VIE ET MILIEU - LIFE & ENVIRONMENT, 2024, 74 (1-2): 99-124 DOI : 10.57890/s10vfv58

BIODEGRADATION OF POLYCYCLIC AROMATIC HYDROCARBONS WITH *ENTEROBACTER ASBURIAE*

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KEYWORDS: POLYCYCLIC AROMATIC HYDROCARBONS, *ENTEROBACTER ASBURIAE*, BIODEGRADATION, TOXICITY ASSESSMENT, ENRICHMENT

ABSTRACT.- Polycyclic Aromatic Hydrocarbons (PAHs) pollution has been found to be toxic, mutagenic, carcinogenic, teratogenic, and immunotoxicogenic. Naphthalene, Anthracene, and Fluorene are among the polycyclic aromatic hydrocarbons (PAHs) that are frequently detected in wastewater from biomass gasification and refineries. In this study, industrial waste water samples were collected from three sites located in Kafr El-Sheikh Governorate, Egypt and showed presence of PAHs in different concentration varying from 0.42 to 253.1 ppb. This study's findings included the isolation and identification of *Enterobacter asburiae* the most proficient bacterial isolate capable of biodegrading PAHs among other 7 studied bacterial isolates. *Enterobacter asburiae strain* No. 1 showed the ability to biologically degrade approximately 79 %, 89.7 % and 82.8 % of Naphthalene, Fluorene and Anthracene active ingredients respectively. The results of toxicity assessment showed that PAHs byproducts after 14 days of incubation had no toxicity; consequently, there was no antibacterial activity detected against *B. subtilis* as a test organism. Such biological approaches used in this study can be further applied in various polluted sites to get rid of ubiquitous organic pollutants.

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are of the ubiquitous environmental pollutants found in different environmental matrices at different concentrations (Nessim *et al.* 2011, Zou *et al.* 2011). Significant accumulation of PAHs in the aquatic ecosystem had been caused by anthropogenic inputs like oil spills, sea navigation, urban runoff, water, and industrial wastes (Wu *et al.* 2011, Westley *et al.* 2010). High concentrations of PAHs are found in marine coastal environment near cities and industrial plants (Opuene *et al.* 2007). PAHs are a class of organic pollutants characterized by the fusion of two or more aromatic rings composed of carbon and hydrogen atoms. These compounds typically exist in the form of colorless, white, or pale-yellow solid substances (Abdel-Shafy & Mansour 2016, Suman *et al.* 2016). The spatial arrangement of the aromatic rings can vary, including linear, angular, or clustered configurations (Abdel-Shafy & Mansour 2016).

PAHs can be categorized based on the number of aromatic rings they contain, resulting in two main groups: light-molecular weight PAHs (LMW PAHs) with two or three rings and high-molecular weight PAHs (HMW PAHs) with four or more rings. Depending on their molecular weight, LMW PAHs are released into the atmosphere in gaseous form, while HMW PAHs are found in particulate matter (Lee & Vu 2010). Furthermore, PAHs can also be classified based on their ring structures. Alternant PAHs consist solely of fused six-carbon benzene rings, whereas non-alternant PAHs, such as fluorene, include six-carbon benzene rings fused with an additional ring containing fewer than six carbon atoms (Gupte *et al*. 2016).

The presence of dense p electrons in the aromatic rings contributes to the chemical persistence of PAHs, making them resistant to nucleophilic attacks (Haritash & Kaushik 2009). In 1983, the United States Environmental Protection Agency (USEPA) identified 16 PAHs as priority pollutants due to their high concentrations, extensive exposure, recalcitrance, and toxicity (Zheng *et al*. 2018, Mojiri *et al.* 2019). PAHs exhibit low water solubility, low vapor pressure, and high melting and boiling points, which vary depending on their specific chemical structures (Lee & Vu 2010). As the molecular weight of PAHs increases, their water solubility decreases, and they become more lipophilic, rendering them more persistent compounds (Okere & Semple 2012).

Anthracene is known to cause direct skin irritation and skin sensitization in both animals and humans (Rengarajan *et al*. 2015). Chronic health effects associated with PAH exposure include conditions like cataracts, kidney and liver damage, respiratory issues, reduced immune function, lung dysfunction, and symptoms resembling asthma (Abdel-Shafy & Mansour 2016). Naphthalene can lead to the breakdown of red blood cells if inhaled or ingested in high concentrations (Rengarajan *et al.* 2015). Fluorene is linked to adverse effects on both wildlife and human health, exhibiting acute toxicity as well as potential mutagenic and carcinogenic properties (Yu 2002).

