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Article

Qualitative Assessment of Biosurfactant Producing Microorganisms in the Cleanup of Crude Oil in Soil

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Abstract

Crude oil contaminates the soil in several ways and its poisonous effect has caused havoc in the environment which can lead to the death of living organisms. Some microorganisms can produce biosurfactants which aid in high biodegradability. This study investigated biosurfactant production by some indigenous microorganisms isolated from crude oil-contaminated soils. Isolates were identified, preliminarily screened for 7 days using the mineral salt medium on agar plates, and then screened further for biosurfactant production. The Drop collapse test, Haemolytic test and Oil spreading test were used to determine the production of biosurfactants. Thirty microorganisms were isolated from the contaminated soil samples and identified as species of *Corynebacterium* (7), *Bacillus* (8), *Lysinibacillus fusiformis* (1) *Aspergillus* (3), *Penicillium* (5), *Fusarium* (3), *Microsporum* (2) and *Cladosporium* (1). Three of the isolates (*Bacillus subtilis*, *Lysinibacillus fusiformis*, and *Aspergillus flavus*) proved to be biosurfactant producers while also utilizing 8 % of crude oil during qualitative screening. These isolates can produce active compounds (biosurfactants) that can emulsify the crude oil which leads to the easy degradation of the crude oil. They could also be potential candidates for Microbial Enhanced Oil Recovery.

Keywords: Crude oil, Biosurfactants, *Aspergillus flavus*, *Bacillus subtilis*, Degradation.

1. Introduction

Researchers made findings about how crude oil was formed and it was stated that over a million years ago, the earth was populated with plants and animals and their locations were near the rivers, lakes and seas. As these plants and animals died, they decay and their remains settle to the bottom of the waters, these waters carried sands in which also contains the remains of these organic matter after, then the thickness of these matters grew to 100s offset over time. The organic matter of the plants and animals that settled in the bottom of these waters was mixed with sands, silt and mud, therefore, as pressure and temperature increased, the sands, mud and silts changes into clay sediment which was called shale rock or sedimentary rock and the organic matter changes into kerogen, this occurs in the absence of oxygen [1]. These kerogens are heated and compressed over a long time, crude oil is thereby formed. Although there are two theories on how crude oil was created which are the organic theory which was just explained above and the inorganic theory which states that hydrogen and carbon far beneath the earth's surface came together under great temperature and pressure to form oil and gas. The organic theory is widely accepted because of some pieces of evidence which are:

1. Molecules in hydrocarbons are thought to be similar to organic matter.
2. Presence of brine (sea water) with crude oil.
3. It is found only associated with sedimentary rocks.

The polarized light that passes through all petroleum resources undergoes a rotation which is also similar to all organic oil.

In environmental remediation, there is a distinction between ex-situ and in-situ remediations. In situ soil remediation concerns techniques like bio-restoration, soil washing or extraction, and soil venting and these techniques prevent the spreading of contaminants [2]. Soil washing uses the solubility of a contaminant, which will dissolve in the percolate and through a special withdrawal system the percolate is pumped up and treated. Soil venting aims at volatilization and biodegradation of the contaminant in the unsaturated zone, followed by a vapour treatment system to remove the contaminants from the vapour. Air sparging involves the injection of air into the saturated zone for the dual purpose of volatilizing organic components and enhancing biodegradation. Ex-situ soil remediation is the removal of contaminated soil, cleaning this soil at an independent location and then returning it after cleaning, possibly at the same location [2].

Bioremediation is a natural method of removing crude oil and other contaminants pollution from the environment. It is a biological method that makes use of living organisms to reduce or completely remove pollutants (organic and inorganic compounds) from polluted areas. In the case of crude oil and other oil-derived products, bioremediation is the most effective way for clean-up [3]. In the past, it has been a serious problem to turn to bioremediation as an implemented policy solution, as the lack of adequate production of remediating microbes led to few options for implementation. Those that manufacture microbes for bioremediation must be approved by the Environmental Protection Agency (EPA); however, the EPA traditionally has been more cautious about the negativity that may or may not arise from the introduction of these species. One of their concerns is that the crude oil or oil-derived products would lead to the microbe's gene degradation, which would then be passed on to other harmful bacteria, creating more issues, if the pathogens evolve the ability to feed off of pollutants [4]. There are different types of bioremediation, in which bio-surfactants is one of the major forms.

