**Futo Journal Series (FUTOJNLS)**
e-ISSN :2476-8456 p-ISSN : 2467-8325
Volume-2, Issue-1, pp- 244 - 254
www.futojnls.org

Research Paper July 2016

# Crude Oil Biodegradation Potential of Bacterial Strains Isolated from Mangrove Ecosystem

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## Abstract

*Crude oil biodegradation potential of rhizosphere bacterial strains isolated from mangrove ecosystem in the Niger Delta of Nigeria was investigated. Total microbial load of rhizosphere soil sample indicated 2.06 x $10^6$, 5.1 x $10^3$ and 1.61 x $10^5$ cfu/g for total aerobic heterotrophic bacterial, total fungal and total hydrocarbon utilizing bacterial counts, respectively. Among 18 oil-degrading bacterial strains, Bacillus sp. RS12, Pseudomonas sp. RS16, Pseudomonas sp. RS23, and Micrococcus sp. RS38 were selected for the study based on their efficiency of crude oil utilization in both BHA-crude oil and NA-crude oil plates. Results showed that the test organisms could utilize crude oil, diesel, petrol, kerosene and engine oil as carbon sources. None of the organisms utilized hexane and xylene but moderate growth was observed in phenol. The test isolates exhibited wide range of adherence to crude oil with values of 46.7 to 65.8%. The adhesion to crude oil was in the following order: Pseudomonas sp. RS23 (58.40%) > Pseudomonas sp. RS16 (55.51%) > Bacillus sp.RS12 (50.70%) > Micrococcus sp. RS38 (48.82%). However, their ability to utilize crude oil varied both in rates of utilization and in growth profile with 45 % of crude oil degraded and 0.30 g/l biomass formed by Pseudomonas sp.RS23. The least crude oil degradation potential was 33 % with 0.25 g/l of biomass formed was observed in Micrococcus sp. RS38. The high crude oil utilization of the test isolates implies that bacteria isolated from contaminated ecosystem are excellent crude oil degraders and can be harnessed for bioremediation purposes.*

***Keywords*** *- Biodegradation, crude oil, mangrove, rhizosphere bacteria*

## 1.0 Introduction

In order to fulfill the energy need of any country's population, various natural resources have to be exploited. The principle source of energy continues to be petroleum hydrocarbon (Sathishkumar *et al*., 2008). Petroleum exploitation and exploration, transportation, consumption, attendant spills as well as disposal often lead to release of hydrocarbon pollutants into the environment with serious ecological problems (Oluwafemi *et al.*, 2009; Sathishkumar *et al.*, 2008). Petroleum consists of crude oils and a wide variety of refined oil products (Westlake *et al.*, 1974). Crude oils vary in chemical composition, color, viscosity, specific gravity, and other physical properties. Color ranges from light yellow-brown to black. Crude oil as a source of energy is a complex mixture of thousands of hydrocarbons and non-hydrocarbon compounds. Hydrocarbons comprise more than 75% of most crude and refined oils. Hydrocarbons in petroleum are divided into four major classes: straight-chain alkanes (n-alkanes or n-paraffins), branched alkanes (isoalkanes/isoparaffins), cycloalkanes (cycloparaffins) and aromatics (Atlas, 1981).

Crude oil as a mixture of different hydrocarbons is highly toxic. Accidental spills of crude oil always results in severe contamination of aquatic and terrestrial environments. Contamination due to spillage of processed petroleum derivates (especially diesel and fuel) is an important problem in water.

