

Catechol-2,3-dioxygenase and Lipase Activities during Degradation of Crude Oil by Hydrocarbon-degrading Bacteria Isolated from Bitumen-polluted Surface Water in Agbabu, Ondo State

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Abstract Environmental pollution arising from petroleum leakages in storage tanks, spillage during transportation of petroleum products, deliberate discharge of petroleum products and various industrial processes is hazardous to soil and water ecosystems. Bioremediation is regarded as a preferable technology for long term restoration of crude oil contaminated sites. This study evaluates activities of some enzymes during degradation of crude-oil degradation by some hydrocarbon-degrading bacteria. Bacterial isolates recovered from bitumen-contaminated surface water were screened for hydrocarbon-degrading potentials by plating them on minimal salt medium (MSM) supplemented with 2% crude-oil over a period of 10 days. Their growth was monitored by measuring the absorbance (OD_{600nm}) and Total viable count (log₁₀ CFU/ml). The isolates were identified by the amplification and sequencing of the 16S rRNA sequences. Degradation procedure was carried out under optimum growth temperature and pH. Catechol-2,3-dioxygenase activity was determined by spectrophotometric method and the unit activity was calculated from standard curve prepared from varying concentrations of 2-hydroxybenzoic aldehyde while lipolytic activity was determined by colorimetric method based on the activity in cleavage of p-nitrophenylpalmitate (p-NPP) at pH 8.0. Five isolates that showed maximum utilization of crude-oil as sole carbon source and were selected for degradative studies. The identities of these isolates as revealed by 16S rRNA sequences were *Pseudomonas aeruginosa*, *Bacillus cereus*, *Dyadobacter koreensis*, *Campylobacter hominis* and *Micrococcus luteus*. Optimum Catechol 2, 3-dioxygenase (C23O) and lipase activities was observed on the 7th and 8th day of degradation respectively. Highest C23O activity (20.0U/ml) was observed with *Bacillus cereus* while *Pseudomonas aeruginosa* had highest lipase activity (8.0U/ml). The temperature optimum of the reaction rate of Catechol 2,3-dioxygenase (C23O) from the selected isolates was between 40 and 50°C while optimum pH for C23O activity was within pH 6 and 8. Optimum activity of lipase recovered from the isolates was within 40°C to 55°C and pH 7 to 8. The degradative and enzymatic activities of these organisms recommend them as potential tools for the bioremediation of crude oil polluted environments.

Keywords: Biodegradation, Crude-Oil, Catechol 2, 3-dioxygenase, Lipase

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1. Introduction

Extensive use of Petroleum and its products is usually accompanied with discharge of hydrocarbons into the environment [1]. Exploration, refinery and transportation of these products have led to their incessant release into the environment. However, other illegal practices like vandalization of oil pipes, oil theft, among others have contributed to the seepage of these compounds into the environment. The negative impacts of these compounds on both aquatic and terrestrial habitats have been well documented [2,3,4,5].

Several approaches have been applied to contain this environmental problem but bioremediation approach, wherein the pollutants can be removed by use of microorganism or their enzymes to return the environment altered by contaminants to its original condition serves as a preferable alternative [6]. Utilization of these compounds by the microorganisms is attributed to their metabolic capabilities for the mineralization of crude oil [7]. Contacts of microbes with complex organic materials usually release extracellular enzymes which convert high molecular weight materials into diffusible fractions, which could be transported through the cell wall for assimilation [8]. Biodegradation of hydrocarbons, both aliphatic and aromatic compounds, may occur under anaerobic or

aerobic conditions [9]. Under aerobic conditions, oxygenase enzymes introduce oxygen atoms into hydrocarbons (mono-oxygenases introduce one oxygen atom to a substrate while dioxygenases introduce two). The anaerobic degradation is catalyzed by anaerobic bacteria, such as sulphate-reducing bacteria, using different terminal electron acceptors [9]. Aerobic catabolism of hydrocarbons can be faster, due to the metabolic advantage of having the availability of O₂ as an electron acceptor [10]. The final product of the oxidation of saturated aliphatic hydrocarbons is acetyl-CoA, which is catabolized in the citric acid cycle, together with the production of electrons in the electron transport chain.

Extracellular enzyme activity is a key step in degradation and utilization of organic polymers, since only compounds with molecular mass lower than 600 daltons can pass through cell pores. Hydrolytic enzymes disrupt major chemical bonds in the toxic molecules and results in the reduction of their toxicity. Recent works have shown that lipase is closely related with the organic pollutants present in the soil. Lipase activity was responsible for the drastic reduction total hydrocarbon from contaminated soil. Lipase activity was found to be the most useful indicator parameter for testing hydrocarbon degradation in soil.

This study evaluates the activities of some extracellular enzymes during crude oil degradation by hydrocarbon-degrading bacteria isolated from bitumen-contaminated surface water.

2. Materials and Methods

2.1. Collection of Samples

Surface water samples were collected aseptically from Bitumen-contaminated sites in Agbabu community Ondo State (E04° 48-50' and N06°34-37'). The samples were labeled appropriately and transported in ice packs to the laboratory for immediate microbiological analyses.

