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(RESEARCH ARTICLE)

Hydrocarbonoclastic potentials of Enterobacteriaceae isolated from the crude oil polluted Iko river estuary and freshwater ecosystem of the Niger Delta Region of Nigeria

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Abstract

Hydrocarbonoclastic potentials of Enterobacteriaceae isolated from the crude oil polluted Iko river estuary and freshwater ecosystem of the Niger Delta was investigated. The isolation of crude oil utilizing bacterial isolates in water sample was carried out by surface spreading technique using diluents prepared with quarter strength Ringers solution and cultured on oil agar medium (OAM). Of the 32 potential hydrocarbon utilizing bacteria from crude oil impacted aquatic ecosystem only eight isolates identified as; *Citrobacter amalonaticus*–Y1 (FSW); *Proteus mirabilis* strain–I(FSW), *Pseudomonas fluorescens*–N (FSE); *Citrobacter farmeri*–Y12 (FSE), *Citrobacter amalonaticus* strain–Y2 (ESWS1), *Enterobacter sp.*–Y8 (ESWS3), *Proteus mirabilis* strain–K (ESWS1), *Proteus penneri* strain–O(ESES3) were found to demonstrate strong hydrocarbonoclastic potentials with variable levels of low pH and increase optical density and free carbon iv oxide production. The study revealed that *Citrobacter amalonaticus* strain–Y2 (ESWS1) of the family Enterobacteriaceae is a good candidate for bioaugmentation technique of bioremediation.

Keywords: Crude oil; Iko river; Hydrocarbonoclastic; Enterobacteriaceae; Bioaugmentation; Bioremediation

1. Introduction

The introduction of large quantity crude oil and other petroleum products occurring mainly during production and transportation processes usually exceeds the self - purification capability of the receiving water ecosystem. The effect of oil pollution on aquatic ecosystem is enormous. Contending oil spillage on water bodies possess a lot of challenges. It is harmful to birds and diverse aquatic lives. It incurs heavy economic and esthetic damage to the environment. Remediating costs of spillage are high often amounting to $10 – 15 per gallon of spilled oil. Physical methods of driving off oil with detergents or dropping down oil with chalk or siliconized sand can only take away oil from the surface, but the exposure of aquatic lives to hydrocarbon pollutants is greatly increased. Predominant climatic and weather conditions can also hinder the cleanup of oil slicks [1].

Ruinous and unmanageable releases of hydrocarbon products result in environmental and ecological reverberation, in that most of the hydrocarbon compounds are toxic and relentless in the terrestrial and aquatic environments. Various physical and chemical methods are used in the removal of contaminants from the environment. However, bioremediation which is biologically mediated process to remove pollutants from contaminated environment is more effective. This involve the microbial mineralization of complex polymers into simple inorganic constituents such as carbon dioxide [2]. This method uses the metabolic capacities of microorganisms to remediate polluted environments. In bioremediation, the schemes used in cleaning up the contaminated ecosystems focus on either stimulating autochthonous microbial population by environmental alteration (biostimulation) or bringing in exogenous microbial species that are known to

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be pollutant degraders to a contaminated site (bioagumentation) [3-4]. Biological degradation is safe and it's application is simple and effective, at such it could be used as economic alternative on large areas. [5].

In aquatic and terrestrial environments, biodegradation of crude oil and other petroleum components predominantly revolves around the action of bacterial and fungal populations [3]. The outcome of an uninterrupted addition of petroleum-based pollutants is an enriched microbial community which has the ability to strive during toxic contamination. Microorganisms are responsive to changes in their environment. At such whenever there is a sudden change in the physical or chemical conditions in their environment, the organisms will experience a lag period during which the organisms is made fit for the new environmental conditions [6-7].This lag period is also called acclimatization period and enables microorganisms to acquire the metabolic repertoire necessary for their survival [8].