Crude oil spills are destructive to both vegetations and animals in the soil not only because of their contact toxicity but also because hydrocarbons in the soil reduce oxygen tension and increases anaerobiosis which is harmful to plant roots (Bossert & Bartha, 1984). The persistence of petroleum pollution depends on the quantity and characteristic of hydrocarbon mixture and on the properties of the affected ecosystem (Ojo, 2005). The ability to isolate high numbers of certain oil-degrading microorganisms from oil polluted environment is commonly taken as evidence that these microorganisms are active degraders in that environment (Amirlatifi *et al.,* 2013; Singh *et al.,* 2013). Many microorganisms have been reported to utilize various petroleum hydrocarbons, including benzene, toluene, ethylbenzene and xylene (BTEX) as their sole carbon and energy substrate, despite their extreme insolubility in the aqueous phase. Biodegradability of crude oil has been reported to depend on the content of n-alkane, asphaltene, nitrogen, sulfur and oxygen containing components of the oil (Westlake *et al.,* 1974). Numerous genera of bacteria are known to degrade hydrocarbons. They tolerate high concentrations of hydrocarbon and have a high capability for their degradation. Most of these bacterial strains belong to *Pseudomonas*, *Sphingomonas*, *Aeromonas*, *Alcaligenes*, *Acinetobacter*, *Arthobacter*, *Brevibacterium*, *Xantomonas*, *Mycobacterium*, *Rhodococcus* and *Bacillus* species (Płaza *et al.,* 2008).

The environmental impact of petroleum exploration in Nigeria in particular and other oil producing countries in general has been on the increase. The increasing concern, however, of the environmental scientist is the destruction caused by oil spill both on cultivated and virgin lands as well as mangrove ecosystem. Crude oil plays an important role in the economy of Nigeria and about 80% of oil exploration and exploitation activities take place in the mangrove areas of the Niger Delta (Ebegbulem *et al.,* 2013). However, mangrove forest clearing and oil spills from operational failures and vandalisation of pipelines, oil well blowouts, tanker seepages and accidents contribute immensely to mangrove species loss and degradation of the ecosystem (Chindah *et al.,* 2007; Ekpo and Udofia, 2008).

Nigeria has the third largest mangrove forest in the world and the largest in Africa covering between 5,400 and 6,000 $km^2$. There are three main mangrove plant families (*Rhizophoraceae*, *Avicenniaceae* and *Combretaceae*) that are rapidly spreading across the Niger Delta (NDES, 2000). The mangrove plants (*Rhizophora mangle* L.) are salt tolerant species that grow on sheltered shores in the tropics and sub-tropical estuaries, where they provide ecosystem functions and several human utility benefits especially for coastal communities of Niger Delta. Their halophytic nature and ability to compensate for low oxygen in the soil allows them to thrive well in the environment. However, their complex breathing roots make them vulnerable to crude oil that can block the openings of the breathing roots (Choudhry, 1997) hence affecting rhizosphere bacterial activities. Mangrove soil rhizosphere provides a unique ecological niche to diverse groups of microbes which play various roles in nutrients recycling as well as various environmental activities. Mangrove rhizosphere as nutritiously rich habitat, also accumulate high concentrations of hydrocarbon and other crude oil products as a result of oil pollution (Emuedo *et al.,* 2014). This has a serious influence on the microbial diversity of the environment particularly on bacterial diversity since it is a more sensitive group than other microorganisms. In such conditions bacteria will react to crude oil pollution by several processes such as using the oil as source of carbon and energy as well as for biomass production.

The aim of this work was to evaluate hydrocarbon utilization by mangrove bacteria and assess their biodegradation potential of crude oil.

## 2.0 Materials and Methods

### 2.1 Chemical reagents

All the chemical reagents used in this study were of analytical grade and were purchased from Sigma-Aldrich (St Louis Missouri, USA), BDH Chemicals (Poole, England) and HACH Chemical Company

(Colorado, U.S,A). Crude oil (Bonny-Light) was obtained from Nigeria Agip Oil Company, Port-Harcourt, River State, Nigeria, which is made up of *n*-alkane-containing oil such as saturates (56 %), aromatics (31 %), polars (11 %), and asphaltenes (2 %). It also has 33.4° API gravity and contains 0.1 % Sulphur content (Norman *et al*., 2004).

2.2 Sample collection

Eagle Island mangrove is located behind Rivers State University of Science and Technology Port Harcourt, Nigeria. It lies within longitude $4^{o}$ $35^{1}$ and $4^{o}$ $5^{1}$ N and latitude $6^{o}$ $00^{1}$ and $7^{o}$ $53^{1}$ E. Like other mangrove swamps of the Niger Delta, the swamp is extensive, covering a wide area of land with a top layer of mud slurry overlaying a hard substratum. The vegetation is as described by Chindah *et al.* (2007). A composite rhizosphere soil (RS) sample of the mangrove vegetation plants of Eagle Island in Port Harcourt, Rivers State of Nigeria, was collected in sterile polyethylene containers. The sample was transported in an ice box to the laboratory for microbial analysis within 6 h of collection.

