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BIODEGRADATION OF ANTHRACENE AND PHENANTHRENE BY *PSEUDOMONAS STUTZERI* (BUK_BTEG1) ISOLATED FROM PETROCHEMICAL CONTAMINATED SOIL

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Abstract: The United States Environmental Protection Agency (USEPA) has identified 16 substances as priority polycyclic aromatic hydrocarbons (PAHs) that are harmful to humans, including anthracene and phenanthrene. These substances are pervasive pollutants introduced into the environment through anthropogenic and natural processes, causing ecological concerns and necessitating the quest for new strains capable of biodegrading these toxins. A novel strain of the genus *Pseudomonas* was isolated and molecularly identified based on partial 16S rRNA and phylogenetic analysis as *Pseudomonas stutzeri* strain BUK_BTEG1 from petrochemical contaminated soil. One factor at a time (OFAT) in Bushnell-Haas (BH) media was used to optimize the strain's biodegradation conditions. The isolate could grow up to 600 mgL^{-1} and 400 mgL^{-1} of anthracene and phenanthrene as the sole carbon source at an optimum pH of 7.0 and 7.5 respectively, inoculum concentration of 4% (v/v), and temperature of 35°C during 72 hours of incubation. The strain could degrade phenanthrene and anthracene to a maximum of 99 and 72 percent, respectively, under ideal conditions. The breakdown products' GC-MS analysis revealed the existence of the pathway's main metabolites, catechol, salicylic acid, and derivatives of phthalic acid. The strain exhibits promising potential for use in the bio-cleansing of environments contaminated by PAHs.metabolites.

Keywords: polycyclic aromatic hydrocarbons (PAHs), biodegradation, anthracene, phenanthrene, bioremediation

1. Introduction

Over time, the understanding of the potential negative effects of pollutants on the environment and public health has grown on a global scale (Ghosal et al., 2016). Anthracene is one of the hazardous chemicals, such as polycyclic aromatic hydrocarbons (PAHs), that are deposited and accumulated in soils due to ongoing pollution with crude oil and its derivatives (Gupte et al., 2016). Organic molecules known as PAHs are made up of two or more fused aromatic rings. The burning of organic materials produces very few of these chemicals. However, since the start of the industrial revolution powered by fossil fuels, they have multiplied in number due to careless human behavior (Chowdhury et al., 2017). Petroleum contamination occurs as a result of above- and below-ground storage tank leaks,

petroleum product spills during transportation, abandoned manufacturing sites for gasoline, other unintentional discharges, and ongoing industrial activities (Nzilaet al., 2018). Petroleum is a source of toxins that can harm the health of plants, animals, and people because it contains risky chemicals including anthracene and phenanthrene, among other PAHs. Exposure to PAHs has been linked to substantial toxicological hazards and has been shown to be genotoxic, mutagenic, and carcinogenic in nature (Fritt-Rasmussen et al., 2015; White et al., 2016; Ibrahimet al., 2018). The US Environmental Protection Agency regarded sixteen PAHs as toxic to humans. Anthracene and its iso-forms have been classified among those sixteen toxic priority PAHs (Zelinkova and Wenzl, 2015). The risks associated with PAH exposure highlight the significance of a comprehensive remediation approach for a PAH-polluted environment (Haritash and Kaushik, 2009).

Bioremediation has grown in popularity in recent decades as a cost-effective, viable, and safe method of cleaning up contaminated places. It makes use of microorganisms, which can feed on dangerous toxins such as PAHs and create innocuous molecules as a result (Ghosal et al., 2016; Oaikhenaet al., 2019; Rabiu and Gimba, 2021). From the beginning of the last decade to date, a number of bacteria strains have been identified as "PAH Degraders" including the common genera of *Pseudomonas, Proteus, Rhodococcus, Stenotrophomonas*, *Bacillus, Alcaligenes,* and *Mycobacterium* (Singh and Tiwary 2016; Ibrahim et al., 2018; Salamat et al., 2018; Suzuki and Takizawa, 2019; Elufisanet al., 2020; Shehu et al., 2021).

Although two basic multistep mechanisms for bacteria to break down PAHs have been reported, depending on whether the process requires oxygen (aerobic) or does not require oxygen (anaerobic) (Ghosal et al., 2016; Li et al., 2021). However, given favourable conditions, the aerobic degradation pathway has been found to be the relevant route in surface layer soils, which contains oxygen and is dependent on gene products, typically via the activity of enzymes such as oxygenases, peroxidases, and hydratases (Ghosal et al., 2016; Gupte et al., 2016)

The persistent nature of these pollutants in the environments coupled with the health problems posed by PAHs and their metabolites, it is, however, important to search for native PAH degrading strains from a microbial community in the natural environment capable of bio-cleaning the environment. The present study focused on the isolation, identification, and characterization of anthracene and phenanthrene degrading bacterium from petrochemical-contaminated soil.

2. Materials and methods

Sample collection

A soil sample was taken from Kwakwachi Mechanic village located in DawakinTofa LGA of Kano State with GPS coordinate N 12°1'18'' E8°31'28''. The sample was taken from the soil's surface to a depth of 15 cm, evenly mixed, and carefully placed in a sterile polyethylene bag stored at room temperature before being transferred to the laboratory for bacteria isolation.

Enrichment and isolation of PAH-degrading bacteria

Enrichment and isolation of anthracene and phenanthrene degrading strain BUK_BTEG1 was carried out using a modified method of Patel et al. (2018), the strain was enriched in Bushnell-Haas (BH) liquid medium containing 1000mgL^{-1} anthracene and phenanthrene (as the sole carbonsource) and

incubated aerobically at 37°C. The medium is made up of 1gNH_4NO_3, 0.02 g $CaCl_2$, 0.2 g $MgSO_4$, 1 g KH_2PO_4, 1 g K_2HPO_4, and 0.05 g of $FeCl_3$ in 100 mL of distilled water. In the instance of Bushnell Hass Agar, 15 g of agar-agar was added and autoclaved. To begin the enrichment, 10 g of sediment was suspended in a 250-mL Erlenmeyer flask with 100 mL BH broth containing 500 mgL^{-1} of each particular PAH as the only carbon and energy source. For 7 days, the strainswere cultured at 37°C in an incubator shaker set to 120 rpm. This procedure was performed several times. Following four successive cultivations, the bacteria were inoculated on BH agar supplemented with 500mgL^{-1} anthracene and phenanthrene as the only carbon and energy source separately using the conventional surface spray-plate technique and incubated at 37°C for 72 hours to examine bacterium growth. The isolate formed was kept for identification and characterization.

