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Bonny Light Crude Oil Degradative Potental of Species of *Citrobacter*

Abigail Nkanang[1*], Sylvester Peter Antai[1] and Atim David Asitok[1]

[1]Department of Microbiology, University of Calabar, Nigeria.

Authors' contributions

This work was carried out in collaboration between all authors. Author AN designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors SPA and ADA managed the analyses of the study. Author AN managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Of the diverse hydrocarbonoclastic bacteria isolated from crude oil polluted IKO River estuarine and freshwater ecosystems, the bacterial isolate, ESW_1 and FSW_2 identified as *Citrobacter amalonaticus* strain – FSW_2 and *Citrobacter amalonaticus* strain - ESW_1.These efficiently degrade Bonny light crude oil sample recording 82.1 and 69.2% degradation respectively after 28 days of incubation. Biodegradability of the components of Bonny light crude oil was determined by gas chromatographic analysis. The chromatographic analysis after 28 days of incubation at 28°C also revealed that during the degradation of Bonny light crude oil, there was a decrease of the total hydrocarbon content (THC) from 10,906.9 mg L^{-1} to 1,947.4 mg L^{-1} and 3,357.9 mg L^{-1} respectively by FSW_2 and ESW_1. These results suggest that *Citrobacter amalonaticus* is a good candidate for microbial seeding of Bonny light crude oil polluted aquatic ecosystem.

Keywords: Biodegradability; Citrobacter amalonaticus; bonny light crude oil.

**Corresponding author: E-mail: abigailnkanang@yahoo.com;*

1. INTRODUCTION

The emergence of crude oil industry has contributed immensely to changing the state of the Nigerian economy and environment. The petroleum sector though has increasingly provided the bulk of current government revenue, on which economic growth largely depends, their operations have impacted adversely on the environment. The oil industry is a major source of environmental pollution and its adverse ecological impacts have been reported [1-3]. The ecological impact is widely spread with more serious damage to the oil producing communities [4]. The tremendous increase in the production, refining and distribution of crude oil and other petroleum products are accompanied by increasing problem of environmental pollution.

Several physico-chemical methods for decontaminating the environment have been established and employed. Biological degradation, a safe, effective and economic alternative method is a complete microbial mineralization of complex materials into simple inorganic constituents such as carbon dioxide [5]. This technology uses the metabolic potential of microorganism to clean up polluted environments. Due to its simple application it can be used on large areas, it is cost effective and removes the contaminants completely [6]. Strategies used in bioremediation of polluted ecosystem revolves around either stimulating indigenous microbial population by environmental modification or inducing exogenous microbial population that are known degraders to a contaminated site, a process known as seeding [7,8].

Although there are studies on the bioremediation and biodegradation of hydrocarbons by various microorganisms on the various facets of the environments [9-12]. However studies on the degradative potential of the hydrocarbon utilizing bacteria in the IKO estuary ecosystem appear to be at the pioneering stagermin. Thus this study was undertaken to determine the degradative potential of *Citrobacter* species.

2. MATERIALS AND METHODS

2.1 Water and Sediment

Samples used were collected from oil producing coastal zone, the IKO River estuary located in IKO Town, Eastern Obolo Local Government Area of Akwa Ibom State in the Niger Delta region, Nigeria in sterile sample bottlles placed in an ice-cooled chest and transported to the microbiology laboratory for analysis.

The mineral salt medium (MSM) of Zajic and Supplison [13] used consisted of dipotassium phosphate (0.8 g/l), potassium dihydrophosphate (0.2 g/l), ammonium chloride (4.0 g/l), magnesium sulphate (0.2 g/l), sodium chloride (0.1 g/l), ferrous sulphate (0.01 g/l).

Crude oil used was Bonny light crude oil collected from Qua Iboe Terminal,Ibeno, Akwa Ibom State and stored at room temperature (28 ±2°C) till when needed.

2.2 Isolation of Crude Oil Utilizing Bacterial Isolates

The isolation of crude oil utilizing bacterial isolates in water and sediments samples was carried out by surface spreading technique [14], using diluents prepared with quarter strength Ringers solution and cultured on oil agar medium (OAM). The medium was supplemented with antimicrobial agent, Nystatin (50 mg L^{-1}) to prevent fungal growth was used.

