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

Ahmed bahgat Adly*[1], Saad EL-Din Hassan[1],Nagwa Mahmoud Sedqi[1]

[1]Department of Botany and Microbiology, Faculty of Science, Al-Azhar University, Nasr City, Cairo, Egypt.

***Corresponding author**: **E-mail:** Ahmed. Bahgat211@azhar.edu.eg.

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 pheneatherene and pyrene separately. The ability of bacterial isolates to grow on high concentrations of pheneatherene and pyrene separately lead to select the high concentrations that used to make a mixture of pheneatherene and pyrene. The selection of the most potent bacterial isolate according to the basis of high biodegradation ratio of pheneatherene 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-paremeter 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 phenanthreneand 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)**:

Degradation % = $(C_i - C_f / C_i) \times 100$ %

Where C_i initial concentration of pheneatherene and pyrene mixture and C_f final concentration of pheneatherene 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 Phenanthreneand 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 pheneatherene 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 and10 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 pheneatherene 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 ^{o}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 ^{o}C for 2 min, programmed to 280 ^{o}C at a rate of 2 ^{o}C/min, and held 280 ^{o}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 ^{o}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 threats the aquatic and terrestrial ecosystems.

Therefore, the isolation and characterization of bacterial isolate which has the ability for biodegradation of pheneatherene 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 sub-lethal 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 (H_2O_2) 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.

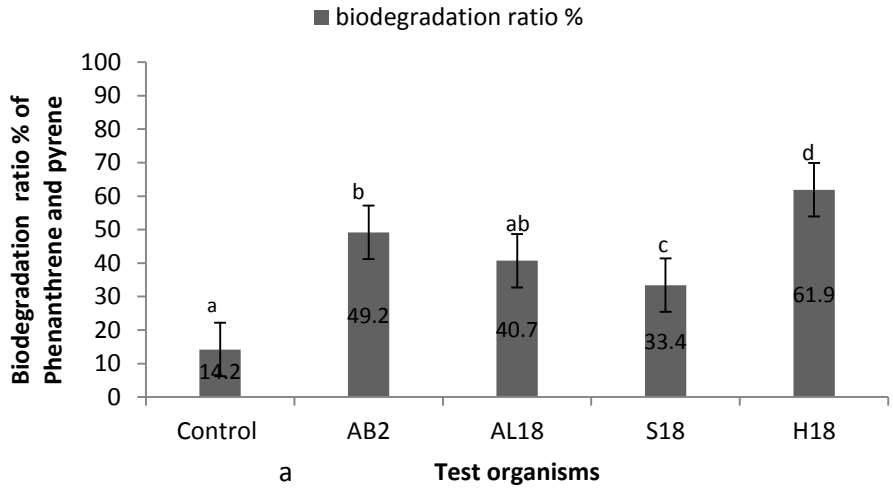


Fig. 1. Biodegradation ratio % of Phenanthrene and pyrene by the bacterial isolates after eight days.

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

No	Code	Cell morphology	Gram reactio	Type of organism	Rate of growt
1	AL	bacilli	Positive	*Bacillus* sp.	+
2	AL1	Rod shape	Negative	*Klebsiella* sp.	+
3	AL1	Short rod	Negative	*Salmonella* sp.	++
4	AL2	Short rod	Negative	*Pseudomonas* sp	+
5	AL2	Rod shape	Negative	*Klebsiella* sp.	+
6	H18	Short rod	Negative	*Pseudomonas* sp	++
7	S 1	Short rod	Negative	*Salmonella* sp.	+
8	S 2	bacilli	Positive	*Bacillus* sp.	+
9	S 5	Short rod	Negative	*Pseudomonas* sp	+
10	S 1	Short rods	Negative	*Pseudomonas* sp	+
11	S 1	bacilli	Positive	*Bacillus* sp.	+
12	S 1	Short rod	Negative	*Salmonella* sp.	+
13	S18	Rod shape	Negative	*Klebsiella* sp.	++
14	AB	cocci	Positive	*Bacillus* sp.	++
15	AB	Short rods	Negative	*Pseudomonas* sp	+
16	AB	bacilli	Positive	*Bacillus* sp.	+
17	AB	Short rod	Negative	*Pseudomonas* sp	+
18	AB 1	Short rods	Negative	*Salmonella* sp.	+
19	AB 1	Short rods	Negative	*Pseudomonas* sp	+
20	AB 1	Rod shape	Negative	*Klebsiella* sp.	+

