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Isolation and Identification of Anthracene Utilizing *Proteus vulgaris* from Oil Spill Contaminated Soil at NNPC Depot Kano State Nigeria

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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

Microbial biodegradation of polycyclic aromatic hydrocarbons (PAHs) like Anthracene, represent an efficient, time, and cost-effective way for bioremediation of the polluted environment. This study was aimed at isolating, identifying and characterizing bacteria with potential to degrade and utilize anthracene as a sole carbon source. A bacteria was isolated from oil spilled contaminated site located in Kano, using an enrichment method on mineral salt media (MSM) following serial dilution (10^{-1}-10^{-6}). Characterization was done by studying the effects of temperature and pH on mineral salt media (MSM) containing anthracene. The isolate was then identified morphologically, biochemically and molecularly based on 16S rRNA partial gene sequence analysis. The morphological and microscopic examination of the isolate from this research shows that the isolate was creamy in color, motile, gram negative, short rod and non-spore forming respectively. The biochemical test of the isolate was found to be positive for these parameters (methyl red, catalase, motility, indole and urease) and negative for (citrate and oxidase). The 16S rRNA sequence and Phylogenetic analysis using neighbor joining tree and 1000 boos trap revealed that, the isolate was closely related (on the same clade) to *Proteus vulgaris* with accession number **MW766369.** Characterization was done by

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studying the effects of temperature and pH. The isolated bacterium was optimal at a temperature of 35ºC and pH 7.5. This isolate is a promising strain that could be used in bioremediation of polycylic aromatic hydrocarbons polluted environment.

Keywords: Isolation; characterization; Proteus vulgaris; anthracene; oil spilled contaminated soil.

1. INTRODUCTION

Contamination of the natural environment with oil, including polycyclic aromatic hydrocarbons (PAHs), is a widespread concern because of the health risks associated with these cancerous and mutagenic compounds [1]. Polycyclic aromatic hydrocarbons (PAHs) constitute a large and diverse group of priority environmental pollutants and can be formed as products during incomplete combustion of organic matter [2]. The increase in environmental pollution via the penetration of polycyclic aromatic hydrocarbon (PAH) into aquatic and terrestrial ecosystems has culminated in progressive degradation of environmental quality. The remediation of such pollutants requires techniques that accelerate degradation and/or coupling bioremediation with other available technologies [3,4,5]. PAHs are considered by US environmental protection agency EPA and European environmental Agency to be priority pollutants, therefore the determination of PAHS in environmental sample is very important [6].

Oil refining industry is one of the major sources releasing/ emitting toxic chemicals into the environment. In past few years, petroleum hydrocarbon pollution has become one of the most serious global concerns due to its toxicity to microorganisms as well as to higher forms of life including humans [7].

The mechanism of mutagenicity of polycyclic aromatic hydrocarbon (PAH) has been extensively investigated. Metabolites of PAHs, e.g. dihydrodiol epoxide, can predominantly bind to nucleic acids at the exocyclic amino groups of adenine and guanine forming DNA adducts that are known to be aetiological agents in cancer development [8]. The potential of reactive metabolites (e.g. epoxides and dihydrodiols) of PAHs, possibly could bind to cellular proteins and DNA and generates toxic effects, can result in biochemical disruptions and cell damages leading to developmental malformations, mutations, and tumors (Kim et al., 2013).

Due to their toxicity, carcinogenicity, and ubiquitous distribution, the US Environmental Protection Agency has listed 16 PAHs as priority pollutants [9]. The low-molecular weight (LMW) PAHs (containing two or three aromatic rings) are acutely toxic [10]. However, research has shown that microbial degradation, with a range of advantages compared to more traditional methods, has been developed as an effective technology for PAH removal [11,12].

Microbial degradation is a method of bioremediation, which consists of seeding microorganisms in polluted environments to achieve the aim of bioremediation, and it is considered a valuable tool for increasing the rate and extent of biodegradation of pollutants [13,14].

Nowadays, in order to eliminate PAHs from the environment by bioremediation, many PAH-degradation microorganisms have been isolated. Most of these bacteria belong to the genera *Pseudomonas*, *Rhodococcus*, *Paenibacillus*, *Acinetobacter*, *Bacillus* and *Mycobacterium* [15,16,2,17]. The aerobic and anaerobic bacteria species have been reported extensively in the literature for the degradation of low molecular weight (LMW) and high molecular weight (HMW) PAHs through pure cultures, consortia and mixed bacterial culture approaches (Patel et al., 2020). Therefore, this research focused on the isolation and characterization of anthracene utilizing *Proteus vulgaris* from oil spilled contaminated soil.

