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Production of Surface Active Glycolipid by *Serratia marcescens* NSK-1 Isolated from Petroleum Contaminated Soil

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Abstract

Biosurfactant-producing *Serratia marcescens* NSK-1 strain was isolated from soil contaminated with petroleum products. The organism, when grown in mineral salts medium with sucrose as sole carbon source, produced a surface-active anthrone-positive glycolipid. The biosurfactant was partially purified by liquid-liquid partition chromatography in chloroform-methanol mixture (1:1) and silica gel chromatography. It reduced the surface tension of distilled water from 72 dynes/cm to 40 dynes/cm and exhibited good emulsification activity with some vegetable oils and hydrocarbons. The biosurfactant exhibited stable surface activity over a wide range of temperature (30-100°C), pH (2-12) and salt concentration. There was no significant toxic effect shown by the biosurfactant towards mice when tested using the World Health Organization guidelines.

***Key words*:** Biosurfactant, bioemulsifier, emulsification, surface tension, *Serratia marcescens*

Introduction

Biosurfactants the surface-active compounds produced by microorganisms comprise a wide range of chemical structures such as glycolipids, lipopeptides, polysaccharide-protein complexes, phospholipids, fatty acids and neutral lipids (Mulligan, 2005; Neto *et al*., 2008). Glycolipids *viz*. rhamnolipids, trehalolipids, and sophorolipids are the most known biosurfactants in which carbohydrates such as rhamnose, sophorose, trehalose or fructose are attached to a long chain fatty acid (Calvo *et al*., 2004). The lipopeptides are the amino acid containing biosurfactants like surfactin produced by *Bacillus subtilis* (Lin, 1996). Other biopolymers of this group include the decapeptide antibiotics (gramicidins) and lipopeptide antibiotics (polymyxins) produced by different species of *Bacillus*. Several bacteria and yeasts have been reported to produce large quantities of fatty acid and phospholipid surfactants when grown on n-alkanes (Cirigliano and Carman, 1984; Desai and Banat, 1997).

Surfactants and emulsifiers are widely used in the pharmaceutical, cosmetic, petroleum, food industries and agriculture. However, most of these compounds synthesized chemically are toxic to environments, not easily biodegradable, and their manufacturing processes and by-products can be environmentally hazardous (Maier and Soberon, 2000). But, biosurfactants commonly have the

advantages of biodegradability, low toxicity and biocompatibility over chemically synthesized surfactants (Mohan *et al*., 2006). In addition, they can potentially be synthesized from renewable and cheaper substrates (Desai and Banat, 1997; Rosenberg and Ron, 1999), and are commonly effective at extremes of pH, temperature and salinity (Georgiou *et al*., 1992). Much interest has currently centered on surface active microbial products i.e., biosurfactants as an alternative source of surfactants.

The range of industrial applications of biosurfactants includes enhanced oil recovery, crude oil drilling, bioremediation of pollutants, health care and food processing (Cameotra and Makkar, 2004; Hickey *et al*., 2007; Dastgheib *et al*., 2008). Biosurfactants have found applications as therapeutic agents and in combating many diseases. In agriculture, biosurfactants are used to dilute and disperse fertilizers and pesticides and to enhance penetration of active compounds into plants. Surface active agents are needed for hydrophilization of heavy soils to obtain good wettability and also achieve equal distribution of fertilizers and pesticides in the soils (Calvo *et al*., 2004). Accordingly, the screening of new microbial surfactants has intensified. They will become important industrial chemicals as yields of biosurfactants in production processes are improved and the potential for the substitution of existing synthetic surfactants is further evaluated.

Many types of surface active agents are synthesized by a wide variety of microorganisms. Mostly, they exhibit the typical amphiphilic character of lipids but they are generally extracellular (Kim *et al*., 2006). The most often isolated and most thoroughly studied biosurfactants are the structurally homogeneous glycolipids e.g., sophorose lipids, rhamnolipids and trehalose lipids. The glycolipids are the microbial surfactants or emulsifying agents that contain carbohydrate in combination with long chain aliphatic acids. Glycolipids have been produced by a variety of microorganisms including *Pseudomonas aeruginosa, Arthrobacter sp*., *Corynebacterium sp., Rhodococcus erythropolis, and Serratia spp* (Mulligan, 2005).

