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Characterisation of PAH-Degrading Endophytes Isolated From Plants Growing Around a Sludge Dam

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Abstract - The degradation of PAHs by bacteria is shown to be performed by dioxygenase enzymes as demonstrated in bacteria endophytes. Isolation of the bacteria endophytes was done using the mineral base medium. A total of 19 distinct isolates that were morphologically identified were isolated from 4 species of plant with a follow-up confirmatory identification using molecular technique. Polymerase chain reaction (PCR) of 16S rRNA gene with specific primers (16S-27F PCR and 16S-1491R PCR). The sequence of the PCR products were carried out, compared with similar nucleotides available in GenBank. Results of the phylogenetic analysis of the isolates indicated their belonging to 4 different clades including Proteobacteria, Actinobacteria, Cyanobacteria and Firmicutes. These were related to the genera Bacillus, Pseudomonas, Terribacillus, Virgibacillus, stenotrophomonas, paenibacillus, Brevibacterium, Geobacillus, Acinetobacter. From the result, Pseudomonas demonstrated a high incidence in the plants sampled. The in-vitro degradation study and the presence of deoxygenase genes indicated that these lists of endophytes are able to use the list of PAHs tested as their source of food and energy leading to their breakdown. This means that the endophytes are responsible for the remediation of petroleum hydrocarbons in planta, a situation that would have been phytotoxic to plant alone. Therefore, these bacteria endophytes could be potential organism for enhanced phytoremediation of PAHs.

***Keywords*:** Biodegradation, Phytoremediation, Oil sludge, PAH, Plants, Endophytes, Bacteria

1. Introduction

Crude oil waste such as sludge generated during the refining of crude oil is a good sink for PAHs. This is referred to as a hazardous organic complex in the 1982 classification of hazardous waste by the United States Environmental Protection Agency (USEPA) [1]. Appropriate management of crude oil wastes could be done when it is dumped in a properly lined pits or dams. However, it could find its way into the environment through the unscrupulous dumping. Storage, landfill, and during transportation, where it will exert its cytotoxic, mutagenic and carcinogenic effects [2]. Various PAHs from crude oil introduced through the anthropogenic activities of man have been implicated in the environmental. This has been reported to occur through seeps from the sludge described as natural sludge leaks from unlined pits, dams, or reservoirs [3]. The rate at which PAHs are identified in the environment and the harmful effects it exacts on nature requires that constant measures be taken to contain it.

Various measures such as physical, chemical, and biological methods has been identified for the control of the toxic effects of crude oil and the constituent PAHs, they include the physical, chemical, and biological methods [4]. These methods are reported to have their limitations leading to the quest for effective and environmentally friendly alternatives. Phytoremediation, which is a green method of using plants to manage the toxic effects of PAHs in soil, and bioremediation (the use of microorganism), has been lauded for its effectiveness [5-6]. However, the recalcitrance properties of most of the long chain PAHs have generally defiled conventional remediation practices and techniques such as phytoremediation or bioremediation [5]. Literature has reported on interests of the synergy between endophytes (microorganisms that lives in plant) and plants, and how they can provide an effective method to remediate recalcitrant organic compounds from the soils [7].

The effectiveness of endophyte enhanced phytoremediation ranges from the ability of the microorganism strain to reach larger population size because of reduced competition, to their ability to degrade any absorbed xenobiotics in planta in such a way as to reduce phytotoxic effects [8-9]. The limitation of phytoremediation is often because of the toxicity of

these chemicals or their toxic end products in plants [10]. This can however be ameliorated through endophyte-assisted phytoremediation either by means of natural colonization or by genetic manipulation of the plant as this has recently drawn attention [11-13]. The negative effect of endophyte-enhanced phytoremediation has minimal effect to the environment and the technique removes organic compounds permanently, without long-term liability; as such, is preferred by the public. The aim of this study was to isolate, test and characterise bacteria endophytes from plants around a sludge dam, with potentials to degrade PAHs present in oil sludge.

