Original Research Article

Screening for biodegradation potential of Endophytic Bacteria isolated from the roots and leaves of Mangrove plants (*Avicennia germinans (*Black mangrove*), Acrostichum aureum (*golden leather fern) and *Rhizophora mangle* (Red mangrove).

ABSTRACT

Aim: The aim of this study is to screen and determine the biodegradation potential of the endophytic bacteria isolated from roots and leaves of mangrove plants.

Methodology: In this work were isolated, identified and screened endophytic bacteria from roots of *Rhizophora mangle*, *Avicennia germinans and Acrostichum aureum; the* three major species of mangrove plants found in the Niger Delta. The roots were transported in a sterile bag to the Microbiology Laboratory, treated using standard Microbiological techniques. The organisms isolated include: *Pseudomonas* sp, *Bacillus* sp, *Staphylococcus* sp, *Micrococcus* sp, *Klebsiella* sp, *Azotobacter* sp, *Nitrobacter* sp. and *Nitrosomonas* sp. These organisms were screened for their ability to degrade crude oil by analyzing them using an ultra-violet spectrophotometer at a wavelength of 600nm and through a colorimetric test that involves the use of 2,6, Dichlorophenol-indophenol (DCPIP) as the metabolic activity indicator for a 14 days period.

Results: According to the colorimetric test, isolates that were positive hydrocarbon degraders were observed by a colour change from blue to colourless, which include *Pseudomonas* sp, *Staphylococcus* sp, *Bacillus* sp, *Klebsiella* sp *and Nitrobacer sp,* while those that changed from blue to pink (an indication of a negative reaction) were: *Nitrosomonas* sp*, Bacillus* sp*, Micrococcus* sp*, Staphylococcus* sp*, Azotobacter* sp *and Klebsiella* sp. Amount degraded and percentage biodegradation ranged from M*icrococcus* sp 278.6 (6.2%) < *Azotobacter* 1242.8 (17.8%) < *Nitrosomonas* 1392.9 (19.9%) < *Staphylococcus* sp 1543.0 (22.1%) < *Bacillus* sp 17927.8 (25.6%) < *Nitrobacter* sp 1935.7 (28%) < *Klebsiella* sp 3392.9 (48.5%) < *Bacillus* sp 3671.4 (52.5%) < *Pseudomonas* sp 4942.8 (70%).

Conclusion: *Pseudomonas* and *Bacillus* species show biodegradation potential above average; they demonstrated the highest potential to degrade crude oil and can therefore be used in bioremediation of water ecosystem impacted by crude oil. Therefore, the scientific contribution of this research is related to identifying several culturable groups of bacteria that might be directed to these further biotechnological approaches.

Keywords: Endophytic bacteria, Mangrove roots, biodegradation

1.0 Introduction

Mangrove ecosystems are of great ecological and economic importance as they play various vital roles at the land-sea interface, provide food, breeding grounds and nursery sites for a variety of terrestrial and marine organisms, used for human sustainability and livelihoods (food, timber, fuel and medicine), and they also offer protection against catastrophic events, such as tsunami, tropical cyclones and tidal bores and can dampen shoreline erosion [1].

Mangrove forests occur at the interface of terrestrial and marine ecosystems portraying a rich biological diversity of plants, animals and microorganisms. Microbes are important part of the mangrove environment as they contribute to the productivity of the mangrove ecosystem. They play a very critical role in creating and maintaining this biosphere and also serve as a source of biotechnologically valuable and important products by participating in various steps of decomposition and mineralization of leaf litter; by being able to recycle nutrients, produce and consume gases that affect global climate, destroy pollutants, treat anthropogenic wastes and can also be used for biological control of plant and animal pests. Microorganisms from mangrove environments are a major source of antimicrobial agents and also produce a wide range of important medicinal compounds, including enzymes, antitumor agents, insecticides, vitamins, immunosuppressants and immune modulators [2].

UNDER PEER REVIEW

Endophytes are microorganisms that live in plant tissues partly or in all their lifecycle without causing any symptoms of disease in the host [3]. These microbes are often bacteria or fungi found in various plant organs such as seeds, roots, stem, leaves, flowers and fruits that colonize host tissues similarly to pathogens. Studies have shown that the endophytic association with the host contributes significantly in accelerated seedling emergence, enhanced plant growth, improved resistance against various phytopathogens and abiotic stresses. Endophytic microorganisms can safeguard the hosts against numerous biotic and abiotic factors such as attack of insects, pathogens and herbivores [4].

