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Effect of biosurfactant produced by *Citrobacter murliniae* AF025369 and a synthetic surfactant on degradation of crude oil

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ABSTRACT

Aims: To compare crude oil degradation by *Citrobacter murliniae* AF025369 in the presence of biosurfactant produced by *Citrobacter murliniae* AF025369 and a named chemical surfactant. Methods: Biosurfactant-producing bacterium was isolated from spent engine-oil polluted soil and analyzed for biosurfactant production in mineral salt medium using 2% glycerol as sole carbon source. The isolate was identified based on 16S rDNA sequence. Biosurfactant production was determined by drop collapse test, oil displacement test and emulsification index measurement. Tensoactive property and bioemulsification activity of the biosurfactant was carried out on various hydrophobic substrates. Crude oil degradation was investigated by gravimetric analysis. Results: The biosurfactant production assay gave an emulsification index (E24) of 66.67%, oil displacement of 1.8 cm and a positive drop collapse. It reduced surface tension of water from 72–42 mN/m, with critical micelle concentration (CMC) of 60 mg/L. Assessment of bioemulsification activities of the biosurfactant produced by *C. murliniae* with various hydrocarbon substrates gave highest emulsification index (E24) of 73.33% with palm oil, 70% E24 with crude oil and least E24 of 33.33% with fuel. Crude oil degradation analysis revealed that *C. murliniae* was able to degrade crude oil by 94%, it attained 96% crude oil degradation when the biosurfactant was introduced into the medium and 78% degradation when supplemented with Tween-80. Statistical analysis indicates that there is significant difference on the degradation rate using one-way ANOVA (p-value 0.0004). Conclusion: The results obtained show that biosurfactant was a better biostimulant and has great potential to be used in bioremediation processes, especially in the petroleum industry.

Keywords: Biosurfactant, *Citrobacter murliniae*, Crude oil degradation, Emulsification index

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INTRODUCTION

Surfactants (surface active agents) are amphipathic molecules with both hydrophilic and hydrophobic (generally hydrocarbon) 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 [1]. Such characteristics confer excellent detergency, emulsifying, foaming, and dispersing traits, which make surfactants one of the most versatile process chemicals [2].

Biosurfactants are surface active agents of microbial origin. Biosurfactants are molecules that exhibit pronounced surface and emulsifying activities, produced by a variety of microorganisms [3]. Biosurfactants are mainly categorized by their chemical composition and microbial origin [4]. Biosurfactants are classified based on chemical nature into glycolipids, lipopeptides, lipoproteins, phospholipid, fatty acids, polymeric biosurfactant and particulate biosurfactants [5].

The enormous market demands for surfactants are currently met by numerous synthetic mainly petroleum based, chemical surfactants. These compounds are usually toxic to the environment and non-degradable [6]. A host of interesting features of biosurfactants such as high biodegradability, low toxicity, and effectiveness at extremes of temperature, pH and salinity have led to a wide range of potential applications in the oil recovery, environmental bioremediation, food processing and medicine fields [3].

Biosurfactants are considered secondary metabolites, however, they confer a wide variety of roles for the survival of the producing organisms such as facilitation of nutrient transport across the membrane, microbe-host interaction and inhibitory activity against pathogenic organisms [7, 8]. Many reviews have summarized the possible roles of biosurfactants [9–11]. These include increasing the surface area and bioavailability of hydrophobic water-insoluble substrates, heavy metal binding, bacterial pathogenesis, biofilm formation and quorum sensing [9, 11]. The largest possible market for biosurfactant is the oil industry, both for enhanced oil recovery and for incorporation into oil formulation [12]. Hydrocarbon degrading and surfactant producing bacteria belong to the genera *Pseudomonas*, *Achromobacter*, *Flavobacterium*, *Micrococcus*, *Bacillus*, *Arthrobacter*, *Klebsiella*, *Acinetobacter*, *Aeromonas*, *Alcaligenes*, *Streptococcus*, *Corynebacterium*, *Moraxella*, and Proteobacteria [13].

MATERIALS AND METHODS

Microorganism

Citrobacter murliniae AF025369, isolated from spent-oil polluted soil and identified based on 16S rDNA sequencing at CAB International, UK was used. The organism was maintained on Nutrient Agar (Lab M) slant at 4˚C.

Inoculum preparation

One loopful of 24h old culture of the isolate was inoculated into 10 ml of sterile Nutrient broth in a test tube and incubated on a reciprocating shaker at 150 rpm and 30˚C for 24h [14]. This served as the seed inoculum.

