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ISOLATION OF BIOSURFACTANT/BIOEMULSIFIER-PRODUCING BACTERIA FROM SOIL AND SEWAGE SLUDGE

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ABSTRACT

Biosurfactant-producing bacteria were isolated from sewage sludge and petroleum contaminated and uncontaminated soils. Isolates were screened for biosurfactant/bioemulsifier production in mineral salts medium with different carbon sources (glucose, sucrose, n-paraffin and kerosene) using the Du-Nouy ring technique. Nine positive isolates were obtained, namely, one *Arthrobacter* sp., three *Bacillus* spp., three *Pseudomonas* spp. and two *Serratia* spp. Glucose and sucrose, as sole carbon source, produced the highest number of positive results but sucrose gave the lowest surface tension value of 39.8 dynes/cm and the most stable emulsion after 24 h with culture supernatant of a *Serratia* sp. Six of the isolates reduced the surface tension of the fermentation broth to less than 45 dynes/cm with sucrose and glucose as carbon source. Kerosene was the least supportive carbon source for biosurfactant production. All the nine isolates produced emulsion with paraffin oil and olive oil while the least emulsification index (EI_{24}) obtained was with kerosene as carbon source.

KEYWORDS: Bioemulsifier; biosurfactant; emulsification; emulsion; surface tension.

INTRODUCTION

Surfactants are amphipathic molecules with hydrophilic and hydrophobic moieties that partition preferentially at the interface between fluid phases with different degrees of polarity and hydrogen bonding such as oil/water or air/water interfaces (Desai and Banat, 1997). Biosurfactants are a structurally diverse group of surface-active compounds produced by microorganisms. Most microbial surfactants are complex molecules, comprising of different structures that include lipopeptides, glycolipids, polysaccharide-protein complex, fatty acids and phospholipids. These molecules reduce surface and interfacial tensions in both aqueous solutions and hydrocarbon mixtures.

Production of microbial surfactants through fermentation of n-alkanes or sugars as carbon sources has generated considerable interest (Ishigami, 1992). In the past few decades, biosurfactants have gained attention because they exhibit some advantages over synthetic surfactants such as biodegradability (Mohan *et al.*, 2006), low toxicity, ecological acceptability and ability to be produced from renewable and cheaper substrates (Desai and Banat, 1997; Rosenberg and Ron, 1999).

The range of industrial applications of biosurfactants includes enhanced oil recovery, crude oil drilling, lubricants, bioremediation of pollutants, health care and food processing (Cameotra and Makkar, 2004; Mulligan, 2005). The use and potential commercial applications of biosurfactants in the medical field has increased recently. The antibacterial, antifungal and antiviral activities of biosurfactants make them relevant molecules for applications in combating many diseases and as therapeutic agents (Rodrigues *et al.*, 2006).

The use of synthetic surfactants in treatment systems of hydrocarbon-contaminated soil improves the restoration of these areas; but they may promote accumulation in the ecosystem causing serious environmental damage (Cunha *et al.*, 2004). A viable alternative to the use of synthetic surfactants in the bioremediation process is the addition of microbial emulsifier (Abalos *et al.*, 2004) or in situ production at the site of contamination by native microorganisms. Mulligan and Wang (2006) have reported the use of rhamnolipid biosurfactant in the remediation of heavy metal-contaminated soils.

There is a wide variety of microorganisms such as bacteria and yeasts, able to produce different kinds of biosurfactants, whose type and amount produced depend on several factors such as the microbial species, carbon and nitrogen sources and other environmental factors. Surface-active properties of biosurfactants depend on the selection of microorganisms, carbon sources and process parameters (Hommel, 1994). The produced biosurfactants, which lower the surface tension of the broth, may remain extracellularly (as water or alkane soluble) (Kim *et al.*, 2006) and/or adsorbed on to the cell surface (Bento *et al.*, 2005). A number of biosurfactants has been characterized in terms of minimum boundary tension (Desai and Desai, 1993).

At present the use of biosurfactants is primarily due to their emulsification property, which include, emulsion forming and stabilizing capacity (Banat, 1995). As the demand for surfactants increases due to population growth and the development of new applications, there will be new emphasis placed upon the development of new types of surfactants. The aim of this study was to screen microorganisms isolated from soil and sewage sludge with potential to produce surface-active compounds during growth with water-soluble and water-insoluble substrates.

