Surface Activity of Extracellular Products of Bacteria Isolated from Petroleum – Contaminated Soil

Samuel Agim Abuh[1]
Mark Agaba Abuh [2]
Chukwudi U. Anyanwu[1]

[1]University of Nigeria, Nsukka, Nigeria
[2]Projects Development Institute (PRODA), Federal Ministry of Science and Technology, Nigeria

Abstract. Soil samples contaminated with petroleum products were obtained from automobile workshops in Nsukka and Coal Camp in Enugu. The waste lubricating oil (carbon source 1), WLO, was similarly obtained alongside the soil. The Waste frying oil was obtained from fried food vendours, all at Enugu State Nigeria. 10 g of the soil sample was measured into 250 ml Erlenmeyer flask containing 40 ml of basal fermentation medium in the different carbon sources. The pH of the medium was adjusted to 7.0 after addition of the carbon sources. A ten-fold serial dilution of the incubated samples was carried out. Basal medium containing the different carbon sources and solidified with agar was plated by titrating 0.1ml of the appropriate dilution of each sample on the plates. The plates were incubated at $30^0 \pm 2^0$C for 48 h, two representative colonies were picked from different plates based on their colonial characteristics. These isolates were streaked on nutrient agar plates for purification and the isolates characterized presumptively. The purified isolates were transferred to slants and stored for biochemical tests, all the isolated strains were tested for haemolytic activity which is an indication of biosurfactant production and used as a rapid method for bacterial screening. The surface activity parameters investigated were surface tension and emulsification index. The bacterial screening showed only *Bacillus and Pseudomonas* as having clear zones of haemolysis on solid blood agar medium which was a positive test for biosurfactant production. The obtained bacteria isolates were now used for the surface activity investigation which showed varying degree of surface activities in the different carbon sources used. The result shows that the isolates (*Bacillus and Pseudomonas* sp) obtained from the soil samples produced biosurfactants using different carbon sources (WLO and WFO), the stability studies shows that the surface activity of the produced biodurfactants were stable over wide range of environmental condition tested, hence their suitability for environmental, pharmaceutical, cosmetics and agricultural application.

Key words: bioemulsifiers, biosurfactants, surface tension, haemolysis, emulsification index Bacillus sp, Pseudomonas sp.

Introduction

Contamination of soil by crude oil occurs around the world because of equipments failure, natural disaster, sabotage and human errors (Yakubo et al., 2009). The release of these contaminants into the environment is one of the main causes of global contamination. These contaminants pose serious risks to plants and animals' health as many of them are carcinogens (Dastgheib et al., 2008: 263-270; Aboused et al., 2008: 1303-1308). These hydrocarbons rich contaminants are released into the environment and are hard to remove as they adsorb to surfaces and are trapped by capillary action in Water-Hydrocarbon immiscible phase. Bioremediation has proven to be alternative in salvaging the effects of contamination of soil and water, using metabolic capacities of microorganisms that can modify them by co-metabolism. The efficiency of removal is

directly related to the compound's chemical structure, its bioavailability (concentration, toxicity, motility and access) and the physiochemical conditions present in the environment (Chen et al., 2004). Bioemulsifiers have been reported as enhancers of hydrocarbon biodegradation in liquid media, soil slurries microcosms (Benincasa et al., 2002: 283-288). For microorganisms, production of biosurfactants is an advantage in soil, giving them advantages in specific conditions (Ron and Rosenbeg, 2002: 249-252; Desai and Banat, 1997: 47-64; Maneerat and Phetrong, 2007: 781-791). Microbial biosurfactants can be intracellular (remain attached to the cell wall) and/or can be excreted into the media (Gerson and Zajic, 1979: 20-29). When biosurfactants are intracellular, their structure includes membrane lipids and promotes the transport of insoluble substrates through the membrane (Ghaly et al., 2004: 631-644).

The term biosurfactants and bioemulsifiers are considered interchangeably, although all bioemulsifiers are considered biosurfactants but not all the biosurfactants produce emulsions. Biosurfactants can reduce the surface tension between two lipids and emulsifiers induce a dispersion of insoluble materials throughout the liquid by formation and stabilization of the dispersed phase (Christofi and Ivshina, 2002: 915-929).

