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CRUDE OIL TOXICITY TOLERANCE OF HYDROCARBONOCLASTIC STRAIN OF *Citrobacter amalonaticus* -Y_2ESW_1 ISOLATED FROM ESTUARINE SEDIMENT IN THE NIGER DELTA OF NIGERIA

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ABSTRACT

Crude oil toxicity possess a major threat to the aquatic ecosystem in the Niger Delta. This study investigated the inherent tolerability potential of hydrocarbon degrading strain of *Citrobacter amalonaticus* - Y_2ESWS_1 to crude oil toxicity. Sediment samples were obtained from selected locations and subjected to bacteriological analysis using standard methods. Bacterial identification result revealed taxonomic group of hydrocarbon utilizing bacteria species including *Citrobacter amalonaticus* strain Y_2ESWS_1. Toxicity testing revealed that the toxicant concentration and exposure time were key variables that mediated toxicity. A second-order polynomial regression model revealed significant (P<0.05, 0.01; R^2=0.9852) relationship between exposure time and bacterial growth at 20% crude oil. *Citrobacter amalonaticus* strain - Y_2ESWS_1 was tolerant to crude oil toxicant concentrations as was evidenced in its prolonged acclimation periods. In this study the toxicity of pollutants to bacteria cultures (*Citrobacter amalonaticus* strain Y_2ESW_1,) was highly dependent on the concentration of the toxicants. The bacterial proliferation was hindered by 20% toxicant. It is a pointer to its poor activity when exposed to hydrocarbon however it can be used in a consortium with other microbes including potent degraders to achieve enhanced degradation of contaminated site.

Keywords: *Citrobacter amalonaticus* strain Y_2ESW_1 , crude oil, toxicity

INTRODUCTION

Pollution is common in aquatic ecosystems. Freshwater, estuarine and marine ecosystems, in recent times, have received attention owing to the considerable stress that they have been subjected to, through deliberate or accidental oil spill, ballast water discharge, untreated sewage and industrial waste water discharges, dredging and agricultural runoff **(Elenwo and Akankali, 2015 ; Antai *et al*.,2016).** Some hydrocarbon components have been known to belong to a family of carcinogenic and neurotoxic organopollutants (**Temitope *et al*., 2015**). Crude oil pollution often results in serious effects on both the biotic and abiotic components of the ecosystem. It has damaging effect on associated microbial community because of their toxic propeies (**Nseabasi and Antai, 2012 ;Iwatt *et al* .,2016).**
Much of crude oil pollution can be eliminated by the activities of hydrocarbon-degrading microbial community especially hydrocarbonoclastic bacteria **(Das and Chandran, 2011**). However, biodegradation of crude oil by these hydrocarbonoclastic bacteria are limited by the resistant and toxic components of the oil itself, low water temperatures, scarcity of mineral nutrients, especially nitrogen and phosphorus, the exhaustion of dissolved oxygen and the scarcity of the hydrocarbon-degrading microorganisms (**Ubani *et al*., 2013**). The pollutants discharged, also pose serious aquatic toxicity problems and affect microorganisms physiological processes, genetic machineries, population density and diversity as well as microbes enzymatic potentials **(Xie *et al*., 2016 and Asitok et al, 2017**). Often, the pollutants inhibit some microbial activities that are important in biogeochemical cycling and productivity in the aquatic ecosystems.
Due to the complex nature of crude oil, biodegradation involves the interaction of many different microbial species. In aquatic environment, it is largely carried out by diverse bacterial populations which are ubiquitously distributed in the water. The most commonly reported genera of hydrocarbon degraders encountered in the crude oil impacted Niger Delta of Nigeria include *Pseudomonas*, *Acinetobacter*, *Nocardia*, *Vibrio* and *Achromobacter* **(Ekpenyong and Antai, 2007; Chikere *et al*., 2016 ; Antai *et al*.,2017a).** *Citrobacter* species are rarely implicated in hydrocarbon degradation nor the tolerance of indigenous strains of oil degraders to hydrocarbon toxicity stress extensively investigated.
Knowledge of the tolerance of hydrocarbonoclastic bacteria to crude oil toxicity on in aquatic ecosystem is critical. This can help in monitoring the efficiency of the process of bioremediation. Examination of the influence of crude oil on the aquatic microbial community will give insight into the vulnerability of this rich ecosystem to low level chronic pollution, which has continued unabated on petroleum oilfields.
This study is therefore intended to examine the tolerance of hydrocarbonoclastic bacteria, *Citrobacter amalonaticus* Y_2ESWS_1 to crude oil toxicity and its oil degrading potential in the aquatic ecosystem.