+, moderately growth ; ++, good growth

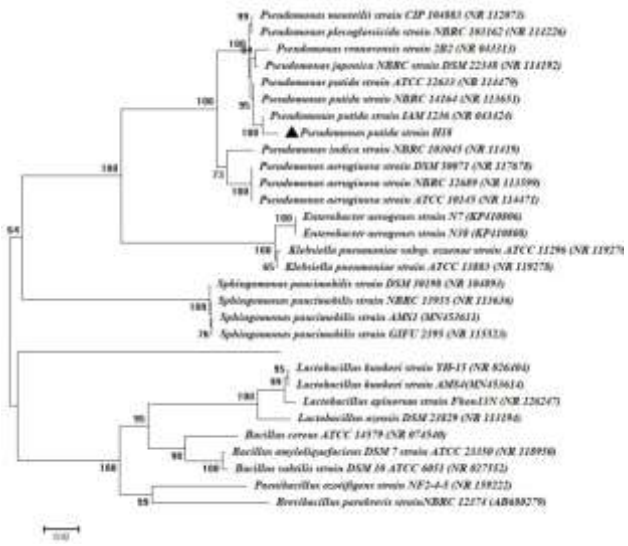


Figure 2. Phylogenetic tree based on bacterial 16s rRNA sequences (1500 bp) for isolate (H18).

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 ^{o}C	+
Growth above 45 ^{o}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 ٣) .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.

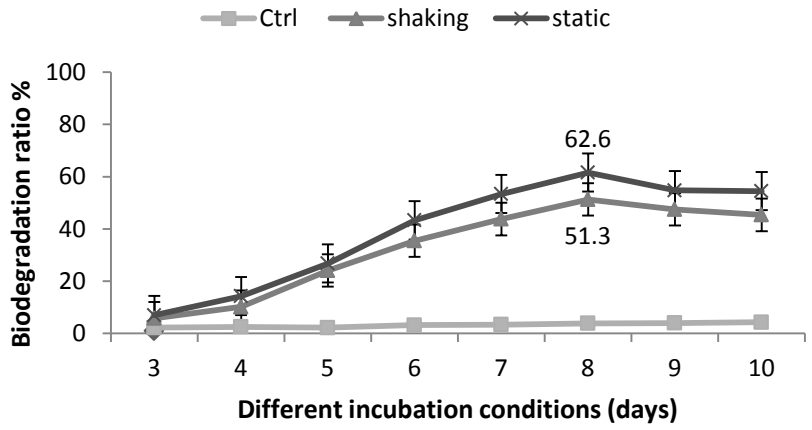


Fig. 3. Effect of incubation periods (days) on biodegradation of phenanthrene and pyrene by the *Pseudomonas putida* (H 18). Ctrl, phenanthrene and pyrene without bacterial inoculation.

Although, the biodegradation ratio of phenanthrene and pyrene was increased with the rising temperature in the medium to 35 ºC 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 ºC and at pH 7. However, there are several of successful bioremediation experiences at extreme temperature and PH (**Kensa, 2011**).

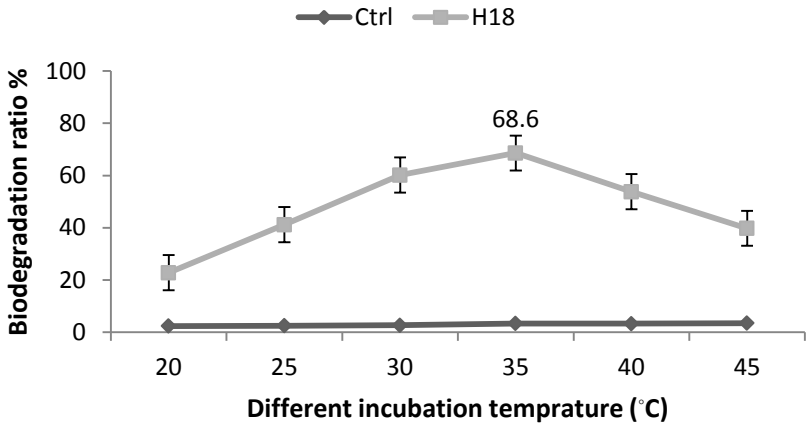


Fig.4. Effect of incubation temperatures (°C) on biodegradation of phenanthrene and pyrene by *pPseudomonas putida* (H 18). Ctrl, phenanthrene and pyrene without bacterial inoculation.