Vollbrecht et al. (1999: 389-394) investigated the production of biosurfactants using domestic vegetable oils in order to convert renewable resources into higher value products, they obtained the best growth using natural vegetable oil rather than a complex media and hydrophobic carbon sources. Mercade et al. (1996: 161-168) reported the screening and selection of microorganisms capable of utilizing waste lubricating oil (WLO) for producing biosurfactants. Makkar and Cameotra (2002: 844-850) in a similar study reported that the strains capable of utilizing WLO represent a valuable source of new compounds with surface active properties and potential for application in bioremediation of soil and water. In the study, *Bacillus* sp and *Pseudomonas* sp utilized both WFO and WLO as carbon sources to produce biosurfactants. This property (ability to utilize WFO and WLO) confers some advantages on microorganisms as making them more versatile and economically relevant in biodegrading used vegetable oil and used motor oil as a sound strategy of waste management for the food and automobile industries to reduce accumulation of waste.

Microbes produce biosurfactants, especially during growth on water-immiscible substrates, which reduces the surface tension and makes the hydrophobic substrates more readily available for uptake and metabolism (Calvo et al., 2000: 238-241; Makkar and Cameotra, 2002: 844-850; Nitschke and Pastore, 2006: 336-341). Cooper et al. (1981: 408-412) reported the inhibition of biosurfactant production by *Bacillus subtillis* due to addition of hydrocarbon to culture medium. This is not in agreement with the present study as WLO and WFO appear to be very good substrates for the production of biosurfactants by *Bacillus* sp and *Pseudomonas* sp. The aim of this work is to determine the emulsifying activity of the extracellular products of bacteria isolated from oil contaminated soil.

Materials and Methods

Sample Collection

Soil samples contaminated with petroleum products were collected from different polluted sites namely: tricycle repairs shop Nsukka, automobile workshop village Nsukka and Coal Camp in Enugu state Nigeria. The soil samples were collected aseptically from the surface (0-15cm) into a sterile polythene bag and transported to the laboratory of the Department of Microbiology University of Nigeria, Nsukka and analyzed within 24 h.

Media Preparation

Basal fermentation medium was prepared according to method described by Anyanwu et al. (2008: 103-107). To 1 liter distilled water was added the following compositions (g/l): K_2HPO_4, 5.0; KH_2PO_4, 2.0; $(NH_4)_2SO_4$, 3.0; $MgSO_4$. $7H_2O$, 0.1; $FeSO_4.7H_2O$ 0.01; carbon source 4.0 %(v/v). The carbon sources were: waste frying oil (WFO), petrol and waste lubricating oil (WLO). The prepared media was autoclaved at 121^0C for 20 minutes.

Microbiological Analysis

This was carried out within 24 hrs of collection. 10 g of the soil sample was measured into a 250 ml Erlenmeyer flask containing 40 ml of basal fermentation medium and different carbon sources in a batch study. The pH of the medium was adjusted to 7.0 and incubated at $30^0 \pm 2^0$C for 96 h in rotary shaker (180rv/minute). A ten-fold serial dilution of the incubated samples was carried out. Basal medium containing the different carbon sources and solidified with agar was plated by titrating 0.1ml of the appropriate dilution of each sample on the plates, using the L-shape spreader, the 0.1 ml of the appropriate dilution was uniformly spread on the surface of the plate. The plates were incubated at $30^0 \pm 2^0$C for 48 h. Biochemical analysis was done according the methods describe by Prescott *et al* 2009

Selection of working isolate

The selection of working isolates was based on their ability to grow on basal media containing 4% (v/v) substrate (WLO, WFO and petrol) solidified with agar and subsequent haemolysis on blood agar. Two distinct colonies were selected each from the agar agar plates containing the different substrate and purified in nutrient agar plate for biochemical analysis. After the biochemical analysis, the organisms were further subjected to screening by plating the isolates in nutrient agar plate containing 5% (v/v) human blood. The organisms with defined zone of haemolysis were then selected for further studies. The use of blood agar lysis is the primary method to screen for biosurfactant activity (Youssef et al., 2004: 9-21).

