BIODEGRADATION OF POLYAROMATIC HYDROCARBON USING LOCALLY PSEUDOMONAS PUTIDA H18 ISOLATED FROM PETROLEUM CONTAMINATED LOCATIONS

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ABSTRACT

Polycyclic aromatic hydrocarbons (PAHs) are the major sources of pollution that cause dangerous effects on human and other organisms. Biodegradation of PAHs in the contaminated area is an engaging remediation technique and its accommodation depends on the optimal condition for the PAH-degrading isolates. In the current study, four bacterial strains were isolated from polluted area with petrochemical compounds with the ability for biodegradation of phenanthrene and pyrene. Only one strain has high biodegradation ratio of phenanthrene and pyrene. The optimization process for biodegradation of phenanthrene and pyrene was executed and qualified under different conditions of shaking, static, pH, temperature, inoculum sizes, salt concentration, carbon and nitrogen sources. Phylogenetic tree based on 16S rDNA genetic analysis sequence indicates that this bacterial isolate was belonged to genus Pseudomonas and identified as Pseudomonas putida (H 18). The optimal conditions for biodegradation were observed in media containing phenanthrene and pyrene as sole carbon source, yeast extract as nitrogen source, and 4% of inoculum size, at pH 8 and 35ºC under static condition for 8 days. The maximum biodegradation efficiency was reached to 92% of phenanthrene and pyrene and was confirmed by using GS-mass spectroscopy.

Keywords: Biodegradation, pseudomonas spp., Poly aromatic hydrocarbon, petroleium contamination and GS-Mass spectroscopy.
1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are aromatic compounds with two or more fused benzene rings. PAHs are hydrophobic compounds and their immutability in the ambience is principally due to their low solubility in water (Bukvic, 2002). This class of compounds is very concerned because of their mutagenic, toxic and carcinogenic vestige (Wang et al., 2017). Consequently, the US Environmental Protection Agency (US EPA) has listed 16 PAHs as precedence pollutants. Although PAHs may submit to photolysis, chemical oxidation, volatilization and microbial degradation, these major process affecting PAH resoluteness in nature (Alabresm, 2020). Bioremediation, prospective to be an economic and efficient alternative method to other remediation processes such as chemical or physical ones, has been progressed as a soil clean-up technique. However, the gaining of PAH bioremediation projects has been restricted by the inability to remove high-molecular weight of PAHs (Dangi et al., 2019). The prominence of PAHs to microbial degradation has been regarded to their hydrophobic nature. These compounds are consequently orderly bounded to soil particles, resulting in low bioavailability to microorganisms (Zhao et al., 2019). This phenomenon is predominately enhanced in aged polluted soils; likewise, many biodegradation studies have focused on isolating microorganisms and study their degradative capability to high molecular weight compounds.

Different polluting anthropogenic activities such as oil spilling, incomplete combustion of fossil fuel, ship traffic, urban runoff and industrial activities have led to significant accumulation of PAHs in marine environments principally those near industrial cities (Alegbeleye et al., 2017). In quintessence it is necessary to eliminate PAHs from environment and diminish their adverse effects. Microbial degradation is primary mechanism in removing of PAHs (Diarra and Prasad, 2021). Many researchers have elaborated the use of mangrove bacteria for bioremediation of PAHs (Imron et al., 2020). In practice, the performance of PAH biodegradation can be influenced by numerous factors, such as bacterial inoculum size, temperature, pH, nutrient, salinity, etc. which may be optimized to obtain a more efficient process (Patel et al., 2020).

Detailed knowledge of the biodegradation of pollutants in the environment is climacteric, in specific to assess the insistence of these chemicals in the environment. One of the main characteristics of pollutant hydrocarbons is that they are most predominating comprise of mixtures of different homologous compounds. It is known that the biodegradation of these complex mixtures in the environment involves various interactions between the components of the mixtures and the varied strains constituting the degradative microfloræ (Behera et al., 2018).

The present study was conducted (i) to isolate and identify of the PAHs-degrading bacteria from pouted area; and (ii) to investigate the effects of various factors such as pH, temperature and bacterial concentration on the degradation of phenanthrene and pyrene by bacterial isolate. Biodegradation ratio was confirmed by using GS-mass spectroscopy.
2. Materials and methods

2.1. Chemicals

All chemicals were of analytical grade. Phenanthrene, 2,6-dichlorophenolindophenol (DCPIP) and pyrene were purchased from Merck (Sigma-Aldrich, Egypt).

