Production of a Peptidoglycolipid Bioemulsifier by *Pseudomonas aeruginosa* Grown on Hydrocarbon

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A strain of *Pseudomonas aeruginosa* isolated from a polluted soil was found to produce an extracellular bioemulsifier when cultivated on hexadecane as sole carbon source. The emulsifier was precipitated with acetone and redissolved in sterile water. Dodecane, crude oil and kerosene were found to be good substrates for emulsification by the bioemulsifier. Growth and bioemulsifier production reached the optimal levels on the fourth and fifth day, respectively. Emulsifying activity was observed over a pH range of 3.5 to 10.0 with a maximum at pH 7.0. The activity of the bioemulsifier was heat stable up to 70 °C while about 50 percent of its activity was retained at 100 °C. The components of the bioemulsifier were determined, it was found to contain carbohydrate, protein and lipid. The protein complex was precipitated with ammonium sulphate and fractionated on a Sephadex G-100. Gel electrophoresis of the bioemulsifier showed a single band whose molecular weight was estimated as 14,322 Da. The bioemulsifier was classified as a peptidoglycolipid. Certain strains of *P. aeruginosa* produce peptidoglycolipid in place of rhamnolipid.

Introduction

The growth of microorganisms on hydrocarbons is often accompanied by the emulsification of the insoluble carbon source in the culture medium (Rosenberg *et al.,* 1979). The spontaneous release and function of biosurfactants are often related to hydrocarbon uptake. Therefore, they are predominantly synthesized by hydrocarbon-degrading bacteria and fungi. Biosurfactants are a structurally diverse group of surface active molecules synthesized by microorganisms which can be accumulated as cell bound or extracellular products. Many hydrocarbon utilizing bacteria and fungi have been reported to possess emulsifying activities due in part to the whole cells or to extracellular surface-active compounds. Emulsifying ability, after removal of cells, often disappear in cells participating in emulsification whereas isolates secreting surface active compounds extracellularly often retain emulsifying ability after removal of cells. Extracellularly produced surface active compounds are the most promising biosurfactants. Some of the compounds with emulsifying properties include phospholipids, fatty acids, lipopeptides, lipoproteins and glycolipids. Polysaccharides have no emulsification activity alone but may become potent emulsifiers when combined with proteins or lipids released during growth (Desai and Banat, 1997). Biosurfactants can be divided into two major categories. The first are the low molecular weight surfactants such as glycolipids, fatty acids and phospholipids. The second type are the high molecular weight polymers (Willumsen and Karlson, 1997). Most known biosurfactants are glycolipids. Among the glycolipids, the best known are rhamnolipids, trehalolipids. Rhamnolipids, produced by *P. aeruginosa* are the best studied and the most reported glycolipids and was first described by Jarvis and Johnson (1949). Several other authors including Hisatsuka *et al.* (1971), Itoh and Suzuki (1972), Reiling *et al.* (1986) and Ochsner *et al.* (1995) have described the rhamnolipids of *P. aeruginosa.* Polymeric emulsifiers include emulsan, liposan, alasan, mannoprotein and other polysaccharide protein complexes (Desai and Banat, 1997). Emulsan is a polyanionic amphipathic heteropolysaccharide bioemulsifier produced by *Acinetobacter calcoaceticus* RAG – 1. Liposan is an extracellular emulsifier produced by *Candida lipolytica*, it is composed of carbohydrate and protein with the carbohydrate portion consisting of glucose, galactose, galactosamine and ga-

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lacturonic acid. Alasan is a heteropolysaccharide – protein biosurfactant produced by *Acinetobacter radioresistens* . Mannoprotein is produced by *Saccharomyces cerevisiae*, the protein has been reported to have excellent emulsifying activity towards several oils, alkanes and organic solvents (Desai and Banat, 1997). Few studies have, however, emerged on the polymeric bioemulsifier of *P. aeruginosa* such as peptidoglycolipid.

In recent years, interest in the isolation and identification of new microbial bioemulsifiers has increased immensely due to their ability to meet most synthetic surfactants' properties including emulsification, wetting, phase separation and viscosity reduction for potential application in the cosmetics, food and agricultural industries. In this paper, we report some properties of a peptidoglycolipid bioemulsifier produced by an isolate of *P. aeruginosa.*

Materials and Methods

Bacterial strain

The organism used in this study was isolated from a Nigerian soil polluted with crude oil using minimal salts agar as described by West *et al.* (1984). The isolate was identified on the basis of Gram reaction, cell morphology and other biochemical tests as described by Holt *et al.* (1994).