2.3 Enumeration and isolation of microorganisms

Ten-fold serial dilutions of the soil sample were prepared in sterile 0.85 % of NaCl solution. One-tenth (0.1 ml) aliquots of the soil suspension were aseptically spread-seeded onto plates of different microbiological media and incubated at ambient temperature (28 ± 2 $^{0}$C). The fungal populations were enumerated on Sabouraud dextrose agar (SDA) plates with streptomycin (50 µg/ml) to suppress bacterial growth. Incubation was done at room temperature for 120 h. The heterotrophic bacterial population was enumerated on nutrient agar (NA) plates after 48 h incubation. Hydrocarbon utilizing bacterial (HUB) population was enumerated on mineral salts agar (Mills *et al.*, 1978). The crude oil source was supplied through the vapour phase by placing filter papers (Whatman No.1) impregnated with 3 ml of filter-sterilized Bonny-Light crude oil on the lids of the plates. The plates were incubated at $30^{0}$C for 168 h. All plates yielding 30 - 300 colonies were counted. The enumerated HUB were isolated and stored in NA slants at 4 °C for further studies and identification.

2.4 Preliminary screening of crude oil degraders

This was done as described by Subathra *et al.* (2013) with little modification. Briefly, hydrocarbon degraders (HUB) which were stored on agar slants were subjected to its efficiency of crude oil degradation. The isolates were single streaked on Bushnell-Haas agar (BHA), (consisting per litre: $KH_2PO_4$, 1.0 g; $K_2HPO_4$, 1.0 g; $NH_4NO_3$, 1.0 g; $MgSO_4.7H_2O$, 0.2 g; $FeCl_3.6H_2O$, 0.085 g; $CaCl_2.2H_2O$, 0.02 g) plates overlaid with 0.1 ml of crude oil (BHA-crude oil) and on plain NA (NA-crude oil) plates and were incubated at 30 °C for 14 days and for 5 days, respectively. Though, Subathra *et al.* (2013) incubated the plate cultures at 25 $^{o}$C for 48 h. Any isolate which grew on NA-crude oil plates but failed to grow on BHA-crude oil plate were confirmed as non-degraders. The isolates which grew on both the agar plates were confirmed as hydrocarbon degraders. The zone of clearance around the colonies of the isolates grown on BHA plate with varying diameters was measured. Isolates with highest zone of clearance around the colonies were selected and were tentatively characterized using a battery of biochemical tests. The isolates were identified to the generic level following Bergey's Manual of Determinative Bacteriology (Holt *et al.*, 1994) and stored at $4^{0}$C on nutrient agar slants for further studies.

2.5 Preparation of inoculum

The test isolates were grown in nutrient broth medium contained in Erlenmeyer flasks (100 ml) at 28 ± 2 $^{0}$C for 48 h. Thereafter, the cells were harvested by centrifugation at 6000 rpm for 10 min and washed in sterile

deionized water. The cell suspensions were standardized by adjusting the turbidity to optical density of 0.1 at absorbance of 540 nm and used throughout the study unless otherwise stated.

2.6 Screening for petroleum hydrocarbon utilization

The screening assay for petroleum products utilization by the test isolates was done as described by Resnick and Chapman (1994) with little modification. Petroleum substrate utilization by the test organisms was determined in sterile Bushnell-Haas (BH) broth medium (9.9 ml in 20 ml culture test tubes) supplemented with various petroleum products [0.1 % (v/v)] such as diesel, petrol, kerosene and hexane. Others included benzene, toluene, xylene and phenol. This was followed by inoculation of the medium with 0.1 ml of bacterial cell suspensions. Controls consisted of inoculated media devoid of petroleum products (positive) as well as with petroleum products but without inoculum addition (negative). The tubes were incubated at $28^0$C for a period of 14 days. Development of turbid culture was indicative of utilization of the petroleum hydrocarbons as carbon source. Cultures without increase in turbidity over initial optical density ($OD_{540nm}$) and non-inoculated control were scored as no growth (-) while cultures with increased turbidity significantly greater than the control ($OD_{540nm}$ reading above 0.2.) were scored as growth (+) (John and Okpokwasili, 2012).

