

Catechol-2,3-Dioxygenase and Crude Oil Biodegradation Screening of Rhizo-Bacterial Endophytes from Bodo-Gokana, Rivers State

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Strain-selection for the biotechnological application is critical in modern environmental bioremediation process design. In this study, twenty-one rhizobacterial isolates were obtained from the rhizosphere soil of *Cyperus sp.*, *Cyperus rotundus*, *Mariscus alternifolius* and *Mariscus ligularis*. Samples were treated using Bushnell-Haas media fortified with Bonny light crude oil plus 1% (v/v) rhizosphere soil from pre-impacted locations in Bodo-Ogoni, Gokana LGA, Rivers state. They were screened and four bacterial isolates were selected on the basis of -2,3 catechol dioxygenase activity and their growth dynamics using the growth function model in XLSTAT v 2019.1.3. Vapour-phase transfer and viable plate count techniques were employed in the determination of microbial dynamics. The order for relative enzyme activity and degradation rates followed *Pseudomonas fluorescens* > *Achromobacter agilis* > *Bacillus thuringiensis* > *Staphylococcus lentus*. The order for growth range were 7.0-10.5 Log₁₀CFU/ml, 6.2-10.3 Log₁₀CFU/ml, 7.1-10.1 Log₁₀CFU/ml and 6.4-10.2 Log₁₀CFU/ml for *Achromobacter agilis* > *Pseudomonas fluorescens* > *Bacillus thuringiensis* >

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Staphylococcus lentus. The growth pattern of these isolates fitted into the 5th order polynomial function ($y = pr_1 + pr_2 * X + pr_3 * X^2 + pr_4 * X^3 + pr_5 * X^4 + pr_6 * X^5$) with R²-values of 0.999, 0.998, 0.991, 0.999 compares to Gompertz and Asymptotic functions that have the least predictability with R²- values of 0.893, 0.599, 0.869, 0.894 and 0.80, 0.545, 0.829, 0.688 for the four isolates respectively. Enzyme activity of the isolates revealed that the isolates were most active on the 6th day of the study and had a lag phase within the first few hours to a day of the study. Statistical analyses revealed a significant difference using two-way ANOVA; $p < 0.001$ for both enzyme activity and growth rate. The results underscore the benefits and richness of rhizobacterial flora as rich in enzymatic activity for ecosystem-recovery. Overall, the study has shown the great potential and feasibility for deploying robust biotechnology for the monitoring of environmental media involving hydrocarbon pollution in the Niger Delta.

Keywords: *Biotechnology; rhizobacterial; catechol dioxygenase; growth function; polynomial function; gompertz; asymptotic; enzymatic activity; ecosystem-recovery.*

1. INTRODUCTION

The bioremediation of hydrocarbon polluted soil takes advantage of nature's own microbial attributes, that carry genes organized into pathways encoding necessary enzymes for the breakdown of pollutants [1,2], Petroleum hydrocarbons undergo multiple but different steps involving various enzymes [3,4] during biodegradation. For instance, the conversion of alkane to alcohol starts with the oxidation of the methyl group by alkane monooxygenases, cyclic alkanes are converted initially to ketones, by an oxidase system, where mono-oxygenase and lactone hydrolase each specific to a different stage of the conversion [4,5]. In the case of aromatic hydrocarbons, which are less responsive to biodegradation than saturated hydrocarbons, cleavage of the benzene ring primarily follows an oxidative attack, then accomplished by different enzymes [6]. Elzobair et al. [7] stated that many soil processes such as nutrient cycling are primarily regulated by microbial communities and their enzymes and changes to microbial community structure and enzyme activity might indicate potential long-term effects of biochar on soil nutrient cycling processes [7]. Soil microorganisms are very sensitive to any perturbation of the ecosystem and their diversity and activity are rapidly altered by such perturbations [8,9]. The measurement of microbiological parameters, such as soil respiration, microbial biomass, carbon sources, and enzyme activities, serves as indicators on the presence and activity of viable microorganisms as well as on the extent, type, and duration of the effects of hydrocarbon pollution on soil metabolic activity. Such measurement may serve as a reliable indication of the impact of pollution on soil health [10,11]. According to Abu [12], microbes are the most

amazing packaging of enzyme and enzyme systems known to man. Enzymes play a key role in the microbial degradation of oil, chlorinated hydrocarbons, pesticide, fuel additives, and many other compounds [13,14]. Monooxygenases and dioxygenases, which are produced by hydrocarbon-degrading bacteria, can catalyze the initial oxidation reactions of n-alkane and aromatic hydrocarbons to primary alcohols (monooxygenase reactions with hydroxylases) and trans-dihydrodiols (monooxygenase reactions) or cis-dihydrodiols (dioxygenase reactions). Further oxidation of the trans-dihydrodiols and the cis-dihydrodiols leads to the formation of catechols, which are substrates for other dioxygenases that catalyze enzymatic cleavage of the aromatic ring [13,15,16]. The alkane hydroxylases play a vital role in the special oxidation of the C-H bond cleavage. Van Beilen & Funhoff [17] reviewed long-chain n-alkane oxidation for two classes of enzymes: (a) the class of cytochrome P450-related enzymes in both yeasts and bacteria, e.g., bacterial CYP153 enzymes, and (b) the class of bacterial particulate alkane hydroxylases. Wentzel et al. [18] summarized the bacterial metabolism of long-chain n-alkanes and stated that enzyme systems involved in the utilization of long-chain n-alkanes have led to an improved understanding of microbial long-chain n-alkane metabolism. Hydrocarbon-oxidizing bacteria capable of growth on aliphatic and aromatic hydrocarbons are found in many genera. In the presence of O₂, the initial steps in the bacterial degradation of hydrocarbons rely on oxygenases. These oxygenases are membrane-bound, and the cell must come into direct contact with their water-insoluble substrates. Given that the oxygenases are group-specific for example, some degrade specific fractions of alkanes, whereas others work on aromatics or cyclic

