

Comparison of Biosurfactants Yield Produced by *Pseudomonas fluorescens* Strain PC20 Isolated from Hydrocarbon Contaminated Soil using Different Agricultural Waste

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Abstract: Biosurfactants are amphipathic compounds produced extracellularly by microorganisms on cell surfaces, or excreted extracellularly. They contain hydrophilic and hydrophobic moieties that reduce surface and interfacial tension between molecules at the surface and interface respectively. The present study focused on isolation, screening, identification of biosurfactant producing bacteria and also development of economical methods for biosurfactant production by the use of unconventional substrates. The research investigated the potential of utilizing agro industrial (Pineapple and cassava) wastes to replace synthetic media for biosurfactant production. Eighteen isolates obtained were screened for biosurfactant activity using Emulsification Index, haemolysis and oil displacement method. The bacteria *Pseudomonas fluorescens* Strain PC20 isolated was able to grow and produce biosurfactant 10ml and 5ml per 200ml of cassava and pineapple peel respectively.

Introduction

Biosurfactants are surface-active compounds naturally derived from microorganisms (Anandaraj and Thivakaran, 2010). They are amphiphilic compounds excreted extracellularly or produced mostly on microbial cell surfaces and contain hydrophobic and hydrophilic moieties that reduce surface and interfacial tensions between two immiscible fluids like oil and water (Anyanwu *et al.*, 2011; Govindammal, 2013).

Biosurfactants are classified based on their physico-chemical properties, molecular weight, chemical structure and mode of action and microbial origin (Calvo *et al.*, 2009). Their chemical structure is very unique in that they contain a hydrophilic moiety, comprising an acid, peptide cations or anions, mono-, di- or polysaccharides and they also contain a hydrophobic moiety comprising of unsaturated or saturated hydrocarbon chains or fatty acids (Saharan *et al.*, 2011). The increase on replacement of synthetic surfactant with their biological counterparts (Biosurfactants) is due to the latter's better features such as low toxicity, higher biodegradability and mild process conditions, higher foaming capacity, temperature, pH and salinity stability and synthesis under user-friendly conditions (Parveen *et al.*, 2011; Chandran and Das, 2010).

On the other hand, different microorganisms are known to synthesize different types of biosurfactants when grown on several carbon sources, therefore the type, quality and quantity of biosurfactant produced are also influenced by the nature of the carbon substrate and the culture conditions such as pH, temperature, agitation and dilution rate in continuous culture (Lakshmipathy *et al.*, 2010). This research work focused on the isolation of bacteria, from crude oil contaminated soil, screening of the isolated bacteria for biosurfactants production ability, identification of the bacteria specie with the best screening result as well as production of biosurfactant from this bacteria. Pineapple and cassava peel were used as the carbon source for the production.

METHODOLOGY

Soil Sample Collection

The soil sample area, Matrix Energy Limited is located at KM 5, New Ode Itsekiri Road, Ifie-Kporo, Warri South Local government area of Delta state, Nigeria. Soil samples were collected at a depth of 0cm to 15cm from three locations; inside the facility by Matrix Truck Park, Matrix tank farm and outside the perimeter fence of LPG (Liquefied Petroleum Gas). The samples were collected at a distance of 20 meters apart in order to get a representative sample. Samples were collected three different times within an interval of three



weeks. The soil samples were obtained using sterile containers and transported to the laboratory for immediate isolation of microorganism. The soil sample were analyzed for pH, Temperature and Total Petroleum Hydrocarbon (TPH). The coordinates of the sampling points are: 5°33'13"N 5°40'56"E, 5°33'15"N 5°41'12"E, 5°33'14"N 5°40'56"E.

Determination of soil pH

The pH of the soil was determined using pH meter. The pH meter was first calibrated before measurement with buffer 4, 7 and 10 solutions. The electrode on the pH meter was rinsed with distilled water. A solution of the soil sample was prepared using deionized water in a beaker the pH meter electrode was then dipped in the solution and readings on the dial which indicates the pH of the solutions were recorded (Nedungadi et al. 2013).

Determination of soil temperature

The soil temperature was obtained from the soil composite prepared and a thermometer was placed in the soil. This was allowed to stand for 5minutes for the temperature to be obtained. The reading was recorded.

Determination of Total Petroleum Hydrocarbon (TPH).

The determination of the total hydrocarbon content (mg/kg) involved a simple extraction process using the solvent hexane.