Concern about PAHs has predominantly focused on their carcinogenic properties (Boffetta *et al.* 1997, Rubin 2001). Recently, there has been increased focus on the potential effects of polycyclic aromatic hydrocarbons on human health. Specifically, their ability to disrupt hormonal functions related to reproduction and depress immune system function has come under examination (Uppstad *et al.* 2011). These concerns have prompted both the World Health Organization (WHO)

(WHO 2006) and the United States Environmental Protection Agency (USEPA) (U.S. EPA 2003) to formulate regulations for the protection of drinking and source water systems in order to safeguard the populace from such harmful pollutants, many of which are considered as potential carcinogens.

Degradation of PAHs in the environment includes: biodegradation, photooxidation, and chemical oxidation adsorption to soil particles, leaching, bioaccumulation (Tolosa *et al.* 1996). Each of these processes affects individual PAHs in a different manner. This is mainly due to the fact that each PAH has a unique structure and a set of physical, chemical, and biological properties. On the other hand; PAHs biodegradation studies focused on aerobic degradation. Nevertheless, anaerobic degradation has been demonstrated (Haritash & Kaushik 2009, Peng *et al.* 2008). In order for bacteria to degrade any given PAH, it must be made available for uptake by the bacteria (Cerniglia 2003, Dandie *et al.* 2004, Fredslund *et al.* 2008). PAHs become bioavailable when they are in either the dissolved or the vapor phase. Bioremediation is a technology that transforms relatively toxic compounds to less hazardous forms using biological processes. Polycyclic aromatic hydrocarbons degradation depends on several factors including: the environmental conditions, number and type of the microorganisms, nature and chemical structure of the chemical compound being degraded. The PAHs-degrading microorganism includes algae, bacteria, and fungi. It involves the breakdown of organic compounds through biotransformation into less complex metabolites, and through mineralization into inorganic minerals, H_2O, CO_2 (aerobic) or CH^4 (anaerobic) (Abdel-Shafy & Mansour 2016).

This study aims to address the knowledge gap in environmental pollution monitoring by investigating polycyclic aromatic hydrocarbons (PAHs) in specific contaminated sites in Egypt that have not been previously monitored for PAH pollution. The hypotheses involve the potential existence of bacteria in the contaminated water capable of effectively reducing polycyclic aromatic compounds. The experimental procedure involves the collection of water samples, isolation of bacteria, screening for the most effective isolates, in vitro evaluation of their efficacy in reducing PAHs, identification of the most effective isolates, and subsequent use of the isolates for in vitro removal of PAHs. The anticipated outcomes include the quantification of PAH concentrations in selected sites, isolation of diverse bacterial isolates with PAH biodegradation capabilities, characterization and identification of these isolates, and conducting biodegradation assays to assess the selected bacterial isolate's proficiency in degrading PAHs. The findings will contribute

to the understanding of microbial remediation strategies for PAH-contaminated environments.

MATERIALS AND METHODS

Sampling and sampling sites: industrial wastewater samples were collected in 2021 from three sites in Kafr El-Sheikh Governorate, Egypt: Neamaa restaurant, Kitchener Drain, and a Total gas station. The samples were collected manually on-site and transported to the laboratory in iceboxes. Table I showed sampling sites locations and coordinates.

	Site	Latitude	Longitude
1	Kitchener Drain	31°07’57.4”N	31° 03’19.2”E
2	Neamaa restaurant	31°06’40.8”N	30° 57’07.6”E
3	Total gas station	31°06’27”N	30°58’09”E

Table I. - Sampling site locations and coordinates.

Chemicals: the tested PAHs active ingredients were anthracene (100.0 % purity), fluorene (99.1 % purity) and naphthalene (99.8 % purity). The active ingredients were supplied by AccuStandard Inc., USA. They were obtained from the Central Laboratory at the Kafr El-Sheikh Company for Water and Wastewater in Kafr El-Sheikh City, Egypt. Gradient grade acetonitrile from Merck Millipore, Germany was used as the exchange solvent for liquid chromatography. Methylene chloride (99.0 % purity) from Sigma-Aldrich, USA was used to extract the PAHs.