Various groups of bacteria are found in the mangrove ecosystem where they are known to perform diverse activities including photosynthesis, nitrogen fixation and methanogenesis [5][6][7]. The over-exploitation of mangroves is of today's concern because it threatened the sustainability of the ecosystems. Microorganisms from mangrove ecosystems contain useful enzymes, proteins, antibiotics and salt tolerant genes, all of which have biotechnological significance [2]. Little is known about bacterial mangrove communities, but these microorganisms may have high biotechnological potential [8]. Recently developed technologies in molecular biology and genetics offer great promise to explore the potential of microbial diversity. The objective of this study is to isolate and screen the endophytic bacteria from the roots of mangrove plants within a crude oil polluted environment and to determine their biodegradability potential.

2 MATERIALS AND METHODS

2.1 Description of Study Area

The roots of mangrove plants used in this study were collected from Creek Road water front (Bonny Jetty) Old Port Harcourt Township, Rivers State, Nigeria. The area has lots of mangrove species and also was an area that witnesses lots of pollution from wastes and activities from speedboats transporting passengers, carpenters building boats, boats transporting wood, diesel, fuel and even crude oil from illegal oil refineries as their products are sometimes discharged, dumped or even burnt in the river. The location is situated at Longitude 4^{0} $45^{'}$10.13976''N and Latitude 7^{0} $1^{'}$4.13012$^{''}$ E.

*Image 1: Acrostichum aureum (*golden leather fern) Image 2: *Rhizophora mangle* (Red mangrove)

2.2 Collection of Materials

The plants collected were placed separately in sterile polyethylene bags and transported first to Department of plant science laboratory for identification and then to Microbiology laboratory, Rivers State University Port Harcourt for processing.

Image 3: Rhizophora racemosa leaves

Image 4: Rhizophora racemosa roots

Image 5 Acrostichum aureum

Image 6:*Acrostichum aureum* roots

Image 7 Avicennia germinans leaves

Imge 8: *Avicennia germinans* roots

MEDIA USED:

Nutrient Agar (TM Media) (g/L); Beef extract (1.50), Yeast extract (1.50), Peptic digest animal tissue (5.00), Sodium chloride (5.00), pH 7.4±0.2 at 25°C.; Phosphate Buffered Saline (g/l); Na_2HPO_4 (1.44), Sodium Chloride (3), Potassium Chloride (0.2), KH_2PO_4 (0.24); Mineral Salt Agar (g/l); K_2HPO_4 (0.5), $MgSO_4.7H_2O$ (0.3), NaCl (0.3), $MnSO_4.H_2O$ (0.02), $FeSO_4.6H_2O$ (0.03), $NaNO_2$ (0.03), Agar (0.5), Fungusol, Distilled water (200ml).; Burks-N-free Agar; Glucose (10), K_2HPO_4 (0.41), K_2HPO_4 (0.52), Na_2SO_4 (0.05), $CaCl_2$ (0.2), $MgSO_4.7H_2O$ (0.1), $FeSO_4.7H_2O$ (0.005), Agar (15.0), Distilled water (1000ml); Winogradsky (Nitrosomonas); $(NH_4)^2SO_4$ (2.0), K_2HPO_4 (1.0), $MgSO_4.7H_2O$ (0.5), NaCl (2.0), $FeSO_4.6H_2O$ (0.4), CaCO3 (0.01), Agar (15.0), Distilled water (1000ml); Winogradsky (Nitrobacter); KNO_2 (0.1), Na_2CO_3 (1.0), NaCl (0.5), $FeSO_4.7H_2O$ (0.4), Agar (15.0), Distilled water (1000ml).

