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Growth Optimization of Naphthalene-Degrading *Proteus vulgaris* Isolated from Oil-spill Contaminated Soil at NNPC Depot in Northern Nigeria

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Abstract

Naphthalene degradation by bacteria represent a green approach for eco-restoration compared to physical or chemical remediation processes. This research aimed to isolate, identify and characterize bacterial isolate capable of degrading and utilizing naphthalene as the sole carbon and energy source. The bacteria was isolated on mineral salt media using an enrichment method. Identification of the isolate was molecularly achieved based on partial sequencing of 16S rRNA and phylogenetic analysis, while the effects of temperature, pH, substrate concentration, inoculum size, incubation time and heavy metals were optimized using one factor at a time (OFAT) approach. The sequence of this isolate was 88% related to several *Proteus* sp., while phylogenetic analysis via the neighbor joining method linked this bacterium to *Proteus vulgaris*, thus it was assigned as strain BTE_BCH. The result shows that growth this bacterium and naphthalene degradation were optimal at a substrate concentration between 400 – 600 mg/L, temperature 35 ºC, pH 7.5 at 72 h of incubation. The isolate was found to tolerate 2 ppm Cu, Hg, Fe, Cr, Zn, Cd, Ni, Pb and As. The degradation efficiency of this bacterium to naphthalene after 120 h of incubation was 97.2%. GC-MS analysis revealed 1,2-naphthalenediol, Cis1,2-dihydroxynaphthalene and 1, 4-Naphthoquinone as metabolites of naphthalene degradation on comparing the fragmentation pattern and mass spectrum obtained with data available on NIST library. This isolate could be used as a promising strain in bioremediation of naphthalene polluted environment.

1. Introduction

Naphthalene as Polycyclic aromatic hydrocarbons (PAHs) is classified amongst the large and diverse group of superiority environmental pollutants, which occur as a result of partial combustion of organic matter such as coal tar, gas, smoked food, organic materials, automobile exhaust, either during industrial and other anthropogenic processes or naturally during biomass reactions associated with the production of fossil fuels [1], volcanic eruptions, forest fires, bacterial reactions and by plant [2]. Physically, PAHs are pale yellow-green solids or colorless with a pleasant and faint odor. They are non-polar organic compounds, chemically made up of carbon and hydrogen atoms. PAHs are generally environmental pollutants commonly found in sediments, air and aquatic environments both surface and ground waters. Their existence in nature is of great environmental concern due to their potential

toxicity, carcinogenicity, and possible mutagenicity [3], [4]. Research has recently proven the carcinogenic and mutagenic potentials of persistent exposure to PAHs sediments in aquatic animals [5].

Naphthalene is listed by USEPA among the major PAHs pollutants due to their ubiquitous distribution, recalcitrant nature and toxicity [6]. Biological system exposure to PAHs resulted in its binding to DNA, RNA or proteins, such covalent interaction determined the rate at which the carcinogenicity is classified [7]. Critical cellular effects may also result from such binding with the PAHs transformed products which at times are more harmful than the parent material [8]. Detoxification of PAHs in in the living system occurs by a group of enzymes (Cytochrome P450 monooxygenases) that oxidizes PAHs to epoxides some of which are highly reactive (such as "bay-region" diol epoxides) with carcinogenic potentials [5].

Photo-oxidation, volatilization, bioaccumulation, chemical oxidation, adsorption in soil particles are routes by which PAHs enters the environments [9]. Their removal through degradation by microbes has been shown by researchers to be efficient means of remediating the environment [10], [11], [12], [13]. Currently, a number of microorganism with PAH-degradation capability have been isolated and characterized [14]–[17]. Most of these bacteria belong to the genera *Pseudomonas*, *Rhodococcus, Paenibacillus, Acinetobacter*, *Bacillus* and *Mycobacterium* with few reported on *Proteus* sp. [6], [18]. Similarly, the search for autochthonous, multi-degrader and more tolerant isolate that could resist high concentration of toxic compounds and degrade/detoxify multiple contaminants is highly desirable. This research focused on isolating and characterizing indigenous bacteria with capability to degrade polycyclic aromatic hydrocarbon (naphthalene).

2. Methodology

2.1 Sample collection

An oil-spilled contaminated soil sample at a of depth of 1-10 cm beneath the top soil was collected using soil auger from NNPC depot, Kano (12.0022N°, 8.5920°E). The samples were aseptically transported to the Microbiology laboratory Bayero University Kano in a sterile polyethene bags for analyses. Analytical grade reagents and chemicals were used throughout the study (Sigma Aldrich, USA). Experiments involving microorganisms were done in a class II biosafety cabinet.

2.2 Isolation and enrichment of naphthalene-degrading bacteria

Into a beaker containing 90 mL of distilled water, 10 g of dried soil sample was transferred. Aliquot (10 mL) was collected and introduced to a conical flask (250 mL) containing 90 mL of mineral salt media (MSM) with the following composition in g/L: K_2HPO_4 1.5 g, NH_4NO_3 3 g, , $MgSO_4.7H_2O$ 0.2 g, KH_2PO_4 0.5 g, NaCl 0.5 g, 0.05 g $CaCl_2$, $FeSO_4$ .02 g, at pH 7.0 [17]. The media was then supplemented with 100 mg/L naphthalene as sole carbon source and incubated at 37 ºC on rotary shaker 120 rpm [17].

2.3 Identification of Isolated Bacteria

Pure culture of the heterotrophic bacterial isolate was molecularly identified.