Sample Preparation: two-liter water samples were collected from each site in cleaned, sterilized, solvent-washed glass bottles. The samples were transported to the laboratory in ice containers. One liter of each sample was filtered through fiberglass -Grade MGC Micro-Glass Fiber Filters, Munktell Filtrak™ , CAT N°. 3.1103.024- to remove turbidity and debris. These filtered samples were stored at 4°C prior to extraction and then tested for PAH concentrations. The remaining one-liter samples were incubated at 30°C for two weeks. After the incubation period, these samples were tested using the same methods in order to detect percentages of PAH biodegradation (Belal *et al.* 2018).

Media: three types of enrichment media were used in this study as described by Brunner *et al.* (1980): minimal medium as mineral salt liquid (MSL), minimal medium as mineral salt agar, and nutrient agar.

Analysis of HPLC: approximately 1 L of each sample was serially extracted three times with 60.0 mL of methylene chloride using a separatory funnel. The methylene chloride extract was dried and concentrated to a volume of 1 mL. Then, 3.0 mL of acetonitrile (the exchange solvent) was added and the extract concentrated again to a final volume of 0.5 mL. The extract analytes were separated by HPLC. Ultraviolet adsorption (UV) and fluorescence detectors coupled to the HPLC system were used to quantitatively measure the PAHs. (Hodgeson 1990).

High performance liquid chromatography (HPLC)

Quantitative determination of polycyclic aromatic hydrocarbons (PAHs) was performed using a Dionex Ultimate 3000 high performance liquid chromatography (HPLC) system (Dionex USA) at the

Central Laboratory, Kafr El-Sheikh Company for Water and Wastewater in Kafr El-Sheikh, Egypt.

Isolation by enrichment culture

Enrichment cultures capable of degrading PAHs were established using water samples collected from the three industrial wastewater sites in Kafr El-Sheikh Governorate, Egypt. The samples were tested to detect PAH concentrations. A 10 mL aliquot of each sample was suspended in 90 mL of sterilized mineral salt medium (MSL) in 500-mL bottles containing 1 µg/L of the PAH active ingredients as the sole carbon source. These enrichment cultures were incubated at 30°C and 150 rpm for 14 days. Next, a 10 mL aliquot from each culture was transferred to fresh 90 mL MSL medium with the same 1 µg/L PAH concentration. This subculture procedure was repeated four times. After the final transfer, dilution series of the enrichment cultures were prepared in glass tubes containing 9 mL MSL medium, to dilutions of 10^{-6}. Then, 100 µL aliquots of each dilution were spread onto MSL medium plates containing 1 µg/L PAHs using Drigalski spatulas. The inoculated plates were sealed in polyethylene bags and incubated at 30°C for 7 days, monitoring for bacterial colony growth. Single colonies appearing on the diluted plates were isolated by picking with sterile inoculation needles. The isolates were further purified using standard spatial streaking onto complex agar media. (Derbalah & Belal 2008, Derbalah *et al.* 2008, Massoud *et al.* 2008).

Screening of bacterial isolates using the viable count technique

The isolated colonies underwent testing for their ability to grow in MSL medium containing the PAHs standard at a concentration of 1 µg/L. Two treatments were employed: one with the medium and PAHs standard, and the other with only the medium as a control (without PAHs). The cultures were agitated at 150 rpm and maintained at a temperature of 30 °C for a duration of 14 days. Screening of bacterial isolates with superior PAH-degrading capabilities was conducted based on the viable count of these isolates. Serial dilution tubes up to $1 * 10^{-6}$ were prepared from each culture, and a 100 µL volume from each tube was spread onto plates of plate count agar medium using a sterilized Drigalski triangle. The plates were then incubated at 30° C for 2 days, and the viable count of each strain was determined using a colony counter. For further identification, single colonies of the selected bacterial isolate (determined to be most proficient in degrading PAHs) were isolated by picking the colonies with a

sterile needle. Subsequently, these colonies were inoculated into fresh plates and slants for identification purposes, following the procedures outlined in Derbalah & Belal (2008), Derbalah *et al.* (2008), and Massoud *et al.* (2008).

Identification

The bacterial isolates capable of degrading selected PAHs were identified through the examination of morphological and biochemical characteristics, following the criteria established by Gordon *et al.* (1973), Krieg & Holt (1984), Chun *et al.* (2001), Logan (2005), and Sharma *et al.* (2015). Additionally, identification was confirmed using the 16S rRNA method outlined by Boye *et al.* (1999).

Morphological and cultural characterization of the selected:

Isolate. Bacterial isolates were examined for their cell shape, motility studies, and gram reaction.

Cell shape. Purified cultures at log phase after 72 h were microscopically examined for the cell morphological characters.