Extraction of genomic DNA and PCR amplification

A loopful of the isolate was inoculated in 5 mL Laurie-Bertani (LB) medium and incubated at 37°C for 24 h. The bacteria suspension formed was centrifuged at 10,000 for 5 min, then the DNA was extracted following the protocol described by Neerajaet al. (2013) and Ya'uet al. (2020). The PCR for the amplification of the 1.5 kb of the partial segment of the 16S rRNA reaction was carried out using KAPATaq DNA polymerase. The total reaction volume was 25 µL. The reaction mixture comprises 2 µL each of the genomic DNA, 2.5 µL of 10 TaqA Buffer, ~0.4 M (0.85 µL) of each of forward 5-*TGGAGAGTTTGATCCTGGCTCAG*-'3 and reverse primer 5'-*TACCGCGGCTGCTGGCAC* -3' from Sigma-Aldrich, United Kingdom, were used (Kumar et al., 2018). 1.5 µL of $MgCl_2$ (1.25mM), 0.2 µL of dNTP (0.25 mM) and 0.2 µL of Taq DNA polymerase made up to 25 µL with ddH_2O. The following conditions were used for the PCR amplification: initial denaturation of 5 min at 95°C, 30s at 60°C of primer annealing and 1 min at 72°C extensions then followed by 10 min of final extension at 72°C (Kumar et al., 2018).The size of the DNA fragment was compared with the Hyper Ladder-2K marker on a 1.5% agarose gel (CSL-AG500, Cleaver Scientific Ltd) and observed using Ingenius' Syngene Gel Documentation System after the last elongation cycle (IG31459). The presence of a product of the predicted size was seen as a favorable outcome.

Sequencing and phylogenetic analysis

The gel extraction kit QIA fast Qiagen was used to extract the 16S rRNA PCR product from the gel (Promega, USA). DNA sequencing was performed according to the manufacturer's instructions using the ABI Prism Big DyeTM 3730/3730XL Terminator Cycle Sequencing Ready Reaction Kit. The sequence was then searched in the nucleotide databases of the National Center for Biotechnology Information (NCBI) to identify the organism. The NCBI/GenBank 16S rRNA gene sequences of type strains of *Pseudomonas* species were obtained, and sequence alignments were performed using the software ClustalW, followed by the building of a neighbor-joining phylogenetic tree. Molecular Evolutionary Genetics Analysis (MEGA 11.0) for Windows was used to compute the evolutionary connection using the Maximum Composite Likelihood technique (Tamura et al., 2021). The sequence was submitted to the NCBI GenBank with the entry number OM039162.

Characterization of anthracene and phenanthrene degradation

Anthracene and phenanthrene degradation by the strain was studied using the One-Factor-at-a time (OFAT) approach by optimizing the growth conditions. The parameters optimized were nitrogen source and concentration, pH, initial substrate (Anth and Phen) concentration, and temperature. In 100 mL BH medium, each parameter was tested progressively while keeping the previously optimized values in mind in 100 mL BH media on an orbital shaker (150 rpm) at 37°C (the temperature was optimized separately) for a period of 120h. The incubation time was examined from 24 h – 120 h. Ammonium chloride, ammonium nitrate, ammonium sulfate, and sodium nitrate were the nitrogen sources used. The best was ammonium nitrate, which was then evaluated at concentrations ranging from 200 mgL^{-1}-1400 mgL^{-1} medium without the addition of any nitrogen source as a control. The influence of initial pH was examined across a range of 5.5 to 8.5. The effect of biomass size was also examined across the range of 1 to 10%. The effect of initial substrate concentrations ranging from 100 mgL^{-1} to 1000 mgL^{-1} were used. Similarly, the influence of temperature was also examined across the range of 25°C to 50°C. The strain's capacity to utilize and breakdown anthracene and phenanthrene in BH media were evaluated using a UV-VIS spectrophotometer (Spectrum-Lab 7525) to measure the rise in turbidity of the BH broth at 24-hour intervals at OD 600 nm.

Quantification of anthracene and phenanthrene degradation

Following the incubation period (72 h), the residual amount of anthracene and phenanthrene in each triplicate flask was extracted with 20 mL of ethyl acetate. The extraction yielded two layers and the upper organic layer was removed and measured using a spectrophotometer by the optical density (OD) at respective λmax (anthracene 380 nm and phenanthrene 280 nm) and the formula below (1) (Rabani et al., 2020) was used to compute the percentage degradation:

$$\% \text{ Degradation Efficiency} = \left(\frac{C_0 - C_f}{C_0}\right) \times 100 \qquad (1)$$

C_0= Initial concentration in control
C_f= final concentration in test

Biodegradation experiment

Isolate grown on nutrient agar plates was inoculated and grown in LB broth for 24 hours before being extracted as pellets after 10 min of centrifugation at 4000 rpm. The pellets were standardized BH medium to make a concentrated bacterial solution (1.00 ± 0.02) with a spectrophotometer. Degradation of each PAH by the bacteria strain was assessed in autoclaved 250 mL Erlenmeyer flask. Anthracene and phenanthrene were dissolved in acetone at 600 mgL^{-1} and 400 mgL^{-1} concentrations, respectively. 1 mL of the solution was pipetted into a sterile empty flask, made up to 99 mL with BH liquid, and was left in a shaker overnight to get rid of the acetone solvent. Then, all flasks (anthracene and phenanthrene) except for control flasks received a 4 % (v/v) inoculum solution of the isolate, and they were incubated for 72 hours at 35 °C in an incubator shaker at 150 rpm. After the incubation period, the medium was passed through UV light to stop the microbial activities and to release the cytoplasmic content, ethyl acetate was used as a solvent to extract the residual PAH (anthracene and phenanthrene) and their degraded products residue from each medium. 20 mL of ethyl acetate was added to the medium to extract the

remaining PAH. The mixture was then sonicated for 10 minutes before being separated using a separating funnel. The medium was separated into two layers, with the upper layer containing the remaining PAH and its breakdown product. The top layer's PAH was collected. Allowing the extract to dry, the volume of each extract was increased to 100 mL by adding more ethyl acetate. The residue was stored in a refrigerator at 40°C before being transferred to GC vials for analysis. Control samples were those that had not been inoculated.