In the present work, we have reported the production of glycolipid biosurfactant by an indigenous tropical strain of *Serratia marcescens* isolated from petroleum-contaminated soil. Some of the properties and initial characterization of the product obtained are also presented.

Materials and methods

Microorganism and culture conditions

A strain of *Serratia marcescens* NSK-1 was used throughout this work. It was isolated in our laboratory from petroleum-contaminated soil as described by Anyanwu *et al.* (2008), and maintained on nutrient agar slants at a refrigeration temperature of 4°C. Each seed culture was prepared by inoculating a loop of the stock culture into 20 ml of nutrient broth, within a 200-ml conical flask. That was incubated at 30°C and 180 rev/min for 8-12 h. An aliquot of 2.5 ml of inoculum was transferred to 250 ml Erlenmeyer flask containing 50 ml of a liquid medium with the following composition (g/l): K_2HPO_4, 4.5; KH_2PO_4, 2.0; $(NH_4)_2SO_4$, 3.0; $MgSO_4.7H_20$, 0.01; $FeSO_4.7H_20$, 0.01 and Sucrose, 20.0. 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°C for three days.

Culture samples were taken at regular intervals during the incubation period and analyzed for viable cell numbers, pH, surfactant concentration and surface tension. Control experiments were set up in parallel with inoculated flasks at the same conditions. Viable cell numbers were determined by the spread plate technique on nutrient agar (Lab M) using serially diluted culture samples and incubated for 24-48 hr at 30°C. All chemicals and reagents used were of analytical grade and obtained from Sigma-Aldrich Chemical Co., Inc.

Biosurfactant isolation

Culture broth of *Serratia marcescens* NSK-1 was centrifuged at 10,000 x g for 15 min to obtain cell-free supernatant. The supernatant was extracted three times with equal volumes of chloroform-methanol (1:1, v/v) mixture. The pooled extracts were evaporated to dryness at 45-50°C and the residue obtained was washed with distilled water and re-extracted after which the solvent was evaporated.

Adsorption chromatography on silica gel

The residue was dissolved in chloroform-methanol mixture and applied to a silica gel 60 (Aldrich, Milwaukee, Wis.) chromatography column (2.0 cm by 30.0 cm) and eluted with chloroform-methanol (5:1) mixture. The column was first washed with chloroform to elute out non-polar compounds and pigments. The effluent obtained was concentrated by evaporating the solvent.

Thin layer chromatography (TLC)

It was carried out by spotting the concentrated residue on precoated TLC plates (Merck, Germany) and developed in the following solvent systems, chloroform-methanol-acetic acid (85:10:5) and n-hexane-ethyl ether-acetic acid (80:20:1) (Suzuki *et al.*, 1974). Detection of materials on the plates was done after air-drying by spraying the developed plates with anthrone-sulphuric acid and α-naphthol-sulphuric acid solutions, respectively, and heated at 100°C for 5 min.

Biochemical analysis

Carbohydrate content of the biosurfactant was estimated by the method of Dubois (1956) using glucose (0-80 μg ml^{-1}) as standard. Protein content was determined by the AOAC method (1980) of Kjeldahl digestion in sulphuric acid while lipid content was determined by extracting a portion of the biosurfactant (0.5 g) with 25 ml of diethyl ether in a water bath at 50°C for 30 min (Pruthi and Cameotra, 1997). The ether extract was dried by filtering through anhydrous Na_2SO_4 and the filtrate clarified and weighed.

Identification of sugar moiety

The sugar moiety of the glycolipid biosurfactant was determined by the method of Suzuki *et al.* (1974). The glycolipid (100 mg) was hydrolyzed with 5 mL of 0.5 M sodium hydroxide (90% ethanol solution) at 55°C for 60 min. At the end of the hydrolysis, the hydrolysate was neutralized to pH 7.0 with hydrochloric acid and extracted three times with equal volumes of diethyl ether. The aqueous and ether extracts were separately pooled together and the aqueous phase evaporated.

The water-soluble fraction of the hydrolysate was subjected to thin layer chromatography in addition to other sugars as standards, in the solvent systems, n-propanol-ethyl acetate-water (65:10:25), phenol-water (4:1) and n-hexane-ethyl ether-acetic acid (80:15:5), respectively. The plates were allowed to air-dry then sprayed with 50% H_2SO_4 and heated at 110°C for 5 min.