Motility. The 72-h grown isolates were microscopically examined using cavity slide for bacterial motility.

Gram reaction. Gram staining was conducted following the procedure outlined by Rangaswami & Bagyaraj (1993).

Biochemical characterization of the selective isolates:

Oxidase test. The isolate was streaked onto trypticase soy agar medium and then incubated at 37° C for 48 hours, following the method described by Cappuccino & Sherman (1996). After the incubation period, 2-3 drops of a 1 % P-aminodimethylaniline oxalate solution were applied to the streaked area. The plates were observed for a color change, transitioning from pink to maroon, and ultimately to purple within 30 seconds, signifying a positive reaction.

Catalase activity. A loopful of a 24-hour-old culture of the isolate was transferred to a glass test tube containing 0.5 mL of distilled water. It was thoroughly mixed with 0.5 mL of a 3 % hydrogen peroxide solution, and the mixture was observed for effervescence, following the method outlined by Gerhardt *et al.* (1981).

Urease test. Urease test was performed on 5 mL of urea broth (20 g/L) in test tubes containing phenol red (0.012 g/L), pH 6.8 as the pH indicator (Cappuccino & Sherman 1996). The cultures were transferred into the sterilized urea broth and incubated for 24 h. The development of red color indicates a positive reaction.

Citrate utilization test. The selective isolate was inoculated into test tubes having Simmons citrate agar medium and incubated for 48 h at 35°C (Seeley & Vandemark 1981). Simmons citrate agar contains citrate as only carbon and energy source. The presence of growth and change of color from green to blue due to pH change indicated positive reaction.

Indole production. The isolate was inoculated into sterilized glucose tryptone broth in test tubes (Seeley & Vandemark 1981). After 48 h of incubation, 0.3 mL of Kovac's reagent was added and mixed well. The reddening of the alcohol layer within few minutes indicates indole production.

Methyl Red. A fresh pure culture of the test culture was grown for 48 hours at 35°C in MR-VP medium. After incubation, 5 drops of a 0.05 % methyl red solution (per 2.5 ml) were added to the culture. The MR-positive shows a red coloration as a result of high acid production and a decrease in the pH of the culture medium to 4.4. The MR-negative culture has a yellow color indicating a less acidic medium. (McDevitt 2009).

Voges-Proskauer. a fresh pure culture of the test culture was grown in MR-VP-broth for 48 hours at 37°C and Barritt's reagents A and B were added. VP-positive shows red coloration on top of the culture as a result of Diacetyl reacts with peptone in the medium, to form a pinkish red-colored product, whereas VP-negative has a yellowish color. (McDevitt 2009).

Molecular characterization of the selective isolate by polymerase chain reaction:

The most efficient bacterial isolates regarding PAHs degradation were identified using 16S rRNA as described by Boye *et al.* (1999). DNA was extracted using protocol of GeneJet genomic DNA, purified, and polymerase chain reaction (PCR) was made by using Maxima Hot Start PCR Master Mix (Thermo).

Biodegradation assay of polycyclic aromatic hydrocarbons

An experiment was conducted to assess the biodegradation capabilities of three polycyclic

aromatic hydrocarbons (PAHs) – Anthracene, Fluorene, and Naphthalene – using the most proficient bacterial isolate capable of degrading these compounds. An initial concentration of 5 ppb for each of the three PAHs was introduced into MSL medium and inoculated with a bacterial cell suspension from the most proficient isolate, with an inoculum size of 10^6 cfu/ml. A control experiment was established without the inoculation of a biological agent to monitor abiotic losses. A duplicate experiment was performed to evaluate biological degradation. All experiments were incubated at pH 7 and 30 °C for 14 days. Average concentrations, standard deviations, and removal averages were calculated for each experiment. The methodology employed aligns with that described by Belal *et al.* (2018).