The crude oil was sterilized by millipore filtration (0.45 µm pore size) and stored in sterile bottles. Sterile Whatman No. 1 filter papers were saturated with 0.5 ml of the of bonny light crude oil. These were then placed aseptically onto the inside lid of the plate, and taped around with masking tape, utilizing vapour phase transfer technique. Inverted agar plates were incubated at room temperature for 5 – 7 days. Descreet colonies developed were picked and purified by repeated subculturing and then stored on nutrient agar slants at 4°C in a refrigerator for further studies.

2.3 Determination Screening for Utilization of Bonny Light Crude Oil by Bacteria Isolates

The bacterial isolates were then screened for their potential to utilize Bonny light crude oil as their source of carbon and energy for growth. The methods of Okpowasili & Okorie [15] and procedure earlier reported by Esin & Antai [16] (modified) and Vanishree, Thatheyus & Ramya [17] were employed.

Mineral salt broth MSB dispensed were prepared in 9.9 ml into test tubes and sterilized by autoclaving. On cooling 0.1 mL of the filtered

Bonny light crude oil was added to the MSB tubes. Then 0.1 mL of the 24 hour nutrient broth culture of the hydrocarbon bacterial utilizing isolates respectively inoculated with the different set of test tubes. Two control tubes were uninoculated 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 14 days, 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°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 broth against 0.05 N NaCl solution using phenolpthalene as an indicator and appearance of a stable pink colour as end point. The amount of CO_2 was calculated using the formula:

Free CO_2 (mg/l) =

$$\frac{\text{Titre value x Normality of NaOH x 1000 x 44}}{\text{Volume of Sample}}$$

2.4 Characterization and Identification of Bonny light Crude Oil Utilization Bacteria Isolates

Characterization and identification of Bonny light crude oil utilization bacteria isolates bacterial isolates followed the determination schemes of Cowan [18] and Bergey's Manual [19].

2.5 Growth Profile Determination of the Most Efficient Bonny Light Crude Oil Utilizing Bacteria

To determine the biodegradability of Bonny light crude oil by the best four hydrocarbon utilizing bacteria isolates from estuarine and freshwater ecosytem, methods earlier reported by Antai [20], Ita & Essien [21] and Esin & Antai [16] (modified) were employed. Mineral salt broth (MSB) prepared in 99 mL amount in thirty five (35) 250 mL conical flasks. Destructive sampling methods were employed. The flasks were then sterilized by autoclaving and on cooling 0.l mL of filtered Bonny light crude oil was measured into the 35 flasks respectively. Then 0.1 mL of 24 hours nutrient broth culture of the 4 bacterial isolates were inoculated into 28 conical flasks, 5 conical flasks were uninoculated and served as controls. The flasks were incubated on a shaker (SGM-300 Gallemkamp, England) operating on 80 oscillation/min at room temperature (28 ± 2°C) for 28 days.

At an interval of 4 days, 20 mL of the sample was taken from each set of the inoculated flasks, representing the 4 different bacterial isolates for determination of (i) optical density i.e. turbidity at 540 nm using HACH RD/2010 spectrophotometer, (ii) pH changes monitored with an electronic pH Meter (HACH Sension 3 p^H Meter) (iii) total viable count (TVC) determined by serially diluting the culture, plating on nutrient agar and incubating at room temperature (28 ± 2°C) for 24 hours after which counts were taken and expressed as colony forming units per milliliter (cfu/ml) (iv) free CO_2 production determined by titrating 1 ml of the fermented broth against 0.05N NaCl solution using phenolphthalein as indicator and appearance of a stable pink colour as end point. The amount of CO_2 was calculated using the formula:

Free CO_2 (mg/l) =

$$\frac{\text{Titre value x normality of NaOH x 1000 x 44}}{\text{Volume of sample}}$$

Percentage utilization as weight loss of the incorporated crude oil was calculated as weight of crude oil (Control) minus weight of crude oil (degraded) divided by weight of crude oil (control) multiplied by 100.

2.6 Biodegradability of Major Component of Bonny Light Crude Oil by Column Chromatographic Analysis

The same procedure and the number of flasks (seventy) already describe in the measurement of Bonny light crude oil biodegradation studies were repeated in parallel for column chromatography. The extraction and column chromatographic analysis were applied to the crude oil before (control) and after degradation by the microbial isolates. The methods of TNRCC [22] and API [23] were employed.

2.7 Extraction

At the end of 28 days, the 10 Erlenmeyer flasks were removed and the residual crude oil in the flask were extracted with dichloromethane, separated by column chromatography and quantified in milligram per litre.