MATERIALS AND METHOD

Source of Test Isolate

The test bacterium *Citrobacter amalonaticus* Y_2ESWS_1 was isolated from the intertidal sediment of Iko River Estuary in Eastern Obolo LGA of Akwa Ibom State, Nigeria. The area, an oil producing community in the Niger Delta region of Nigeria lies within latitude 7° 30' N and 7° 45' N and longitude 7° 30' E and 7° 30' E. The Iko River Estuary 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 7m (**Udotong *et al*., 2008**). Sampling points were geo-referenced with a hand-held Garmine Trek-type (Garmin 760F) GPS.

Collection of sediment samples

The intertidal sediment samples were obtained by scooping the top (1 - 5 cm depth) using a short core sampler (**Artiola and Warrick, 2004; Forstner and Solomons, 1980**). The collected sediments were scooped from the buckets, mixed together to have a composite sample. All samples were collected in duplicates. Samples were then placed in an ice-cooled chest and transported immediately to the Microbiology Laboratory for analysis. Crude oil was employed as the toxicants. Two liters of Qua Iboe light crude oil were collected from Qua Iboe Terminal into 2 liter- capacity sterile glass bottle and stored at room temperature until needed for use. The toxicant concentrations of 1%$^{v}/_{v}$,

5%$^{v}/_{v}$, 10%, 15%$^{v}/_{v}$ and 20%$^{v}/_{v}$ were employed according to the methods of **Zajic and Supplison (1972**).

Enumeration and Isolation of the hydrocarbon-utilizing bacteria (HUB)

The vapour phase transfer method described by **Asitok *et al.* (2017**) was employed in this analysis using the mineral salt medium (MSM) of **Zajic and Supplison [1972**] as the analytical medium. The medium comprises dipotassium phosphate (0.8g/l), potassium dihydrophosphate (0.2g/l) ammonium chloride (0.4 g/l), magnesium sulphate (0.2 g/l), sodium chloride (0.1 g /l) and ferrous sulphate (0.01 g/l). Hydrocarbon utilizing bacteria in the sediment sample were estimated by the viable plate count method using the spread plate technique. After a 10 - fold serial dilution of the water and sediment samples, 0.1ml of the various dilutions were plated in triplicates onto mineral salt medium supplemented with nystatin to inhibit fungal growth. After inoculating the agar plated with samples, sterile filter paper (Whatman no 1) were asceptically placed on to the inside of the covers of the inverted petri dishes, saturated with 2.0 mls of filtered Qua Iboe light crude oil and sealed around with a masking tape. This was to ensure the supply of hydrocarbons by vapour phase transfer as the sole source of carbon and energy for growth of the organisms that developed on the agar surfaces. The plates were incubated at room temperature (28±2^{o}C) for 5 to 7 days and discrete colonies that developed were counted and expressed as colony forming unit per gram (cfu g^{-1}) of the sediment samples. The discrete colonies were picked and purified by repeated sub-culturing and then stored on nutrient agar slants at 4 ^{0} C in a refrigerator for further studies.

Molecular characterization of hydrocarbon-utilizing bacteria

DNA extraction was performed using a 24 hours grown isolates in BHI broth harvested by centrifugation at 14, 000 x g for 10 minutes. The cells were washed three times in 1 ml of Ultra pure water by centrifuging at 12,000 rpm for 5 min. DNA extraction and purification was done using ZR Fungal/Bacterial DNA MiniPrep™50 Preps Model D6005 (Zymo Research, California, USA). The Ultra-pure resulting filtrate (DNA) obtained was used as a template during the assay. This was transported in ice – cooled chest to the laboratory for sequencing. PCR amplifications were performed on a thermocycler (A & E Laboratories, UK Model Cyl-005-1). Amplified products (10 µl) were separated using 2 % agarose gel electrophoresis in TAE buffer (40mM Tris-acetate, 2 mM EDTA [pH 8.3]) performed at 70 V for 1 hour. Gels were stained with 0.5 µg/ml of ethidium bromide for 45 min and de-stained with water for 20 min. Stained gels were examined under ultra-violet (UV) trans-illuminator in a photo-documentation system (E- box). Major bands corresponding to the expected band size considered in the analysis. A DNA ladder digest of 1 kb (Fermenters USA) was used as a molecular weight marker