hydrocarbons it follows that only a mixture of different microorganisms can efficiently degrade crude oil and petroleum fractions. The polycyclic aromatic hydrocarbons (PAHs) are a minor constituent of crude oils; however, they are among the most toxic to plants and animals. Bacteria can completely convert PAHs to biomass, CO₂, and H₂O, but they usually require the initial insertion of O₂ via dioxygenase enzymes. This study was designed to use the dynamics of catechol-2,3-dioxygenases (C23O) as a marker for the bioremediation screening of high throughput bacterial strains mined from rhizosphere of aged polluted soil.

2. METHODOLOGY

2.1 Study Site and Sample Collection

The sample collection was from an aged oil-polluted soil site in B-dere, Gokana of Ogoni, Rivers state (4°36'N and 7°15'E). Plant and their rhizosphere soil obtained from the study location was transported aseptically, in sample bags to the Greenhouse belonging to the Department of Plant Science and Biotechnology, University of Port Harcourt Rivers state. The service of an herbarium curator was sought to identify plants as *Cyperus sp.*, *Cyperus rotundus*, *Mariscus alternifolius*, and *Maricus ligularis*.

2.2 Enumeration and Isolation of Rhizobacterial

The total bacterial population in the oil-polluted rhizosphere soil sample was enumerated and isolated adopting serial dilution and the standard plate counts technique using the pour plate method [19]. Ten grams of the soil sample was measured into a conical flask and 90ml of sterile normal saline was mixed with the sample. The suspension was properly shaken for three hours to homogenize the solution and this served as the stock solution. Ten-fold serial dilution of all the homogenized mixture was carried out using sterile normal saline as diluents. Seven test tubes containing 9ml of normal saline were used for the serial dilution. Aliquots of 1 ml from 10⁻⁵ and 10⁻⁷ dilutions were introduced into duplicate sterile petri dishes and 20 ml of molten nutrient agar incorporated with nystatin to suppress fungal growth was poured into the plates and swirled to allow homogenization. The plates were incubated at 37°C for 24 hours after which colonies on the plates were enumerated and subculturing of bacterial isolates was done to obtain a pure culture. Bacterial colonies were

picked with a sterile inoculating loop and streaked on freshly prepared nutrient agar plates [20]. The plates were incubated at 37°C for 24 hrs. Aliquots of 1ml from dilutions of 10⁻⁵ and 10⁻⁷ were also plated in duplicates on Bushnell Haas Agar, using the spread plate technique; 100 µgml⁻¹ Nystatin were added to the Bushnell Haas Agar to suppress fungal growth. A filter paper saturated with sterile crude oil was aseptically placed on the inside of the inverted Petri dishes and the culture plates were incubated for 14 days at 37°C. Plates containing colonies were afterward enumerated for the bacterial load [19].

2.3 Determination of Hydrocarbon Utilizing Bacteria

Bushnell Haas medium was prepared by dissolving 3.2 g of BHM (LabM, China) salt in 1000 ml of deionized water and the media were solidified with 15 g of agar. The media were preheated and allowed to cool. A 1.0% (v/v) of lactic acid with pH of 2.56 was introduced into the media to inhibit fungal contaminants [21]. Bonny-light crude oil was autoclave-sterilized along with media. Vapour phase method reported by Amanchukwu et al. [22] was adopted. Pre-sterilized Whatman filter paper was impregnated with Bonny light crude oil (BLCO) and placed in the lid of the petri dishes. The plates were incubated at 37°C for 48 hours with the lid in an inverted position [23].

2.4 Identification of Isolates

Pure bacterial isolates were identified based on their cultural, morphological and biochemical features using the taxonomic scheme of Bergey's Manual of Determinative Bacteriology (1984) [24,20].