1.1 Biosurfactants

Despite the composition and toxicity of hydrocarbons, several groups of microorganisms are found to synthesize chemical compounds called biosurfactants that enhance the emulsification of hydrocarbons. Biosurfactants are produced by microorganisms to decrease the tension at the hydrocarbon water interface which aimed to falsely solubilize the hydrocarbons thereby increasing mobility and consequent biodegradation [5].

Several microorganisms (*Pseudomonas aeruginosa*, *Bacillus subtilis*) have been reported to produce biosurfactants such as glycolipids, lipoprotein, and surfactin which can emulsify petroleum hydrocarbons and this plays a great role in the uptake and assimilation of hydrocarbons [6]. Hence this study aimed at the qualitative assessment of biosurfactant production by microorganisms isolated from crude oil-contaminated soil. Specific objectives include: isolation, characterization and identification of microorganisms from crude oil-contaminated soil, preliminary screening for oil-degrading microorganisms, and biosurfactant screening of the oil-degrading isolates.

2. Methods

2.1 Study Area

This study was carried out in Oyo town, which is in the South-Western part of Nigeria. The town is situated on the latitude 7^{O} North of the equator and longitude 3^{O} East in which having a daily temperature between the ranges of 25^{o}C and 35^{o}C, covering an area of 28,454 square kilometres precisely, the landscape is made up of old rocks and dome-shaped hills.

2.2 Sample Collection

The soil samples used in this study were collected at a depth of 2-3cm from Ogoni town in Gokana Local Government Area of Rivers State situated at latitude 4° 40′ 5″ N and 4° 43′ 19.5″N and longitude 7° 22′ 53.7″ E and 7° 27′ 9.8″ E. and Warri South Local Government area at Latitude: 5.6133 and Longitude: 5.6106 in Delta State, Nigeria. Also, a soil sample was taken from Ajayi

Crowther University, Oyo town field area, this served as a control sample. A sterile hand spade was used to collect soil samples into a sterile plastic bag. The samples were taken to the microbiology laboratory for further analysis. This research was carried out in two stages. Stage 1 was to screen the indigenous fungal and bacterial isolates for 7 days. In stage 2, these isolates were subjected to biosurfactants screening.

2.3 Preliminary Screening of Fungal and Bacterial Isolates (Qualitative Analysis)

Microorganisms (Fungal and bacterial isolates) were screened by a modified method [7]. The medium was a composition of (g/L) KH_2PO_4, 7.584; K_2HPO_4, 0.80; $MgSO_4.7H_2O$, 0.80; $CaCl_2$, 0.16; $(NH4)_2NO_3$, 0.80; $FeSO_4$, 0.16; Agar, 20; and 2 % Crude oil, pH was maintained at 7.0, 1mL of the inoculum of each isolate was introduced into 10mL of the sterilized medium in test tubes, after which they were poured into Petri dishes, incubation was at room temperature 27^{o}C - 30^{o}C for 7 days. After incubation, agar plates were observed for growth, the organisms degraded the crude oil; showing signs of growth, with the crude oil on the medium surface slightly cleared.

2.4 Screening of Biosurfactant

Biosurfactant screening is an essential concept used to detect surfactant properties produced by different types of microorganisms especially bacteria and fungi using carbon as a substrate. The three methods used in screening for biosurfactants in this study included: Haemolysis activity test, Oil displacement assay, Drop collapse test etc. [8].

