Journal of Oleo Science
Copyright ©2010 by Japan Oil Chemists' Society
J. Oleo Sci. **59**, *(11) 601-605 (2010)*

JOURNAL OF OLEO SCIENCE
JOS

NOTE

Biosurfactant Production from n-Paraffins by an Air Isolate *Pseudomonas aeruginosa* OCD_1

Sahoo, S., Datta, S.* , Biswas, D. and Banik Choudhury, R.

Department of Chemical Technology, University of Calcutta (92, A.P.C. Road, Kolkata – 700 009, INDIA)

Abstract: The potential production of biosurfactant was investigated with a strain of *Pseudomonas aeruginosa* OCD_1, which was isolated from air in our laboratory. The degradation of different hydrocarbons was studied with this microorganism. The values of surface tension and emulsification index of culture broth were very promising when n-octadecane was used as substrate. Characterization of biosurfactant revealed that the biosurfactant was rhamnolipid in nature. The surface tension of water was reduced to 31.5 mN/m from 72 mN/m with the critical micelle concentration of 35 mg/L. A low rhamnolipid concentration (< 5 mg/L) had a strong effect on reduction of surface tension.

Key words: air isolate, biosurfactant, *Pseudomonas aeruginosa*, rhamnolipid

1 INTRODUCTION

Microbially-derived surfactants or biosurfactants are heterogeneous group of surface active molecules produced by a wide variety of bacteria, yeast and filamentous fungi, which either adhere to cell surface or are excreted extracellularly in the growth medium. Increasing environmental concern had lead to consider biological surfactants as alternative to chemically synthesized surfactants. The most important advantage of biosurfactant when compared to synthetic surfactants is their ecological acceptance, owing to their low toxicity and biodegradable nature[1]. Other advantages are ease of synthesis, specific action, and effectiveness at extreme conditions viz., temperature, pH and salinity[2]. Microbial surfactants are complex molecules, comprising a wide variety of chemical structures, such as glycolipids, lipopeptides, fatty acids, polysaccharides-protein complexes, peptides, phospholipids and neutral lipids[3]. Biosurfactant have many industrial applications in different areas like oil industry, food industry, agricultural sector, pharmaceutical industry, paper and pulp industry[4]. About a 30% increase in total oil recovery from underground sandstone by using trehalolipids from *Nocardia rhodochrous* has been documented[5]. A commercialized biosurfactant obtained from an isolate of *B. licheniformis* JF-2 is potentially useful for in situ microbial enhanced oil recovery[6]. Microbial remediation of hydrocarbon and crude oil contaminated soils is an emerging technology involving the application of biosurfactants[7, 8]. Biosurfactants are also useful in bioremediation of sites contaminated with heavy metals such as uranium, cadmium, lead etc[9]. In the food processing industries, improvement in dough stability, texture, volume and conservation of bakery products is obtained by addition of rhamnolipid[10]. Biosurfactants are also very attractive in the health care and cosmetic industries[11]. Some antimicrobial action against bacteria, fungi, algae, and viruses are observed by several biosurfactants. The lipopeptide iturin from *B. subtilis* showed potent antifungal activity[12].

This paper demonstrates the isolation of a potent biosurfactant producer from air and corresponding production of biosurfactant which reduces the surface tension of culture media when grown on n-paraffins especially n-octadecane. Characterization of isolated biosurfactant was also conducted.

2 EXPERIMENTAL

2.1 Materials

Diesel was procured from the retail market and pure hy-

*Correspondence to: Datta, S., Department of Chemical Technology, University of Calcutta, 92, A.P.C. Road, Kolkata-700 009, INDIA
E-mail: sriparna_d@yahoo.com
Accepted April 23, 2010 (received for review February 25, 2010)
Journal of Oleo Science ISSN 1345-8957 print / ISSN 1347-3352 online
http://www.jstage.jst.go.jp/browse/jos/

drocarbons were procured from Lancaster. Other chemicals and solvents were of LR grade and purchased from local suppliers. Bushnell-Haas (BH) and Nutrient agar media of Hi Media Laboratories Pvt. Ltd. were used for isolation, cultivation and maintenance of culture.