2.7 Bacterial adhesion to hydrocarbons (BATH)

Cell hydrophobicity of the selected bacterial strains was determined by bacterial adherence to hydrocarbon (BATH) according to a method similar that of Rosenberg (1984). The isolates were enriched in 20 ml BH broth medium supplemented with crude oil (1 %, w/v) for 7 days in an orbital shaker (120 rpm) at 30 $^O$C. Cells were harvested by centrifugation and re-suspended in sterile BH medium with crude oil. The optical density ($OD_{540nm}$) of the cell suspensions was adjusted to 0.5. Crude oil (0.5 ml) was added to cell suspension of 4.0 ml contained in screw capped glass test tubes and vortexed for 3 min. Change in $OD_{540nm}$ was recorded for cell suspension after allowing for the formation of crude oil and aqueous phases. Cell hydrophobicity was expressed as cell adherence (%) to the crude oil which was calculated as given in equation 1.

$$\text{Bacterial adherence}(\%) = \left(1 - \frac{A_C}{A_O}\right) \times 100 \qquad (1)$$

Where Ac and Ao were $OD_{540nm}$ of cell suspension after and before being shaken with crude oil, respectively.

2.8 Emulsification index

The emulsification index ($EI_{24}$) was measured using the method as described by Ilori *et al.* (2005). The emulsification stability of the isolates was determined by adding 2 ml (instead of 3 ml as used by Ilori *et al.*, 2005) of crude oil to equal volume of cell-free supernatant contained in 15 ml screw capped culture glass test tubes. The tubes were properly vortexed at high speed for 2 min and allowed to stand for 24 h. The emulsification index was calculated using the expression as shown in equation 2.

$$\text{Emulsification index}(EI_{24}) = \frac{\text{Height of emulsion}}{\text{Height of supernatant}} \times 100 \qquad (2)$$

2.9 Biodegradation assay

A 48 h old of each of the selected culture was adjusted with sterile distilled water to give absorbance of 0.5 at a wavelength of 540 nm with spectrophotometer (Bausch and Lomb). Two milliliter (2.0 ml) of the cell suspensions was transferred into 250 ml Erlenmeyer flasks containing 98 ml of sterile BH medium with 2% of Bonny-Light crude oil. The flasks were then incubated in a shaker at 200 rpm at $30^0$C for 20 days. Flask containing sterile BH medium and 2 ml of crude oil but without test organisms served as control. At every 4-day interval, aliquots of the culture were used for the estimation of cell density and residual crude oil. Three milliliter (3.0 ml) of culture sample was used to estimate the cell biomass by spectrophotometer technique while same volume of uninoculated sample served as a blank. Absorbance value was then converted to dry cell mass (g/l) by a calibration curve (y = 0.008x) which was obtained by plotting dry cell weight against absorbance. Residual crude oil in both inoculated and uninoculated flasks was determined as adopted from Rahman *et al*. (2002). Cell free culture samples were mixed with equal volume of n-hexane (3.0 ml) in screw capped glass test tubes to extract crude oil. The tubes were vortexed thoroughly for 60 sec and thereafter the extracted crude oil was detected spectrophotometrically at 340 nm. A standard curve prepared using known concentrations of crude oil was used to estimate the amount of hydrocarbons in the sample. The percentage biodegradation of the crude oil was determined using the expression in equation 3:

$$\%\,\text{Degradation} = \left(\frac{A-B}{A}\right)\times 100 \qquad (3)$$

Where A and B were the residual crude oil in the control and test samples, respectively.

2.10 Statistical analysis

All the experiments were done in duplicates and the data mean values obtained were statistically compared using Analysis of Variance (ANOVA) with significance assumed if $P < 0.05$.

