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Enhanced Degradation of C_{17} and C_{34} of Bonny Light Crude Oil by *Enterobacter* sp.

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Investigation of enhanced potential of *Enterobacter sp*. in biodegradation of Bonny light crude oil components, C_{17} and C_{34} was carried out. Samples of water and sediment were collected from predetermined locations and subjected to microbiological analyses using standard methodologies. Of the 32 potential hydrocarbon-utilizing bacteria isolated from the water and sediment samples that were exposed to crude oil contamination, only one isolate,*Enterobacter sp*. (Y_8ESWS_3) was found to effectively biodegrade 99.8% of C_{17} and 93% of C_{34} of the aliphatic hydrocarbon after 28 days of degradation. Analysis of variance assessed by gas chromatography revealed that bacterial species (P=9.05E-05<0.05) and nature of hydrocarbon fraction (P=8.55E-08<0.05) significantly influence the hydrocarbonoclastic potential. The high growth rate of *Enterobacter* sp. and its ability to considerably reduce the hydrocarbon component, C_{17} and C_{34} of Bonny light crude oil makes it, an impressive candidate for consideration in bio – augmentation.

Keywords: Aliphatic-compound, Bacteria, Biodegradability, Gas-chromatography, Hydrocarbonoclastic

Introduction

With increasing industrialization and exploitation of natural resources in the Niger Delta and its environs in recent years, the region's aquatic ecosystem is threatened with increasing ecological and toxicological problems resulting from the release of pollutants. The major sources of pollutants include oil exploration and production activities, gas flaring, dumping of human and industrial waste and the use of fish poison by artisanal fishermen.[1] Hydrocarbon contamination of the marine environment is a continuous problem irrespective of the origin, whether it is from low-level discharge from refineries and drilling platforms or from disaster tanker spills. Hydrocarbon spills result in considerable contamination of marine and estuarine sediments[2], however, it is challenging to predict the effect of such oil contamination due to its complex nature. After release from a damage tanker, weathering processes substantially transformed the oil composition within few hours at sea, with subsequent loss of many of the toxic components.[2]

Bioremediation strategies of polluted ecosystems could either be by stimulating indigenous microbial population through environmental modification or by introduction of exogenous population of microorganisms with known degradation potential to the contaminated site, a process known as seeding.[3,4] Because of its simplicity in application, it can be used on wide areas.[5] Although there are several reports on microbial utilization and biodegradation of hydrocarbon products[6–11] there are no reports on the biodegradation of C_{17} and C_{34} component of Bonny light crude oil. Thus the investigation was to determine the potential of *Enterobacter* sp. in enhancing biodegradation of C_{17} and C_{34} component of Bonny light crude oil.

Materials and Methods

Water and sediment samples used were collected from the crude oil producing coastal zone at the IKO River estuary located in IKO Town, Eastern Obolo Local Government Area of AkwaIbom State in the Niger Delta region, Nigeria. Water samples were collected (10–25 cm) below the water surface. Surface water samples were aseptically collected into clean 1 litre capacity plastic bottles while nanseen water bottle samplers were opened to fill and close below the water for sub-surface water samples.[12] The intertidal sediment samples were obtained by scooping the top (1–5 cm depth) using a short core sampler.[13] Samples were then placed in an ice-cooled chest and transported to the microbiology laboratory for analysis.

Bonny light crude oil used was collected from Qua Iboe Terminal, Ibeno, AkwaIbom State, Nigeria and stored at room temperature (28 ± 2°C) till when needed. The mineral salt medium (MSM) of Zajic & Supplison[14] used had the following chemical compositions, dipotassium phosphate (0.8 g/l), potassium dihydrophosphate (0.2 g/l), ammonium

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chloride (4.0 g/l), magnesium sulphate (0.2 g/l), sodium chloride (0.1 g/l), ferrous sulphate (0.01 g/l).

Isolation of Crude Oil -Utilizing Bacteria

Vapour phase method described by Asitok *et al.*[15] was used in isolating hydrocarbonoclastic bacteria from water and sediment samples. Mineral salt medium (MSM) was used to estimate the numbers of hydrocarbonoclastic bacteria in the water and sediment samples by viable plate count method using the surface spreading technique.