5g of the soil sample was dissolved in 25ml of hexane in a beaker containing a stirring bar. The mixture was placed on a stirrer until there was an obvious separation of the soil sample from the solvent. During the course of the separation, the oil content of the soil was extracted by the solvent which resulted to the supernatant (the mixture above) being a mixture of the solvent (hexane) and the oil contained in the soil. After this process, the supernatant was transferred into a beaker, and a filter paper was used to separate the oil from the solvent. The oil was trapped as the residue.

The UV-SPECTROPHOTOMETER was first standardized using pure hexane. After standardization, the oil was then transferred to a cuvette. The cuvette was placed in the sample compartment and the read out icon was activated to obtain the absorbance of the sample.

The formula below was used to obtain the Total hydrocarbon content of the soil sample in mg/kg: $0.5546 + 420.26 X$ – where X is the absorbance.

Media Preparation.

Preparation of Mineral Salt Media.

The mineral salt medium was prepared using 2.2g of Na_2HPO_4 , 1.4g KH_2PO_4 , 0.6g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01g $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05g NaCl , 0.02g CaCl_2 ,

0.02g yeast extract, and 0.1ml of trace element solution containing (g/l): 2.32g $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 1.78g $\text{MnSO}_4 \cdot 2\text{H}_2\text{O}$, 0.56g H_3BO_3 , 1.0g $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.39g $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 0.42g COCl_2 and 0.66g KI and all the constituents were dissolved in 1000ml of distilled water one at a time and mixed thoroughly. The solution was autoclave at 15psi for 15minutes and at a temperature of 121°C.

Preparation of Nutrient Agar.

Nutrient agar is a general purpose medium supporting the growth of wide range of non-fastidious microorganism. The nutrient agar is composed of peptones, beef extract, agar and distilled water. The nutrient agar was prepared by dissolving 7g of agar in 250ml of distilled water. The mixture was shaken gently to dissolve and autoclaved for 15minutes at a pressure of 15psi and at a temperature of 121°C.

Isolation of Microorganism.

1g from each of the soil sample were inoculated in 250ml of mineral salt medium with 0.05ml of crude oil added to the conical flasks as the sole carbon source and then the solution were placed in an orbital shaker for 48hours at 120rpm at room temperature.

Thereafter, the samples from the flasks were then serially diluted using 9ml of peptone water. The samples were serially diluted up to 10^{-6} dilution. 1ml of 10^{-6} dilution was then transferred to 3 different sterile Petri dishes and about 15-20 ml of the already prepared nutrient agar was poured over each of the 3 Petri dishes. These plates were allowed to solidify and then inverted in an incubator for 24 hours after which bacteria growth was seen and counted using colony counter. Morphologically distinct colonies were streaked on another Petri plate. The streaked plate were incubated for 24hours after which pure isolate of the distinct colonies were obtained. Slants were prepared by preparing nutrient agar and pouring in a McCarty bottle and allowing it to solidify. The pure isolate obtained from streaked plates were placed in the slant and stored in the refrigerator for further analysis.

Screening of Microorganism for Biosurfactant Production

The isolated microorganism were screened for the production of biosurfactant using the following methods;

Haemolysis

Biosurfactants can cause lysis of erythrocytes. This principle is used for the haemolysis assay which was developed by Mulligan et al, 1984. Cultures are inoculated on blood agar plates and incubated for 2 days at 25°C. Positive strains will cause lysis of the blood cells and exhibit a colorless, transparent ring around the colonies.

Oil Spreading Assay

The oil spreading assay was developed by Morikawa *et al*, 2000. For this assay, 10 µl of crude oil was added to the surface of 40 ml of distilled water in a Petri dish to form a thin oil layer. Ten (10) µl of culture supernatant are gently placed on the center of the oil layer. If biosurfactant is present in the supernatant, the oil is displaced and a clearing zone is formed. The diameter of this clearing zone on the oil surface correlates to surfactant activity, also called oil displacement activity. For pure biosurfactant a linear correlation between quantity of surfactant and clearing zone diameter is given.