2. Materials and Methods

2.1. Plants

Plants were collected from the neighbourhood of a sludge dam at the South African Petroleum Refinery (SAPREF), making sure that all the different plant species were sampled. The collection of the plants did not involve protected or endangered species, hence was safe to the environment. The GPS coordinates of the sampling point are E 29.99’42.33”, N 30.94’ 88.68”.

Soil was also collected from the same point that plants were collected to obtain the concentration of the total PAH at the sampling sites.

Figure 1: Satellite image of the sampling site E 29.99’42.33”, N 30.94’ 88.68”.

2.2. Reagents

Standard samples of pyrene, chrysene and perylene and other analytical reagents were purchased from Sigma Aldrich South Africa. The entire reagents were of analytical grade.

2.3. Extraction of PAHs from plants and soil

The dried plant samples collected from the sludge dam were ground using commercial blender, sieved at 2 mm and were stored prior to extraction while the soil samples were ground using a commercial mortar and were sieved at 2 mm. The extraction process adopted was ‘Method 3540 Soxhlet Extraction’ [14] which was formally used by Chen et al, [15]. 5 g of 2 mm sieved dry soil as well as 5 g of 2 mm sieved plant samples were extracted using soxhlet apparatus for 4 hrs at 4-6 cycles per hour with 150 ml mixture of hexane-acetone (1:1, v/v), after which the extracted solution was concentrated to 2 ml in rotary evaporator (Buchi Rota vaporTM Japan model R-200 with heating bath B-490 and heating intensity of 20-180 ºC). USEPA Method 3630B: Silica Gel Cleanup was used as this method has been shown to specifically address organics (Erickson, 1998).The extract from soxhlet extraction was diluted with hexane to a volume of 10 ml and passed from a glass chromatographic column (i.d 20 mm and 400 mm height) parked with layers of silica gel and anhydrous sodium sulphate and then eluted with 100 ml of hexane. The eluent was finally concentrated with rotary evaporator for the second time to about 1ml and was analyzed using GC-MS.

2.4. Isolation of PAH-degrading endophytic bacteria

Mineral salt medium (MSM) was used in this study, the content of which include 500 mg KH_2PO_4, 500 MG $MgSO_4.7H_2O$, 500 mg NaH_2PO_4,H_2O, 500 mg NH_4Cl, 4000 mg NaCl, 500 mg $NaHCO_3$, and 500 mg Na_2CO_3, which was mixed with 1 ml trace elements solution containing 1500 mg $FeCl_2.H_20$, 9000 mg NaCl, 197 mg $MnCl_2.4H_2O$, 900 mg $CaCl_2$, 238 mg $CoCl_2.H_2O$, 17 mg $CuCl_2.H_2O$, 287 mg $ZnSO_4$, 50 mg $AlCl_3$, 62 mg H_3BO_3, 24 mg NiCl2.6H_2O, 10 ml 10.18 M HCl per liter of distilled water. All the reagents were dissolved in 1 litre of distilled water.

2.5. Enrichment culture

Soil collected at the area around the sludge dam was sampled. In each of a 250 ml flat-bottom flasks containing 100 ml of sterile MSM flooded with 10ml each of the three tested PAHs of initial concentration of 50 mg/l, 10 g of the soil was added. The mixture was made in triplicate for each type of PAH, and a control experiment was also prepared similar to the ones described, but without PAHs. The flask was stoppered with cotton wool, and covered with aluminium foil and left in the incubator in the dark at 28 ^oC on a rotary shaker at 120 rpm for 21 days. One millilitre of each was transferred from each flask into another set of flasks of equal volume as before containing 100 ml of sterile MSM also spiked with the PAHs. Each subsequent transfer was incubated at the same condition. At the end of each incubation period, samples were withdrawn from each flask for determination of concentrations of the PAHs by gas chromatography/mass spectrometry (GC/MS). The final culture was however, used for the isolation and identification of the PAH degraders.

2.6. Mineral salt agar (MSA)

In a 2 litre bottle containing 900 ml of MSM, 20 g of bacteriological agar was added and swirled to mix. The mixture was autoclaved at 121 ^oC for 15 min. the medium was then allowed to cool at the laminar flow to about 50 ^oC, followed by the addition of 1 ml of trace element solution filtered through a 0.2 µm membrane filter before dispensing in the Petri dishes under aseptic conditions. Each plate was overlaid with 50 µl of filter-sterilized PAHs.