Fermentation

A fermentation process was carried out following a method described by [14]. A mineral salt medium containing the following components was used: Basal medium [composition (g/L): KCl, 1.1; NaCl, 1.1; $FeSO_4.7H_2O$, 0.00028; KH_2PO_4, 3.4; K_2HPO_4, 4.4; $MgSO_4.7H_2O$, 0.5; Yeast extract, 0.5]; 2 ml of Trace element solution [composition (g/L): $ZnSO_4.7H_2O$, 0.29; $CaCl_2.4H_2O$, 0.24; $CuSO_4.5H_2O$, 0.25; $MnSO_4.\ 7H_2O$, 0.17]; $NaNO_3$, 1.5 and Glycerol, 2% w/v. The pH of the medium was adjusted to 7.2 with 1N NaOH. A 50 ml of the medium in 100 ml Erlenmeyer flask was sterilized in autoclave at 121˚C for 15 min, cooled and inoculated with 1 ml (2.15×10^6 cell/ml) of the seed inoculum. The flask was incubated for 72 h on orbital shaker at 150 rpm and 30°C. Duplicate flasks were used and uninoculated flasks served as control

Biosurfactant production assay

Biomass estimation (growth). The sample aliquot of 3 ml was collected from the culture flasks at the end of fermentation and growth determined using spectrophotometer (PerkinElmer Lambda 35 UV-VIS) at a wavelength of 600 nm.

Drop collapse method of screening

The method of Tugrul and Cansunar [15] was used. A polystyrene microwell plates with a diameter of 8 mm and 0.03 mm depth was coated with 7 µl mineral oil and left for 24h at room temperature. A 20 µl supernatant of the broth culture was then added to each well using a sterile syringe at an angle of 45˚C. Sterile distilled water was used as control. After one minute, the drops were examined visually for positive or negative result.

The oil displacement technique

According to the method of Morikawa et al. [16], 40 ml of distilled water was placed in a large Petri dish, followed by the addition of 15 µl of crude oil to the surface of the water. 10 µl of the supernatant of the broth culture was slightly put on the surface of oil film. The diameter of the clear zone on the oil surface was measured.

Emulsification index

The emulsifying activity of biosurfactant was determined according to the method described by Cooper and Goldenberg [17]. A mixture of 2 ml supernatant and 2 ml kerosene was vertically stirred for 2 min and the height of emulsion layer was measured after 24 h to determine the emulsification index. Emulsification index

was calculated thus: E24 = (H_e/H_t) ×100, where E24 = Emulsification index, H_e = Height of the emulsion layer, H_t = Total height.

Surface tension measurement

The surface tension of the cell free culture broth was determined by capillary rise method [18]. The cell free culture broth was added to 1 L of sterile distilled water in increasing concentration (1–8 mg). A capillary tube (0.01 cm diameter) was placed inside the water. Surface tension was measured from height of the water in the capillary tube using the equation: surface tension (γ) = [(ρga) /2]h

The concentration at which micelles began to form was represented as the Critical micelle concentration. The CMC value was determined by plotting the surface tension as a function of the biosurfactant concentration

Assessment of emulsifying activity

Bioemulsification activity was assessed by measuring the emulsification index (E24) of cell free broth with various substrates: kerosene, fuel, crude oil, groundnut oil, palm oil.

Purification of biosurfactant

The biosurfactant produced after fermentation was purified following the method described by Gnanamani et al. [19]. The culture broth was centrifuged twice at 4000 rev/min for 20 min to remove bacterial cells. The supernatant which served as the source of crude biosurfactant was adjusted to pH 2.0 with 1 N HCl and allowed to stand for 10h at 4˚C to precipitate the biosurfactant. The residual pellet obtained upon centrifugation was dissolved in 10 ml sterile distilled water.

Crude Oil Degradation

The degradation of crude oil was investigated following the method of Latha and Kalaivani [20]. 50 ml of the mineral salt medium supplemented with 5 g/l of crude oil was dispensed into three 100 ml Erlenmeyer flasks. The first flask was inoculated with 1 ml of the seed inoculum, the second flask contained 1 ml of the seed inoculum and 1% Tween 80 (chemical surfactant), 1 ml of the seed inoculum and 1% of the biosurfactant was added to the third flask. The flasks were incubated at 30°C for seven days on an orbital shaker at 150 rpm. Thereafter, residual oil was extracted and oil degradation rate was determined.