DNA sequencing was performed by Sanger (dideoxy) sequencing technique to determine the nucleotide sequence of the specific microorganism isolated. This was done with Big dye kit. The labelled products were cleaned with the ZymoSeq clean-up kit. The cleaned products were injected with a 50cm array, using POP7 into automated PCR cycle- Sanger Sequencer™ 3730/3730XL DNA Analyzers from Applied Biosystems (**Russell, 2002; Metzenberg, 2003**).The result was obtained as nucleotides IN FASTA format. Identification of the species present was done using the resultant nucleotides base pairs. This was performed by BLAST analysis by direct blasting on http://blast.ncbi.nlm.nih.gov. For every set of isolate, a read was BLASTED and the resultant top hits with minimum E-score for every BLAST result showing species name was used to name the specific organism.

Growth response of bacterial isolate to different concentrations of crude oil toxicant

Bacterial response to varying concentrations (1, 5, 10, 15 and 20% v/v) of crude toxicant was assayed using the modified method reported by **Nseabasi and Antai (2012)**. Twenty four (24) h broth culture of bacterial species were serially diluted to dilutions of 10^{-4} to tease out bacterial population. One milliliter (1 mL) from the dilution tube was introduced into 49.5, 47.5, 45, 42.5 and 40 mL of MSM flasks at pH 7.0 to constitute 1, 5, 10, 15 and 20 % $^{v}/_{v}$ respectively of crude oil toxicant concentrations (Zajic and Supplision, 1972). The flasks were incubated at room temperature (28 ± 2^{o} C) for 24 h. At an interval of 8, 16, 24, 32, 40, 48, 56, 64 and 72 h the number of viable bacterial cells in the flasks were determined by pour plate technique and recorded as colony forming unit per ml (CFU mL^{-1}).

Acclimation periods of bacterial growth

The bacterial isolates were subjected to acclimation to the various toxicant concentrations. The methods reported by **Silva *et al.*(2015**)(modified) and **Nseabasi and Antai (2012)**(modified) were employed. Twenty-four (24) h broth culture of bacterial species were serially diluted to dilutions of 10^{-4} to tease out bacterial population. One milliliter (1 mL) from the dilution tube was introduced into 49.5, 47.5, 45, 42.5 and 40 mL of MSM flasks at pH 7.0 to constitute 1, 5, 10, 15 and 20 % $^{v}/_{v}$ respectively of crude oil toxicant concentrations (Zajic and Supplision, 1972). The flasks were incubated at room temperature (28 ± 2^{o}C) for 24 h. At an interval of 8, 16, 24, 32, 40, 48, 56, 64 and 72 h, the number of viable bacterial cells in flasks were estimated by the pour plate technique and bacterial counts were recorded as colony forming unit per ml (CFU mL^{-1}).

Statistical analysis

The data collected were subjected to statistical analysis using SPSS version 17. Two factor analysis of variance was used to compare the mean counts among the physiological groups while a second order polynomial regression was used to determine the goodness of fit in the toxicity studies.

RESULTS AND DISCUSSION

The homology sequence and phylogenetic analysis of the 16S rRNA of the isolates from this study showed that they are closely related *Citrobacter* sp. (Figure 1).The organisms isolated in this study include some of the commonly isolated degraders of hydrocarbon (Atlas and Bartha, 1992).The test isolate, Citobacter sp is rarely encountered in the area. The toxicity of crude oil on *Citrobacter amalonaticus* strain Y_2ESWS_1 is presented in Figure 2**.** The result revealed that the higher the concentration of the toxicant, the higher the toxic effect on the bacterial cells. It showed that the exponential growth of the bacterium in the control was observable within 8 h of incubation and growth reached peak log-transformed concentration at 16 h. Trends in the test systems were different with lag phases of bacterial growth extended when cells were exposed to various concentrations of the crude oil toxicant. At 1 and 5 % toxicant concentrations, there was an extended lag phases until 40 h and growth reached peak log-transformed concentration of cells of 7.03 at 56 h. At 15% toxicant concentration, the organism grew marginally until 40 h with log-transformed concentration of cells of 6.10. At 20% concentrations, the lag phase was up to 24 h after which the toxicant inhibited the growth of the bacterium. A two factor analysis of variance revealed that the toxicant concentration significantly ($p< 0.05$) influenced the growth of *Citrobacter amalonaticus* strain Y_2ESWS_1 but the duration of exposure did not significantly ($p> 0.05$) influence the growth of the bacterium. This shows that despite its tolerance to crude oil toxicity, the bacterium may not have used the medium effectively for growth." The qualitative and quantitative differences in hydrocarbon content of petroleum influence its susceptibility to degradation, a major consideration in determining the toxicological effect of the petroleum