Screening Test for Hydrocarbonoclastic Potential of Bacteria Isolates

The ability of bacterial isolates to use Bonny light crude oil as their source of carbon and energy was determined. The method of Okpokwasili and Okorie reported by Esin & Antai[11] was employed. Mineral salts broth (MSB), 9.9 mL were measured into test tubes and autoclaved for sterilization. The filtered Bonny light crude oil, 0.1 mL was added to the MSB tubes on cooling. Into the test tubes were inoculated, 0.1 mL of the 24 h nutrient broth culture of the hydrocarbonoclastic bacteria isolates respectively. Two (2) tubes were un-inoculated which serve as control. All the test tubes were incubated for 14 days at room temperature ($28 \pm 2^{o}C$).

To assess the utilization of Bonny light crude oil by the bacterial isolates, the tubes were visually compared with the control after every 4 days. The isolates potential to biodegrade the crude oil was recorded as strong (+++), moderate (++), and weak (+) while no growth (−) was regarded as inability to grow. To read the final turbidity of the medium, HACH 2100P Turbidimetre was used while HACH RD/210 spectrophotometer was used to read optical density at 540 nm.

Molecular Characterization of Crude Oil -Utilizing Bacteria

In the extraction and purification of bacterial DNA, ZR Fungal/Bacterial DNA MiniPrep™50 Preps Model D6005 (Zymo Research, California, USA) through PCR (Polymarase Chain reaction) amplification was used. The resulting Ultra-pure filtrate (DNA) was used as the assay template and was transported in ice – cooled chest to Inqaba Biotechnology, laboratory, Pty South Africa for sequencing. A thermocycler (A & E Laboratories, UK Model Cyl-005-1) was used for performing PCR amplifications. The primer pairs used the amplified position 1492–1510 of bacteria 16S rRNA, were 27-F 5'-AGA GTT TGA TYM TGG CTC AG-3, and 1492-R 5′-TAC CTT GTT AYG ACT T-3. Agarose gel (2.0%) electrophoresis in TAE buffer (40 mM Tris-acetate, 2 mM EDTA [pH 8.3]) performed at 70 V for 1 hour was used to separate the amplified products (10 µl). Ultra-violet (UV) transilluminator in a photodocumentation system was used to examine the stained gels. Consideration was given to a major band corresponding to the expected band size in the analysis. One (1) kb (Fermentas, USA) DNA ladder digest was used as a molecular weight marker.

Sequencing of DNA was done at Inqaba Biotechnology Pty South Africa. ExoSAP was used in cleaning the PCR products. The nucleotide sequence of the specific isolated microorganism was determined using Sanger (dideoxy) sequencing technique done with Big dye kit. ZymoSeq clean-up kit was then used to clean the labelled products. The cleaned products were then injected with a 50 cm array, using POP7 into automated PCR cycle- Sanger Sequencer™ 3730/3730XL DNA Analyzers from Applied Biosystems.[16,17]

The result was obtained as nucleotides IN FASTA format. The resultant nucleotide base pairs was used in specie identification by direct blasting on http://blast.ncbi.nlm.nih.gov. using BLAST analysis. A read was BLASTED for every set of isolate and the resultant top hits with minimum E-score showing species name was used to name the specific organism. The homology sequence and phylogenetic tree was constructed.

Assessment of Biodegradability of Bonny Light Crude Oil by Bacterial Isolates

Methods earlier reported by Antai[10], Itah & Essien[18], Esin & Antai.[11(modified)] were employed in assessing the biodegradation of Bonny light crude oil by the four best hydrocarbonoclastic bacteria isolated from the ecosystem. Thirty five (35) numbers of 250 mL Erlenmeyer flasks of 99 mL mineral salt broth (MSB) were prepared. The flasks were sterilized by autoclaving, cooled and 0.l mL of filtered Bonny light crude oil was introduced in each flask flasks. Into 28 flasks, 0.1 mL of 24 h nutrient broth culture of the 4 bacterial isolates was inoculated. Seven (7) un-inoculated flask served as control. This was followed by the incubation of the flasks on a rotary shaker (SGM-300 Gallemkamp, England) on 80 rpm at temperature, $28 \pm 2^{o}C$ for 28 days. At an interval of 4 days, 20 mL of the sample from each set of the inoculated flasks, corresponding to the 4 hydrocarbonoclastic bacteria were taken to assess the Total Viable Count (TVC), pH changes, optical density and Free CO_2 production. To determine TVC, serially diluted culture were plated on nutrient agar and incubated at room temperature ($28 \pm 2^{o}C$) for 24 h after

which counts were taken, expressed as colony forming units per milliliter (cfu/mL). Electronic pH Meter (HACH Sension 3 pH Meter) was used to read pH changes and HACH RD/2010 spectrophotometer was used for reading optical density at 540 nm. For the determination of free CO_2 production, 1 mL of the fermented broth was titrated against 0.05N NaOH solution using Phenolphthalein as indicator to notice stable pink colour at end point. The amount of CO_2 was calculated using the formula:

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

$$\text{Percentage utilization} = \frac{\text{Weight of crude oil in control} - \text{weight of the degraded crude oil}}{\text{Weight of crude oil in control}} \times 100$$

Assessment of Biodegradability of Bonny Light Crude Oil Component, C_{17} and C_{34} by Gas Chromatography

For gas chromatographic analysis, the same procedure for assessing the biodegradation of Bonny light crude oil was carried out in parallel. The extraction and gas chromatographic analysis was done before and after degradation by bacterial isolates.

The technique outlined by TNRCC[19] and API[20] were used. Ten (10) Erlenmeyer flasks were removed after 28 days. The residual crude oil were extracted with dichloromethane, separated by column chromatography and measured in milligram per litre.

Extraction of Crude Oil

Sample containers were rinsed with dichloromethane. Then 100 mL of the sample broth was measured into a separating funnel, after which 10 mL of dichloromethane was added and shaken vigorously to mix to obtain the organic solvent extracts in the organic material. The organic extract was made to pass through a column containing glass wool, silica – gel and anhydrous sodium sulphate and collected into a receiving vial[19,20.]

Gas Chromatography

Organic extract obtained from crude oil extraction was injected into the gas chromatograph. Hypodermic syringe was used to inject 1 µL of the concentrated sample extract into the column thru a rubber septum. There was a vapor constituent partition between the gas and liquid phases as separation occurs. Several fractions of the aliphatic compounds ($C_8 - C_{40}$) were detected automatically as they come out from the column by the FID detector. The results was expressed in mg/l.[19,20]

Statistical Analysis

For statistically analysis of the collected data, SPSS version 17 was used. Analysis of variance was used to assess the influence of bacterial specie and nature of hydrocarbon fraction on hydrocarbonoclastic bacteria.

Results and Discussion

The screen test for the hydrocarbonoclastic potential revealed that most bacteria isolates encountered in the Iko river estuary are capable of utilizing crude oil as their carbon and energy source (Table 1). However, *Enterobacter* sp. designated as

Table 1—Screening test for hydrocarbonoclastic potential by bacteria isolates

S/N	Code	Isolate Code	Turbidity (Visual)	Turbidity (NTU)	Optical Density (540nm)
1	Control	a	−	1.292	0.082
2	$ESWS_1B_{1MSM3}$	f	+	148.2	0.0963
3	$ESWS_2B_{1MSM3}$	e_5	++	159.1	0.187
4	$ESWS_2B_{1MSM1}$	y	+	147.0	0.193
5	$ESWS_3B_{1MSM3}$	C_3	++	205.1	0.18
6	$ESWS_1B_{1MSM1}$	b_2	++	214.0	0.246
7	$ESWS_2B_{1MSM2}$	Act_2	++	163.1	0.179
8	$ESWS_1B_{2MSM1}$	h	+	127.3	0.393
9	$ESWS_1B_{2MSM3}$	Y_2	+++	250.1	1.77
10	ESW_1B_{1MSM3}	J	+	132.2	0.197
11	$FSWB_{2MSM3}$	Y_1	+++	267.1	1.988
12	$ESWS_1B_{1MSM2}$	K	+++	230.1	1.413
13	$ESWS_1B_{2MSM2}$	L	++	161.2	0.306
14	$ESWS_3B_{1MSM1}$	Y_8	+++	258.0	1.42
15	$FSWS_2B2_{MSM3}$	i	+++	250.3	1.467
16	$ESWS_3B_{2MSM1}$	M	+	128.1	0.294
17	$ESWS_3B_{1MSM2}$	p	+	121.0	0.282
18	$ESSWS_1B_{2MSM2}$	Q	+	118.1	0.241
19	$ESSWS_1B_{2MSM3}$	r	+	147.2	0.317
20	$ESSWS_1B_{1MSM3}$	s	+	120.1	0.148
21	$ESSWS_3B_{1MSM1}$	t	++	156.2	0.317
22	$ESSWS_1B_{1MSM1}$	Y_9	++	168.1	0.325
23	$ESES_2B_{1MSM1}$	V	++	130.2	0.313
24	$ESES_3B_{1MSM2}$	O	+++	228.1	1.412
25	$FSES_1B_{1MSM2}$	W	+	129.1	0.247
26	$FSES_1B_{1MSM2}$	X	+	146.2	0.252
27	$FSES_2B_{1MSM3}$	d_4	++	163.3	0.323
28	$FSES_2{}^1B_{1En}$	Y_{12}	+++	215.1	1.375
29	$FSES_1{}^1B_{1En}$	Z	+	139.1	0.342
30	$FSES_3{}^1B_{1En}$	n	+++	243.0	1.414
31	$FSES_2{}^2B_{1En}$	Y_7	++	165.1	0.328
32	$FSES_1{}^3B_{1En}$	Act_3	++	135.0	0.315