2. MATERIALS AND METHODS

2.1 Sample Collection and Isolation of Anthracene-tolerating Bacteria

A sample of oil-spilled contaminated soil was collected from NNPC depot, Kano (12.0022N°, 8.5920°E). The samples (3) at depth of 1-10 cm beneath the top soil were collected using soil auger and transferred into a sterile polyethene bags, and transported to the Microbiology laboratory Bayero University Kano for analyses. About 10 g contaminated soil samples were suspended in 90 ml of distilled water, and then 10 ml was transferred into a 250 ml conical flask containing 90 ml of mineral salt media (MSM)

containing (g/L): 3 g NH_4NO_3, 1.5 g K_2HPO_4, 0.5 g KH_2PO_4, 0.2 g $MgSO_4.7H_2O$, 0.5 g NaCl, 0.02 g $FeSO_4$, 0.05 g $CaCl_2$, pH 7.0 [8]. The media was then supplemented with anthracene at concentration of 100 mg/L as a sole carbon source. The flasks were incubated on an orbital shaker at 150 rpm at 37ºC [8]. After 7 days of incubation, an aliquot (10 ml) of the enriched culture was transferred into another 250 ml conical flasks containing 90 ml freshly autoclaved MSM medium supplemented with anthracene on plate MSM agar and 48 hours at 37ºC. After the required incubation, colonies formed were sub-cultured on a sterile MSM agar. The colonies formed were picked up for further analysis [8].

2.2 Screening of Isolates with High Utilization of Anthracene

A loop-full of each isolate was inoculated into flasks containing 50 ml of screening medium (MSM broth). The screening medium was same as enrichment medium, except that 1000 mg of anthracene were separately dissolved in acetone and only added to each flask after autoclaving the media, as sole source of carbon. The ability of each isolate to utilize anthracene were indicated by an increase in turbidity of the medium measured at 600 nm using UV-Visible spectrophotometer [18].

2.3 Identification of Isolated Bacteria

Pure cultures of the heterotrophic bacterial isolates were identified on the basis of cultural, morphological and biochemical characteristics. Biochemical test was conducted following the method of Oyeleke and Manga, [19] while morphological characteristics was determined as described by Cheesbrough, [20].

2.4 Molecular Identification

Genomic DNA was extracted from pure bacterial culture; 24 h grown in MSM medium at 30˚C, centrifuged for 2 min at 5000 rpm. Bacterial lysis was performed according to the manufacture instructions using the Gene JET™ genomic DNA purification kit (Thermo Fisher Scientific, USA). The obtained purified DNA was re- suspended in 100 µL of Tris-EDTA (TE) buffer (Sambrook and Russel, 2001). The A260/A280 absorbance ratio was used to determine purity. The extracted DNA (5 µL) was loaded on 1% agarose gel (Invitrogen, California, USA), which contained ethidium bromide (1 µg/mL) for DNA staining [8].

16S rRNA amplification was carried out according to Promega GoTaq kit with universal primers: F:5-'TGGAGAGTTTGATCCTGGCT CAG-3′ and R:5′-TACCGCGGCTGCTGGCAC-3′ in a final volume of 25 µL containing 5 µL 5x buffer with 20 mM magnesium, 0.5 µL 2.5 mM nucleotide mix, 0.125 µL Polymerase Promega M830B, 0.113 µL from each forward and reverse primer with concentration 100 pmol and 1 ng/µL genomic DNA (Linxiang et al., 2014). Amplification was performed in an Applied Biosystems ABI 9901 veriti 96-well fast PCR thermal cycler according to the following program: 2 min denaturation at 95°C, followed by 35 cycles of 40 s denaturation at 95°C, 40 s annealing at 55°C 90 s extension at 72 °C, and a final extension step of 10 min at 72°C. 5 µL of the amplified mixture was then analyzed using 1.5% 1X TBE agarose gel electrophoresis. The gel was stained with ethidium bromide, visualized under UV light, and photographed. The amplified products with the correct size were then purified and sequenced in both directions using an ABI automated sequencer [8].