Measurement of biosurfactant concentration

Biosurfactant concentration of the culture broth was estimated by determining the critical micelle concentration (CMC) which involves measuring the surface tension of serially diluted broth samples as described by Cooper *et al*. (1981). The CMC is defined as the concentration of surfactant necessary to initiate micelle formation. If more of the surfactant is present, there will be no further decrease in surface tension. The dilution factor obtained, which is the reciprocal of the CMC (CMC^{-1}), is proportional to the amount of surfactant present.

Surface tension and critical micelle concentration

Surface tension of the culture broth and purified biosurfactant was determined by the ring method (Kim *et al*., 2000) using Du-Nouy ring tensiometre (K6, Krüss, Hamburg, Germany) at room temperature (30±2°C). The CMC of the purified biosurfactant was determined by measuring the surface tension of serially diluted known weight of the biosurfactant and plotting the surface tension as a function of the biosurfactant concentration (Gerson and Zajic, 1979). The critical micelle concentration value is defined as that point at which purified surface active compound no longer aggregates to form micelles.

Assay of emulsification activity

The emulsification activity of the biosurfactant was measured as described by Cooper and Goldenberg (1987). Briefly, 3.0 ml of different oil samples was added to 2.0 ml of the biosurfactant solution in a graduated test tube and vortexed at high speed for 2 min. This was allowed to settle for 24 hours and the emulsification index (E_{24}) was calculated by dividing the measured height of emulsion layer by the mixture's total height and multiplying by 100.

Stability studies

The effect of pH on surface activity was carried out by introducing the biosurfactant solution (0.1%, w/v) in 0.1M-phosphate buffer, pH 7.0, into graduated test tubes and adjusting the pH to various values (2-12) by the addition of HCl or NaOH at room temperature (Zhang and Miller, 1992). The heat stability study was carried out by incubating the biosurfactant solution in 0.1M phosphate buffer, pH 7.0 at different temperatures (30-100°C) for 30 min and cooling to room temperature. The effect of salt 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. The emulsification index, measured using paraffin oil, and surface tension were respectively determined at the end of each treatment period.

Acute toxicity test

Acute toxicity studies were carried out following World Health Organization guidelines as adapted by Ashtaputra and Shah (1995). Ten mice (22-27 g each), five of each sex, were given 5.0 g biosurfactant in water/kg body weight per *os*. Control mice were given only water in a similar manner. All the mice were weighed immediately before the administration of the biosurfactant solution or water and periodically after 4, 10, 16, 23 and 30 days, respectively. The mice were observed for any toxic symptoms during the experimental period after which they were sacrificed. The liver and kidney indices of sacrificed mice were determined at the end of the treatment period. The indices were determined as the ratio of weight of the liver or kidney to the weight of the animal.

Soil column study

A sandy loam soil obtained from the University of Nigeria, Nsukka demonstration farm was used in this study. The soil column study was performed by the method of Pruthi and Cameotra (1997). Glass columns (40.0×2.0 cm), each packed with 80.0 g of the soil, were saturated with 25 ml of engine oil and 22 ml of kerosene, respectively. The efficiency of the biosurfactant solution in releasing the oil from the soil was tested by adding 100 ml aqueous solution of 0.5% (w/v) of the biosurfactant solution to the column. Distilled water only was used as control in flushing the soil column. Efficiency of oil recovery was estimated by measuring the volume of each, oil released.

Statistical analysis

All experimental set-ups and analyses were performed at least three times with at least three replicates of each test under each condition in all experiments. 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.

Results

The surface-active glycolipid was isolated from the cell-free culture supernatant of *Serratia marcescens* NSK-1 cultivated in a sucrose-mineral salt medium. Figure 1 shows the production of the biosurfactant and growth characteristics of *Serratia marcescens* NSK-1 on minimal medium containing 2% (w/v) sucrose at different time intervals. The surface tension, pH values and biosurfactant concentration recorded during growth of the organism are also shown in figure 1. The results indicated that as cell growth and biosurfactant production continued, there was reduction in the surface tension and pH of the medium. Maximum cell count of 8.10×10^{9} cfu/ml from an initial of 1.32×10^{7} cfu/ml and minimum surface tension value of 36.8 dynes/cm of the culture filtrate were achieved at 36 h at which maximum biosurfactant concentration of $2.7gL^{-1}$ was obtained.