Key to Codes: E = Estuary, SW = Surface water, SE = Sediment, SS = Subsurface,

Fig. 1 — Neighbor – joining Phylogenetic tree showing the relationships among *Enterobacter sp.*

Y_8 (Table 1) was among the best bacteria isolates that exhibited higher optical density reading of 1.42 and strong visual turbidity (+++) on the crude oil minimal medium indicating a strong crude oil utilization potential. Others encountered in the ecosystem demonstrated weak or fairly strong oil degrading capabilities. The phylogenetic tree for *Enterobacter* sp. as shown in Fig. 1 is distinct from other *Enterobacter* sp from the Gen Bank especially those from Asian origin *Enterobacter* sp.

The result of biodegradability of Bonny light crude oil by the bacterial isolate, *Enterobacter* sp.- Y_8, is presented in Table 2. It was observed that the total viable count of the organism increased with increase in optical density and free CO_2 evolution while pH decreased as the incubation went on. This suggest that *Enterobacter* sp - Y_8 has active and competent crude oil degradative enzyme.[11] The result revealed that the medium containing the growth of *Enterobacter* sp.- Y_8, resulted in the highest free CO_2 production (803 mg L^{-1}) after 20 days of degradation. The capability of the bacterial isolate to utilization crude oil

Table 2 — Biodegradation of Bonny light crude oil by *Enterobacter* sp. Y_8ESWS_3

Day	Isolate	pH	Optical Density (540nm)	Total Viable Count (TVC)	TVC	Free CO_2 (mg/l)
0	Control	7.10	0.04	NG	0	22.0
	Y_8	7.11	0.08	2.02×10^5	5.31	44.0
4	Control	7.40	0.08	NG	0	39.60
	Y_8	7.55	0.37	2.0×10^6	6.30	609.4
8	Control	7.60	0.09	NG	0	46.20
	Y_8	7.39	0.38	2.5×10^5	5.40	576.4
12	Control	7.62	0.03	NG	0	55.5
	Y_8	6.88	0.65	1.9×10^5	5.28	633.6
16	Control	7.58	0.07	NG	0	72.60
	Y_8	6.70	0.50	2.0×10^5	5.30	750.0
20	*Control*	7.58	0.09	NG	0	81.40
	Y_8	6.64	0.8	2.0 x 105	5.30	803.0
24	*Control*	7.55	0.08	NG	0	99.0
	Y_8	6.25	0.42	2.5 x 105	5.40	748.0
28	*Control*	7.54	0.09	NG	0	105.6
	Y_8	6.66	0.49	1.7 x 105	5.23	765.6

fractions as carbon and energy source is evidenced by the high amount of free carbon-dioxide evolved from the incubated substrates. This is similar to earlier observation by Nkanang *et al*.[21], who asserted that CO_2 and water, as bye-product of hydrocarbon degradation are measures of microbial respiration activity in the ecosystem. The result obtained agrees with earlier work carried out by Darsa *et al*.[22] who stated that in contaminated environments, measurements of total carbon dioxide production can provide outstanding information on the biodegradability potential of hydrocarbons in mineralization studies.

Determination of degradability of C_{17} and C_{34} components of the Bonny light crude oil by gas chromatography analysis after 28 days of incubation are presented in Figs 2–4 and Table 3. The total