Toxicity assessment of biodegradation byproducts

The toxicity bioassay for byproducts resulting from the biodegradation of PAH compounds was conducted on aqueous solutions after a 14-day incubation period with *Enterobacter asburiae* (Isolate Nº 1). A bioassay test was performed using *Bacillus subtilis*, a gram-positive bacterium chosen for its sensitivity to PAHs, serving as a suitable indicator to assess the remaining toxicity of PAHs following treatment with the tested microbial isolate. The selection of *Bacillus subtilis* as the target organism was based on its demonstrated sensitivity to PAHs, as indicated by Wulandari *et al.* (2021). To determine toxicity, the inhibition zone in the growth of *B. subtilis* was recorded and compared to the control treatment (untreated). *Bacillus subtilis* was treated with supernatants that had been incubated with the tested bacterial isolate for 14 days. The test was standardized as follows: Nutrient agar medium was poured into Petri dishes (9 cm in diameter, 15 ml/dish). After solidification, 100 µl of *B. subtilis* (10^8 c.f.u/ml) was evenly spread onto the nutrient agar plate using sterilized glass beads. Wells (5 mm in diameter) were punched in each plate. Subsequently, 50 µl of the supernatant was added to the punched holes (5 mm in diameter) in the nutrient agar medium. For the three PAHs compounds, each incubated with *Enterobacter asburiae* (Isolate Nº 1), broth cultures were filtered through a sterile membrane filter (0.2 µm). Conversely, a volume of 50 µl of sterilized liquid medium was placed in the punched holes and used as a control treatment. The experiments were conducted in triplicate. The plates were incubated at 30 °C until full growth of the control treatments was achieved (2 days). The diameter (mm) of the inhibition zone surrounding each hole, where the growth of *Bacillus subtilis* was inhibited, was recorded. Toxicity was determined as a percentage of inhibition in the growth of the tested bacteria

compared to the control treatment. Similar bioassay protocols for assessing metabolite toxicity were employed by Massoud *et al.* (2008), Hauka *et al.* (2014), Derbala & Belal (2008), and Derbalah *et al.* (2008).

RESULTS AND DISCUSSION

This study conducted an analysis of three PAH (Polycyclic Aromatic Hydrocarbon) compounds present in water samples collected from three distinct sites in Kafr El-Sheikh governorate, Egypt. The selected sites were Neamaa restaurant, Kitchener Drain, and Total gas station. The compounds under investigation comprised Naphthalene, Anthracene, and Fluorine. Table II presents the data, revealing variable concentrations of PAHs in the water samples.

Table II shows the concentration (in ppb) of three PAHs compounds - Anthracene, Fluorene, and Naphthalene - in water samples collected from three industrial wastewater sources - Kitchener drain, Neamaa restaurant, and total gas station - before and after incubation period. It also shows the degradation percentage of these compounds. Before incubation, the concentrations ranged from 0.424 to 253.108 parts per billion (ppb). The highest recorded value was 253.108 ppb for Anthracene found at the Total gas station site. In contrast, the lowest recorded concentration was 0.424 ppb for Fluorene detected in the Kitchener Drain samples. For Anthracene, there is a high degradation percentage in all three sources after the incubation period, ranging from 99.54 % in Kitchener drain to 100 % in Neamaa restaurant and total gas station samples. This indicates that Anthracene was efficiently degraded in the wastewater from all sources. Fluorene shows varying degradation percentages - 46.23 % in Kitchener drain, 100 % in Neamaa restaurant, and 100 % in total gas station. While it was completely degraded in samples from Neamaa and the gas station, the Kitchener drain sample retained over 50 % of initial Fluorene after incubation. Naphthalene degradation ranges from 60.12 % in the gas station sample to 90.68% in the Kitchener drain. The Neamaa restaurant sample shows 80.9 % reduction. While degradation is occurring, Naphthalene persists after incubation most out of the three PAHs across the samples. Additional measures may be required to improve removal of this recalcitrant compound. Overall, the results demonstrate the efficiency of degradation of PAH contaminants in wastewater during the incubation period. However, persistence of compounds like Fluorene and Naphthalene in some samples highlight the need to further optimize treatment conditions.

	PAHs	Kitchener drain			Neamaa restaurant			Total gas station		
		Before	After	Degradation percentage %	Before	After	Degradation percentage %	Before	After	Degradation percentage %
1	**Anthracene**	7.896	0.036	99.54 %	1.6	0.0	100 %	253.108	0.0	100 %
2	**Fluorene**	0.424	0.228	46.23 %	15.64	0.0	100 %	11.816	0.0	100 %
3	**Naphthalene**	42.832	3.992	90.68 %	5.12	0.98	80.9 %	6.68	2.664	60.12 %

Table II.- Concentration (ppb) of PAHs in water samples collected from three sites of industrial waste water sources

Figures 1 and 2 shows chromatograms of three PAHs compounds - Anthracene, Fluorene, and Naphthalene - in water samples collected from Kitchener drain before and after incubation period respectively, figures 3 and 4 shows chromatograms of three PAHs compounds - Anthracene, Fluorene, and Naphthalene - in water samples collected from Neamaa restaurant before and after incubation period respectively and figures 5 and 6 shows chromatograms of three PAHs compounds - Anthracene, Fluorene, and Naphthalene - in water samples collected from total gas station before and after incubation period respectively.

