INTERNATIONAL JOURNAL OF ENVIRONMENTAL SCIENCES Volume 1, No 2 ,2010

Research article ISSN 0976 – 4402

Surface activity of extracellular products of a *Pseudomonas aeruginosa* isolated from petroleum-contaminated soil

Anyanwu[1], Chukwudi U[2].
Department of Microbiology, University of Nigeria, Nsukka, Nigeria.
chudizoma@yahoo.com

ABSTRACT

Pseudomonas aeruginosa isolated from petroleum-contaminated soil of Nsukka, South-East Nigeria produces surface-active compounds when grown in different carbon sources tested. The surface properties of the culture supernatants of the isolate, using water-soluble and water-insoluble carbon sources as substrates are described in terms of surface tension and emulsification index. Water-soluble substrates showed better surface activity than water-insoluble substrates. The surface tension of the culture supernatants was lowest with glucose (34.5 dynes/cm) followed by sucrose (38.6 dynes/cm), glycerol (40.2 dynes/cm), fructose (41.8 dynes/cm), hexadecane (50.4 dynes/cm) and paraffin oil (56.4 dynes/cm). The culture supernatants showed emulsification index of between 52% and 85% and the emulsion formed remained stable during an extended ageing period of 30 days. The biosurfactants formed by the isolate retained surface active properties after exposure to high temperature (100^0C), a wide range of pH (4 – 12) and high salinity (16%).

Key words: Biosurfactants, extracellular, surface tension, emulsification.

1. Introduction

Biosurfactants are a diverse group of surface-active chemical compounds that are produced by a wide variety of microorganisms, which include bacteria, yeasts and filamentous fungi (Abouseoud and Yataghene, 2008; Mulligan, 2005). Most microbial surfactants are complex molecules, comprising different structures that include lipopeptides, glycolipids, polysaccharide-protein complex, fatty acids and phospholipids (Nitschke and Pastore, 2006). The different types of biosurfactants include lipopeptides synthesized by many species of *Bacillus* and other species, glycolipids synthesized by *Pseudomonas* species and *Candida* species, phospholipids synthesized by *Thiobacillus thiooxidans*, polysaccharide-lipid complexes synthesized by *Acinetobacter* species, or even the microbial cell surface (Mousa *et al*., 2006).

Due to their amphiphilic nature in possessing both polar and non-polar domains, biosurfactants are able to partition preferentially at the interface between phases of different degrees of polarity and hydrogen bonding such as water-oil, water-air or solid-water interfaces and are thus able to reduce the interfacial or surface tension (Banat *et al*., 2000; Rosenberg and Ron, 1999). These molecules have tremendous potential for application in the bioremediation of soil and sand (Van Dyke *et al*., 1991), or in the cleanup of hydrocarbon contaminated groundwater and enhanced oil recovery (Ron and Rosenberg, 2001), or wide variety of industrial processes involving emulsification, foaming, detergency, wetting, dispersing or solubilization (Desai and Banat, 1997). Some biosurfactants are known to have therapeutic applications as antibiotics and antiviral or antifungal agents (Mousa *et al*., 2006).

Currently, almost all the surfactants being produced are chemically derived from petroleum (Banat *et al*., 2000). However, production of microbial surfactants through fermentation of n-alkanes, sugars

Research article ISSN 0976 – 4402

and other substrates has generated considerable interest because of their many important advantages. Biosurfactants show many advantages over chemical surfactants as regards biodegradability, low toxicity, and effectiveness at extreme temperatures, pH, or salinity (Abu-Ruwaida *et al*., 1991). Biosurfactants are attracting attention in recent years because of the several advantages they offer over chemical surfactants and also their ability to be produced from renewable and cheaper substrates (Ishigami, 1997).

The objective of this work was to isolate and demonstrate a bacterial species with potential for the production of extracellular biosurfactant with emulsification and surface tension reduction property.

2. Materials and Methods

2.1 Microorganism and culture conditions

The microorganism used in this study, a strain of *Pseudomonas aeruginosa*, was isolated from a soil sample collected from a vehicle mechanic village where the soil usually remains soaked with spilled petroleum products. Details of the isolation, identification and maintenance of the organism have been described earlier (Anyanwu *et al*., 2008). The carbon sources used in the shake flask culture were fructose, glucose, sucrose, glycerol, hexadecane, and paraffin oil.