MATERIALS AND METHODS

Collection of samples

The soil samples were collected in sterile containers from the "motor mechanic village", Nsukka and the University of Nigeria agricultural farm. The soil sample from the motor mechanic village was contaminated with petroleum products while the agricultural farm soil had no history of petroleum contamination. The sewage sludge was collected from the University of Nigeria sewage treatment plant.

Isolation from soil and sewage sludge.

Ten grammes of the soil and sewage sludge samples were, respectively, suspended in 90ml sterile distilled water contained in 250ml Erlenmeyer flasks and agitated for 10 min in a Gallenkamp rotary shaker at 180 r.p.m. The flasks were allowed to stand for 1min to allow heavier particles to settle. Serial ten-fold dilutions were made of the samples. A 0.1ml aliquots of 10^{-5} dilution were spread-inoculated on sterile plates of nutrient agar fortified with 0.1% (w/v) yeast extract. The plates were incubated at ambient temperature (30 ± 2^{0}C) for 48 h. Several developed colonies were repeatedly sub-cultured to obtain pure isolates.

Screening for biosurfactant production

Several pure isolates obtained as described earlier were screened for biosurfactant/bioemulsifier production in mineral salts medium differently supplemented with glucose and

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kerosene as sole carbon sources. The mineral salts medium had the following composition: (gL^{-1}): K_2HPO_4, 4.5; KH_2PO_4, 2; $(NH_4)_2SO_4$, 2; $CaCl_2$, 0.01; $MgSO_4.7H_2O$, 0.5; pH, 7.0. The kerosene was filter-sterilized and both substrates separately added to a final concentration of 2% (w/v, v/v).

The cultures were incubated in a Gallenkamp orbital incubator with shaking at 180 rev. min^{-1} at 30°C for three days. Biosurfactant production during batch growth was monitored by measuring the surface tension of the whole fermentation broth (air-liquid broth interface) using a Du-Nuoy ring tensiometre (K6, Kruss, Harmburg, Germany) (Margaritis *et al.*, 1979). At the end of the screening, the bacterial isolates capable of producing biosurfactants, by lowering the surface tension of the fermentation broth, were assembled and stored at 4°C on nutrient agar slants for further studies.

Preliminary production of biosurfactant/bioemulsifier

The bacterial isolates exhibiting surface tension reduction were screened further for preliminary production of biosurfactants and emulsification of some oils. An inoculum of each slant culture was introduced into 2 ml nutrient broth and incubated at room temperature overnight. Each of these served as the source of inoculum for 50ml mineral salts medium containing sucrose, glucose, n-paraffin and kerosene as the respective carbon sources in 250 ml Erlenmeyer flasks. The flasks were shaken in a Gallenkamp orbital incubator at 180 rev. min^{-1} at 30°C for three days. At the end of the fermentation, the broths were centrifuged at 10,000 g for 15 minutes to separate the cells. The cell- free supernatants were assayed for biosurfactant/bioemulsifier production by measuring the surface tension of the fermentation broth and also the emulsification of paraffin oil and olive oil by the broths.

Identification of the isolates

The taxonomic identification of the isolates was based on their morphological and cultural characteristics and standard biochemical tests as described in the identification schemes of Barrow and Feltham (1993) and Holt *et al.* (1994).

Measurement of surface tension

The surface tension of the biosurfactant solution was measured by the method of Margaritis *et al.* (1979) using a Du-Nouy ring tensiometre (K6, Krüss, Hamburg, Germany) at room temperature. A 5-mL aliquot of the broth sample was placed in a watch glass and the ring of the tensiometre was completely immersed into the solution with the aid of the micrometer screw. The surface of the solution was allowed to form by allowing the liquid to remain at rest for about 1min. The micrometer screw was rotated in the anticlockwise direction thereby increasing the torsion applied to the ring. The increase in the torsion was continued until the ring breaks off in the upward direction from the surface of the solution. The surface tension at this point was read out from the micrometer and vernier scales.

Determination of emulsification index

Emulsifying activity was measured by the modified method of Cooper and Goldenberg (1987). Three millilitres each of kerosene and olive oil were separately added to 2mL of the aqueous culture supernatant in graduated test tubes. The mixture was vortexed in a vortex mixer for 2min and kept for 24h. Emulsification index measurements were made based on the volume of the emulsion layer. The emulsification index (EI_{24}) was calculated as the height of the emulsion layer, divided by the total height, multiplied by 100.