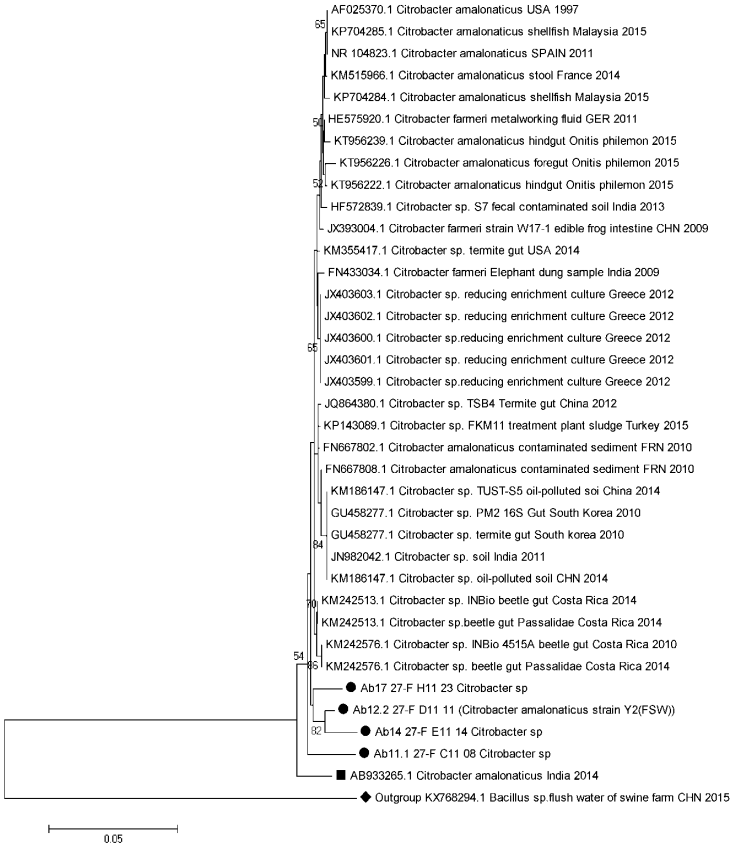

Figure 1 Phylogram of *Citrobacter sp.*

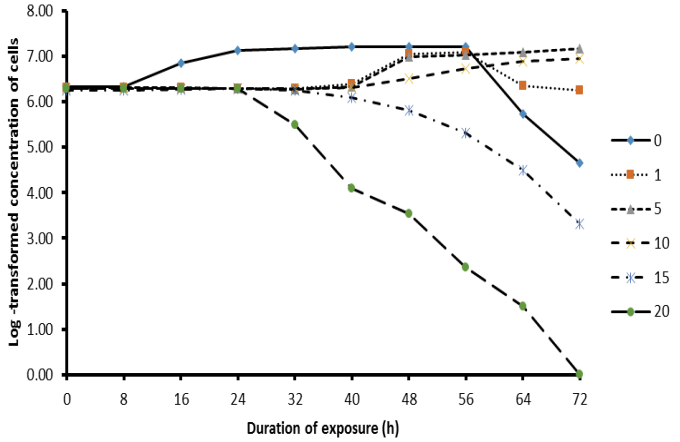

Figure 2 Periodic toxicity of crude oil to *Citrobacter amalonaticus strain* Y_2 $ESWS_1$. Crude oil concentrations are expressed as *per cent* (%)

The acclimation periods of *Citrobacter amalonaticus* strain Y_2ESWS_1 growth responses in glucose-minimal medium (control) is presented in Table 1.The result revealed that the acclimation period for the bacteria isolates *Citrobacter amalonaticus* strain Y_2ESWS_1, was 8 h. Two-factor analysis of variance revealed that both the nature of the organism and the duration of exposure significantly ($p<0.05$) influence the growth of the organism. Figure 3 shows the regression analysis result of the plot of mean log-concentration of cells against the incubation time in the control. The result revealed the second order polynomial regression showing R^2 value of 0.8456 for *Citrobacter amalonaticus* strain Y_2ESWS_1, indicating significant ability of the model to explain the glucose effect on the growth of the isolate.

