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SURFACTANT PROPERTIES OF AN EXOPOLYMERIC SUBSTANCE PRODUCED BY A GLUCOSE AND HYDROCARBON-UTILIZING BACTERIUM ISOLATED FROM A BRACKISH ENVIRONMENT IN THE NIGER DELTA

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ABSTRACT

A bacterium was isolated from water samples collected from the New Calabar river in Port Harcourt in the Niger Delta region of Nigeria. It was identified as a *Bacillus* sp. based on phenotypic, physiological and biochemical characteristics. Surfactant property of an exopolymeric substance produced by the isolate was tested using the emulsification of crude oil and surface tension reduction methods. The isolate grew in crude oil (Bonny light and Bonny medium) and glucose as sole sources of carbon; the spent culture filtrates of the crude oil and the glucose media emulsified the crude oil. Further more the surface tension of the glucose enriched culture broth was reduced by up to 40%. The biomolecule was precipitated, from culture supernatants as an exocellular polymeric substance, using cold ethanol and further extracted in a 1:1 mixture of chloroform and water, where it collected at the interface, and was shown to contain protein, carbohydrate and lipid. The critical micelle concentration (CMC) of the extract was determined to be $0.2gl^{-1}$. As a further check a *Bacillus* sp. that had been isolated from a soil sample was tested for ability to degrade hydrocarbons. The soil isolate did not degrade hydrocarbons, it did not produce an exocellular polymeric substance and the surface tension of the growth media did not change even though it grew luxuriantly in glucose enriched medium, as did the river isolate. It is concluded that the river isolate which is able to utilize glucose and petroleum hydrocarbons has potential application in bioremediation of oil-polluted environments.

KEYWORDS: surfactant, exopolymeric substance, hydrocarbon utilization, surface tension

INTRODUCTION

The poor solubility of hydrocarbons in water poses special problems for their use for microbial growth. The growth of the microbes on hydrocarbons is thought to involve specific processes, including the interaction of the microbial cells with, and uptake of the substrate. The immiscible hydrocarbon must be transported to the surface in such a way that it may achieve effective cell to substrate contact. Some microorganisms have developed two general strategies for enhancing contact with hydrocarbons, namely, emulsification and adherence (Rosenberg *et al.*, 1992).

The increase in occurrence of biodeterioration of petroleum derived products by microorganisms led to research on the possibility of using them for the treatment of hydrocarbon pollution, which triggered the study of different aspects of petroleum microbiology. Thus, there has been a growing interest in biosurfactants. Biosurfactants are known to affect the rate of biodegradation of hydrophobic compounds by increasing the solubilization and dispersion of the hydrocarbons and by changing the affinity between microbial cells and hydrocarbons by inducing increase in cell surface hydrophobicity (Zhang and Miller, 1994).

The biosurfactants could be simple glycolipids (Wasko and Bratt, 1991), lipopeptides (Yakimov *et al.*, 1995), sophoroselipids, rhamnolipids, trehalose lipids and sucrose lipids or simply polysaccharide/protein lipid complexes. These products could be extra cellular, and are found in the spent culture medium (Asha *et al.*, 1993), or cell bound (Wasko and Bratt, 1991). Their presence in the culture medium could be detected through reduction in surface and interfacial tension (Yakimov *et al.*, 1995) of spent culture medium and measurement of emulsification of hydrocarbons (Wasko and Bratt, 1991).

Surfactants being surface-active agents have amphiphilic structure; they have groups (moieties) which are lipophilic (oil soluble) and hydrophilic (water soluble). This amphiphilic structure results in the surface-active nature of surfactants and causes them to concentrate at interfacial regions. Thus, this brings about the reduction in the surface tension and brings about an oil-water emulsion. Hence, it is common practice to detect the presence of biosurfactants in a liquid culture medium by measuring the surface tension (Asha *et al.*, 1993, Yakimov *et al.*, 1995, Deziel *et al.*, 1996), which decreases with increase in the concentration of the biosurfactant until the critical micelle concentration (CMC) is reached or by measuring the emulsification of hydrocarbons by the liquid culture media (Asha *et al.*, 1993, Deziel *et al.*, 1996, Marin *et al.* 1996). The critical micelle concentration is the amount of biosurfactant needed to achieve the lowest possible surface tension.

