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Identification of Bacteria Isolated from Crude Oil Polluted Fish Pond Undergoing Bioremediation

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Authors' contributions

This work was carried out in collaboration between the two authors. Author OFO designed the study and wrote the protocol. Author OOO wrote the first draft of the manuscript and managed literature searches. Both authors read and approved the final manuscript.

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ABSTRACT

The study was carried out to identify bacteria associated with crude oil polluted water collected from the Fish pond, Department of Fisheries and Aquaculture, Federal University of Technology Akure, Ondo State Nigeria. The water samples were aseptically collected into several capped bottles and transported to the laboratory, Department of Microbiology, for microbiological analyses and were analyzed at interval of two weeks using pour plate techniques. Nutrient agar (NA) was used for the isolation of bacteria. The bacterial isolates were identified by morphological and biochemical characterization using the taxonomic scheme of Bergey's manual of Determinative Bacteriology. The bacteria obtained from the polluted water were *Bacillus cereus, Bacillus subtilis, Pseudomonas aeruginosa* and *Micrococcus luteus.* The total bacterial counts obtained from the polluted samples ranged from $2.5x10^6$ to $6.0 x10^6$ Cfu/mL at 0.25% and $4.0x10^6$ to $7.0x10^6$ Cfu/mL in 1.5% crude oil polluted water while unpolluted samples ranged from $5.2 x 10^4$ to $5.8x10^4$ Cfu/mL. *B. cereus* and *B. subtilis* had the highest frequency of occurrence of 26.3% and 21% respectively while *Staphylococcus* spp., *Streptococcus* spp. and *Escherichia coli* had the least. The bacterial species obtained in this work can be further subjected to degradation tests.

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1. INTRODUCTION

The world depends on oil. Vast amount of this oil is used, transported, processed and stored around the world. In 2003, the total world consumption of petroleum was over 13.1 billion liters per day. According to the United States Energy Information Administration projects, the world consumption of oil would increase to 98.3 million barrels per day ($15.63x10^6$ m^3 day^{-1}) in 2015 and 118 million barrels day^{-1} ($18.8x10^6$ m^3 day^{-1}) in 2030 [1]. With such a high consumption, oil spills are inevitable. An oil spill is the release of liquid petroleum hydrocarbon into the environment, especially marine areas, due to human activity and is a form of pollution. Oil spillage has a major impact on the ecosystem into which it is released, destroying crops and aquacultures through contamination of the groundwater and soils [2]. People in the affected areas are prone to health issues including breathing problems and skin lesions; many have lost basic human rights such as health, food accessibility, clean water and an ability to work. A diverse group of microorganisms are implicated in biodegradation of waste products in both aquatic and terrestrial habitats [3]. Accidental releases of petroleum products are of particular concern in the environment and this has led to a concerted effort in studying the viability of using oil-degrading microorganisms for bioremediation [4].

Bioremediation has long been applied as a treatment technology that is cost-effective, ecologically friendly and efficient for the decontamination of hydrocarbon polluted soils [5-7]. Microorganisms are capable of utilizing the petroleum hydrocarbons as their carbon and energy source, thereby, degrading the contaminants [8]. The ability to degrade hydrocarbon substrates is exhibited by a wide variety of bacteria genera and therefore contributes to bioremediation.

2. MATERIALS AND METHODS

2.1 Sample Collection

The source of the water samples collected from the Fish pond in the Fisheries and Aquaculture Department, Federal University of Technology Akure, Ondo State, Nigeria was Ala River, Akure, Nigeria. (Twenty) 20 ml of the water sample was collected using a sterile universal bottle and was taken to the laboratory for microbiological analyses.

2.2 Enumeration of Total Bacteria in Water Samples

One ml of the water sample was diluted with 9ml of sterile distilled water to make the stock solution and the progressive serial dilution of stock solution of 10^{-1} to 10^{-9} was done. This was cultured using pour plate method. One ml of the solution was pipetted into labelled sterile Petri dishes, after which cooled and molten agar medium was poured on it and swirled gently for even distribution of bacteria in the plate. Then the plates were allowed to set or solidified and immediately incubated upside down to avoid condensation for 24 hrs at 37℃. After incubation, the plates were observed for bacterial growth [9].

