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Characterization of biosurfactants from indigenous soil bacteria recovered from oil contaminated sites

Govind Kumar[1]*, Rajesh Kumar[2] and Anita Sharma[1]

[1]Department of Microbiology, College of Basic Science and Humanities, G.B.P.U.A.T, Pantnagar-263 145, India
[2]Department of Environmental Microbiology, Babasaheb Bhimrao Ambedkar University, Lucknow-226 025, India
*Corresponding Author E-mail: govind34093@gmail.com

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Abstract

Three bacterial isolates (G1, G2 and G3) characterized as *Pseudomonas plecoglossicida, Lysinibacillus fusiformis and Bacillus safensis* were recovered from contaminated soil of oil refinery. These bacterial isolates produced biosurfactants in MSM medium in stationary phase. Biosurfactants were characterized on the basis of their emulsifying properties with petrol, diesel, mobil oil and petrol engine oil. Reduction in surface tension (below 40 mN m^{-1}) and blood hemolysis were also included in biosurfactants characterization. Emulsification indices of G1, G2 and G3 were in the range of 98.82, 23.53 and 58.82 for petrol; 29.411, 1.05 and 70.588 for diesel; 35.31, 2.93 and 17.60 for mobil oil and 35.284, 58.82 and 17.647 for petrol engine oil respectively. Dry weight of the extracted biosurfactant was 4.6, 1.4 and 2.4 g l^{-1} for G1, G2 and G3 respectively. Structural analysis of the biosurfactants by Fourier Transform Infrared Spectroscopy (FTIR) revealed significant differences in the bonding pattern of individual biosurfactant.

Key words

Biosurfactants, Emulsification index, FTIR, Surface tension

Introduction

Biosurfactants are structurally diverse group of surface-active substances produced by different groups of microorganisms during late log or stationary phase of growth. Biosurfactant production is regulated by available carbon source, pH, salinity and temperature (Saikia *et al.,* 2012). They are amphipathic molecules and consist of a polar (hydrophilic) moiety and a non polar (hydrophobic) moiety, which partition preferentially at the interface between fluid phase with different degrees of polarity and hydrogen bindings. A hydrophilic group of biosurfactant consists of mono-oligo- or polysaccharides, peptides or proteins and hydrophobic moiety, usually containing saturated, unsaturated and hydroxylated fatty acids or fatty alcohol. These molecules reduce surface and interfacial tension in aqueous solutions and hydrocarbon mixtures (Joice and Parthasarathi, 2014). The range of pollution in soil extends from waste, including polychlorinated biphenyls, trichloroethylene, pentachlorophenyl and dioxin via polyaromatic hydrocarbons, crude oil, refinery products (kerosene, gasoline, diesel fuel, benzene, toluene) and pesticide to heavy metals. Pollution of sea water and coast with aromatic containing crude oil resulting from oil tanker discharge and accident is a worldwide problem (Rosenfeld and Feng, 2011). In comparison to their chemically synthesized equivalents, they have many advantages. They are environment friendly, biodegradable, less toxic and non-hazardous, hence preferred over chemical surfactants (Singh *et al.,* 2013).

Biosurfactants have better foaming properties and higher selectivity. They are active at extreme temperatures, pH and salinity (Kebria *et al.,* 2009), and can be produced from industrial wastes and by-products. This feature favours their cheap production and also allows utilizing waste substrates and reducing their polluting effect at the same time (Kosaric, 2001; Rahman *et al*., 2003; Das and Mukherjee, 2007; Das *et al*., 2008). Because of their potential advantages, biosurfactants are widely used in many industries such as agriculture, food, cosmetics and pharmaceutics (Muthusamy *et al.,* 2008; Banat *et al.,* 2010; Soberón-Chávez and Maier, 2011). Characterization of bacterial isolates, recovered from oil contaminated soil of Jhansi and their biosurfactants were characterized on the basis of their

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emulsifying potential for different oil.

Materials and Methods

Sample collection, isolation and identification of micro-organisms : For the isolation of biosurfactant producing bacteria, soil samples were collected from an oil refinery at Haldia (W.B.). Dilute samples were plated on Kings B agar to obtain segregated bacterial colonies. Purified bacterial cultures were maintained in Kings B medium.

Identification of test isolates : Recovered bacterial isolates were identified based on morphological (Bergeys Manual, 1994) and 16S rDNA sequence analysis. Purified bacterial isolates were sent to Chromous Biotech India Ltd., Banglore.

Screening of biosurfactant production : Recovered bacterial isolates were tested for biosurfactant production in mineral salt medium g l^{-1} (sodium nitrate- 2.5, K_2HPO_4-1.0, KH_2PO_4-0.5, $MgSO_4$ (anhydrous)- 0.5, KCl-0.1, $FeSO_4$-0.01, $CaCl_2$-0.01, Na_2HPO_4-5.67, $MnSO_4$-0.002, NH_4NO_3- 0.39, *Dextrose-30, pH 7.0 (*Dextrose autoclaved separately). Biosurfactants produced by the test bacteria were characterized on the basis of following tests.