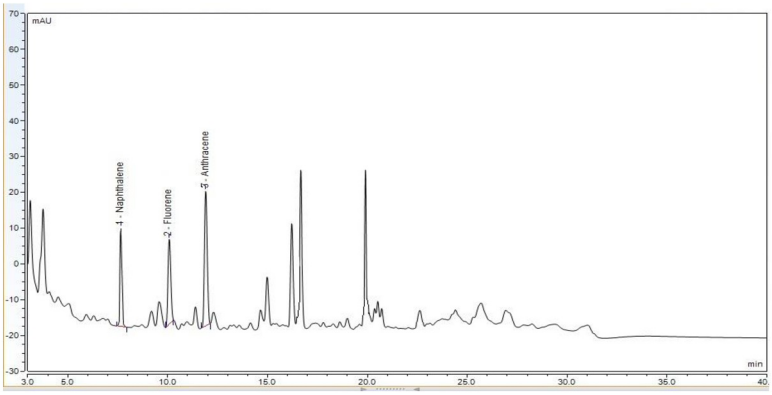

Fig.1.- Chromatogram of three PAHs compounds - Anthracene, Fluorene, and Naphthalene - in water sample collected from Kitchener drain before incubation period

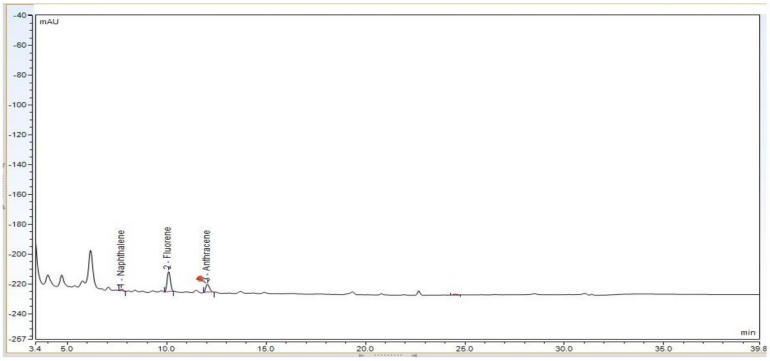

Fig. 2. - Chromatogram of three PAHs compounds - Anthracene, Fluorene, and Naphthalene - in water sample collected from Kitchener drain after incubation period

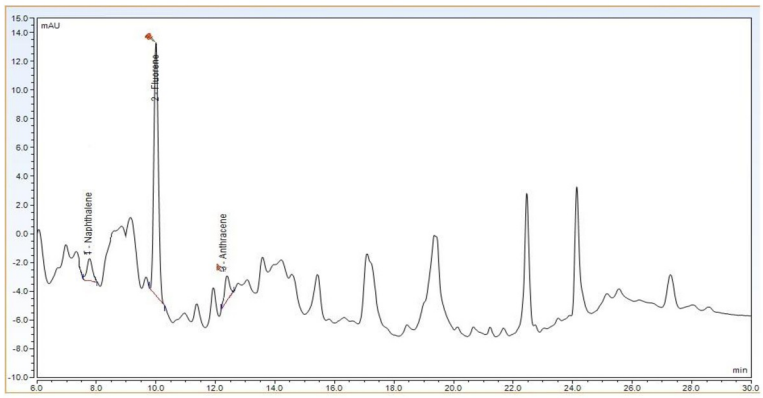

Fig. 3. - Chromatogram of three PAHs compounds - Anthracene, Fluorene, and Naphthalene - in water sample collected from Neamaa restaurant before incubation period

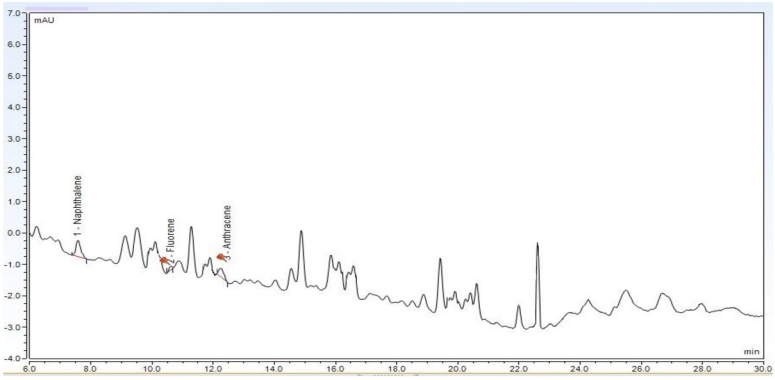

Fig. 4.- Chromatogram of three PAHs compounds - Anthracene, Fluorene, and Naphthalene - in water sample collected from Neamaa restaurant after incubation period