Each seed culture was prepared by inoculating a loopful of the stock culture into 20 ml of nutrient broth, contained within a 200 ml conical flask. This was incubated at 30^0C and 180 rev/min for 8-12 h. An aliquot (1.0 ml) of inoculum was transferred to 250 ml Erlenmeyer flask containing 50 ml of basal fermentation medium with the following composition (g/l): K_2HPO_4, 5.0; KH_2PO_4, 2.0; $(NH_4)_2SO_4$, 3.0; $MgSO_4.7H_2O$, 0.10; $FeSO_4.7H_2O$, 0.01; Carbon source, 4.0% (w/v or v/v). The pH of the medium was adjusted to 7.0. All experiments were carried out in triplicates. The incubation was carried out on a rotary shaker (Gallenkamp) at 180 rev/min and 30^0C for 96 h. All chemicals and reagents used were of analytical grade. Culture samples were taken at the end of the incubation period and analyzed for pH, biomass, surface tension and emulsification index. Culture supernatant of the broth at zero hour was considered as the control for each of the carbon source treatments.

2.2 Biomass determination

Biomass was determined by the dry weight method. The culture broth (250 ml) was centrifuged at 10,000 x g for 20 min. The cell pellet obtained was dried overnight at 105^0C and weighed.

2.3 Surface tension measurement

Surface tension was determined by the ring method (Kim *et al*., 2000) using Du Nouy ring tensiometre (K6, Kruss, Hamburg, Germany) at room temperature (30 ± 2^0C) at the end of incubation. Measurements were made on whole broth and cell-free broth samples after centrifugation.

2.4 Assay of emulsifying activity

The emulsifying activity of the biosurfactants was measured as described by Cooper and Goldenberg (1987). Briefly, 6.0 ml of kerosene was added to 4.0 ml of the broth supernatant solution from each carbon source in a graduated test tube and vortexed at high speed for 2 min. This was allowed to stand for 24 h and the emulsification index (E_{24}) calculated by dividing the measured height of emulsion

Research article ISSN 0976 – 4402

layer by the mixture's total height and multiplying by 100. Emulsions formed were observed for an extended period of time for 30 days. Distilled water was used as control.

2.6 Effect of pH on surface activity

The effect of different levels of pH regime on surface tension and emulsifying activity was carried out by adjusting the pH of the supernatants to various values (4 - 12) by addition of HCl or NaOH at room temperature (Zhang and Miller, 1992). The surface tension was determined as earlier described. The emulsifying activity was measured by vortexing a mixture of the supernatant and kerosene and measuring the E_{24}.

2.7 Effect of temperature on surface activity

The heat stability of the culture supernatants was studied by heating the supernatants in a boiling water bath at 100^0C for 15 min and cooled to room temperature. The surface tension and emulsifying activity were, respectively, determined as described earlier.

2.8 Effect of sodium chloride on surface activity

The effect of sodium chloride concentration on biosurfactant activity was determined by adding different concentrations (0 – 20%, w/v) of NaCl to the biosurfactant solution and allowed to stand for 30 min. The surface activity was determined in terms of surface tension and emulsification index as described above.

2.9 Statistical analyses

All experimental set-ups and analyses were performed at least three times with at least two replicates of each test under each condition. Means and standard errors were calculated for pooled results in all experiments for each test. Analysis of variance was performed on some of the data obtained to determine significant differences among the means on the basis of 5% level of significance.

3. Results

The isolated *Pseudomonas aeruginosa* LS-1 produced extracellular surface-active compounds when cultivated in batch cultures on mineral salts medium containing fructose, glucose, sucrose, glycerol, n-hexadecane and paraffin oil as sole carbon sources. The average values of the surface tension, emulsification index, pH and biomass of the cultures for the different carbon sources at the end of the cultivation period are shown in Table 1.

The surface tension values of the culture supernatants ranged from 34.5 dynes/cm to 56.4 dynes/cm. The surface tension values did not show any differences between the whole broth and cell-free broth samples, respectively. The lowest surface tension value of 34.5 dynes/cm was obtained with glucose as the carbon source while the highest value of 56.4 dynes/cm was obtained with paraffin oil. The surface tension values of the controls ranged between 70.6 and 71.1 dynes/cm. There was, therefore, a significant lowering ($p<0.05$) of the surface tension of the culture supernatants from their respective controls. The decrease in surface tension indicated the production of extracellular surface-active compounds by the *Pseudomonas aeruginosa* LS-1 using the different carbon sources.

Table 1: Biosurfactant production by *Pseudomonas aeruginosa* LS-1 with various

Research article ISSN 0976 – 4402

carbon sources.

Carbon source	Surface tension (dynes/cm)	Emulsification index (%)	Biomass (g/l)	pH
Fructose	41.8	68	2.4	5.5
Glucose	34.5	85	3.2	5.6
Sucrose	38.6	70	3.0	5.4
Glycerol	40.2	68	3.5	5.6
n-Hexadecane	50.4	72	3.0	6.1
Paraffin oil	56.4	52	2.5	6.5

The emulsification activity of the culture supernatants was measured in terms of percent emulsification and was found to be maximum (85%) with glucose and lowest (52%) with paraffin oil as carbon source (Table 1). The *Pseudomonas aeruginosa* LS-1 biosurfactants were effective in producing very good emulsification with kerosene. The emulsions formed were stabilized by the surfactants and did not revert to separate oil and water phases even after an extended period of observation of 30 days, whereas no stable emulsions were formed at the zero hour. Table 1 also shows that the pH of the culture supernatants were all in the acid range, between 5.4 and 6.5.