2.3 Sub Culturing of Bacterial Isolates

Sub culturing of bacterial isolates were done after 24 hrs. Bacterial colonies were picked with sterile inoculating loop and were streaked on freshly nutrient agar plates. The plates were incubated at 37℃ for 24 hrs.

2.4 Cultural, Biochemical and Morphological Characterization of Bacterial Isolates

2.4.1 Gram Stain

A loopful of sterile distilled water was dropped on a clean grease free slide by using a sterile inoculating loop after which an inoculum from the culture was mixed with the water on the slide. The smear was allowed to air dried and then heat fixed gently by passing it quickly over a Bunsen flame. The smear was flooded with crystal violet solution for 60 seconds (one minutes) and rinsed with water. The smear was again flooded with Lugol's iodine for 30 seconds and rinsed with water, 70% alcohol was poured on the slides for 15 seconds until the crystal violet had been completely washed off. It was then counterstained with Safranin for 60 seconds and allowed to dry. The slides were then observed under oil immersion objective. Gram positive cells remained purple while Gram negative cells appeared red or pink [9].

2.4.2 Catalase test

A drop of hydrogen peroxide solution was placed on a clean grease free slide. A flamed inoculating loop was used to place a loopful of an inoculum on the slide and gently mixed after which it was observed for bubbles or effervescence which is an indication of catalase positive organism [9].

2.4.3 Motility test

A little immersion oil was placed round the edge of the depression of a cavity slide and then a loopful of the bacterial colony was transferred to the centre of a clean dry cover slip with a sterile inoculating loop. The cavity slide was inverted over the cover slip such that the culture drop was in the centre of the slide depression. The culture drop appeared hanging. This was examined immediately for motility under the oil immersion microscope [9].

2.4.4 Spore staining

A heat fixed smear was prepared after which malachite green solution was added and steamed for 10 minutes ensuring that the stain did not dry out. It was washed with cold water, counter stained with Safranin solution for 15 seconds after which it was washed with water. It was then blot dried and examined for spores under the microscope with the oil immersion objective. Spores stained green while bacteria cells stained red [9].

2.4.5 Indole test

Three millimeters of 1% Tryptone broth was placed into different tubes after which a loopful of the bacterial isolates were inoculated into different test tubes leaving one of the tubes uninoculated to serve as the control. The test tubes were then incubated at 37℃ for 48 hours. After incubation, 0.5 ml of Kovac's reagent was added and shaken gently after which it was allowed to stand for 20 minutes to permit the reagent to rise to the top. A red colour at the surface of the tubes indicated a positive result while a yellow colouration of the surface layer indicated a negative result [9].

2.4.6 Coagulase test

A loopful of normal saline solution was placed on each glass slide and was emulsified. Human plasma was added to one of the suspension and was stored properly for 15 minutes while the other was left as control. Coagulase positive was indicated by clumping which did not re- emulsify [9].

2.4.7 Sugar fermentation test

Nine milliliters of nutrient broth was placed into different test tubes and 1 ml of the sugars was added into the different test tubes after which Durham tubes were placed inside the test tubes. The test tubes were then covered with cotton wool, sterilized and then allowed to cool. The organisms were then inoculated into the test tubes and they were incubated at 37℃ along side an uninoculated test tube which serves as a control. It was checked at 24 hrs and 48 hrs for colour change and gas production [9].

3. RESULTS

3.1 Bacterial Load from Crude Oil Polluted Water Sample

Bacterial loads obtained from unpolluted and polluted water samples collected from the fish pond from the Department of Fisheries and Aquaculture, Federal University of Technology Akure, Ondo State, Nigeria are presented in Table 1. The bacterial loads ranged from 5.2 $x10^4$ to $5.8x10^4$ Cfu/mL in the unpolluted water samples while in 0.25% of crude oil polluted water, it ranged from $2.5x10^6$ in week 2 to 6.0 $x10^6$cfu/ml in week 6 and from $4.0x10^6$ in week 10 to $7.0x10^6$ CFU/mL in week 2 in 1.5% crude oil polluted water.