2.2. Sample collection, isolation and biodegradation screening

Nine samples were collected from Gharbia drain (Kafr El Sheikh Governorate) Damietta Governorate – Spanish Egyption Gas Company (SEGAS), and El Dakahlia Governorate – Misr gas station, Egypt. The GPS positions were 31° 34' 20.52" N, 31° 10' 49.94" E, 31°27' 27.63" N, 31°44'41.44" E and 30°50'12.05" N, 31°18'49.60" E, where all samples are represented as soil, sediment and water. All samples were transported to the laboratory and screened for isolation of the most potent biodegradable organisms of phenanthrene and pyrene. Bacterial isolation conducted on Bushnell-Has [BH] media (Roostan et al., 2015). The final pH was adjusted at 7.0. The cultural medium supplemented with 1 g/L of soil sample or 5 mL of water sample and incubated at 37°C for 8 days under static condition. The purified bacterial isolates which grown on [BH] media supplemented with different concentration of phenanthrene and pyrene separately. The ability of bacterial isolates to grow on high concentrations of phenanthrene and pyrene separately lead to select the high concentrations that used to make a mixture of phenanthrene and pyrene. The selection of the most potent bacterial isolate according to the basis of high biodegradation ratio of phenanthrene and pyrene mixture at the selected concentrations. This strain was maintained on slants of Nutrient Agar. The bacterial identification was based on standard morphological, physiological and biochemical tests as described by Bergey’s Manual of Systematic Bacteriology (2009)(Ludwig et al., 2009) and 16S rRNA gene sequence analysis.

2.3. Molecular identification of bacterial isolates

Isolate H18 identification was confirmed by 16S rRNA gene sequence. The DNA was purified using the Qiagen genomic DNA buffer set. PCR amplification was performed as described by (Mirnejad et al., 2012). The 16S rRNA sequencing was done by Beijing Liuhe Huada Genomic Company (Beijing, China). The sequences with the highest 16S rDNA partial sequence similarity were selected and compared by CLUSTAL W. Phylogenetic and molecular evolutionary analyses were conducted by MEGA 4.0 software with the Kimura 2-parameter model and the neighbor joining algorithm (Haws et al., 2011). Confidence estimates of branching order were determined by bootstrap resampling analysis with 1000 replicates.

2.4. Biodegradation of phenanthrene and pyrene mixture measurement

The most potent bacterial isolate was inoculated in a 250 mL Erlenmeyer flask containing 120 mL nutrient broth, then incubated at 37°C for 24h. Cells were harvested by centrifugation at 5000 rpm for 10 min. The cell pellets were washed with 0.85% normal saline and finally suspended in the same buffer to obtain a cell suspension with
an absorbance ($A_{620}$) of 1.0. This cell suspension was used as the inoculum as previously mentioned by (Hassan et al., 2015). PAHs biodegradation was carried out using 2,6 dichlorophenolindophenol (DCPIP) assay. Concentrations of phenanthrene (1000 mg/L) and pyrene (500 mg/L) which supplemented to Bushnell-Has [BH] media. After incubation, the biodegradation was examined with 200 mg/L DCPIP using spectrophotometer (Umar et al., 2017). The degrading efficiency was calculated using the equation of (Arun and Bhaskara, 2010):

\[
\text{Degradation} \% = \left( \frac{C_i - C_f}{C_i} \right) \times 100 \%
\]

Where $C_i$ initial concentration of phenanthrene and pyrene mixture and $C_f$ final concentration of phenanthrene and pyrene mixture.

2.5. **Optimization of biodegradation ability for the most potent selected bacterial isolate**

The effect of various culture conditions such as pH, temperatures, inoculum sizes and incubation periods, on biodegradation ratio of phenanthrene and pyrene mixture by the most potent bacterial strain was examined.

2.5.1. **Effect of different incubation period and incubation condition (static and shaking status) on biodegradation ratio of Phenanthrene and pyrene mixture**

This experiment was carried out to examine the effect of different incubation periods and conditions on biodegradation process. The potent bacterial isolate was allowed to grow on [BH] media containing phenanthrene and pyrene mixture as a sole source of carbon, allowed to grow for 3-10 days at static and shaking (150 rpm) to determine the best incubation conditions and time.