The effect of pH on the surface tension of the biosurfactants is presented in Figure 1 while Figure 2 shows the effect of pH on the emulsification activity of the biosurfactants. Both the surface tension and emulsification index were not very sensitive to pH. The surface activity was retained over the pH range of 4 – 12 with minimal deviation in surface activity.

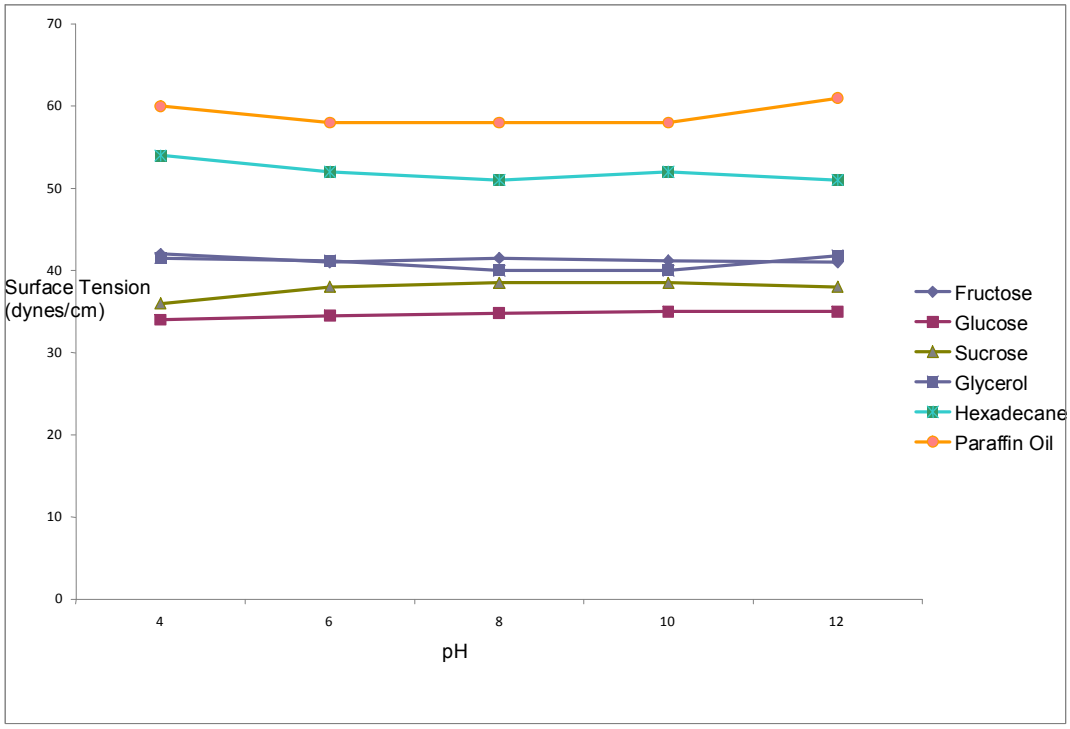

Figure 1: Effect of pH on Surface Tension of Culture supernatant

Research article ISSN 0976 – 4402

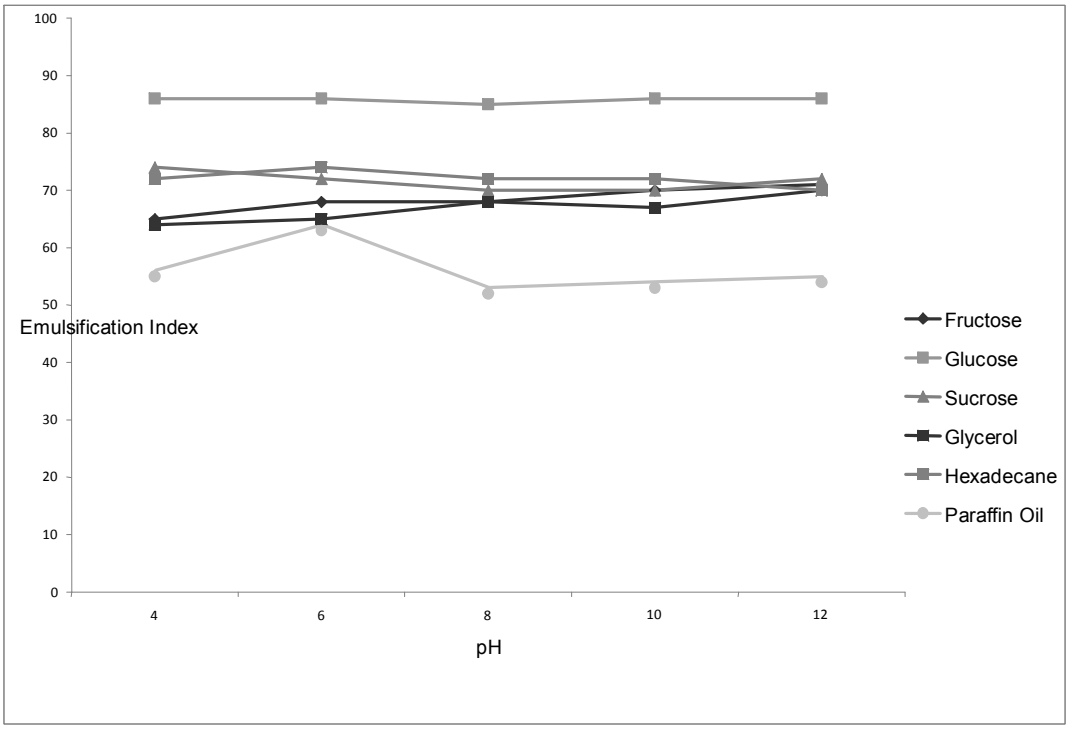

Figure 2: Effect of pH on Emulsification Index of Culture Supernatant

Thermal stability study of the biosurfactants was done by heating the culture supernatants to 100^0C for 10 min and cooling to room temperature. The heating did not cause any significant effect on the biosurfactant performances. Figures 3 and 4 showed that the surface tension and emulsification activity were quite stable after exposure to high temperature (100^0C).

