 5%, 7%, 10% and 12% sodium chloride concentrations). The culture was inoculated, incubated for 24 h at 32 ± 2°C and salt tolerance was recorded. Salt tolerance was noted in terms of occurrence of growth on the solid medium.

The extracellular polymeric substance produced by *Planococcus maitriensis* Anita I was recovered by inoculating a loopful of the bacterial culture in 100 ml of Zobell Marine broth, incubating at 30 ± 2°C for 48 h, centrifuging the broth (10,000 rpm, 15 min, 10°C) to collect the supernatant, precipitating the supernatant by alcohol (isopropanol) followed by air-drying. The air-dried exopolymer was dialysed (12,000 Da cut off dialysis tubing, Sigma) and lyophilized. This crude extracellular polymeric substance, designated as EPS, was subjected to chemical investigation. The total sugar (Dubois et al. 1956), sulfate (Dodgson and Price 1962), protein (Lowry et al. 1951) and uronic acid (Kuntson and Jeanes 1968) contents of the EPS were estimated spectrophotometrically using glucose, potassium sulfate, bovine serum albumin and galacturonic acid as standards, respectively.

Biosurfactant production was examined using oil-spreading technique (Youssef et. al. 2004). Here, 200 µl of crude oil (Kuwait crude oil) was added onto the surface of distilled water filled in a 50 ml beaker to form a layer on the surface. The cell free supernatant (20 µl) was gradually added on the centre of this layer. The spreading of oil indicated a positive surface active test. A single screening experiment included (1) a negative control or blank using 20 µl distilled water, (2) a positive control using 20 µl of standard surfactant (0.1% Triton X 100 and 0.1 % Tween 80) and (3) tests with 20 µl of 0.1% EPS solution having pH 5.6. Efficiency of biosurfactant production was judged by the zone of oil spreading or dispersion which was observed macroscopically, the larger the zone of spreading of oil, the higher is the surface-active property of the sample. Effect of pH (3 and 9) on oil spreading efficacy was also studied.

The ability of the EPS to emulsify hydrophobic substrates (oil /hydrocarbon) such as cotton seed oil, cedar wood oil, silicone oil, olive oil, jojoba oil, jatropha oil, parrafin oil, sunflower oil, machine oil, coconut oil, kerosene, carbon tetrachloride, hexane, diethyl ether and xylene was studied. The oils were procured from the local market, whereas, the other hydrocarbons were of analytical grade purity. Emulsifying activity was measured using a modified method of Cooper and Goldenberg (1987). Here, the hydrocarbon or oil was added to a graduated test tube containing 0.5% exopolymer solution with hydrocarbon:exopolymer ratio of 3:2(v/v), followed by vigorous agitation on a cyclo-mixer for 2 min. The oil, emulsion and aqueous layers were measured at every 24 h interval (1, 24, 48, 72, etc.) and an emulsification index (E) was calculated as the {(volume of the emulsion layer × total volume^{-1}) × 100 and represented as E_{24}, E_{48}, E_{72}, etc. respectively till 1,080 h.

The surface tension measurements were determined using a dataphysics dynamic contact angle meter and tensiometer (DCAT 21), Dataphysics Instruments GmbH, Germany using Wilhelmy plate (PT 11) made of platinum–iridium. The cell free supernatant (CFS) of the culture grown in Zobell Marine broth for different incubation period (24–72 h) was subjected to surface tension measurement and later precipitated with 3 volumes of alcohol (isopropanol) to obtain EPS.

Interfacial tension of the CFS (obtained after 24 h of incubation period) was measured using with a standard platinum–iridium Du-Nouy ring RG 11 mode. It was measured by dipping the ring into the aqueous sample, layering an equal volume of hydrocarbon on the surface and then measuring the interfacial tension at the CFS-hydrocarbon interface. These studies were carried out

using hydrocarbons like toluene (density 0.86), xylene (density 0.863) and hexane (petroleum fraction, density 0.665). The reagents used in the study were of analytical grade.

Results and Discussion

The exopolymer producing bacterium selected for this study was banked as *Planococcus maitriensis* Anita I (NCBI GenBank Accession number EF467308) as it displayed homology with *Planococcus maitriensis.* The bacterium grew well on media containing 0.5–12% salt and hence it could be classified as a moderately halophilic bacterium.

The chemical nature of a biosurfactant/bioemulsifier plays a vital role in its function. The extracellular polymeric substance (EPS) produced by *Planococcus matriensis* Anita I contained substantial amount of carbohydrate (12.06%), protein (24.44%), uronic acid (11%) and sulfate (3.03%). Thus, it could be classified as a polymeric biosurfactant. Few of the best studied polymeric biosurfactants include emulsan, liposan, mannoprotein, and other polysaccharide–protein complexes (Desai and Banat 1997). Naitali et al. (1989) reported that, although no glycolipidic biosurfactants were produced by *Alcaligenes* Vi1, interfacial tension of hexadecane was notably decreased, suggesting the production of another class of biosurfactants. He also reported a Gram-negative bacterium, HeB2, producing glycosides which moderately reduced surface and interfacial tensions.