Genomic DNA isolation and amplification of 16S rRNA sequence

Pure bacterial culture grown on Luria broth at 37 °C was centrifuged for 2 min at 5000 rpm to extract genomic DNA from the isolated bacterium. Ice-cold 70% ethanol (100 µL) was used to rinse the pellet and suspended in 100 µL dH_2O incubated for 10 min at 65 °C. The concentration of DNA was

determined using Nano drop spectrophotometry (2000/2000c, Thermo Fisher Scientific, USA) [17]. The PCR reaction was carried out using KAPATaq DNA polymerase. Total volume of 25 µL and comprise of 2 µL genomic DNA, 2.5 µL of 10 TaqA Buffer, ~0.4 M (0.85 µL) of each of forward primer Bact1442-F (AGAGTTGATCCTGGCTCAG) and reverse primer Bact1492-R (GGTTACCTTGTTACGACTT) 1.25 mM (1.5 µL) of MgCl2, 0.25 mM (0.2 µL) of dNTPs mixes and 0.2 µL of Taq DNA polymerase, in ddH2O [17]. PCR amplificon was viewed using agarose gel electrophoresis. A 14-well comb was set in placed and the gel was allowed to set for 30 min. After the gel solidified, the comb was removed and the gel tray placed in the buffer tank. The gel was submerged to a depth of 2 to 5 mm by pouring 0.5X TBE into the tank [6].

Sequence alignment and phylogenetic analysis

Sequencing of amplified PCR product was carried out with an ABI 3130 Genetic Analyzer. The 16S rRNA gene sequence of the isolate was analyzed with nucleotide BLAST search in GenBank. Phylogenetic relationship was analyzed with other closely related in GenBank [6].The sequences were obtained in FASTA format, checked for Multiple Sequence Alignment (MSA) using T-Coffee tool from EBI (EMBL), and a tree was created sequence of the isolated bacteria was also deposited to Genebank for accession number [17].

2.4 Optimization of Naphthalene-degrading Bacteria

The effect of process parameters on the growth and degradation of naphthalene by this isolate was optimized based on one-factor-at-a-time (OFAT) [19].

Effect of substrates (naphthalene) concentration on the degradation

The effect of concentration of substrate (naphthalene) on growth of this isolate was studied by varying its concentration from 50 mg/L – 1000 mg/L in MSM. Briefly, into conical flasks (250 mL) containing 100 mL MSM supplemented with various concentration of the substrate, 100 µl of bacterial suspension was inoculated and incubated at 37 ºC under shaking condition (120 rpm). 1 mL of the bacterial aliquot was collected at regular time intervals (24 h) for up to 120 h and bacterial growth was measured at 600 nm.

Effect of temperature on naphthalene degradation

To study the effect of temperature on growth of this isolate, 100 mL of freshly prepared MSM containing 100 mg/L naphthalene, was transferred into conical flasks (250 mL), inoculated with 100 µL bacterial suspension, then incubated at varied ranged from 25 – 50 ºC. 1 mL of the bacterial aliquot was collected at regular time intervals (24 h) for up to 120 h and bacterial growth was measured at 600 nm.

Effect of initial pH on naphthalene degradation

The effect of pH on growth of naphthalene-degrading bacteria was studied by separately adjusting the pH of MSM (100 mL) containing 100 mg/L naphthalene to range between 5.5 – 8.0 using 1M HCl or NaOH. The culture media was then inoculated 100 µL bacterial suspension and incubated at 37 ºC under shaking condition (120 rpm). The control, was kept under similar condition. 1 mL of the bacterial aliquot was collected at regular time intervals (24 h) for up to 120 h and bacterial growth was measured at 600 nm.

Effect of inoculum size on naphthalene degradation

The effect of inoculum on naphthalene degradation was studied by adding different amount of inoculum ranging from 50 µL-1000 µL to MSM (100 mL) containing 100 mg/L naphthalene and incubated at 37 ºC. 1 mL of the bacterial aliquot was collected at regular time intervals (24 h) for up to 120 h and bacterial growth was measured at 600 nm.

Effect of heavy metals on naphthalene degradation

The effect of these metals (Ni, Cr, Zn, Hg, Ar, Cd and Fe) on the growth of naphthalene-degrading isolate was studied by dissolving the corresponding salts in deionized water (concentration 1 – 10 mg/L) and each separately introduced into freshly prepared 100 mL MSM containing 100 mg/L of naphthalene, then inoculated and incubated at 37 ºC for 120 h. 1 mL of the bacterial aliquot was collected at regular time intervals (24 h) for up to 120 h and bacterial growth was measured at 600 nm.

GC-MS analysis of *Proteus vulgaris* degraded naphthalene products

Modified method of [17] was used for the process. *Proteus vulgaris* was subjected to optimum conditions achieved through one-factor-at-a-time optimization which include, pH value of 7.5, temperature 35 ºC, naphthalene concentration 400 mg/L, inoculum size 200 µL and incubated for 120 h. To determine the percentage degradation, GC-MS analysis was performed by taking 5 mL of the cultures centrifuged at 4000 rpm for 10 min and the supernatant was extracted with 3 times half volume of ethyl acetate as a solvent in separating funnels to remove cellular material. The vials were kept at 4 ºC until the GC-MS analysis. Uninoculated control was incubated in parallel to monitor abiotic losses of the substrate.

The degradation pattern and metabolites of naphthalene were detected by GC-MS (Thermo GC- Trace Ultra ver: 5.0, Thermo MS DSQ II), which was equipped with a DB 35- MS Capillary Standard Non-polar column (30 m×0.25 mm×0.25 µm). 1 µL of the organic phase was analyzed using GC-MS equipped with a split–split less injector (split ratios of 50:1). The oven temperature was initially at 40 ºC and then programmed to 270 ºC at a rate of 8 ºC /min where it was held for 5 min. The temperatures of injector, transfer line and ionization source were all 250 ºC. The electron impact ionization was tuned at 70 eV and Helium was used as carrier gas with an average linear velocity of 1.0 mL/min.