Liquid-liquid partitioning in chloroform-methanol mixture and silica gel adsorption chromatography resulted in an enrichment of the active compound. The extract from the sucrose-grown cells was identified as glycolipid based on its positive chromogenic reactions with anthrone-sulphuric acid and α-naphthol-sulphuric acid spray reagents after development in chloroform-methanol-acetic acid solvent. The isolated biosurfactant after purification was found to contain about 46.3% carbohydrate and 50.2% lipid. The thin-

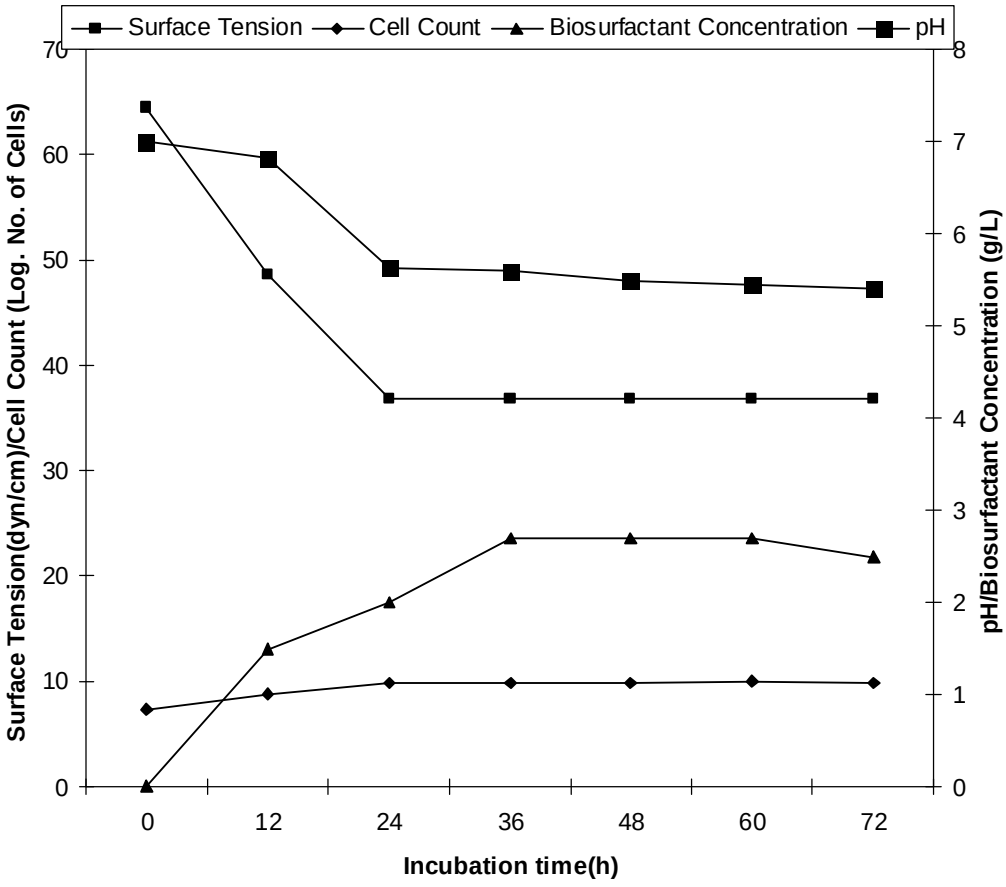

Figure 1: Time Course of Biosurfactant Production with Sucrose as Carbon Source

layer chromatogram of the glycolipid biosurfactant exhibited an R_f value of 0.56. The biosurfactant exhibited surface activity at low concentrations, reaching a critical micelle concentration of 52 mgL^{-1}.

The glycolipid was deacylated as described in the methodology section. The water soluble fraction from the saponified mixture was examined by thin layer chromatography. A single major spot was obtained, in phenol-water solvent, with R_f of 0.59 and colour reaction very identical with those of standard sucrose. This revealed that the sugar moiety of the glycolipid was sucrose.

The biosurfactant obtained from *Serratia marcescens* NSK-1 was capable of forming stable emulsions with several oils tested. Emulsification activity of the biosurfactant was greater with vegetable oils than hydrocarbon oils (Tab. 1). Olive oil, a vegetable oil, showed the most stable

emulsion with emulsification index of 100%, while kerosene, hydrocarbon oil, exhibited the least stable emulsion after 24 h with emulsification index of 56%.

Table 1. Emulsifying activity of biosurfactant with different oils.

