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Research Article

Preliminary Screening of Biosurfactants Produced by a *Pseudomonas* sp. Isolated from Mud Drill Cuttings

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Abstract

Biosurfactants are a group of surface-active molecules synthesized by microorganisms. They either adhere to cell surfaces or are excreted extracellularly into the growth medium. A bacterial isolate identified as *Pseudomonas* sp. was cultured in a synthetic medium adjusted to varying acidic to alkaline conditions with varying concentrations of sodium chloride. Screening tests were carried out on the bacterial isolate to verify its ability to produce biosurfactants. The effect of the media on the emulsification index and surface tension of the free product showed that although the isolate was found to be osmotolerant, withstanding and growing in media containing high sodium chloride concentration, biosurfactant was produced optimally at pH 7.2 and sodium chloride (NaCl) concentration of 3%. The cell-free media recorded optimum emulsification index of 66.5% and reduced the surface tension of the culture media from 59.7 mN/m^2 to 28.1 mN/m^2.

Keywords: Biosurfactant; Drilling Mud; Emulsification Index; *Pseudomonas* sp

Introduction

Surfactants are amphiphilic molecules with hydrophobic and hydrophilic sections that can partition between fluid phases interface with different degrees of polarity and hydrogen bonding such as air/water or oil/water interfaces [1]. Hence, surfactants adsorb and alter the conditions prevailing at surfaces. These properties render surfactants capable of reducing surface and interfacial tension and forming micro emulsion where hydrocarbons can solubilize in water or where water can solubilize in hydrocarbons. Such characteristics confer excellent detergency, emulsifying, foaming, and dispersing traits, which makes surfactants some of the most versatile process chemicals [2,3]. Hydrophobic pollutants present in petroleum hydrocarbons, and soil and water environment require solubilization before being degraded by microbial cells. Mineralization is governed by desorption of hydrocarbons from soil. Biosurfactants are a structurally diverse group of surface-active molecules synthesized by microorganisms [4]. Chemically synthesized surfactants have been used in the oil industry to aid clean-up of oil spills as well as to enhance oil recovery from oil reservoirs. These compounds are not biodegradable and can be toxic to environment. Biosurfactants are a group of surface - active molecules synthesized by microorganisms. They have a special advantage over commercially manufactured chemical surfactants because of their lower toxicity, biocompatibility (biodegradable nature), plasticity and environmental compatibility (effectiveness at extreme temperature, pH, salinity and ease of synthesis) [5,6].

A number of attempts have been made to increase biosurfactant productivity by manipulating physiological conditions and medium composition. A good surfactant can lower surface tension (ST) of water from 72 to 35 mN/m^2 and the interfacial tension (IFT) of water/hexadecane from 40 to 1 mN/m^2. Surfactin from

Bacillus substilis can reduce the surface tension of water to 25 mN/m^2 and interfacial tension of water/hexadecane to <1 mN/m^2. Rhamnolipids from *Pseudomonas aeruginosa* decrease the surface tension of water to 26 mN/m^2 and the interfacial tension of water/hexadecane to <1mN/m^2. The sophorolipids from *Candida bombicola* have been reported to reduce ST o 28.56 + 0.42 mN/m and and IFT to t2.13 + 0.09 mN/m within 72 h [7].

Materials and Methods

Sample collection

Composite sample of drilling mud cuttings was collected from UBIT well 4 offshore well in Akwa Ibom State, Nigeria, explored by Mobil oil Company using a sterile bottle.

Bacteriological analysis

Isolation of potential biosurfactant producing bacteria from mud drill cuttings

The vapour phase hydrocarbon transfer method as described by [8] was adopted for the isolation of potential biosurfactant producing bacteria. The various components of the mineral salt medium were weighed and dissolved in 1.0 litre of deionized water in a 2.0 litre capacity conical flask. Then the media was sterilized by autoclaving at 121^0C and 15psi for 15 minutes, dispensed in 20 ml quantities into sterile Petri dishes and allowed to cool.

Ten milliliters (10 ml) of the sample (drilling mud) was mixed with 90 ml of sterile physiological saline and diluted serially to a final dilution of 10^{-5} (into test tubes containing 9 ml of sterile physiological saline). The Petri dishes containing the set mineral salt agar medium were inoculated with 0.1 ml of each dilution by spread plate technique. The inoculated plates were inverted unto the lids of the respective Petri dishes in which 9.0 cm filter papers (Whatman No. 1) saturated with crude oil were placed aseptically and incubated at 30^0C for 7 days.