Sample Preparation/Processes

The roots of the mangrove plants were treated to obtain only the endophytic bacteria. The roots were first washed under running water thoroughly to remove surface adhering debris. They were cut into small pieces, which were washed in sterile distilled water for 5 mins, surface-sterilized with 70% ethanol for 1 min, 3% sodium hypochlorite (NaOCl solution) for 3 mins and then rinsed 6 times in sterile distilled water in different containers. They were then grounded separately with already sterilized mortar and pestle to make slurries. Ten fold serial dilution of the plant slurry was made up 10^6. Thereafter, an aliquot of 0.1ml of the appropriate dilutions was inoculated aseptically into different properly dried media; Burk's N-Free Agar(10^3), Winogradsky Agar (10^3) (for both *Nitrosomonas* and *Nitrobacter*), Mineral Salt Agar (10^3) and Nutrient Agar (10^6). Using vapour phase diffusion method, filter paper were soaked with 0.2ml crude oil and placed on the cover of the plate containing Mineral Salt Agar. The cultured plates were incubated at 37°C for 24 hours for the nutrient agar, the modified Winogradsky by [6] used was incubated at 37°C for 3 days while the previous Winogradsky agar was incubated for 5-7 days and finally the Mineral Salt Agar was incubated at same 37°C for 5-7 days. After incubation, bacterial colonies were differentiated and counted based on their morphological characteristics. Individual colonies were picked randomly and sub-cultured by streaking them onto nutrient Agar plates using the streak plate technique and incubated at 37ºC [9][6].

Chart 1: Screening Setup

Isolates	Volume of MSA (ml)	Volume of Crude oil (ml)	Volume Broth of Organisms (ml)	Total volume (ml)
Control	0.5	0.5 (0.45g)	0.5	10
Pseudomonas aeruginosa (MW369466)	0.5	0.5 (0.45g)	0.5	10
Pseudomonas aeruginosa (MN314747).	0.5	0.5 (0.45g)	0.5	10
Micrococcus sp	0.5	0.5 (0.45g)	0.5	10
Azotobacter sp	0.5	0.5 (0.45g)	0.5	10
Staphylococcus sp	0.5	0.5 (0.45g)	0.5	10
Brevibacillus brevis	0.5	0.5 (0.45g)	0.5	10
Staphylococcus sp	0.5	0.5 (0.45g)	0.5	10
Bacillus sp	0.5	0.5 (0.45g)	0.5	10
Nitrosomonas sp	0.5	0.5 (0.45g)	0.5	10
Bacillus amyloliquefaciens	0.5	0.5 (0.45g)	0.5	10
Nitrobacter sp	0.5	0.5 (0.45g)	0.5	10
Klebsiella sp	0.5	0.5 (0.45g)	0.5	10

UNDER PEER REVIEW

Klebsiella Pneumoniae	0.5	0.5 (0.45g)	0.5	10

Screening of Isolates

The microbial isolates were screened twice. Overnight pure culture was used for the screening. Broth of the organisms was first prepared by transferring the pure culture into properly labelled test tubes containing normal saline (that has been autoclaved and allowed to cool and agitated).

The method described by [10] was applied. 9ml of MSA (======), tween 8 (surfactant), Dichlorophenol-indophenol (indicator) and 0.5ml of crude oil were put into test tubes. After capping, all the test tubes were autoclaved at 121°C at 15PSI for 15 mins, then allowed to cool and labeled, 0.5ml of the broth (standardized bacterial cell suspension of the respective bacterial isolates) was transferred into the labelled test tubes containing crude oil. The test tubes which served as controls were not inoculated. All test tubes were incubated for 14 days and their potential to utilize hydrocarbon was determined by observing the colour changes in the test tube on day 7 and 14 and then each tube was scored for optical density (OD) using a spectrophotometer on preparation day (Day1), day 7 and 14 respectively to measure the amount of light absorbed by the solution so as to determine the organism that has more potential to degrade hydrocarbon as described by [11]. The optical density of the culture was measured at 600nm. The change in colour from blue to colourless was an indication that hydrocarbon degradation had occurred in those test tubes while those that changed from blue to pink was indication of a negative result for hydrocarbon degradation. The endophytic bacteria with more potential to degrade crude oil were used for the remediation study.

Three of the endophytic bacteria that showed more potential to degrade crude oil were used for the remediation study.

The concentration of absorbance was calculated using the calibration curve for THC(=====) as adopted by [6][12] as follows:

$$Y = 0.28 + 0.0609$$

Y = absorbance

X = mg/ml + conc of oil in xylene

$$X = \frac{Y - 0.0609}{0.28}$$

$$THC = \frac{X \times 1000}{0.5}$$

Where X = concentration calculated from absorbance

1000 = Value used to convert from g to mg

0.5 = quantity of crude oil used — equation 1

Percentage (%) Biodegradation Rate

This was calculated using the formula adopted by [12] as follows.