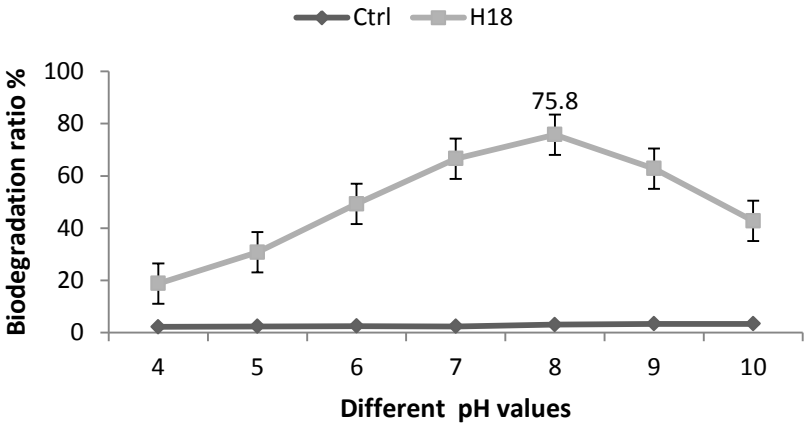


Fig. 5. Effect of pH values on biodegradation of phenanthrene and pyrene by the most potent strain *pseudomonas putida* (H 18). Ctrl, phenanthrene and pyrene without bacterial inoculation.

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.

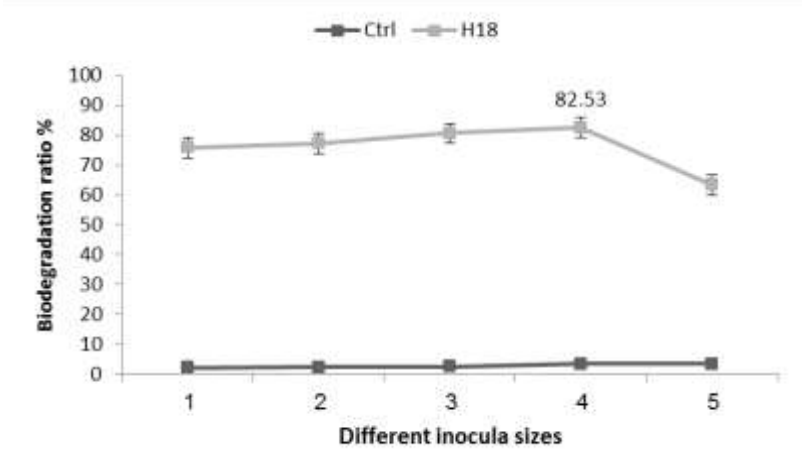


Fig. 6. Effect of different inoculum sizes (mL) for biodegradation of phenanthrene and pyrene by *Pseudomonas putida* (H 18).). Ctrl, phenanthrene and pyrene without bacterial inoculation.

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/Land 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.

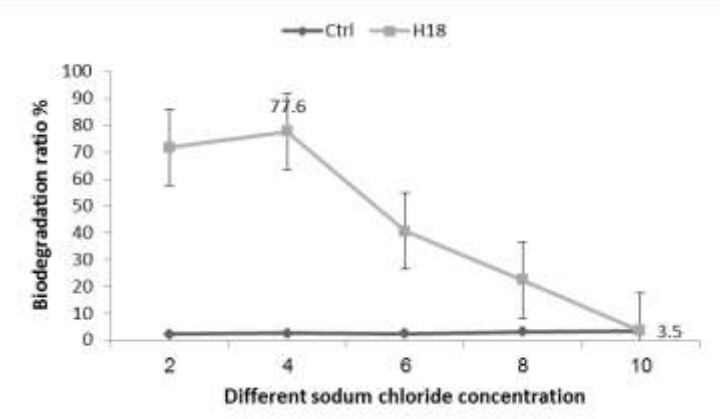


Fig.7. Effect of different sodium chloride concentrations (g) for biodegradation of phenanthrene and pyrene by *Pseudomonas putida* (H 18). Ctrl, phenanthrene and pyrene without bacterial inoculation.

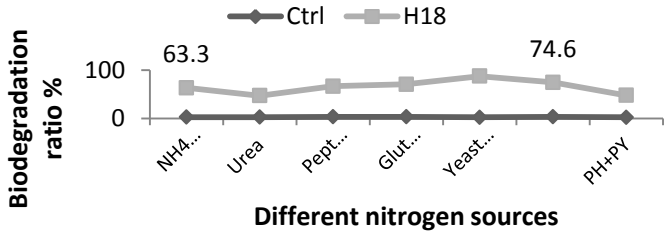


Fig.8. Effect of different nitrogen sources for biodegradation of phenanthrene and pyrene by *Pseudomonas putida* (H 18). Ctrl, phenanthrene and pyrene without bacterial inoculation.