Discrete colonies that developed on the plates were further subcultured unto sterile nutrient agar plates by streaking technique and subcultured unto sterile nutrient agar slant surfaces in McCartney bottles by streaking, incubated at 30^0C for 24 hours and preserved in a refrigerator as pure isolates for further tests and analysis.

Characterization and identification of potential biosurfactant producing bacteria

Microscopic examination of isolates to determine the cell morphology, biochemical tests, substrate utilization and motility tests were carried out to characterize and identify *Pseudomonas* Sp.

Screening tests for potential biosurfactant producing bacteria from drilling mud cuttings

Exactly 950 ml mineral salt medium of Mill., *et al*. (1978) as modified by [9]. [8] supplemented with kerosene (0.3%, w/v) as carbon source was composed as shown in the appendix 1. The medium was adjusted to pH 7.2 and sterilized at 121^0C and 15psi for 15 minutes. This was then, inoculated with 50 ml of 3 -day old culture of the potential biosurfactant producing bacterial isolates and incubated at room temperature for seven days.

Red blood cell lysis test

The method of [10] was adopted, seventy two (72) hours old cultures of the potential biosurfactant producers were inoculated unto blood agar (see appendix 2 for composition) by streak plate method and incubated at 37^0C for 24-48 hours. The plates were examined for positive hemolysis (clear zones around the colonies), which is indicative of biosurfactant production [11,12]). Some of the isolates showed positive alpha or beta hemolysis while others showed negative hemolysis. Those showing positive hemolysis (alpha or beta) were subjected to further testing.

Oil spreading test

The methods of [12,13] were adopted. To 5.0 ml of distilled water 0.2 ml of crude oil and subsequently by 0.1 ml of the biosurfactant cell free culture were introduced. This was left for 30 seconds after which the diameters of the clear zones were measured.

Effect of salinity and pH on emulsification index and surface tension of biosurfactant cell free culture

The kerosene supplemented mineral salt medium with varying concentrations of sodium chloride (1.0%, 3.0%, 6.0%, 12.0% and 15.0%) was employed. The pH of the media was adjusted to 7.0 and sterilized 121^0C at 15psi for 15minutes, inoculated with the test isolates and incubated for seven (7) days at 29 ± 2^0C in an orbital incubator at 150RPM after which the emulsification index and surface tension were determined.

The kerosene supplemented mineral salt medium with varying pH of (4.2, 5.2, 6.2 7.2 and 8.2) was employed. The media were sterilized 121^{0}C at 15psi for 15minutes, inoculated with the test isolates and incubated for seven (7) days at 29 ± 2^{0}C in an orbital incubator at 150RPM. After seven days the emulsification index and surface tension of the biosurfactant cell free culture were determined.

Determination of emulsification index

A mixture of 2 ml of kerosene and 2 ml of cell -free filtrate of the broth cultures into a test tube and vortexed at a high speed of 4000 rpm for 2minutes [13] and left to stand for 24 hours at room temperature. The 24 hours emulsification index (E_{24}) was calculated thus;

Determination of surface tension

The method of employed by [14,15] was adopted. To 5 ml of cell free filtrate broth cultures in test tubes submerged in a water bath at a constant temperature of 28^{0}C, a capillary tube was aseptically inserted. The height reached by the broth when freely ascended through the capillary tube was measured and the surface tension was calculated thus;

where,

y = Surface Tension (MN/m)

r = Capillary radius (0.05 Cm)

h = height

d = Density

g = Gravity (980 Cm/S^2)

Result and Discussion

Potential biosurfactant producers

Figure 1 shows the percentage biosurfactant producing bacteria. Out of the five bacteria isolated, only 20% of *Pseudomonas* sp. is able to produce biosurfactant at pH 7.2 + 0.3.