Step 1: amount of pollutant remediated equals initial concentration of pollutant (Day 1) minus Final concentration of pollutant at the end of experiment.

Step 2: percentage (%) bioremediation = amount of pollutant remediated divided by initial concentration of pollutant (Day 1) multiplied by 100.

$Bc = Ic - Fc$

$Bx = Ic - Io$

Where,

Bc = Amount of pollutant degraded

Ic = initial concentration of pollutant (Day 0)

Fc = final concentration of pollutant at end of experiment

Bx = Actual amount of pollutant in test medium

Io = = initial concentration value of control of crude oil polluted water at Day 0

$$\%Bioremediation = \frac{Bc \times 100}{Bx}$$

UNDER PEER REVIEW

RESULTS AND DISCUSSION

Microbial Isolation of the test Organisms

The mangrove plant samples used were identified in the department of plant science laboratory as: *Avicennia germinans (*Black mangrove*), Rhizophora mangle* (Red mangrove) and *Acrostichum aureum (*golden leather fern).

Characteristics and identification of bacterial isolates

Eight (8) bacterial genera were isolated from the mangrove plant which include; *Pseudomonas* sp*, Micrococcus* sp*, Nitrobacter* sp*, Nitrosomonas* sp*, Staphylococcus* sp*, Azotobacter* sp*, Klebsiella* sp *and Bacillus* sp.

Screening of the isolates using spectrophotometer

The results of the screening using a spectrophotometer to determine the organisms with the highest biodegradation potential is presented below in table 1. According to the results, *Pseudomonas aeruginosa* (MN314747) degraded the hydrocarbon most at a percentage (%) value of 71% (4942.8), followed by *Brevibacillus brevis* at 59% (4100), then *Bacillus amyloliquefaciens* at a value of 53% (3671.4), followed by *Klebsiella Pneumoniae* at a value of 49% (3392.9), then *Pseudomonas aeruginosa* (MW369466 at 35% (2450), then *Klebsiella* sp with a degradation value of 34.1% (1957.1), followed by *Nitrobacter* sp with a value of 28% (1935.7), followed by *Bacillus* sp with a degradation value of 26% (1792.8), then *Staphylococcus aureus* with a degradation value of 21.1% (1473.1), then *Nitrosomonas* sp with a value of 20% (1392.9), followed by *Azotobacter* sp with a degradation value of 18% (1242.8), then *Staphylococcus* sp with a value of 17.3% (1210.7) while *Micrococcu*s sp had the lowest biodegradation potential at a value of 6% (434.6).

Table 1 biodegradation potential

Isolates	Day 1	Day 7	Day 14	Amt degraded (mg/L)	% degraded
Control	6993.6	5672.1	5429.3	1564.4	23.5%
Pseudomonas aeruginosa (MW369466)	4993.6	3036.4	2543.6	**2450**	**35%**
Pseudomonas aeruginosa (MN314747).	6036.4	3050.7	1093.6	**4942.8**	**71%**
*Micrococcu*s sp	4122.2	3842.9	3687.6	434.6	6%
Azotobacter sp	4529.2	4065	3286.4	1242.8	18%
Staphylococcus sp	5978.5	5075.6	4767.8	1210.7	17.3%
Brevibacillus brevis	5993.6	4865	1893.6	**4100**	**59%**
Staphylococcus sp	5922.7	4927.5	4449.6	1473.1	21.1%
Bacillus sp	5779.2	6350.8	3986.4	1792.8	26%
Nitrosomonas sp	6107.9	5229.3	3715	1392.9	20%
Bacillus amyloliquefaciens	6272.1	4315	2600.7	**3671.4**	**53%**
Nitrobacter sp	5908.5	4321.6	3951.4	1957.1	28%
Klebsiella sp	5707.9	4993.6	3322.1	2385.8	34.1%
Klebsiella Pneumoniae	5186.4	6536.4	1793.5	**3392.9**	**49%**

Screening using surfactant and indicator

For the screening using tween 80 (surfactant) and Dichlorophenol-indophenol (indicator) the result is presented in Table 2; the colour change from blue to colourless indicates a positive result to hydrocarbon degradation and those that changed from blue to pink is an indication of a negative result. According to the results *Pseudomonas aeruginosa* (MN314747), *Brevibacillus brevis*, *Bacillus amyloliquefaciens*, *Klebsiella Pneumoniae*, *Pseudomonas aeruginosa*, *Nitrobacter* sp and *Staphylococcus aureus* were

UNDER PEER REVIEW

positive as the turned colourless by day 14, while *Azotobacter* sp, *Staphylococcus* sp, *Micrococcu*s sp, *Bacillus* sp and *Nitrosomonas* sp turned pink by day 14 showing a negative result.