Gas Chromatography-Mass Spectrometry (GC-MS) analysis

Agilent Gas Chromatography (GC 7890B, MSD 5977A, Agilent Tech) with a DB 35- MS Capillary Standard Non-polar column (30 m×0.25 mm×0.25 μM) film thickness was used to measure PAH degradation. The organic phase was examined by GC-MS using 1 microliter of sample. The GC-MS analysis was performed using a gas chromatograph outfitted with a split-split less injector (split ratios of 50:1). The carrier gas had a steady flow of 1 mL/min and was helium. The oven was first set at 40°C for five minutes. The injection, transfer, and ionization source temperatures were all 270°C at 37.1 Kpa. Over the course of 6.5 to 85 minutes, the mass spectrometer was run in full scan mode in electron impact (EI) mode at 70 electron volts (EV) from 85 to 450 m/z. Temperatures for the injector and detector were 270° C and 280°C, respectively. To calculate the percentage degradation efficiency of anthracene and phenanthrene by strain BUK_BTEG1 from the chromatogram generated, equation (2) below was used (Mohan et al., 2019). On the other hand, to confirm and identify the metabolites of various PAHs (anthracene and phenanthrene) degradation, the NIST GC–MS library program search was used to relate the m/z fragmentation pattern of the detected metabolite to the ones from the reference Database (NIST, 2017).

$$\% \text{ Degradation efficiency} = = \left[\frac{\text{Standard AUC} - \text{Sample AUC}}{\text{Standard AUC}}\right] \times 100 \quad (2)$$

AUC = Area under the curve/peak area

3. Results and discussions

In the current study, Bushnell-Haas (BH) supplemented with either anthracene or phenanthrene as the only sole source of carbon and energy was used for isolation and screening for a potential PAH-degrading bacteria strain obtained from petrochemical-contaminated soil samples. The biodegradation potential of the strain was indicated by the formation of turbidity as an index of biomass growth in the BH enrichment medium.

For bacterial identification, it has been discovered that 16S rRNA gene sequencing maintains a respectable level of accuracy and dependability (Hong and Farrence, 2015). In order to identify the isolate, a small segment of the 16S rRNA gene was amplified using PCR. When the amplified product for the isolate was run on 1.5 percent agarose gel electrophoresis, it revealed around 1500 bp (**Fig. 1A**). Depending on the species, segment amplified, or kind of primers used, other studies have previously reported comparable ranges of 16S rRNA gene amplification products for the genus Pseudomonas between the ranges of 1200 and 1500 bp (Görtler and Stanisich, 1996; Widada et al., 2002; Godini et al., 2019). The gene amplicon was successfully sequenced and blasted. The isolate was identified as a member of the genus Pseudomonas according to blast results in the NCBI GenBank revealing that the isolate showed high similarities to the first forty representatives that were of genus

Pseudomonas, revealing the highest similarity of 99.6% to Pseudomonas stutzeri strain AKVG5, followed by Pseudomonas stutzeri strain FN9 98.8%.The sequence was deposited underthe accession number OM039162 into the NCBI GenBank database. The 16S rRNA gene sequence underwent phylogenetic analysis using the neighbor-joining tree technique (**Fig. 1B**).

The phylogeny and evolutionary analysis showed parent clads with Pseudomonas stutzeri strain VKMB-97 5and sub-clads of Pseudomonas stutzeristrain NERC 14165, Pseudomonas stutzeri strain DM51990, Pseudomonas stutzeristrain P2and BUK-BTEG1 with a bootstrap valueof 95% for the parent, indicating a closer relatedness of these organisms (**Fig. 1B**).