The crude exopolymer (EPS) produced by *Planococcus maitriensis* Anita I gave a positive oil spreading test when used at 0.1% concentration at pH 5.6. It also successfully caused dispersion of oil at pH 3 and 9. The efficiency of 0.1% exopolymer to disperse oil was equivalent to Tween 80; where as, Triton X 100 exhibited lower oil spreading efficiency at the same concentration (Table 1). Morikawa et al. (2000) reported the area of displacement by a surfactant-containing solution to be directly proportional to the concentration of the biosurfactants. Youseff et al. (2004) reported oil spreading values ranging from 1–1.5 cm for 100 mg l^{-1} surfactin.

Studies on emulsifying properties of EPS (5 mg ml^{-1}) with xylene (Table 2) indicated that the product exhibited good emulsifying activity (E_{24} = 70). An equivalent emulsifying index has been reported at lower concentration of EPS (1 mg ml^{-1}) produced by *E. cloacae* (Iyer et al. 2006). Karaya and tragacanth gums are reported to exhibit an equivalent emulsification index at a much higher concentration (35 mg ml^{-1}) (Ashtaputre and Shah 1995). Gum arabic is reported to have a lower emulsification index even at higher concentration. Thus this exopolymer can be considered as a more effective emulsifier with respect to some of the standard gums.

Table 1 Comparison of oil spreading efficiency of standard surfactants and crude exopolymer at different pH

Surfactant concentration (0.1%)	Zone of oil spreading (cm)
Distilled Water	0
Tween 80	[a]
Triton X 100	1.7
EPS[b]	
pH 5.6	[a]
pH 3	[a]
pH 9	[a]

[a] Complete dispersion of oil layer

[b] Crude exopolymer produced by *Planococcus maitriensis* Anita I

Table 2 Comparison of the emulsifying properties of crude exopolymer and other standard gums with xylene

Gum (mg ml^{-1})	Emulsification index (E_{24})
Karaya (35.0)	69
Arabic (35.0)	33
Tragacanth (35.0)	67
Xanthan (3.5)	61
Exopolysaccharide produced by *E cloaceae* (1.0)	70
EPS[a] (5.0)	70

[a] Crude exopolymer produced by *Planococcus maitriensis* Anita I

The crude exopolymer emulsified jatropha, silicone and paraffin oil with emulsification index, $E_{1,080}$ = 100 (Table 3). Relatively stable emulsions could also be achieved with cedar wood oil ($E_{1,080}$ = 97). An emulsification index of 85 was recorded for hexane even after a period of 1,080 h. Stable emulsions (E_{24} = 70–75) were also obtained with diethyl ether, xylene and carbon tetrachloride. Less stable emulsion were obtained with machine, cottonseed and groundnut oils as the emulsification indices recorded after 1 h was 100 which decreased with time. The exopolymer formed weak but stable emulsions with kerosene ($E_{24–1,080}$ = 50). *Ralstonia picketti* (BP 20) and *Alcaligenes piechaudii* (CZOR L-1B), isolated from petroleum hydrocarbon-contaminated soil, are also reported to produce stable emulsifiers with several hycrocarbons except diesel oil (Plaza et al. 2005).

Several bioemulsifiers containing a polysaccharide moiety attached to lipid and/or protein have been reported. The hydrophobic lipid portion of emulsan, the nature and concentration of the protein in acacia gums and exopolysaccharide synthesized by *S. paucimobilis* are responsible for emulsifying properties. In the present investigation also, the presence of protein may be responsible for the

Table 3 Emulsifying properties of the crude exopolymer with various hydrocarbons/ oils

Hydrocarbon/oil	Emulsification Index (E_h)											
	E_1	E_{24}	E_{48}	E_{72}	E_{96}	E_{120}	E_{144}	E_{168}	E_{192}	E_{216}	E_{240}	$E_{1,080}$
Diethyl ether	75	70	70	70	70	70	70	70	70	70	70	70
Xylene	70	70	70	70	70	70	70	70	70	70	70	70
Hexane	90	85	85	85	85	85	85	85	85	85	85	85
Carbon tetrachloride	70	70	60	60	60	60	60	55	55	55	55	55
Kerosene	50	50	50	50	50	50	50	50	50	50	50	50
Cottonseed oil	100	55	55	55	50	50	50	50	50	50	50	50
Cedar wood oil	100	100	100	100	100	100	100	97	97	97	97	97
Groundnut oil	100	60	60	60	60	50	50	50	50	50	50	50
Jojoba oil	100	100	100	80	70	70	70	70	70	70	70	70
Jatropha oil	100	100	100	100	100	100	100	100	100	100	100	100
Machine oil	100	95	80	70	50	50	50	50	50	50	50	50
Paraffin oil	100	100	100	100	100	100	100	100	100	100	100	100
Silicone oil	100	100	100	100	100	100	100	100	100	100	100	100