The varying degradation percentages of PAHs compounds across the three industrial wastewater sources indicate that the efficacy of bacterial remediation is impacted by site-specific conditions. As noted by Gallego *et al.* (2001), the composition and complexity of the microbial population, presence of additional contaminants, and physico-chemical properties of water can all affect bioremediation. Kitchener drain water likely contained high organic content which can inhibit microbial activity and lowered Anthracene and Naphthalene degradation despite adequate bacterial populations (Yu *et al.* 2005). The Neamaa restaurant wastewater, on the other hand, showed near complete PAHs degradation, suggesting favorable microbial community and optimum growth conditions (pH, temperature, nutrients etc.) (Chang *et al.* 2002). The differences also highlight that while bacteria may efficiently metabolize some PAHs like Anthracene, recalcitrant compounds like Naphthalene persist due to their complex chemical structure. According to Samanta *et al.* (2002), the presence of alkyl side chains inhibits enzyme activities during microbial metabolism. Pre-exposure to low Naphthalene levels can induce these enzymes over time, increasing degradation capacity (Juhasz *et al.* 1997). Thus, further acclimatization

of native bacteria through incremental exposure can potentially improve degradation rates for stubborn PAH contaminants at the sites. Overall, careful assessment of site-specific conditions and adjustment of bioremediation strategy is imperative to achieve consistent PAHs removal across diverse industrial wastewater sources.

Isolation by enrichment culture

Seven morphologically distinct microorganisms with the capability to degrade PAHs were isolated from the previously mentioned three sources. Fig. 7 provides visual representation of the intricate growth patterns of PAHs-degrading bacteria on minimal salt medium plates supplemented with PAHs (1 µg/L). Table III presents compiled data on the numbers, codes, and sources of these seven isolated microorganisms, each identified and distinguished for their proficiency in PAH degradation.

Screening of PAHs degrading bacterial isolates using the viable count technique

A total of seven morphologically distinct bacterial isolates with the capability to degrade PAHs were obtained (see Table III). Table IV presents the viable count of bacterial isolates demonstrating the capability to biodegrade PAHs, with Bacterial Isolate N^{o} 1 exhibiting the highest count.

No.	Code	Sources of isolates
1	Strain 1	Kitchener drain
2	Strain 2	Total gas station
3	Strain 3	Kitchener drain
4	Strain 4	Neamaa restaurant
5	Strain 5	Total gas station
6	Strain 6	Neamaa restaurant
7	Strain 7	Total gas station

Table III. - Numbers, codes, and sources of 7 microorganisms that were isolated and marked for their abilities to degrade PAHs

The viable count technique is commonly used for the initial screening of PAHs-degrading bacteria from environmental samples (Juhasz *et al.* 1997, Dean-Ross *et al.* 2002). This involves plating serial dilutions of bacterial enrichments from sources like wastewater or petroleum-contaminated soil onto basal

mineral media agar plates coated with target PAHs as sole carbon source (Tejeda-Agredano *et al.* 2011).

No.	Bacterial isolates	Count (CFU/mL)
1	Strain 1	**$12*10^7$**
2	Strain 2	**$8*10^7$**
3	Strain 3	**$4*10^5$**
4	Strain 4	**$3*10^4$**
5	Strain 5	**$2*10^5$**
6	Strain 6	**$6*10^4$**
7	Strain 7	**$10*10^7$**