2.5.2. **Effect of different incubation temperatures, pH values and inoculum sizes on biodegradation ratio of Phenanthrene and pyrene mixture by most potent bacterial strains.**

The effect of different incubation temperatures on biodegradation ratio of phenanthrene and pyrene mixture was examined. The bacterial strain was allowed to grow on the medium as previously mentioned. The microbial isolate was incubated at different incubation temperatures of 20, 25, 30, 35, 40 and 45°C with a triplicate for each temp. Similarly, the effect of different pH values of 4, 5, 6, 7, 8, 9 and 10 bacterial inoculum sizes applied as 1%, 2%, 3%, 4% and 5% (v/v) on biodegradation ratio of Phenanthrene and pyrene mixture was tested. At the end of each incubation period, the biodegradation ratio (%) of phenanthrene and pyrene mixture was assayed after 96 hrs according to the result of incubation time.
2.5.3. Effect of different carbon and nitrogen sources on biodegradation by bacterial isolate

In order to evaluate the effect of different carbon and nitrogen sources on biodegradation ratio of phenanthrene and pyrene mixture, different carbon sources were introduced to [BH] media at 0.51 g/L concentration with equimolecular level for each sugar and supplemented by pheneatherene and pyrene mixture. The media without carbon source was used as PH+PY (containing only Phenanthrene and pyrene mixture as carbon source one inoculated and other not inoculated). The carbon sources were represented by glucose, glycerol, maltose, starch and lactose. Similarly, with the equivalent amount of nitrogen level located at 1g/L, the effect of different organic and inorganic nitrogen source such as potassium nitrate, ammonium chloride, urea, peptone, tryptophan, glutamic acid and yeast extract on biodegradation ratio of Phenanthrene and pyrene mixture were evaluated in media. In each case, all previously mentioned optimal conditions of temperature, pH, and inoculation size were taken into the consideration.

2.6. Determination of pheneatherene and pyrene mixture degradation using GC-MS spectroscopy.

Biodegradation experiments were conducted in 250 ml Erlenmeyer flasks containing 100 ml of BH broth media supplemented with Phenanthrene and pyrene mixture as a sole source for carbon and energy, at optimum condition which pH 8, inoculation size with (4%, v/v) of cell suspension of isolate then incubation period at 35°C on a under static condition were carried out for 8 days. After growth on phenanthrene and pyrene mixture, contents of the flasks were extracted with three equal volumes of ethyl acetate. The aqueous fraction after extraction was acidified with concentrated HCl to pH 2 and extracted again with three equal volumes of ethyl acetate. The residual extracts were dried over anhydrous sodium sulfate and evaporated with rotatory evaporator at 40° C to 10 mL (Kotoky et al., 2017). The samples were dried in vacuum and stored at -20°C until used.

2.6.1. Identification of degradation metabolites

GC-MS analysis of Phenanthrene and pyrene mixture degradation metabolites was performed on a Varian 3800 gas chromatograph (GC) apparatus with a Saturn 2000 ion trap mass spectrometer system (ITMS) (Varian Inc., Walnut Creek, CA), at Regional Center of Mycology and Biotechnology, Al-Azhar University, Cairo, Egypt. An aliquot of 2.0 mL of sample was injected in splitless mode with an AS8400 autosampler. The purge valve was activated 3 min after the sample injection. Helium was the carrier gas as a flow rate of 2 ml/min. The column temperature was started at 120 °C for 2 min, programmed to 280 °C at a rate of 2 °C/min, and held 280 °C for 10 min. To remove any remaining compounds, the analysis was finished with a ramp of 20 °C/min to 320 °C held for 20 minutes. Injector and transfer line temperatures were set to 270 and 280 °C, respectively. The compounds were identified on the basis of their mass spectra and using the National Institute of Standards and Technology (NIST) library. Numerous American Society for Testing and Materials (ASTM) standards that cover GC/MS are also utilized for routine determinations.
2.7. Statistical analysis

Data were statistically analyzed by SPSS v17, one-way analysis of variance (ANOVA) test was used for multiple sample comparison, when normality and homogeneity of variance were satisfied, followed by multiple comparison Tukey test.

3. Results and discussion

3.1. Isolation and identification of bacterial strain

The contaminated location resulted from petroleum industries contains different hydrocarbon aromatic compounds which are difficulty degraded. The liberation of these compounds into the environment without treatment considers a serious source for environmental pollution which threatens the aquatic and terrestrial ecosystems.

Therefore, the isolation and characterization of bacterial isolate which has the ability for biodegradation of phenantherene and pyrene mixture is potentially important for bioremediation.