RESULTS

Forty-two different bacterial isolates were obtained from soil and sewage sludge samples. Preliminary screening of the isolates for biosurfactant/bioemulsifier-production resulted in nine positive isolates. There were one *Arthrobacter* sp., three *Bacillus* spp.,

Table 1: Microbial isolates positive for biosurfactant production

Isolate	Source of isolate	Carbon source: Glucose	Sucrose	n-paraffin	Kerosene
		Surface tension (dynes/cm)[c]			
Arthrobacter sp.	Soil[a]	46.4 ± 0.72	48.4 ± 0.57	43.0 ± 0.31	47.2 ± 0.50
Bacillus sp.	Soil[b]	48.2 ± 0.60	47.1 ± 0.44	50.3 ± 0.59	55.6 ± 0.55
Bacillus sp.	Soil[b]	45.3 ± 0.70	45.8 ± 0.40	46.4 ± 0.53	48.4 ± 0.32
Bacillus sp.	Sewage	43.4 ± 0.53	46.1 ± 0.47	44.6 ± 0.47	51.3 ± 0.50
Pseudomonas sp.	Soil[a]	42.8 ± 0.21	44.2 ± 0.51	47.4 ± 0.49	49.8 ± 0.35
Pseudomonas sp.	Sewage	42.3 ± 0.31	45.5 ± 0.46	46.2 ± 0.60	48.1 ± 0.60
Pseudomonas sp.	Soil[a]	46.7 ± 0.50	44.1 ± 0.42	45.3 ± 0.58	51.8 ± 0.45
Serratia sp.	Soil[b]	43.2 ± 0.40	39.8 ± 0.38	44.6 ± 0.35	50.8 ± 0 60
Serratia sp.	Soil[b]	46.3 ± 0.47	42.5 ± 0.35	45.8 ± 0.42	52.7 ± 0.36

a------ Agricultural soil
b ------ Oil-contaminated Soil
c------ Means of triplicates ± standard deviation.

three *Pseudomonas* spp. and two *Serratia* spp. (Table 1). Three of the positive isolates were obtained from the agricultural soil, four from the petroleum-contaminated soil and two from the sewage sludge.

The carbohydrates, glucose and sucrose, as carbon sources, produced the highest number of positive isolates for biosurfactant/bioemulsifier production. Sucrose was the best carbon source for biosurfactant production followed by glucose. The lowest surface tension value of 39.8 dynes/cm was obtained with a *Serratia* sp. isolated from petroleum-contaminated soil and utilized sucrose as the sole carbon source. Kerosene was the least supportive carbon source for biosurfactant/bioemulsifier production, which gave the highest surface tension value of 55.6 dynes/cm with a *Bacillus* sp. (Table 1). Thus *Serratia* sp. isolated from petroleum-contaminated soil was the most promising biosurfactant producer while *Bacillus* sp. isolated from the same soil was the least potent biosurfactant producer.

All the nine positive isolates for biosurfactant/bioemulsifier production were able to form emulsions with a hydrocarbon oil,

paraffin oil and a vegetable oil, olive oil. With paraffin oil, the *Serratia* sp. gave the highest emulsification index of 90.41%, with sucrose as carbon source, while a *Bacillus* sp. gave the least emulsification index of 45.24% with kerosene as carbon source (Table 2). The culture filtrate of the *Serratia* sp. with sucrose as carbon source gave an emulsification index of 100% with olive oil while the *Bacillus* sp. using kerosene as carbon source gave an emulsification index of 47.12% with olive oil (Table 3). Thus, sucrose as carbon source gave the most stable emulsion with both paraffin oil and olive oil while kerosene gave the least stable emulsion.

Table 2: Emulsifying activity of biosurfactants on paraffin oil

	Carbon source			
	Glucose	Sucrose	n-Paraffin	Kerosene
Isolate	Emulsification Index (%)			
Arthrobacter sp.	68.24	64.85	70.26	64.85
Bacillus sp.	60.18	70.13	60.32	45.24
Bacillus sp.	71.82	70.25	79.86	55.25
Bacillus sp.	71.87	74.86	63.32	57.35
Pseudomonas sp.	86.30	76.32	68.27	59.86
Pseudomonas sp.	90.16	80.16	69.87	62.32
Pseudomonas sp.	74.32	83.84	82.31	50.14
Serratia sp.	84.92	90.41	85.24	51.80
Serratia sp.	80.34	86.18	67.88	50.34