Emulsification Capacity Assay

Another popular assay based on the emulsification capacity of biosurfactants was developed by Cooper and Goldenberg (1987) for measuring this trait, kerosene is added to an aqueous sample. The mixture is vortexed at high speed for 2 minutes. After 24 hours, the height of the stable emulsion layer is measured. The emulsion index E_{24} is calculated as the ratio of the height of the emulsion layer and the total height of liquid as indicated in the formula below: (Cooper and Goldenberg, 1987)

$$E_{24} = \frac{\text{height of emulsion layer}}{\text{total height of liquid}} \times 100\%$$

Extraction of Biosurfactant

20ml of the MSM was added to 200ml of the carbon source (Pineapple peel and cassava peel) each in a 250ml conical flask and 2ml of the aqueous isolate was added. The solution was placed in an orbital shaker for 7 days at 120rpm at room temperature. At the end of the seven days, the culture broth was centrifuged at 4000rpm for 15min to remove the cells as well as debris and the supernatant was used for the extraction. The supernatant was then precipitated by acidification with hydrochloric acid to pH 2.0. Equal volume of chloroform: methanol (2:1) was added. This mixture was shaken well for mixing and left overnight for evaporation. White coloured sediment was obtained as a result i.e. the crude biosurfactant. Anandaraj and Thivakarn, (2010).

Identification of isolated Microorganism.

Biochemical and phenotypic characterization was carried out on the positive biosurfactant-producing isolate using Bergey's Manual of Determinative Bacteriology as a guide (Buchanan and Gibbons 1974).

The bacteria isolates that passed the screening test were further sent for DNA identification at the International Institute for Tropical Agriculture, (IITA) Ibadan. The sequences generated by the sequencer were visualized using Chromaslite for base calling. BioEdit was used for sequence editing; Basic Local Alignment Search Tool (BLAST) was performed using NCBI (National Center for Biotechnology Information) database. Similar sequences were downloaded.

RESULTS AND DISCUSSIONS

Physiochemical analysis of the soil sample.

The physiochemical characteristics of the soil sample are presented in table 1, below. The hydrocarbon contaminated soil had a pH of 7.53 ± 0.2 . The temperature of the soil was 29.6 ± 0.4 and the type of soil collected was Humus mixed with hydrocarbon. TPH value was 109.1mg/kg.

Table 1.0. Physiochemical properties of the soil sample

| Parameter | Hydrocarbon contaminated soil |
|------------------------------------|-----------------------------------|
| pH | 7.53 ± 0.2 |
| Temperature ($^{\circ}\text{C}$) | 29.6 ± 0.4 |
| Type of soil | Humus soil mixed with hydrocarbon |
| TPH (mg/kg) | 109.1 |

Screening of Isolates for biosurfactant production

A mean count of $256 \pm 40 \times 10^6$ cfu/g was obtained from the samples. A total of 18 isolates obtained were subjected to three screening methods (Blood agar haemolysis, Emulsification index test, Oil displacement test) to select biosurfactant-producing strains. Table 2 below shows the result of the blood haemolysis of the isolates

Table 2: Result for blood haemolysis of the isolates

| S/N | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 |
|-----------------------|-------|------|-------|------|------|------|------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| Isolate | A1 | A2 | A3 | A4 | B1 | B2 | B3 | B4 | B5 | B6 | B7 | B8 | C1 | C2 | C3 | C4 | C5 | C6 |
| Blood Agar Haemolysis | Alpha | Beta | Alpha | Beta | Beta | Beta | Beta | Gamma | Gamma | Gamma | Gamma | Gamma | Alpha | Gamma | Gamma | Gamma | Gamma | Gamma |

The data in figure 2 below shows the result of the oil displacement of the isolates, figure 3, 4 and 5 shows the Emulsification index in crude oil, olive oil and kerosene respectively.