Effect of sodium chloride concentration on emulsification index and surface tension of biosurfactant cell free culture

Figure 2 shows the effect of percentage sodium chloride concentration on the emulsification index and surface tension

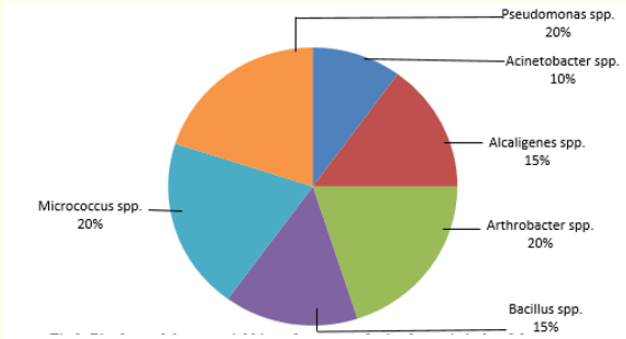

Figure 1: Percentage of potential biosurfactant producing bacteria isolated from mud drill cutting.

of *Pseudomonas* sp. at pH 7.2 + 0.3. The emulsification index ranged from 68% to 54.3% at NaCl concentrations of 1% and 6% respectively. The surface tension ranged from 57.1 mNm^2 to 28.1 mNm^2 at NaCl concentrations of 6% and 1% respectively. The ANOVA showed a high level of significance at 95% confidence level.

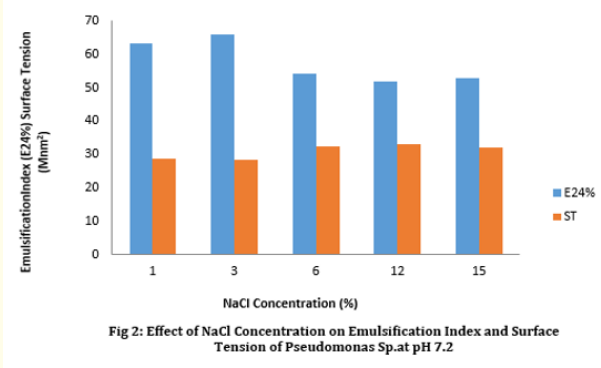

Figure 2: Effect of NaCl concentration on emulsification index and surface tension of *Pseudomonas* Sp. at pH 7.2.

The bacterial isolates were inoculated into sterile mineral salt broth as modified by [9] with varying concentrations of salinity ranging from 1% - 15%. The emulsification index was significantly highest at 66.1 E24% for *Pseudomonas* sp at 3% NaCl concentration. This means that biosurfactant yield in mineral salt broth as modified by [9] was optimal at 3% NaCl concentration and at the pH value of 7.2. Figure 2 shows a significant reduction of surface tension (28.32 mN/m) by *Pseudomonas* Sp. at 3% NaCl concentration which indicates the production of surfactants.

Similar results were obtained by [10]; they observed surface tension values of below 40 mN/m by isolated bacteria from Iranian crude oil reservoirs. The results in Figure 2 indicates that at 1% sodium chloride concentration, emulsion and surface tension activity is optimized for the three biosurfactant producing bacteria. According to studies done, salt concentrations also affect biosurfactant production depending on its effect on cellular activity [10]. According to [19], *Bacillus lichenformis* (BAS50) isolate grew and produced a lipopeptide surfactant when cultured on a variety of substrates at varying degrees of salinity ranging from 1% - 15% sodium chloride (NaCl) concentration. The results showed that biosurfactant production by *Bacillus lichenformis* was optimal at 5% sodium chloride concentration. In this study, the production of biosurfactant was optimal at 3% sodium chloride concentration.

The ANOVA for the effect of NaCl concentration on the emulsification index profile of biosurfactant producing bacteria indicates that F^c = 10.81 > P = 0.001 hence, it means that the NaCl concentration of the media affected the emulsification index profile of the biosurfactant producing bacteria. The least significant difference multiple range test indicates that the effect of NaCl concentration on the emulsification index is significant since the difference between the mean of emulsification index at 1% (66.5) and 3% (65.1) and the mean emulsification index at 15% (53.63) is > 5.33, hence there is a significant difference between the effect of the sodium chloride concentrations. The ANOVA for the effect of NaCl concentration on surface tension of biosurfactant producing bacteria also indicates that the NaCl concentration affected the surface tension of the biosurfactant producers at Fc = 10.81 > P = 0.001. The least significant difference multiple range test indicates that, the effect of NaCl concentration on the surface tension is significant since the difference between the mean of surface tension at 1% (66.5) and 3% (65.1) and the mean of emulsification index at 15% (53.63) is > 5.33, hence there is a significant difference between the effect of the sodium chloride concentrations.