At the present study, twenty bacterial isolates were tested for the ability of biodegradation of phenanthrene and pyrene mixture at the selected concentration. all bacterial isolates are listed in (Table 1), which showed that, 15 bacterial strains were Gram negative, while 5 bacterial strains were Gram positive and identified as five (5) bacterial strain were Bacillus sp., four (4) bacterial strain were Klebsiella sp., four (4) bacterial strain were Salmonella sp. and seven (7) bacterial strain were Pseudomonas sp. Lethal dose of phenanthrene was 1000 ppm, while for pyrene was 600 ppm; thus, the pheneatherene and pyrene mixture concentration of 1000-500 ppm for each compound respectively as a sublethal dose was chosen in the present study. Four bacterial isolates have the ability to degrade of phenanthrene and pyrene mixture but, the highest biodegradation ratio of phenanthrene and pyrene mixture was observed by one of the which isolated from the petroleum polluted industrial area (Figure 1). The morphological, physiological and biochemical identification of the bacterial strain was similar to Pseudomonas putida (Table 2) The morphological characters of the most potent bacterial appeared as rod shape, negative for Gram reaction, non-spore forming and the oxidase test positive. The bacterial isolate has the ability to motile and can be grow under aerobic condition. The bacterial isolate could degrade hydrogen peroxide (H₂O₂) by producing catalase enzyme. The bacterial isolate under study can ferment of glucose, sucrose and lactose. The isolate could not produce gas and grow in presence of NaCl 10%, confirmed these identification by molecular identification based on DNA extraction followed by amplification and sequence analysis of 16S rDNA gene fragments showed that the bacterial strains was identified pseudomonas putida (H 18) with similarity of 100 % (accession numbers of MW577023). The phylogenetic analysis showed the topology of pseudomonas species (Fig. 2).

Similarly, Hassan et al., (2015) isolated two strains of Klebsiella sp. isolated from the effluent samples of textile dyeing process. Also, Ponaraj et al., (2011) isolated four bacterial strains including Bacillus sp. Klebsiella sp, Salmonella sp and Pseudomonas sp from the textile dyeing effluent. Mohan et al., 2013 isolated six bacterial strains from the effluent samples of textile dyeing process. Hassan et al.,
(2015) uses this technique to identify of *Klebsiella* sp. that Biodegrade of phenanthrene.

![Biodegradation ratio % of Phenanthrene and pyrene by the bacterial isolates after eight days.](image)

**Fig. 1.** Biodegradation ratio % of Phenanthrene and pyrene by the bacterial isolates after eight days.

**Table 1.** Preliminary screening for bacterial strains growing on phenanthrene and pyrene

| No | Code  | Cell morphology | Gram reaction | Type of organism | Rate of growth |
|----|-------|-----------------|---------------|------------------|----------------|
| 1  | AL3   | bacilli         | Positive      | *Bacillus* sp.   | +              |
| 2  | AL13  | Rod shape       | Negative      | *Klebsiella* sp. | +              |
| 3  | AL18  | Short rod       | Negative      | *Salmonella* sp. | +              |
| 4  | AL24  | Rod shape       | Negative      | *Klebsiella* sp. | +              |
| 5  | AL2   | Short rod       | Negative      | *Pseudomonas* sp.| +              |
| 6  | H18   | Short rod       | Negative      | *Pseudomonas* sp. | +              |
| 7  | S1    | Short rod       | Negative      | *Salmonella* sp. | +              |
| 8  | S2    | bacilli         | Positive      | *Bacillus* sp.   | +              |
| 9  | S5    | Short rod       | Negative      | *Pseudomonas* sp. | +              |
| 10 | S11   | Short rods      | Negative      | *Pseudomonas* sp. | +              |
| 11 | S14   | bacilli         | Positive      | *Bacillus* sp.   | +              |
| 12 | S16   | Short rod       | Negative      | *Salmonella* sp. | +              |
| 13 | S18   | Rod shape       | Negative      | *Klebsiella* sp. | ++             |
| 14 | AB2   | cocci           | Positive      | *Bacillus* sp.   | ++             |
| 15 | AB1   | Short rods      | Negative      | *Pseudomonas* sp. | +              |
| 16 | AB1   | bacilli         | Positive      | *Bacillus* sp.   | +              |
| 17 | AB1   | Short rod       | Negative      | *Pseudomonas* sp. | +              |
| 18 | AB1   | Short rods      | Negative      | *Salmonella* sp. | +              |
| 19 | AB1   | Short rods      | Negative      | *Pseudomonas* sp. | +              |
| 20 | AB1   | Rod shape       | Negative      | *Klebsiella* sp. | +              |

+, moderately growth ; ++, good growth
Figure 2. Phylogenetic tree based on bacterial 16s rRNA sequences (1500 bp) for isolate (H18).