2.2. Isolation and Characterization of Hydrocarbon-utilizing Bacteria

One hundred milliliter (100ml) of Mineral salt medium (0.2g of KCL, 6g Na₂HPO₄, 2.8g of NaH₂PO₄, 0.1g of MgSO₄ and 5g of NaCl) supplemented with 1% crude sterilized by autoclaving at 121°C for 15 minutes. Ten milliliters (10ml) of each of the samples was introduced into the minimal salt medium in 250 ml Erlenmeyer flask and incubated at 37°C for 7days. After 7days, 10ml of the culture from the minimal salt medium (MSM) was transferred into a freshly prepared minimal salt medium (MSM) and incubated for 37°C for another 7 days. Pure cultures were obtained from the minimal salt medium by pour plate method.

2.3. Purification of Bacterial Isolates

Discrete colonies from each of the cultured plates were picked and sub-cultured into r nutrient agar plates and incubated at 37°C for 24hours.

2.4. Characterization and Identification of Isolates

The pure cultures of bacteria isolated were subjected to morphological and biochemical characterization tests. The identities of these isolates were confirmed by using molecular techniques.

2.5. Degradation Studies

Degradation of crude oil was conducted by inoculating the selected bacteria into MSM (6g of Na₂HPO₄, 5g of NaCl, 2.8g of NaH₂PO₄, 0.2g of KCl, 0.1g of MgSO₄.7H₂O) supplemented with 2% crude oil at optimum growth conditions for each isolate. The set up was done in duplicates and incubated for 15 days.

2.5.1. Growth Profile of Hydrocarbon-utilizing Bacteria during 15 day Course Crude Oil Degradation

Growth profile of each bacterial isolate in the degradation medium was determined by reading the absorbance OD_{600nm} of the medium at 3days interval using a JENWAY 6850 spectrophotometer.

2.5.2. pH Changes during a 15 day Course Crude Oil Degradation by Hydrocarbon-Utilizing Bacteria

The pH of the minimal salt medium was monitored at an interval of every two (0, 3, 5, 7 10 and 15) days throughout the process of degradation.

2.5.3. Catechol 2,3-dioxygenase Activity of Hydrocarbon-utilizing Bacteria during a 15days Course Degradation of Crude Oil

Catechol 2,3-dioxygenase activity was determined spectrophotometrically from the rate of product accumulation using a Jenway 6850 spectrophotometer. The reaction mixture contained 3ml of of 50 mM potassium phosphate buffer (pH 7.5), 0.2 ml of 10 mM Catechol 0.1ml cell free extract. The content was mixed in a test tube. The increase in absorbance at 375nm caused by the formation of the reaction product (2-hydroxybenzoic semi-aldehyde) was monitored for a 1h. One unit of enzyme activity was defined as the amount of enzyme catalyzing the production of 1 μmol 2-hydroxybenzoic aldehyde per minute [11].

2.5.4. Lipolytic activity of Hydrocarbon-utilizing Bacteria during a 15day Course Degradation of Crude-oil

The experimental set-up was centrifuged at 10,000rpm for 20minutes at 4°C, the supernatant served as extracellular lipase enzyme was determined using the supernatant, Lipase activity was determined spectrophotometrically at 30°C using p-nitrophenolpalmitate (p- NPP) as the substrate. One hundred and eighty microlitres (180μl) of solution A (0.062g of p-NPP in 10ml of 2-propanol, sonicated for 2minutes before used), 1620μl of solution B (0.4% triton X-100 and 0.1% gum Arabic in 50ml TrisHCl, pH 8.0) and 0.2ml of the enzyme extract, incubated at 37°C for 15minutes. One unit of enzyme activity was expressed as 1 nmol of p-nitrophenol released per minute under the assay conditions [12].

Effect of pH on Catechol 2, 3-dioxygenase

The effect of pH on the catechol 2,3-dioxygenase activity was determined by measuring the enzyme activity at 30°C over the pH range of 3.0 -11.0 using the following buffers: 50mM sodium acetate (pH 3.0-5.0), 50mM phosphate (pH 6.0 and 7.0), 50mM Tris-HCL (pH 8.0 and 9.0) and 50mM carbonate (pH 10 and 11) [13].

Effect of temperature on Catechol 2, 3-dioxygenase

The optimum temperature was determined by measuring the enzyme activity at varying temperatures (30°C – 90°C) in 50mM Tris/HCL buffer (pH 8.0). The enzyme and the substrate solutions were pre-incubated at 30°C in a water bath, mixed and the enzymatic reaction was then determined [13].

Effect of pH on lipase enzyme

The effect of pH on the lipase activity was determined by measuring the activity at 30°C over the pH range of 3.0-11.0 using the following buffers: p-nitrophenolpalmitate (p-NPP) which is the substrate. One hundred and eighty microliter (180µl) of solution A (0.062g of p-NPP in 10ml of 2-propanol, sonicated for 2minutes before used), 1620(µl) of solution B (0.4% triton X-100 and 0.1% gum Arabic in 50ml TrisHCl, pH 8.0) and 0.2ml of enzyme sample [14].