Screening for surface activity

The pure seed culture was prepared by inoculating a loopful of solid stock culture into 20 ml nutrient broth, contained within a 200 ml conical flask. This was incubated at 30^0C and 180rev/min for 8-12hrs (Anyanwu, 2008: 103-107). An aliquot (1.0 ml) of the inoculums was transferred into a 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; $MgSO_4$. $7H_2O$, 0.1; $FeSO_4.7H_2O$ 0.01; carbon source 4.0 %(v/v). Ring method (Kim et al., 2000: 249-253) was applied to measure the surface tension(S.T) using Du Nouy ring tensiometre (Kruss, Hamburg and Germany) at room temperature (30 ± 2^0C).

Assay for emulsifying activity

The emulsifying activity of the supernatant was measured as described by Cooper and Goldenberg (1987: 224-229). Briefly, 4.0 ml of kerosene was added differently to 4.0ml 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}), E.I, calculated by dividing the measured height of emulsion layer by the mixture total height and multiply by 100.

$$E_{24} = \frac{Height\ of\ emulsion\ layer}{(Total\ Height)}\ x\ 100$$

Measurement of process parameters

The surface tension was determine using the Du Nouy ring tensiometer as described earlier and the emulsifying activity was measured by vortexing a mixture of the supernatant and kerosene and measuring the E_{24} for every process parameter measured. The pH regime was carried out by adjusting the pH of the supernatant to various values (4,6,7,8,10,12) by addition of HCl or NaOH at room temperature (Zang and Miller, 1992: 3276-3282). The thermal stability was carried out by heating the supernatant in water bath at temperatures, 30^0C, 40^0C, 60^0C, 80^0C and 100^0C for 15 min. Sodium chloride concentration effect was determined at different concentrations of NaCl (0%, 5%, 10%, 15% and 20% (w/v)) at 30 min of standing. Metal ions effects were determined at different concentrations (0, 1, 2, 3 and 4 mM) of metal ions (Cu, Zn, Ni and Mn) in the biosurfactant solution.

Statistical analyses

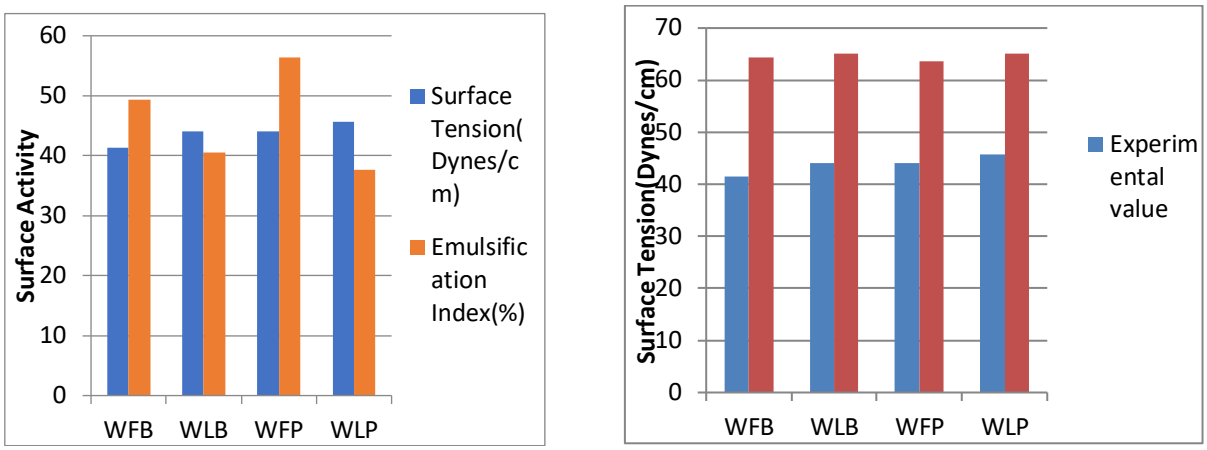
All experiments were performed in duplicate. Means and standard errors were calculated for pooled results in all experiments for each test. ANOVA was performed to determine significant differences among the means on the basis of 5% level of significance using spss.

Results and Discussion

The next Table 1 presents surface activity of surfactant produced by Bacillus sp and Psedomonas sp using Waste Frying Oil(WFO) and Waste Lubricating Oil (WLO), Fig. 1 is devoted to the comparism of surface activity of the bacteria in the substrates.