2.5 In-vitro Screening for Bacterial Hydrocarbon Degradation Potential

Crude oil Biodegradation screening of the organism was determined by the method of Shekhar et al. [25]. *In vitro* hydrocarbon degradation potential was determined using 18 h old bacterial inoculum (1 ml) transferred into 98 ml Bushnell-Hass medium at pH 7.0 and was supplemented with 1 ml of Bonny light crude oil as the carbon source and incubated at 37°C at 170 rpm in a shaker incubator for a period of 7 days. The hydrocarbon degradative potential of the bacterial isolates was both monitored by viable plate count and optical density (OD) methods. The growth of the bacterium was

measured by taking the optical density (OD) readings at 600nm for 7 days at regular 1-day intervals using a spectrophotometer, against the Bushnell Haas medium as blank. A corresponding 5 ml aliquot sample was collected out of which a 1 ml was used as serial dilution for viable plate count culture and 4 ml was used in crude cell free extract for the determination of total protein. Corresponding daily reading of pH is taken and recorded. All experiments were performed in duplicate [26].

2.6 Determination of Bacterial Specific Growth Rate

Growth kinetic parameters for the isolates were determined by using the total viable counts obtained with time from the batch culture experiment and assuming first-order kinetics. Batch culture occurs in a closed system that contains an initial limited amount of substrate, in this case, the isolates were inoculated in Bushnell-Hass medium amended with 1ml crude oil as the only carbon source. The inoculated microorganism will pass through a number of growth phases. During the log phase, cell numbers increase exponentially at a constant maximum rate. In practical laboratory data, the specific growth rate for a microorganism is calculated from the linear portion of a semi-log plot of growth versus time

In mathematical terms, we can write the first-order rate equation:

$$r = dN/dt = \mu N \quad (1)$$

Where, N = the concentration of microbial biomass, t = the time in hours, and μ = specific growth rate of microorganisms in hours⁻¹

On integrating equation (1), within the limit; at t = 0, N = N₀ and at t = t, N = N:

$$\ln(N/N_0) = \mu t \quad (2)$$

To obtain the specific growth rate (μ) of the bacterial isolates for each batch culture concentration of crude oil amended, the graph of $\ln(N/N_0)$ against time t, is plotted. The slope of the graph gives the specific growth rate at the initial concentration of crude oil being amended.

2.7 Total Protein Estimation from Cell Free Extract (Bradford Method)

A standard curve was constructed by developing various standard protein concentrations of

Bovine Serum Albumin (BSA) from a stock concentration of 0.3 gml⁻¹. About 1 ml of stock BSA was first diluted in 100 ml of distilled water having a concentration of 3 mgml⁻¹ of the stock protein. Further serial dilution was carried out by preparing 9 empty sterilised test tubes and labeled 1 to 9. Then a micropipette was used to pipette sample from the prepared 3 mgml⁻¹ stock solution in 1000 μ l, 750 μ l, and 500 μ l into tube 1, 2 and 3 respectively, this was made up to 1000 μ l with distilled water. Then 500 μ l each was pipetted from tubes 2 and 3 into the empty tubes 4 and 5. This was also made up to 1000 μ l with distilled water. Similarly, 500 μ l was pipetted from tube 5 to tube 6 and made up with distilled water to 1000 μ l. The process is then repeated from tube 6 to tube 7 and from tube 7 to finally tube 8. Test tube 9 contained only 1000 μ l of distilled water as the zero (blank) sample. The standard curve was prepared using sample from tubes 4 to 9.

The standard samples of tubes 4 to 9 were suspended in a 4 ml lysis buffer. The lysis buffer was prepared from 50mM Tris phosphate pH 8.0, 1% glycerol for the stabilization of the protein and prevention of aggregation, and 0.1% SDS for the prevention of aggregation of hydrophobic and membrane proteins. Next, 5 ml of Coomassie brilliant blue reagent was added. The Coomassie brilliant blue reagent was prepared by adding 100 mg of Coomassie brilliant blue G-250 in 50 ml of 95% ethanol and 100 ml of 85% phosphoric acid and made up to 600 ml with distilled water; then the mixture solution was then filtered and 100 ml of glycerol was added, then made up to 1000 ml with distilled water. Then following the addition of the 5 ml Coomassie brilliant blue reagent, the tubes were mixed by gentle inversion to avoid foaming and allowed to wait for 5 to 30 minutes before readings were taken. A Cary 55B UV-Vis Spectrophotometer was already powered on for 15 minutes, then auto zero by taking the absorbance reading of the blanks sample (test tube 9) at 595 nm wavelength. The absorbance reading at 595 nm wavelength was also taken for test tubes 4 to 8. The results were used to construct a standard curve of absorbance versus concentration.

The free cell extract from bacterial isolates (previously enriched by centrifuging at 5000 g, for 10 min at 4°C. Approximately, 1 ml of the supernatant was suspended in a 4 ml of lysis buffer and kept in an ice bath) was mixed with the Coomassie brilliant blue reagent, gently inverted to avoid foaming and allowed to wait for 5 minutes. The free cell extract samples from the

bacterial isolates were then read using the UV-Vis spectrophotometer at 595 nm wavelength. The results of the corresponding absorbance or optical density (OD) were recorded against samples and their total protein concentration was read off the standard curve.