2.7. Isolation of PAH-degrading bacteria from enrichment cultures

PAH-degrading bacteria were isolated from the enrichment cultures using serial dilution (10-6). An aliquot of 0.1 ml taken from the 10-5 to 10-6 dilutions was inoculated on the MSM plates. The plates were then incubated for 21 days at 28 ^oC, being checked daily for bacteria growth. Distinct colonies from the plates were stroke into nutrient agar plates and incubated for three days at 28 ^oC. Pure cultures were made from distinct colonies from nutrient agar plates by serial dilution (10-3), plated on nutrient agar plates and incubated for three days at 28 ^oC. The colonies were further purified by streaking unto new nutrient agar plates. Identification was done using gram testing, followed by the molecular techniques.

2.8. Nutrient broth

In a two litre bottle containing 1 I distilled water was poured 15 g of nutrient broth. The mixture was sterilized at 121 ^oC for 15 min and allowed to cool in the laminar flow, followed by the dispensing of 10 ml each into centrifuge test tubes with caps and stored at 4 ^oC.

Preparation of overnight cultures

The colonies from the streaked plates were inoculated into the nutrient broth in the test tubes, by carefully picking the colonies with sterile wire loop in the Lamina flow. The test tubes were capped and incubated overnight at 28 ^oC.

2.9. Molecular characterization of bacteria isolates

2.9.1. Morphological characterization

Pure colonies of the bacteria isolates from the soil as well as that of the plants were identified and characterized using the results of their gram reaction tests and the features of their morphology when compared to the Bergey's manual according to Cerqueira et al. [16].

2.9.2. Extraction of genomic DNA from bacteria isolates

The genomic DNA extraction was conducted using the commercial DNA extraction kits (Quiagen kits from Whitescience South Africa) with the provided extraction protocol. The protocol follows the preliminary harvesting of bacteria and incubation with lysosome to lyse the cell walls of the bacteria preceding the proper purification. Cleaning of the DNA was also done using the commercial kits (Qiagen from Whitescience SA).

After extraction of the genomic DNA, it was separated using agarose gel electrophoresis with 1 % agorose stained with 0.1 µg/ml ethidium bromide running at 100 V for 50 mins on tris acetate EDTA (TAE) electrophoresis buffer. Visualization of the DNA was done with UV fluorescence.

2.9.3. Polymerase chain reaction (PCR) and sequencing

Aliquots of the master mix of PCR was dispensed into individual PCR tubes and the samples of DNA were added to each tube. A control was set up without a DNA template used to check for contamination. In each of the PCR tubes were containing 50 µl, made up of 5 µl of buffer, 1.5 µl of MgCl2, 2 µl each of the primers (forward and reverse), 1 µl of dNTP mix, 0.25 µl of Dream Taq DNA Polymerase, 3 µl of the DNA samples, and sterile water used to make up the volume. The reaction of the PCR was run using MJ mini thermal cycler (BioRad, Hercules-CA, USA). Using Shannon Diversity index (H), the community structure of the isolated organisms was defined based on their abundance and evenness (Shannon, 1948; Obi et al., 2016). Five of the organisms were selected for each bacterium from the GenBank for phylogenetic analysis.

Shannons Diversity Index (H) = $\Sigma - (Pi^* \ln Pi)$. i = 1

Where: Pi = fraction of the entire population made up of specie i

2.9.4. Phylogenetic analysis

The bacteria sequences from the five treatments: soil, root, stem, leaf and control were aligned using online Muscle software in MEGA. The phylogenetic analyses were done using MEGA 7 software and the evolutionary distance of the isolates was computed using neighbour-joining methods. The tree was drawn from 1000 replicates and all positions containing gaps as well as missing nucleotide data were eliminated from the data-set. *Escherichia coli* (E-coli) was used as the out-group.