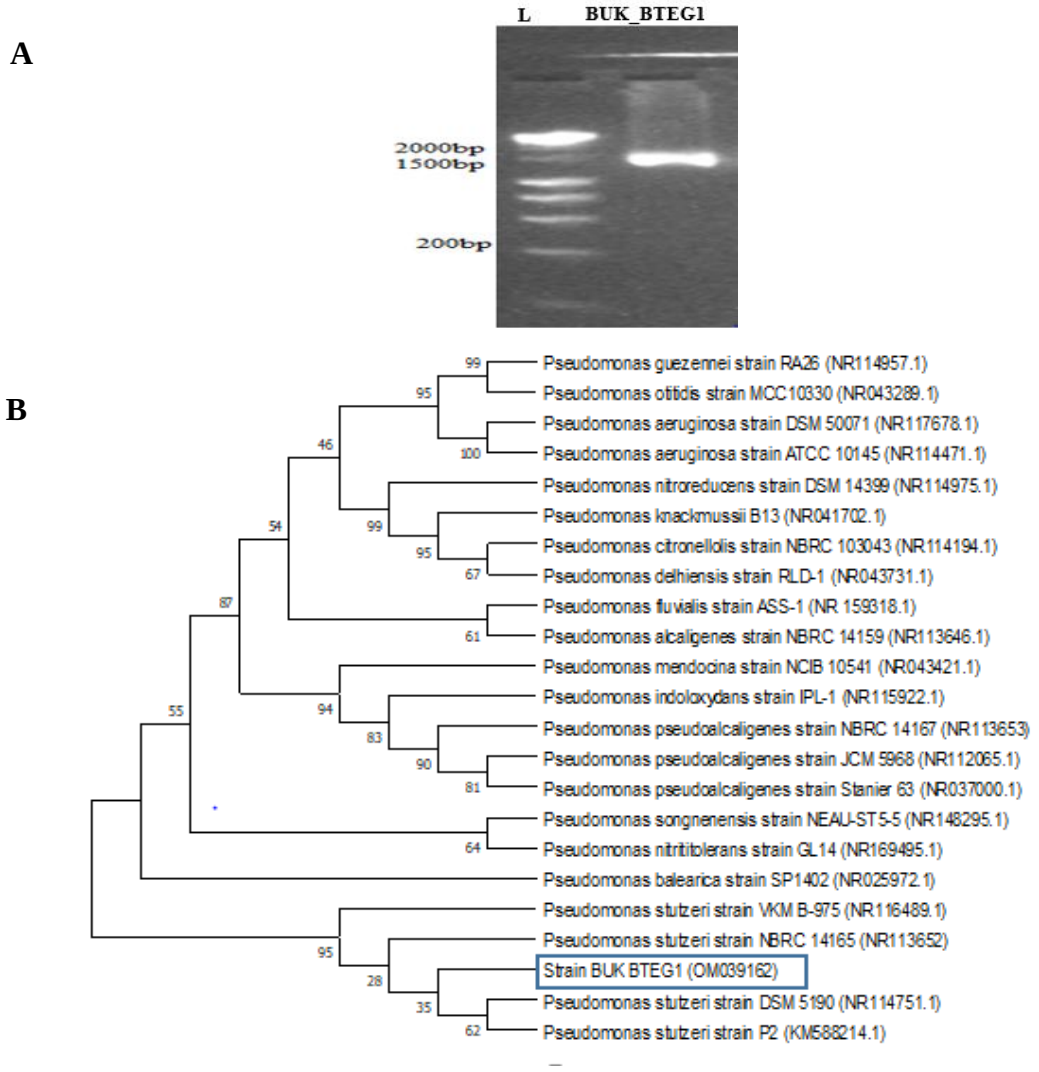

Fig. 1. Visualization of the 16S rRNA PCR amplification product on the BUK BTEG1 strain's Agarose gel electrophoresis. L stands for Bioline's Hyper Ladder-2K marking (Lot Number H4-q111B) (**A**); Phylogenetic and evolutionary relationships of taxa of strain BUK-BTEG1. The rectangle denoted the strain's evolutionary position. The Neighbor-Joining approach was used to infer the evolutionary history. The accession numbers are included beside the species names (**B**)

Therefore, strain BUK-BTEG1 was tentatively designated as *Pseudomonas stutzeri* strain BUK-BTEG1. Several studies for anthracene and phenanthrene-degrading bacteria belonging to the genera Pseudomonas were reported (Li et al., 2021; Singh and Tiwary, 2017; Nwinyiet al., 2016).

Bioremediation is often limited by environmental, physical, and chemical factors (Naik and Duraphe, 2012). The most important factors that can be detrimental to bacterial growth and ability to digest PAHs effectively and efficiently include nitrogen source, pH, substrate concentration, bacteria inoculum size, and temperature. Controlling these factors is important for biodegradation by microbial strain (Fareezet al., 2021).

The availability of suitable nitrogen sources, in addition to the carbon source, is one of the most crucial factors in the biodegradation processes (Ibrahim et al., 2020). Nitrogen-containing compounds are being integrated into biodegradation byproducts as well as enzymes and cofactors in the metabolic pathways involved in PAH biodegradation. Different nitrogen sources may hinder or stimulate bacterial growth and degradation.Any microorganism needs nitrogen for growth and metabolism, as well as for the production of RNA, DNA, and proteins. The strain BUK BTEG1 showed maximal growth and PAH consumption when ammonium sulphate, ammonium chloride, or sodium nitrate was utilized, with the highest performance seen when utilizing ammonium nitrate (out of the four organic and inorganic nitrogen sources evaluated in this study) (**Fig. 2A**)

The findings of this study on the impacts of various nitrogen sources are consistent with those of Merike et al. (2017) and Mujahid et al. (2015).The outcome, however, defies the claims made by Dudhagara et al. (2018), Sachaniya et al. (2010), and Al-Dossary et al. (2021) that sodium nitrate was the optimum nitrogen source for degradation.

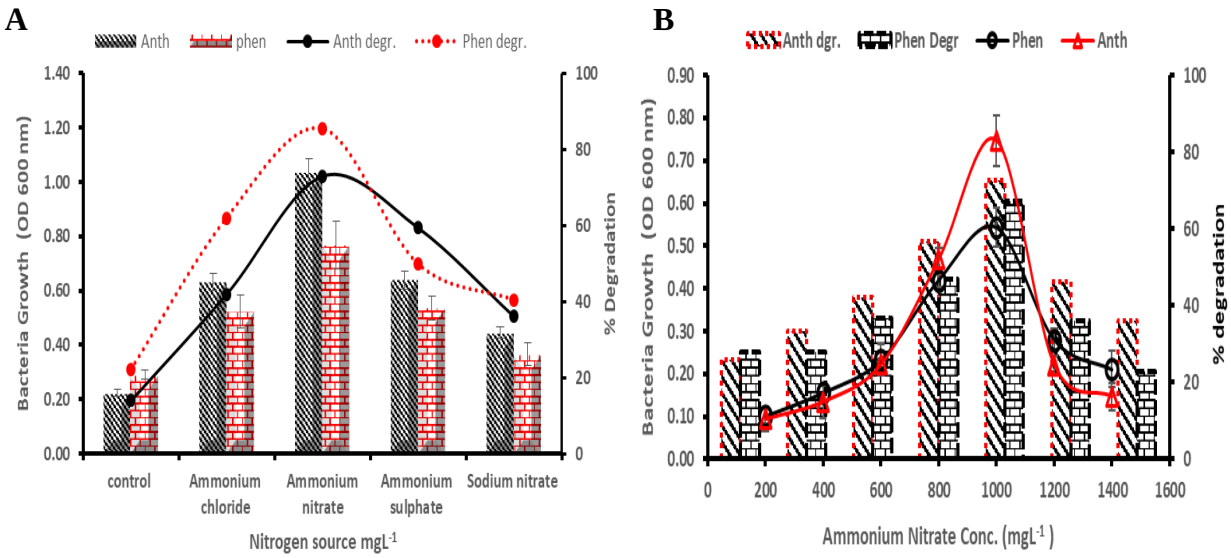

Fig. 2. The effect of nitrogen sources (**A**) and ammonium nitrate concentration (**B**) on bacterial growth and degradation of various PAH (anthracene and phenanthrene) by strain BUK_BTEG1 Data represent mean ± standard deviation of triplicate determination.