The crude oil was extracted by measuring out 100 mL of the broth sample into a separate funnel after rinsing the container (sample) with dichloromethane. Ten (10) mL of dichloromethane was added to 100 mL part of the broth sample then shaken to mix vigorously to have the organic solvent extract all the available organic material. The organic extract was collected into a receiving container (vial) passing the organic extract through a column containing glass wool, silica – gel and anhydrous sodium sulphate. The silica-gel aids the clean-up of the extract by disallowing the passage of debris from the extract while the anhydrous sodium sulphate acts as a dehydrating agent to rid the extract of every form of moisture/water from the sample since they are two immiscible liquids.

2.8 Gas Chromatographic Analysis

The collected organic extract was injected into the gas chromatograph, then 1 µL of the concentrated sample extract was injected by means of hypodermic syringe through a rubber septum into the column. Separation occurs as the vapour constituent partition between the gas and liquid phases. The various fractions of the aliphatic compounds (C_8 – C_{40}) were automatically detected as it emerges from the column by the FID detector whose response is dependent upon the composition of vapour. The results were expressed in mg/l which is equivalent unit.

3. RESULT AND DISCUSSION

The result of the screening of the bacteria isolates from the fresh and estuarine ecosystem, for their ability to utilize Bonny light crude oil as the sole energy and carbon source is presented in Table 1.

From the table, out of the 32 hydrocarbon utilizing bacteria isolated from the water and sediment samples, two bacteria isolates (FSW_2) and (ESW_1) had high optical density reading of 1.790 and 1.987 respectively as compared to the control of 0.082. Due to the efficient ability of EW_2 and FW_1 to utilize Bonny light crude oil, the two isolates were subjected to biodegradation studies and identified as Citrobacter amalonaticus strain- FSW_2 and Citrobacter amalonaticus – ESW_1.

Figs. 1 and 2 shows the growth profile of Citrobacter amalonaticus strain- EW_2 and Citrobacter amalonaticus – FW_1 determined using the growth indices of p^H, turbidity(optical density), carbon iv oxide evolution and total viable count (TVC) of the culture as it utilize Bonny light crude oil as the sole energy and carbon source.

The figures revealed an increased; optical density, carbon iv oxide evolution and total viable count with decreased in optical density), carbon iv oxide evolution and total viable count with decreased in pH of the culture as the biodegradation days progressed.

The high increase in the amount of free carbon-dioxide evolved in the incubated substrates is an indication of the utilization of petroleum hydrocarbon fractions as a source of carbon and energy by the microbial isolates. The end-product of petroleum hydrocarbon degradation is carbon-dioxide and water which are also measures of microbial respiration and activity in ecosystem. The utilization of crude oil by species of *Citrobacter* resulted in their growth with accompanying reduction in the pH, changing the medium to acidic metabolic product. The increase in optical density may be attributed to the ability of the organisms to proliferate while utilizing the pollutant as its sole carbon source.

The spectacular degradative potential of *Citrobacter amalonaticus* suggest that the organism posses an active and competent hydrocarbon degradative enzymes. The efficiency of *Citrobacter amalonaticus* in degrading Bonny light crude oil agrees with the result of some researchers. Irshaid & Jacob [24] reported the role of *Citrobacter amalonaticus* in biodegradation of crude oil. Also this agrees with earlier findings of Esumeh, Akpe & Eguagie [25] who isolated more of Gram negative organisms suggesting that they are better degraders of crude oil when compared with their Gram positive counterparts. The higher ability of Gram negative bacteria to utilize hydrocarbon may be connected with the possession of plasmid-borne or chromosomal genes involved in hydrocarbon degradation and porins in their cell wall which helps in the uptake of certain substances by the

cell or extrusion of others which may be harmful [26].

Table 2 represent the level of Bonny light crude oil components left after 28 days of degradation by bacterial species as revealed by Gas chromatographic analysis. The result revealed that *Citrobacter amalonaticus* was able to degrade more than 75% of the Bonny light crude oil fractions including $C_{14,}$ $C_{17,}$ C_{24}, C_{26} and C_{27} except C_{10} and C_{20} which recorded only 20% degradation. Citrobacter amalonaticus strain-EW2 enhanced degradation from 10,906.9 mg/l to 1947.4 mg/l thereby recorded the best degradation percentage of 82.1% while *Citrobacter amalonaticus* – FW1 had 69.2%

Table 1. Hydrocarbonoclastic potential of bacterial isolates from IKO estuarine and fresh water ecosystem

S/N	Isolate code	Isolates	Optical density (540 nm)	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	*Proters 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	*Kliebsiella* 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

degradation. This findings agree with the observations of Irshaid & Jacob [24] that *Citrobacter amalonaticus*– $Y_1(ESWS_1)$ has the capability to use xylene as the sole source of carbon and energy. Analysis of variance of degradation data assessed by gas chromatography revealed significant influence of bacterial species (P=9.05E-05<0.05) and nature of hydrocarbon fraction (P=8.55E-08<0.05) on hydrocarbonoclastic potential.