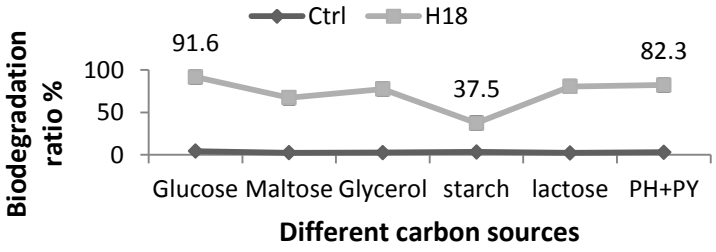


Fig.9. Effect of different carbon sources for biodegradation of phenanthrene and pyrene by *Pseudomonas putida* (H 18).Ctrl, phenanthrene and pyrene without bacterial inoculation.

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)**							
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±0.092 a	24.1±0.016b	35.5±0.019b	43.8±0.016 c	51.3±0.02d	47.5±0.012c	45.4±0.013c
	Biodegradation % of phenanthrene and pyrene at static incubation tie (days)							
H18	7.03±0.0 1a	14.3±0.04a	26.8±0.06b	43.3±0.04c	53.4±0.03d	62.6±0.01e	54.8±0.04d	54.5±0.03d
	Biodegradation % of phenanthrene and pyrene at different incubation temperatures							
Temperatu rs	**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	22.8±0.0 49a	41.2±0.08b	60.2±0.16d	68.6±0.03d	53.8±0.036 c	39.8±0.056b		
	Biodegradation % of phenanthrene and pyrene at different pH values							
PH values	**4**	**5**	**6**	**7**	**8**	**9**	**10**	
Ctrl	2.2±0.03 1a	2.4±0.056a	2.5±0.044b	2.4±0.071a	3.1±0.013b	3.4±0.045b	3.4±0.036a	
H18	18.8±0.0 61a	30.8±0.049 b	49.3±0.036c	66.6±0.03d	75.8±0.076 e	62.8±0.069	42.8±0.049c	
	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±0.091 b	80.6±0.016c	82.5±0.19c	63. 3±0.017			
	Biodegradation % of phenanthrene and pyrene at different sodium chloride concentrations g/ L							
Sodium chloride	**2**	**4**	**6**	**8**	**10**			
Ctrl	2.3±0.04 1a	2.7±0.052a	2.5±0.046a	3.1±0.061b	3.3±0.041b			
H18	71.8±0.0 42d	77.6±0.031 e	40.6±0.062c	22.5±0.042b	3.5±0.051a			
	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	82.3±0.0 57d	63.3±0.017 b	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							
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 ($p \leq 0.05$) 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 befor 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* .

.

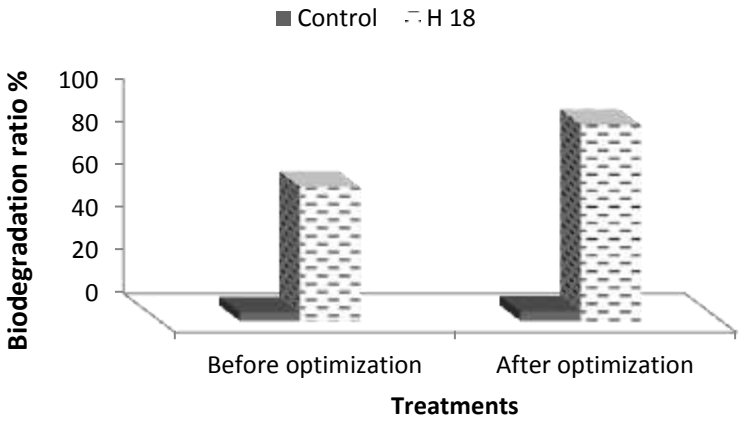


Fig.10. Biodegradation ratio % of phenanthrene and pyrene by the most potent strain *pseudomonas putida* (H 18) before and after optimization.where, Ctrl, phenanthrene and pyrene without bacterial inoculation.