16S rRNA PCR product was extracted from gel using gel extraction kit QIA quick Qiagen (Promega, USA). DNA sequencing was conducted using ABI Prism BigDyeTM Terminator Cycle Sequencing Ready Reaction Kit (Malaysia) according to manufacturer's instructions. ABI PrismTM 3730/3730XL DNA Sequencer (Malaysia). The 16S rRNA gene sequences of the isolates obtained in this study were aligned and compared with the known 16S rRNA gene sequences in Genebank database using the BLAST search at the National Center for Biotechnology Information (http://www.ncbi. nlm.nih.gov/BLAST/) to determine the closest available database sequences. Sequences were finally submitted to GeneBank [8].

2.5 Characterization of Anthracene-Utilizing Bacteria

2.5.1 Effect of initial pH on anthracene degradation

To study the effect of pH on degradation, MSM supplemented with 100 mg/L of anthracene was adjusted to different initial pH 5.5 – 8.0 using 1M HCl or NaOH. 100 µL of the bacterial suspension were inoculated and incubated at 37°C under shaking condition (120 rpm). In the control, without the inoculation of bacterial species was kept under similar condition. 1 mL of the bacterial aliquot was collected at regular intervals of time

(24 h) and absorbance was measured at 600 nm for up to 120 h.

2.5.2 Effect of temperature on degradation of anthracene

To study the effect of temperature on degradation, 100 ml of MSM was freshly prepared into 250 ml of conical flasks and supplemented separately with 100 mg/L of anthracene. The isolated bacterial suspension of 100 µL was added into various conical flask and was then incubated at various different temperatures ranging from 25-50°C (25,30, 35,37,40,45 and 50). 1 mL of the bacterial aliquot was collected at regular intervals of time (24 h) for up to 120 h and OD was measured using spectrophotometer at 600 nm.

3. RESULTS

Table 1. Screening of anthracene utilizing bacteria (1000 mg/L)

Name of isolate	OD (600nm)
An1	**0.314**
An2	**0.876****
An3	**0.234**
An4	**0.561**
An5	**0.214**
An6	**0.156**
An7	**0.412**
An8	**0.365**
An9	**0.113***
An10	**0.154**

*Key: An= Anthracene, OD= optical density **=highest OD *= Lowest OD*
Values in the above table are mean triplicate