2.9.5. Identification of bacteria isolates with ó-cresol indophenol (ó-CIP)

In order to screen the bacteria cultures, their pure cultures were inoculated into a nutrient agar in tubes prepared in a slant form. The components of the test tube include Bushnell Haas broth that was incubated for 24 h at 37 ºC at 180 rpm. A mixture of 0.5 % (w/v) ó-cresol indophenol (ó-CIP), 0.1 % Tween 50 and 3 % (v/v) PAHs was introduced into the tubes. The experiment was monitored daily for change. The control experiment was also prepared without the inoculum and the set up were made in triplicate in accordance with that of Varjani et al. (2013). The experiment was allowed for 7 days in a rotatory incubator at 28 ºC observing the colour change. Afterwards the experiment was filtered to separate the biomass. The filterate was then centrifuged at 8000 rpm for 15 min, and the supernatant analysed at 609 nm using the ultra violet-visible (UV-VIS) spectrophotometer (UNICAM S675). The percentage of biodegradation was subsequently estimated as follows:

Degradation % = 1- Absorbance of treated sample/Absorbance of control x 100

3. Result

3.1. Isolation, identification and characterisation of PAH-degrading bacteria

In this study, plants collected around petroleum sludge dam were cultured for three days and soil through enrichment cultures for 21 days. The isolated organisms include gram-positive and gram-negative bacteria. A total of 19 distinct colonies that were able to colonize with clear zones were isolated and selected from the plants and soil cultures for molecular characterization. The variations of the isolates were 12 from plants, 5 from the soil, and 2 from the control varied into 10 Gram-negative and 9 Gram-positive bacterial of which bacilli represented 94.74 %, while the remaining 5.26 % was coccobacilli isolated from one of the control samples. The oligonucleotide primers used were able to amplify 1489 base pairs of 16S rRNA gene fragments using 3 µl DNA sample. The homology sequence and phylogenetic analyses of the 16S rRNA of the isolates indicated that they belong to 2 different clades namely *Proteobacteria and Firmicutes* drawn

from different class (Table 1-2). According to the distinct grouping of these organisms, they tend to exist phylogenetic relationships between them with high bootstrap. This result showed that the isolates represented distinct groups that are closely related hence forming a coherent group existing in a synergistic relationship, as to be able to initiate degradation of the target compounds. According to the results, the variations of the isolates as were drawn from the two clades are

phylogenetic analyses indicated that the isolates are related closely in 7 genera. Their relations are shown in Figure 3. These isolate were allocated the accession numbers (CP008876 - CP026048), and their closely related genera compared from the GenBank are shown in the tree (Figure 3). The result of the enrichment cultures of soil are presented in Table 7. The results are means of 3 replicates and the results are focussing on the 16 priority PAHs in soil and the selected 3 PAHs in plants. From the enrichment technique bacteria isolates capable of resisting the toxicity of PAHs as they use the compounds as their source of carbon and energy were isolated and used to compare with the plants isolates as shown in the Tables. E. coli were used as the out-group.