Turbidity, foaming and cell biomass : Bacterial isolates were checked for biosurfactant production in mineral salt medium. Sterile 10 ml medium was inoculated with bacterial isolate and incubated on a rotary shaker at 150 rpm for 48 hr at 28ºC. Turbidity was measured using spectrophotometer at 600 nm. Foam formation was a positive test for biosurfactant production. For cell biomass, 48hr grown cultures were centrifuged and pellet obtained was weighed for biomass estimation. Dry weight of biomass, left after drying at 60ºC for 1 hr was recorded.

Surface tension : Bacterial cultures growing in mineral salt medium for 5 days were centrifuged at 8000 rpm for 20 minutes at 4ºC. Supernatant obtained was used to study surface tension. Tensiometer was calibrated using two standards. One standard was water, which had a surface tension of 72.8 mN m^{-1} with a ring of 6 cm, while the second standard was ethyl alcohol having a surface tension of 22.3 mN m^{-1}. Surface tension of cell free broth was determined using Du Nuoy Ring detachment method according to Zaric and Stefens (1984), with Fischer Autotensiomat Model-21, Fisher Scientific and Co., USA.

Hemolytic activity : Hemolytic activity of the biosurfactant producing bacterial isolates was tested on blood agar plates. Bacterial cultures were stabbed on blood agar and zone of clearance around the colonies was observed according to Plaza *et al.* (2006).

Emulsification index : A mixture of 2 ml culture supernatant (obtained by centrifuging for 96 hrs MSM grown bacterial cultures at 8000 rpm for 20 min at 4ºC) and 2 ml petrol/ diesel/ mobil oil/ petrol engine oil was taken in a test tube. Height of the mixture was measured and then the contents were vortexed for 2 min. Tubes were allowed to stand for 24 hrs and then the height of emulsion layer was measured to determine the emulsion index (Cooper and Goldberg, 1987; Das *et al.*, 1998).

Biosurfactant extraction and their structural analysis by FTIR : Biosurfactants from 48 hr old bacterial cultures was extracted in diethyl ether. For FTIR analysis, biosurfactant samples and potassium bromide were dried overnight at 60ºC in an oven to remove traces of moisture. Sample (20 mg) was mixed with KBr (120 mg) and then grinded using a paestle and mortar. Finely grinded sample was analysed using FTIR model Bruker, Vertex 70 Fourier transform IR spectrophotometer at Dept. of Biophysics of the University.

Results and Discussion

Among the recovered surfactant producing bacterial isolates, G1 was Gram negative while G2 and G3 were Gram positive. Morphological features of the bacteria was observed on King' s B agar media. As per sequence analysis using NCBI (www.ncbi.nlm.gov/BLAST), bacterial isolates G1, G2 and G3 showed 98% homology with *Pseudomonas plecoglosicida, Lysinibacillus fusiformis* and *Bacillus safensis* respectively. The accession number of G1, G2 and G3 were: JX149549, JX149544 and JX149547 respectively.

With turbidity, cell yield of all the bacterial cultures increased gradually. Maximum turbidity and cell yield were observed in G2 (Fig 1). To test foaming characterstics, bacterial isolates were grown in 10 ml minimal salts and carbohydrate medium for 48 hrs. All the test isolates were found to foam. On blood agar, isolates G1 and G2 showed %-hemolysis whereas G1 and G2 showed %-hemolysis. Culture, showing haemolysis was able to produce biosurfactants. These results are in accordance to the results reported by Rashedi *et al*. (2005) and Kumar *et al.* (2013).

Five-days-old mineral salt medium broth of G1, G2 and G3 showed a reduction in surface tension from 72.8 to 25.89, 27.82 and 30.16 mN m^{-1}, respectively (Fig. 2 a,b,c). Mulligan,

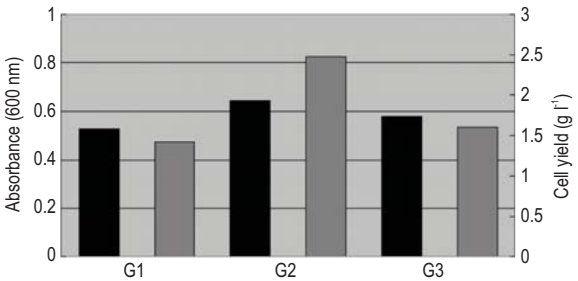

Fig 1 : Absorbance (black bar) and cell yield (g l^{-1}) (grey bar) of bacterial strains isolated from soil of oil refinery