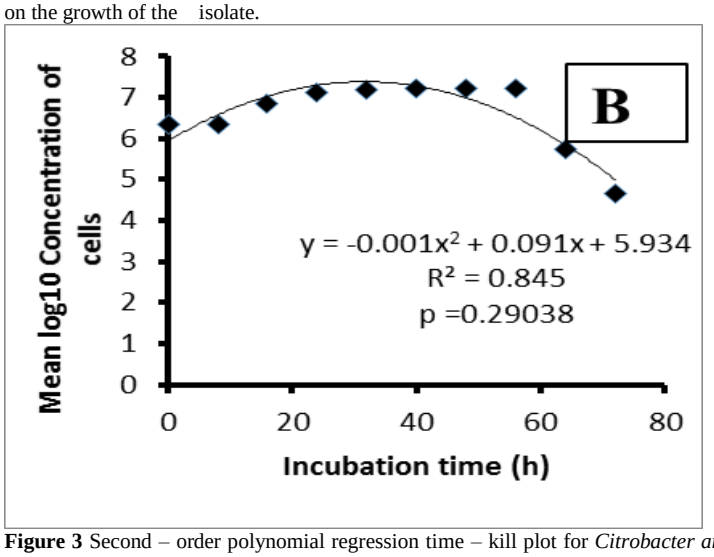

Figure 3 Second – order polynomial regression time – kill plot for *Citrobacter amal* mediu

Table 2 revealed that at 1% concentration of crude oil**,** there was a prolonged acclimation period of 40 h for *Citrobacter amalonaticus* strain Y_2ESWS_1. Figure 4a showed the regression analysis of the plot of the mean log-concentration of cells against incubation time in 1% concentration of crude oil. It showed that the second-order polynomial regression showing R^2 of 0.2227 for *Citrobacter amalonaticus* strain Y_2ESWS_1, indicating significant ability of the model to explain the event in 1% crude oil concentration effect on the growth of the bacteria isolate. Similar prolonged acclimation period was observed for 5% concentration of crude oil (Table 2). Figure 4b shows the regression analysis of the plot of the mean log-concentration of cells against the incubation time in 5% concentration of crude oil which depicts a second-order polynomial regression R^2 value of 0.8538 *Citrobacter amalonaticus* strain Y_2ESWS_1 indicating significant ability of the model to explain the events in 5% crude oil concentration effect on the growth of the bacterium .

Table 2 shows that at 10% concentration of crude oil there was an extended acclimation period for *Citrobacter amalonaticus* strain Y_2ESWS_1 up to 72 hours. Figure 4c shows the regression analysis result of the plot of the mean log concentration of cell against the incubation time in 10% concentration of crude oil.

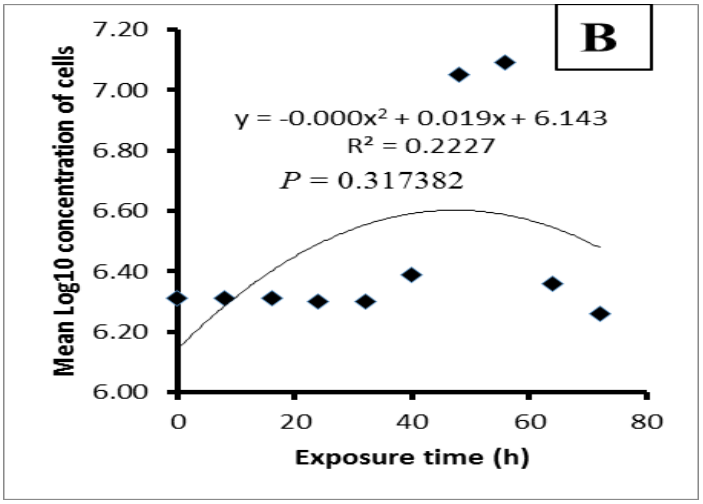

Figure 4a Second-order polynomial regression time-kill plot for *Citrobacter amalonaticus* strain Y_2ESWS_1 grown in 1% crude oil-minimal medium