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 cisdihydrodiols 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 petuda	7.01	1.86	BENZENE, (CHLOROMETHYL)	C_7H_7Cl	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

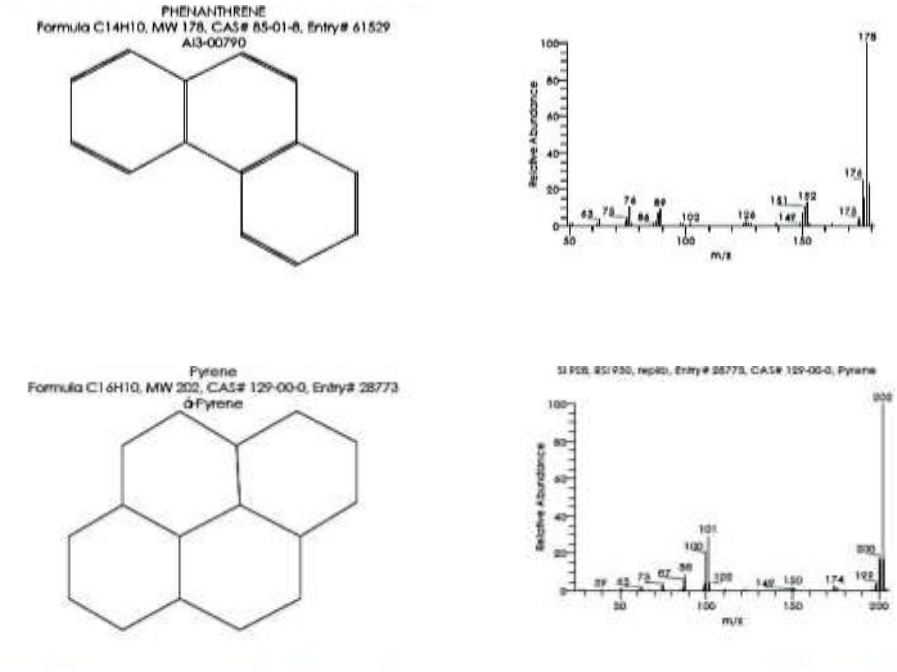
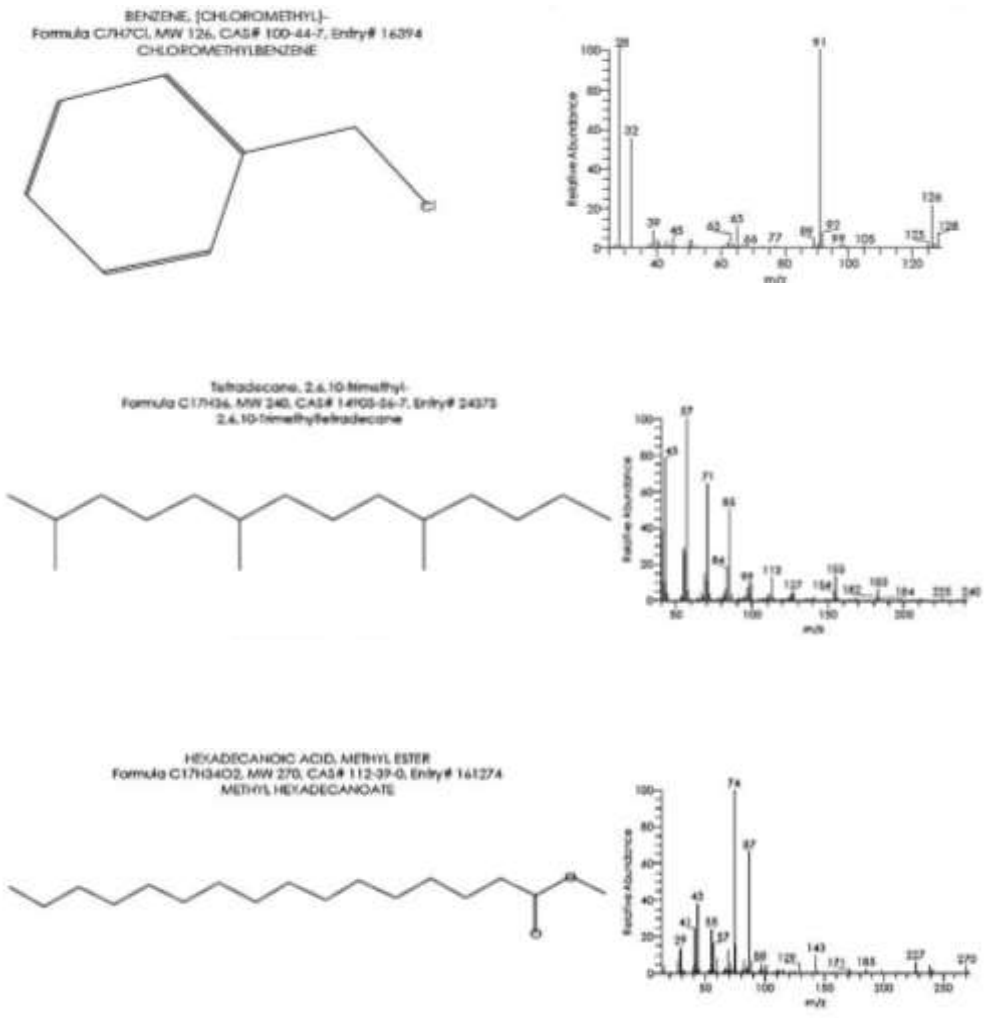


Fig.11. GC-MS analysis of phenanthrene and pyrene (Control).