Growth conditions

The medium for the production of bioemulsifier contained per litre: NH_4N0_3, 4.0 g; Na_2HP0_4, 2.0 g; KH_2P0_4, 0.53 g; K_2S0_4, 0.17 g, $MgS0_4$. $7H_20$, 0.10 g and n-hexadecane (1.0% w/v) as sole carbon and energy source. The pH of the medium was adjusted to 7.1 before autoclaving at 121 °C for 15 min. Trace elements solution (2.5 ml per litre) described by Bauchop and Elsden (1960) was sterilized separately and then added aseptically to the medium. The medium was inoculated with the organism and incubated with shaking (120 rev/min) at 30 °C.

Growth of organism, bioemulsifier production and purification

Studies on growth of the organism and bioemulsifier production over a time course was carried out. Samples were aseptically withdrawn, diluted in sterile distilled water and aliquots plated in triplicate on nutrient agar for total viable counts. The cells were removed from the culture broth by centrifugation (10,000×*g*, 15 min, 4 °C) while the clear supernatant was used as the source of crude bioemulsifier.

The bioemulsifier was recovered from the cell free culture supernatant by acetone precipitation. Three volumes of chilled acetone was added and allowed to stand for 10 h at 4 °C. The precipitate was collected by centrifugation and evaporated to dryness to remove residual acetone after which it was redissolved in sterile water.

Emulsification measurement

The activity of the purified bioemulsifier was measured twice in all cases by adding 2.5 ml of n-dodecane to 2.5 ml of bioemulsifier in a test-tube. The tube was vortexed at high speed for 2 min. and left undisturbed for 24 h. Measurements were made manually thereafter to get the average emulsion index. The emulsion index (E24) was the height of the emulsion layer divided by the total height multiplied by 100. Hexadecane, octane, kerosene, crude oil and hexane were also assayed for their ability to serve as substrates for emulsification.

Effect of pH and temperature; protein determination

The pH of the purified bioemulsifier was adjusted to a range of 3.5–10.0 with dilute HCl or Na0H after which its activity was determined. Samples of the bioemulsifier were also exposed to heat for 30 min at a temperature range of 27–121 °C and its activity determined thereafter.

The protein content of the purified bioemulsifier was assayed by the Bradford (1976) method with bovine serum albumin as standard.

Carbohydrate and lipid analysis

The carbohydrate content of the purified bioemulsifier was determined as total hexoses by the

anthrone reagent (Spiro, 1966). To the sample (1.0 ml) was added 5.0 ml of cold anthrone reagent in a test tube. The tubes were shaken vigorously to ensure mixing and heated in a boiling water bath for 15 min at the end of which the tubes were cooled in a water bath and allowed to stand for 20 min followed by absorbance measurement at 620 nm. The blank contained 1.0 ml of distilled water and 5.0 ml of the anthrone reagent. The carbohydrate content in samples were extrapolated from a standard calibration curve prepared with known concentrations of glucose.

Thin layer chromatography (TLC) was carried out on a 20 × 20 cm precoated silica gel plate using petroleum ether, diethyl ether and acetic acid (90:10:1) as running solvent. The plate was developed by staining with 5% H_2S0_4 in 95% ethanol followed by heating at 150 °C for 30 min. The Rf value of the developed spot was calculated and compared with values of standard compounds in similar solvent as described by Kates (1972).

Protein purification and molecular weight determination

The protein complex of the bioemulsifier was precipitated with $(NH_4)_2SO_4$ (analytical grade) at 80% saturation. The precipitate was collected by centrifugation, redissolved in sodium phosphate buffer (0.05 M, pH 7.0) and dialyzed against the buffer at 4 °C for 12 h. The dialyzed sample was fractionated on a Sephadex G-100 column as described by Ilori *et al.* (1995). The protein content of fractions was determined after Bradford (1976) while the apparent molecular weight was estimated using sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE, 30%). The gel was stained with Coomassie blue reagent. The following marker proteins (Sigma, USA) were used: bovine serum albumin (66,000 Da), egg albumin (45,000 Da), glyceraldehyde-3-phosphate-dehydrogenase (36,000 Da) bovine carbonic anhydrase (29,000 Da), bovine pancreas trypsinogen (24,000 Da), soybean trypsin inhibitor (20,000 Da) and bovine milk α-lactalbumin (14,200 Da).

The hot phenol method described by Navon – Venezia *et al.* (1995) was used to ascertain whether or not the protein moiety was essential for emulsifying activity.