Research article ISSN 0976 – 4402

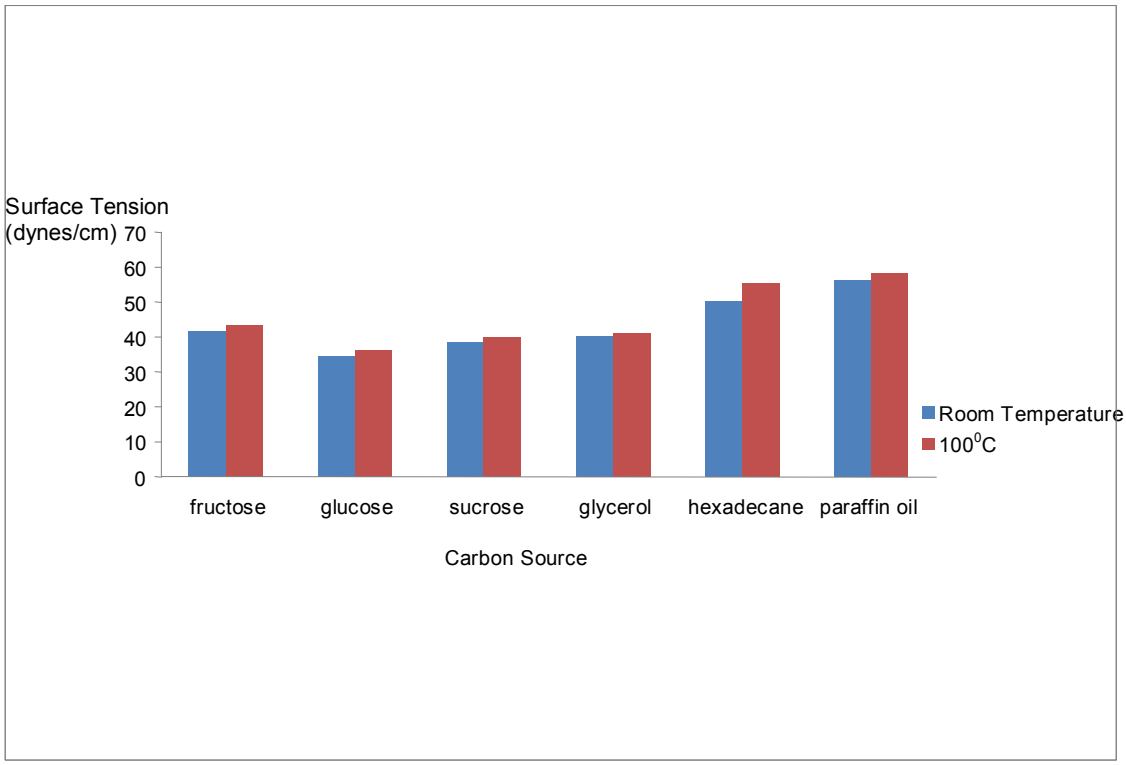

Figure 3: Effect of Temperature on Surface Tension of Culture Supernatant

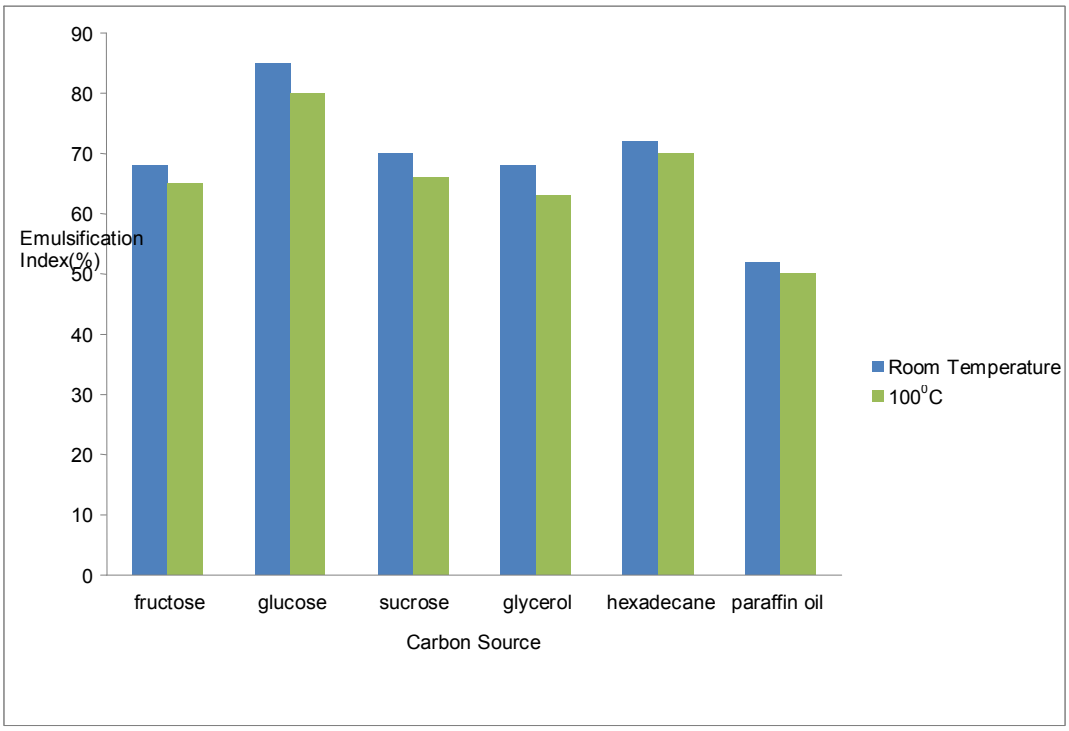

Figure 4: Effect of Temperature on Emulsification index

Research article ISSN 0976 – 4402

Figures 5 and 6 indicated that the addition of NaCl in the range of concentrations tested (0 – 20%) had only a weak effect on surface tension and emulsification index of *Pseudomonas aeruginosa* LS-1 biosurfactants. A significant effect ($p < 0.05$) was observed at NaCl concentration of 16% and above.