2.2 Isolation of organism

The diesel degrading organism was isolated from air in our laboratory as air is also rich in microorganisms like soil and water. Isolation of microorganisms was done in five 250 mL sterilized conical flasks, containing 100 mL BH media. 2% (v/v) diesel was added aseptically as the sole C-source for microorganisms and four flasks were left open in the laboratory environment for isolating strain from air against a blank which was in sterile condition. The flasks were left open for fifteen days and manual shaking was done at regular intervals. After fifteen days, growth was observed in all the flasks in which two flasks showed greenish color broth. These cultures were then plated on nutrient agar media by streak plate method. The single colonies isolated were maintained on nutrient broth at 4℃ and subcultures were made in every two week.

2.3 Screening for biosurfactant producer

Shake flasks studies were performed for screening of most effective biosurfactant producer in a shaker incubator (ORBITEK-LJE, Scigenics Biotech Pvt. Ltd., Chennai, India) at 35℃ and 100 r.p.m in 250 mL conical flasks containing 100 mL of sterile BH media and 2% (v/v) diesel. 1% (v/v) fresh overnight cultures were inoculated in the media aseptically and incubated for four days. The pH of the medium was 7.0. The experiment was performed in triplicate. For screening of most effective biosurfactant producer, surface tension, emulsification index were measured and cell surface hydrophobicity test (BATH assay) were performed with the specific culture broths respectively. Surface tension of cell free and oil free supernatent was measured at 30℃ by the application of du Nouy ring tensiometer[13] locally made, graduated to 0.1 mN/m. Culture broth was centrifuged at 10,000 rpm, (Remi C 30) and then separated from the oil phase in a separating funnel. Emulsification index (E_{24}) was measured using the method described by Cooper and Goldenberg[14]. The BATH test was used to determine changes in cell surface hydrophobicity during growth on liquid BH media with diesel as the C-source. This test was carried out using a method of Rosenberg *et al.*[15].

2.4 Identification of selected organism, OCD_1

Initially, some biochemical tests were performed in our laboratory following directions of the Bergey's manual[16]. Then genomic identification was carried out based on 16S r-DNA gene-sequencing from Bangalore Genei, Bangalore, India.

2.5 Treatment of hydrocarbons of N-P-A series

The organism was treated with some single hydrocarbons of naphthene-paraffin-aromatic (N-P-A) series like nonane, isooctane, dodecane, hexadecane, octadecane, eicosane, octacosane, decalin, benzene, naphthalene, anthracene to establish the degradation effect of the microorganism on the particular type of hydrocarbons.

2.6 Production and extraction of biosurfactant

Production of biosurfactant was done with OCD_1 in a liquid BH media containing 2% (v/v) n-octadecane at 35℃, 125 r.p.m and pH 7.0. After four days incubation, cell free supernatant was taken for partial purification of the surfactant. The biosurfactant was precipitated by acidifying culture supernatant to pH 2 with concentrated HCl and kept at 4℃ overnight, then recovered by centrifugation at 12,000 r.p.m for 1 h. The precipitate was dissolved in deionized water. Two volumes of chloroform and methanol (2:1, v/v) were added to biosurfactant solution and shaken 30 min for extraction[17]. The organic phase was separated and evaporated to dryness using a rotary vacuum evaporator (EYELA, Rikakikai Co. Ltd., Tokyo, Japan) to give the crude biosurfactant at 45℃.

2.7 Characterization of biosurfactant

Three different chemical tests and one spectroscopic analysis were performed for structural characterization of the isolated biosurfactant.

2.7.1 Chemical tests

Molisch's test was done with alcoholic sample and 2 drops of Molisch's reagent, a 5% solution of α-naphthol in alcohol. A reddish-violet ring at the junction of the two liquids after addition of conc. H_2SO_4 indicated the presence of sugar in the sample. The presence of carbohydrate groups in the biosurfactant molecule was also determined by rhamnose test using the method described by Dubois *et al.*[18]. Again, the orcinol-test[19] was used for detection of rhamnolipid present in the culture broth.

2.7.2 FTIR spectra analysis

The extracted biosurfactant was dried with anhydrous Na_2SO_4 and used for FTIR spectra analysis. The IR spectra were recorded on the JASCO FTIR-670 Plus spectrophotometer using KBr discs, in the 4000～400 cm^{-1} spectral region at a resolution 2 cm^{-1}.

2.8 Determination of the critical micelle concentration (CMC)

Determination of CMC was performed by measuring surface tension of serial dilution of the surfactant solution (0.1 %, w/v) at 30℃.