Results

The microbial strain used in this study, *Pseudomonas aeruginosa*, was an anthracene degrader isolated from soil polluted with crude mineral oil. The organism was rod shaped, Gram negative, catalase positive, oxidase positive, motile and did not produce indole. It however produced pyocyanin and pyoverdin pigments. The organism grew at 42 °C and utilized glucose, raffinose, sucrose, mannitol and maltose but did not utilise trehalose and sorbitol as sources of carbon. The organism grew in minimal salts medium containing n-hexadecane as sole carbon source and secreted extracellular compounds with surface active properties. The growth of the organism on n-hexadecane was accompanied by an increase in viable cell counts. Maximum production of the bioemulsifier occurred on the 5th day (Fig. 1A). A decrease in the viable cell counts was observed on the 5th and 6th day.

Emulsifying activity was observed over the entire pH range studied. The purified bioemulsifier was very active at pH 6 and 7 and fairly active at pH 4–5 and 8–9. Low emulsifying activity was however observed at pH 3.5 and 10 (Fig. 1B). The activity of the purified bioemulsifier was stable up to 70 °C after which a sharp drop in activity occurred (Fig. 1C). At 100 °C, about 50% of emulsifying activity was retained.

The ability of a range of selected liquid hydrocarbons to serve as substrates for emulsification was investigated. The results presented in Fig. 2 showed that n-dodecane was the best substrate emulsified. Crude oil and kerosene also served as good substrates for emulsification. The least emulsified substrate was n-hexane. Hexadecane which was the substrate used in cultivation of the cells was fairly emulsified. Emulsion formed with kerosene, crude oil, and dodecane were stable for at least 48h. The emulsion formed with crude oil separated when allowed to stand undisturbed for more than 48h into an upper turbid emulsion and a lower organic phase. On agitation the two phases mixed immediately forming a creamy emulsion.

The purified bioemulsifier of *P. aeruginosa* had protein and carbohydrate components of approximately 28% and 34% respectively. A single SDS – PAGE band was obtained from the purified pro-

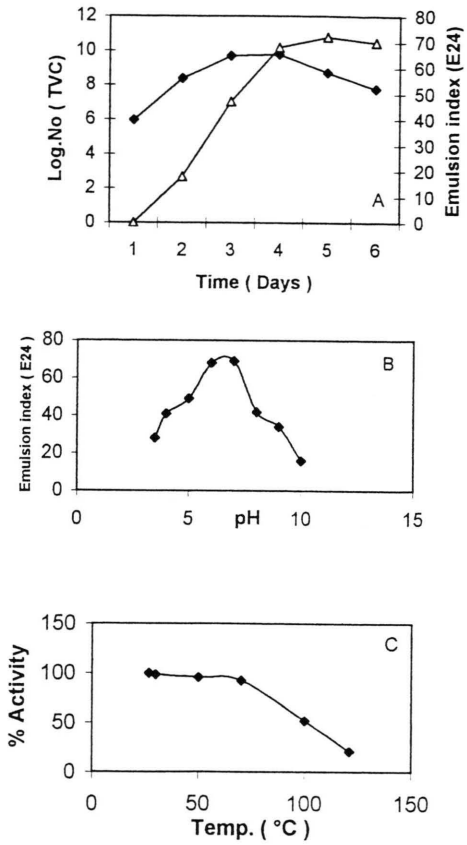

Fig. 1A. Time course production of bioemulsifier by *P. aeruginosa* △, bioemulsifier; ◆, Log. number of viable cells. Total viable cells (TVC) ranged from 9.3×10^5 to 6.03×10^9.
B. Effect of pH on bioemulsifier production by *P. aeruginosa.*
C. Effect of temperature on bioemulsifier production by *P. aeruginosa.*
100% value = emulsion index (E24) 70.

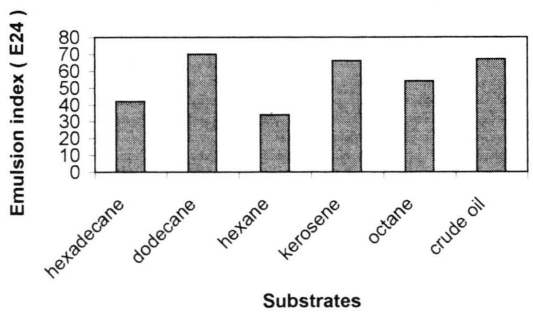

Fig. 2. Substrate specificity of the bioemulsifier.