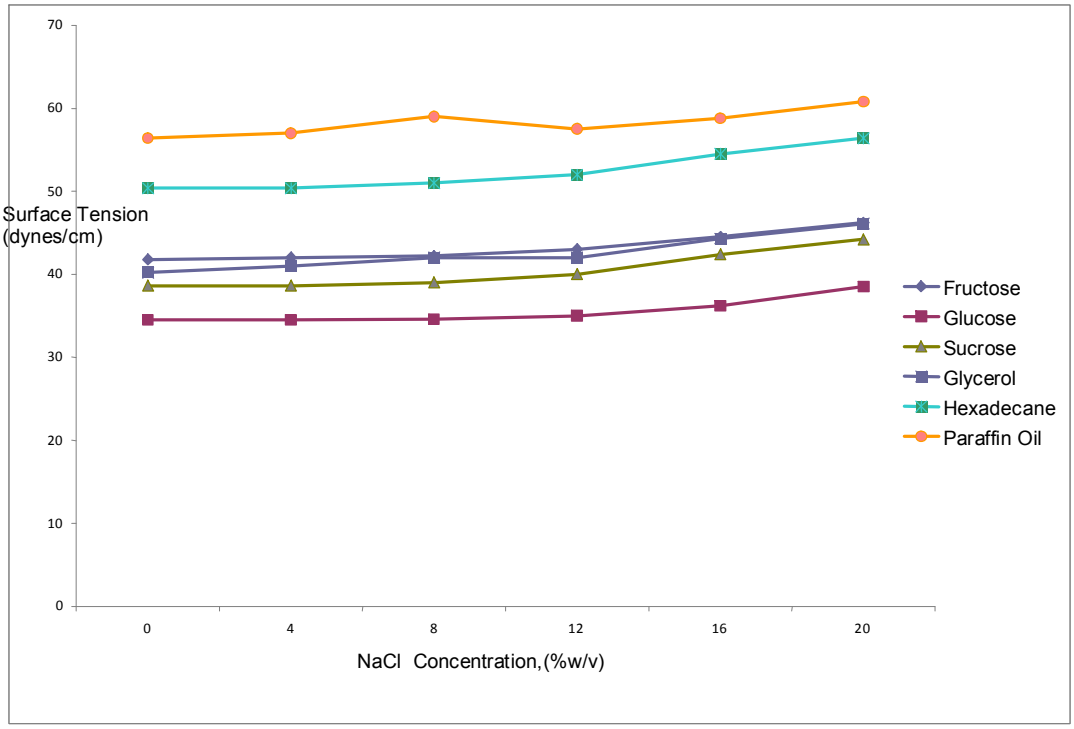

Figure 5: Effect of NaCl on surface tension

4. Discussion

The *Pseudomonas aeruginosa* LS-1 strain used in the present study produced biosurfactants when grown in batch culture with fructose, glucose, sucrose, glycerol, hexadecane, and paraffin oil as carbon and energy sources. The isolate grew on the various carbon sources tested. However, the biosurfactant production varied as shown by the differences in surface tension values and emulsification activity. The organism was able to produce extracellular water-soluble biosurfactants during growth on these substrates. The products were extracellular since the removal of the biomass did not affect the activity. In a study, Wu and Ju (1998) reported that biosurfactants were either adhered to, or an integral part of, the cell surface of isolates that only reduced the surface tension in the presence of cells. Bento *et al*. (2005) had shown that no substantial emulsification was achieved with cell-free extracts, indicating that the emulsification activity was not extracellular. Glazyrina *et al*. (2008) reported that biosurfactants are present on the cell surface or excreted extracellularly.

Research article ISSN 0976 – 4402