Extraction of Crude Oil

Oil degradation rates by gravimetric analysis was done according to the method of Mbachu [21]. 5 ml of n-hexane was added to the fermentation flasks of oil degradation and the contents transferred to a separating funnel extraction. Extraction was carried out twice to ensure complete recovery of oil. The extract was treated with 0.4 g of anhydrous sodium sulfate to remove the moisture and decanted into a beaker leaving behind sodium sulfate. This was evaporated to dryness by heating in a water bath.

The amount of residual oil was measured after extraction of oil from the medium and evaporating it to dryness.

The percentage of degradation of oil was calculated as follows:

Weight of Residual crude oil= Weight of beaker containing extracted crude oil – Weight of empty beaker.

Amount of crude oil degraded = Weight of crude oil added in the media – Weight of residual crude oil

%degradation = (Amount of crude oil degraded/ Amount of crude oil added in the media) x100

Statistical analysis

Data obtained were subjected to statistical analysis (one-way ANOVA) using Graphpad Prism 6.0 for window evaluation version 2003–2012.

RESULTS

The biosurfactant production assay showed bacterial growth of 1.102. Biosurfactant produced by *Citrobacter murliniae* AF025369 had emulsification index of 66.67%, maximum oil displacement of 1.8 cm and a positive drop collapse (Table 1). The biosurfactant produced reduced surface tension of water from 72–42 mN/m, with critical micelle concentration (CMC) of 60 mg/L (Figure 1). The bioemulsification assay is shown in Figure 2. Highest emulsification was obtained with palm oil substrate at E24 of 73.33%, followed by crude oil at E24 of 70%. Petrol gave least emulsification (E24 = 33.33%). Crude oil degradation analysis is given in Table 2. *C. murliniae* AF025369 obtained 94% degradation of crude oil. In the presence of biosurfactant, it obtained 96% degradation and least degradation rate with Tween-80.

DISCUSSION

Microorganisms of the class Enterobacteriaceae have been discovered to be biosurfactant producers. In this study, *Citrobacter murliniae* AF025369, isolated from spent engine-oil polluted soil was identified as a biosurfactant producer. This is supported by the works of Thavasi et al. [22] and Mandal et al. [23]. While Thavasi et al. [22], isolated *Citrobacter intermedius* alongside *Klebsiella ozaenae* as biosurfactant producers, Mandal et al. [23], isolated and characterized *Citrobacter* and *Enterobacter* as lipopeptide biosurfactant producer. The screening procedures used were consistent with previous works [24, 25]. In the biosurfactant production assay (Table 1), the cell free culture broth gave positive drop collapse result, 1.8 cm oil displacement diameter and 66.67% emulsification index measurement. The oil

Table 1: Biosurfactant production assay of C. murliniae AF025369

Organism	OD (600nm)	Emulsification index (%)	Oil displacement (cm)	Drop collapse
C. murliniae	1.102	66.67	1.8	+++

Table 2: Crude oil degradation analysis of Citrobacter murliniae AF025369

Combination	Initial weight (g)	Residual (g)	% degradation
C	0.5	0.03	94
C+B	0.5	0.02	96
C+T	0.5	0.11	78

Abbreviations: C = Culture only ; C + T = Culture + Tween 80; C + B = Culture + Biosurfactant

displacement diameter result is quite contrary to the works of Hesham et al. [26] and Jaysree et al. [27]. While Hesham et al. [26] obtained rate of oil displacement ranging from 2.8 cm to 4.1 cm in the screening of Candida species for biosurfactant production, Jaysree et al. [27] recorded displacement diameter ranging from 3.0 cm to 4.2 cm in their work on biosurfactant production by halophilic bacteria. Emulsification activity is one of the criteria to support the selection of potential biosurfactant producers [25]. The cell-free culture broth used in the emulsification index assay contains biosurfactant; it emulsified the kerosene present in the test solution by 66.67%.

Surface tension measurement of the cell-free culture broth obtained in this study (Figure 1), showed that *C. murliniae* AF025369 was able to reduce surface tension of water from 72–42 mN/m. Critical micelle concentration (CMC) was reached at 60 mg/L This is indicative of the tensoactive property of the produced biosurfactant.

Bioemulsification results (Figure 2), show that biosurfactant produced by the *C. murliniae* AF025369 formed stable emulsions with the hydrophobic substrates tested. It gave considerable emulsification activity with crude oil, thus showing potential for crude oil bioremediation. Maximum emulsification of 73.33% was obtained with palm oil substrate. Thavasi et al. [22], in their study, observed that the biosurfactant produced by *Pseudomonas aeruginosa* was able to emulsify several hydrophobic substrate (waste motor lubricant oil, crude oil, peanut oil, kerosene, diesel, xylene, naphthalene and anthracene) better than the synthetic surfactant tested.