Statistical Analysis

GraphPad InStat 3.10 was used to analyze all data, values presented as means ±SD. Comparison between means was performed using one-way analysis of variance with Tukey's test post hoc analysis. Statistically significance was considered at $P<0.05$.

3. Results and Discussion

3.1 Isolation and Screening of Naphthalene-Degrading Bacteria

Following spread plating of the serially diluted sample, twenty colonies of bacteria were obtained, out of which only one isolate was found to degrade, utilized and grow on naphthalene as sole carbon source. The bacterial isolate was screened by streaking on fresh MSM agar supplemented naphthalene. Hence, utilized for the characterization work.

3.2 Identification of the bacterial isolate

The morphology of the isolated bacteria viewed under microscope was found to be gram negative, rod-shaped, motile and non-spore forming. Molecularly, the isolate was identified on the basis of 16S

rRNA partial sequencing and subsequent phylogenetic analysis. A total of 1444 bases of the ribosomal gene were obtained and compared with GenBank database using blast sever at National center for biotechnology information NCBI. The analysis show that the DNA sequences obtained were closely related to the partial sequence of several *Proteus* sp. with 88% similarity. Molecular phylogenetic studies using the neighbor joining method linked the identity of the obtained bacterium sequence to *Proteus vulgaris*. Thus, this bacterium is tentatively assigned as *Proteus vulgaris* strain BTE_BCH with accession number MW766369. Detailed of the molecular phylogenetic studies are presented in **Figure** 1.

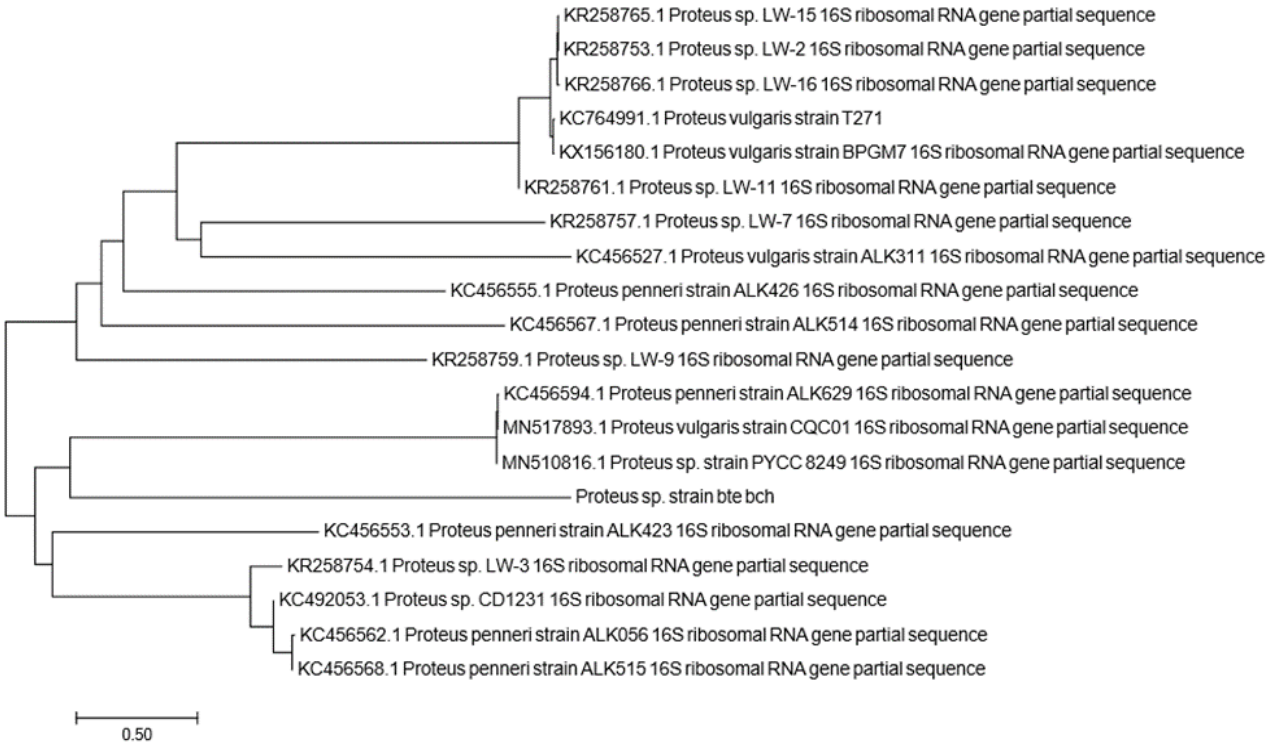

Figure 1. Cladogram (neighbor-joining method) indicating the evolutionary relationship between *Proteus* sp. strain BTE_BCH and referenced related microorganisms based on 16S rRNA gene sequence analysis using molecular evolutionary Genetics Analysis software version 7.0 (MEGA7). Accession numbers are accompanied by the species names of their 16S rRNA sequences.

3.3 Optimization of naphthalene-degrading bacterium

Effect of substrate concentration on *Proteus* sp. strain BTE_BCH

In this study, the growth of the isolate increases as the substrate concentration increases until it reaches optimum as shown in **Figure** 2. The optimum substrate (naphthalene) concentration was found to be between 400 and 600 mg/L. However, as the substrates increases above 400 mg/L, the growth of the isolate significantly decreases (P<0.05). The observed decrease in growth observed at higher concentrations beyond the optimum may be due to the toxicity of the substrate (naphthalene) to the isolate. This finding agrees with work of [23] who reported an optimum substrate concentration to be 600 mg/L, this could be attributable to the specie similarity, source of isolation and the existence of related pathway for detoxification.