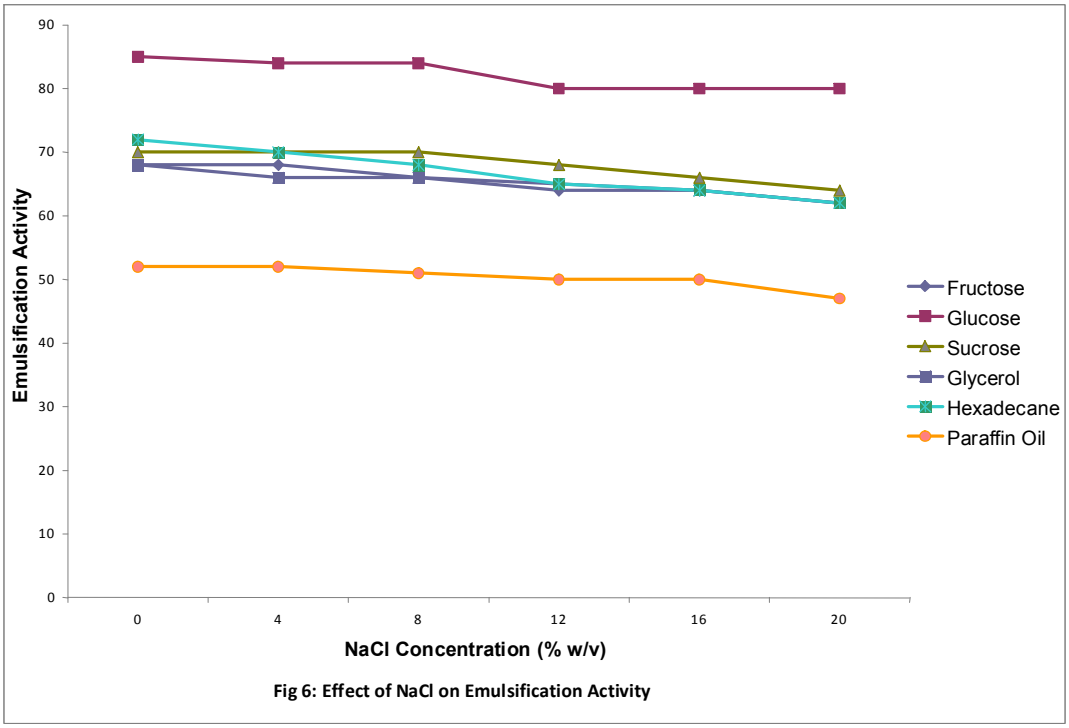

Figure 6: Effect of NaCl on Emulsification activity

It has been reported by Makkar and Cameotra (2002) and Nitschke and Pastore (2006) that microbes produce biosurfactants, especially during growth on water-immiscible substrates. This permits the microorganisms to grow on such substrates by reducing the surface tension thereby making the hydrophobic substrate more readily available for uptake and metabolism (Calvo *et al*., 2004). Very often, the growth of microorganisms on hydrocarbons is accompanied by the emulsification of insoluble carbon sources in the culture medium and, in most cases, this has been attributed to the production of emulsifying agents in the presence of these substrates (Kim *et al*., 2000). However, Cooper *et al*. (1981) reported that the addition of a hydrocarbon to culture medium completely inhibited surfactant production by *Bacillus subtilis*. Meanwhile, the microorganism used in the present study, *Pseudomonas aeruginosa* LS-1, utilized both water-miscible and water-immiscible substrates to produce biosurfactants. This property confers some advantage on the microorganism as it makes it more versatile and more ecologically and economically relevant.