Table 2 Screening using surfactant and indicator

Isolates	Reaction	Colouration
Pseudomonas aeruginosa (MW369466)	+ve	Colourless
Pseudomonas aeruginosa (MN314747).	+ve	Colourless
*Micrococcu*s sp	-ve	Pink
Azotobacter sp	-ve	Pink
Staphylococcus sp	-ve	Pink
Brevibacillus brevis	+ve	Colourless
Bacillus sp	-ve	Pink
Staphylococcus aureus	+ve	Colourless
Nitrosomonas sp	-ve	Pink
Bacillus amyloliquefaciens	+ve	Colourless
Nitrobacter sp	+ve	Colourless
Klebsiella sp	-ve	Pink
Klebsiella Pneumoniae	+ve	Colourless

Evaluating Hydrocarbon Utilizing Potential of the Endophytic Bacteria

Screening of the isolates was done using two different methods to evaluate their hydrocarbon utilizing potential. The readings from the spectrophotometer were used to determine the isolates with the highest biodegradation potential by subtracting the reading of Day 14 from the reading on Day 1 using the formula adopted by [6]. All the isolates showed ability to degrade hydrocarbon as its been reported that more than 79 bacterial genera e.g *Pseudomonas*, *Staphylococcus, Acinetobacter, Streptococcus, Enterobacter etc* have been found to play vital roles in petroleum hydrocarbon degradation [13][14][15][16]. According to the results, the amount degraded and percentage degradation rate are shown in figure 1 and 2 respectively. The rate of crude oil degradation is as follows: *Pseudomonas aeruginosa* (MN314747), which showed the highest hydrocarbon degradation potential most with a value of 4942.8mg/l at 71% > *Bacillus amyloliquefaciens* with a value of 4100mg/l at 59% > *Brevibacillus brevis* with a value of 3671.4mg/l at 53 > *Klebsiella Pneumoniae* with a value of 3392.9mg/l at 49% > *Pseudomonas aeruginosa* (MW369466) with a value of 2450mg/l at 35% > *Nitrobacter* sp with a value of 1957.1 at 28% > *Bacillus* sp 1792.8 (26%) > *Staphylococcus* sp 1473.1 (21.1%) > Nitrosomonas 1392.9 (20%) > *Azotobacter* sp 1242.8 (18%) > *Staphylococcus* sp 1210.7 (17.3%) > *Micrococcu*s sp 434.6 (6%). This confirms or emphasizes the thought of [17] that *Pseudomonas* and *Bacillus* species are more adapted to survival and biodegradation in marine environments. [18] also reported *Pseudomonas* sp as the best degrader when compared with others in their study.

The surfactant and indicator were also used to identify the hydrocarbon degraders by monitoring the colour change at the end of the 14 days. Seven of the thirteen isolates (*Staphylococcus* sp, *Pseudomonas aeruginosa* (MW369466), *Klebsiela pneumonia*, *Bacillus amyloloquefaciens*, *Brevibacillus brevis*, *Pseudomonas aeruginosa* (MN314747), *Nitrobacter* sp) showed a positive reaction as they turned colourless while six (*Micrococcus* sp, *Klebisella* sp, *Azotobacter* sp, *Staphylococcus* sp, *Bacillus* sp and *Nitrosomonas* sp) showed a negative reaction as they turned pink.

The screening using surfactant and indicator were compared against the screening with spectrophotometer and the organisms with highest degradation rate showed a positive result with a colour change from blue to colourless. The five (5) isolates that showed higher degradation rate changed to colourless confirm the hydrocarbon degrading ability. They were then picked for further study.

