Abstract

Surfactants are amphipathic molecules, which reduce surface and interfacial tensions and confer excellent detergency, emulsifying, foaming and other versatile chemical properties. Biosurfactants are surface active molecules produced by various microorganisms which exhibit structural diversity, low toxicity and biodegradability. In addition to surface tension reduction, these exhibit additional antimicrobial, antiadhesive, anti-tumor and antiviral activities of biological interest. The marine environment covering more than two-third of the earth's surface, is an unexplored area. It represents a huge and exhaustive resource for discovery of novel natural products especially biosurfactants. In recent years there has been an increasing surge from research communities for search of biosurfactants of marine origin and the results are overwhelming. This prompted us to screen for novel biosurfactant/ bioemulsifier producing marine bacteria and study the potent producers in greater detail. Thus the present study on screening, production, characterization, and applications of biosurfactant/ bioemulsifier from marine bacteria was undertaken.

A total of 16 samples comprising of marine water, sediment, mussels and ship scrapings were collected from coastal areas in India and Iran. Four hundred isolates were obtained in the preliminary screening. Gram negative flora comprising of 337(84.25%) isolates dominated over Gram positive flora comprising of 15.75%. Four Gram negative strains (SW1, SSC3, SS4 and CS6) were identified to belong to the genus Acinetobacter by the chromosomal DNA transformation assay. Analysis by the API 32GN system identified all four marine Acinetobacter as A. baumannii. Physicochemical tests delineated all strains except SSC3 (A. jiinii) into A. baumannii. Closest match for all four strains with the 16S rDNA sequencing and gyrB gene were with A. baumannii. However, CS6 could probably represent a new species as percent similarity with both 16s rDNA gene and the gyrB gene was less than 97%. All four Acinetobacter strains produced enzymes, exhibited 8-11% salt tolerance values and utilized different sugars and hydrocarbons effectively. These formed effective biofilm on glass (A<sub>590</sub> upto 1.3 units) as well as polypropylene surfaces around 48-72 h which in most cases coincided with the maximum bioemulsifier production stage. Except for Acinetobacter sp. CS6, all Acinetobacter spp. exhibited comparable or more biofilm formation (A<sub>590</sub> upto 1.5 units) than Pseudomonas aeruginosa PA01 (A<sub>590</sub> between 0.7 to 1.4 units) on polypropylene surfaces.

Acinetobacter minimal media supplemented with peptone (AMMP) was the best medium for bioemulsifier production. A. baumannii SW1 produced maximum bioemulsifier in AMMP supplemented 1% (w/v) NaCl and 0.01% (w/v) MgSO<sub>4</sub> when incubated at 30 °C, 150 rpm. Maximum bioemulsifier was produced at the onset of stationary phase Emulsification indices at 72 h were 68.8, 65.52 and 62 for xylene, toluene and benzene respectively. A. baumannii SSC3 produced maximum bioemulsifier at in the late log phase of incubation. Optimised medium was AMMP supplemented with 1% (w/v) NaCl, 0.01% (w/v) FeCl<sub>3</sub> and MgSO<sub>4</sub> and incubated at 45 °C and 150 rpm. A. baumannii CS6 was poorest in terms of bioemulsification activities. Maximum
emulsification indices were 68.4, 66.0 and 59.0 at 96 h of incubation. The optimized media contained 0.005% (w/v) FeCl₃ and 0.001% (w/v) MgSO₄; pH of the medium was adjusted to 8. The flasks were incubated at 45 °C and 200 rpm.

Maximum bioemulsifier was produced by *A. baumannii* SS4 at stationary phase in AMMP supplemented with 0.01% (w/v) FeCl₃ and MgSO₄. The flask was incubated at 30 °C and agitation of 150 rpm. Emulsification indices were 76.47, 75.35 and 71.42% for xylene, toluene and benzene respectively. Bioemulsifier from *A. baumannii* SS4 was purified by solvent extraction and was composed of 65.84% non reducing sugars, 1.15% reducing sugars, 30.43% protein and 2.58% lipids with yield of 300 mg/L. Thin layer chromatographic studies revealed that the bioemulsifier probably contained either fructose or glucose or both. DL-alanine, L-glutamic acid and L-lysine were present as a part of the protein component of the bioemulsifier structure. The bioemulsifier was stable at pH between 2 to 12 and temperature up to 100 °C and emulsified a wide range of hydrocarbons.