2.8 Estimation of Specific Enzyme (catechol-2,3-dioxygenase) Activity

The specific enzyme is quantifiable due to the enzymatic interaction between the protein from the cell-free extract and the intermediate hydrocarbon degradation product, catechol. Meta-Cleavage dioxygenase activity was assayed by monitoring the increase in absorbance at the corresponding wavelength of the meta-cleavage product with the use of a UV-Vis spectrophotometer. The procedure involved adding a 0.2 ml cell-free extract (previously enriched by centrifuging at 5000 g, 10 min at 4°C and preserved in a cool ice bath) to 2 ml of 50 mM Tris-HCl buffer pH 7.5 (standard stock solution), 6 ml of distilled water was added and mixed by inversion. Then a 0.2 ml catechol was added. The increase in absorbance or optical density (OD) at 375nm wavelength caused by the formation of the reaction product 2-hydroxymuconic semialdehyde was monitored. The results of the absorbance or OD were recorded for each isolate. One unit of the specific activity is defined as the amount of enzyme that converts 1 μ l of meta-cleavage product per minute at 30°C.

2.9 Statistical Analysis

Data obtained during the benchwork were analyzed using XLSTAT version 2019.1.3 and ANOVA by SPSS. The analysis was considered at p-value <0.05 significance. The data were presented in graphs and tables using Microsoft Excel 2016.

3. RESULTS AND DISCUSSION

Biochemical characterization of the bacteria as shown in Table 1, revealed the isolates as *Achromobacter agilis*, *Pseudomonas fluorescens*, *Bacillus thuringiensis*, and *Staphylococcus lentus*. The biochemical characterization indicates three (3) of the isolates were Gram-positive and one Gram-negative. The result presented in Fig. 1 shows the changes in microbial population during the *in-vitro* screen of bacteria for hydrocarbon degradation potential. The biomass and cell count accumulation pattern as presented in Fig. 1 describes an increase in

the bacterial population between the first minute of inoculation in day 1 to day 7. The result suggests there was a very short lag phase of growth between the inoculum time of first minute and day 1 as summarized in Table 2, of the growth pattern as monitored by the plate count approach and cell optical density. The growth entered a log or exponential phase between 2nd to 5th day as shown in Fig. 3, of the monitoring which was significantly different ($p < 0.05$) from day one. However, *Achromobacter agilis* was observed to have increased in cell count from 6.9 to 10.5 Log₁₀ Cfu/ml between the inoculum time of first minute to 5th day and steadily fell or declined between day 5 to 7th of the growth monitoring which then reduced to 9.7 Log₁₀ Cfu/ml on the 7th day of the monitoring. The specific growth rate μ of 0.07 h⁻¹ as shown in Table 2, of the hydrocarbon degradation growth pattern, confirmed *Achromobacter agilis* as having the highest activity. This is followed by *Pseudomonas fluorescens* as shown both in Fig. 1 and Table 2. The cell count of *Pseudomonas fluorescens* increased from 6.2 to 10.3 Log₁₀ Cfu/ml between the inoculum time of first minute to 5th day and steadily fell or declined between day 5 to 7th day of the growth monitoring which reduced to 8.3 Log₁₀ Cfu/ml on the 7th day of the monitoring and has a specific growth rate μ of 0.068 h⁻¹. The activity was then followed by the pattern presented by *Bacillus thuringiensis* and its specific growth rate μ of 0.058 h⁻¹. Furthermore, *Bacillus thuringiensis* was observed to have an increase in the cell count from 7.1-10.1 Log₁₀ Cfu/ml between the inoculum time of first minute to 6th day and declined on day 7th to 9.4 Log₁₀ Cfu/ml. Lastly, *Staphylococcus lentus* shows the least activity from the growth pattern and the specific growth rate value. The Line graphs clearly put both *Achromobacter agilis* and *Pseudomonas fluorescens* as the lead in the utilization of BLCO and the growth pattern also suggests the utilization of BLCO by the entire isolates during the study.

The changes in the pH of the media were recorded during the study and the results are presented in Fig. 2. The study shows that all four bacterial isolate hydrocarbon degradation led to pH decline from about pH 7.2 to 6.4. The decline in pH is most likely due to bacterial metabolism.

The result presented in Fig. 4, reveals the calibration of protein activity using the Bradford approach. The optical density was observed to vary between 0.0165 to 1.8871 Abs with Bovine Serum Albumin concentration between 0 and 1.25 Abs. The experiment was designed to

enable the detection of very low enzyme activity during the screening of bacterial hydrocarbon degradation potential. The result presented in Fig. 5 reveals the variation in the enzyme activity for catechol-2,3-dioxygenases (C23O) during the *in-vitro* screening for bacterial hydrocarbon degradation potential. The variation suggests that *Pseudomonas fluorescens* had the highest enzymatic activity, especially on the 6th day. This is followed in the order of *Achromobacter agilis*, *Bacillus thuringiensis*, and *Staphylococcus lentus*

of decreasing enzymatic activity. The enzyme activity followed a sinusoidal growth pattern during the *in vitro* degradation of the crude oil. There was a steady increase in the enzyme activity during the study but this was more conspicuous between days 3 to 6 and declined on the 7th day. The decline in the biomass at the 7th day Fig. 1 was observed to have a corresponding effect on enzyme synthesis described in Fig. 5.