Effect of salinity and pH on emulsification index and surface tension of biosurfactant cell free culture

Figure 3 shows the effect of changes in pH of the medium on the emulsification index and surface tension of *Pseudomonas* sp. at 3% NaCl concentration. The emulsification index profile ranged from 68% to 54.2% at pH 7.2 and 8.2 ± 0.2 respectively. The surface tension ranged from 32.52 mNm^2 to 28.1mNm^2 at pH 4.2 and 7.2 respectively. The ANOVA showed a high level of significance at 95% confidence level. Figure 3 showed that for *Pseudomonas* sp, the emulsification index increased from 54.3% at pH 4.2 to 68% at pH 7.2 but decreased to 54.2 at pH 8.2. The effect of pH on the surface tension was such that the surface tension was observed to decrease steadily from 32.52 mNm^2 at pH 4.2 to 28.1 mNm^2 at pH 7.2 after which it increased at pH 8.2 to 32.5 mNm^2. *Pseudomonas* sp. exhibited optimum emulsification index and surface activity at pH 7.2. This indicates that emulsion and surface tension activity were optimal at pH 7As shown on Figure 3, the surface tension of the bacterial isolates were low between pH 4.2 and 7.2, indicating that pH has no appreciable effect on the surface tension of the bacterial isolates ([10], but maximum surface tension reduction occurred at pH ranging from 6.2 - 7.2. Also, [16] observed maximum reduction in the surface tension of *Rhodococcus* sp. at a pH of 7.2..2.

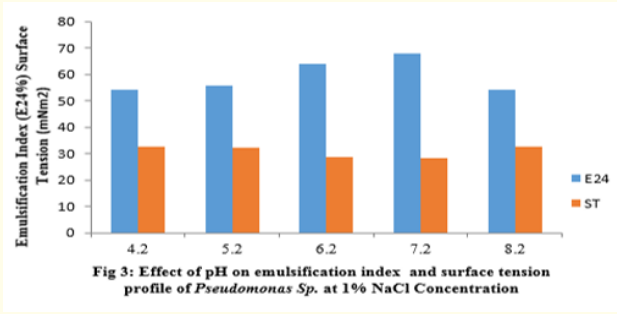

Figure 3: Effect of pH on emulsification index and surface tension profile of *Pseudomonas* Sp. at 1% NaCl concentration.

The bacteria isolates were inoculated into sterile mineral salt media with pH values varying from 4.2 - 8.2 and they showed significant increase in emulsification index with increase in pH. The emulsification index was seen to be highest at the pH levels of 6.2 - 7.2. This can be attributed to the fact that bacteria thrive at pH values near neutrality and bacterial cell growth has been found to affect biosurfactant cell-free production cultures emulsification index. The effect of pH on surface activity has been reported for biosurfactants from different microorganisms, this could be caused by a better stability of fatty acids-surfactant micelles in the presence of NaOH and the precipitation of secondary metabolites at higher pH values [17]. Environmental factors and

growth conditions such as pH, temperature, agitation and oxygen availability were found to affect biosurfactant production through their effects of cellular growth or activity [10]. Comparable results were obtained by [18] who observed that the surface tension reducing activity of selected strains of *Bacillus* Sp., *Pseudomonas* Sp., *Acinetobacter* Sp., *Gluconobacter* Sp. and *Arthrobacter* Sp. was stable to pH over the range of pH of 4.0 - 8.0 and optimal at pH range 6.0 - 7.0. Also [16] observed biosurfactant production of *Rhodococcus* optimized at pH 6.2 - 7.2 (determined by surface tension).The bacteria isolates were inoculated into sterile mineral salt broth [8]. 2006) with concentrations of salinity ranging from 1% - 15% and the pH was adjusted to 7.2 ± 0.2. The emulsification index for *Pseudomonas* sp was significantly high at 68%. At 1% NaCl concentration. Identification of biosurfactant producing bacteria was further confirmed by measurement of surface tension. Reduction of surface tension measurements by bacteria isolated from mud drill cutting indicates the production of surface active compounds. Similar results obtained from [10] showed surface tension values of below 40 mN/m by isolated bacteria from Iranian crude oil reservoirs.

Conclusion

The bacterial isolate identified as *Pseudomonas* sp. was found to be capable of producing biosurfactant. The effect of the media on the emulsification index and surface tension of the cell- free product showed that although the isolate was found to be osmotolerant, withstanding and growing in media containing high sodium chloride concentration, biosurfactant was produced optimally at pH 7.2 and at a sodium chloride (NaCl) concentration of 3%. The cell free media recorded optimum emulsification index of 66.5% and reduced the surface tension of the culture media from 59.7 mN/m^2 to 28.1 mN/m^2.

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