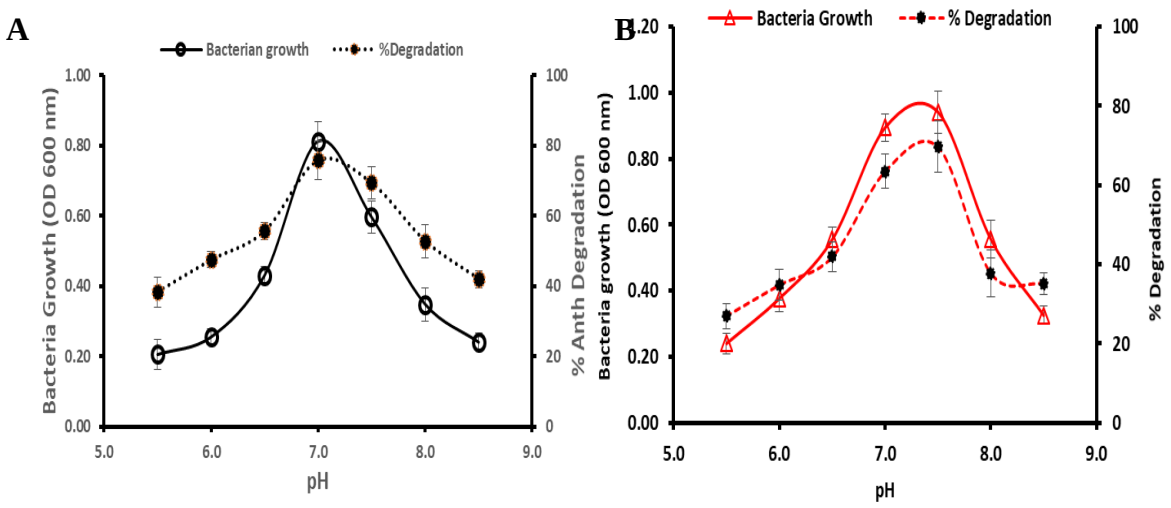

Fig. 3. The effect of initial pH on growth of strain BUK_BTEG1 and anthracene (**A**) and phenanthrene (**B**) degradation. Data represent the mean ± standard deviation of triplicate determinations.

It is critical to have access to a suitable nitrogen supply, but the concentration is equally necessary (Fareezet al., 2021). Investigation into the effects of various ammonium nitrate concentrations on the strain growth and the degradation of various PAHs revealed that 1000 mgL^{-1} was the ideal concentration (**Fig. 2B**). The rate of PAH breakdown slows down when the concentration is below the optimum. This might be because there isn't enough ammonium nitrate available to support the bacterium's rate of replication, or it might be because the media's increased pH is a result of the presence of ammonia. The atmosphere becomes more alkaline when there is too much ammonia, which slows down the degradation rate. Similarly, the results agree with Singh and Tiwary (2017), who reported optimum degradation with 1000 mgL^{-1} ammonium nitrate, but differ from Amani et al. (2020) and Fareez et al. (2021), who described 3000 mgL^{-1} ammonium nitrate as the optimum for degradation by bacteria consortium and Bacillus species.

During the microbial breakdown of PAHs as a solitary source of carbon and energy, the pH of the surrounding environment plays an important role in the process (Abatenhet al., 2017). Most microorganisms thrive at pH levels close to neutral. Microbial tolerance to pH stress is frequently aided by the ability of the microbes to physiologically alter their cell membrane to aid intracellular pH control. At varied initial pH values ranging from pH 5.5 to pH 8.5, the effects of pH on the bacterium growth and various PAH degradation were examined., and an optimum pH of 7.0 was identified for anthracene **(Fig. 3B**) while pH 7.5 was identified optimum for degradation of phenanthrene (**Fig. 3A)**. In the current investigation, microbial growth and PAH breakdown were both at their peak between pH 7 and 7.5, with performance rapidly declining at higher alkaline pH values. pH fluctuations in a shake flask setup are mostly caused by the buildup of metabolic wastes, which may be compensated for by using appropriate buffer systems. The inclusion of phosphorus-containing chemicals in the experimental buffer solution may have provided nutritional support to the microbial cells, promoting bacterial growth (Fareezet al., 2021). The ideal pH for bacteria consortiums or individual strains to break down hydrocarbons is between 6.5 to 7.5,

according to other studies, which is consistent with the present findings (Al-Dossaryet al., 2021; Bibi et al., 2018; Elufisanet al., 2020; Singh and Tiwary, 2017).

Biomass dosage also played a major role in the PAH degradation process, the amount of bacteria population in the medium affects the acclimatization of the cell and the enzyme levels synthesized to facilitate cell metabolism (Koutsoumanis and Sofos, 2005). It is essential to determine the volume of the inoculum to achieve maximum PAH degradation. Over a 1–10% (v/v) range, the effect of the initial biomass population was investigated. From the result presented in (**Fig**. **4A** and **B**). Bacterial growth and breakdown were optimum for both PAHs with a biomass dosage of 4%, after which there was a decline.

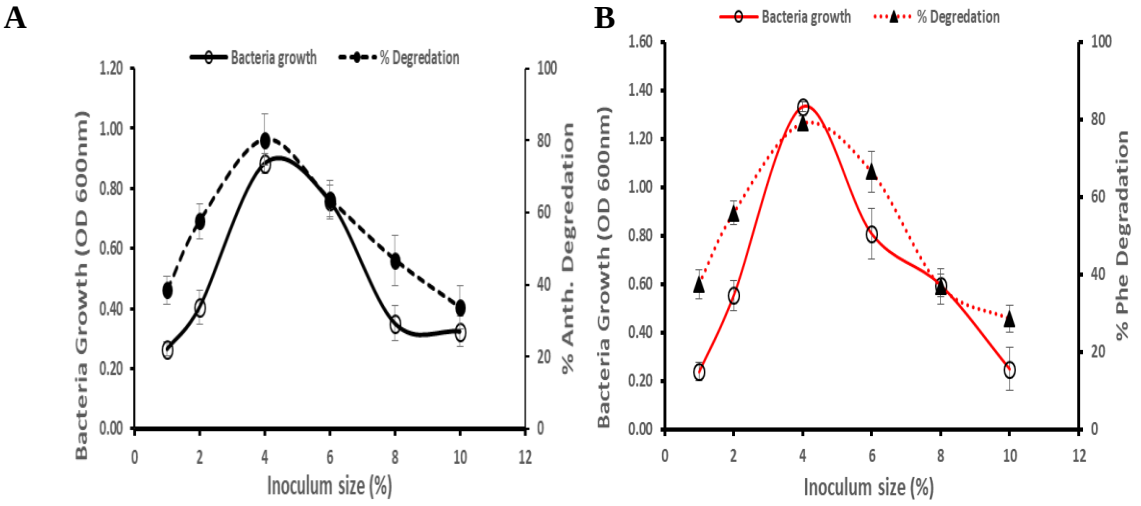

Fig. 4. The effect of inoculum size on bacterial growth and degradation of anthracene (**A**) and phenanthrene (**B**) by strain BUK_BTEG1. Data represent the mean ± standard deviation of triplicate determination.