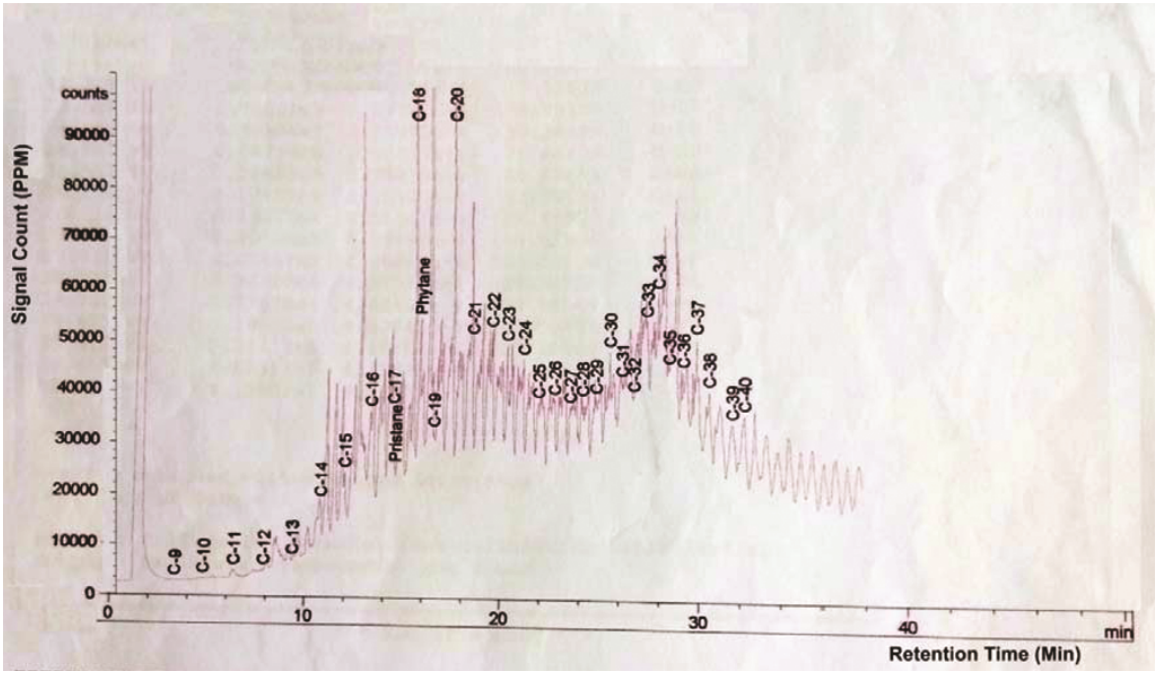

Fig. 2 — Chromatogram of total petroleum hydrocarbon (TPH) of Bonny light crude oil by *Citrobacter amalonaticus*-Y_1

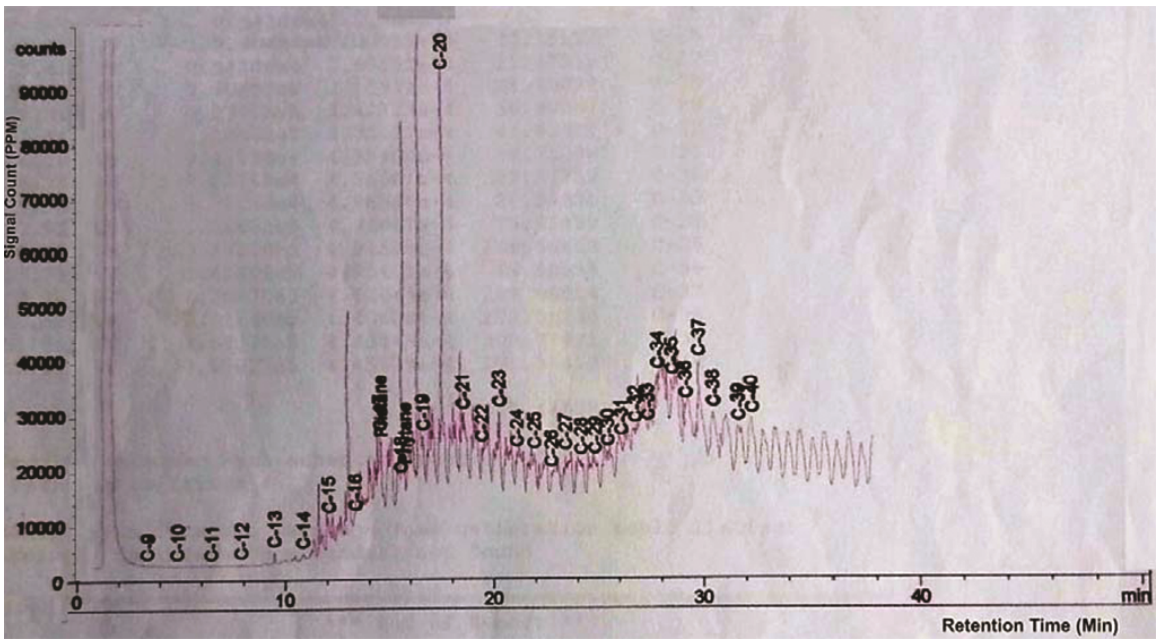

Fig. 3 — Chromatogram of total petroleum hydrocarbon (TPH) of Bonny light crude oil by *Citrobacter amalonaticus* strain -Y_2