It has been demonstrated that some microorganisms which utilize hydrocarbons produce reduced amounts of biosurfactants when they are growing on water-soluble substrates (Hommel *et al*., 1987). This is not in agreement with the results of the present study as glucose appeared as the best substrate for biosurfactant production by the *Pseudomonas aeruginosa* LS-1. According to Sandrin *et al*. (1990), glucose, fructose and sucrose, which are water-soluble substrates, were the best carbon substrates for the synthesis of biosurfactant by *Bacillus subtilis*. Ghojavand *et al*. (2008) showed that surface tension reduction was not under influence of crude oil as the source of carbon. The production of surface-active substances by the isolates did not necessarily occur in response to the presence of an insoluble substrate in the growth medium. These results support the conclusion that, in general, bioemulsifiers are not necessarily produced by microorganisms to facilitate the uptake of an insoluble substrate (Cooper and Goldenberg, 1987).

Research article ISSN 0976 – 4402

The emulsification properties of *Pseudomonas aeruginosa* LS-1 studied showed that the culture supernatant of the isolate from different carbon sources formed good, but varied stable emulsions with kerosene. The emulsifying activity of glucose growth medium was better than all other substrates. These results show that the isolate produced different amounts of extracellular biosurfactants when it grew in the presence of different carbon sources. Thus the isolate demonstrated the ability to utilize different carbon sources to different extent.

Stability studies of the culture supernatants indicated that the surfactant was thermostable as heating up to 100^0C caused no significant effect on the biosurfactant performances. The surface tension reduction and emulsification activity were quite stable, despite the high temperature heating. Abouseoud *et al*. (2008) had shown that the biosurfactant produced by *Pseudomonas fluorescens* was stable even at an autoclaving temperature of 120^0C. Similarly, the biosurfactants produced by *Pseudomonas aeruginosa* in the present study were pH-stable as a range of pH values (4 – 9) had minimal effect on the surface activity of the culture supernatants. The activity of the rhamnolipid produced by *Rhodococcus* (Abu-Ruwaida *et al*., 1991) and surfactin produced by *Bacillus subtilis* (Kim *et al*., 1997) was also pH-stable in a wide range from 4 to 11. However, Abousseoud *et al*. (2008) had shown that pH increase had a positive effect on surface tension and emulsion stability. The positive effect could be caused by a better stability of fatty acids-surfactant micelles in presence of NaOH and the precipitation of secondary metabolites at higher pH values. The addition of sodium chloride in the range 0 – 20% to the culture supernatants had weak effect on the surface tension and emulsification index. This finding revealed that the products obtained had a high level of tolerance to ionic strength. The report is in agreement with the reports of Abouseoud *et al*. (2008) and Nitschke and Pastore (2006).

5. Conclusion

The results of the present study showed that the isolated *Pseudomonas aeruginosa* could produce extracellular biosurfactants utilizing both hydrocarbons and sugars. The surface tension of the culture medium was lowered to different extent depending on the carbon source. Glucose was a better carbon source than all other carbon sources for biosurfactant production. The emulsifying activity of the biosurfactants revealed that they could be used as emulsion forming agents for hydrocarbons and oils, giving stable emulsions. The products exhibited a high level of thermal and pH stability and demonstrated a high level of tolerance to ionic strength. These observations show clear perspectives for the use of the products in extreme environmental conditions in bioremediation, pharmaceutical formulations and other industrial fields.

6. References

1. Abu-Ruwaida, A.S,. Banat, I.M., Salam A. and Kadiri, M. (1991). Isolation of biosurfactant producing bacteria: product characterization and evaluation. Acta Biotechnol. 11:315-324.

2. Abouseoud, M., Yataghene, A., Amrane, A. and Maachi, R. (2008). Biosurfactant production by free and alginate entrapped cells of *Pseudomonas fluorescens*. J. Ind. Micr. Biotechnol. 35:1303 – 1308.

3. Anyanwu, C.U., Obi, S.K.C and Okolo, B.N (2008). Isolation of biosurfactant/bioemulsifier-producing bacteria from soil and sewage sludge. Global Journ. Pure Appl. Sci. 14(1): 103-107.

Research article ISSN 0976 – 4402

4. Banat, I.M., Makkar, R.S. and Cameotra, S.S. (2000). Potential commercial applications of microbial surfactants. Appl. Microbiol. Biotechnol. 53: 495 – 508.