2.4.1 Haemolysis Activity

Biosurfactants can cause the lyses of erythrocytes. This was carried out according to the method of [9]. Cultures were inoculated on sheep blood agar plates and incubated for 2 days at 25°C. Positive strains caused lyses of the blood cells, this was indicated by a colourless, transparent ring around the colonies.

2.4.2 Oil Displacement Assay

One mL of crude oil was added to over 30 mL of distilled water in a Petri plate. Thereafter, 20ɥl of the supposed biosurfactant sample obtained from the supernatant of the microorganism was added to the distilled water. The increase in the diameter of the oil proves that the microorganism produced biosurfactants [10].

2.2.3 Drop Collapse Test

Few drops of crude were placed on a clean slide and 10ɥl of the supernatant was added using a micropipette. The drop collapsing within 1 min indicated that the microorganism produced biosurfactant [10].

3. Results

Bacillus subtilis strain DU1 and *Lysinibacillus fusiformis* strain KL2-13 visibly showed growth and utilization of 2 %, 6 % and 8 % crude oil on mineral salt agar. At 2 % concentration, *Corynebacterium xerosis* showed visible growth on the mineral salt agar plate but there was no sign of visible utilization of the crude oil. On the other hand, there was a visible sign of growth as well as visible utilization of the crude oil by *Corynebacterium kutsceri* on the mineral salt agar plate (Table 1). At a 6 % concentration of crude oil, *Corynebacterium kutsceri* and *Bacillus cereus* showed visible growth but there was no sign of visible utilization of the crude oil. However, at 8 %of crude oil, these bacterial isolates showed slightly visible growth but there was no visible sign of crude oil utilization. *Corynebacterium* sp. and *Bacillus cereus* grew visibly at 6 % of crude oil but there was no visible utilization of the crude oil on the mineral salt medium. At 8%, *Corynebacterium* sp. and *Bacillus cereus* also showed slight growth and there was no visible utilization of the crude oil, however, *Lysinibacillus fusiformis* and *Bacillus subtilis* showed visible signs of growth and crude oil utilization (Table 1).

Furthermore, *Microsporum* sp., *Aspergillus flavus*, *Penicillium* sp., and *Penicillium citrinum* could visibly utilize the 8 % crude oil on mineral salt medium in this study. *Cladosporium* sp. proved capable of growth and crude oil utilization at 2 % crude oil concentration on mineral salt agar plate

but at 6 % concentration, the growth was not visible. At the increase of crude oil (8 %) in the mineral salt medium, *Cladosporium* sp. showed no sign of growth. *Fusarium* sp. was able to grow and utilize 2 % crude oil, however, at a 6 % concentration of crude oil, there was no sign of visible growth on the mineral salt agar plate. *Aspergillus niger* showed signs of growth at 6 % and 8 % concentrations of crude oil on the mineral salt agar plate, although there was no sign of visible utilization of crude oil. All the fungal isolates grew at 2 % of crude oil on the mineral salt agar plate (Table 2). The utilization of crude oil is evidenced by the clear zones on the plates.

Table 1: Qualitative screening of bacterial isolates in the degradation of crude oil at different concentrations (2, 6 and 8 %)

Bacterial isolates	2%	6%	8%
Corynebacterium kutsceri	+++	++-	+ - -
Corynebacterium xerosis	++-	+- -	+- -
Bacillus subtilis strain DU1	+++	+++	+++
Bacillus cereus	+ + -	++ -	+- -
Lysinibacillus fusiformis strain KL2-13	+++	+++	+++

***KEY**: (+++): Growth and Visible utilization of crude oil; (++-): Growth and no visible utilization of crude oil; (+- -): Slight Growth and no visible utilization of crude oil; (- - -): No growth and no visible utilization of crude oil.*

Table 2: Qualitative screening of fungal isolates in the degradation of crude oil at different concentrations (2, 6 and 8 %).