Table 2. A summary of morphological, physiological and biochemical properties of most potent *Pseudomonas putida*.

| Test                          | H18    |
|-------------------------------|--------|
| Cell shape                    | Rod    |
| Gram’s stain reaction         | -ve    |
| Endospores produced           | Non-Sporing |
| Motility                      | Motile |
| Growth in NaCl agar 10% (w/v) | -      |
| Growth at 30 °C               | +      |
| Growth above 45 °C            | -      |
| Gelatin hydrolysis            | +      |
| Nitrate reduction             | +      |
| Catalase                      | +      |
| Indol production              | -      |
| Methyl red                    | -      |
| Citrate utilization           | +      |
| Starch hydrolysis             | -      |
| Lipid hydrolysis              | +      |
| Voges Proskauer               | -      |
| H2S production                | -      |
| Hemolysis                     | Complete hemolysis |
| Oxidase                       | +      |
| Urease                        | +      |
| Fermentation of glucose       | +      |
| Fermentation of lactose       | +      |
| Fermentation of sucrose       | +      |
| Fermentation of fructose      | -      |
| Fermentation of starch        | -      |
| Acetate Utilization           | -      |

+,positive result; -, negative result.
3.2 Effect of environmental Factors on Phenanthrene and pyrene Degradation.

The bioremediation of PAH compounds in the environment is mainly carried out through microbial processes, but there are number of environmental factors affect the prospect degradation of PAH by bacteria (Ghosal et al., 2016). Therefore, in the present study, the effect of pH, temperature, inoculum size and different incubation conditions such as incubation period, static and shaking were evaluated to achieve the maximum degradation ratio of phenanthrene and pyrene in broth media with bacterial inoculation and without bacterial inoculation as a control. The optimal biodegradation ratio of phenanthrene and pyrene occurred by Pseudomonas putida (H18), after 8 days of incubation periods was (62.6%) under static condition (Fig.3), while the optimal biodegradation ratio under shaking condition was (51.1%). The biodegradation ratio of phenanthrene and pyrene increased gradually to eight days (Table 3). These results differed with Kuppusamy et al., (2016) which was reported that the ability of novel bacterial consortia to degrading of PAH after 60 days achieving the maximum degradation.

![Graph showing biodegradation ratio](image.png)

**Fig. 3.** Effect of incubation periods (days) on biodegradation of phenanthrene and pyrene by the Pseudomonas putida (H 18). Ctrl, phenanthrene and pyrene without bacterial inoculation.

Although, the biodegradation ratio of phenanthrene and pyrene was increased with the rising temperature in the medium to 35 °C but decreased its ratio at high temperature (Fig.4). Highly acidic pH and highly basic pH bigger than pH 8 (Fig.5) lead to negative impact of these conditions on the enzymatic activity which is vital for growth might be the reason for the observed reduction in biodegradation ratio (Fouda et al., 2016). Hassan et al., (2015) reported that, the biodegradation ratio of Phenanthrene increased with increasing temp. to 30 °C and at pH 7. However, there are several of successful bioremediation experiences at extreme temperature and PH (Kensa, 2011).
Fig. 4. Effect of incubation temperatures (°C) on biodegradation of phenanthrene and pyrene by *pPseudomonas putida* (H 18). Ctrl, phenanthrene and pyrene without bacterial inoculation.

In order to find out the optimum inoculum size of *Pseudomonas petuda* (H18) which needed for faster and higher degradation ratio of phenanthrene and pyrene, the degrading ability was tested at different inoculum concentrations starting from 1% to 5% (v/v) (Fig. 6). The rate of degradation increased with increase the inoculum size, reaching maximum value at 4% (v/v). maximum biodegradation ratio of Phenanthrene and pyrene reached to (82%). As the inoculum of *Pseudomonas petuda* (H18) was increased above 4%, it resulted in decreasing degradation. This agrees with Hassan *et al.*, (2015) who reported that, maximum biodegradation ratio of Phenanthrene was occurred at 2% (v/v) of inoculum size of *Klebsiella sp.* (SB_2.1). Also, Chen *et al.* (2008) reported that, inoculum size was the key factor affecting the speed of
Phenanthrene biodegradation by *Sphingomonas* sp. isolated from mangrove sediment. An *et al.*, (2020) reported that, maximum biodegradation ratio of hexaconazole by degrading strain *Sphingobacterium multivorum* was occurred by using 4g/L of inoculum size.

![Graph showing biodegradation ratio](image)

**Fig. 6.** Effect of different inoculum sizes (mL) for biodegradation of phenanthrene and pyrene by *Pseudomonas putida* (H 18). Ctrl, phenanthrene and pyrene without bacterial inoculation.