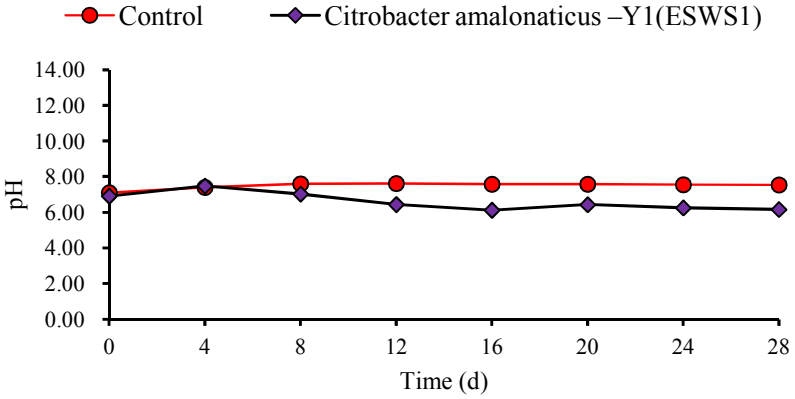

Fig. 1a. Changes in pH during biodegradation of Bonny light crude oil by bacterial isolates obtained from IKO estuarine water ecosystem

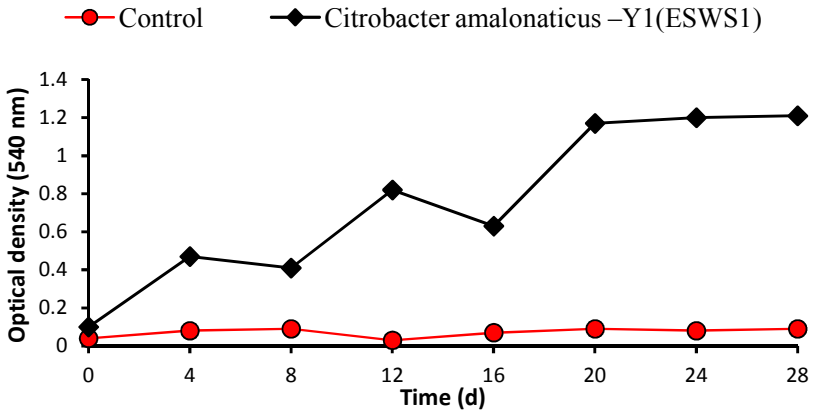

Fig. 1b. Changes in microbial population during biodegradation of Bonny light crude oil by bacterial isolates obtained from IKO estuarine water ecosystem

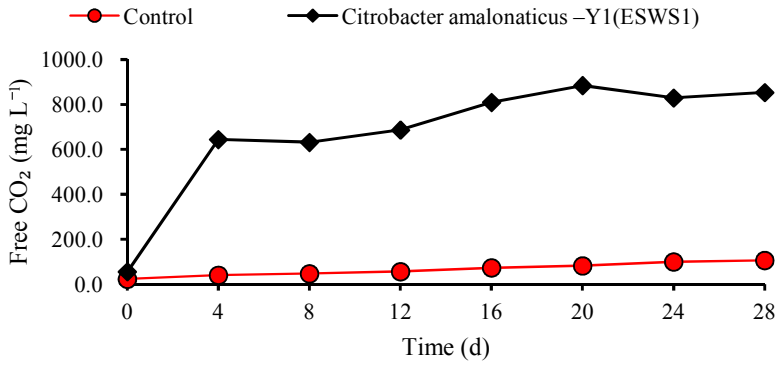

Fig. 1c. Changes in Free CO_2 evolution during biodegradation of Bonny light crude oil by bacterial isolates obtained from IKO estuarine water ecosystem