Table 1 Biosurfactant producing activity of three isolated strain, OAD_4, OBC_3 & OCD_1

Microorganisms	Surface Tension[a] (mN/m)	E_{24} (%)	Hydrophobicity (%)
OAD_4	53.3	3.57	30.43
OBC_3	59.6	0.00	22.62
OCD_1	36.8	47.45	65.75

[a]Surface tension of culture broth was measured at 30℃.

3 RESULTS AND DISCUSSION

3.1 Isolation of organisms and screening of biosurfactant producers

Three different single colonies were isolated as OAD_4, OBC_3 and OCD_1 from air. Among which strain OCD_1 was the best biosurfactant producing microorganism based on the values of surface tension reduction, E_{24} and BATH test (Table 1) using diesel (2%, v/v) as the substrate. Therefore, strain OCD_1 was studied further.

3.2 Identification of strain OCD_1

The biochemical tests resulted (Table 2) that the strain OCD_1 was tentatively *Pseudomonas* sp. This result was also supported with the result of 16S r-DNA sequences. Based on nucleotides homology and phylogenetic analysis the nearest homolog of the strain OCD_1 was found to be *Pseudomonas aeruginosa* strain J007 (Accession No. FJ227280).

3.3 Treatment of single hydrocarbon of N-P-A series

The organism OCD_1 degraded only n-paraffins viz., dodecane, hexadecane, octadecane, eicosane and octacosane, other hydrocarbons of naphthene and aromatic series were not degraded by the organism. Measuring optical density (O.D) and surface tension (data not shown) of the culture broth, the maximum growth and maximum biosurfactant production were observed respectively with n-octadecane as the C-source. Therefore, n-octadecane was used as substrate for production of biosurfactant.

Table 2 Biochemical characteristics of strain OCD_1

Characteristics	Test Results
Gram reaction	(-)ve
Shape	Rod
Oxidase Test	(+)ve
Non-fluorescent diffusible blue-green pigment formation	(+)ve
Growth at 40℃	(+)ve
Growth at 4℃	(-)ve
Starch hydrolysis test	(-)ve
Nitrate reduction test	(+)ve

Table 3 Biosurfactant production capability of strain OCD_1 using n-octadecane as the substrate

Surface Tension[a] (mN/m)	CMD^{-1} [a] (mN/m)	CMD^{-2} [a] (mN/m)	E_{24} (%)
32.4	40.8	56.7	66.67

[a]Surface tension, CMD^{-1} and CMD^{-2} of culture broth were measured at 30℃.

3.4 Production of biosurfactant

The effective biosurfactant production on n-octadecane with OCD_1 was observed by the values of surface tension (ST), critical micelle dilution (CMD^{-1} & CMD^{-2}) and E_{24} (Table 3). CMD^{-1} and CMD^{-2} denote the surface tension of the culture broth diluted 10-times and 100-times with distilled water respectively. Dimensions of CMD^{-1} and CMD^{-2} are same as the dimension of surface tension, mN/m. ST, CMD^{-1}, CMD^{-2} and E_{24} are the indication of biosurfactant concentration in liquid broth. Surface tension of culture broth was reduced to 32.4 mN/m. The CMD^{-1} and CMD^{-2} were obtained as 40.8 mN/m and 56.7 mN/m respectively. The lower the CMD^{-1} and CMD^{-2} values, the higher is the dilution needed to cause a significant change in surface tension, thus higher is the biosurfactant concentration in culture media. The emulsification index of 66.67% is a good indication of high biosurfactant production by OCD_1.

3.5 Characterization of biosurfactant

The Molisch' s test, rhamnose test and orcinol-test were positive which indicate that the presence of sugar moieties in the isolated biosurfactant i.e., the biosurfactant could be of rhamnolipid type. This finding was confirmed by FTIR analysis of extracted biosurfactant. Figure 1 shows the characteristics bands of different groups. In the region 3000～2700 cm^{-1} were observed stretching bands of CH_2 and CH_3 groups. Carbonyl stretching band was found at