Hydrocarbonoclastic bacteria are hydrocarbon degrading bacteria. Hydrocarbonoclastic organisms exhibit inherent capability to utilize and assimilate polluting petroleum hydrocarbon as a sole source of carbon and energy with the evolution of carbon dioxide and water by oxidative process. This reaction leads to eventual disappearance of oil in the water bodies [9]. Hydrocarbon-degrading bacteria and fungi are widely distributed in aquatic and terrestrial habitats. Lee *et al*., [10] recommended the use of microbial seeding of oil slicks as a cleanup technique where physical removal is impracticable or as a concluding stage during cleanup of residual oil. The most common hydrocarbon degraders in aquatic ecosystems include members of the bacterial genera *Pseudomonas*, *Mycobacterium, Bacillus, Flavobacterium, Alcaligenes, Streptomyces, Nocardia, Desulfovibrio, Acinetobacter, Corynebacterium, Thiobacillus, Klebsiella, Achromobacter, Serratia, Peptococcus, Peptostreptococcus, Azotobacter, Clostridium, Brevibacterium, Vibrio, Halobacterium, Bacteriodes and Proteus* [11-12].

Hydrocarbon contamination of aquatic ecosystem is among the most evident negative impacts of the petroleum industry. Oil spills, oil transportation, drilling operation, refineries and local fuel filling stations are some of the causes responsible for hydrocarbon contamination [13]. Due to the oily nature and inability of hydrocarbons to dissolve in water, they are refractory. At such their persistence in the environment could cause perilous effect on plants and living organism of the terrestrial and aquatic ecosystems.

Hydrocarbonoclastic bacteria are important constituents of oil degrading consortia and are usually present in hydrocarbon contaminated sites. Due to their high degradation potentials, they have been utilized in remediating oil contaminated soil and aquatic ecosystems. Beside bacteria, some fungal species are known to have hydrocarbonoclastic potential. (Potential hydrocarbonoclastic Bacteria, https://www.biotecharticles.com/Applications-Article/Potential-Hydrocarbonoclastic-Bacteria-730.html, last accessed on 03/10/2011).

One of the interesting features of hydrocarbonoclastic bacteria is their ability to proliferate rapidly in the presence of hydrocarbon. They proliferate effectively in the presence of aliphatic and aromatic hydrocarbons since they are heterotrophic in nature. Though most of the hydrocarbonoclastic bacteria can only grow in the presence of hydrocarbon, some members of these group sometimes utilize other substrate like acetate and pyruvate which are not hydrocarbon substrates. Sampling of hydrocarbonoclastic bacteria are often from hydrocarbon contaminated environments since they are not common in unpolluted environments (Potential hydrocarbonoclastic Bacteria, https://www.biotecharticles.com/Applications-Article/Potential-Hydrocarbonoclastic-Bacteria-730.html, last accessed on 03/10/2011).

Oil contamination of aquatic ecosystem remains an on-going problem whether it originates from low-level discharge from refineries and drilling platforms or from more dramatic spills from tanker disasters. Spills result in considerable contamination of aquatic environment [14], but the impact of oil contamination is difficult to predict due to its complex nature. Although there are reports on hydrocarbon utilizing microorgannisms in various ecosystem [15-17], reports on the hydrocarbonoclastic potentials of bacteria is still at the toothing stage. Thus this study was undertaken to determine the hydrocarbonoclastic potentials of bacteria species of the family Enterobacterieceae isolated from Iko river estuary and freshwater ecosystem in the Niger Delta in order to determine their efficiency in bioaugmentation technique of bioremediaton.

2. Material and methods

2.1. Study area

The study area lies within latitude 7° 30' N and 7° 45' N and longitude 7° 30' E and 7° 30' E. The river takes its rise from Qua Iboe River catchment and drains into the Atlantic Ocean at the Bright of Bonny. The river is characterized by flood and ebb tides with shallow depth ranging from 1 to 7 m [17].

The Iko River estuary is formed by adjourning tributaries, creeks and channels. This provides an appropriate site for petroleum exploration and production activities, good fishing ground for artisan fishermen as well as breeding sites for diverse aquatic resources in the area. The Iko River shoreline is also characterized with tidal mud flats and mangrove vegetation – Neppa palm (*Protium pneumaphores* and exposed during low tides [17].