Oil	Emulsification index (%)
Soybean	98 ± 0.8
Olive	100 ± 0.0
Palm	80 ± 2.2
Groundnut	95 ± 1.4
Engine	78 ± 2.2
Kerosene	56 ± 2.4
Paraffin	90 ± 1.6
Crude	60 ± 2.4

The effects of pH, temperature and sodium chloride on the surface tension of the biosurfactant are shown in table 2. The biosurfactant retained surface tension activity over wide pH and temperature ranges. The variations in surface tension values of the biosurfactant over the pH and temperature ranges were not significant ($p<0.05$). However, sodium chloride concentration of 16% (w/v) and above significantly ($p<0.05$) inhibited the surface tension of the biosurfactant. In table 3 are shown the effects of pH, temperature and sodium chloride concentrations on the emulsifying activity of the biosurfactant. The biosurfactant exhibited stable emulsion formation over wide ranges of pH, temperature and sodium chloride concentration values. At pH 4 and below and pH 12, emulsification activity was significantly inhibited ($p<0.05$) but no significant effect was shown by temperature. Sodium chloride concentration of up to 12% (w/v) showed no appreciable effect on biosurfactant activity. However, at sodium chloride concentration of 16% and above, emulsifying activity was significantly ($p<0.05$) inhibited.

Table 2. Effects of pH, temperature and sodium chloride on surface tension of biosurfactant.

Parameter	Value	Surface tension (dynes/cm)
pH	2	43.2±0.7
	4	43.0±0.6
	6	41.5±0.5
	7	40.2±0.6
	8	39.0±0.5
	10	38.7±0.5
	12	38.5±0.9
Temperature (°C)	30	39.3±0.5
	40	39.5±0.6
	50	40.0±0.7
	60	40.8±0.5
	80	42.2±0.4
	100	43.1±0.6
NaCl (%, w/w)	0	40.2±0.7
	4	40.4±0.4
	8	41.2±0.6
	12	42.2±0.7
	16	43.7±0.8
	20	44.0±0.7

Table 3. Effects of pH, temperature and sodium chloride on emulsifying activity of biosurfactant.

Parameter	Value	Surface tension (dynes/cm)
pH	2	78.0±2.2
	4	78.5±0.2
	6	82.5±1.9
	8	85.5±1.6
	10	84.5±2.1
	12	80.0±1.4
Temperature (°C)	30	85.5±1.2
	40	85.0±1.5
	50	84.0±2.4
	60	84.0±1.6
	80	82.5±2.3
	100	81.0±2.8
NaCl (%)	0	85.0±0.8
	4	82.0±1.4
	8	80.5±2.5
	12	78.0±2.4
	16	73.0±2.2
	20	67.5±2.5

Acute toxicity study of the biosurfactant on mice demonstrated that none of the mice given the biosurfactant *per os* died or showed any toxicity syndrome. The apparent increase in body weight of the test animals was not significant ($p<0.05$) and was comparable with that of control animals (Tab. 4a). There was also no significant difference ($p<0.01$) in the liver and kidney indices of the sacrificed experimental and control mice (Tab. 4b).

Table 4a. Effect of oral administration of biosurfactant on the growth of mice.

Days of growth	Body weight (g)*	
	Experimental	control
0	25.35 ± 1.04	25.28 ± 1.57
4	25.98 ± 0.65	26.05 ± 1.65
12	26.82 ± 1.61	26.70 ± 1.53
20	27.84 ± 1.75	27.68 ± 1.77
30	29.15 ± 1.72	28.75 ± 1.83

*Values are means of body weight ± standard deviation for 10 mice.

Table 4b. Organ indices of sacrificed mice fed with biosurfactant.

Organ	Organ index	
	Experimental	control
Liver	0.050	0.049
Kidney	0.007	0.007

Values are means of organ indices

The soil column study, which tested the effectiveness of the biosurfactant in possible microbial enhanced oil recovery, shows that 52% of engine oil and 62% of kerosene were recovered by the addition of the biosurfactant solution. Only 26% of engine oil and 30% of kerosene were recovered with distilled water.