Effect of Temperature on Lipase

The optimum temperature was determined by assaying the enzyme activity at various temperatures (30°C- 90°C) in 50mM Tris/HCL buffer (pH8.0). The enzyme and the substrate solutions were pre-incubated at 30°C in a water bath, mixed and the enzymatic reaction was then carried out. The residual activity was calculated after incubation period of 1hr [14].

Statistical analysis: Statistical tests (mean and standard deviation) were performed using Microsoft Excel Package.

3. Results and Discussion

In the present study, fifty two (52) strains of hydrocarbon-degrading bacteria were isolated from bitumen contaminated surface water. The hydrocarbon-degrading bacteria in the samples analyzed were predominated by gram-negative. In the samples collected, 65% were gram negative while the remaining 35% were gram positive. The gram negative isolates belonged to five genera namely: *Pseudomonas* spp., *Enterobacter* spp., *Citrobacter* spp., *Alcaligenes* spp. and *Proteus* spp. accounting to 20, 18, 12, 10 and 5% respectively. The gram positive isolates belonged to two genera viz, *Bacillus* spp. and *Micrococcus* spp. accounting for 25 and 10% respectively (Table 1). Prevalence of gram negative bacteria in this study corroborate previous studies which reported that the gram negative bacterial strains isolated from hydrocarbon contaminated soil was higher than gram positive bacteria [15,16].

Bacillus spp. accounted for the highest frequency of occurrence (25%) of the total hydrocarbon-degrading bacteria. The prevalence of *Bacillus* species in hydrocarbon polluted sites have been reported by several authors [17,18,19]. *Bacillus* spp., due to their genetic make-up, they are able to tolerate unfavorable environmental conditions and high levels of hydrocarbons which could have eliminated other organisms. Their adaptive features

could be attributed to their ability to survive periods of unfavorable conditions by switching to their spore-producing stage which becomes vegetative upon the return of favourable conditions. *Pseudomonas* spp., *Micrococcus* spp., *Citrobacter* spp. and *Alcaligenes* spp. recovered in this study have also been isolated from hydrocarbon-contaminated environments [20,21].

The hydrocarbon-degrading bacteria were screen down to five isolates based on their crude oil utilization potentials. These bacterial strains were identified on the basis of morphological and biochemical characteristics by using Bergey's Manual of Determinative Bacteriology. They were further identified as *Campylobacter hominis*, *Bacillus cereus*, *Dyadobacter koreensis*, *Pseudomonas aeruginosa* and *Micrococcus luteus* based on 16SrRNA Sequencing (Table 2). The genus *Dyadobacter* have been isolated from hydrocarbon contaminated site Zhang *et al.*, [22] but this study reports the hydrocarbon-degrading potential of *Dyadobacter koreensis*.

Table 1. Percentage distribution of Hydrocarbon-degrading Bacteria Isolated from Bitumen-contaminated Surface water samples from Agbabu, Ondo State

| Isolates | Number of isolates | Frequency (%) |
|-----------------------------|--------------------|---------------|
| <i>Pseudomonas</i> spp. | 11 | 20 |
| <i>Enterobacter</i> spp. | 9 | 18 |
| <i>Citrobacter freundii</i> | 6 | 12 |
| <i>Alcaligenes faecalis</i> | 5 | 10 |
| <i>Proteus vulgaris</i> . | 3 | 05 |
| <i>Bacillus</i> spp. | 13 | 25 |
| <i>Micrococcus</i> spp. | 5 | 10 |
| Total | 52 | 100 |

Table 2. TAXONOMIC AFFILIATION AND % SEQUENCE SIMILARITIES OF BACTERIAL ISOLATES WITH CLOSEST RELATIVES FROM THE GENBANK DATABASE

| Identities | Query code | (%) Similarity | Accession no |
|-------------------------------|--------------|----------------|--------------|
| <i>Campylobacter hominis</i> | ATCC BAA-381 | 100 | NR_074141.1 |
| <i>Bacillus cereus</i> | ATCC 14579 | 90 | NR_074540 |
| <i>Dyadobacter koreensis</i> | NBRC 101116 | 91 | NR_113977 |
| <i>Micrococcus luteus</i> | Sn1-1 | 86 | AB109983.1 |
| <i>Pseudomonas aeruginosa</i> | aab51a04 | 85 | DQ815168.1 |

The growth profile of the selected bacterial isolates in minimal salt medium supplemented with 2% Bonny light crude oil for a 15days incubation period is shown in Figure 1. The absorbance readings show that all the isolates utilized crude oil as sole carbon source throughout the degradation process. Growths of all the isolates increased gradually with time till their optimum growths were achieved however, their growths later declined as incubation period progressed. *Dyadobacter koreensis*, *Bacillus cereus* and *Micrococcus luteus* had optimum growth of 1.071, 1.003 and 0.881 respectively at day 10 while *Pseudomonas aeruginosa* and *Campylobacter hominis* grew optimally with 0.983 and 0.81 respectively at day 7. The growth profile of the five selected

hydrocarbon-degrading bacteria in MSM supplemented with 2% crude oil showed that the bacteria utilized the crude-oil as their sole carbon source. Gradual increase in growth indicates gradual utilization of crude-oil. Optimum growth achieved within the degradation process and eventual decline in growth implies that the bacteria were utilizing the oil for their cell metabolism and division. Over time, metabolic products released can reduce the bacterial growth. Also, exhaustion of the crude-oil which serves as their carbon source can lead to declination of their growths.