2.2. Collection of water samples

The surface water samples were aseptically collected into clean 1 L capacity plastic bottles. All containers were rinsed three times with water sample before collection [18]. All water samples were collected from the surface (10 to 25 cm). The Nansen water bottle samplers were opened to fill and close below the water for subsurface water sample. Samples were then placed in an ice-cooled chest and transported immediately to the Microbiology Laboratory for analysis.

The analytical media employed in this research included nutrient agar (NA) and mineral salt medium (MSM) of Zajic and Supplison, [19]. Crude oil used was Bonny light crude oil collected from Qua Iboe Terminal, Ibeno, Akwa Ibom State and stored at room temperature.

2.3. Isolation, enumeration and purification of crude oil utilizing bacterial isolates

The vapour phase transfer technique method described in Asitok *et al*. [20] was used. Isolation of hydrocarbon utilizing bacteria in the water sample was carried out using the surface spread technique. Tenfold serial dilutions of the samples were made. Then 0.1 mL of the various dilutions were then plated in triplicates into mineral salt medium (MSM) supplemented with nystatin (50 mg L^{-1}) to inhibit fungal growth. Thereafter, sterile filter paper (Whatman no. 1) saturated with 2.0 mL of filtered Bonny light crude oil were aseptically placed inside the covers of the inverted Petri dishes. The inoculated plates were then sealed around with a masking tape. This process enable the supply of hydrocarbons by vapour phase transfer as the carbon and energy source for colonies that developed on the agar surfaces. Inoculated plates were incubated at room temperature (28±2 ^{o}C) for 5 to 7 days.

Developed colonies were enumerated and expressed as colony forming units per milliliter (cfu ml^{-1}). Distinct colonies were purified by repeatedly transferring to freshly prepared nutrient agar plates by streak-plate method and subsequent storing on agar slants in the refrigerator (4 °C) for further use.

2.4. Molecular characterization of hydrocarbon utilizing bacterial isolates

DNA extraction was carried out with a 24 hours BHI broth microbial isolates harvested by centrifugation at 14,000 g for 10 minute. The cells were washed three times in 1mL of ultra-pure water by centrifugation at 12,000 rpm for 5 minutes.

DNA extraction and purification was done using ZR fungal/bacterial DNA Miniprep™ 50 Preps. Model D6005 (Zymo Research, California, USA). 50 – 100 mg of bacterial cells were re-suspended in 200 µL of sterile water. This was transferred to a ZR Bashing Bead™ Lysis Tube. Exactly 750 µL analysis solution was added to the tube. The bead containing the solution was secure in a bead beater fitted with a 2 mL tube holder assembly and processed at maximum speed for 5 minutes. The ZR Bashing Bead™ Lysis Tube containing the bacterial cells was then centrifuged in a micro-centrifuge at 10,000 g for 1 min. 400 µL of the supernatant was then pipetted into a zymo-spin™ IV spin filter in a collection tube and centrifuged at 7,000 g for 1 min. This was followed by the addition of 1,200 µL of fungal/bacterial DNA binding buffer into the filtrate in the collection tube. After this 800 µL the mixture was transferred into a Zymo-spin™ 11C column in a collection tube and centrifuge at 10,000 g for 1 min. The flow through was discarded from the collection tube and the process was repeated to obtain the products. The 200 µL DNA pre-wash buffer was added to the column in a new collection tube and then centrifuge at 10,000 g for 1 min. 500 µL fungal/bacterial DNA wash buffer was added into the Zymo-spin™ IIC column and centrifuged at 10,000 g for 1 min. This was then transferred into a clean 1.5 mL micro centrifuge tube and 100 µL of DNA elution buffer was then added to the column matrix. DNA was eluted by centrifuging at 10,000 g for 30 seconds. The resulting ultra-pure filtrate (DNA) obtained was then transported in ice pack to the biotechnology laboratory for sequencing.

DNA sequencing was performed by Sanger (dieoxy) sequencing technique to determine the nucleotide sequence of the specific microorganism isolated using automated PCR cycle – Sanger sequencer™ 3730/3730 XL DNA Analyzer from Applied Biosystems. The results were obtained as nucleotide in FASTA format. Identification of the species were done using nucleotide base pairs. These were formed by BLAST analysis by direct blasting on http://blast.ncbi.nim.nih.gov. For every set of isolate, a read was BLASTED and the resultant top limit with minimum E-score for every BLAST result showing species name was used to name the specific organism.