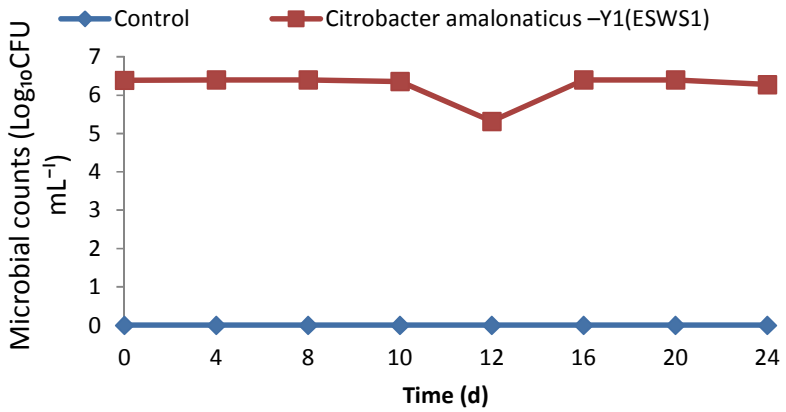

Fig. 1d. Changes in total viable counts during biodegradation of Bonny light crude oil by bacterial isolates obtained from IKO estuarine water ecosystem

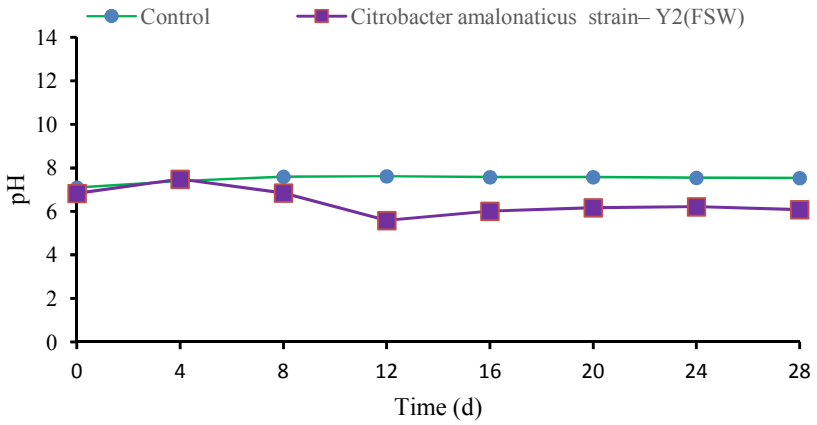

Fig. 2a. Changes in pH during biodegradation of Bonny light crude oil by bacterial isolates obtained from IKO freshwater ecosystem

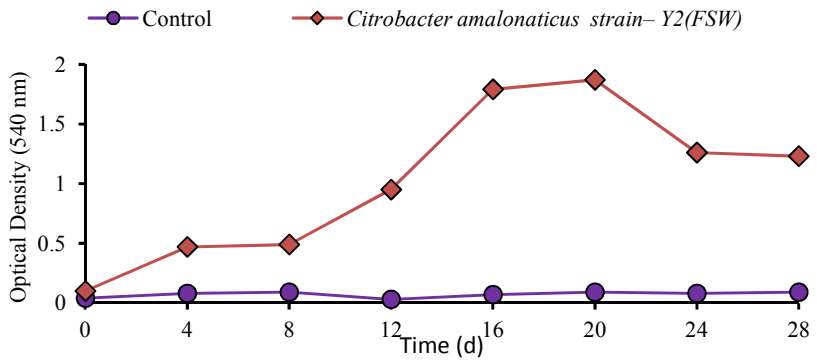

Fig. 2b. Changes in microbial load during biodegradation of Bonny light crude oil by bacterial isolates obtained from IKO freshwater ecosystem

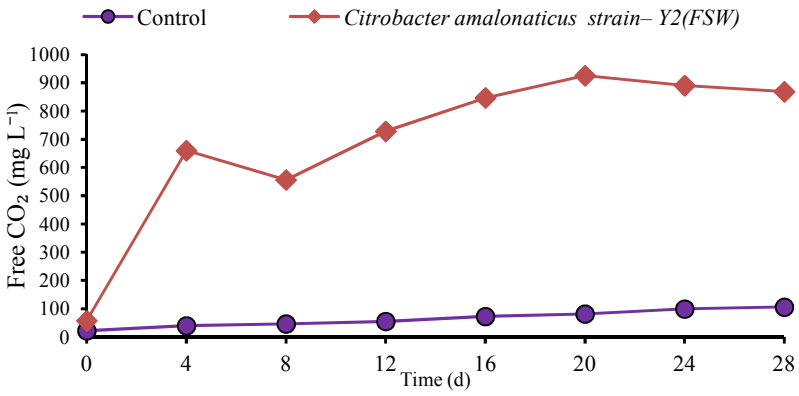

Fig. 2c. Changes in free CO_2 evolution during biodegradation of Bonny light crude oil by bacterial isolates obtained from IKO freshwater ecosystem