Figure 2 illustrates the changes in pH during the degradation of crude-oil by the selected hydrocarbon-degrading bacteria. Gradual decline in the pH till the end of the study was observed by all the isolates. Decrease in pH of the culture was observed as the biodegradation days progressed. The utilization of crude oil by the bacterial isolates resulted in their growth with reduction in the pH, changing the medium to acidic metabolic product. This agrees with the reports of Nkanang *et al.*, [23]. Microbial degradation of hydrocarbons often leads to production of organic acids and other metabolic products [24,25].

Catechol 2, 3 -dioxygenase activity was measured during the degradation of crude oil by the selected

hydrocarbon-degrading bacteria. Substantial activity of C23O was observed during the degradation. C23O activities of *Dyadobacter koreensis*, *Bacillus cereus* and *Campylobacter hominis* increased gradually with time to reach a peak value of 20.2U/ml, 18.2U/ml and 9.5 U/ml respectively on day 7 which declined a bit till the end of the degradation process while *Micrococcus luteus* and *Pseudomonas aeruginosa* followed the same trend but had optimum C23O activities of 14.8 U/ml and 12.2 U/ml respectively on the 10th day of incubation (Figure 3). Catechol-2, 3-dioxygenases are homo-tetramers and extracellular enzymes and they have been detected from many Gram-negative bacteria such as *Pseudomonas* and Gram-positive bacteria such as *Nocardia* and *Bacillus* strains [26]. Catechol 2, 3 -dioxygenase activities of the selected hydrocarbon-degrading bacteria during crude-oil degradation shows that all the bacteria had appreciable C23O activity during the degradation process but highest activity was recorded by *Dyadobacter koreensis*. Optimum C23O activities observed between day 7 and day 10 correlates with when the bacteria were actively growing (Figure 1). One could infer from this that this enzyme was actively involved in the degradation of crude-oil by these bacteria.

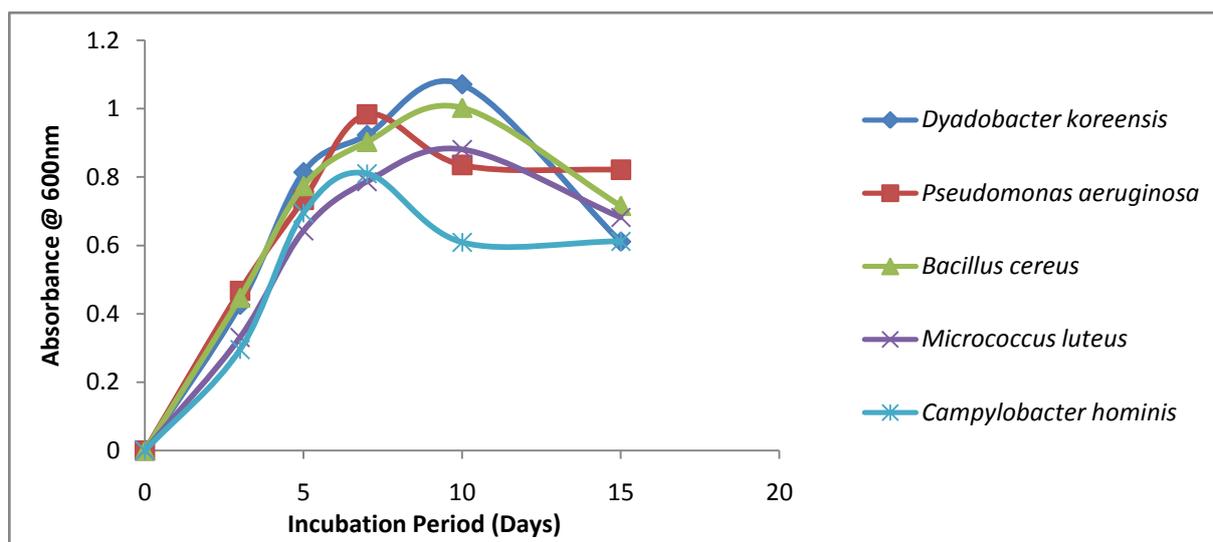


Figure 1. Growth profile of hydrocarbon-degrading bacteria in MSM supplemented with 2% Crude-Oil