2.5. Determination of hydrocarbonoclastic potential of bacteria isolates

The methods of Okpokwasili and Okorie [21] (modified) and procedure earlier reported by Esin and Antai [22] and Vanishree *et al.* [23] were employed. 9.9 mL of mineral salt broth (MSB) were dispensed were into test tubes and then autoclaved for sterilization. 0.1 mL of the filtered Bonny light crude oil was then added to the cooled MSB tubes. Thereafter, 0.1 mL of the 24 hour nutrient broth culture was then inoculated into different set of test tubes. Two control tubes were not inoculated and all the test tubes were incubated at room temperature (28 ± 2 °C) for 14 days.

At an interval of 4 days, the tubes were compared with the control and observed visually for turbidity as an index of utilization of the Bonny light crude oil. At the end of 14days, the ability to degrade oil were regarded as strong (+++), moderate (++), and weak (+) while inability to grow was recorded as no growth (-). The final turbidity of the medium was read using HACH 2100P Turbidimetre, pH was measured by an electronic pH meter (HACH sension 3 pH meter); optical density at 540 mm using HACH RD/210 spectrophotometer; Total viable count by serially diluting the culture, plating on nutrient agar and incubating at (28 ± 2 ^{o}C) for 24 hours and expressed as colony forming units per milliliter (CFU mL^{-1}) and free CO_2 determined by titrating 1 ml of the fermented culture against 0.05 N NaCl solution using phenolphthalein as an indicator and appearance of a stable pink colour as end point. The amount of CO_2 was calculated using the formula:

$$\text{Free } CO_2 \left(\frac{\text{mg}}{\text{L}}\right) = \frac{\text{Titre value x Normality of NaOH x 1000 x 44}}{\text{Volume of Sample}}$$

3. Results

The sequencing result of hydrocarbonoclastic bacteria showing sequence homology and accession number of specie and strain in the gene bank is shown in table1.

The hydrocarbonoclastic potentials of bacteria isolated from the estuarine and freshwater ecosystems are presented in Table 2. The screening test revealed that most of the microorganisms encountered in the Iko river estuary and freshwater were able to use the hydrocarbons as carbon and energy source. Table 2 shows *Citrobacter amalonaticus* – Y_1(FSW); *Proteus mirabilis strain* – I(FSW), *Pseudomonas fluorescens* – N(FSE); *Citrobacter farmeri* – Y_{12}(FSE), *Citrobacter amalonaticus* strain – Y_2($ESWS_1$), *Enterobacter* sp – Y_8($ESWS_3$), *Proteus mirabilis* strain – K($ESWS_1$), *Proteus penneri* strain – O($ESES_3$) which exhibited abundant growth on the crude oil minimal medium, lower pH and caused the evolution of a higher amount of free CO_2 indicating a strong hydrocarbonoclastic potential. Others encountered in the ecosystem demonstrated weak or fairly strong oil degrading capabilities.

Table 1 Sequencing result showing the sample numbers and the corresponding accession numbers and identity of organism