UNDER PEER REVIEW

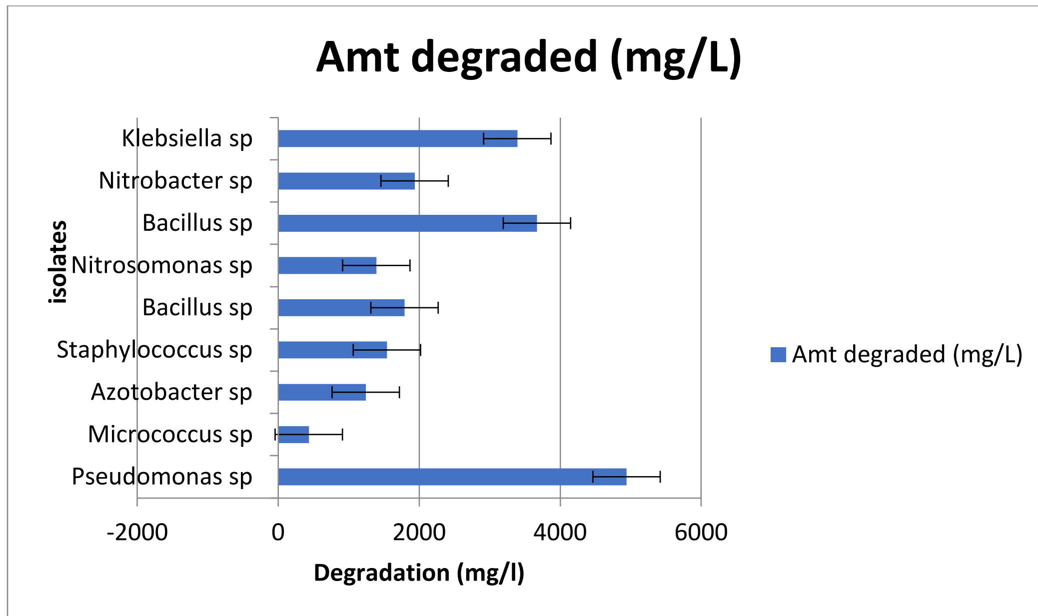

Figure 1: Amount of crude oil degraded by the isolates

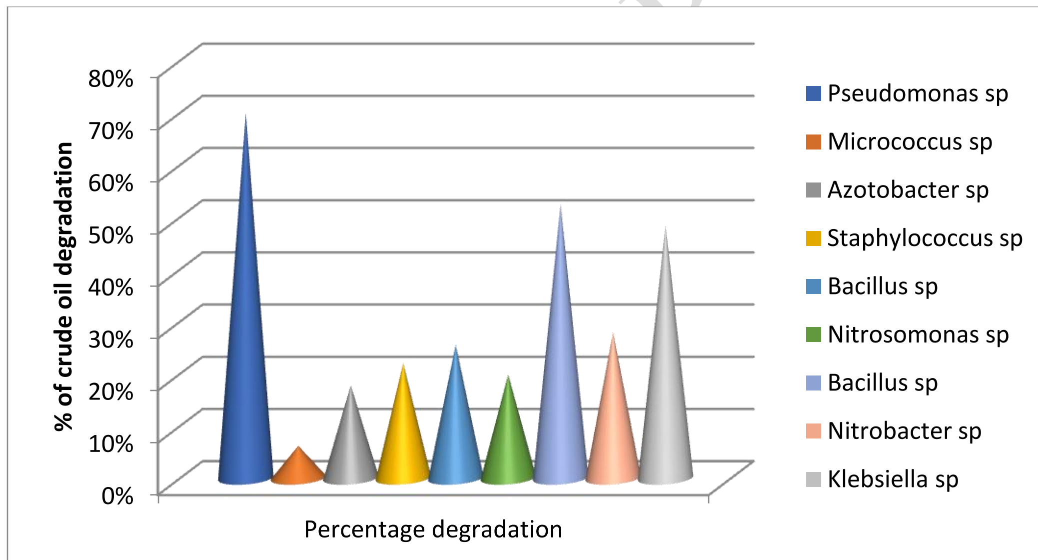

Figure 2: percentage (%) degradation rate

Conclusion

The results revealed high numbers of active endogenous bacteria in the roots of the mangrove plants, many of which are known to possess catabolic abilities and have the ability to degrade crude oil. These mangrove roots may harbour bacterial genera that may play important role in the nitrogen cycle and can also bring about bioremediation of polluted environment. Therefore a consortium of these bacteria can be used to clean up oil spills in hydrocarbon polluted environment.

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IJSER PEER REVIEW

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