Table 3: Emulsifying activity of biosurfactants on olive oil

	Carbon source			
	Glucose	Sucrose	n-Paraffin	Kerosene
Isolate	Emulsification index (%)			
Arthrobacter sp.	79.86	70.21	68.42	64.85
Bacillus sp.	68.23	74.42	60.24	47.12
Bacillus sp.	75.28	80.18	64.86	56.80
Bacillus sp.	88.16	75.86	75.26	60.42
Pseudomonas sp.	79.68	88.36	70.38	62.26
Pseudomonas sp.	90.34	86.28	69.86	59.86
Pseudomonas sp.	74.25	73.84	85.32	65.14
Serratia sp.	86.16	100	90.41	53.38
Serratia sp.	81.82	90.46	86.36	50.28

There was a positive correlation (r = 0.98) between surface tension reduction and ability to stabilize emulsions for the majority of the isolates.

DISCUSSION

The carbon source is known to be critical for the structure and yields of biosurfactants. Depending on the physiology of the producing microbe, the production of biosurfactants can either be induced or inhibited by the addition of certain substrates.

In the present study, water-soluble substrates were better than water-immiscible substrates for biosurfactant production. Many known biosurfactants are synthesized from water-immiscible substrates such as n-alkanes and olive oil (Cunha *et al.*, 2004) and water-soluble substrates such as glucose, sucrose and glycerol (Cooper and Goldenberg, 1987; Das and Mukherjee, 2005; Wei *et al.*, 2005; Batista *et al.*, 2006). Cooper *et al.* (1981) reported that the addition of a hydrocarbon to culture medium of *Bacillus subtilis* completely inhibited surfactin production. On the other hand, addition of hydrocarbons to culture media has been shown to result in significant increase in the yield of biosurfactants by organisms grown in water-soluble substrates (Banat, 1995). However, water-soluble substrates are cheaper than hydrocarbons and are preferred because single-phase fermentation is simpler than biphasic fermentation (Makkar and Cameotra, 1997).

From an engineering point of view, hydrocarbon substrates require more sophisticated equipment and more power input to achieve an adequate dispersion of the insoluble hydrocarbons (Guerra-Santos *et al.*, 1984). In addition, the availability of hydrocarbons is limited if applications of biosurfactants other than in enhanced oil recovery are envisaged. According to Sandrin *et al.* (1990), glucose, fructose and sucrose were the best carbon substrates for the synthesis of surfactin by *Bacillus subtilis*.

In the present study, kerosene was the least supportive substrate for biosurfactant production. Wei *et al.* (2005) had shown that *Pseudomonas aeruginosa* J4 was able to assimilate seven carbon substrates examined whereas it grew less sufficiently in mineral oils, especially kerosene. Of the 19 bacterial isolates selected for biosurfactant production, Batista *et al.* (2006) reported that 17 tested positive when grown on glucose as the sole carbon source and only two when grown on sucrose. It was further shown that growth was observed on fructose but no biosurfactant was produced while in kerosene only six isolates grew on 0.5% of the substrate with only two producing biosurfactants. Thus, a variety of carbon substrates can be incorporated into the biosurfactant by various microorganisms. Mounting evidence leads to the conclusion that the available carbon source has a great bearing on the type of biosurfactant produced.

The biosurfactant-producing isolates in this study liberated biosurfactants into the culture medium. Such isolates that liberate biosurfactants into the culture medium are interesting from an industrial point of view, because the product recovery process can be simplified (Cameotra and Makkar, 1998; Kuyukina *et al.*, 2001).

All the nine biosurfactant/bioemulsifier-producing isolates were able to form emulsions, with minimum emulsification

index of 45.24% with paraffin oil. Of all the substrates tested, sucrose broth gave the most stable emulsion with olive oil after 24 h., while kerosene gave the least stable emulsion. The positive relationship between surface tension reduction and ability to stabilize emulsions for the majority of the isolates indicated that the main stabilizing effect of the culture supernatants was due to their surface-active properties. It has been shown by Das *et al.* (1998) that culture filtrate of sucrose had excellent stabilizing capacity giving 100% emulsion stability up to 48h. The ability of the biosurfactants to form more stable emulsions with vegetable oil than hydrocarbon oil suggests potential application as cleaning and emulsifying agent in food industry if the organisms are proven to be non-pathogenic.

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