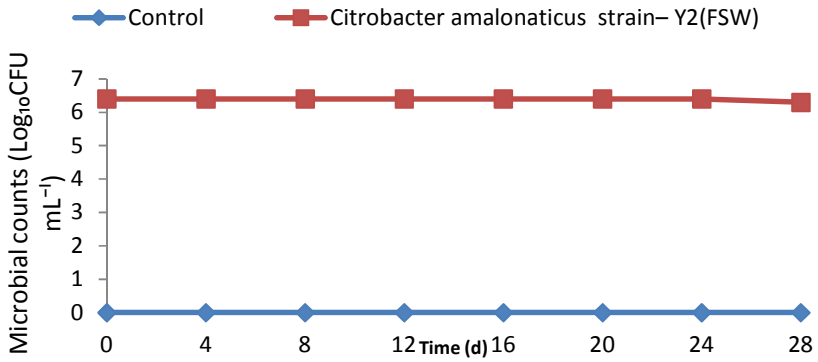

Fig. 2d. Changes in total viable count during biodegradation of Bonny light crude oil by bacterial isolates obtained from IKO freshwater ecosystem

Table 2. Levels of petroleum hydrocarbons components left after 28 days degradation by bacteria species from estuarine and freshwater ecosystem

Parameter		Microorganism and amount of hydrocarbon (Mg L^{-1})			
Hydrocarbon	Control	*Citrobacter amalonaticus* – Y_1(ESWS$_1$)		*Citrobacter amalonaticus* – Y_2(FSW)	
		Residual	% Deg	Residual	% Deg
C8		-			
C9	0.01	0.2	-1900	0.02	-100
C10	0.04	1.1	-2650	0.03	25
C11	0.3	10.9	-3533.33	0.02	93.33
C12	4.2	2.8	33.33	0.4	90.48
C13	22.4	3.3	85.27	3.1	86.16
C14	182.1	31.6	82.65	13.0	92.86
C15	407.5	83.5	79.51	11.3	97.23
C16	238.7	130.3	45.41	17.9	92.50
C17	616.2	80.0	87.01	51.1	91.71
Pristine	319.8	66.1	79.33	74.4	76.74
C18	94.5	273.1	-188.99	7.7	91.85
Pytane	862.4	338.1	60.79	60.2	93.02

Parameter		Microorganism and amount of hydrocarbon (Mg L^{-1})			
Hydrocarbon	**Control**	***Citrobacter amalonaticus* – Y_1(ESWS$_1$)**		***Citrobacter amalonaticus* – Y_2(FSW)**	
		Residual	**% Deg**	**Residual**	**% Deg**
C19	862.4	29.0	96.64	32.8	96.19
C20	64.5	300.5	-365.89	119.3	-84.96
C21	106.2	86.5	18.55	22.0	79.28
C22	338.7	49.7	85.33	34.7	89.75
C23	119.5	77.9	34.81	50.8	57.49
C24	371.1	78.4	78.87	18.4	95.04
C25	47.9	86.8	-81.21	14.4	69.94
C26	229.1	17.3	92.45	30.2	86.82
C27	717.7	78.8	89.02	25.7	96.42
C28	111.7	30.3	72.87	24.4	78.16
C29	799.4	71.4	91.07	36.9	95.38
C30	325.3	86.9	73.29	43.9	86.50
C31	587.1	34.0	94.21	38.8	93.39
C32	509.3	28.5	94.40	33.0	93.52
C33	1001.6	109.5	89.07	28.5	(97.15
C34	1000.0	233.3	76.67	75.5	92.45
C35	707.7	26.3	96.28	166.6	76.46
C36	657.2	134.8	79.49	69.8	89.38
C37	55.9	147.5	-163.86	289.7	-418.24
C38	434.6	229.6	47.17	172.7	60.26
C39	82.0	215.0	-162.19	208.8	-154.63
C40	37.7	184.4	-389.13	171.6	-355.17
Total	**10,906.9**	**3357.9**	**69.2**	**1947.4**	**82.1**

% Deg. indicate percentage degradation

4. CONCLUSION

In conclusion, the result presented in this study showed that *Citrobacter* species has higher crude oil degradative potential a good candidate in the bioremediation of hydrocarbon polluted environment.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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