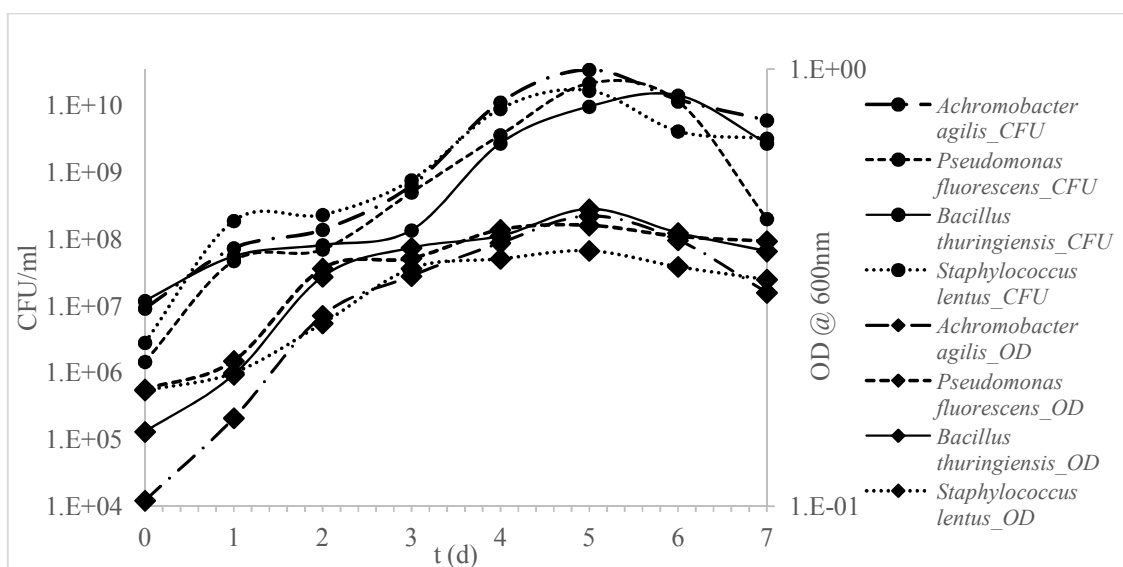


Fig. 1. Bacterial hydrocarbon degradation potential growth pattern- showing cell count/ biomass concentration of *Achromobacter agilis*, *Pseudomonas fluorescens*, *Bacillus thuringiensis*, *Staphylococcus lentus* measured in the colony-forming unit (cfu/ml) and optical density (OD) over time in days in a semi-log scale

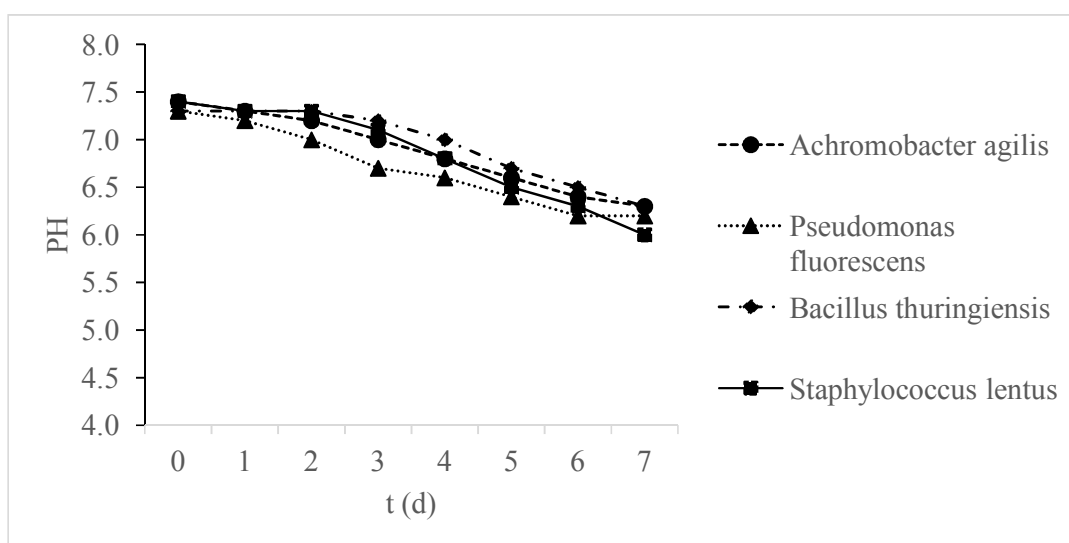


Fig. 2. pH readings during bacterial hydrocarbon degradation potential study

Table 1. Morphological and biochemical characteristics of bacterial Isolated from aged rhizosphere soil Collected from B dere Gokana, Ogoni, Rivers state