3.2 Morphological and Biochemical Characteristics of Bacterial Isolate from Unpolluted, Polluted and Inoculated Water Sample

3.2.1 Unpolluted samples

Morphological and biochemical characteristics of bacterial isolates from non-contaminated water sample are presented in Table 2. The isolates identified were *Staphylococcus aureus, Escherichia coli, Bacillus cereus*, *Pseudomonas aeruginosa*, *and Streptococcus* sp. All the isolates were Gram positive with the exception of *P. aeruginosa* and *Escherichia coli*. *Bacillus cereus* was positive to spore staining test while *Staphylococcus aureus* was coagulase positive.

Table 1. Bacterial load (Cfu/mL) from crude oil polluted water sample

Concentration (%)	Dilution factor	Week 2	Week 4	Week 6	Week 8	Week 10	Week 12	Week 14
Unpolluted	10^{-2}	$7.0x10^{3}$	$5.6x10^{3}$	$6.0x10^{3}$	$6.5x10^{3}$	$6.8x10^{3}$	$6.6x10^{3}$	$6.5x10^{3}$
	10^{-3}	$5.5x10^{4}$	$5.8x10^{4}$	$5.4x10^{4}$	$5.3x10^{4}$	$5.6x10^{4}$	$5.2x10^{4}$	$5.5x10^{4}$
Polluted 0.25%	10^{-4}	$8.0x10^{5}$	$7.5x10^{5}$	$10.0x10^{5}$	$8.5x10^{5}$	$8.0x10^{5}$	$7.0x10^{5}$	$6.5x10^{5}$
	10^{-5}	$2.5x10^{6}$	$3.5x10^{6}$	$6.0x10^{6}$	$4.5x10^{6}$	$4.0x10^{6}$	$3.0x10^{6}$	$3.0x10^{6}$
1.5 %	10^{-4}	$7.5x10^{5}$	$5.0x10^{5}$	$8.0x10^{5}$	$5.5x10^{5}$	$5.0x10^{5}$	$4.8x10^{5}$	$4.7x10^{5}$
	10^{-5}	$7.0x10^{6}$	$4.5x10^{6}$	$5.5x10^{6}$	$4.5x10^{6}$	$4.0x10^{6}$	$4.6x10^{6}$	$4.4x10^{6}$

Table 2. Morphological and biochemical characteristics of bacterial isolate from the unpolluted water samples

Probable bacteria	*Staphylococcus aureus*	*Escherichia coli*	*Bacillus cereus*	*Pseudomonas aeruginosa*	*Streptococcus* spp.
Colour	Yellow	Yellow	White	Green	Grayish
Surface		Smooth	Smooth	Smooth	
Edge	Entire	Rhizoid	Rhizoid	Entire	Entire
Elevation	Raised	Flat	Flat	Flat	Raised
Shape	Cocci	Rod	Rod	Rod	chain cocci
Gram reaction	+	-	+	-	+
Catalase	+	-	+	+	+
Motility	-	+	+	+	-
Spore	-	+	+	-	-
Indole	-	+	-	-	
Coagulase	+		-	-	-
Glucose	AG	AG	A	AG	AG
Lactose	AG	A	-	-	-
Mannitol	AG	A	-	AG	AG
Galactose	-	AG	AG	AG	
Fructose		AG	AG	AG	
Maltose	-	A	-	-	AG
Sucrose	AG	AG	A	AG	AG

Key:+ -- Positive, - --Negative, A –Acid production, AG –Acid and gas production

3.2.2 Polluted samples

Morphological and biochemical characteristics of bacterial isolates from unpolluted water sample are presented in Table 3. The isolates identified were *Bacillus cereus, P. aeruginosa, Micrococcus luteus and Bacillus subtilis.* All the isolates were Gram positive with the exception of *P. aeruginosa*. *Bacillus cereus* and *B. subtilis* were positive to spore staining test.