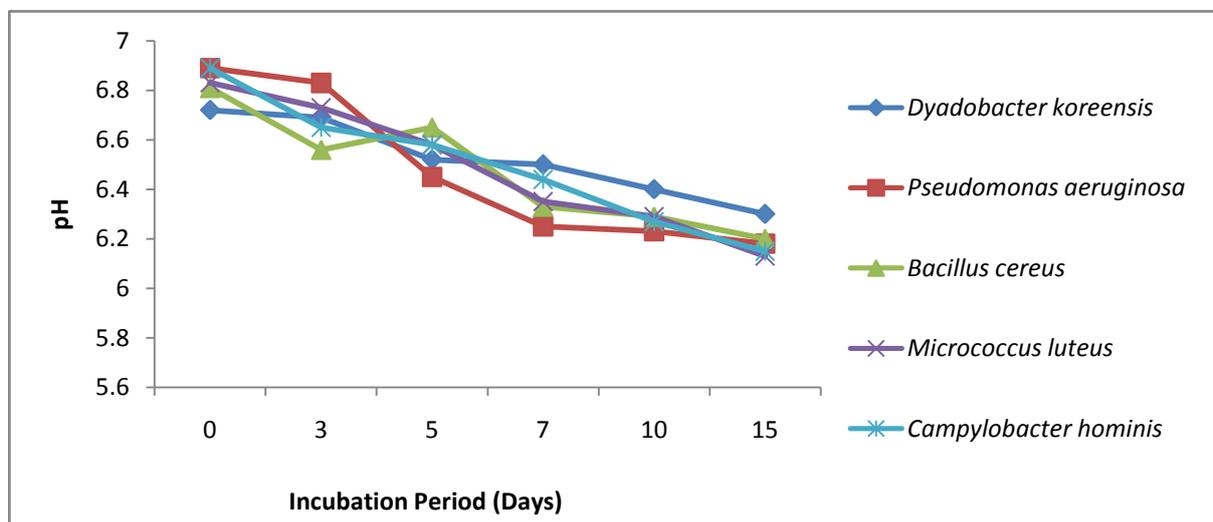


Figure 2. pH changes during 15 days degradation of crude oil by hydrocarbon-degrading bacteria

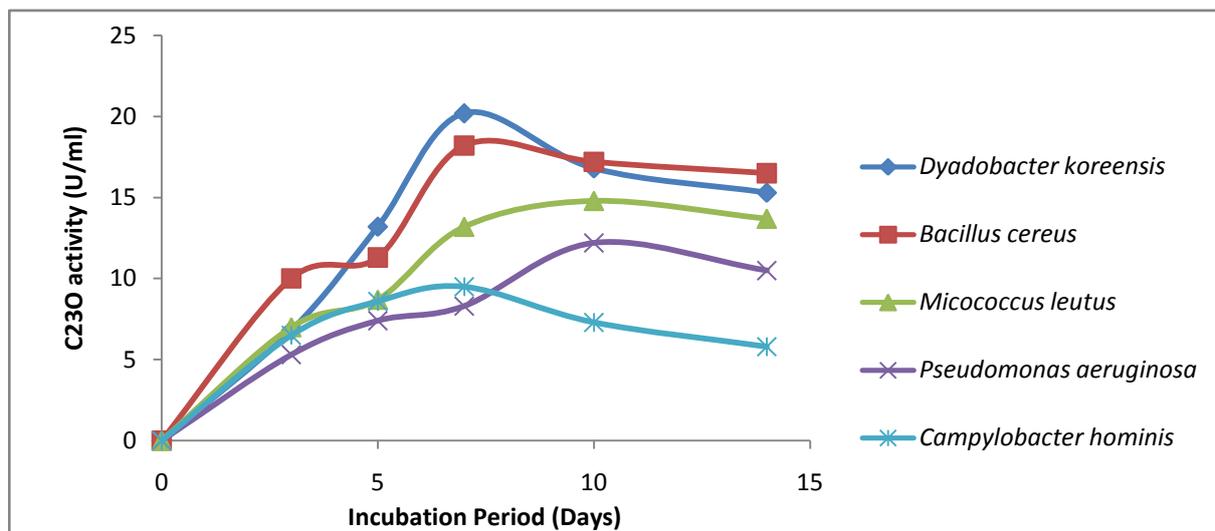


Figure 3. Catechol 2,3-dioxygenase activities of Hydrocarbon-degrading Bacteria during 15 days degradation of Crude Oil