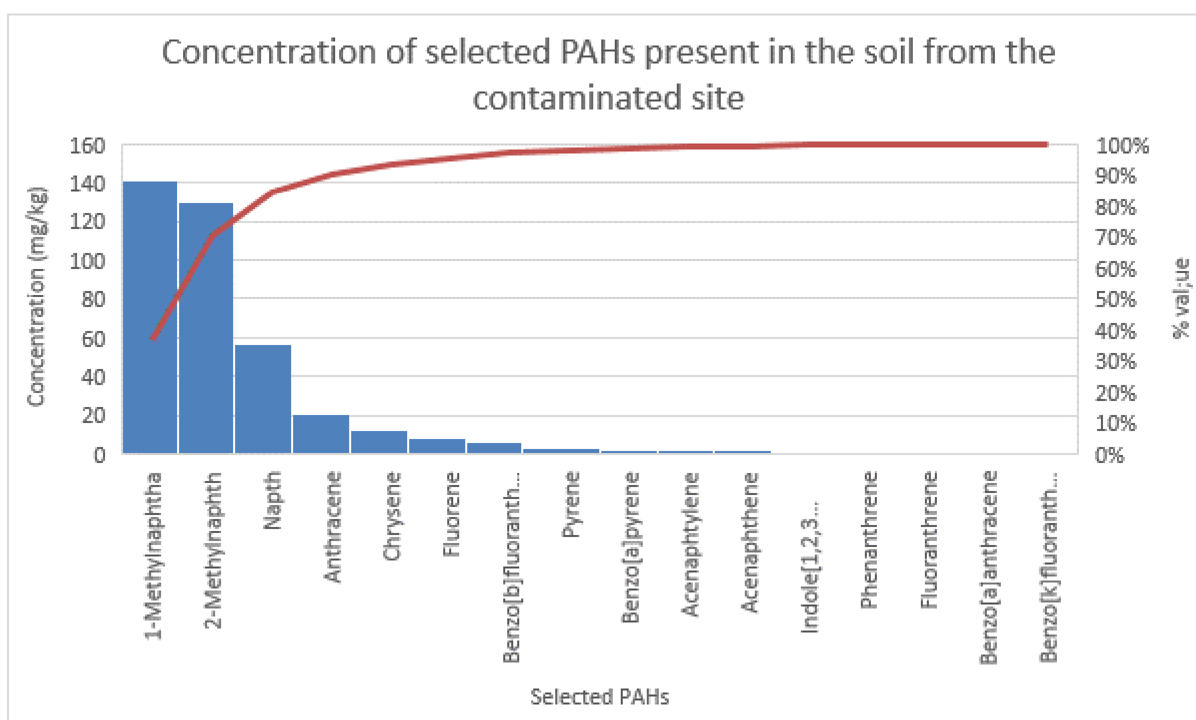

Figure 2: Concentration of the selected PAHs in the soil collected from the sludge dam. Values are means of three replicates.

Table 1: Morphological and molecular identification of bacterial endophytes isolated from plants from the contaminated environment

ID of bacteria isolates	Shape of bacteria	Gram reaction test	Molecular identification	Percentage of homology (%)
NPSI	Bacilli	+	*Terribacillus aidingensis*	100
NPLI	Bacilli	+	*Bacillus safensis*	100
NPR4	Bacilli	+	*Bacillus safensis*	100
NPR6	Bacilli	+	*Bacillus altitudinis*	100
NPL2	Bacilli	-	*Pseudomonas parafulva*	100
NPS2	Bacilli	-	*Pseudomonas parafulva*	100
NPS5	Bacilli	-	*Stenotrophomonas maltophilia*	99
NPR2	Bacilli	+	*Bacillus altitudinis*	99
$S_3$1	Bacilli	+	*Bacillus sp.*	100
R12	Bacilli	+	*Bacillus sp.*	99

R1-13	Bacilli	+	*Bacillus safensis*	100
S12	Bacilli	-	*Pseudomonas parafulva*	93
R1-11	Bacilli	+	*Bacillus safensis*	100
NPR3	Bacilli	-	*Acinetobacter junii*	100
NPR1	Bacilli	-	*Stenotrophomonas maltophilia*	99
L1-13	Bacilli	-	*Pseudomonas putida*	93
S31	Bacilli	-	*Pseudomonas sp.*	93
Control 1	Coccobacilli	-	*Pusillimonas sp.*	95
Control 3	Bacilli	-	*Burkholderia stabilis*	99

3.2. Bacteria molecular characterization

From the sequences of the bacteria isolates, operational taxonomic units (OTU) were generated for a more thoroughfare classification. Using the BLAST program, species identification was done based on the DNA nucleotide sequences. The sequences of the 16S rRNA gene were group into 11 OTU with 93 % and above similarity (Table 2). However, using Bootstrapping for 1000 repetitions, only values higher than 50 % are shown.

Using Shannon Diversity index (H), the species was calculated to be 0.325. Bacillus sp was found to be higher in Shannon Diversity Index than the rest species, the higher the value of H, the greater the diversity. However, the distribution of the bacteria were heterogeneous in nature, with the most dorminant isolates selected from OUT 2, 3 and 6 genus levels were closely related to Bacillus, and represented about 42.1 % of all the isolates. The list of level representation is shown in Figure 4 and Table 2.