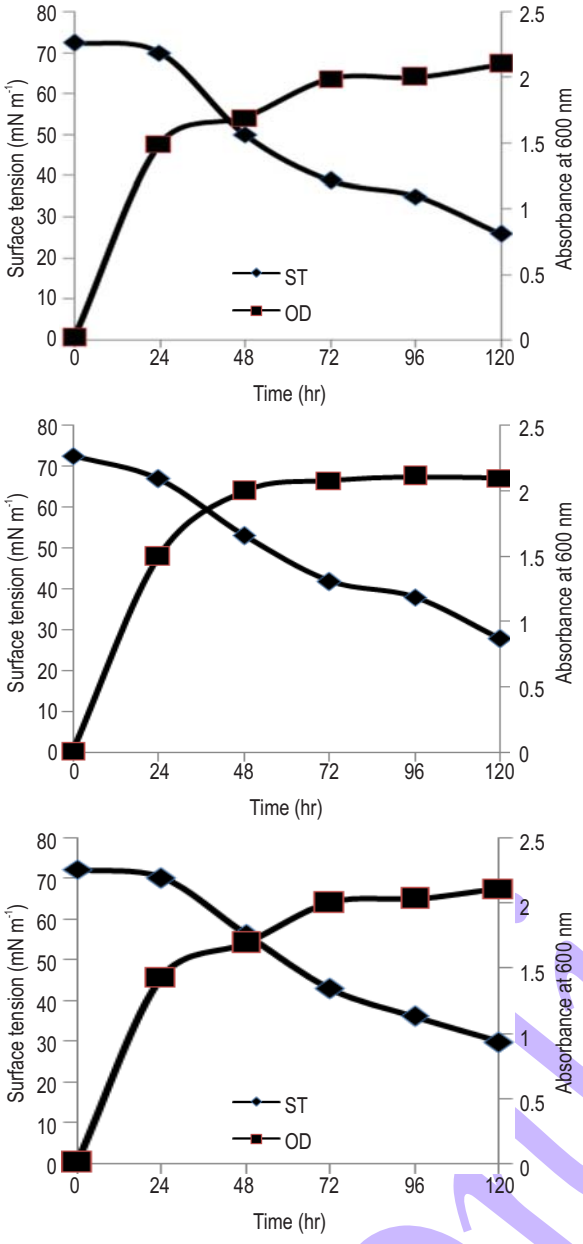

Fig 2 : Surface tension reduction and growth pattern in MSM medium by culture G1 (a), G2 (b) and G3 (c)

(2005) mentioned that a good surfactant can lower down the surface tension of water from 72 to 35 mN m^{-1}. Similar results were observed by Vaz *et al.* (2012).

Stranded mixture of petroleum products (petrol, diesel, mobil oil and petrol engine oil) and cell free extract of 48 hr grown bacterial culture was used for checking the emulsification index of crude biosurfactants. Cell free extracts of all bacterial isolates emulsified values for petrol efficiently as compared to petrol, diesel,mobil oil and petrol engine oil. For petrol, mobil oil and petrol engine oil, cell free extract of G1 showed maximum emulsification index (E24) of 98.82, 35.31 and 35.254, respectively as compare to other test strains. For diesel max, E_{24} *i.e* 70.588 was showed by G3 strain. Emulsification is an indicator of the surfactant's ability to chelate oil or hydrocarbon and this ability varies with the type of hydrocarbon. Similar type of study was also reported by Anyanwu and Chukwudi (2010). More the percentage of emulsification better is the surfactant for chelation of oil and subsequently it can be used effectively for remediation of petroleum contaminated soil.

IR spectra of biosurfactant of G1 showed the presence of different bonds. Position of secondary amide was observed at 3360.432, CH_2 stretching frequency at 2930.361, carbonyl stretching frequency at 1721.132, C-C stretching vibration at 1128.171. C-H deformation frequency was observed at 1384.304. IR spectra of biosurfactant of G2 showed CH_2 stretching frequency at 2868.582 and C-C stretching vibration at 1140.653. IR spectra of biosurfactant of G3 showed CH_2 stretching at 2865.747, carbonyl stretching frequency at 1736.553, C-C stretching vibration at 1139.692 and N-H deformation of secondary amide at 1648.865. C-H deformation frequency was observed at 1378.876.

Different types of biosurfactants have different bonds, Presence of at least one ester bond and / or one carboxylic group was reported in all. Similar types of bond of biosurfactants have been reported in *P. aeruginosa* by Pacwa *et al.* (2011). CH_2 and C-C stretching vibration were observed in all the biosurfactants while C-H deformation frequency was observed in the biosurfactants of G1 and G2 only. Among the surfactant producing bacterial isolates, G1 and G2 showed maximum similarities with *P. aeruginosa.* Biosurfactant produced by these organisms (G1 strain) can be rhamnolipid as evident by Sadoudi *et al.* (2014). Biosurfactant produced by G2 and G3 could be compared with biosurfactant produced by *Bacillus sp.* (Thaniyavaran *et al.,* 2006). A similar kind of study was reported by Chandankere *et al.,* (2014).

Biosurfactants having higher foaming, emulsification of hydrocarbon with a wide range of reduction in surface tension, can be used for *in situ* bioremediation of ground water, oil spill clean up, oil contaminated soil and microbial enhance oil recovery.

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