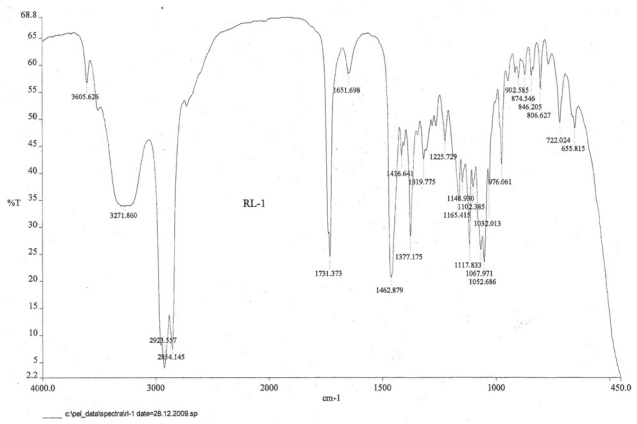

Fig. 1 FTIR spectrum of extracted rhamnolipid

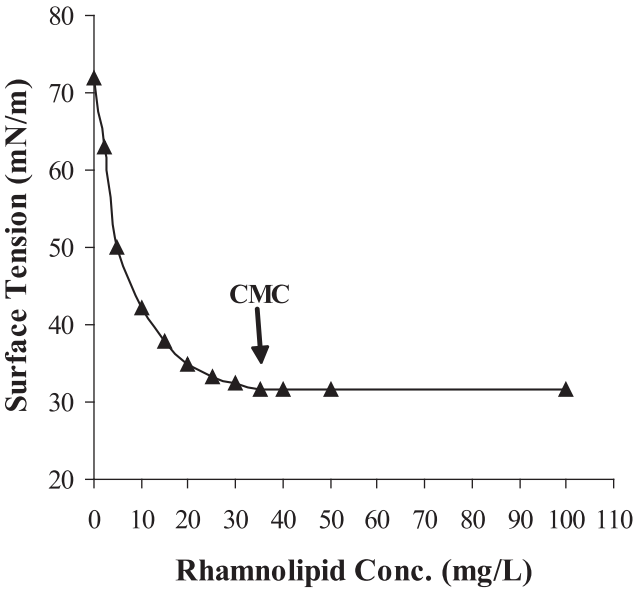

Fig. 2 Surface tension (mN/m) versus rhamnolipid concentration (mg/L)

1731 cm^{-1} which was characteristic for ester compounds. Several C-O stretching band were also observed at 1118, 1053, 1032 cm^{-1}. The C-H and O-H deformations of carbohydrates were found at 1462, 1416, 1319 and 1225 cm^{-1} i.e., in the region 1462-1225 cm^{-1}.

3.6 Effect of rhamnolipid on surface tension and CMC determination

The dependence of surface tension on the biosurfactant concentrations studied is shown in Fig. 2. Surface tension decreased rapidly from 72 mN/m to 33.8 mN/m with small increase in the rhamnolipid concentration up to 20 mg/L. Further increase in the rhamnolipid concentrations only slowly reduced the surface tension from 33.8 mN/m to 31.5 mN/m. Once the surface tension reached 31.5 mN/m, the further addition of rhamnolipid had no effect on surface tension. Low rhamnolipid concentrations (<5 mg/L) had a strong effect on surface tension, while high concentration (>35 mg/L) had a negligible effect. It was observed that the rhamnolipid concentration of 35 mg/L, corresponding to surface tension of 31.5 mN/m, was the point after that surface tension remains constant. Therefore, 35 mg/L was assumed to be CMC of rhamnolipid obtained from *Pseudomonas aeruginosa* OCD_1 when grown on n-octadecane.

4 CONCLUSION

Pseudomonas aeruginosa OCD_1, isolated from air, was able to produce rhamnolipid type biosurfactant using higher n-paraffins i.e. C_{12}-onwards.The surface tension of culture broth reduces remarkably using n-octadecane as the substrate. The isolated rhamnolipid has good emulsifying properties also. In literature, values from 5 to 200 mg/L have been reported for CMC in water for rhamnolipids produced by *Pseudomonas* strains[20, 21]. A CMC value of 35 mg/L obtained for the isolated rhamnolipid, which is satisfactory as it indicates that small quantity of biosurfactant may reduce surface tension significantly. Therefore, the isolated strain may be capable of reducing environmental pollution both by taking hydrocarbons as substrate and simultaneously producing good quality of environment friendly biosurfactant, which may have potential industrial applications.

ACKNOWLEDGEMENT

The authors gratefully acknowledge University Grants Commission (UGC) for financial support.