Result on crude oil degradation (Table 2), shows that *Citrobacter murliniae* AF025369 is a crude oil-degrading bacterium. It utilized the crude oil as source of energy. Although *C. murliniae* AF025369 showed maximum degradation in the presence of biosurfactant. It also showed high degrading capacity in the absence of any stimulation by surfactant. Comparatively, biosurfactant produced by *C. murliniae* AF025369 stimulated its crude oil degradation potential than Tween-80. The organism without the biosurfactant also performed optimally than in the presence of the chemical surfactant. This could be that the chemical surfactant had inhibitory effect on the organism.

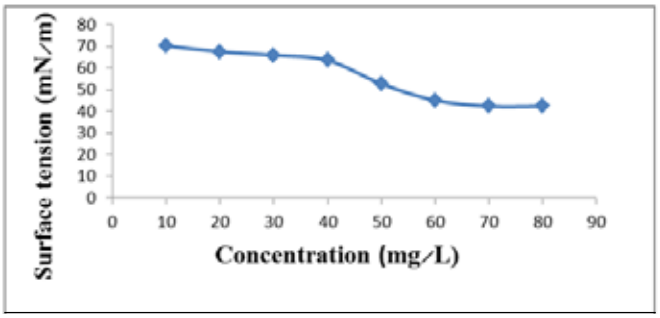

Figure 1: Surface tension measurement of biosurfactant produced by Citrobacter murliniae AF025369.

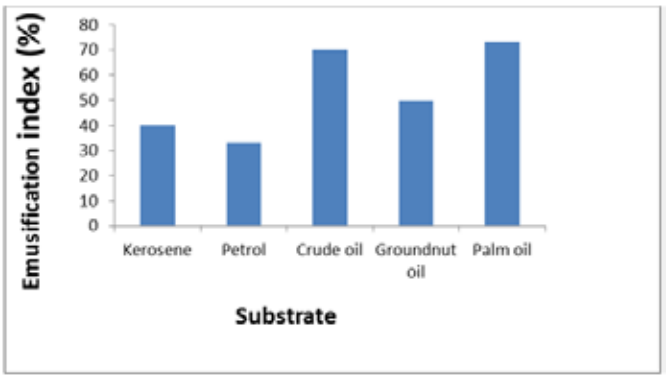

Figure 2: Emulsification assay (E24) of biosurfactant produced by Citrobacter murliniae AF025369.

CONCLUSION

From the results obtained in this study, *Citrobacter murliniae* AF025369 is a crude oil-degrading bacterium. The biosurfactant produced had low critical micelle concentration (CMC). The emulsification study showed that *Citrobacter murliniae* AF025369 can utilize varieties of hydrophobic substrate as energy source. The biosurfactant produced performed better than synthetic surfactant in crude oil biodegradation. The overall results suggest that *C. murliniae* AF025369 is a good producer of biosurfactant which possess potential for biostimulation in crude oil bioremediation.

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Author Contributions

Chikodili Gladys Anaukwu – Substantial contributions to conception and design, Acquisition of data, Drafting the article, Revising it critically for important intellectual content, final approval of the version to be published
Chinyere Constance Ezemba – Substantial contributions to conception and design, Acquisition of data, Drafting the article, Revising it critically for important intellectual content, Final approval of the version to be published
Vivian Nonye Anakwenze – Substantial contributions to conception and design, Acquisition of data, Drafting the article, Revising it critically for important intelolectual content, Final approval of the version to be published
Kingsley Chukwuebuka Agu – Substantial contributions to conception and design, Acquisition of data, Drafting the article, Revising it critically for important intellectual content, Final approval of the version to be published
Nsikak Sunday Awah – Ssubstantial contributions to conception and design, Acquisition of data, Drafting the article, Revising it critically for important intellectual content, Final approval of the version to be published
Benjamin Chidi Okeke – Substantial contributions to conception and design, Acquisition of data, Drafting the article, Revising it critically for important intellectual content, Final approval of the version to be published
Ikechukwu Amechi Ekwealor – Substantial contributions to conception and design, Acquisition of data, Drafting the article, Revising it critically for important intellectual content, Final approval of the version to be published

Guarantor

The corresponding author is the guarantor of submission.

Conflict of Interest

Authors declare no conflict of interest.

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