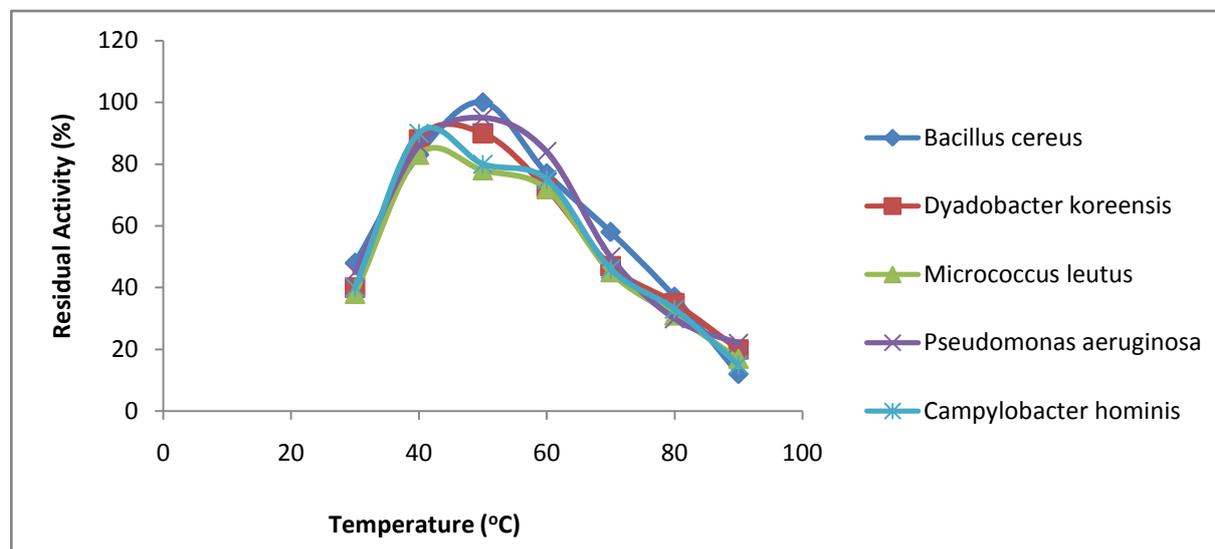


Figure 4. Effect of temperature on activity of Catechol 2,3-dioxygenase produced from selected hydrocarbon-degrading bacteria

The temperature optimum of the reaction rate of Catechol 2,3-dioxygenase (C23O) from *Bacillus cereus*, *Pseudomonas aeruginosa* and *Dyadobacter koreensis* were estimated to be at 50°C while Catechol 2,3-dioxygenase isolated from *Micrococcus luteus* and *Campylobacter* showed the highest activity at 40°C (Figure 4). The effect of temperature on the activities of catechol 2,3-dioxygenase produced from the bacterial isolates showed that catechol 2,3-dioxygenase activities were influenced by temperature. Temperature has been reported to influence enzyme activity as a result of its effect on the stability of the enzyme because increase in temperature increases the frequency of molecular motions [27].

The optimum temperature observed for catechol 2,3-dioxygenase isolated from the bacterial isolates was within the range of 40°C to 50°C. This agrees with the findings of Zou *et al.*, [28] who reported catechol 2,3-dioxygenase isolated from *Pseudomonas* strains ZJF08 and S-47 to be optimally active at 40°C and Fernandez-Lafuente *et al.*, [29] who determined the optimum temperature for the activity of the enzyme C23O as 50°C from *Bacillus stearothermophilus*. However, their C23O activities declined as the temperature increased

until the activity was eventually lost between 80°C-90°C. The loss of C23O activity could be due to denaturation of the proteins at elevated temperature.

Figure 5 shows the effect of pH on activity of C23O produced by selected hydrocarbon-degrading bacteria. It was observed that all the isolates had C23O activity within the pH range studied. Although low activity was observed within the acidic range while the activity was optimum within the neutral to alkaline range. Optimum Catechol 2,3-dioxygenase (C23O) activity from *Pseudomonas aeruginosa*, *Dyadobacter koreensis* and *Campylobacter hominis* were observed at pH 6, while Catechol 2,3-dioxygenase isolated from *Micrococcus luteus* and *Bacillus cereus* showed optimum activities at pH 7 and 8 respectively. Activities of the enzyme were also influenced by changes in pH (Figure 5). Optimum activity was observed within neutral to alkaline range while low activity was observed within the acidic range. Catechol 2,3-dioxygenase isolated *Pseudomonas aeruginosa*, *Dyadobacter koreensis* and *Campylobacter hominis* were observed at pH 6, while Catechol 2,3-dioxygenase isolated from *Micrococcus luteus* and *Bacillus cereus* showed optimum activities at pH 7 and 8 respectively. This agrees with Olukunle *et al.*, [30]

who reported that C23O isolated from *Bacillus cereus* that had its optimum relative activity at pH 8.0 while other isolates had their optimum C23O activities at pH 7.0.

Lipase activities of the selected hydrocarbon-degrading bacteria during degradation of crude oil is reported in Figure 6. All the bacterial isolates showed appreciable lipase activity during the course of degradation except *Dyadobacter koreensis* which had insignificant lipolytic activity. Lipase activities of *Bacillus cereus* and *Pseudomonas aeruginosa* rose steadily with time to optimum activities of 8.5 U/ml and 8.0 U/ml respectively on day 7. Optimum activities lower than the above (5.3 U/ml and 3.0 U/ml) was observed with *Micrococcus luteus* and *Campylobacter hominis* respectively on day 7 and day 5 while *Dyadobacter koreensis* had the least optimum activity (1.3U/ml) on day 5. The entire bacterial genus used in the study have been reported to produce lipase except *Dyadobacter koreensis* which has been reported to lack the ability to produce lipase [31]. This supports its insignificant lipase activity observed in the study. Lipases have been produced from *Bacillus* [32,33], *Pseudomonas* sp. [34].

The effect of temperature on activity of lipase produced by selected hydrocarbon-degrading bacteria is shown in Figure 7. The result shows that lipase produced from the bacteria had different temperatures for optimum activity. The activities increased gradually to an optimum between 40°C and 55°C and declined gradually until activity was lost at 90°C. Lima *et al.*, 2004 also reported that optimum lipase activity of *Bacillus megaterium* was observed at 55°C.