Discussion

The *Serratia marcescens* NSK-1 strain isolated and used in this study produced glycolipid biosurfactant when grown in batch cultures with sucrose as the sole carbon and energy source. The production of biosurfactant and biomass during growth of *Serratia marcescens* NSK-1 in the sucrose medium was accompanied by decrease in surface tension of the fermentation broth. The decrease in surface tension indicates production of surface-active compounds by the bacterial strain. The results of the present study indicate that the biosurfactant production is growth-associated because a parallel relationship existed between growth and biosurfactant production as shown by surface tension reduction. The production of surface-active agent by *B. cereus* IAF-346 and biodispersan by *Bacillus* sp. strain IAF-343 (Cooper and Goldenberg, 1987) are examples of growth-associated biosurfactant production.

The majority of known biosurfactants are synthesized from water-immiscible hydrocarbons (Nitschke and Pastore, 2006). However, it was reported by Cooper *et al.* (1981), that the addition of a hydrocarbon to culture medium completely inhibited surfactant production by *Bacillus subtilis.* Meanwhile, the *Serratia marcescens* NSK-1 produced biosurfactant from water-soluble substrate, sucrose. According to Sandrin *et al*. (1990), glucose, fructose and sucrose were the best carbon substrates for the synthesis of biosurfactant by *Bacillus subtilis*. Moreover, it has been shown (Makkar and Cameotra, 1997) that water-soluble substrates are cheaper than hydrocarbons and are preferred because single-phase fermentation is simpler than biphasic fermentation.

Stability studies of the biosurfactant indicated it to be thermostable and also pH stable. The surfactant showed salt tolerance of up to 12%. These findings suggest that

the product obtained has potential application over a wide range of temperature and pH and relatively high salt environment. As such it could be very useful in situations where extreme conditions of temperature, alkaline pH and salinity are present such as enhanced oil recovery and bioremediation of soil and marine environments. Some biosurfactants have been found to be less stable over extreme pH range (Cirigliano and Carman, 1984; Zhang and Miller, 1992).

The ability of the *Serratia marcescens* NSK-1 biosurfactant to emulsify and stabilize the emulsion of a number of vegetable and hydrocarbon oils was examined. The oils did form emulsions with water in the absence of the biosurfactant; however, these emulsions rapidly separated in less than 10 min. The addition of the biosurfactant to the emulsions stabilized them for over a 24 hr period. The ability of biosurfactants to emulsify hydrocarbon-water mixtures has been demonstrated to increase hydrocarbon degradation significantly and is thus potentially useful in oil spill management (Abu-Ruwaida *et al*., 1991; Atlas and Bartha, 1992). The ability to form emulsions with vegetable oils and fats suggests potential application as cleaning and emulsifying agent in the food industry (Neto *et al*., 2008). A surfactant obtained from *Rhodococcus* strain AT-5 had lower emulsifying index towards short-chain hydrocarbons than for long-chain hydrocarbons (Abu-Ruwaida *et al*., 1991). The emulsifying activity of *Serratia marcescens* NSK-1 surfactant seemed not to be related to hydrocarbon chain length as it formed stable emulsions with oils of varying hydrocarbon chain lengths. The ability of the obtained biosurfactant to form stable emulsions with some hydrocarbons and different oils suggests considerable potential application in the petroleum industry as well as food and pharmaceutical industries.

The glycolipid biosurfactant was non-toxic to mice at the 5.0 g/kg body weight dose tested, which was the highest dose recommended by the Food and Agricultural Organization/World Health Organization for a food additive. This is indicative of its non-toxic nature even when used as food additive or accidentally consumed. Moreover, the concentration of the surfactant, which could be consumed in whatever form, would be significantly less than the dose tested (5 g/kg body weight). The low toxicity of biosurfactants has been recommended as a veritable advantage over synthetic surfactants (Desai and Banat, 1997; Kim *et al*., 2000).

The isolated biosurfactant, which removed 52% and 62% of engine oil and kerosene, respectively, is relatively effective in removing oil from a soil column saturated with known amounts of these hydrocarbons. However, Pruthi and Cameotra (1997) reported the recovery of 85-90% of the oil from a sand pack column when biosurfactant solution from *Arthrobacter protophormie* was used. The *Serratia marcescens* NSK-1 biosurfactant, therefore, has potential application in microbial enhanced oil recovery and bioremediation.

The results obtained in the present study showed the presence of indigenous bacteria in the tropical Nigerian soil capable of producing biosurfactant. The study also led us to suggest that the *Serratia marcescens* NSK-1 may have a significant application in the environmental, crude oil recovery and food industries.

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