Table 1. Surface activity of surfactant produced by *Bacillus* sp and *Psedomonas* sp using Waste Frying Oil(WFO) and Waste Lubricating Oil (WLO)

			Process Parameter			
			Surface Tension(Dynes/cm)		Emulsification Index(%)	
	Carbon Source	Code	Obtained value	control	Obtained value	Control
Bacillus SPP	Waste Frying Oil	WFB	41.40	64.42	49.40	0.00
	Waste Lubricating Oil	WLB	44.10	65.03	40.50	0.00
Pseudomonas Spp	Waste Frying Oil	WFP	44.10	63.60	56.40	0.00
	Waste Lubricating Oil	WLP	45.70	65.01	37.60	0.00



A B

Fig. 1. Comparism of surface activity of the bacteria in the substrates

The surface activity profile as shown in figure1 shows that the emulsification index was higher in WFO than in WLO irrespective of bacteria isolate (Bacillus spp, Pseudomonas spp) used. This is due to the light weight molecular hydrocarbons of predominantly C_8 – C_{16}, high degree of unsaturation, absence of synthetic additives which makes the oil more biodegradable to produce emulsifiers. WLO contains predominantly heavy molecular weight hydrocarbons of above C_{20} and are more saturated due to the C – C single bonds, high proportion of synthetic additives for thermal stability and hence resistance to biodegradation. The surface tension was higher in WLO which is due to the presence of synthetic additives. The results in Table 2 show the isolates which were presumptively identified as species of: *Bacillus, Proteus, Pseudomonas and Citrobacter.*

Table 2. Haemolysis test

Sample	Carbon Source	Haemolysis test
Citrobacter	WLO	-
Bacillus Spp	WLO	+
Bacillus Spp	P	+
Proteus Spp	WFO	-
Pseudomonas Spp	WFO	+
Pseudomonas Spp	WLO	+
Bacillus Spp	WFO	+
Citrobacter Spp	P	-
Bacillus Spp	WFO	+
Pseudomonas Spp	P	+
Proteus Spp	WLO	-
Pseudomonas Spp	P	+

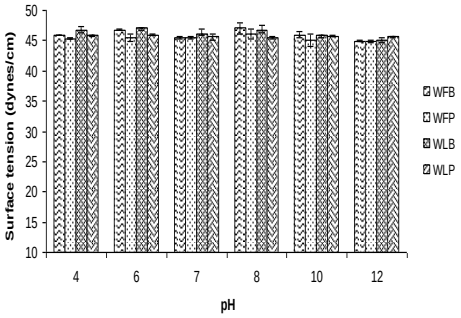
Key: Carbon source

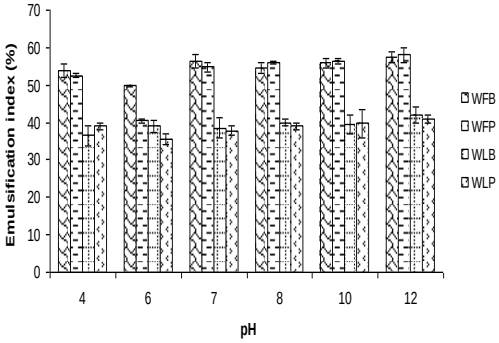
P	Petrol
+	Positive
-	Negative

From Table 2, Isolates that were positive were used for further studies. In the table *Pseudomonas* sp and *Bacillus* sp showed positive test and were used for further analysis.

Effect of Process parameters

Effect of pH





C D

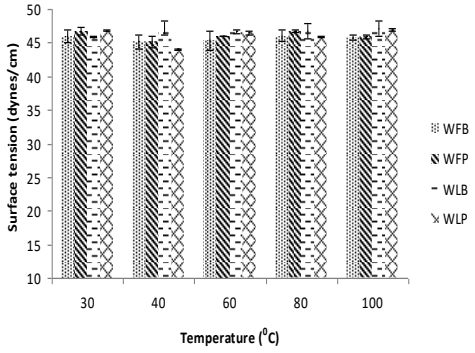
Fig.1. Effect of pH on [C] surface tension (S.T) [D] *emulsification index (E.I)* of biosurfactant produced by *Pseudomonas* sp and *Bacillus sp*. using waste frying oil and waste lubricating oil as substrate