Effect of temperature on *Proteus* sp. strain BTE_BCH

The effect of temperature on the growth of naphthalene-degrading *Proteus vulgaris* on MSM was presented in **Figure** 3. The growth of this bacterium was found to be linear from 25 – 30 ºC, reaching

optimum at 35 ºC following 72 h incubation, and decreased significantly (P<0.05) above the optimum temperature. The significant decrease in growth observed could be due to denaturation of the proteins responsible for growth and survival of the microbe. In general, microbes have specific temperature at which rapid increased in growth and metabolic activity involving enzymes proteins is optimum.

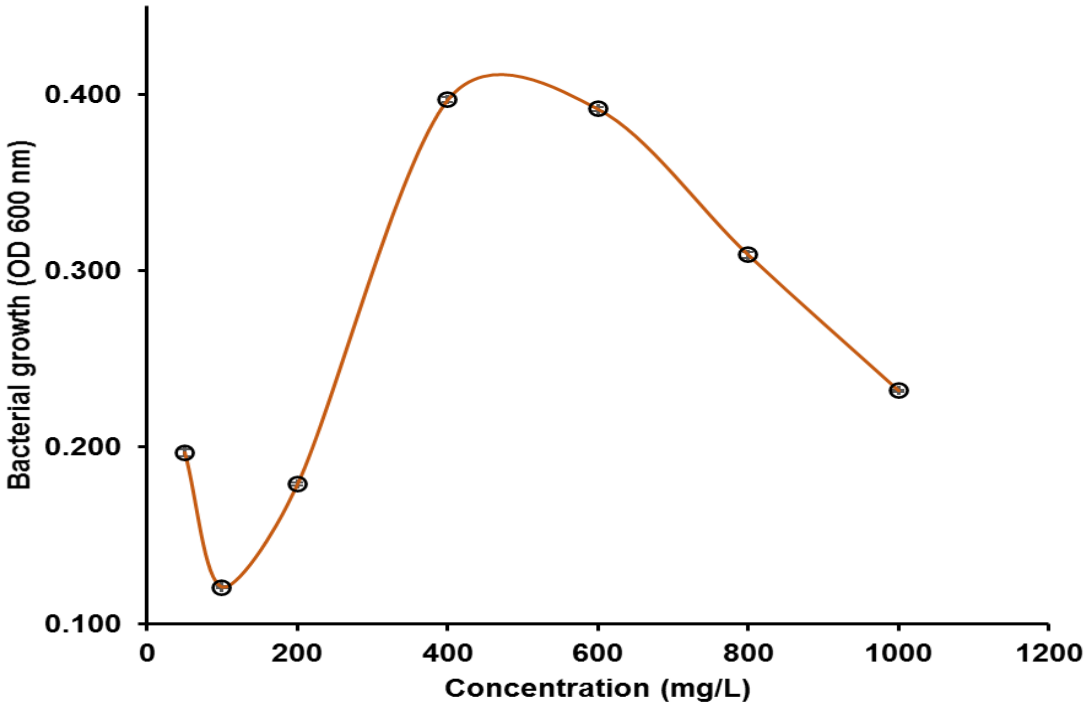

Figure 2. Effect of substrate concentrations (naphthalene) on the growth of *P. vulgaris* on MSM media incubated for 72 h at 37 ºC. Data are mean ±SD of triplicate determinations.

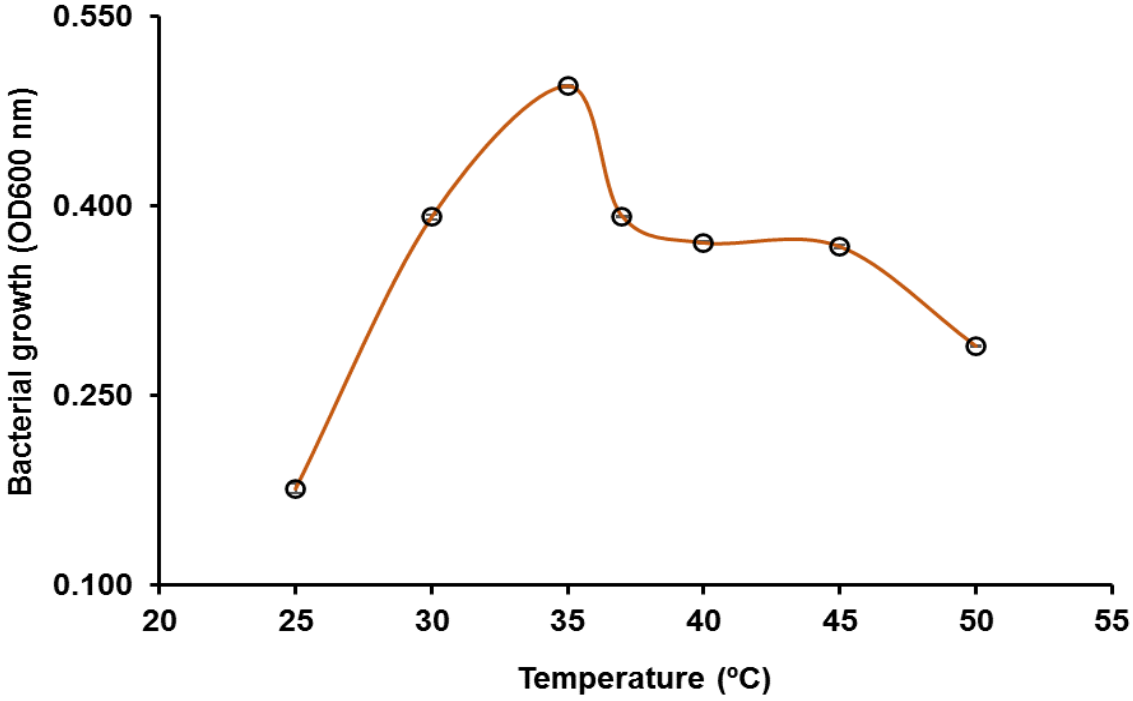

Figure 3. Effect of temperature on the growth of naphthalene-degrading *P. vulgaris* in MSM media at various temperature for 72 h. Data are mean ±SD of triplicate determinations.