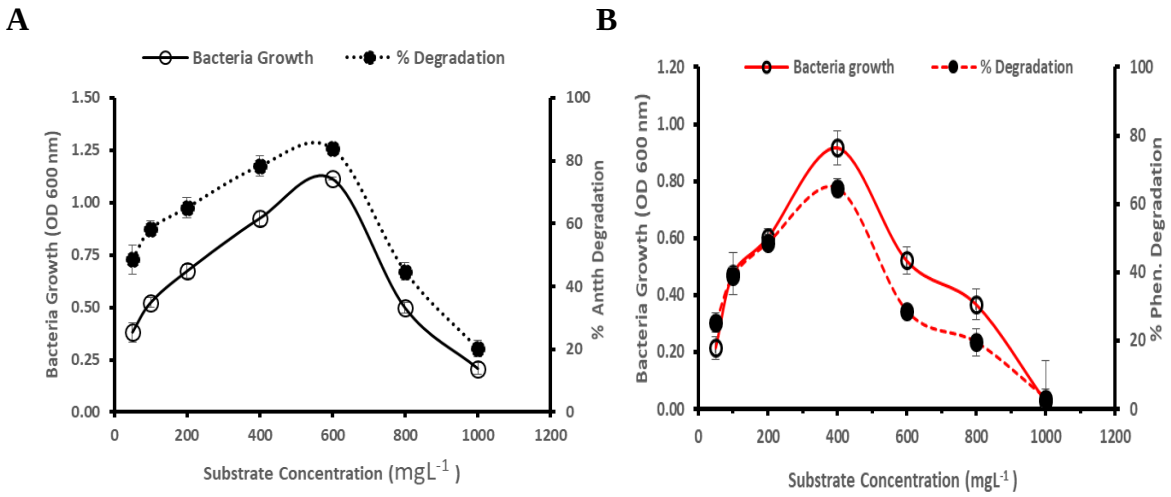

Fig. 5. The effect of anthracene (**A**) and phenanthrene (**B**) concentration on bacterial growth and degradation by strain BUK_BTEG1. Data represent mean ± standard deviation of triplicate determination

The decline in growth and degradation observed at higher inoculum beyond the optimum may be attributed to a rapid increase in cell density that competes for the limited nutrients, resulting in the death of less competent cells, or it may most likely result from nutritional depletion and a lack of total dissolved oxygen accessible to the cells (Ghosal et al., 2016).

It is known that some microbial species can withstand PAH concentrations that are high (Bibi et al., 2018). Substrate concentration plays an essential role in bacteria growth and PAH degradation. Any viable biodegradation bacterial strain should be able to tolerate and break down a PAHs high concentration. Anthracene and phenanthrene initial concentrations ranging from 100 mgL^{-1} to 1000mgL^{-1}were examined for their impact on the strain growth rate and degradation. Optimum growth and degradation by the strain were achieved at a concentration of 600 $mgL^{-1}$and 400 $mgL^{-1}$for anthracene **(Fig. 5A**) and phenanthrene **(Fig. 5B)** respectively following 72 h of incubation.

For each of the PAHs, the strain's growth and degradation decreased quickly at starting PAH concentrations above the optimal levels. The data obtained indicate that concentrations above the optimum may have been toxic to the strain, leading to a proportional decrease in growth and degradation efficiency. Similar results were reported by Patel et al., (2018) for high degradation at initial concentrations of 50 mgL^{-1}–1000 mgL^{-1}, which dramatically decreased at a concentration over 500 mgL^{-1}. Furthermore, a similar result is also reported by Bibi et al., (2018) whose optimum concentration was 1000 mgL^{-1}, also with an observed proportional decline in growth and degradation at a concentration greater than the optimum. This further suggests that a higher concentration of PAH may be toxic to the metabolic activity which in turn affects the growth and degradation efficiency of the bacteria. Several other findings also reported optimum degradation at low PAH concentration in contrast to the findings of this study (Singh and Tiwary, 2016; Praveen, 2019).

One of the most significant physical factors impacting the growth and proliferation of microorganisms is temperature. There is an ideal temperature for each enzyme-mediated breakdown process (Abatenh et al, 2017). The desolations of PAHs and bacteria's capacity to metabolize them may be impacted by an increase in temperature (Bhattacharya et al., 2011). For example, high temperatures can make PAHs more soluble and accessible while decreasing oxygen solubility, which can primarily impair the activity of aerobic bacteria (Bibi et al., 2018). As a result, earlier studies have focused on intermediate temperatures rather than high or low temperatures. Similar to the previous research, high anthracene and phenanthrene degradation were seen at 35 °C in the current investigation (**Fig. 6A** and **B**), which can be related to the optimal growth conditions for the strain. As the research organism was a mesophilic bacterium, additional investigations have discovered that the ideal temperatures for PAH breakdown were between 30 and 40°C. Bisht et al. (2015) and Nzila et al. (2018) similarly found that *Pseudomonas citronellolis* strain PHC3Z1A and a mixed culture of *Pseudomonadales*, Actinobacteria, *Caulobacterales*, *Rhizobiales*, and *Xanthomonadales* degraded best at 35°C (Ashok-Kumar et al., 2018).