In our experimental conditions, testing different salt concentration, the results showed that *Pseudomonas petuda* (H18) have the ability to grow on different concentrations of sodium chloride up to 8 g/L and capable to biodegrade of Phenanthrene and pyrene. While at higher concentration, the test organisms do not grow and the biodegradation ratio approximately equal to control (Fig. 7). Testing different nitrogen sources showed that *Pseudomonas petuda* (H18) had the ability to utilize different organic and inorganic nitrogen sources (Fig. 8). Data analysis showed that yeast extract and potassium nitrate were the best nitrogen sources utilized by *Pseudomonas* species to increase degradation ratio of phenanthrene and pyrene. This result agrees with Mohanrasu *et al.*, (2020) thus, use potassium nitrate as nitrogen source for increasing of Poly Cyclic Aromatic Hydrocarbons (PAHs) degradation. Although glucose was the best carbon source for *Pseudomonas petuda* (H18) for higher degradation ratio of phenanthrene and pyrene, but other carbon source can be *Pseudomonas* species. At the presence of glucose, the bacterial growth was enhanced and increased for degradation ratio contrast to other carbon source, except phenanthrene and pyrene as a carbon source, degradation ratio of phenanthrene and pyrene was reached to (82%) (Fig. 9). Zhao *et al.*, (2019) uses glucose for enhancement of PAH and oil degradation. Govarthanan *et al.*, (2020) uses glucose for increasing of PAH by using *Halomonas* sp.
Fig. 7. Effect of different sodium chloride concentrations (g) for biodegradation of phenanthrene and pyrene by *Pseudomonas putida* (H 18). Ctrl, phenanthrene and pyrene without bacterial inoculation.

Fig. 8. Effect of different nitrogen sources for biodegradation of phenanthrene and pyrene by *Pseudomonas putida* (H 18). Ctrl, phenanthrene and pyrene without bacterial inoculation.

Fig. 9. Effect of different carbon sources for biodegradation of phenanthrene and pyrene by *Pseudomonas putida* (H 18). Ctrl, phenanthrene and pyrene without bacterial inoculation.
Table 3. A summary of the optimal environmental conditions for biodegradation ratio % of phenanthrene and pyrene by the most potent strain *Pseudomonas putida* (H

| Isolate code | Biodegradation % of phenanthrene and pyrene at shaking incubation time (days) |
|--------------|--------------------------------------------------------------------------------|
|              | 3                               | 4                               | 5                               | 6                               | 7                               | 8                               | 9                               | 10                              |
| Ctrl         | 2.5±0.03 1a                      | 2.5±0.058a                      | 2.2±0.031b                      | 3.2±0.043b                      | 3.4±0.016c                      | 3.9±0.055c                      | 4.0±0.035c                      | 4.3±0.011c                      |
| H18          | 5.8±0.06 1a                      | 10.2±0.092a                     | 24.1±0.016b                     | 35.5±0.019b                     | 43.8±0.016c                     | 51.3±0.02d                      | 47.5±0.012c                     | 45.4±0.013c                     |

Biodegradation % of phenanthrene and pyrene at static incubation time (days)

| Isolate code | Biodegradation % of phenanthrene and pyrene at different incubation temperatures |
|--------------|--------------------------------------------------------------------------------|
|              | Temperatures                                                                     |
|              | 20 ºC                             | 25 ºC                             | 30 ºC                             | 35 ºC                             | 40 ºC                             | 45 ºC                             |
| Ctrl         | 2.4±0.016a                        | 2.5±0.013a                        | 2.7±0.014a                        | 3.4±0.07b                         | 3.3±0.013b                        | 3.5±0.033b                        |
| H18          | 42.1±0.08b                        | 60.2±0.16d                        | 68.6±0.03d                        | 53.8±0.036c                       | 39.8±0.056b                       | 45.4±0.013c                       |

Biodegradation % of phenanthrene and pyrene at different pH values

| Isolate code | Biodegradation % of phenanthrene and pyrene at different inoculum sizes |
|--------------|------------------------------------------------------------------------|
|              | Inoculum sizes                                                         |
|              | 1                        | 2                        | 3                        | 4                        | 5                        |
| Ctrl         | 2.1±0.03 3a               | 2.3±0.014a               | 2.4±0.013a               | 3.3±0.043b               | 3.3±0.053b               |
| H18          | 75.8±0.0 76b              | 77.2±0.091b              | 80.6±0.016c              | 82.5±0.19c               | 63.3±0.017               |