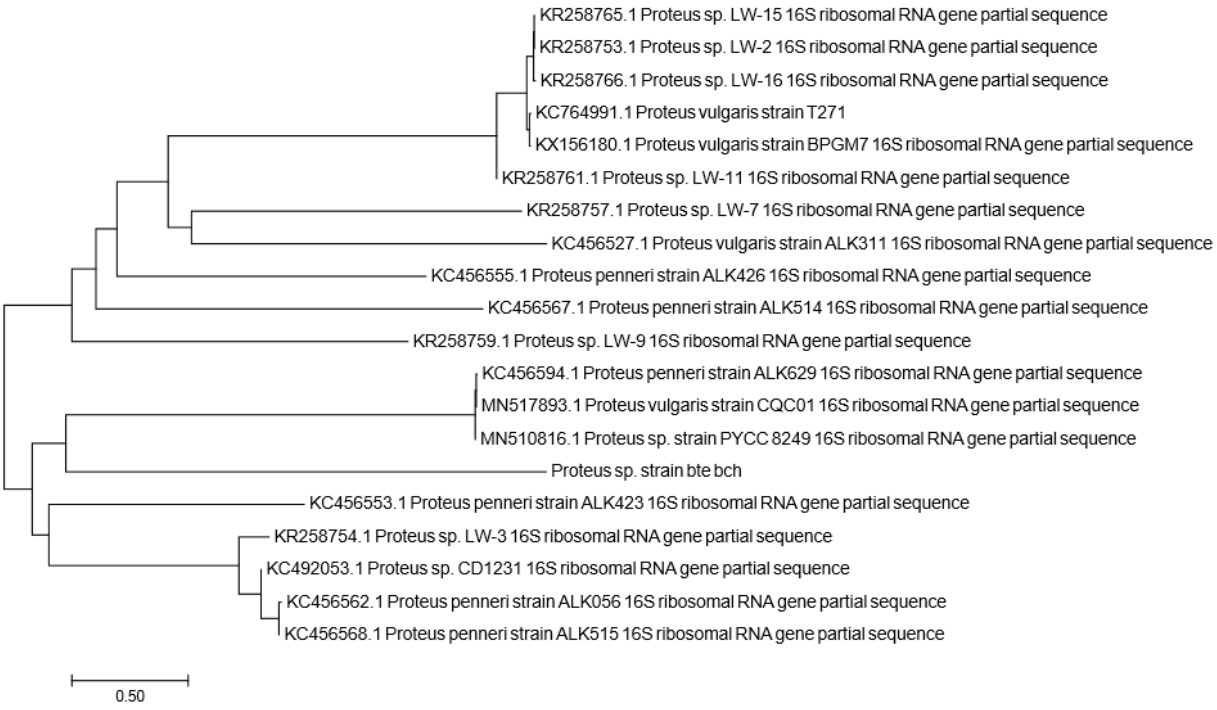

Fig. 1. Evolutionary relationship of bte_buk using molecular evolutionary Genetics Analysis software version 7.0 (MEGA7). Phylogram (neighbor-joining method) indicating the genetic relationship between unknown and referenced related microorganisms based on 16S rRNA gene sequence analysis. Accession numbers are accompanied by the species names of their 16S rRNA sequences

Table 2. Biochemical and morphological characteristics of the isolate

Tests	Biochemical test
Methyl Red	+
Catalase production	+
Oxidase production	-
Urease production	+
Motility test	+
Indole production	+
Citrate production	-
Gram reaction	-
Color	Creamy
Shape	Short rod

+=positive test, -=negative test

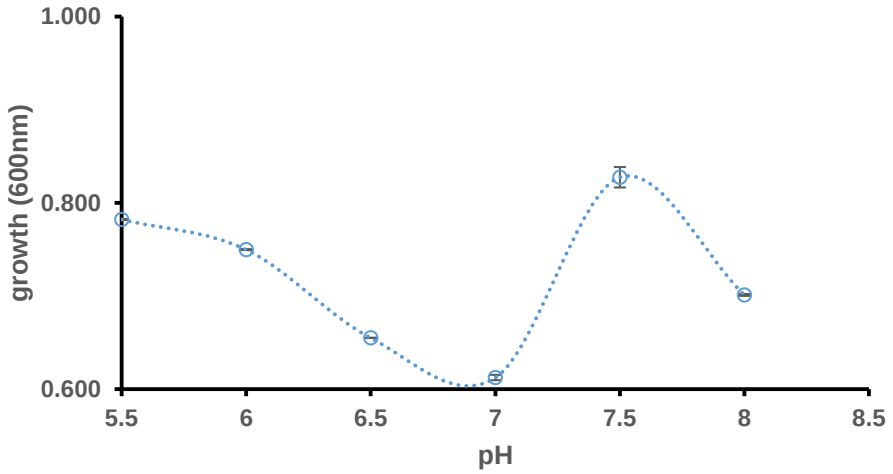

Fig. 2. Effect of various pH on the growth of naphthalene and anthracene degrading proteus vulgaris in MSM media after 72 h of incubation at 37°C. Data are mean ±SD of triplicate determination

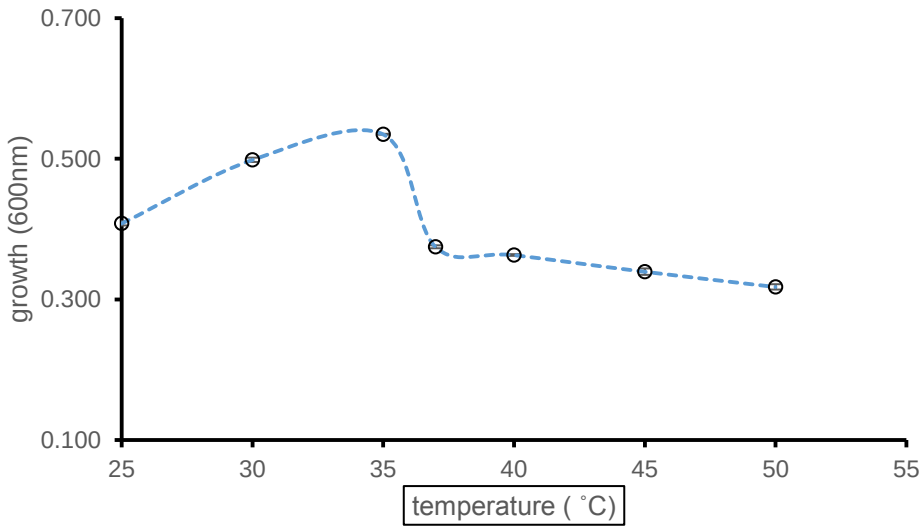

Fig. 3. Effect of various temperature on the growth of naphthalene and anthracene degrading *Proteus vulgaris* in MSM media after 72h of incubation at 37°C. Data are mean ±SD of triplicate determination