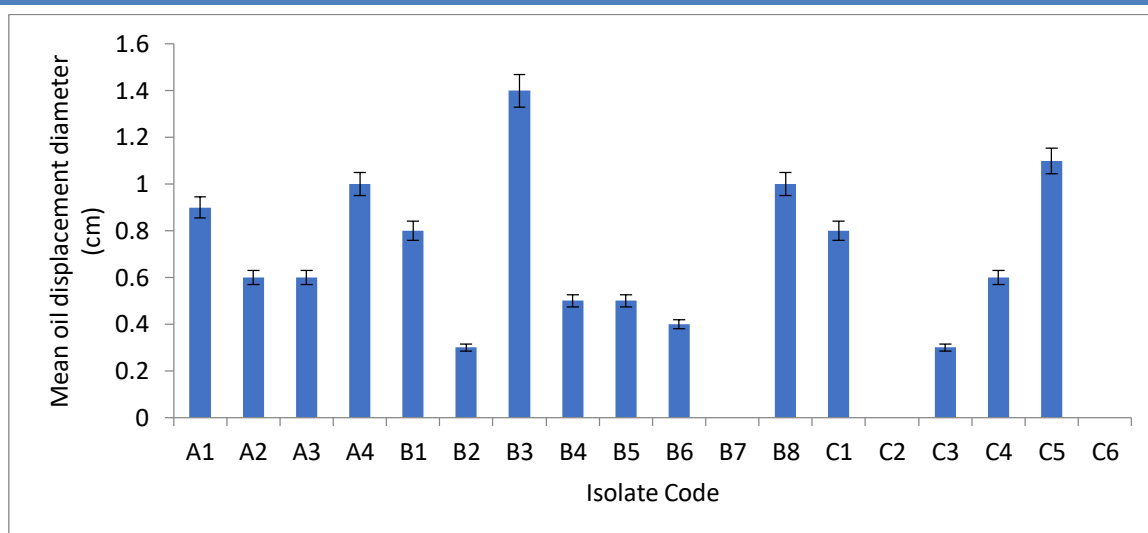


Figure 2. Showing Oil displacement of the Isolates

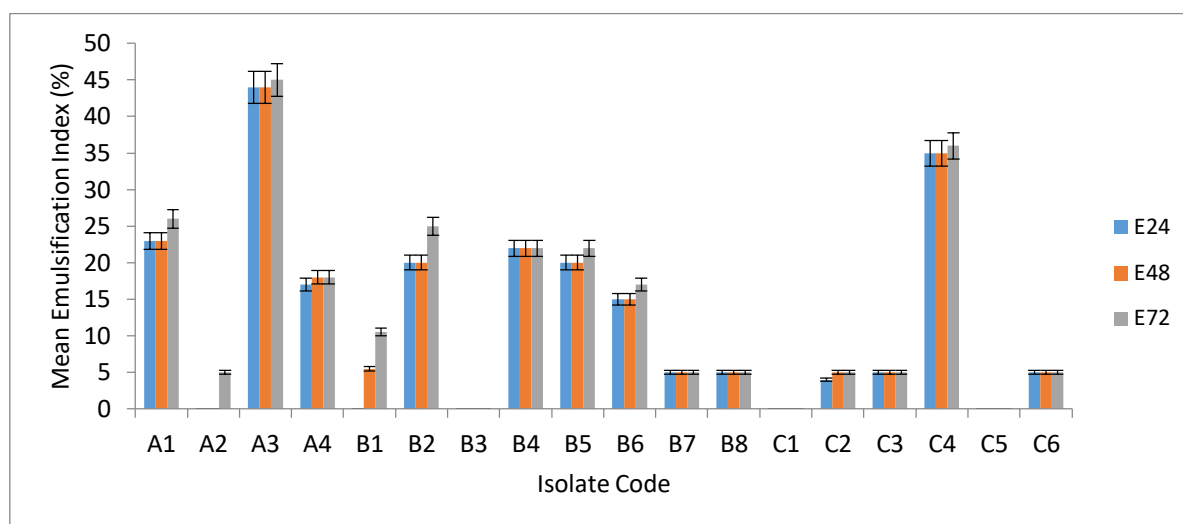


Figure 3. Showing Emulsification Index of the isolates at 24, 48 and 72hours using Crude Oil