Biodegradation % of phenanthrene and pyrene at different sodium chloride concentrations g/L

| Isolate code | Biodegradation % of phenanthrene and pyrene at different nitrogen sources |
|--------------|--------------------------------------------------------------------------|
|              | Nitrogen sources                                                         |
|              | KNO3                      | NH4CL2                    | Urea                      | Peptone                   | Glutamic acid             | Yeast extract             | Tryptophan                | PH+PY                      |
| Ctrl         | 2.3±0.05 3a                | 2.8±0.081a                | 2.6±0.064a                | 3.4±0.023b               | 3.4±0.063b               | 2.4±0.013a                | 3.4±0.043b                | 2.4±0.043a                |
| H18          | 73.7±0.0 76d               | 63.3±0.017b               | 47.2±0.091a               | 66.6±0.076               | 70.6±0.048               | 87.6±0.036               | 74.6±0.019c               | 47.8±0.019a               |

Biodegradation % of phenanthrene and pyrene at different carbon source

| Isolate code | Biodegradation % of phenanthrene and pyrene at different carbon source |
|--------------|------------------------------------------------------------------------|
|              | Carbon sources                                                         |
|              | Glucose                   | Maltose                   | Glycerol                  | Starch                    | Lactose                   | PH+PY                     |
| Ctrl         | 4.4±0.06 6c               | 2.6±0.013a                | 2.8±0.053a                | 3.4±0.038b               | 2.4±0.063a               | 3.1±0.038b               |
| H18          | 91.6±0.036               | 67.3±0.066               | 77.6±0.016c               | 37.5±0.046               | 80.6±0.056               | 82.3±0.046               |

Different letters between columns denote that mean values are significantly different (p≤0.05) by Tukey LSD test, means ± SE (n=3). Ctrl, control without bacterial inoculation; H 18, *pseudomonas putida*. 
3.3 The Optimum Condition of phenanthrene and pyrene Biodegradation

The rate of biodegradation is influenced by pH, temperature, inocula size, and incubation condition. Therefore, biodegradation of of phenanthrene and pyrene by *Pseudomonas petuda* (H18) was studies by providing those critical factors at the optimum level. The results showed that increasing the biodegradation ratio from 62.6% at starting experiment to reach 91.6% under the optimum condition within eight days (Table 4) and represented in (Fig. 10).

Table (4). Biodegradation ratio % of phenanthrene and pyrene by the most potent strain *pseudomonas putida* (H 18) after and before optimization

| Treatments | Before optimization | After optimization |
|------------|--------------------|--------------------|
| Control    | 3.9±0.055          | 4.4±0.066          |
| H 18       | 62.6±0.01          | 91.6±0.03          |

Means ± SE (n=3). Ctrl, control without bacterial inoculation; H 18, *pseudomonas putida*.

Fig.10. Biodegradation ratio % of phenanthrene and pyrene by the most potent strain *pseudomonas putida* (H 18) before and after optimization where, Ctrl, phenanthrene and pyrene without bacterial inoculation.

3.4. Determination of GC-MS analysis of pheneatherene and pyrene mixture biodegradation

The biodegradation ratio of phenanthrene and pyrene by bacterial strain *Pseudomonas petuda* (H18) was evaluated using GC-MS analysis to determine the biodegradable compounds and comparable with controls. Since bacteria initiate PAH degradation by the action of intracellular dioxygenases, the PAHs must be taken up by the cells before degradation can take place. Bacteria most often oxidize PAHs to cis-dihydrodiols by incorporation of both atoms of an oxygen molecule. The cis-dihydrodiols are further oxidized, first to the aromatic dihydroxy compounds (catechols) and then channeled through the ortho- or meta cleavage pathways. (*Sinha et al., 2017*). Among the many different enzymes that are involved in PAH degradation, the initial
dioxygenases that enable aerobic bacteria to attack the aromatic ring structures are key enzymes that serve as useful markers for PAH degradation activity. These enzymes are multimeric and are comprised of three components including a reductase, a ferredoxin, and an iron-sulfur protein (ISPnap) (Dhar et al., 2019). Results showed that, phenanthrene and pyrene residue at control treatment without any bacterial inoculation was appeared at R.T. 27.53 with percent area 16.13 % for phenanthrene. While, pyrene residue appeared at R.T. 32.72 with percent area 15.17% and presence of other compounds at different R.T (Fig.11), with low different percent area. The major peaks of compounds resulting from phenanthrene and pyrene biodegradation by Pseudomonas putida (H18) appeared at different R.T. of 7.01, 24.41, 28.99 and 33.54 as a result for biodegradation (Fig.12). and (Table 4). Varjani and Upasani, (2016) examined biodegradable compound of petroleum hydrocarbons occurred by Pseudomonas aeruginosa NCIM 5514 by using GC-MS analysis. Also, Masika et al., (2020) used GC-MS analysis for detect the biodegradable compounds of petroleum hydrocarbon waste using consortia of Bacillus sp.