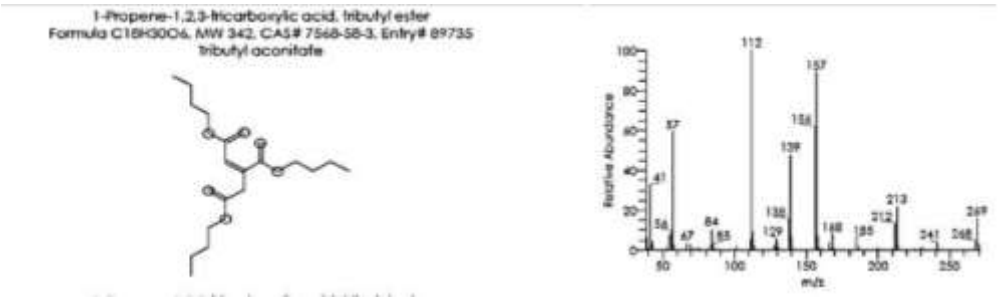


Fig.12. GC-MS analysis of biodegradation metabolites of phenanthrene and pyrene occurred by *Pseudomonas putida*.

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, 35ºC 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 *seudomonas putida* (H 18) has applicable role in the degrading of sediment resulted from various process contain pheneatherene and pyrene mixture.

REFERENCES

Alabresm, A. (2020). The Use of Polymer-Coated Magnetic Nanoparticles for Amelioration Oil Toxicity and Enhancement Microbial Remediation. ,p. 1-130.

Alegbeleye, O. O., Opeolu, B. O. and **Jackson, V. A. (2017).** Polycyclic aromatic hydrocarbons: a critical review of environmental occurrence and bioremediation. *Environmental Management*, *60*(4), 758-783.

An, X., Tian, C., Xu, J., Dong, F., Liu, X., Wu, X. and Zheng, Y. (2020). Characterization of hexaconazole-degrading strain *Sphingobacterium multivorum* and analysis of transcriptome for biodegradation mechanism. *Science of The Total Environment*, *722*, 137171.

Arun, P. A. S. and Bhaskara, R. K. V. (2010). "Physico chemical characterization of textile effluent and screening for dye decolorizing bacteria". *Global Journal of Biotechnology and Biochemistry.* 5, 80 – 86.

Behera, B. K., Das, A., Sarkar, D. J., Weerathunge, P., Parida, P. K., Das, B. K., .. and Bansal, V. (2018). Polycyclic Aromatic Hydrocarbons (PAHs) in inland aquatic ecosystems: Perils and remedies through biosensors and bioremediation. *Environmental Pollution*, *241*, 212-233.

Bukvic, A. (2002). *Phytoremediation of Metals and PAHs Occurring as Mixtures* (Doctoral dissertation, University of Cincinnati). Retrieved from https://etd.ohiolink.edu/.

Dangi, A. K., Sharma, B., Hill, R. T. and Shukla, P. (2019). Bioremediation through microbes: systems biology and metabolic engineering approach. *Critical Reviews in Biotechnology*, *39*(1), 79-98.

Dhar, K., Subashchandrabose, S. R., Venkateswarlu, K., Krishnan, K., and Megharaj, M. (2019). Anaerobic microbial degradation of polycyclic aromatic hydrocarbons: A comprehensive review. *Reviews of Environmental Contamination and Toxicology Volume 251*, 25-108.

Diarra, I. and Prasad, S. (2021). The current state of heavy metal pollution in Pacific Island Countries: a review. *Applied Spectroscopy Reviews*, *56*(1), 27-51.

Fouda, A., Hassan, S. E. D., Azab, M. S. and Saied, E. (2016). Decolorization of different azo dyes and detoxification of dyeing wastewater by *Pseudomonas stutzeri* (SB_13) isolated from textile dyes effluent. *Biotechnology Journal International*, 1-18.

Ghosal, D., Ghosh, S., Dutta, T. K. and Ahn, Y. (2016). Current state of knowledge in microbial degradation of polycyclic aromatic hydrocarbons (PAHs): a review. *Frontiers in Microbiology*, *7*, 1369.