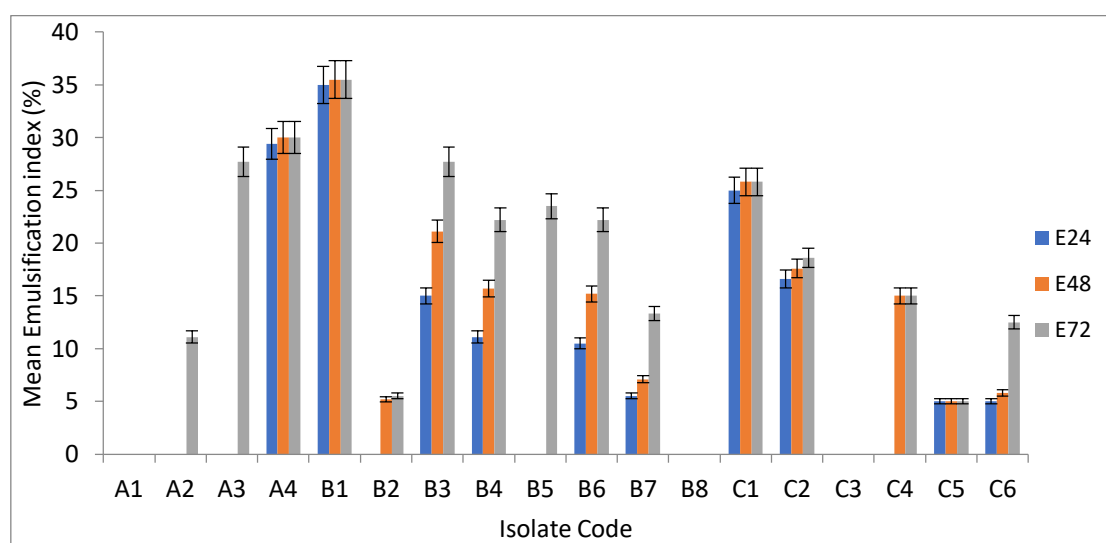


Figure 4. Showing Emulsification Index of the isolates at 24, 48 and 72 hours using Kerosene

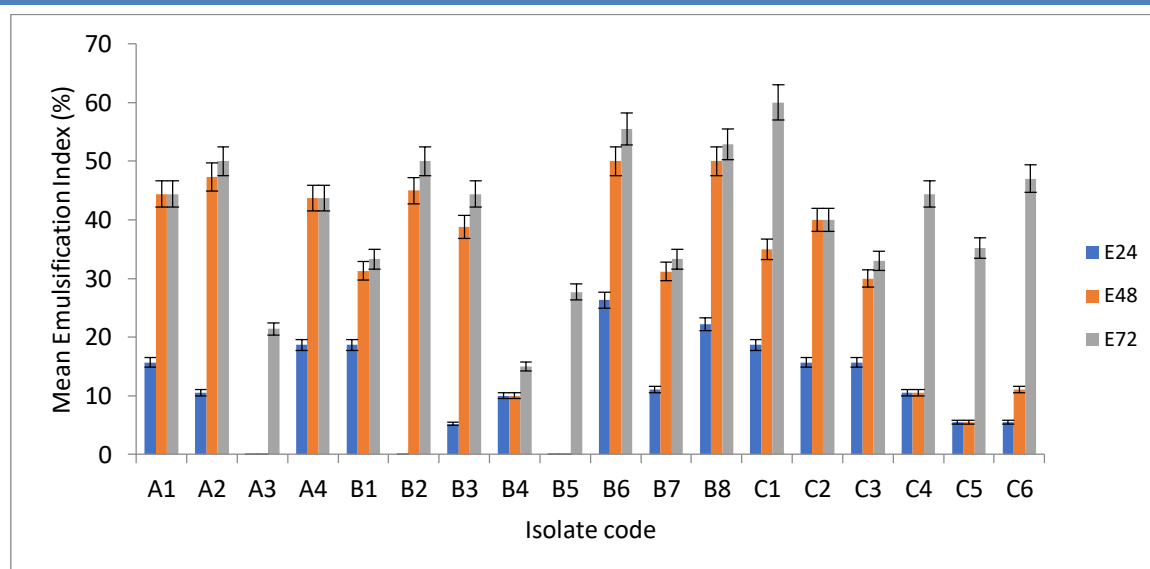


Figure 5: Showing Emulsification Index of the isolates at 24, 48 and 72 hours using olive oil

Production And Extraction Of Biosurfactant

The isolate that showed best result in the entire three screening method was used for the production of biosurfactant using pineapple peel and cassava waste carbon source. The data in table 3.0 shows the volume of the culture used, carbon source added and the quantity of biosurfactant produced by the isolate, A₄ which displayed positive result from the three screening test.

Table 3.0: Quantification of Biosurfactant

| Quantity of MSM (ml) | Quantity of Carbon Source (ml) | Quantity of crude biosurfactant produced (ml) |
|----------------------|--------------------------------|---|
| 20 | 200 | 10 |
| 20 | 200 | 5 |

Identification of Isolate.

The morphological and biochemical characteristics of the isolate A₁₁ placed it in the genus *Pseudomonas*. The 16sR sequence generated from the isolate gave 98% similarity to *Pseudomonas fluorescens* strain PC 20.