References

1. Karanth, N. G. K.; Deo, P. G.; Veenanadig, N. K. Microbial production of biosurfactants and their importance. *Curr. Sci.* **77**, 116-126 (1999).
2. Tabatabaee, A.; Mazaheri Assadi, M.; Noohi, A. A.; Sajadian, V. A. Isolation of biosurfactant producing bacteria from oil reservoirs. *Iranian. J. Env. Health Sci. Eng.* **2**, 6-12 (2005).
3. Banat, I. M.; Makkar, R. S.; Cameotra, S. S. Potential commercial application of microbial surfactants. *Appl. Microbiol. Biotechnol.* **53**, 495-508 (2000).
4. Mukherjee, S.; Das, P.; Sen, R. Towards commercial production of microbial surfactants. *Trends Biotechnol.* **24**, 509-515 (2006).
5. Rapp, P.; Bock, H.; Urban, E.; Wagner, F.; Gebetsberg-

er, W.; Schulz, W. Use of trehalose lipids in enhanced oil recovery. *DESCHEMA Monogr. Biotechnol.* **81**, 177-185(1977).
6. Javaheri, M.; Jenneman, G. E.; McInerney, M. J.; Knapp, R.M. Anaerobic production of a biosurfactant by *Bacillus licheniformis* JF-2. *Appl. Environ. Microbiol.* **50**, 698-700(1985).
7. Banat, I. M. Characterization of biosurfactants and their use in pollution removal-state of the art. *Acta Biotechnol.* **15**, 251- 267(1995).
8. Bartha, R. Biotechnology of petroleum pollutant biodegradation. *Microb. Ecol.* **12**, 155-161(1986).
9. Miller, R. M. Biosurfactant-facilitated remediation of metal-contaminated soils. *Environ. Health Perspect* **103**, 59-62(1995).
10. Van Haesendonck, I. P. H.; Vanzeveren, E. C. A. Rhamnolipids in bakery products. *International Application Patent*(*PCT*) WO 2004/040984(2004).
11. Klekner, V.; Kosaric, N. Biosurfactants for cosmetics. in *Biosurfactants: Production, Properties, Applications*(Kosaric, N. ed.) Marcel Dekker Inc. NY. pp. 329-372(1993).
12. Besson, F.; Peypoux, F.; Michel, G.; Delcambe, L. Characterization of iturin A in antibiotics from various strains of *Bacillus subtilis. J. Antibiot.* **29**, 1043-1049 (1976).
13. Adria, A.; Bodour, K.; Drees, P.; Raina, M. M. Distribution of biosurfactant-producing bacteria in undisturbed and contaminated Arid South Western soils. *Appl. Environ. Microbiol.* **69**, 3280-3287(2003).
14. Cooper, D. G.; Goldenberg, B. G. Surface active agents from two *Bacillus* Species. *Appl. Environ. Microbiol.* **53**, 224-229(1987).
15. Rosenberg, M.; Gutnick, D.; Rosonberg, E. Adherence of bacteria to hydrocarbons: a simple method for measuring cell surface hydrophobicity. *FEMS Microbiol. Lett.* **9**, 29-33(1980).
16. Holt, J. G.; Krieg, N. R.; Sneat, P. H. A.; Staley, J. T.; Williams, S. T. *Bergey's Manual of Determinative Bacteriology*. 9th ed. Williams & Wilkins. Baltimore (1994).
17. Wang, Q.; Fang, X.; Bai, B.; Liang, X.; Shuler, P. J.; Goddard, W. A. Ⅲ; Tang, Y. Engineering bacteria for production of rhamnolipid as an agent for enhanced oil recovery. *Biotechnol. Bioeng.* **98**, 842-853(2007).
18. Dubois, M.; Gills, K. A.; Hamilton, J. K.; Rebers, P. A.; Smith, F. Colorimetric method for determination of sugar and related substances. *Anal. Chem.* **28**, 350-356(1956).
19. Chandrasekaran, E. V.; Bemiller, J. N. Constituent analysis of glycosaminoglycans. in *Methods in Carbohydrate Chemistry*(Whistler, R. L.; Wolfrom, M. L. eds.) Academic Press Inc. NY. pp. 89-96(1980).
20. Bognolo, G. Biosurfactants as emulsifying agents for hydrocarbons. *Colloids. Surf. A* **152**, 41-52(1999).
21. Mata-Sandoval, J. C.; Karns, J.; Torrents, A. High-performance liquid chromatography for the characterization of rhamnolipid mixtures produced by *Pseudomonas aeruginosa* UG2 on corn oil. *J. Chromatogr. A* **864**, 211-220(1999).