Govarthanan, M., Khalifa, A. Y., Kamala-Kannan, S., Srinivasan, P., Selvankumar, T., Selvam, K. and Kim, W. (2020). Significance of allochthonous brackish water *Halomonas* sp. on biodegradation of low and high molecular weight polycyclic aromatic hydrocarbons. *Chemosphere*, *243*, 125389.

Hassan, S. E. D., Desouky, S. E., Fouda, A., El-Gamal, M. S. and Alemam, A. (2015). Biodegradation of phenanthrene by *klebsiella* sp isolated from organic contaminated sediment. *Journal of Advances in Biology & Biotechnology*, 1-12.

Hassan, S. E., Fouda, A., Salah, M. A. and Saied, E. (2015). Biological decolorization of different azo dyes using two bacterial strains of *Klebsiella* spp. and their consortium. *International Journal of Environmental Biology*, *5*(4), 104-114.

Haws, D. C., Hodge, T. L. and Yoshida, R. (2011). Optimality of the neighbor joining algorithm and faces of the balanced minimum evolution polytope. *Bulletin of Mathematical Biology.*, 73(11), 2627-2648.

Imron, M. F., Kurniawan, S. B., Ismail, N. I. and Abdullah, S. R. S. (2020). Future challenges in diesel biodegradation by bacteria isolates: A review. *Journal of Cleaner Production*, *251*, 119716.

Kensa, V. M. (2011). Bioremediation-an overview. *I Control Pollution*, *27*(2), 161-168.

Kotoky, R., Das, S., Singha, L. P., Pandey, P. and Singha, K. M. (2017). Biodegradation of Benzo (a) pyrene by biofilm forming and plant growth promoting *Acinetobacter* sp. strain PDB4. *Environmental Technology & Innovation*, *8*, 256-268.

Kuppusamy, S., Thavamani, P., Megharaj, M. and Naidu, R. (2016). Biodegradation of polycyclic aromatic hydrocarbons (PAHs) by novel bacterial consortia tolerant to diverse physical settings–assessments in liquid-and slurry-phase systems. *International Biodeterioration & Biodegradation*, *108*, 149-157.

Ludwig, W., Schleifer, K. H. and Whitman, W. B. (2009). Revised road map to the phylum Firmicutes. In *Bergey's Manual® of Systematic Bacteriology* (pp. 1-13). Springer, New York, NY.

Masika, W. S., Moonsamy, G., Mandree, P., Ramchuran, S., Lalloo, R. and Kudanga, T. (2020). Biodegradation of petroleum hydrocarbon waste using consortia of Bacillus sp. *Bioremediation Journal*, 1-8.

Mirnejad, R., Babavalian, H., Moghaddam, M. M., Khodi, S. and Shakeri, F. (2012). Rapid DNA extraction of bacterial genome using laundry detergents and assessment of the efficiency of DNA in downstream process using polymerase chain reaction. *African Journal of Biotechnology*., 11(1), 173-178.

Mohan, V. Madhumitha, M. Menon, S and Saranya devi, K. (2013). Isolation and Screening of potential Dye decolorizing bacteria from Textile dye effluents in Tamil Nadu, India. *Journal of Academia and Industrial Research (JAIR)*. 2 (2). 3678 – 3783.

Mohanrasu, K., Rao, R. G. R., Dinesh, G. H., Prakash, G. S., Pugazhendhi, A., Jeyakanthan, J., ... and Arun, A. (2020). Effect of C/N Substrates for enhanced Extracellular Polymeric Substances (EPS) Production and Poly Cyclic Aromatic Hydrocarbons (PAHs) degradation. *Environmental Pollution*, 116035.

Patel, A. B., Shaikh, S., Jain, K. R., Desai, C. and Madamwar, D. (2020). Polycyclic Aromatic Hydrocarbons: Sources, Toxicity and Remediation Approaches. *Frontiers in Microbiology*, *11*, 2675.

Ponaraj, M., Gokila, K. and Vasudeo Z. (2011). Bacterial decolorization of textile dye- Orange 3R, *International Journal of Advanced Biotechnology and Research*, 2(1), 168-177.

Roostan, Z., Safahieh, A., Mojodi, F., Zolgharnein, H., Ghanemi, K. and Abiar, H. (2012). Phenanthrene biodegradation by *Pseudomonas aeruginosa* and

Bacillus subtilis isolated from Persian gulf sediments. *African Journal of Microbiology Research*, *6*(21), 4585-4591.