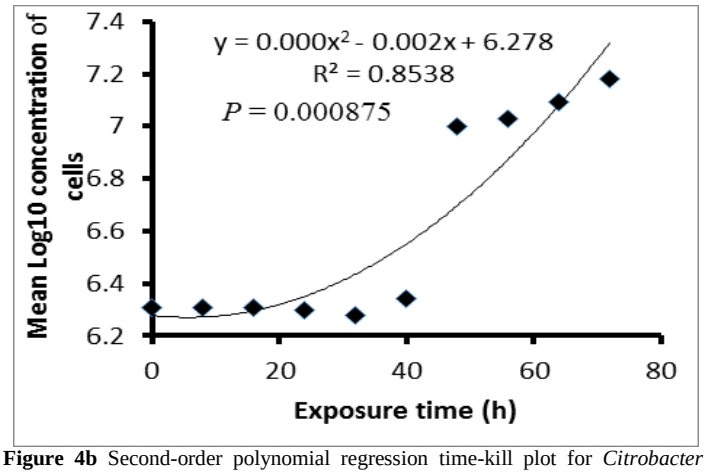

Figure 4b Second-order polynomial regression time-kill plot for *Citrobacter amalonaticus* strain Y_2ESWS_1 grown in 5% crude oil-minimal medium

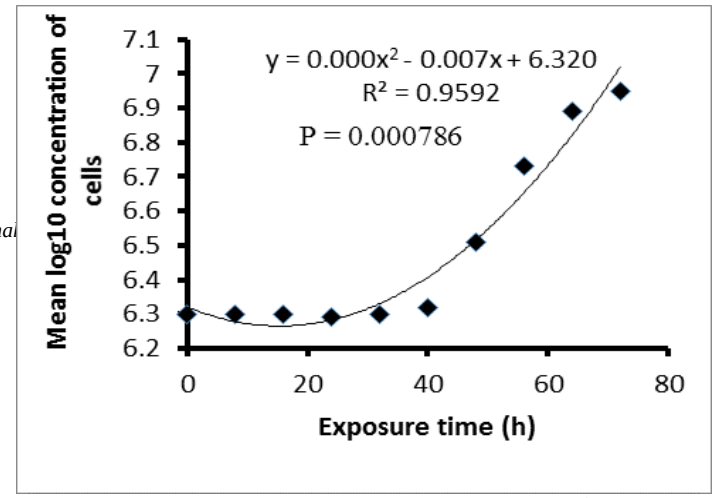

Figure 4c Second-order polynomial regression time-kill plot for *Citrobacter amalonaticus* strain Y_2ESWS_1 grown in 10% crude oil-minimal medium

The result presents the second-order polynomial regression showing R^2 values of 0.9592 for *Citrobacter amalonaticus* strain Y_2ESWS_1, indicating significant ability of the model to explain the events in 10% crude oil concentration effect on the growth of the bacteria isolate.

Results in Table 2.also revealed that at 15%, *Citrobacter amalonaticus* strain Y_2ESWS_1 recorded marginal growth until 40 hours of exposure before there was a decline in the growth after exposure to the toxicant.

Figure 4d shows the regression analysis result of the plot of the mean log concentration of cells against the incubation time in 15% concentration of crude oil. The result presents the second-order polynomial regression showing R^2 values of 0.9688, for, *Citrobacter amalonaticus* strain Y_2ESWS_1 indicating significant ability of the model to explain the events in 15% crude oil

concentration effect on the growth of the bacteria isolate. However at 20% concentration, *Citrobacter amalonaticus* strain Y_2ESWS_1 recorded a marginal growth until after 24 h and entered death phase at the 72 h of exposure (Table 2). Figure 4e shows the regression analysis result of the plot of the mean log concentration of cells against the incubation time in 20% concentration of crude oil.