Several surfactants have been studied. *Pseudomonas aeruginosa* produces a simple rhamnolipid surfactant (Hisatsuka et al., 1997) whereas *Acinetobacter calcoaceticus* RAG-1 produces a complex lipopolysaccharide. A lipopeptide surfactant produced by *Bacillus licheniformis* BAS 50 has been characterised (Yakimov et al., 1995), and a tentative structure and composition for the surfactant has been described as lichenysin A. The lipid moiety contains a mixture of 14 linear and branched β-hydroxy fatty acids ranging in size from C_{12} to C_{17} and contains seven amino acids. Another well characterized lipopeptide surfactant is surfactin obtained from several strains of *Bacillus subtillis and Bacillus pumilus*, (Arima et al., 1968).

In this work a *Bacillus* sp. was isolated from water samples of a stream that receives periodic releases of petroleum hydrocarbons. Its hydrocarbon and

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glucose utilizing and biosurfactant producing ability were studied with the aim of establishing its potential usefulness as a bioremediation tool (Water Quality International, 1997) in tackling environmental pollution arising from petroleum hydrocarbon contamination.

MATERIALS AND METHODS

Isolation of Hydrocarbon utilizers

The organism used was isolated from water samples collected using sterile sample bottles, from the New Calabar River in Port Harcourt in the Niger Delta, Nigeria. The sampling point of the river is characterized by frequent discharges of oily wastes from construction and oil servicing companies. Aliquots (0.1 ml) of the water samples that had been diluted to extinction using normal saline (0.85% NaCl in distilled water), were spread onto sterile mineral salts agar plates prepared in a litre of distilled water as follows: $MgSO_4$. $7H_2O$, 0.42; KH_2PO_4, 0.83; NaCl, 10.00; KCl, 0.29; Na_2HPO_4, 1.25; NH_4NO_3, 2.0 and agar 15g. Filter paper soaked in Bonny light crude oil was aseptically placed on the covers of the petri dishes. The plates were incubated with the agar base up, allowing the hydrocarbon to reach the agar surface in the vapour phase. The plates were kept at ambient temperature for at least 5 days. Discrete colonies appearing after 5 days were presumed as hydrocarbon degraders.

Screening for Hydrocarbon Utilizing Ability

Pure cultures of the hydrocarbon utilizers were made by picking from discrete colonies and inoculated into 100ml mineral salts broth contained in conical flasks and enriched with 1ml Bonny light and Bonny medium crude oil as the only carbon source. During growth, dispersion of oil, increase in turbidity and disappearance of the oil were monitored by physical observation comparing with un-inoculated controls.

Screening for Biosurfactant Production

The isolates were grown with intermittent shaking in mineral salts medium supplemented with glucose (1% w/v) or crude oil (1% v/v) as sole carbon sources. These were incubated at ambient temperature (ca. 28-30°C) for four days. The cells were removed by centrifugation at 2500 rpm for 20 minutes. The crude oil enriched culture was first filtered using whatman no. 3 filter paper before centrifugation. The supernatants from the two setups were used to test for emulsification. The growth profile was monitored by viable cell count on mineral salt agar.

Emulsification Test

The cell-free filtrate of the culture broth was added into test tubes in 10ml amount. The absorbance of the supernatant was read at 470 nm (JENWAY 6100 spectrophotometer) Sterile mineral salts medium was used as reference. Bonny light crude oil at 0.1ml amount was added into the tubes containing the supernatant culture broth and vortexed for about 30 seconds. This was then incubated at ambient temperature for 15 minutes after which the absorbance of the lower aqueous phase was read in the spectrophotometer. Emulsification ability was expressed as percentage increase in optical absorbance of the lower aqueous phase.

Characterization and Identification of the Test Organism

The characterization and identification of the organism was based on cell morphology: cell shape, presence of spore, Grams reaction, and biochemical/physiological tests which included, catalase, oxidase, indole production, methyl red (MR), Voges-Proskauer (VP), citrate and urease tests, sugar fermentation test for sucrose, glucose, maltose, lactose and starch hydrolysis (Cruickshank *et al.*, 1980, Bergey's Manual of Determinative Bacteriology, 1984).