Activities of lipase produced by the bacterial isolates were optimum between pH 7.0 and 8.0 while their activities were relatively low between pH4 and 5 and eventually lost at pH 3 (Figure 8). For every enzyme, there is an optimum pH value, at which the specific enzyme functions most actively. Any change in this pH significantly affects the enzyme activity and the rate of reaction. In this study, activities of lipase recovered from the selected hydrocarbon-degrading bacteria were influenced by changes in pH. However, optimum activities were recorded at pH7.0 to 8.0. This partly agrees with Kambourova *et al.*, [35] who reported the optimum lipase activity of *Bacillus stearothermophilus* at pH 7.0 to 9.0.

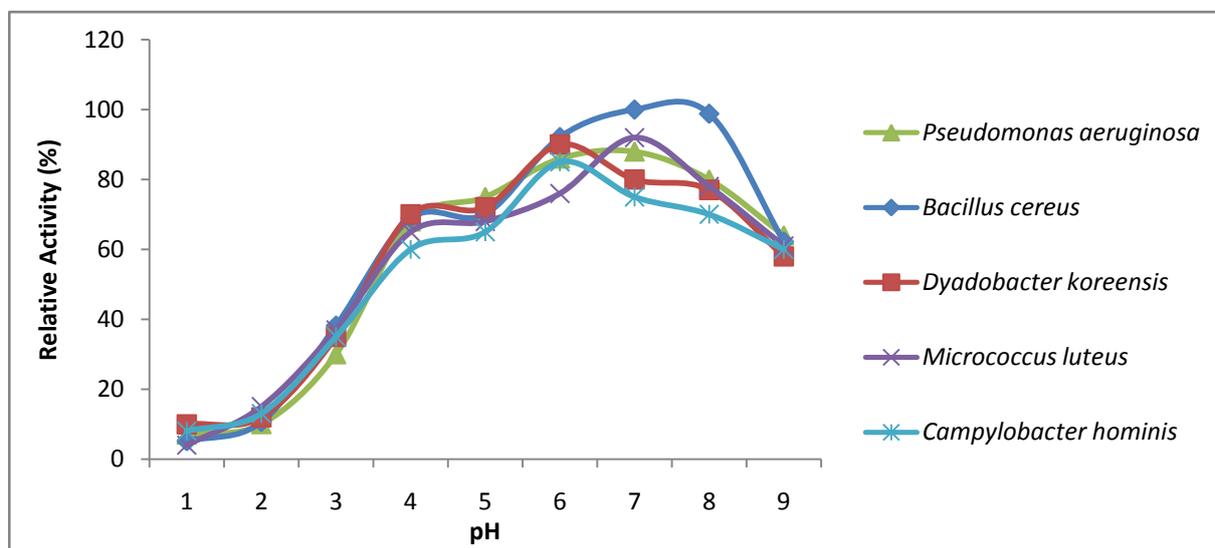


Figure 5. Effect of pH on catechol 2,3 –dioxygenase activity produced from selected hydrocarbon-degrading bacteria

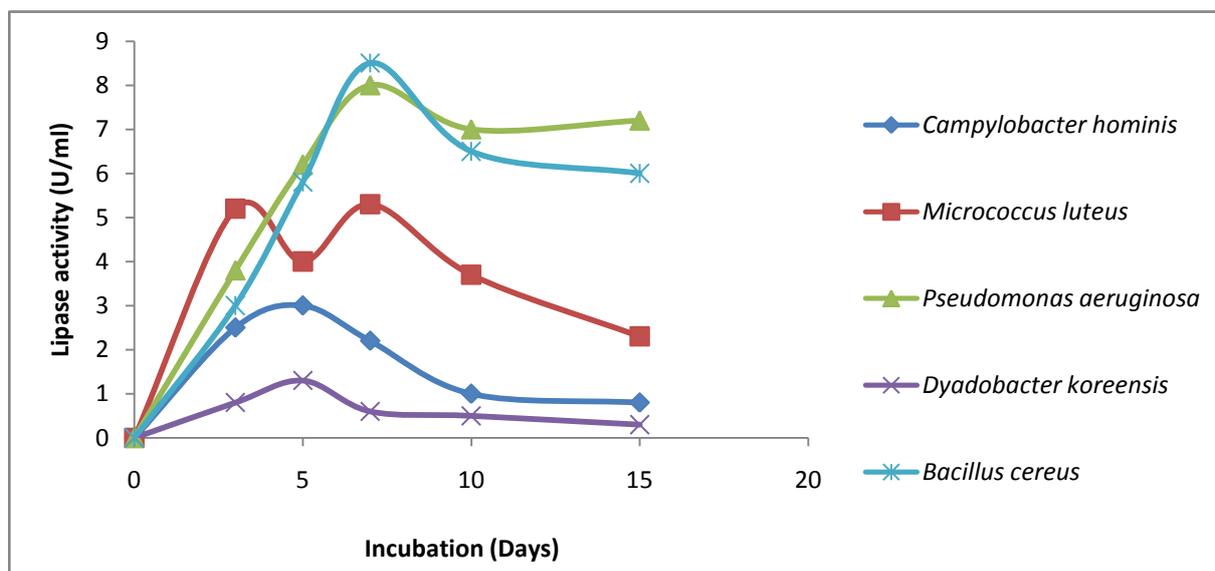


Figure 6. Lipase activities of hydrocarbon-degrading Bacteria during 15days degradation of Crude-Oil