## 3.0 Results and discussion

The microbial load of Eagle Island mangrove rhizosphere soil sample on different microbiological isolating media are depicted in Table 1. The microbial load of 2.06 x $10^6$, 5.1 x $10^3$ and 1.61 x $10^5$ CFU/g isolated on nutrient agar, sabouruad dextrose agar and mineral salt agar were total aerobic heterotrophic bacterial count, total fungal count and total hydrocarbon utilizing bacteria count, respectively. Similar total aerobic heterotrophic bacterial counts have been reported by Okpokwasili *et al*. (2012) after assessing the microbial flora of the site of this study. Generally, there was more bacterial count than fungal count.

Out of sixty-one (61) bacterial strains isolated from rhizosphere soil sample of Eagle Island mangrove, eighteen (18) of the isolates were able to grow on both NA-crude oil medium and BHA-crude oil media, respectively, with varying diameters of zone of clearance around the colonies. The diameter of zone of clearance ranged from 1.7 to 3.4 mm (Table 2). This study has shown that the isolates from the mangrove ecosystem exhibited strong crude oil degradability as indicated by the formation of halo zones around their colonies. The largest and smallest diameter of zone of clearance was exhibited by RS12 and RS46, respectively. Among the test isolates that developed colonies on both NA-crude oil and BHA-crude oil media, four of the isolates were able to exhibit large halo zones and were identified as *Bacillus* sp. RS12, *Pseudomonas* sp. RS16, *Pseudomonas* sp. RS2, *Micrococcus* sp. RS38, respectively.

**Table 1: Total microbial load of rhizosphere soil sample of Eagle Island mangrove ecosystem**

| Total aerobic heterotrophic bacterial count (CFU/g) | Total fungal count (CFU/g) | Total Hydrocarbon-utilizing bacterial count (CFU/g) |
|---|---|---|
| $2.06 \times 10^6$ | $5.1 \times 10^3$ | $1.61 \times 10^5$ |

**Table 2: Diameter of zone of clearance on BHA plate by hydrocarbon degraders**

| Isolate | Diameter (mm) |
|---|---|
| RS12 | 3.4 |
| RS13 | 2.5 |
| RS15 | 2.4 |
| RS16 | 3.1 |
| RS19 | 2.4 |
| RS22 | 2.2 |
| RS23 | 3.1 |
| RS25 | 2.3 |
| RS26 | 2.1 |
| RS28 | 2.1 |
| RS31 | 2.2 |
| RS33 | 2.8 |
| RS34 | 2.5 |
| RS36 | 2.6 |
| RS38 | 3.2 |
| RS40 | 2.4 |
| RS46 | 2.7 |
| RS47 | 2.3 |

Table 3 shows the level of different petroleum hydrocarbon substrates utilized by the test isolates. The results indicated that the organisms could utilize crude oil and its petrochemical products, such as diesel, petrol, kerosene, engine oil and phenol, for growth. This agreed with the reports of Ezekoye *et al.* (2015), John and Okpokwasili (2012) and Nweke *et al.* (2006) that mangrove bacteria was an excellent degrader of crude oil and had ability to utilize crude oil and its products.

**Table 3: Mangrove bacteria growth test on crude oil and different hydrocarbons**

| Substrates | Bacteria | | | |
|---|---|---|---|---|
| | *Bacillus* sp. RS12 | *Pseudomonas* sp. RS16 | *Pseudomonas* sp. RS23 | *Micrococcus* sp. RS38 |
| Crude oil | ++ | ++ | ++ | ++ |
| Diesel | ++ | ++ | ++ | ++ |
| Petrol | ++ | ++ | ++ | ++ |
| Kerosene | ++ | ++ | ++ | ++ |
| Engine oil | ++ | ++ | ++ | ++ |
| Hexane | - | - | - | - |
| Benzene | - | + | + | - |
| Toluene | + | + | + | - |
| Xylene | - | - | - | - |
| Phenol | + | + | + | + |

Growth was followed by measuring the increase in optical density ($OD_{540nm}$) of the culture after 14 days incubation