Comparison of Surface Tension Reduction as a Test For Biosurfactant Production

In two, 1 litre conical flasks each containing 300ml of mineral salts medium, pH 7.0, enriched with glucose as the only carbon source, were inoculated the river isolate and a soil isolate which has been shown to metabolize azo dyes and also identified to be a *Bacillus* sp. but which had tested negative for ability to utilize hydrocarbons. The flasks were incubated at 37°C in an incubator with intermittent shaking. The total viable count and surface tension of the two setups were monitored. This experiment helped to ascertain whether reduction in surface tension was associated with an organism's ability to produce surface active substances or bioemulsifiers.

Surface Tension Measurement.

The surface tension of cell-free filtrates of the spent culture for the two organisms was measured using the Griffin and George student Veneer Travelling Microscope (Okeke *et al.*,1993) in which 10ml of the filtrate was added into a glass tube. A capillary tube of known diameter was dipped into the filtrate. The height of the liquid in the capillary and that in the tube were measured using the veneer travelling microscope. The difference in the two heights was used to calculate the surface tension (τ) using the formula;-

$$\tau = \frac{drhg}{2} \text{kgm}^{-2} \text{ or } \mu\text{m}^{-1}$$

Where d = density, r = radius of the capillary tube, g = gravitational force, h = difference in height.

Isolation and Partial Purification of the Biosurfactant

The cell-free filtrates of the spent culture of the river and soil isolates were subjected to cold ethanol precipitation over night (Abu *et al.*, 1991) in a refrigerator. The precipitate, separated through centrifugation, was dried at 60°C. The dried material was further extracted with a 50:50 mixture of water and chloroform in a separatory funnel. The material partitioned at the interface and was collected into a pre-weighed beaker and allowed to evaporate to dryness, at ambient temperature, and then further dried at 60°C in a drier. This was termed partially purified biosurfactant and was used for the determination of critical micelle concentration as well as other tests such as protein and carbohydrate analysis.

Determinatin of Critical Micelle Concentration of Partially Purified Biosurfactant

Graded concentrations of the partially purified biosurfactant were made in distilled water contained in test tubes starting from 0 - 0.5 gl^{-1}. The surface tension of these concentrations was measured using the traveling microscope method. A graph of surface tension values against concentrations of the partially purified material was plotted from where the critical micelle concentration (CMC) was determined.

Chemical Analysis of the Partially Purified Biosurfactant

Protein measurement was by the Lowry method (Lowry *et al.*, 1951). Carbohydrate determination was by Anthrone Method (Osborne and Voogt, 1978)..

RESULT AND DICUSSION

Detection of Presence of Biosurfactants

The river isolate was identified as a *Bacillus* sp. Its ability to grow on crude oil was by physically observing increase in turbidity, dispersion and disappearance of the crude oil compared with un-inoculated controls. Biosurfactant production was authenticated by emulsification of crude oil by the cell free filtrate of spent cultures when grown on both crude oil and glucose enriched mineral salts broth (Figure 1). Bonny light and Bonny medium crude oil were respectively emulsified at 141% and 189% when grown in glucose enriched medium

and 14% and 70% when grown on crude oil hydrocarbon. Figure 2 compares surface tension reduction with time, when the test organism and a non-hydrocarbon degrading, non-biosurfactant producing *Bacillus* sp. which was isolated from soil and shown to utilize Azo dyes as the carbon source were grown in the mineral salts broth enriched with glucose as carbon source. Virtually no reduction in surface tension was noticed for the non-biosurfactant producing organism while the surface tension was reduced by up to 40% for the river isolate that also produced the bioemulsifier. Biosurfactant production is associated with crude oil utilizers (Arino *et al.* 1996). Figure 3 shows that the critical micelle concentration of the partially purified biosurfactant was 0.2g/l. The chemical analysis of the partially purified surfactant revealed that it contained 60% protein and 15 % carbohydrate. The remaining 25% is attributable to associated lipids and inorganics (Abu *et al.*, 1991).

Emulsification of hydrophobic substances like crude oil by the spent culture filtrates of the test organism is an indication that the bioemulsifier or biosurfactant was excreted into the medium when the organism was growing. It is evident that the production is associated with growth. The reduction of surface tension of the growth medium of the surface active-producing organism, in contrast to that of a non-producing organism (Figure 2) is an indication that the substance, which reduced the surface tension was from the hydrocarbon degrader.