Table 2: Classification of the bacteria endophytes based on their OTU representatives and sequences.

OTUs	Number of sequences	OTU Representatives	Phylum	Class
1	1	*Terribacillus aidingensis*	Firmicutes	Bacilli
2	2	*Bacillus safensis*	Firmicutes	Bacilli
3	2	*Bacillus altitudinis*	Firmicutes	Bacilli
4	3	*Pseudomonas parafulva*	Proteobacteria	γ-Proteobacteria
5	2	*Stenotrophomonas maltophilia*	Proteobacteria	γ-Proteobacteria
6	2	*Bacillus sp.*	Firmicutes	Bacilli
7	1	*Acinetobacter junii*	Proteobacteria	γ-Proteobacteria
8	1	*Pseudomonas putida*	Proteobacteria	γ-Proteobacteria
9	1	*Pseudomonas sp*	Proteobacteria	γ-Proteobacteria
10	1	*Pusillimonas sp.*	Proteobacteria	β-Proteobacteria
11	1	*Burkholderia stabilis*	Proteobacteria	β-Proteobacteria

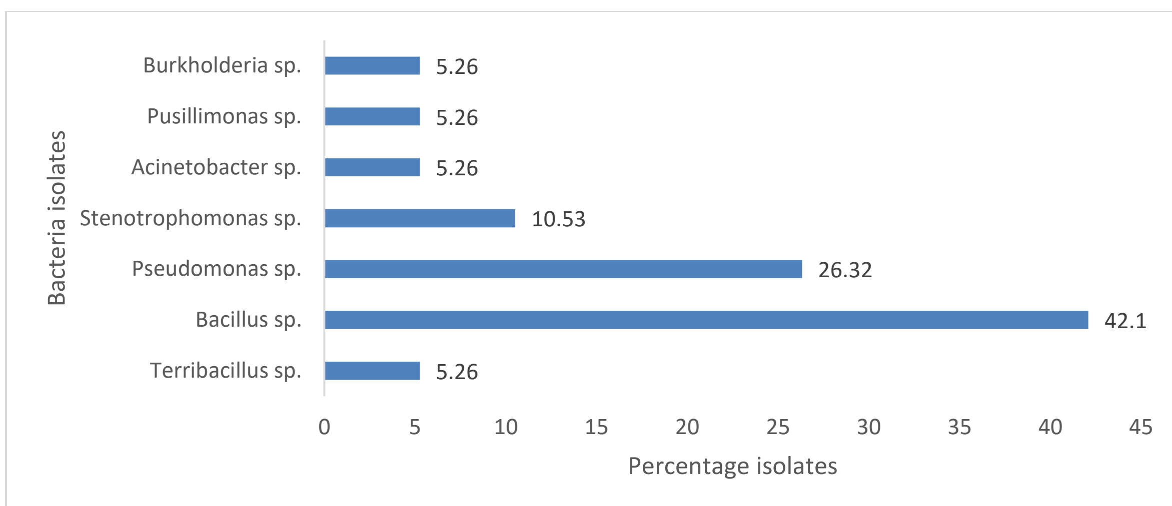

Figure 3: Percentage diversity of the bacteria genus isolated in the study

3.3. Screening of bacteria isolates for PAHs degradability

From the results obtained, the entire bacteria isolates could grow on the MSA flooded with the three selected PAHs as the only source of carbon. Meanwhile, using o-CIP as an indicator, showed a positive response on 17 of the 19 isolates with Pseudomonas sp. response as the best degrader. This was shown by the by the rate at which the bacteria was able to decolourise the compound indicator. The sequence of the reaction is in this order Pseudomonas sp.> Bacillus sp.>Terribacillus sp.>Stenotrophomonas sp.>Acinetobacter sp.