Temperature above 40 ºC, activity decreases as the enzyme start denaturation. The results from this study also found that the growth and degradation of naphthalene strongly depend on temperature. Similar observations were also reported by [21]. However, the findings in this research was not in conformity with the works of [17], [22] who reported the temperature of 42 ºC and 40 ºC, respectively to be optimum for degradation of naphthalene. The difference in optimum temperature was due to the differences in bacterial specie and the region of isolation.

Effect of initial pH on *Proteus* sp. strain BTE_BCH

The effect of initial pH on growth of naphthalene-degrading *Proteus* sp. strain BTE_BCH was evaluated at pH ranging from 5.5 – 8.0. The growth of this isolate was found to be optimum at pH between 7.0 and 7.5 when naphthalene was utilized as substrate, with significantly lower ($P < 0.05$) growth at pH below and above the optimum (**Figure** 4). pH plays a substantial role in microbial growth and biodegradation of compounds. The different pH conditions affected the degradative activity of *Proteus vulgaris* significantly over the whole incubation period. In addition, the growth and utilization of naphthalene by *Proteus vulgaris* was found to increase as pH increases. However, as the pH exceeds optimum level (pH 7.0 – 7.5), the growth of the isolate decreases. This finding was similar to that of [20], who reported alteration in pH might resulted in changed of electrical charge on various chemical groups in enzymes molecules, and can probably hinder 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.

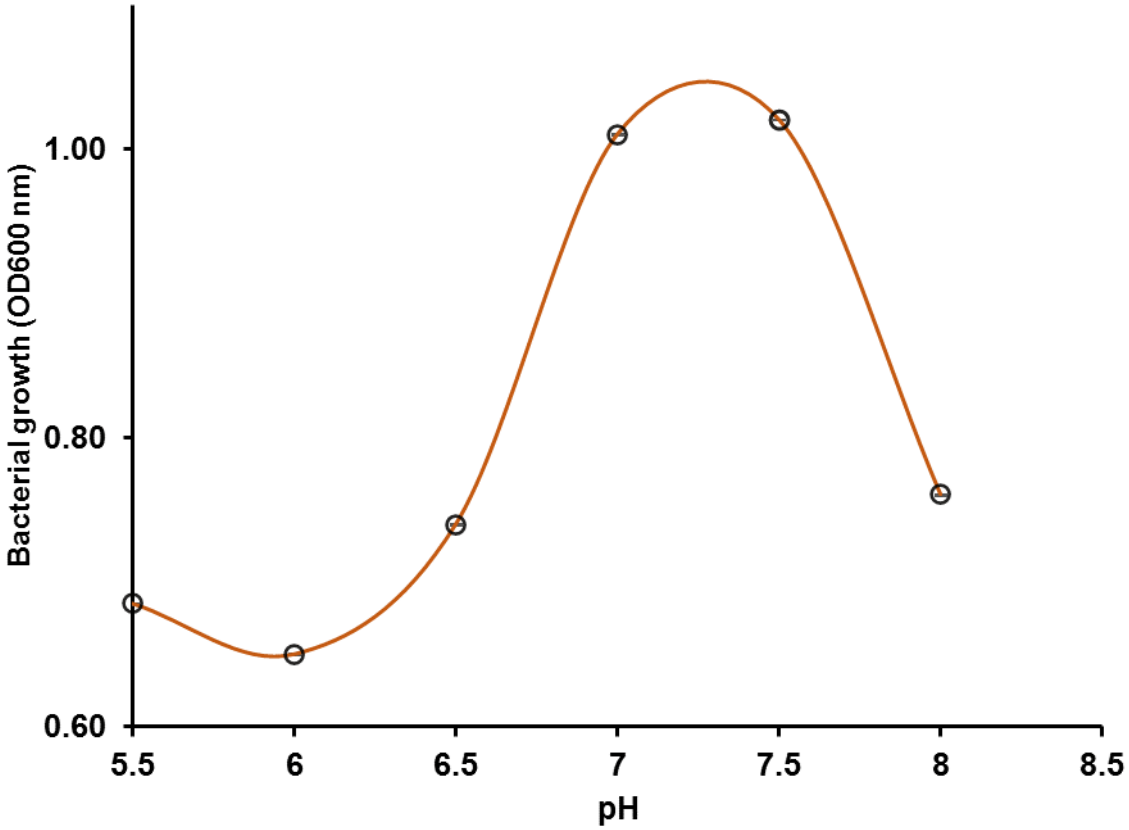

Figure 4. Effect of various initial pH on growth of naphthalene-degrading *P. vulgaris* in MSM media incubated for 72 h at 37 ºC. Data are mean ±SD of triplicate determinations.

Effect of inoculum size on *Proteus* sp. strain BTE_BCH

The effect of inoculum size on the growth of naphthalene-degrading *P. vulgaris* revealed that the isolate grew best at optimal inoculum of 200 µL. The growth was significantly higher ($P < 0.05$) at this optimal inoculum, which significantly ($P < 0.05$) decreased as inoculum was raised to 400 µL. The growth of naphthalene-degrading *Proteus vulgaris* increased with increasing inoculum. Microbial degradation of organic compounds is generally growth associated process. The maximum observed degradation was achieved with the optimum bacterial cell density of 200 µL with naphthalene as substrate as shown in **figure 5.** The decreased growth and degradation observed at higher inoculum beyond the optimum may be attributed to rapid increase in the cell density that competes for the limited nutrients causing death of the less competent cells [17].