Sr. No.	Original Number	Sample Type	DNA Type	Gene Bank Accession Number	Percentage Match (%)	Organism in the Gene Bank	ID of Organism
1	I	DNA	Genomic	KR133627	92	*Proteus mirabilis* strain RBX1	*Proteus mirabilis* strain–I(FSW)
2	M	DNA	Genomic	JX393013	85	*Morganella morganii* strain ZW45-1	Morganella morganii
3	N	DNA	Genomic	KM206793	87	*Pseudomonas fluorescens* WN-1	*Pseudomonas fluorescens* – N(FSE)
4	O	DNA	Genomic	KC456589	95	*Proteus penneri* strain ALK624	*Proteus penneri* strain– O(ESES3)
5	S	DNA	Genomic	CP009450	84	*Pluralibacter gergoviae* strain FB2	*Pluralibacter gergoviae* -
6	T	DNA	Genomic	CP001407	86	*Bacillus cereus* 03BB102	*Bacillus cereus*– T(ESSWS3)
7	B2	DNA	Genomic	AY279207	80	*Bacillus substilis* strain HA401	*Bacillus substilis* strain– B2(ESWS1)
8	C3	DNA	Genomic	KR133627	88	*Proteus mirabilis* strain RBX1	*Proteus mirabilis* strain– C3(ESWS2)
9	D4	DNA	Genomic	HE575920	100	*Citrobacter farmeri*	*Citrobacter farmeri*– D4(FSE)
10	E5	DNA	Genomic	KP260658	96	*Enterobacter hormeachei* strain DD3	*Enterobacter hormeachei* strain– E5(ESWS2)
11	Y1	DNA	Genomic	DQ187379	93	*Citrobacter amalonaticus*	*Citrobacter amalonaticus*–1(FSW)
12	Y2	DNA	Genomic	FN667808	96	*Citrobacter amalonaticus* strain ADR61	*Citrobacter amalonaticus* strain– Y2(ESWS1)
13	Y3	DNA	Genomic			No amplification	
14	Y7	DNA	Genomic	DQ187383	94	*Citrobacter farmeri*	*Citrobacter farmeri* - Y7 (FSE)
15	Y8	DNA	Genomic	HQ268733	97	*Enterobacter s*p P2B	*Enterobacter* sp – Y8(ESWS3)
16	Y9	DNA	Genomic	KC456538	97	*Proteus mirabilis* strain ALK043	*Proteus mirabilis* strain – Y9(ESSWS1)
17	Y12	DNA	Genomic	DQ187383	96	*Citrobacter farmeri*	*Citrobacter farmeri* – Y12(FSE)
18	K	DNA	Genomic	KJ206073	99	*Proteus mirabilis* strain 13a	*Proteus mirabilis* strain– K(ESWS1)
19	Act3	DNA	Genomic	KP117097	99	*Proteus mirabilis* strain CIFRI H-TSB-70	*Proteus mirabilis* strain– Act3
20	Act2	DNA	Genomic	JF775415	97	*Proteus mirabilis* strain YCG36	*Proteus mirabilis* strain– Act2

Table 2 Hydrocarbonoclastic potential of bacterial isolates from Iko estuarine and fresh water ecosystem

S/N	Isolate Code	Isolates	Optical Density (540nm)	Turbidity (Visual)	pH	Free CO_2 mg/L
1	A	Control	0.082	-	7.23	11
2	F	*Vibrio* sp	0.0964	+	7.26	294.25
3	E_5	*Enterobacter hormeachei* strain -$E_5(ESWS_2)$	0.186	++	6.5	349.25
4	Y	*Corynebacterium* sp	0.194	+	7.13	255.75
5	C_3	*Proteus mirabilis* strain – $C_3(ESWS_2)$	0.18	++	6.26	437.25
6	B_2	*Bacillus substilis* strain– $B_2(ESWS_1)$	0.247	++	6.21	442.75
7	Act_2	*Proteus mirabilis* strain– Act_2	0.179	++	6.18	363
8	H	*Chromatum* sp	0.394	+	7.3	250.25
9	Y_2	*Citrobacter amalonaticus* strain -Y_2(FSW)	1.79	+++	6.15	517
10	J	*Actinomyces* sp	0.198	+	7.18	277.75
11	Y_1	*Citrobacter amalonaticus* –$Y_1(ESWS_1)$	1.987	+++	6.02	563
12	K	*Proteus mirabilis* strain– $K(ESWS_1)$	1.412	+++	6.19	459.25
13	L	*Micrococcus* sp	0.307	++	6.14	352
14	Y_8	*Enterobacter* sp – $Y_8(ESWS_3)$	1.42	+++	6.2	467.5
15	I	*Proteus mirabilis* strain I(FSW)	1.469	+++	6.15	473
16	M	*Morganella morganii*	0.293	+	7.21	269.5
17	P	*Chromatium* sp	0.281	+	7.25	247.5
18	Q	*Staphylococcus aureus*	0.243	+	7.27	228.75
19	R	*Serratia* sp	0.316	+	7.14	291.5
20	S	*Plualibacter gergoviae*	0.148	+	7.55	198
21	T	*Bacillus cereus* - $T(ESSWS_3)$	0.318	++	6.82	302.5
22	Y_9	*Proteus mirabilis* strain – $Y_9(ESSWS_1)$	0.325	++	6.66	379.5
23	V	*Klebsiella* sp	0.312	++	7.63	361.25
24	O	*Proteus penneri* strain – $O(ESES_3$	1.413	+++	6.23	440
25	W	*Staphylococcus aureus*	0.247	+	7.35	211.75
26	X	*Norcardia* sp	0.251	+	7.16	280.5
27	D_4	*Citrobacter farmeri*– D_4(FSE)	0.324	++	6.12	393.25
28	Y_{12}	*Citrobacter farmeri* – Y_{12}(FSE)	1.375	+++	6.1	404.25
29	Z	*Serretia* sp	0.343	+	7.51	272.25
30	N	*Pseudomonas fluorescens* – N(FSE)	1.414	+++	6.13	451
31	Y_7	*Citrobacter farmeri* - Y_7 (FSE)	0.327	++	6.5	376.75
32	Act_3	*Proteus mirabilis* strain - Act_3	0.315	++	6.47	361.25