Fungal Isolates	2%	6%	8%
Microsporum sp.	+++	+++	+++
Aspergillus flavus	+++	+++	+++
Aspergillus niger	+++	++ -	++ -
Cladosporum sp.	+++	+- -	- - -
Fusarium sp.	+++	- - -	- - -
Penicillium sp.	+++	+++	+++
Penicillium citrinum NKM6	+++	+++	+++

***KEY**: (+++): Growth and Visible utilization of crude oil; (++-): Growth and no visible utilization of crude oil; (+- -): Slight Growth and no visible utilization of crude oil; (- - -): No growth and no visible utilization of crude oil.*

Table 3: Biosurfactant production by the microbial isolates

Organisms	Haemolysis Test	Oil displacement assay	Drop collapse test
Penicillum sp.	-	-	-
Microsporum sp.	+	-	-
Microsporum sp.	-	-	-
Aspergillus flavus	+	+	+
Penicillium citrinum NKM6	-	-	-
Lysinibacillus fusiformis strain KL2-13	+	+	+
Bacillus subtilis strain DU1	+	+	+

***KEY**: +: Positive; -: Negtive*

Precisely three analyses were carried out to screen for biosurfactant production by the indigenous microorganisms from the soil collected from Warri town and Ogoni land. Results showed that *Lysinibacillus fusiformis* strain KL2-13 and *Bacillus subtilis* strain DU1 were positive for all the analyses carried out to screen for biosurfactant production (Haemolytic test, Oil displacement assay and Drop collapse assay). *Aspergillus flavus* (OGSF) and *Fusarium* sp. (NDSH) were positive for two out of the three analyses, still indicating biosurfactant production (Positive for the oil

spreading test and Drop collapse test; Negative for the Haemolytic test) (Table 3).All the *Penicillium* and *Microsporum* spp. in this study proved negative to biosurfactant production (Table 3).

4. Discussion

Indigenous isolates were screened for oil degradation in this study, many other researchers have screened for potential oil degraders using 1 %, 2 % or 5 % of crude oil as the sole carbon source [11]; [12]; [13]; [14]. [15] carried out preliminary screening on fungal using minimal salt media supplemented with 1 % crude oil as the sole carbon source in which *Cladosporium and Fusarium* were the potential candidate for oil degraders, contrary to what was observed in this study, these two isolates could not utilize the crude oil at 6 and 8 %. *Lysinibacillus fusiformis* was isolated as a potential oil degrader in this study, [12] also isolated *Lysinibacillus fusiformis* as a potential oil degrader (Tables 1 and 2).

Some of the microbial isolates used in this study proved capable of biosurfactant production. Indeed biosurfactant has been screened for pure cultures of all the microbial genera used in this study [12]; [16]; [17]; [18]. Biosurfactant production is an important criterion in recommending isolates for biotreating hydrocarbon-contaminated areas [19].

The formation of clear zones around the colonies of the selected isolates on the blood agar is an indication of biosurfactant production by the organisms. This was reported by [8]. Literature have confirmed that it is possible to isolate fungal and bacterial strains capable of producing biosurfactant by using the medium selected in this study [8]. Most organisms especially bacteria in polluted soils have been proven by different researchers that they are emulsifiers having the ability to emulsify petroleum hydrocarbons and this plays a great role in the uptake and assimilation of hydrocarbon [6]. As observed in this study, [16] and [12] also used the hemolytic assay method to screen through the appearances of clear zones for the bacterial strain *Bacillus subtilis* and *Lysinibacillus fusiformis* respectively. *Fusarium* sp. showed the inability to produce biosurfactants in this study, however, contrarily, [20] reported *Fusarium* sp. as a biosurfactant-producing fungus (Table 3).

Biosurfactants can emulsify petroleum hydrocarbons and this plays a great role in the uptake and assimilation of hydrocarbons, hence the isolates (*Bacillus subtilis*, *Lysinibacillus fusiformis,* and *Aspergillus flavus*) used in this study are potential candidates for hydrocarbon degradation. They could also be potential sources of oil recovery (MEOR). Further work needs to be carried out to authenticate this claim.

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