Table (4). Suggested compounds resulted from biodegradation of phenanthrene and pyrene by Pseudomonas putida.

| Treatments         | RT    | percent | compounds                     | molecular formula | Molecular weight |
|--------------------|-------|---------|--------------------------------|-------------------|-----------------|
| Control            |       |         |                                |                   |                 |
|                    | 27.53 | 16.13   | Phenanthrene                   | C\(_{14}\)H\(_{10}\) | 178             |
|                    | 32.72 | 15.17   | Pyrene                         | C\(_{16}\)H\(_{10}\) | 202             |
| Pseudomonas putida |       |         |                                |                   |                 |
|                    | 7.01  | 1.86    | BENZENE, (CHLOROMETHYL)        | C\(_{7}\)H\(_{7}\)Cl | 126             |
|                    | 24.41 | 0.66    | Tetradecane, 2,6,10-trimethyl   | C\(_{17}\)H\(_{36}\) | 240             |
|                    | 28.99 | 0.64    | HEXADECANOIC ACID, METHYL ESTER| C\(_{17}\)H\(_{34}\)O\(_{2}\) | 270             |
|                    | 33.54 | 0.96    | 1-Propene-1,2,3-tricarboxylic acid, tributyl ester | C\(_{18}\)H\(_{30}\)O\(_{6}\) | 342             |
Fig. 11. GC-MS analysis of phenanthrene and pyrene (Control).
Fig.12. GC-MS analysis of biodegradation metabolites of phenanthrene and pyrene occurred by *Pseudomonas putida*.

4. Conclusions

At this work, *Pseudomonas putida* (H 18) was isolated from PAHs contaminated soil. The optimum conditions for biodegradation efficiency of pheneatherene and pyrene mixture by using *Pseudomonas* sp. was found in vitro with glucose, yeast extract supplementation, and 4% bacterial inoculum size, at pH=8, 35°C and after 96 hrs. under static condition. The maximum biodegradation ratio of pheneatherene and pyrene mixture was confirmed by using GS-mass spectroscopy petroleum industries. It is suggested that *pseudoomonas putida* (H 18) has applicable role in the degrading of sediment resulted from various process contain pheneatherene and pyrene mixture.

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التكسير الحيوي الهيدروكربونات العطرية متعددة الحلقات باستخدام سلالة Pseudomonas putida H18

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الملخص العربي:
التكسير البيولوجي للمبيدات العطرية متعددة الحلقات (PAHs) في المناطق الملوثة هو تقنية حيوية يعتمد عليها بشرط توافر الظروف المثلى للعزلات المستخدمة للتكسير الحيوي للمبيدات العطرية. تم عزل سلالة بكتيرية واحدة من منطقة ملوثة مربكية بتروكيميائية قادرة على التحلل الحيوي للمبيدات العطرية. تم دراسة الظروف المثلى للكائن المستخدم في التكسير الحيوي للمبيدات العطرية من حيث الظروف المختلفة من الاحترام والطversions ودرجة الحرارة وأحمق تحت مختلف وبين التتروجين.

تشير الشجرة الوترائية المستندة إلى تسلسل التحلل الجيني إلى أن كائن الاختبار هذا كان ينتمي إلى جنس Pseudomonas putida (H 18). كانت الظروف المثلى للكائن المستخدم للتحليل الحيوي في الوسط الذي يحتوي على للفينيثرين والباربرين كمصادر وحيد للتكسير، استخدام مستخلص الخميرة كمصدر للتتروجين ، وتركيز بكتيري 4% ، عند الأس الهيدروجيني = 8 و 35 درجة مئوية تحت ظروف ثابتة لمدة ثمانية أيام. تم الوصول إلى أقصى كفاءة للتحليل الحيوي بنسبة 49% من للفينيثرين والباربين وتم تأكيده هذه النسبة من التحلل البيولوجي باستخدام التحليل الطيفي للكلثة GS. على حد علمنا ، هذا هو أول تقرير يفيد بأن السلالة البكتيرية من جنس Pseudomonas. تم تسجيله بقدرية تحلل حيوى مقابل الفينيثرين والباربين وبذلك تكون قد استطاع تقليل المدة الزمنية لتكسير هذه المواد مقارنة بالدراسات السابقة.

الكلمات المفتاحية: التحلل البيولوجي ، جنس Pseudomonas ، الهيدروكربونات العطرية متعددة الحلقات ، المناطق الملوثة بالمواد البترولية والتحليل الطيفي - GS.