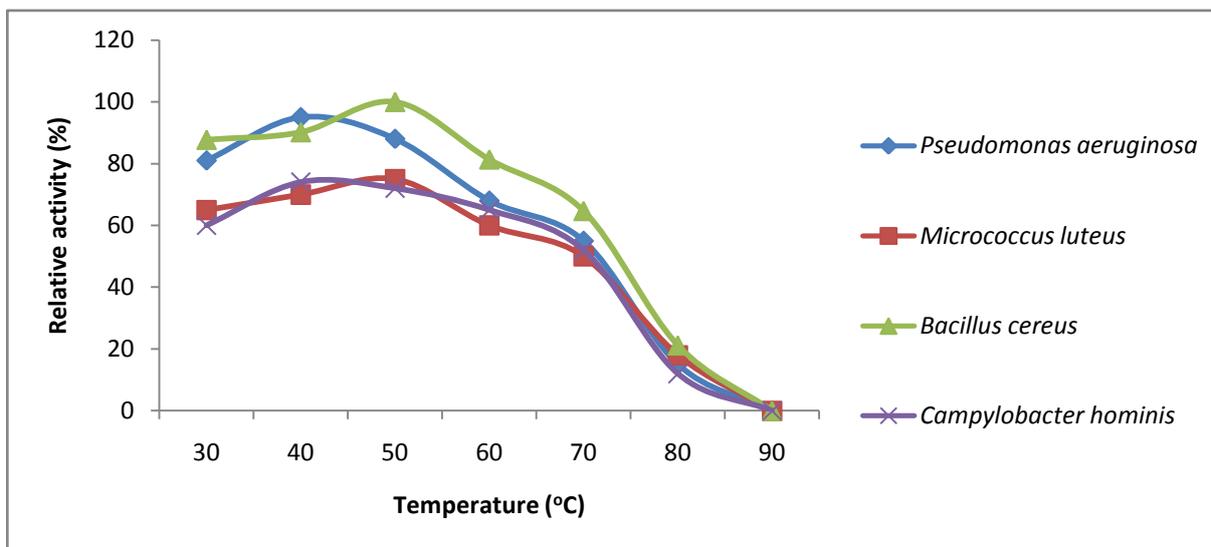


Figure 7. Effect of Temperature on activity of Lipase produced from selected hydrocarbon-degrading bacteria

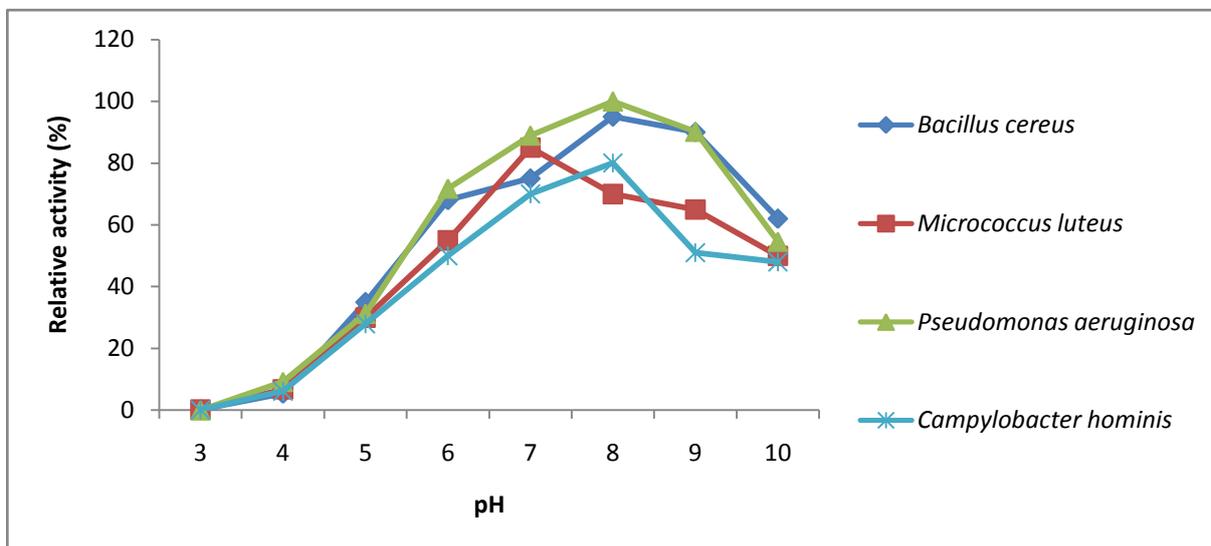


Figure 8. Effect of pH on activity of Lipase produced from selected hydrocarbon-degrading bacteria

4. Conclusion

Findings of this study revealed the dominance of crude-oil degrading bacteria in the study area. These bacteria have been shown to possess significant enzymatic activities during their degradative process. They have shown their optimum catechol-2, 3-dioxygenase activities between 40°C and 50°C and pH 6 to 8 while their optimum Lipase activities were within the temperature range of 40°C and 55°C and pH 7 to 8. Crude-oil degrading potentials and detection of catabolic enzymes activities in these bacteria make them essential tool with respect to microbial bioremediation of crude-oil polluted environments.

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