The two substrates used viz: glucose and crude oil supported biosurfactant production. The production of surfactant by *Ochrobactrum anthropii* (Wasko and Bratt, 1991) on a simple sugar medium without the presence of hydrocarbons suggests that biosurfactant production might be constitutive rather than inducible It is also likely that the production is initially induced by the presence of hydrophobic substances, and once induced the cells can continue to produce irrespective of the substrate.

The surface tension of distilled water with the graded concentration of the partially purified biosurfactant reduced with increasing concentration of the surfactant until there was little or no further reduction with increasing surfactant concentration. This concentration that gave the highest surface tension reduction and on which additional amount of surfactant has
no effect on surface tension, is termed the critical micelle concentration (cmc). It was found for this biosurfactant to be $0.2gl^{-1}$. Lipopeptide biosurfactants have been reported in *Bacillus subtillis* and *Bacillus licheniformis* with critical micelle concentrations of $0.025gl^{-1}$ and $0.012gl^{-1}$, respectively (Yakimov *et al.*, 1995).

The precipitated exopolymer surfactant from the growth medium was further extracted with chloroform and called partially purified surfactant. This was collected at the interface between water and chloroform phase. This suggests that it has both hydrophilic and hydrophobic ends (Zhang and Miller, 1994). The hydrophobic end might be a lipid terminal having more affinity to the chloroform. The extract was analyzed and found to contain carbohydrate and protein. Whether the protein or carbohydrate components separately could have surfactant activity is not yet known.

The production of biosurfactants or bioemulsifiers has been attributed mostly to hydrocarbon degraders (Arino *et al.*, 1996) or by organisms growing on immiscible hydrophobic substances and is usually connected with organisms isolated from hydrocarbon-contaminated environments. Several authors have recorded production of biosurfactants while the organisms were growing on simple hexadecane, vegetable oil and gasoline, pristane, insoluble aliphatic compounds, crude oil, soybean and coconut oil, polycyclic aromatic hydrocarbons (PAH) (Deziel, 1996), Arino *et al.*, 1996, Marin *et al.*, 1996). This suggests that production of the surface active substances could be inducible by the presence of hydrophobic substances. However, there have been instances where production of the biosurfactants has been found to be constitutive as they can be produced when growing on water soluble compounds like carbohydrates (Wasko and Bratt, 1991).

Though oil degraders are ubiquitous, they are found at lower percentage of the total heterotrophic count in an uncontaminated environment, while they are found to be at higher percentage in a contaminated environment. Exposure to hydrophobic pollutants in contaminated soil therefore, appears to select biosurfactant producers, which could emulsify the hydrophobic compounds. Under these specific circumstances, different species of microbes appear to exhibit the capacity to produce biosurfactants and so their production is often associated with the capacity of the microorganism to utilize hydrocarbon as substrate.

For example production of biosurfactants has been reported for *Klebsiella oxytoca* that was isolated from soil contaminated by oil (Hwang 1993), *Ochrobactrum anthropii* that had been isolated from contaminated marine diesel fuel (Wasko and Bratt, 1991), *Pseudomonas aeruginosa* that had been isolated from soil contaminated by petroleum waste (Desiel *et al.*, 1996), *Pseudomonas aeruginosa* that had been isolated from oil spillage sample (Shafeeq *et al.*, 1989), *Acinetobacter calcoaceticus* MM5 that had been isolated from a sample of altered heating oil which was collected in a storage tank (Marin *et al.*,1996), a *Pseudomonas* strain isolated from the soil of an ancient manufactured gas plant contaminated by PAH of the U.S. environmental protection agency (Arino *et al.*, (1996), a *Pseudomonas* from contaminated soil (Deschenes *et al.*, 1996) and a *Bacillus subtilis* that had been isolated from a petroleum reservoir at a depth of 1500 meters (Yakimov *et a.*, 1995).

It is important to search for and research into the capabilities of microorganisms from various localities to produce biosurfactants. This will lead to discovery of biosurfactants with greater effectiveness and wider application The ability of biosurfactant-producing microorganisms to utilize simple sugars such as glucose is an attractive feature in bioremediation technology. The release of surface active agents into culture media represents additional advantage for large scale production using both hydrocarbon and water soluble substrates. Under such circumstances the trophophase can also be manipulated to enhance production of the growth-associated exocellular materials. Bioremediation holds great promise for Nigerian oil pollution problem (Water Quality International , 1997), especially in the Niger Delta region (Abu and Ogiji, 1996).

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