Isolates Code #	Grams Rxn	Spore Test	Catalase	Oxidase	Gram Morp	Citrate	Indole	MR	VP	Motility	Slant	Butt	H2S	Gas	Trehalose	Mannose	Rafinose	Celbiose	Ribose	Urease	Xylose	Galactose	Mannitol	Arabinose	Starch	Probable bacteria
A4	-	-	+	+	Rod	-	+	-	+	+	K	K	-	-	-	-	A	A	A/G	+	-	A		-	<i>Achromobacter agilis</i>	
A3	-	-	+	+	Rod	+	-	+	-	+	A	A	-	-	A	A/G	A/G	A	A/G			A	A/G	A/G	-	<i>Pseudomonas fluorescens</i>
D2	+	+	+	+	Rod	-	+	-	+	-	K	A	-	-	A	A	-	A	-	+	-	-	-	A/G	-	<i>Bacillus thuringiensis</i>
L1	+	-	+	+	Cocci	+	-	-	+		A	A	-	+	A/G	A	A/G	A/G	A/G	+		A/G	A	A/G	+	<i>Staphylococcus lentus</i>

A4, A3, D2, and L1 represents *Achromobacter agilis*, *Pseudomonas fluorescens*, *Bacillus thuringiensis*, and *Staphylococcus lentus*

Table 2. Summary of kinetics parameter of bacterial hydrocarbon degradation potential growth pattern

	Specific growth rate (μ) h ⁻¹	Growth lag time (λ) d	Asymptote-max bacterial count (A)	R ² value
<i>Achromobacter agilis</i>	0.070	0.6	3.42E+10	0.957
<i>Pseudomonas fluorescens</i>	0.068	0.5	2.15E+10	0.967
<i>Bacillus thuringiensis</i>	0.058	0.7	1.41E+10	0.895
<i>Staphylococcus lentus</i>	0.053	1.0	1.67E+10	0.926

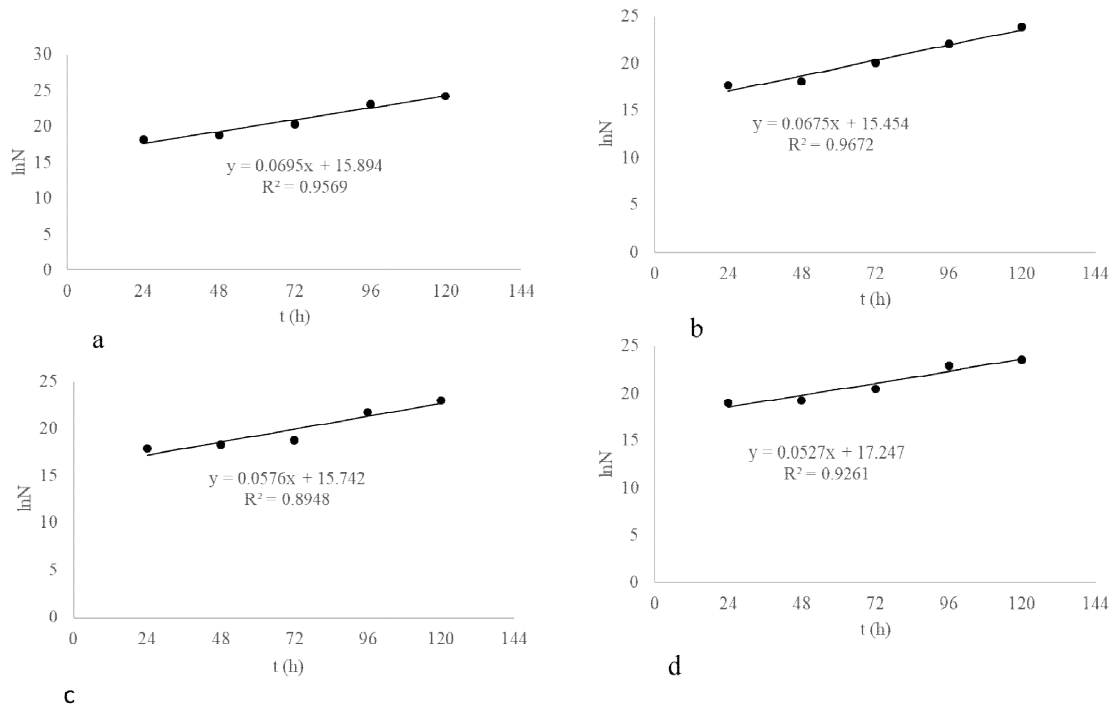


Fig. 3. shows the natural log vs time of the exponential growth of (a) *Achromobacter agilis*, (b) *Pseudomonas fluorescens*, (c) *Bacillus thuringiensis*, and (d) *Staphylococcus lentus*. The slope of the line is equal to the specific growth rate (μ)