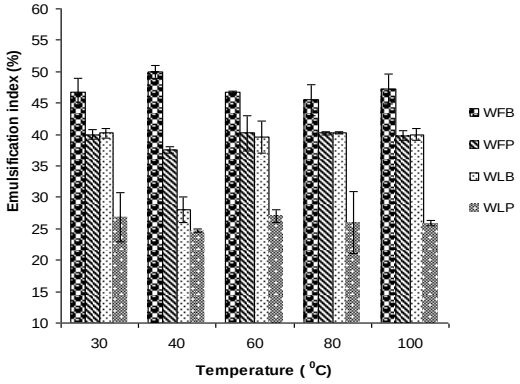
From figure 1, the surface tension and E.I of the biosurfactants produced by *Bacillus and Pseudomonas* sp displayed a high degree of stability over a wide range of pH (4-12). This is consistent with the work of Kim et al. (1997: 41-46) which reported the stability of surfactants produced by *Bacillus subtillis* over a similar range of pH of 4-11. The figure shows no significant difference ($p \leq 0.05$) of surface tension at pH of 4 and 12 when compared with the controlled pH of 7.

Leonie et al. (2006) reported a positive effect on surface tension and emulsification index with increase in pH. This could be caused by a possible transformation of less surface active species into more active emulsifiers by increased ionization at higher pH (Pavitran, 2004: 811-816). From the results, emulsification activity for both *Bacillus* sp and *Pseudomonas* sp on the samples (WFO and WLO) was independent of pH (4–12) but dependent on the nature of the substrates(samples). The WFO contains $C_8 - C_{22}$ molecular weight hydrocarbons which are biodegradable hence could produce higher amount of biosurfctants when compared with WLO which is primarily C_{22}-C_{70} heavy molecular weight hydrocarbons with lower biosurfactant production and hence lower emulsification index.

Effect of temperature

The results are presented in Fig. 2.





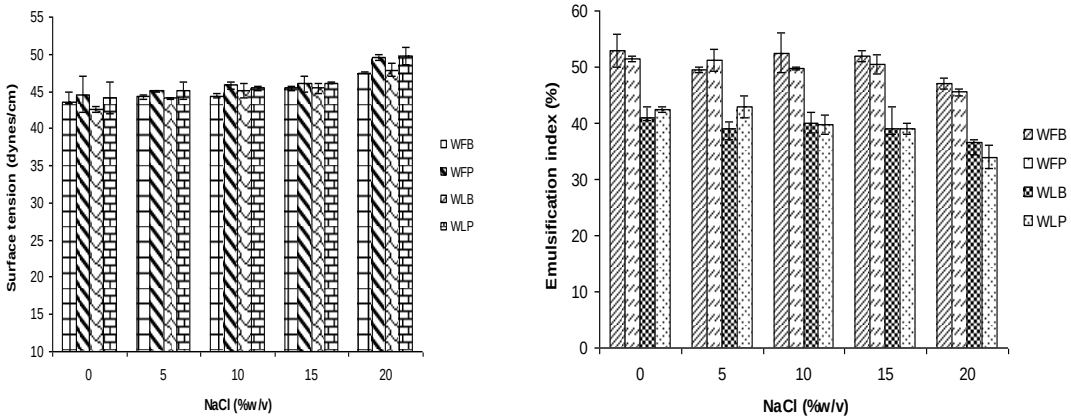
E F

Fig. 2. Effect of temperature on [E] surface tension [F] emulsification index of biosurfactant produced by Pseudomonas sp and Bacillus sp using waste frying oil and waste lubricating oil as substrate

When the surface activity of the two organism in the different substrates (WFO and WLO) were compared, there was a significant difference in S.T though the organisms performed better in WFO than in WLO as shown in figure 2. This is probably due to the lower molecular weight and ease of biodegradation hence higher yield of biosurfactants in WFO when compared with WLO with higher molecular weight hydrocarbons. Thus, the isolates demonstrated their ability to utilize renewable carbon sources to different extents. E.I values obtained for *Bacillus sp* in WFO were relatively higher than that for pseudomonas. Also, no significant effect on performance of the surfactant was observed at 30^0 C when compared with the temperatures at 60^0 C and 100^0 C. the difference in surface activity of the two waste oils is probably due to the presence of antioxidants and low molecular weight hydrocarbons in WFO which increases the yield of biosufactants when compare with WLO with high molecular weights hydrocarbons and additives

Effect of NaCl

The results of Effect of NaCl are presented in Fig. 3.