++ = Heavy growth: OD 540nm > 0.2

+ = Moderate growth: OD 540nm > 0.1

- = No growth: OD 540nm < 0.02

The adhesion to crude oil by test isolates ranged between 58.82 to 68.40 %. Among the strains studied, *Micrococcus* sp. RS38 and *Pseudomonas* sp. RS23 had the lowest and highest percentage of adhesion to crude with adhesion values of 58.82 and 68.40 %, respectively (Table 4). Strains are normally divided into three groups according to their percentage of adhesion (% Adh) to crude oil: % Adh < 20 describes low adhesion, 20 < % Adh < 40 describes middle adhesion and % Adh > 40 describes high adhesion (Vasileva-Tonkova & Gesheva, 2004). Stelmack *et al.* (1999) in their report on bacterial adhesion to soil contaminants in the presence of surfactant said that hydrophobicity is an important factor in the initial adhesion of microorganisms to the interface between the non-aqueous and the aqueous phase. A number of species of

bacteria are able to degrade liquid hydrocarbons after adhering to the surfaces of droplets. This direct contact between a bacterial cell and a target hydrocarbon plays a major role in the rate of diffusion into the cell, thereby enhancing growth and increasing the rate of dissolution of the hydrocarbon (Zhang *et al.*, 2009). Such high values of adhesion to crude oil existed for the test isolates in this study.

**Table 4: Estimation of percentage adhesion and emulsification index of the bacterial strains to crude oil**

| Bacteria | % Adhesion | Emulsification index (%) |
|---|---|---|
| *Bacillus* sp. RS12 | 50.70 | 54.63 |
| *Pseudomonas* sp. RS16 | 55.51 | 57.70 |
| *Pseudomonas* sp. RS23 | 58.40 | 60.32 |
| *Micrococcus* sp. RS38 | 48.82 | 53.57 |

Emulsification assay is an indirect technique used to assess for biosurfactant production by microbial cells. It was assumed that if the cell-free culture broth used in this assay contained biosurfactant, then it would emulsify the hydrocarbons present in the test solution (Thavasi *et al.*, 2011). In this study, crude oil was used as the hydrophobic substrate. Results observed in this study revealed that all the test isolates showed positive emulsification activity. Table 4 also depicts the various ranges of emulsification index of the organisms with *Pseudomonas* sp. RS23 exhibiting the highest emulsification index value of 60.32 % while the lowest emulsification index value of 53.57 % was found in *Micrococcus* sp. RS38. The emulsification index of crude oil by test organisms were in the following order: *Micrococcus* sp. RS38 (53.57 %) > *Bacillus* sp. RS12 (54.63 %) > *Pseudomonas* sp. RS16 (57.70 %) > *Pseudomonas* sp. RS23 (60.32 %).

The utilization of the crude oil as sole carbon and energy source by the test organisms resulted in their growth with time. However, the percentage of crude oil degraded was observed to increase with increase in incubation period but varied with different bacterial strains tested, as shown in Fig. 1. *Pseudomonas* sp. RS23 exhibited the highest capability to degrade crude oil with 45.8 % degraded after 20 days of incubation. The crude oil degrading capability of *Pseudomonas* sp. RS16 was also remarkable (42.2 %) over the same period of incubation. The least was *Micrococcus* sp. RS38 with 36.4 % crude oil degraded. The statistical analysis indicated that biodegradation of crude oil did not differed significantly among the rhizophere bacteria of Eagle island mangrove ecosystem but differed significantly from the control ($P < 0.05$). Though *Micrococcus* sp. RS38 showed impressive level of growth during screening in crude oil and other petroleum products (Table 3) where the organisms grew at equal optical densities of > 0.2 within 14 days of incubation. The results obtained on the degradative ability of the test organisms indicated that they maximally utilized the crude oil .This might be as a result of the ability of the organisms to utilize the hydrocarbons as substrates for growth by probably releasing extracellular enzymes which were capable of breaking down the recalcitrant hydrocarbon molecules by dismantling the long chains of hydrogen and carbon thereby converting them into simpler products that can be absorbed for their growth. The growth of the 4 isolates showed that all the isolates found to have had their exponential phase between 4 and 12 days with the stationary phase occurring at day 20. This showed that the test isolates could take up crude oil to produce biomass.