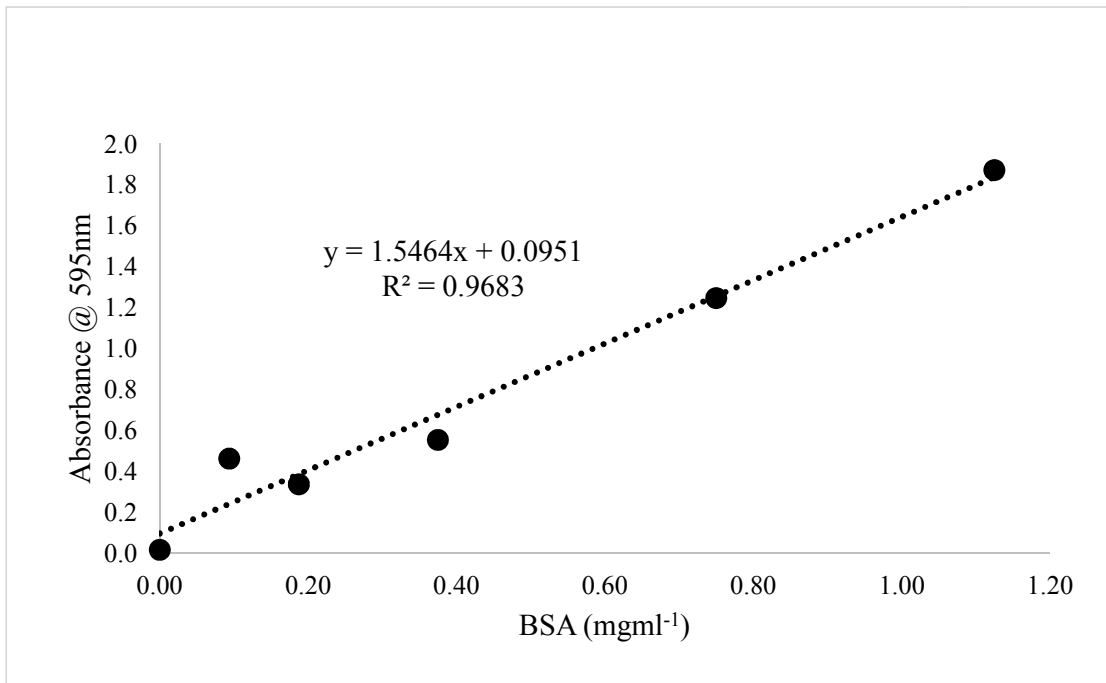


Fig. 4. Standard protein calibration curve for the estimation of total protein concentration in bacterial cell free extract

Table 3. Kinetic model of bacterial hydrocarbon degradation potential growth pattern

Growth model	Goodness of fit stat indices	Biomass optical density				Heterotrophic cell count Log10cfu/ml			
		<i>Achromobacter agilis</i>	<i>Pseudomonas fluorescens</i>	<i>Bacillus thuringiensis</i>	<i>Staphylococcus lentus</i>	<i>Achromobacter agilis</i>	<i>Pseudomonas fluorescens</i>	<i>Bacillus thuringiensis</i>	<i>Staphylococcus lentus</i>
Gompertz = $pr1 * \exp(-\exp(-pr2 * (X - pr3)))$	R ²	0.743	0.929	0.889	0.777	0.893	0.599	0.869	0.894
	SSE	0.031	0.005	0.011	0.032	29.165	71.717	35.962	14.927
Asymptotic regression = $pr1 * \text{Exp}(pr2 * X) + pr3$	R ²	0.600	0.723	0.665	0.656	0.800	0.545	0.829	0.688
	SSE	0.043	0.019	0.031	0.015	2.299	6.152	1.594	3.251
Polynomial equations: second Order = $pr1 + pr2 * X + pr3 * X^2$	R ²	0.930	0.953	0.959	0.924	0.920	0.864	0.872	0.926
	SSE	0.007	0.003	0.004	0.003	0.917	1.843	1.193	0.767
Polynomial equations: Third Order = $pr1 + pr2 * X + pr3 * X^2 + pr4 * X^3$	R ²	0.991	0.957	0.971	0.961	0.967	0.944	0.951	0.927
	SSE	0.001	0.003	0.003	0.002	0.380	0.755	0.455	0.758
Polynomial equations: Fourth Order = $pr1 + pr2 * X + pr3 * X^2 + pr4 * X^3 + pr5 * X^4$	R ²	0.991	0.971	0.975	0.993	0.968	0.990	0.979	0.935
	SSE	0.001	0.002	0.002	0.000	0.369	0.136	0.195	0.676
Polynomial equations: Fifth Order = $pr1 + pr2 * X + pr3 * X^2 + pr4 * X^3 + pr5 * X^4 + pr6 * X^5$	R ²	0.991	0.974	0.977	0.993	0.999	0.998	0.991	0.999
	SSE	0.001	0.002	0.002	0.000	0.013	0.030	0.080	0.007
Exponential growth = $pr1 * \exp(pr2 * X)$	R ²	0.504	0.650	0.580	0.592	0.768	0.508	0.810	0.654
	SSE	0.054	0.024	0.039	0.017	2.660	6.664	1.770	3.618