3.2.3 Inoculated samples

The bacterial isolates from water samples undergoing bioremediation are shown in Table 4. These include *Bacillus cereus, Bacillus subtilis, Pseudomonas aeruginosa, Aerococcus viridians* and *Micrococcus luteus.*

3.3 Frequency of Occurrence of Bacteria Obtained During Bioremediation

The frequency of occurrence of bacteria obtained from this study is presented in Table 5. *Bacillus cereus, B. subtilis, Pseudomonas aeruginosa, Aerococcus viridans and Micrococcus luteus* occured through out the bioremediation process from week 8 to 14.

3.4 Percentage (%) Occurrence of Bacteria Obtained in This Study

The percentage occurrence of bacteria obtained from this study is shown in Table 6. *Bacillus cereus* had the highest percentage occurrence of 26.3% while *Staphylococcus aureus, Escherichia coli* and *Streptococcus* sp. had the least percentage occurrence of 1.8% each.

4. DISCUSSION

The bacterial loads in crude oil polluted water samples had higher colony forming units than in unpolluted samples. The high number of bacteria in crude oil polluted water was due to the organic matter present in the crude oil. The bacteria might have utilized the organic compound present in the crude oil as source of carbon and energy for their metabolic properties. The frequency of *Bacillus cereus* was higher in contaminated water samples than non-contaminated water samples. This is likely to be as a result of the metabolic ability possesed by *B. cereus*, which enables the utilization of hydrocarbon present in the crude oil as source of carbon and energy [10-12]. The highest occurrence of *Bacillus* spp. in contaminated water, obtained in this study, is in conformity with the report of [13] that *Bacillus* sp. and *Pseudomonas* sp. are of high predominance in hydrocarbon polluted water as a result of their ability to utilize it. *Bacillus* sp. produces spores which may shield it from the toxic effects of hydrocarbon. *Staphylococcus aureus, Streptococcus* sp. and *Escherichia coli* were absent in the crude oil polluted water. This may be due to their inability to utilize petroleum products and the fact that they have no spores for protection. Microbial degradation of organic contaminants normally occurs as a result of microorganisms using the pollutants for their metabolism, growth and reproduction [14].

The frequency of occurrence of bacteria showed that *Bacillus cereus* had the highest occurrence (26.3%) followed by B*acillus subtilis* (21%) while *Staphylococcus aureus, Streptococcus* sp. and *Escherichia coli* were least in occurrence with value of 1.8% each. This also corroborates the findings of [15], who studied the potential of *Pseudomonas* sp., *Bacillus* sp., and *Proteus* sp. isolated from rivers polluted by hydrocarbons and refinery effluents to degrade petroleum [16]. The result of this investigation showed the occurrence of high numbers of certain oil degrading microorganisms from oil polluted environment as evidence that these microorganisms are the active degraders of such environment [2]. The majority of these organisms isolated were *Pseudomonas*, *Bacillus, Micrococcus* sp. and the dominance of these organisms has been reported by different researchers as crude oil degraders [11,13].

Since these organisms were isolated from the hydrocarbon-polluted water samples, it shows that they were able to exist in the oil spilled environment [17,18] while microorganisms that could not survive in this environment were eliminated by the unfavorable condition caused by the crude oil spill. Most of the isolates are predominantly indigenous microorganisms of coastal region, which are constantly exposed to different petroleum contaminants [19]. The presence of oil-degrading organisms in the polluted water is a clear indication that the indigenous microbes were carrying out their metabolic activity. The activities of these microorganisms could be responsible for the bioremediation of the environment [20,21].