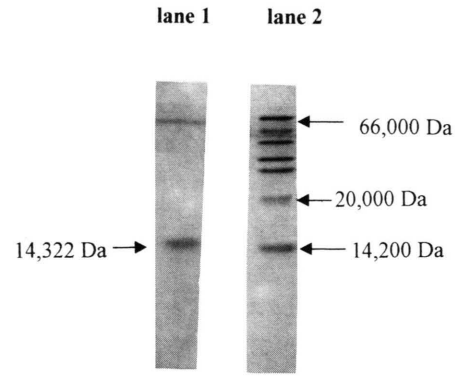

Fig. 3. Polyacrylamide gel electrophoresis.
Lane 1, 10 µl of purified bioemulsifier produced by *P. aeruginosa*,
Lane 2, 10 µl of standards: bovine albumin (M_r = 66,000), egg albumin (M_r = 45,000), glyceraldehyde-3-phosphate-dehydrogenase (M_r = 36,000), bovine carbonic anhydrase (M_r = 29,000), bovine Pancreas trypsinogen (M_r = 24,000), Soybean trypsin inhibitor (M_r = 20,000) and bovine milk α-lactalbumin (M_r = 14,200).

tein complex preparation (Fig. 3, lane 1). The apparent molecular weight was estimated as 14,322 Da.

After hot phenol treatment, no emulsifying activity was found in the aqueous deproteinized fraction. This indicated that the emulsifying activity was totally associated with the denatured protein fraction. The analysis of the purified bioemulsifier by TLC showed a single spot of lipid that was not identified. The bioemulsifier was subsequently classified as a peptidoglycolipid.

Discussion

Biosurfactants are a heterogenous group of surface – active molecules synthesized by microorganisms such as bacteria, yeasts and moulds. Biosurfactant producers among the bacteria include *Acinetobacter* sp, *Pseudomonas* sp, *Bacillus* sp and *Corynebacterium* sp. A soil isolate of *Pseudomonas aeruginosa* used in this work grew on n-hexadecane and produced bioemulsifier. The production of the bioemulsifier was growth associated. Growth associated production of surface active compounds have been reported from several other microorganisms such as *Bacillus stearothermophilus* VR-8 (Gurjar *et al.*, 1995), *Corynebacterium lepus* (Duvnjak and Kosaric, 1985), *Candida lipolytica* (Cirigliano and Carman,

1984) and *Arthrobacter* RAG-1 (Rosenberg *et al.*, 1979).

Most microbial emulsifiers are substrate specific, solubilizing or emulsifying different hydrocarbons at different rates. Dodecane, crude oil and kerosene served as good substrates for emulsification by the peptidoglycolipid of *P. aeruginosa.* The emulsification of crude oil by the peptidoglycolipid was not surprising because crude oil is a mixture of hydrocarbons and would naturally present the emulsifier with substrates of choice. Emulsification of crude oil by the peptidoglycolipid makes it suitable for oil and storage tank clean up.

The peptidoglycolipid was most active at pH 7.0, very active at pH 6.0 and fairly active at acidic and alkaline pH. Different emulsifiers are known to have different pH optima. The optimum pH for the emulsifier of *Acinetobacter radioresistens* was reported to be 5.0 (Navon – Venezia *et al.*, 1995). The emulsifier of *Bacillus cereus* was only active at pH below 7.0 while at a pH above 7.0, a pronounced loss of activity was recorded (Cooper and Goldenberg, 1987). The wider pH range of activity of the peptidoglycolipid of *P. aeruginosa* makes it more suitable for use in soil and aquatic environment bioremediation relative to those with narrower pH range. The peptidoglycolipid was heat stable and retained about 50% of its activity at 100 °C. In terms of heat stability, the peptidoglycolipid of *P. aeruginosa* appeared better than liposan produced by *Candida lipolytica* reported to be stable at temperatures up to 70 °C and with about 60% loss in activity after heating at 100 °C (Cirigliano and Carman, 1984).

The peptidoglycolipid had a protein activator that was involved in emulsification. Hisatsuka *et al.* (1972, 1977) described the isolation from *P. aeruginosa* of a protein activator that was involved in emulsification of hydrocarbons, it had a molecular weight of 14,300 and contained 147 amino acids of which 51 were serine and threonine. The protein component of a bioemulsifier produced by *P. aeruginosa* P-20 was reported to bear 52 amino acids (Koronelli *et al.*,1983). The molecular weight of the emulsifying proteins produced by *P. cepacia* N1 was estimated as 14,000 Da (Goswami *et al.*, 1994). In terms of molecular weight, the protein complex produced by *P. aeruginosa* reported in this work appeared closely similar to that described in *P. aeruginosa* by Hisatsuka *et al.* (1972). The bioemulsifier had a lipid component that was not identified. Some known lipids reported to be involved in bioemulsification include monoglycerides, 1,2-diglyceride and corynomycolic acids (Cooper and Goldenberg, 1987). Lipids improve emulsion stability by reducing interfacial tension between two immiscible phases.

The peptidodglycolipid reported in this work was found to be different from rhamnolipid produced by many strains of *P. aeruginosa*. The broad pH and temperature stability of this bioemulsifier indicate its potential for exploitation in areas such as soil and water clean up, cosmetic and food industries.

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