Singh, P., Jain, R., Srivastava, N., Borthakur, A., Pal, D. B., Singh, R., ... and Mishra, P. K. (2017). Current and emerging trends in bioremediation of petrochemical waste: A review. *Critical Reviews in Environmental Science and Technology*, *47*(3), 155-201.

Umar, Z. D., Aziz, N. A. A., Zulkifli, S. Z and Mustafa, M. (2017). Rapid biodegradation of polycyclic aromatic hydrocarbons (PAHs) using effective Cronobacter sakazakii MM045 (KT933253). *MethodsX*, *4*, 104-117.

Umar, Z. D., Aziz, N. A. A., Zulkifli, S. Z. and Mustafa, M. (2017). Rapid biodegradation of polycyclic aromatic hydrocarbons (PAHs) using effective *Cronobacter sakazakii* MM045 (KT933253). *MethodsX*, *4*, 104-117.

Varjani, S. J. and Upasani, V. N. (2016). Biodegradation of petroleum hydrocarbons by oleophilic strain of *Pseudomonas aeruginosa* NCIM 5514. *Bioresource technology*, *222*, 195-201.

Wang, C., Wang, Y. and Herath, H. M. S. K. (2017). Polycyclic aromatic hydrocarbons (PAHs) in biochar–Their formation, occurrence and analysis: A review. *Organic Geochemistry*, *114*, 1-11.

Zhao, L., Deng, J., Hou, H., Li, J. and Yang, Y. (2019). Investigation of PAH and oil degradation along with electricity generation in soil using an enhanced plant-microbial fuel cell. *Journal of Cleaner Production*, *221*, 678-683.

Zhao, L., Yang, S. T., Yilihamu, A. and Wu, D. (2019). Advances in the applications of graphene adsorbents: From water treatment to soil remediation. *Reviews in Inorganic Chemistry*, *39*(1), 47-76.

التكسيير الحيوى الهيدروكربونات العطرية متعددة الحلقات باستخدام سلالة *Pseudomonas putida* H18المعزولة من المواقع الملوثة بالمواد البترولية

[1]*احمد بهجت عدلى , [1]سعد الدين حسن عفيفى , [1]نجوى محمود صدقى

[1]قسم النبات والميكروبيولوجى - كلية العلوم -جامعة الأزهر- القاهرة , مصر

البريد الالكتروني للباحث الرئيسي : Ahmed. Bahgat211@azhar.edu.eg.

الملخص العربى:

التكسير البيولوجي للهيدروكربونات العطرية متعددة الحلقات (PAHs) في المناطق الملوثة هو تقنية حيوية يعتمد عليها بشرط توافر الظروف المثلى للعزلات المستخدمة للتكسر الحيوى للهيدروكربونات العطرية. تم عزل سلالة بكتيرية واحدة من منطقة ملوثة بمركبات بتروكيماوية قادرة على التحلل الحيوي للفينانثيرين والبايرين. تم دراسة الظروف المثلى للكائن المستخدم فى التكسير الحيوي للفينانثيرين والبايرين من حيث ظروف مختلفة من الاهتزاز والثبات ودرجة الحموضة ودرجة الحرارة وأحجام حقنات مختلفة ويضا مصادرمختلفة من الكربون والنيتروجين. تشير الشجرة الوراثية المستندة إلى تسلسل التحليل الجيني إلى أن كائن الاختبار هذا كان ينتمي إلى جنس الذيدوموناس وتم تحديده على أنه (*pseudomonas putida* (H 18). كانت الظروف المثلى للكائن المستخدم للتحلل الحيوي في الوسط الذي يحتوي على للفينانثيرين والبايرين كمصدر وحيد للكربون ، استخدام مستخلص الخميرة كمصدر للنيتروجين ، وتركيز بكتيري ٤٪ ، عند الأس الهيدروجيني = ٨ و ٣٥ درجة مئوية تحت ظروف ثابتة لمدة ثمانية ايام. تم الوصول إلى أقصى كفاءة للتحلل الحيوي بنسبة ٩٢٪ من للفينانثيرين والبايرين وتم تأكيد هذه النسبة من التحلل البيولوجي باستخدام التحليل الطيفي للكتلة GS. على حد علمنا ، هذا هو أول تقرير يفيد بأن السلالة البكتيرية من *جنس النيدوموناس*. تم تسجيله بقدرة تحلل حيوي مقابل الفينيثرين والبايرين وبذلك نكون قد استطعنا تقليل المدة الزمنية لتكسير هذه المواد مقارنة بالدراسات السابقة.

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