Table 3 — GLC analysis of levels of C_{17} and C_{34} component of Bonny light crude oil after 28 days degradation by bacteria species

Parameter		Bacteria species and quantity of hydrocarbon ($mg \cdot L^{-1}$)							
Hydrocarbon	Control	*Proteus mirabilis*- I		*Citrobacter amalonaticus*-Y_1		*Citrobacter amalonaticus* -Y_2		*Enterobacter* sp.- Y_8	
		Residual	% Deg	Residual	% Deg	Residual	% Deg	Residual	% Deg
C17	616.2	97.7	84.14	80.0	87.01	51.1	91.71	1.0	99.84
Pristine	319.8	122.6	61.66	66.1	79.33	74.4	76.74	230.1	28.05
Pytane	862.4	686.1	20.44	338.1	60.79	60.2	93.02	20.3	97.65
C34	1000.0	118.1	88.19	233.3	76.67	75.5	92.45	70.0	93

% Deg indicate percentage degradation

petroleum hydrocarbon resolves 35 aliphatic hydrocarbon component which are identifiable from C_8 to C_{40} with pristine and phytane. The height of the peak is directly proportional to the concentration of the hydrocarbon fraction. The absence of a component from the chromatogram depicts its non-detection and the loss of the volatile hydrocarbon could be due to the differences in the molecular weight; the higher the molecular weight of the hydrocarbon, the longer the retention time. The results showed an excellent decrease in the petroleum hydrocarbon content of the substrates.

Among the organisms assessed for biodegradation of the C_{17} and C_{34} component of Bonny light crude oil, *Enterobacter sp*.-Y_8 emerged as the best degrader of the crude oil components, C_{17} and C_{34} with (99.8% and 93.0%) degradation rate demonstrated by its rapid discoloration of the redox indicator (in free CO_2 production) (Table 2). The other three microbial isolates also exhibited their potential in degrading C_{17} and C_{34} with *Citrobacter amalonaticus* -Y_2 (91.7% and 92.5%), *Proteus mirabilis*-I (84.1% and 88.2%) and *Citrobacter amalonaticus*-Y_1 (87.0% and 76.6%) respectively. Although the isolate *Proteus mirabilis strain* – I exhibited high turbidity of 1.46, it recorded a lower % degradation of 84.1% for C_{17} as compared to *Enterobacter* sp.-Y_8 with 99.84%, and 88.2% for C_{34} as compared to *Enterobacter* sp.-Y_8 with 93%.

Additionally, researchers have reported on the degradation of C_{17} and C_{34} by other microorganisms. Wang,[23] reported that *Acinobacter pittii* H9 -3 strain was able to degrade n –alkane C_{17} after 21 days, the reduction of n-C_{34} compound by the same organism was not obvious. Al –Wasify and Hamed,[24] reported that *Pseudomonas auriginosa*, *Bacillus substilis* and *Acinetobacter iwofii* that was isolated from hydrocarbon contaminated water and soil samples were able to degrade C_{17} and C_{34} minimally. However, no report has been has been on the degradation of C_{17} and C_{34}. Interestingly, this research has shown that *Enterobacter* sp.-Y_8 could degrade C_{17} and C_{34} successfully with high degradation percentage. The ability of *Enterobacter* sp.-Y_8 to degrade crude oil components is not unexpected since the result of other research work, Saadoun[25] have shown the role of *Enterobacter* sp. in degradation of crude oil. Although *Enterobacter sp* is an enteric organism, many studies have reported the ability of strains of this specie to degrade environmental pollutants.[25–27] Katsivela[28] reported the capability of some *Enterobacter sp*. including strain EK 3.1 to grow on a combination of aliphatic, mono-aromatic and poly-aromatic hydrocarbons, including toluene, acenaphthene and acenaphthalene. The ability of *Enterobacter sp*. to degrade faster with higher percentages than others may also be due to environmental factors which include pH, temperature and bioavailability of oil sludge.[29]

Conclusions

Therefore, the high percentage of degradation of C_{17} and C_{34} component of Bonny light crude oil, high growth rate and the capability to reasonably decrease the aromatic component of Bonny light crude oil by *Enterobacter sp.* in this study, makes it a candidate for consideration in bio-augmentation.

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