G H

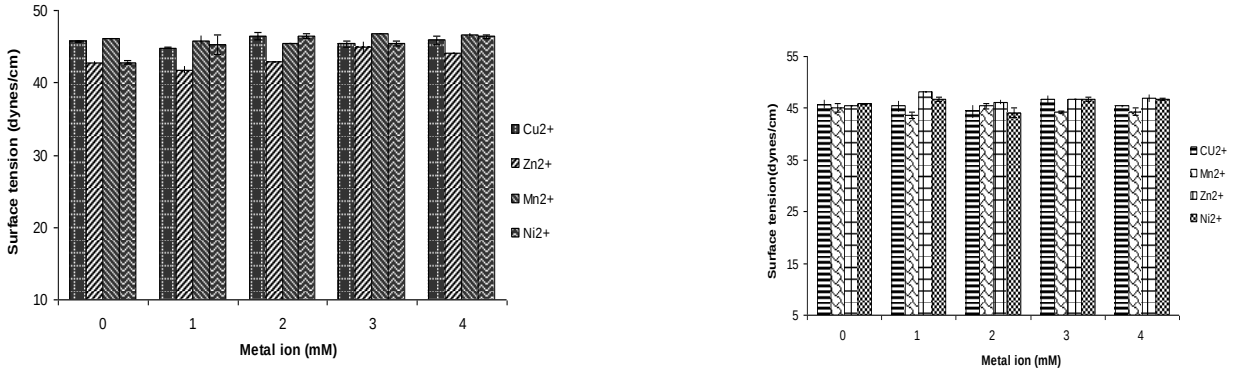
Fig. 3. Effect of NaCl on [G] surface tension [H] emulsifying activity of biosurfactant produced by *Pseudomonas* sp and *Bacillus* sp

The addition of sodium chloride had weak effect on the S.T and E.I of the biosurfactants of the cultured supernatant as shown in fig 3, the surfactant displayed a high level of stability to NaCl up to concentrations of 16% w/v. The effect of NaCl on the surfactant became significant at high salt concentration of 20% (w/v). These findings revealed that the products obtained had a high level of tolerance to ionic strength up to a concentration of 16% (w/v). However, at sodium chloride concentration of 20%, emulsifying activity was significantly ($P<0.05$) inhibited. It is possible that the increase in NaCl concentration decrease the viscosity of the produce emulsion which possibly reduced the emulsification capacity. This agrees with the work of Anyanwu et al. (2008: 103-107) which showed that very high salinity affected the emulsification activity of surfactant produce by *Serratia marcescens* isolated from petroleum contaminated site in Nsukka.

Effect of heavy metal ions (Cu, Zn, Mn and Ni)

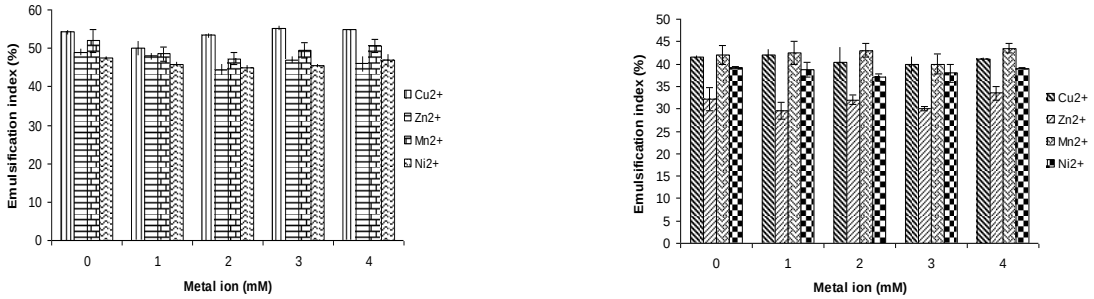
The effects of metal ions (Cu, Zn, Mn and Ni) on the surface activity of the biosurfactants produced by the two organisms were assayed by exposing the culture supernatant to metal salt concentrations in the range 0-4 mM. The metal ions showed stability over a wide range of the

concentration tested. There was therefore no significant (P≤0.05) difference when the concentration at 2 mM and 4 mM were compare with the control which had no metal ion concentration. Figs 4 and 5 show the effect of the metal ions (Cu, Mn, Zn and Ni) on S.T and E.I of biosurfactant produced by *Pseudomonas* sp. The biosurfactant produced by the organisms showed no significant difference in stability to the varying concentration of metal ion used.