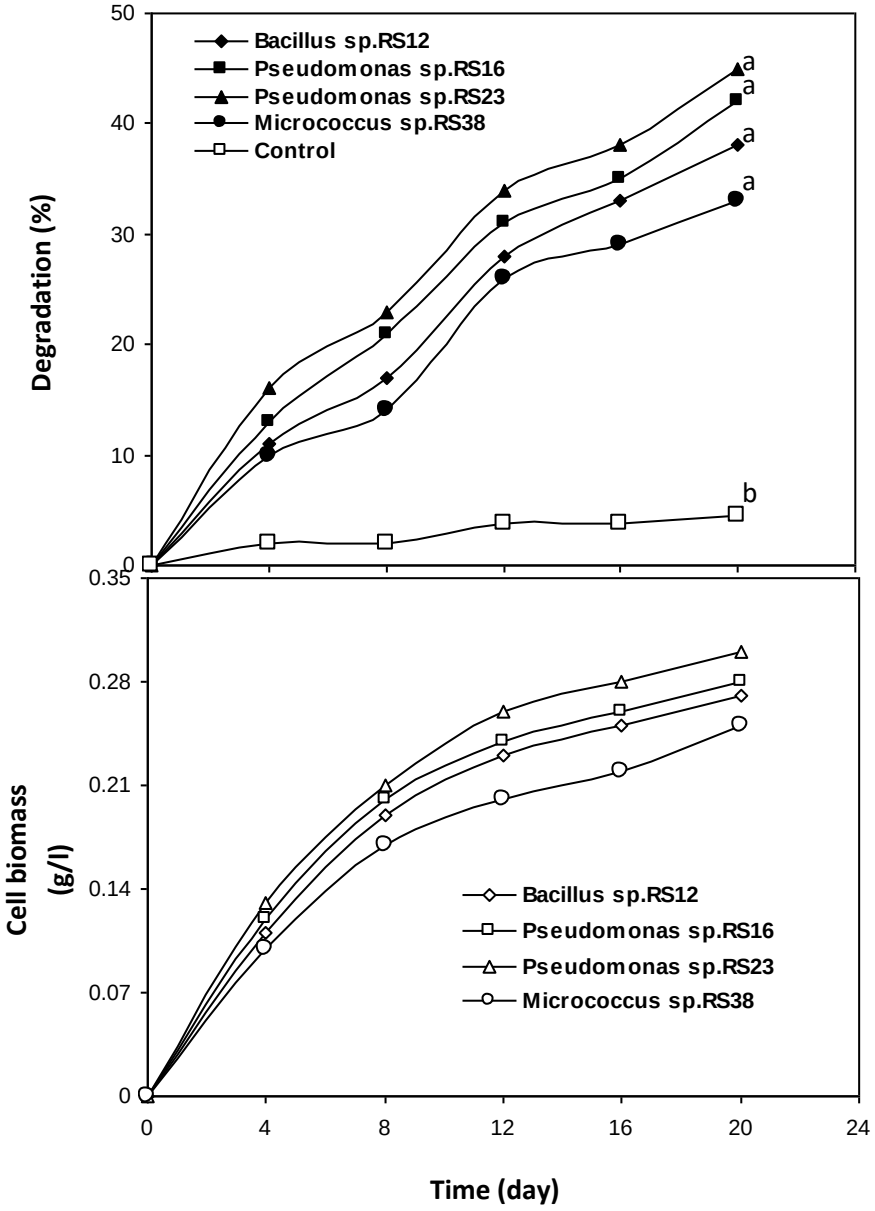


**Figure 1: Time course of crude oil reduction by mangrove bacteria and their biomass formation during biodegradation of crude oil in BH medium. Values with the same letter (a, b) are not significantly different ($p < 0.05$)**

In conclusion, the findings in this study showed that the test isolates could be useful in bioremediation of sites and wastewaters that are contaminated with crude oil and other petroleum products.

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