4. DISCUSSION

The growth of the bacteria on mineral salt media MSM (Table 1) is measured using spectrophotometer and it was fond that AN2 has the highest OD which indicates high growth and was used for the study, and this was in conformity with that of the Abdulhaleem et al. [8] who selected the highest bacteria utilizing the pollutant for further studies. The morphological characteristics of the isolate from this research (Table 2) show that the isolate was creamy in color and motile. Microscopic examination showed that the isolate was Gram negative, short rod and non-spore forming. The biochemical test of the isolate was found to be positive for these parameters (methyl red, catalase, motility, indole and urease) and negative for (citrate and oxidase). However, the parameters were compared with Bergeys manual and showed that it belongs to the genus of *Proteus*. This finding was in agreement with that of Abdelhaleem et al. [8] except that, the difference is based on the biochemical test (urease and indole). It was reported by Abdelhaleem et al. [8] that the urease and indole test were negative which conformed with that of *Bacillus* spp, while this study found that the isolate was positive for both urease and indole which conformed with that of *Proteus vulgaris*.

The molecular analysis using 16S rRNA partial gene sequence (Fig. 1) confirmed the earlier finding on biochemical test. The 16S rRNA gene sequence obtained was submitted to the National Centre for Biotechnology Information (NCBI) database using BLAST algorithm and compared with submitted gene sequences for similarity. The phylogenetic analysis proved that the isolate has the same clade with *Proteus vulgaris*.

pH plays an important role in growth and biodegradation of the microbes. The effect of initial pH on anthracene degradation was evaluated at various pH (5.5 – 8.0) (Fig. 2). The different pH conditions affected the degrading activity of *Proteus vulgaris* significantly over the whole incubation period. In addition, the growth and utilization of anthracene by *Proteus vulgaris* was found to increase as pH increases. However, as the pH exceeds optimum level (pH 7.5), the growth of the isolate decreases. This study was similar to that of Othman and Abdul-Talib, [21] who reported that changes in pH can alter the electrical charge on various chemical groups in enzymes molecules, and can probably alter the enzyme's ability to bind its substrate and catalyze a reaction. This imbalance of the electrical charges in very acidic and alkali condition can disrupt hydrogen bonds and other weak forces that maintain enzyme structure. Such disruption of enzyme structure is called denaturation. Thus, this phenomenon resulted in poor biodegradation process.

The effect of temperature on anthracene-utilizing *Proteus vulgaris* was carried out at different temperatures (25 – 50ºC) (Fig. 3). However, this study found that at lower temperatures (25 – 30ºC) the growth was slow and hence the degradation process. The slow growth observed at low temperature suggests that the temperature is not sufficient for the enzyme activity. Therefore, increased in temperature will result in faster reaction rate in degrading anthracene. In general, enzymes as catalysts have certain temperature that it withstands and normally at optimum temperature will catalyzed the reaction rapidly. Microbial enzymes likewise function best at optimum temperature which related to an organism's normal environment. Thus, reaction such as degradation process is normally at optimum temperature in microbial cell. Above 40ºC, enzyme start being denatured, and its activity decreased accordingly. The results from this study also found that the degradation of anthracene strongly depend on temperature.

Similar observations with these findings were also reported by Neelofur et al. [22]. Furthermore, this research was not in conformity with the works of Abdelhaleem et al. [8] who reported the temperature of 42ºC and 40ºC to be the optimum temperature for degradation of anthracene respectively. The difference in optimum temperature was due to the differences in the organisms isolated and the region from which the isolation was made [23,24].

5. CONCLUSIONS

In this study, a bacterium identified as *Proteus vulgaris* was isolated, identified morphologically, biochemically and molecularly based on 16S rRNA partial gene sequence analysis, phylogenetic analysis revealed that the isolated bacterium has the same clade with *Proteus vulgaris*. The Proteus vulgaris was characterized base on pH and temperature with potential to utilize anthracene as sole carbon source from oil-spilled contaminated soil. The isolate grows optimally at 35 ºC and pH 7.5, the ability of bacterium to utilize anthracene as sole source of carbon and energy provides an environmental

friendly approach for dealing with environmental pollution and toxicity caused by these chemical.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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