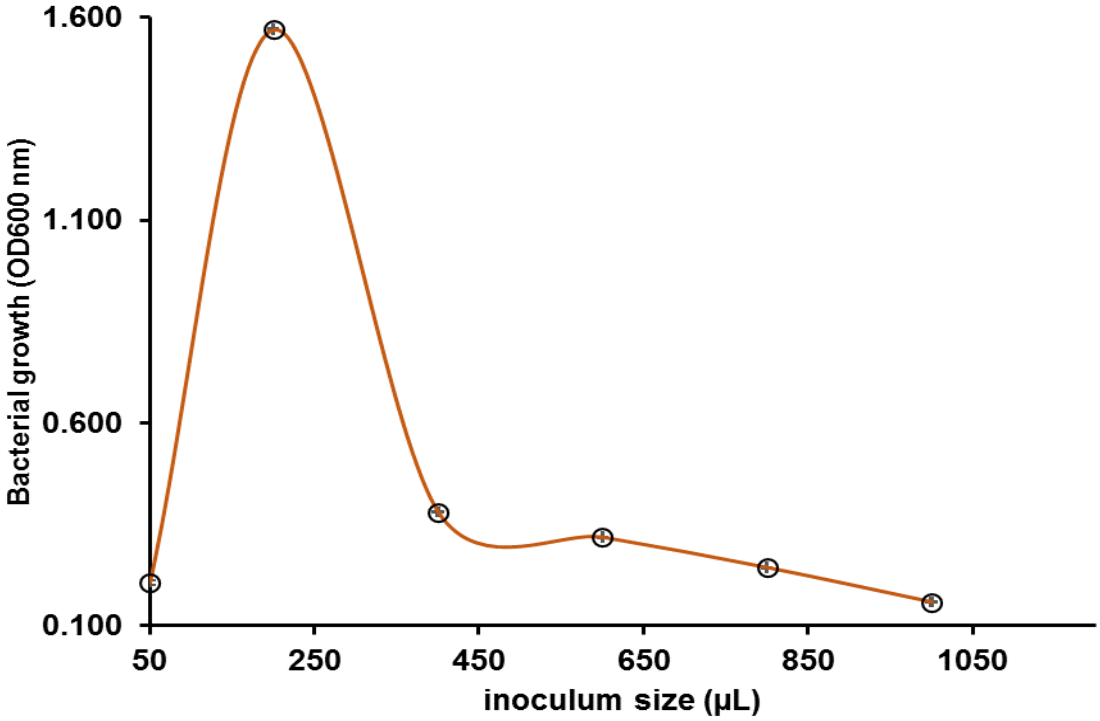

Figure 5. Effect of inoculum size on the growth of naphthalene-degrading *P. vulgaris* in MSM media incubated for 72 h at 37 ºC. Data are mean ±SD of triplicate determinations.

Effect of heavy metals on *Proteus* sp. strain BTE_BCH

Heavy metals effect on the growth of naphthalene-degrading *Proteus vulgaris* shows that in the presence of 400 mg/L of naphthalene, the isolate tolerated 2 ppm of all the tested metals solutions supplemented in the media. Zinc and cadmium did not show effect on the growth and naphthalene degradation. However, chromium and nickel slightly inhibited the growth of this bacterium relative to control (**Figure** 6). Generally, higher concentrations of heavy metals are known to diminish microbial activity, through inhibition of microbial enzymes, causing DNA mutation. The toxicity of heavy metals at the high concentration may be responsible for the rapid decrease in the *Proteus vulgaris* population upon increase in concentration which is similar with the findings of [24].

3.4 Biodegradation study

The percentage degradation was determined by subjecting the isolate (*Proteus vulgaris*) to optimum conditions achieved through one-factor-at-a-time optimization which include, pH value of 7.5, temperature 35 ºC, naphthalene concentration 400 mg/L, inoculum size 200 µL and incubated for 120h.

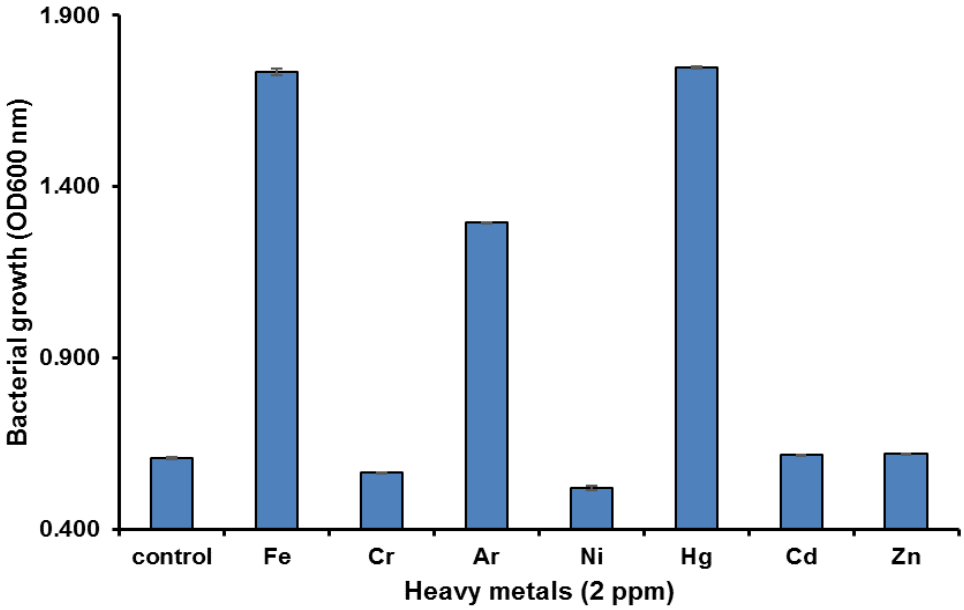

Figure 6. Effect of interaction heavy metals (2 ppm) on growth of *Proteus vulgaris* supplemented with 400 mg/L of naphthalene.