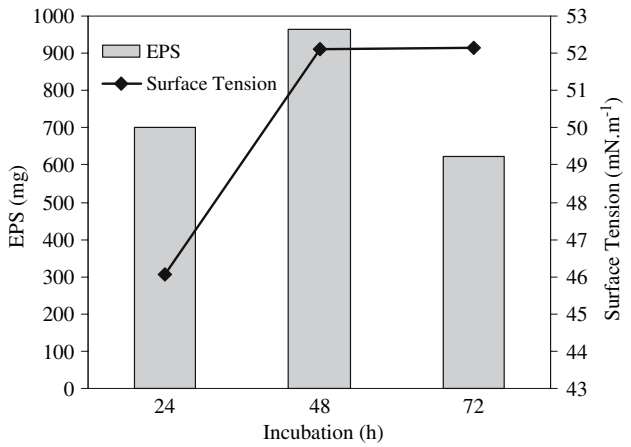

Fig. 1 Crude exopolymer production by *Planococcus maitriensis* Anita I and surface tension of its cell free supernatant (CFS) with respect to time

production of stable emulsions as reported for *E. cloaceae* (Iyer et al. 2006). The presence of protein in the EPS was also confirmed by IR spectroscopy (unpublished data).

In the present investigation, the co-relation between the exopolymer production and reduction in surface tension was studied (Fig. 1). Maximum exopolymer production was recorded at 48 h of incubation. Moreover, minimal surface tension values were recorded between 24 and 48 h of incubation which coincides with initiation of exopolymer secretion and its maximum production. Least surface tension value (46.07 mN m^{-1}) was recorded for CFS collected after 24 h of incubation. Further incubation (after 48 h) led to an increase in the surface tension of the CFS i.e. 52.48 mN m^{-1}. Surface tension values ranging from 65–70 mN m^{-1} has been reported for *T. acidophilus* after 100–500 h of growth (Kingma et al. 1979). In the present investigation, the bacterium proved to be more efficient in reducing surface tension as compared to *T. acidophilus*.

No reduction in interfacial tension could be obtained when the CFS was tested with toluene. In case of hexane, a significant reduction in interfacial tension from 45.57 ± 0.07 to 20.96 ± 1.56 mN m^{-1} was achieved (Table 4). *Planococcus maitriensis* Anita I reduced the interfacial tension of xylene from 33.84 ± 0.19 to 22.95 ± 0.11 mN m^{-1}. Naitali et al. (1989) reported a reduction in interfacial tension of hexadecane from 26.1 to 23 mN m^{-1} by *Pseudomonas fluorescens* and 10 mN m^{-1} by *Alcaligenes faecalis* respectively. Biosurfactant produced by *Rhodococcus* sp., is known to decrease both, surface and interfacial tension to 27 and 1.8 mN m^{-1}, respectively (Abu-Ruwaida et al. 1991).

Planococcus maitriensis Anita I not only produced an extracellular biosurfactant but also demonstrated promising emulsifying activity with numerous hydrocarbons and oils. Contrarily, Menezes et al. (2005) reported surface active and emulsifying properties of *Acinetobacter junii* where the emulsification activity was not due to extracellular products. Pyaza et al. (2006), studied biosurfactant/bioemulsifier production by 16 thermophilic bacteria. According to them, although the surface tension reduction was a good measure of biosurfactant production, it did not correlate well with emulsion ability. Based on the salt tolerance, tensiometric and emulsification characteristics of *Planococcus maitriensis* Anita I, one can suggest the use of either this bacterium or its product as a bioemulsifier/biosurfactant for various applications like microbially enhanced oil recovery (MEOR) and removal of hydrocarbon pollutants, remediation of organics and metals and as cosmetic additives (Desai

Table 4 Reduction in interfacial tension by cell free supernatant of *Planococcus maitriensis* Anita I

Hydrocarbon	Interfacial tension (mN m^{-1}) with distilled water	Interfacial tension (mN m^{-1}) with cell free supernatant
Toluene	30.66 ± 0.18	32.10 ± 0.21
Xylene	33.84 ± 0.19	22.95 ± 0.11
Hexane	45.57 ± 0.07	20.96 ± 1.56

and Banat 1997; Herman et al. 1995; Miller 1995; Miller and Zhang 1997; Stanghellini and Miller 1997; Van Dyke et al. 1993; Zhang and Miller 1995).

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