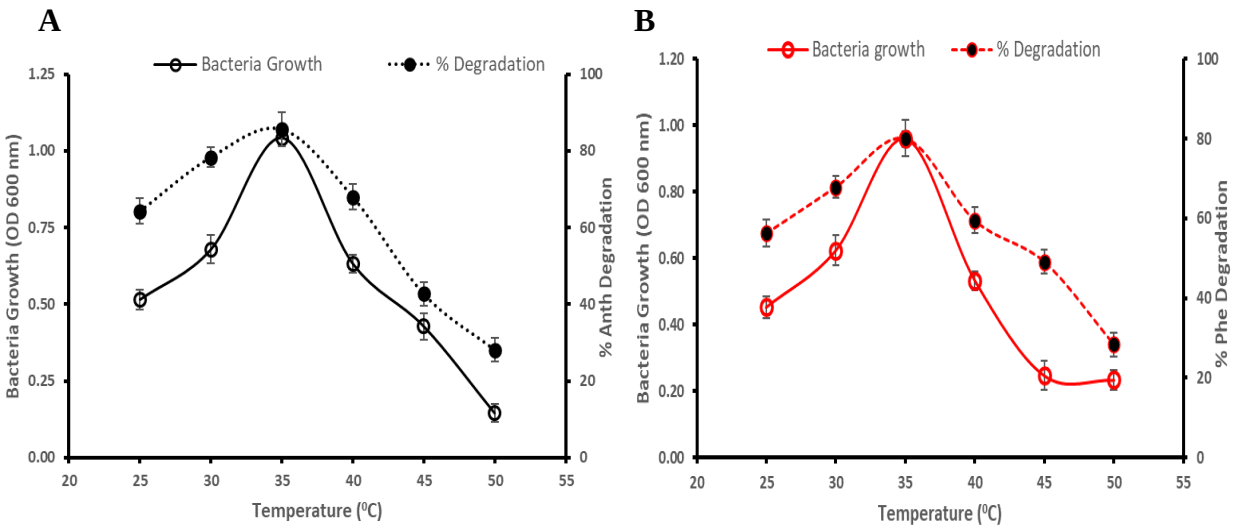

Fig. 6. The effect of temperature on the growth and degradation of anthracene (**A**) and phenanthrene (**B**) by strain BUK_BTEG1. Data represent the mean ± standard deviation of triplicate determination.

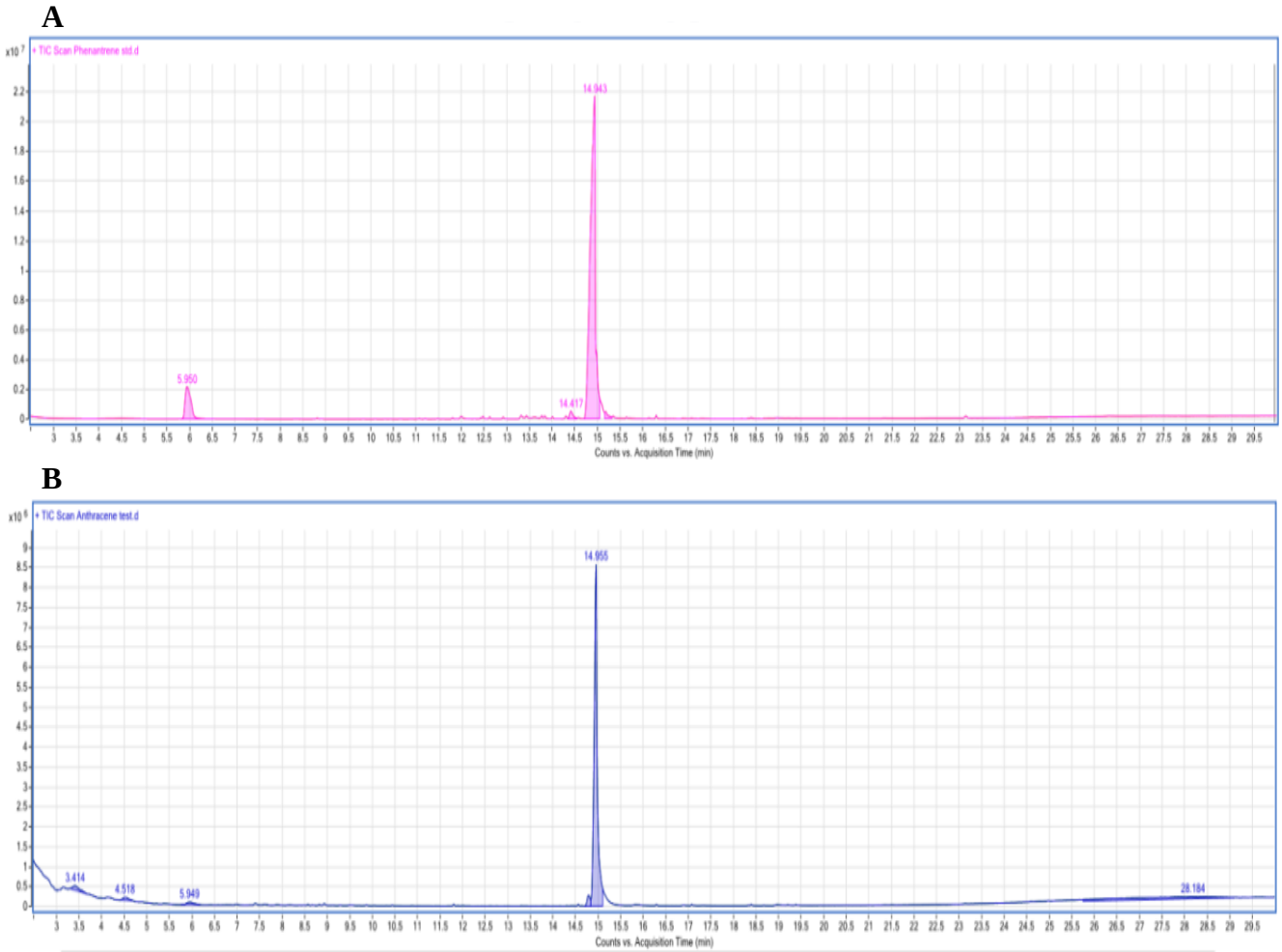

Fig.7. GC-MS chromatogram of anthracene (uncultured medium (**A**) and cultured medium (**B**))