GC-MS Analysis

The GC-MS chromatogram for naphthalene standard and degraded samples were presented in **Figures** 7 and 8, respectively. Naphthalene standard with retention time of 20.642 had an area under the curve (AUC) of 9863862 (**Figures** 7), while the degraded naphthalene sample with RT of 20.672 had AUC of 274138 (**Figures** 8) indicating degradation efficiency of 97.2% within 120 h of incubation under optimized conditions.

The metabolites of naphthalene degradation by this isolate were presented in **Figure 9**. Comparing the metabolites profile of naphthalene degradation in this study with the data available on NIST library based on mass spectrum and fragmentation pattern, compounds like 1,2-naphthalenediol, Cis1,2-dihydroxynaphthalene and 1, 4-Naphthoquinone were identified as byproducts of naphthalene degradation.

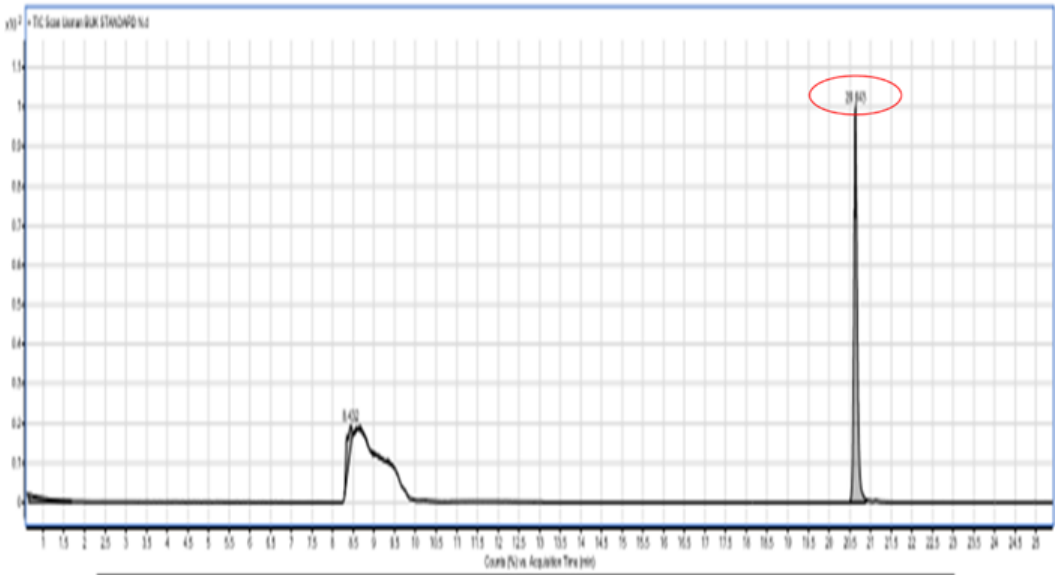

Figure 7. S GC-MS chromatogram of naphthalene standard with retention time (RT) at 20.642

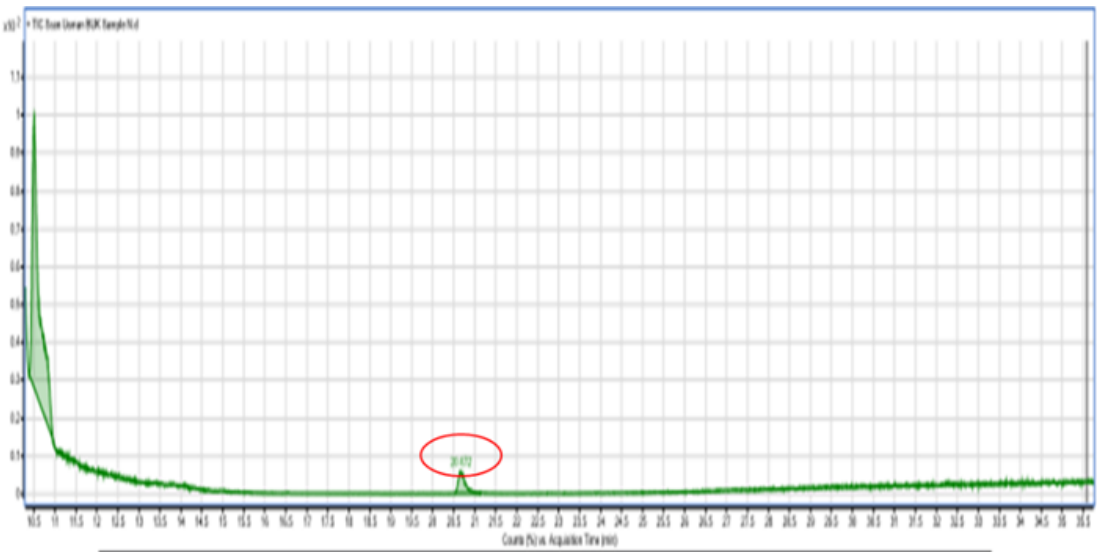

Figure 8. S GC-MS chromatogram of naphthalene sample (400 mg/L) with RT at 20.672 degraded by *Proteus vulgaris* (200 µL) after incubation at 35 ºC for 120 h.