Discussion Of Result

A total of 18 bacteria were isolated from 3 samples of hydrocarbon contaminated soil using pour plate and serial dilution technique of isolating microorganisms. The total number of organism gotten from sample A is 333, while sample B was 242, and sample C was 195 making it a total of 770. Four (4) isolate were isolated from sample A, 6 isolate were obtained from sample B and 8 isolate were gotten from sample C. The 18 isolate gotten were subjected to screening test for production activities. Three screening test was employed, the oil displacement test, emulsification index test and the blood agar haemolysis test.

The oil displacement test used was developed by Morikawa *et al* (2000). Isolate A1, A2, A3, B1, B3, B4, B5, B8, C1, C4, C5 showed significant oil displacement zone and the other isolate B2, B7, C2 and C6 showed very little or no oil displacement zone. This assay was also applied by Huy *et al*, 1999 which is a rapid and easy method to carry out. In the second screening method, the isolates were subjected to emulsification index also known as emulsification capacity test. For measuring this capacity, crude oil, kerosene and olive oil was used. Using crude oil, isolate A3 showed the highest emulsification (44%) after 24hours of suspension in mineral salt medium and isolate C2 showed the lowest emulsification (4%) after 24hours. Using Kerosene, isolate B6 showed the highest emulsification (26%) after 24hours of suspension in mineral salt medium and isolate B3 showed the lowest emulsification (5.2%) after 24hours of suspension in mineral salt medium. Using Olive oil, isolate B1 showed the highest emulsification (35%) after 24hours of suspension in mineral salt medium while isolate C5 and C6 showed the lowest emulsification (5.5%) after 24hours. This screening method was developed by Cooper and Goldenberg, 1987 who used kerosene for measuring the emulsification capacity and he also stated that the kerosene can be replaced with other hydrophobic compounds such as hexadecane.

The third screening method used is the blood agar haemolysis which was developed by Mulligan *et al*, 1984. In this test, 5 isolates were able to show clear zone indicating beta haemolytic activity on blood agar plate. 10 isolates showed a lack of haemolysis indicating gamma haemolytic activity on blood agar plate while 3 isolates showed alpha haemolytic activity on blood agar plate which indicate partial haemolysis Schulz *et al*, 1991 also used this screening method for a preliminary

screening of microorganisms for the ability to produce biosurfactant on hydrophilic media. One isolate A₄ passed the three screening methods used for this study. The isolate was able to displace the oil giving good result while on blood agar hemolysis and it was able to emulsify in aqueous solution using crude oil, kerosene and olive oil after 24 hours. This correlates with the work done by Plaza *et al*, 2006 who used the three screening methods for biosurfactant production. The isolate that passed the screening test was used for the production of biosurfactant using pineapple and cassava peel as carbon source which are agricultural by product. Other agricultural by product have been reported as substrate for the production of biosurfactant by Lima *et al*, 2009, such as soybean hull. The production of biosurfactant is affected by some environmental factor such as temperature, pressure and agitation speed. The effect of pH on biosurfactant production was studied by Zinjarde and Pant (2002) who reported that the best production occurred when the pH was 8.0 which is the natural pH of sea water. The effect of pH on biosurfactant produced was studied by Zinjarde and Pant (2002) who reported that the best production occurred when the pH was 8.0 which is the natural pH of sea water.

The quantity of biosurfactant produced is 10ml/200ml of carbon source (blended pineapple peel) and 5ml/200ml of carbon source (cassava peel). Okuda Frank (2014), was able to produce 1g/1000ml of crude biosurfactant from *Pseudomonas aeruginosa* using red cashew pomace waste as the carbon source. Similar work done by El-Sheshtawy and Doheim (2013) on the yield of biosurfactants by *Pseudomonas aeruginosa* also yielded 1g/1000ml of synthetic carbon source. Although the conditions were not optimized, the yield from these carbon sources if properly harnessed may increase with a little modifications of the organism and also the use of consortium of organisms.

Conclusion

This study has shown that the bacterium *Pseudomonas fluorescens* strain PC20 isolated from hydrocarbon contaminated soil is capable of producing biosurfactant using pineapple and cassava peel as the carbon source. The biosurfactant produced was also found to demonstrate emulsification activity against the following hydrocarbons: diesel, kerosene and petrol, a feature, which is attractive for application in the biodegradation of petroleum hydrocarbons.

Recommendation

More research work on the conditions suitable for the optimal production using the above organism should be studied. Other Agricultural substrates

should also be used for production of biosurfactants using the *Pseudomonas* spp. Also the use of consortium of organisms and characterization of the biosurfactant produced is recommended.

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