Large number of marine bacteria were identified as bioemulsifier producers by a battery of tests. 10 cultures were strongly positive for bioemulsifier production as indicated by emulsification activity greater than 150 EU/ml. 96 cultures exhibited excellent emulsification index values. 94 cultures exhibited either alpha or beta haemolysis patterns. 28 cultures were positive though very slightly by the CTAB plate assay. Two cultures (BS8 & BS72) detected as potent biosurfactant producers were selected amongst evaluated marine bacteria for further studies. BS72 reduced the surface tension of zobbell marine medium by 15 mN/m; and emulsified all hydrocarbons and hydrocarbon mixtures with maximum emulsification of toluene (263.5 EU/ml) and mixture of xylene and hexadecane (198.4 EU/ml) at 48 h of incubation. BS8 emulsified aniline maximally (E₂₄ value of 64%) when grown in Zobell marine medium and reduced the surface tension by 9 mN/M. BS8 shared a 100% homology with *Pseudomonas aeruginosa*, while BS72 shared 99% homology with *Brevibacterium halotolerans*.

*Pseudomonas aeruginosa* BS8 produced maximum biosurfactant in peptone glucose medium, pH 7.2 incubated at 30°C, 200rpm at 96 h of incubation. The biosurfactant, a secondary metabolite was a water soluble brown powder with a yield of 100 mg/L. It reduced the surface tension of water from 72.12 mN/m to 49.45 mN/m, had low CMC value of 24 mg/L and good interfacial tension reduction ability. The biosurfactant was stable over wide range of pH and temperature. Biosurfactant produced by *P. aeruginosa* BS8 was glycolipid in nature and was composed of two fractions. Both fractions contained glucose as the sugar moiety. One fraction was a mixture of two lipids with 2-decenonic acid as the major fraction and hexadecanoic acid, 2,3-dihydroxypropyl ester as the minor lipid component. Molecular weight of the compound was found to be 674 amu. The second glycolipid biosurfactant fraction, namely the higher glycolipid fraction also contained a mixture of two lipids with 2-decenonic acid as the major component and 3-hydroxydecanoic acid as the minor fraction.
B. halotolerans BS72 produced a potent biosurfactant in Bushnell Haas minimal medium supplemented with 2% (w/v) mannitol, 3% NaCl (w/v), at 200 rpm and 30°C at 72 h of incubation. The biosurfactant was partially purified by acid precipitation using 6N HCl followed by extraction with chloroform: methanol (2:1). The biosurfactant was an off white water soluble powder and emulsified large number of hydrocarbons. The biosurfactant was a lipopeptide with yield of 280 mg/L. It reduced the surface tension of water by 35 mN/m and had low CMC value of 18 mg/L. It also reduced interfacial tension between xylene water interface by 5.578 ± 0.026 mN/m, toluene water by 7.815 ± 0.029 mN/m, benzene water by 9.478 ± 0.03 mN/m and kerosene water to 8.381 ± 0.03 mN/m. The biosurfactant was stable at pH values from 5 to 14 and temperature of 100°C.

Biosurfactants from B. halotolerans BS72 and P. aeruginosa BS8 exhibited strong antimicrobial activity against both Gram positive and Gram negative bacteria, while biosurfactant from Brevibacterium was antifungal. Toxicity testing and MTT viability assay to test the anticancer potential of the partially purified biosurfactants against human cancer HeLa cells indicated that both the biosurfactants were potential anticancer agents. Percent survival of HeLa cells was as low as 16.1% with partially purified biosurfactant from Pseudomonas and 17.93% with that from B. halotolerans BS72 at a concentration of 500 μg/ml. Partially purified biosurfactant from Brevibacterium exhibited excellent anti-diabetic activity as indicated by an inhibition of 4.4% of pancreatic amylase, 87.92% of liver amylase and 89.47% of intestinal amylase indicating tremendous anti-diabetic potential. Antioxidant activity of the partially purified biosurfactant from Pseudomonas was found to be 83.55% per 20 minutes while that of B. halotolerans biosurfactant was 50%. Partially purified biosurfactants from Brevibacterium and Pseudomonas exhibited strong anti-biofilm activities against pathogenic and detrimental environmental biofilm formers A. baumannii AIIMS 7, P. aeruginosa PA01 and Y. lipolytica 3589. Both biosurfactants disrupted preformed biofilms of these biofoulers by more than 50% and inhibited biofilm formation by more than 70%. Results were reconfirmed by fluorescence and scanning electron microscopic imaging.

Overall, this work represents a detailed study on screening, production, optimisation and characterisation of bioemulsifier from marine Acinetobacter strains. Production, characterisation and applications of biosurfactants from two potent marine bacteria Pseudomonas and Brevibacterium has been evaluated. These biosurfactants can be further exploited commercially for their biomedical applications.

Keywords: Marine, Acinetobacter sp, identification, halotolerant, biofilm, emulsification, Brevibacterium, Pseudomonas, surface tension, interfacial tension, Critical micelle concentration, glycolipid, lipopeptide, antimicrobial, anti-diabetic, anticancer, antioxidant biofilm disruption.