5. Bento, F.M., De Olivera, F.A., Camargo, Okeke, B.C. and Frankenberger, W.T. (2005). Diversity of biosurfactant producing microorganisms isolated from soils contaminated with diesel oil. Mirobiol. Res. 160: 249 – 255.

6. Calvo, C., Toledo, F.L., Pozo, C,.Martinez-Toledo, M.V. and Gonzalez-Lopez, J.(2004). Biotechnology of bioemulsifiers produced by microorganisms. J. Fd Agric. Environ. 2 (3&4): 238 – 243.

7. Cooper, D.G. and Goldenberg, B.G. (1987). Surface-active agents from two *Bacillus* species. Appl. Env. Microbiol. 53: 224-229.

8. Cooper, D.G., MacDonald, C.R., Duff, S.J.B. and Kosaric, N. (1981). Enhanced production of surfactin from *Bacillus subtilis* by continuous product removal and metal cation addition. Appl. Env. Microbiol. 42: 408-412.

9. Desai, J.D. and Banat, I.M. (1997). Microbial production of biosurfactants and their commercial potential. Mcrobiol. Mol. Biol. Rev. 61: 41-64.

10. Ghojavand, H., F. Vahabzadeh, M. Mehranian, M. Radmehr, K.A. Shahraki, F. Zolfagharian, M.A. Emadi and E. Roayaei, 2008. Isolation of thermotolerant , halotolerant, facultative biosurfactant-producing bacteria. Appl. Microbiol. Biotechnol. 80: 1073 – 1085.

11. Glazyrina, J., Junne, S., Thiesen, P., Lunkenhelmer, K.and Goetz, P. (2008). Insitu removal and purification of biosurfactants by automated surface enrichment. Appl. Micrbiol. Biotechnol. 81: 23 – 31.

12. Hommel, R.K., Stower, O., Staber, W., Haferburg, D., and Kleber, H.P. (1987). Production of water-soluble surface-active exolipids by *Torulopsis agricola*. Appl. Microbil. Biotechnol. 26: 199 – 205.

13. Ishigami, Y. (1997). Characterization of biosurfactants. In Esumi, K. and Ueno, M. (Eds). Structure performance relationships in surfactants. Dekker, New York, pp 197 – 226.

14. Kim, S.H., Yoon, B., Lee, C., Suh, H., Oh, H., Katsuragi, T. and Tani, Y. (1997). Production and properties of a lipopeptide biosurfactant from *Bacillus subtilis* C9. Journ Ferment. Bioeng. 84: 41 – 46.

15. Kim, S.H., Lim, E.J., Lee, S.O., Lee, J.D. and Lee, T.H. (2000). Purification and characterization of biosurfactants from *Nocardia* sp. L-417. Biotechnol. Appl. Biochem. 31: 249-253.

16. Makkar, R.S. and Cameotra, S.S. (2002). An update on the use of unconventional substrates for biosurfactant production and their new applications. Appl. Microbiol.Biotechnol. 58: 844 – 850.

17. Moussa, T.A.A., Ahmed, A.M. and Abdel-hamid, S.M.S. (2006). Optimization of cultural conditions for biosurfactant production from *Nocardia amarae*. Journ Appl. Sc. Res. 2: 844 -850.

Research article ISSN 0976 – 4402

18. Mulligan, C.N. (2005). Environmental application for biosurfactants. Environ. Poll. 133:183-198.

19. Nitschke, M. and Pastore, G.M. (2006). Production and properties of a surfactant obtained from *Bacillus subtilis* grown on cassava wastewater. Biores. Technol. 97: 336-341.

20. Ron, E.Z. and Rosenberg, E. (2001). A review of natural roles of biosurfactants. Environmental Microbiology 3: 229 – 236.

21. Rosenberg, E. and Ron, E.Z. (1999). High and low molecular mass microbial surfactants. Appl. Microbiol. Biotechnol. 52: 154-162.

22. Sandrin, C., Peypous, F. and Michel, G. (1990). Coproduction of surfactin and iturin A lipopeptides with surfactant and antifungal properties by *Bacillus subtilis*. Biotechnol. Appl. Biochem. 12 : 370 – 375.

23. Van Dyke, M.I., Lee, H. and Trevors, J.T. (1991). Application of microbial surfactants. Biotechnol. Adv. 9: 241 -252.

24. Wu, J. and Ju, I.K. (1998). Extracellular particles of polymeric material formed in n-hexadecane fermentation by *Psudomonas aeruginosa*. Journ. Biotechnol. 59:191-202

25. Zhang, Y and Miller, R.M. (1992). Enhanced octane dispersion and biodegradation by a *Pseudomonas* rhamnolipid surfactant (biosurfactant). Appl. Environ. Microbiol. 58: 3276-3282.