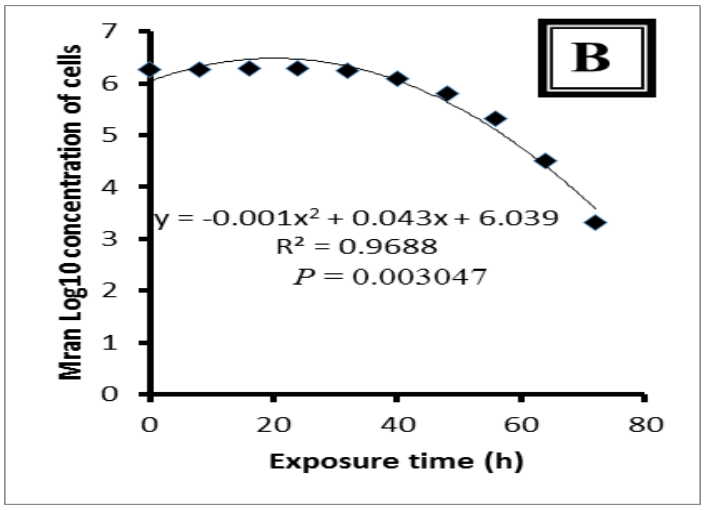

Figure 4d Second-order polynomial regression time-kill plot for *Citrobacter amalonaticus* strain Y_2ESWS_1 grown in 15% crude oil-minimal medium

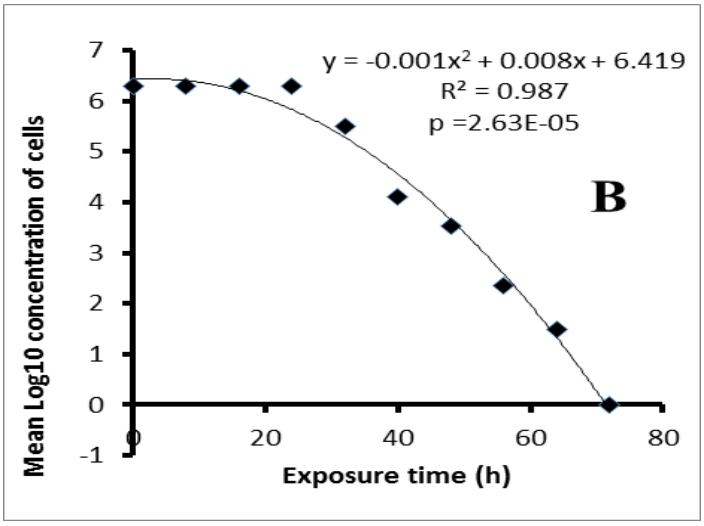

Figure 4e Second-order polynomial regression time-kill plot for *Citrobacter amalonaticus* strain Y_2ESWS_1 grown in 20% crude oil-minimal medium

Table 1 Comparative acclimation periods of *Citrobacter amalonaticus* strain Y_2ESWS_1 growth response to Glucose-minimal medium (control)

Organism	0 hr	8 hrs	16hrs	24 hrs	32 hrs	40 hrs	48 hrs	56 hrs	64 hrs	72 hrs
Citrobacter amalonaticus strain Y_2ESWS_1	2.12×10^6	2.12×10^6	7.13×10^6	1.34×10^7	1.47×10^7	1.63×10^7	1.6×10^7	1.63×10^7	5.5×10^5	4.5×10^4

Table 2 Comparative acclimation periods of *Citrobacter amalonaticus* strain Y_2ESWS_1 growth response to concentrations (%) of crude oil.

Toxicant Conc.(%)	**0 hr**	**8 hrs**	**16hrs**	**24 hrs**	**32 hrs**	**40 hrs**	**48 hrs**	**56 hrs**	**64 hrs**	**72 hrs**
1%	2.05×10^6	2.05×10^6	2.05×10^6	1.99×10^6	2.0×10^6	2.4×10^6	1.11×10^7	1.24×10^7	2.3×10^6	1.81×10^6
5%	2.02×10^6	2.02×10^6	2.02×10^6	2.0×10^6	1.89×10^6	2.2×10^6	9.9×10^7	1.08×10^7	1.2×10^7	1.5×10^7
10%	1.99×10^6	1.99×10^6	1.99×10^6	1.96×10^6	2.01×10^6	2.11×10^6	3.2×10^6	5.4×10^6	7.8×10^6	8.9×10^6
15%	1.8×10^6	1.8×10^6	1.89×10^6	1.97×10^6	1.78×10^6	1.26×10^6	6.5×10^5	2.1×10^5	3.2×10^4	2.1×10^3
20%	1.93×10^6	1.93×10^6	1.93×10^6	2.0×10^6	3.1×10^5	1.26×10^4	3.4×10^3	2.3×10^2	3.1×10^1	0

The result presents the second order polynomial regression showing R^2 values of 0.9872 for *Citrobacter amalonaticus* strain Y_2ESWS_1 indicating significant ability of the model to explain the events in 20% crude oil concentration effect on the growth of the bacteria isolate.