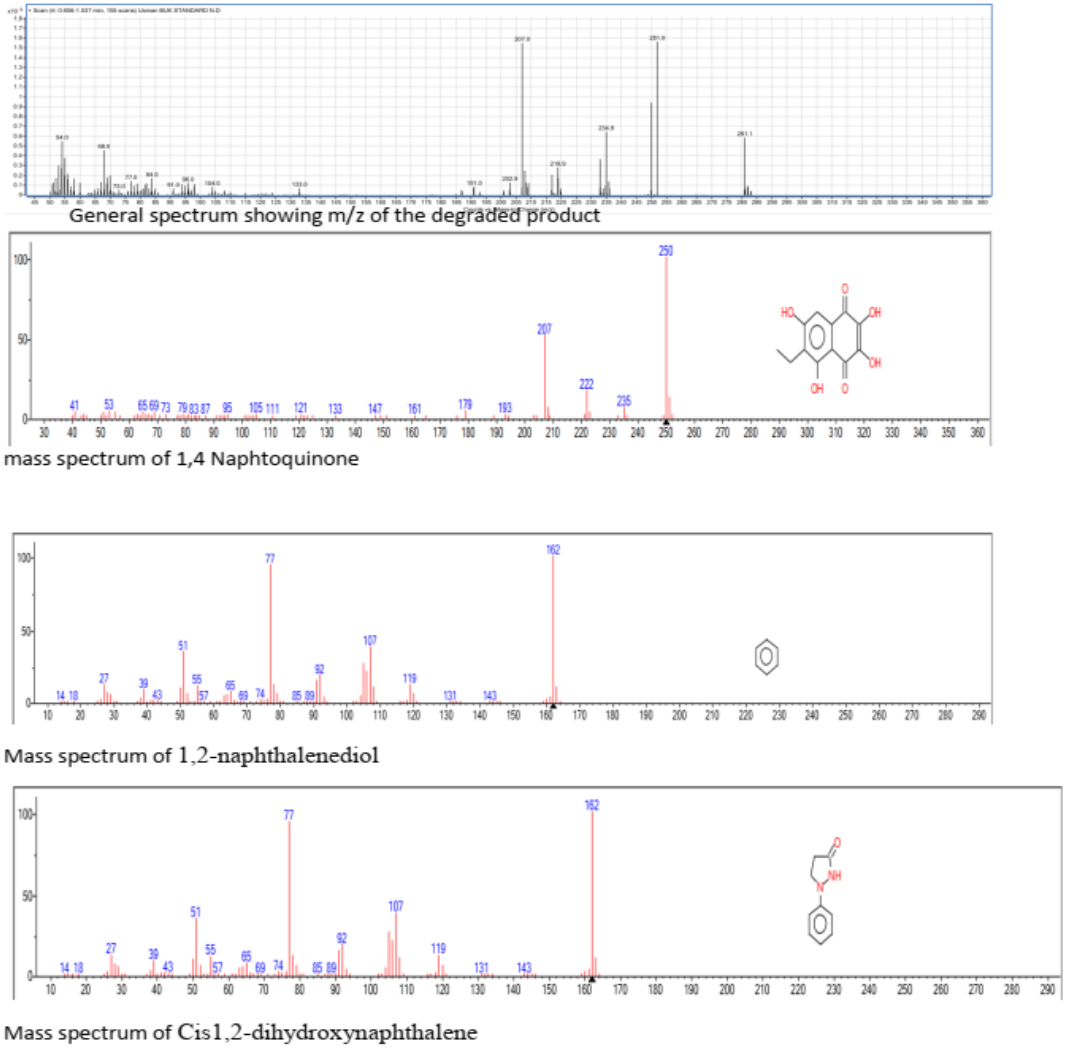

Figure 9. Mass spectrum of metabolites naphthalene biodegradation

From the result of this analysis, retention time and area under the curve (AUC) was used to calculate the degradation percentage of substrate (naphthalene) by the isolate. GC-MS analysis revealed that the percentages of degradation increased as concentration of substrate increase up to 400

mg/L. Although there was a dropped in the growth of the isolate before it reaches the steady phase. Thus, the degradation process was optimum at 400 mg/L of substrate or is known as a steady state for naphthalene. At this point, the degrading isolate has sufficient carbon sources for energy and growth has high efficiency to mineralize naphthalene. Therefore, the percentage degradation of naphthalene was found to be 97.2% at concentration of 400 mg/L following incubation at 35 ºC for 120 h. A similar finding on naphthalene degradation was reported by [5], though on different bacterial specie. At a certain concentration, naphthalene was beneficial to the growth of isolated bacteria because it serves as sole carbon and energy source for its growth. The degradation of naphthalene at higher concentration shows an increasing trend because the increase will increase the affinity of substrate toward product formation. Therefore, low concentrations would become a limiting factor for the bacterial culture to grow. The metabolites of naphthalene biodegradation found in this study were similarly reported [17], [25].

Conclusion

The deployment of bacterium with competence to degrade and utilize naphthalene as sole source of carbon and energy provides an environmentally friendly approach for dealing with pollution and toxicity caused by hazardous chemicals. Naphthalene degrading bacteria was isolated from contaminated soil and identified as *Proteus vulgaris* strain BTE_BCH. The isolate grew optimally at 35 ºC, pH 7.5, substrate concentration between 400 – 600 mg/L, with degradation efficiency of 97.2%, within 120 h when 200 µL was used as inoculum. The isolate was found to tolerate 2 ppm solution of metal ions. The metabolites obtained following GC-MS analysis were classified to be less harmful to humans and causes less pollution to the environment than the parent material. Thus, this isolate could be a suitable candidate for future bioremediation of naphthalene polluted environment.

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Disclosure statement: *Conflict of Interest:* The authors declare that there are no conflicts of interest. *Compliance with Ethical Standards:* This article does not contain any studies involving human or animal subjects.

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