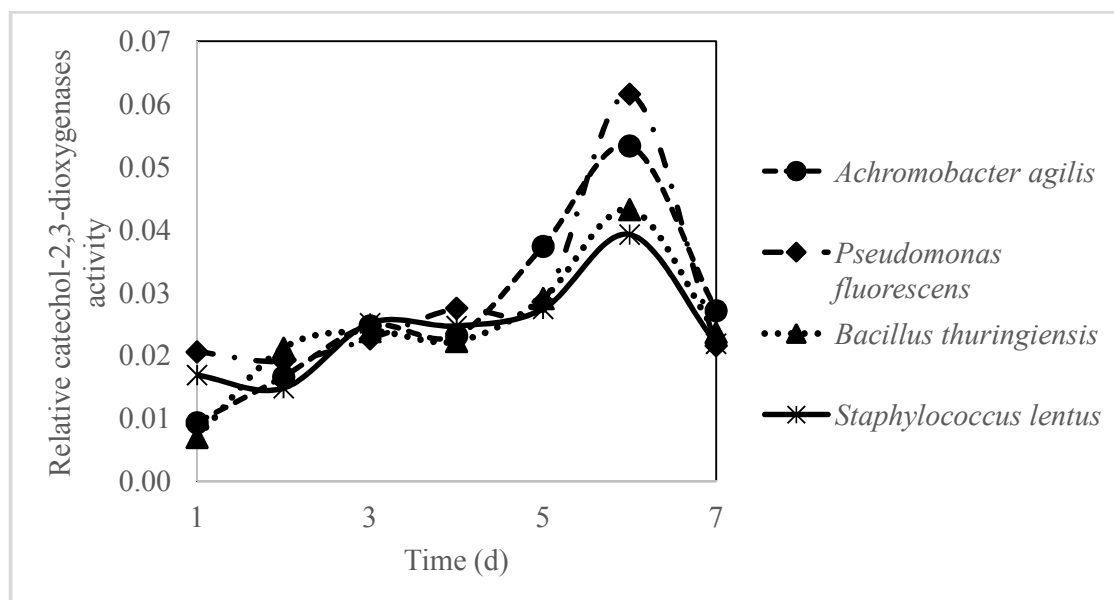


Fig. 5. Catechol-2,3-dioxygenases activity monitoring during *in-vitro* screening for bacterial hydrocarbon degradation potential

Model studies observation suggests that the polynomial function was the best fit for the growth pattern of the isolates in the BLCO-amended media. The basis of consideration was the low Root mean square values and R^2 value > 98% as seen in Table 3, with the 5th order degradation equations of BLCO (Biomass cell count increase) = $pr_1 + pr_2 \cdot X + pr_3 \cdot X^2 + pr_4 \cdot X^3 + pr_5 \cdot X^4 + pr_6 \cdot X^5$. Where as the Gompertz and Exponential growth models function failed to fit the growth kinetics of the bacterial isolates in the crude oil media as they are described with low p-values and R^2 -values < 90% predictability index.

4. CONCLUSIONS

Petroleum has been publicized as the most terrestrial pollutant [27] and its presence has been stated to change the community structure of pristine soil bacterial in both population and genetic-enzyme process for degradation of hydrocarbon pollutant [28]. The work of several researchers has confirmed that bacterial isolated from hydrocarbon impacted soil have gene-coded with hydrocarbon-degrading enzymes. Ajayi and Abiola [19] reported *Acinetobacter iwofii*, *Micrococcus luteus*, *Bacillus cereus*, and *Pseudomonas aeruginosa*; Stephen et al. [29] reported *Pseudomonas* sp. and *Bacillus* sp.; Ijah & Abioye [30] observed *Bacillus* sp. and *Pseudomonas* sp. from a diesel polluted soil and

[31] identified *Proteus* sp. as active degrader of petroleum pollutants. Okerentugba & Ezeronye [32] reported the predominance *Micrococcus* sp., *Bacillus* and *Proteus* to have the ability to degrade Nigerian Crude. These all support the findings of this work that the rhizobacterial *Achromobacter agilis*, *Pseudomonas fluorescens*, *Bacillus thuringiensis*, and *Staphylococcus lentus* isolated from aged hydrocarbon polluted soil are hydrocarbon degraders as demonstrated by their growth pattern and catechol 2,3- dioxygenase activity. In this light, *Pseudomonas fluorescens* was observed to have the peak activity for enzyme biodegradability and crude oil metabolism, in contrast to being second in terms of cell count increase due to hydrocarbon utilization. This finding was in consonance with the report of Okerentugba & Ezeronye [32] reporting activity peak for *Pseudomonas aeruginosa*. This study also observed that the C23O activity peak on the 6th day of the study which corroborates the report Chikere & Ughala [33].

The variations in structural conformations of this enzyme and its synthesis mechanisms may vary among isolates. Olukunle et al. [28] studied the impact of temperature on C23O activity and reported temperature to act as a promoter of C23O activity with the enzyme to be stable at 30-40°C. This study was carried out in the temperature range of 30-37°C.

The study also, observed an increase in the enzyme activity as the pH declined around pH6-7 and this is consistent with the submission of Okerentugba & Ezeronye, [32] who reported that the microbes are responsible for the flux in acidic condition of the media. However, acidic pH conditions of the biodegradations could be as a result of the presence of organic acids and precursor compounds secreted during the process of bacterial metabolism.

In conclusion, it is necessary to confirm that the isolates are able to secrete relevant enzymes with the highest activity of degrading hydrocarbons. The degrading ability demonstrated by the microorganisms is a clear indication that they can be used in hydrocarbon degradation. The selected organisms have shown their optimum catechol 2,3- dioxygenase activity within the temperature range of 30°C to 37°C during degradation.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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