Crude oil spills constitute a world-wide problem because of the toxic components in the oil. The toxicity of crude oil varies widely, depending on their composition and concentration. The scale of pollution depends on the quantity of oil and the damage done to the environment (**Cowell and Walker, 1977**). In heavily polluted areas, there will be an immediate detrimental effect on biological forms (**Obire and Anyanwu, 2009**). **Tiku *et al*,(2016)** and **Paniagua-Michel and Rosales (2015**) emphasized that microorganisms play key role in the remediation of petroleum pollution. However, it cannot be said that all the oil will disappear in a proportionate time, since the remaining fraction may be more retractile to microbial attack. The qualitative and quantitative differences in hydrocarbon content of petroleum influence its susceptibility to degradation, a major consideration in determining the toxicological effect of the petroleum. Although microorganisms are capable of degrading petroleum and petroleum by-products, there is a great deal of variability in the extent of degradation of the petroleum components, with the more refractory components, such as aromatics and polynuclear aromatics accumulating in the aquatic ecosystem **(Das and Chandran, 2011; Antai *et al*.,2017b**). In the present study the toxicity of crude oil to *Citrobacter amalonaticus* strain Y_2ESWS_1, revealed that toxicity increased with time, leading to the reduction in the counts of the bacterial isolate. This could be attributed to the inhibitive effect of crude oil components (**Yemashova *et al*., 2007).** The study also revealed that the toxicity of crude oil to the isolates was highly dependent on crude oil concentration. However at higher concentration (10, 15 and 20 %) of crude oil, *Citrobacter amalonaticus* strain Y_2ESWS_1 was still able to grow marginally. The crude oil toxicant was toxic as it could terminate growth of the bacterium at 20% toxicant concentration within 72 h. This could be attributed to the presence of high concentration of short chain biologically-available fractions as well as long and un-metbolizable hydrocarbon chains (C_2 - C_{55}) in the compound. The statistical analysis revealed that the concentration of crude significantly affected the growth of the various bacteria isolates ($p \leq 0.005$). Like other microorganisms, bacteria are not insensitive to the changes in their environment. At such whenever there is any alteration in their chemical or physical environment, there is acclimatization period, during which the microbial community adapts to the new environmental conditions (**Chu and Barnes, 2016; Oyedeji, 2016)**. This acclimatization period enables microorganisms to possess the metabolic repertoire necessary for their survival (**Etuk *et al*., 2012**). Acclimation periods of *Citrobacter amalonaticus* strain Y_2ESWS_1 growth response in crude oil concentrations revealed that at crude oil concentrations of 1, 5 ,10 and 15 %, there was acclimation for the bacterial isolates. This result agrees with **Chu and Barnes (2016)** who stated that, whenever the chemical or physical environment is suddenly altered, there is a lag period during which the microbial community adapts to the new conditions. At 20% toxicant concentration, the acclimation period was 24 h after exposure, however, the organism was able to survive until 72 h of exposure. This agrees with the findings of **Nseabasi and Antai, (2012)** who reported that microbial community decreases in species richness in the presence of a selective pollutant. The statistical analysis revealed that the nature of the organism and the exposure time influences the growth of the organism ($P<0.05$).

CONCLUSION

This study has revealed that *Citrobacter amalonaticus* strain Y_2ESWS_1 is not adversely affected by the duration of exposure to the toxicants, rather the toxicity to the organism is just a function of the concentration of the toxicant. In this study the toxicity of pollutants to bacteria cultures was highly dependent on the

concentration of the toxicants. The bacterial proliferation was hindered by 20% toxicant. It is a pointer to its poor activity when exposed to hydrocarbon however it can be used in a consortium with other microbes including potent degraders to achieve enhanced degradation of contaminated site

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