Key to Codes: E = Estuary, SW = Surface water, SE = Sediment, SS = Subsurface, F = Freshwater, Sx = Station, Bx = Bacteria Isolate number

4. Discussion

Many microorganisms are able to use various petroleum products as their major carbon and energy source though these hydrocarbon are insoluble in aqueous phase. Itah and Essien [24] reported that the hydrocarbonoclastic activities of microorganisms is greatly determined by the ability of the organisms to elaborates the vital enzymes required for the decomposition of the recalcitrant components of hydrocarbons. Most of these organisms belong to genera *Pseudomonas, Bacillus, Aeromonas*, Alcaligenes, *Acinetobacter*, *Mycobacterium*, *Rhodococcus* and *Sphingomonas* sp [25]. In this study, hydrocarbonoclastic potential of estuarine and freshwater microorganisms was accessed using indirect (the optical density, pH, viable count and free carbon dioxide evolution) procedures. *Citrobacter amalonaticus* strain - $Y_2(ESWS_1)$, *Proteus mirabilis strain* - I(FSWS), *Pseudomonas fluorescens*– N(FSE), *Citrobacter farmer*– Y_{12}(FSE), *Citrobacter amalonaticus* -–Y_1(FSW) *Enterobacter* sp– $Y_8(ESWS_3)$, *Proteus mirabilis* strain– K($ESWS_1$), *Proteus penneri* strain– O($ESES_3$) were found to be the bacteria with high hydrocarbonoclastic potential.

Citrobacter amalonaticus strain -Y_2 ($ESWS_1$), exhibited a very strong hydrocarbonoclastic potential, the role of *Citrobacter amalonaticus* in utilization of crude oil and its products has previously been reported by Irshaid and Jacob [26]. Obst *et al.,* [27] reported that *Citrobacter amalonaticus* utilized dipeptides released from cyanophycin granule polypeptide by another bacterium. *Pseudomonas fluorescens* – N (FSE), also demonstrated the ability of utilizing crude oil. This corroborates with the report of Gomathy and Senthilkumar [25] who reported that *Pseudomonas fluorescens* ability to utilize crude oil could be due to the haemolytic and emulsification of crude oil by the species. *Proteus mirabilis*- I (FSWS), and *Proteus penneri* strain -O ($ESES_3$) were observed in this study to utilize crude oil. Similar findings have revealed that *Proteus* species are more tolerant to high levels of hydrocarbons [28].

5. Conclusion

The study has shown that of the eight bacterial isolates of the family Enterobacterioceae that had strong hydrocarbonoclastic potential in hydrocarbon degradation, *Citrobacter amalonaticus* strain - $Y_2(ESWS_1)$ was outstanding. *Citrobacter amalonaticus* is added to the long list of hydrocarbon degrading bacteria species in the Niger Delta area of Nigeria and therefore set it apart as a good candidate for bioaugmentation technique of bioremediation.

Compliance with ethical standards

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Disclosure of conflict of interest

Authors have declared that no competing interest exist.

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