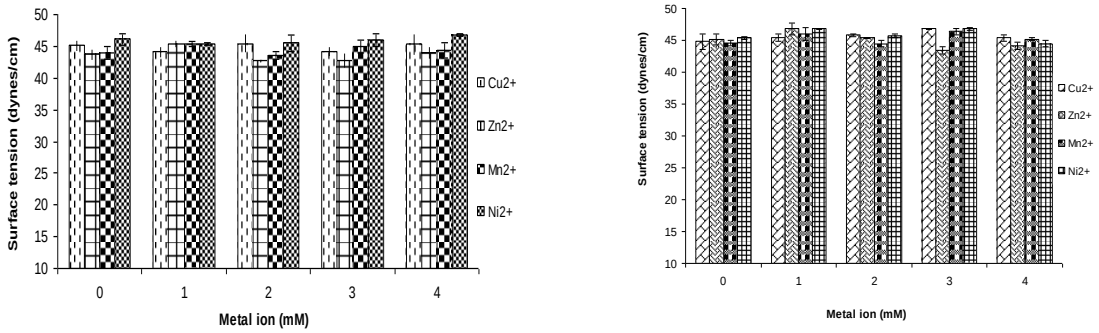
I J

Fig. 4. Effect of metal ions (Cu, Mn, Zn and Ni) on surface tension of biosurfactant produced by *Pseudomonas* sp on [I] WFO and on [J] WLO



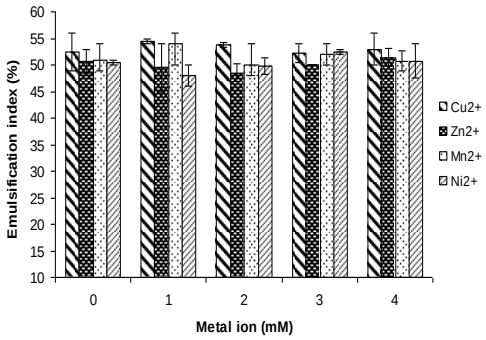
K L

Fig. 5. Effect of metal ions (Cu, Mn, Zn and Ni) on emulsifying activity of biosurfactant produced by *Pseudomonas* sp on [K] WFO and [L] on WLO

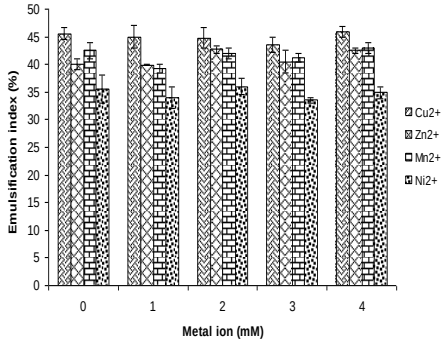


M N

Fig 6. Effect of metal ion on surface tension of biosurfactant produced by *Bacillus* sp on [M] WFO and [N] on WLO



O



P

Fig. 7. Effect of metal ion on emulsifying activity of biosurfactant produced by *Bacillus* sp on [O] WFO [P] WLO

The addition of metal ions (Cu,Zn,Ni and Mn) in the range 0-4mM to the culture supernatants had no significant effect on the surface tension and the emulsification index as shown in Figs. 6-7. The emulsion produced by the other metal ions (Mn, Zn, and Ni) as indicated in the results above showed stability of the emulsion produced to the metal ion tested. This stability could be due to high polysaccharide concentration within the emulsion Bandyodpadhyay et al. (2012: 592) This agrees with the work of Phale et al. (1995: 424-431) which shows that the emulsions produce by *Pseudomonas maltophilia* were stable to metal ion concentration and does not require metal ion for emulsion.

Conclusion

The isolates utilized WFO and WLO to produce biosurfactants. The emulsion produced by the two organisms in the different carbon sources showed stability over a wide range of environmental conditions such as pH, temperature, salinity and metal ion concentrations. The stability study of the surfactant suggests their suitability for environmental, pharmaceuticals, cosmetic, health, food and agricultural application.

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