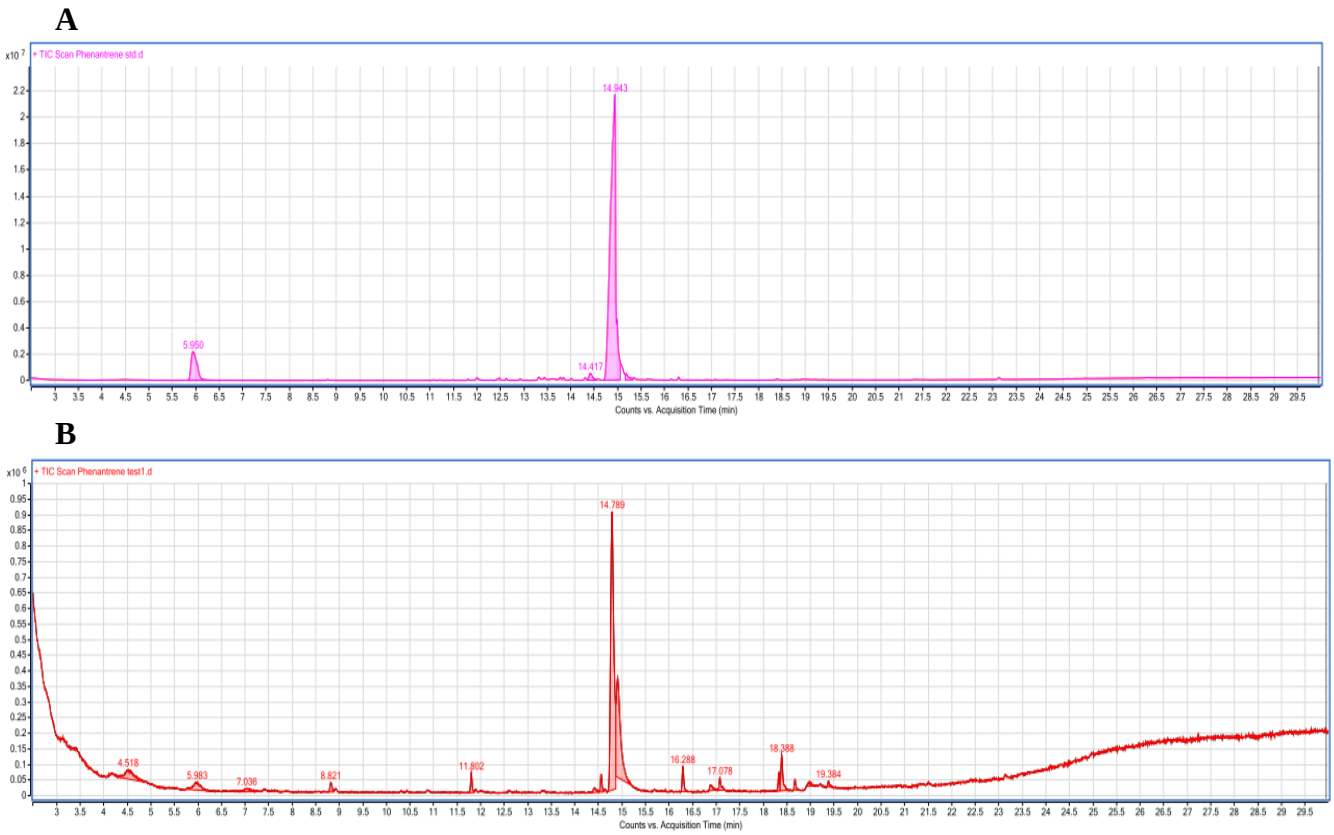

Fig. 8. GC-MS chromatogram of phenanthrene (uncultured medium (**A**) and cultured medium (**B**)) 400 mgL^{-1}at pH 7.0 ± 0.2 shaken at 120 rpm incubated at 35 ºC for 120 hours)

The biodegradation of PAHs may be significantly enhanced by optimizing environmental factors. The modification of environmental factors to promote the development and enzymatic activity of the existing bacteria is known as bio-stimulation (Bibi et al., 2018). The effects of 1000 mgL^{-1} NH_4NO_3, 35°C, and pH 7.0 on each of the PAH (600 mgL^{-1} anthracene, and 400 mgL^{-1} phenanthrene) degradation by strain BUK_BTEG1 were evaluated here using GC-MS to get the area under the curve (AUC) for both non-culture (**Fig. 7A** and **8A**) and cultured (**Fig. 7B** and **8B**) samples of the anthracene and phenanthrene respectively. Under bio-stimulation, the total percentage of anthracene and phenanthrene degradation was 72.09%, and 98.81% respectively following 72 h incubation. A substantial literature has reported high PAH degradation within that range and that corresponds to those shown by this strain (Singh and Tiwary, 2017; Salamat et al., 2018; Shehu et al., 2021).

The use of GC-MS methods is crucial for highlighting certain structural characteristics that might indicate intermediary metabolites in the biodegradation of anthracene and phenanthrene. The primary metabolites of the anthracene and phenanthrene biodegradation pathway were found in the current investigation to be derivatives of salicylic acid, catechol, benzyl-pyruvic acid, and phthalic acid (**Table 1**). These findings suggested that the PAHs degradation route by strain BUK BTEG1 followed a similar course to that described for *Pseudomonas* sp. strain Jpyr-1 and strain CECT 930 (Moscoso et al., 2015) *Pseudomonas otitidis* strain P4 (Singh and Tiwary, 2016) and *Pseudomonas stutzeri* P2 (Singh and Tiwary, 2017).

Table 1.GC-MS data for the metabolites of anthracene and phenanthrene obtained from the organic extracts of the cultures and resting cell incubations ofstrainBUK_BTEG1

Substrate	Retention Time	m/z	Suggested metabolites	Suggested structure
Anthracene	8.542	210.28	1,2-Dihydroxy anthracene	$C_{14}H_{10}O_2$
	5.949	122.12	Salicylaldehyde	$C_7H_6O_2$
	4.844	110.11	Catechol	$C_6H_6O_2$
	28.18	196.21	6,7-Benzocoumarin	$C_{13}H_8O_2$
Phenanthrene	14.532	262.41	3,4-Dihydroxyphenanthrene	$C_{14}H_{10}O_2$
	16.849	334.43	Phthalic acid derivatives	$C_{15}H_{13}NO_3$
	18.926	188.25	1-Hydroxy-2-naphthoic acid	$C_{11}H_8O_3$
	19.052	160.17	1,2-Naphthalene diol	$C_{10}H_8O_2$
	4.844	110.12	Catechol	$C_6H_6O_2$

Conclusions

Interaction *Pseudomonas* strain BUK BTEG1 was isolated from petrochemical-contaminated soil. The isolate effectively uses and degrades anthracene and phenanthrene as its only source of carbon and energy. The optimum degradation rate of anthracene and phenanthrene were achieved at ammonium nitrate, substrate concentration of 600 mgL^{-1} and 400 mgL^{-1} respectively, 35°C temperature and pH of 7.0 for anthracene while 7.5 for phenanthrene, inoculum size of 4% (v/v) and 72 hours of incubation time. Under optimal conditions, the strain could degrade around 72% and 99% of anthracene and phenanthrene at concentrations of 600 mgL^{-1} and 400 mgL^{-1}, respectively, in 72 hours. The presence of major metabolites of the pathway, namely catechol, salicylic acid, and phthalic acid derivatives, was revealed by gas chromatography-mass spectrometry analysis of anthracene and phenanthrene degradation products, indicating the representative of *Pseudomonas* as the significant PAHs degrader. As a result, the strain has excellent potential for bioremediation of polycyclic aromatic hydrocarbon polluted settings.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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