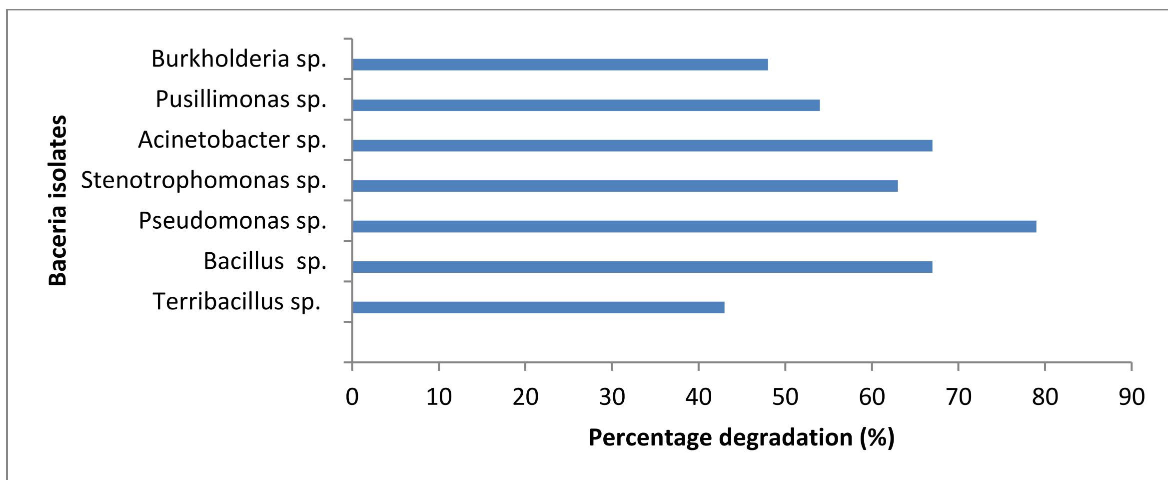

Figure 4: Estimated percentage degradation rate of perylene by the bacterial isolates using ó-CIP.

4. Discussion

This study was aimed at isolating and characterizing bacteria that has the ability to use PAHs as their source of carbon and energy. This phenomenon could find its use in the phytoremediation of such compounds in plants. From the characterisation of the sampled bacteria, several isolates belonging to the Gram-negative type of bacteria. Reports of several Gram-negative bacteria being involved in the degradation of petroleum hydrocarbon are found in the literature [13,17-18]. From the results of this study, the dominant specie was from the phylum Proteobacteria drawn mostly from the y-Proteobacteria class and few β-Proteobacteria (Table 2). The result of this study is synonymous with that of Obi et al. [19], which reported that these classes of bacteria has the ability to use nitrogen-sulphur-oxygen fractions of petroleum hydrocarbons as their source of nutrients.

PAH-degrading bacteria from our finding belong to these genera: *Pseudomonas, Bacillus, Terribacillus, Stenotrophomonas,* and *Acinetobacter.* The studies of Guzik et al. [20], Alquati et al. [21], Liu et al. [22], and Mishra et al. [23], were all in agreement with the findings reported thus, stating that these list of bacterial endophytes could make use of petroleum hydrocarbons as their source of carbon and energy. *Bacillus* and *Pseudomonas sp.* forms the greatest population with diversity level of over 42 and 26 % respectively while *Pseudomonas* were the genus with the highest degradation ability. Past literatures indicated that the excessiveness of *Bacillus* and *Pseudomonas* in a toxic organic environment as is found in the PAH-contaminated environment could have rendered them the ability to possess the highest substrate-degradation potential [19,24-25].

5. Conclusion

For pure bacteria endophyte cultures to grow in and utilize PAH as sole source of carbon and energy has provided a greener method of dealing the PAHs. Considering the menace caused by the toxicity of PAHs resulting from the presence of petroleum product in the atmosphere, could now be controlled by the isolation of endophytic bacterial from plants growing around petroleum-contaminated environment. This study was able to isolate, test and characterize bacteria from plants collected from the surrounding of a PAH-contaminated soil. Degradation of PAHs by these microbial consortia as reported in this study showed that they have specialized cometabolic capacities, as they were able to reduce the concentration of the tested PAHs. In oil-polluted environments, specialised bacterial consortiums are abundant because of their adaptation ability to pollutants. These bacteria are the most predominant microorganism among others in either *in situ* or *ex situ* bioremediation processes, indicating that they are the main agents responsible for the degradation of oil. All these characteristics were demonstrated by the endophytes isolated in this study.

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