Table 3. Morphological and biochemical characteristics of bacterial Isolate from the polluted water samples

Probable bacteria	*Bacillus cereus*	*Pseudomonas aeruginosa*	*Micrococcus luteus*	*Bacillus subtilis*
Colour	White	Green	Yellow	White
Surface	Smooth	Smooth	Smooth	Rough
Edge	Rhizoid	Entire	Entire	Rhizoid
Elevation	Flat	Flat	Flat	Flat
Shape	Rod	Rod	Cocci	Rod
Gram reaction	+	-	+	+
Catalase	+	+	-	+
Motility	+	+	-	+
Spore	+	-	-	+
Indole	-	-	-	-
Coagulase	-	-	-	-
Glucose	A	AG	A	AG
Lactose	-	-	A	AG
Mannitol	-	AG	A	A
Galactose	AG	AG	-	AG
Fructose	AG	AG	A	A
Maltose	-	-	A	A
Sucrose	A	AG	A	A

Key: + -- Positive, - --Negative, A --Acid production, AG –Acid and gas production

Table 4. Morphological and biochemical characteristics of bacterial Isolate from polluted water sample undergoing bioremediation

Probable bacteria	*Bacillus cereus*	*Bacillus subtilis*	*Pseudomonas aeruginosa*	*Aerococcus viridians*	*Micrococcus luteus*
Colour	White	White	Green	Cream	Yellow
Surface	Smooth	Rough	Smooth	Smooth	Smooth
Edge	Rhizoid	Rhizoid	Entire	Entire	Entire
Elevation	Flat	Flat	Flat	Flat	Flat
Shape	Rod	Rod	Rod	Cocci	Cocci
Gramreaction	+	+	-	+	+
Catalase	+	+	+	-	-
Motility	+	+	+	-	-
Spore	+	+	-	-	-
Indole	-	-	-	-	-
Coagulase	-	-	-	+	-
Glucose	A	AG	AG	A	A
Lactose	-	AG	-	AG	A
Mannitol	-	A	AG	AG	A
Galactose	AG	AG	AG	AG	-
Fructose	AG	A	AG	A	A
Maltose	-	A	-	A	A
Sucrose	A	A	AG	A	A

Key: + -- Positive, - --Negative, A –Acid production, AG –Acid and gas production

Table 5. Bacterial occurrence during bioremediation

Weeks/bacterial isolates	*Bacillus cereus*	*Bacillus subtilis*	*Pseudomonas aeruginosa*	*Aerococcus viridians*	*Micrococcus luteus*
8th	✓	✓	✓	✓	✓
10th	✓	✓	✓	✓	✓
12^{th}	✓	✓	✓	✓	✓
14th	✓	✓	✓	✓	✓

Table 6. Percentage (%) frequency of occurrence of bacteria obtained in this study

Bacteria	1st	2nd	3rd	4th	5th	6th	7th	Total frequency	Percentage occurrence
Pseudomonas aeruginosa	2	1	2	1	2	1	2	11	19.3%
Bacillus cereus	2	2	3	2	1	2	3	15	26.3%
Micrococcus sp.	1	1	1	2	1	1	1	8	14%
Aerococcus sp.	1	1	1	1	2	1	1	8	14%
Bacillus subtilis	1	1	2	3	2	1	2	12	21%
Staphylococcus aureus	1	Nil	Nil	Nil	Nil	Nil	Nil	1	1.8%
Escherichia coli	1	Nil	Nil	Nil	Nil	Nil	Nil	1	1.8%
Streptococcus sp.	1	Nil	Nil	Nil	Nil	Nil	Nil	1	1.8%
Total	10	6	9	9	8	6	9	57	100

5. CONCLUSION

The information obtained from this study reveal a population growth of the bacteria possessing the ability to survive the crude oil pollution. Its proliferation in crude oil implies the utilization of the crude oil as a possible source of energy for the bacteria. Thus, the bacteria are able to breakdown the crude oil. In turn, this makes the crude oil less harmful to the activities of plants and animal in the environment. Therefore, the bacteria could be used as agent of bioremediation, useful for the breakdown of crude oil pollution in the environment.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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