Table IV.- Viable count of bacterial isolates

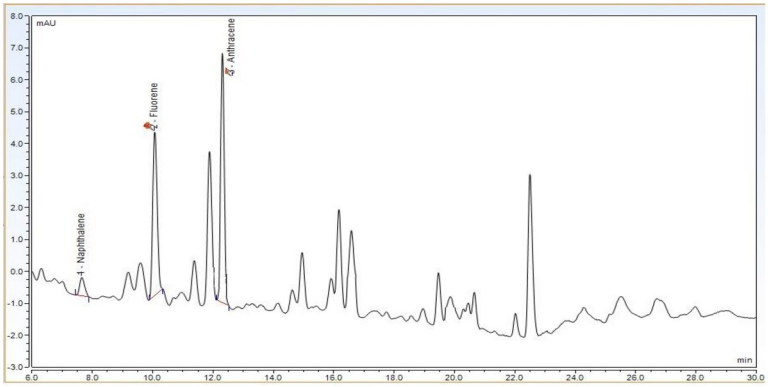

Fig. 5.- Chromatogram of three PAHs compounds - Anthracene, Fluorene, and Naphthalene - in water sample collected from total gas station before incubation period

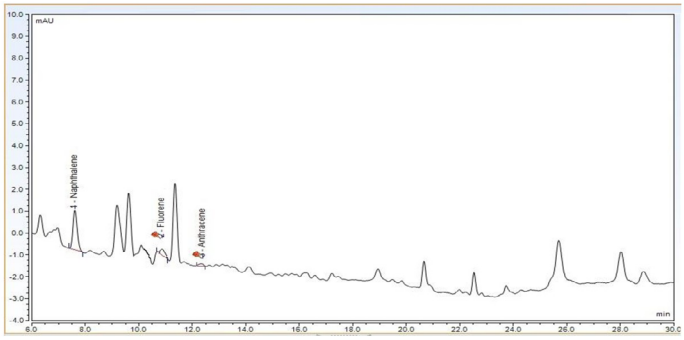

Fig 6.-. Chromatogram of three PAHs compounds - Anthracene, Fluorene, and Naphthalene - in water sample collected from total gas station aftier incubation period

Colonies showing growth after incubation period are considered to utilize the PAH substrate. These candidates can then be identified through biochemical tests or 16S rRNA sequencing and evaluated for PAHs degradation efficiency through methods like gas chromatography. According to Muller *et al.* (1996), combining viable counts with most probable number analysis provides both qualitative and

quantitative assessment of pollutant-degrading isolates for bioremediation applications. The viable counting approach allows rapid preliminary profiling of the PAHs-degradation potential in bacterial communities prior to further characterization of promising isolates.

Identification of efficient PAHs degrading bacterial isolates

The cultural, morphological, and physiological properties of the most efficient PAH-degrading bacterial isolates were investigated for organism identification using Bergey's Manual of Systematic Bacteriology (Krieg & Holt 1984), as well as drawing upon insights from various studies focusing on the morphological and biochemical characterization of microorganisms (Zhang *et al.* 2013, Guo *et al.* 2012, Bahamdain *et al.* 2015). Isolate No. 1 was found to be gram Negative, motile, rod shaped, 1.5 µm in length with rough surface and yellow in color. These morphological attributes are summarized in Table V. The biochemical examination of the selected bacterial isolate revealed negative results for endospore formation, growth at 40°C, oxidase activity, indole production, urease activity, and Voges Proskauer test. Conversely, positive results were observed for growth at 5 % NaCl, catalase activity, citric acid utilization, and methyl red test. A summary of the biochemical characteristics can be found in Table VI.

	Character	Isolate No. 1
1	Gram stain	Negative
2	Colony shape	Small rods
3	Color	Yellow
4	Margin	Rough
5	Elevation	Raised
6	Opacity	Transparent
7	Motility	Motile
8	length	1.5 micrometers

Table V.- Morphological Characteristics of Colonies of Most Efficient PAHs Degrading Bacterial Isolate No. 1

	Character	Isolate No. 1
1	Gram stain	Negative
2	Endospore	Negative
3	Growth at 5% NaCl	Positive
4	Growth at 40°C	Negative
5	Catalase	Positive
6	Oxidase	Negative
7	Citric acid utilization	Positive
8	Indole production	Negative
9	Urease	Negative
10	methyl red	Positive
11	Motility	Positive
12	VP (Voges Proskauer)	Negative

Table VI.-. Biochemical Characteristics of Most Efficient PAHs Degrading Bacterial Isolate No.1

Characterizing the cultural, morphological and metabolic features of putative PAH-degrading isolates facilitates their precise taxonomic classification, beyond the genus-level identification afforded by 16S rRNA sequencing alone (Singleton *et al.* 2009). Parameters including cell shape, colony pigment, growth temperature/pH optima and salt tolerance can pinpoint the isolate to species-level when matched to databases like Bergey's Manual of Determinations for systematic assignment (Gallego *et al.* 2001). Analyzing different biochemical and physiological patterns can also indicate closeness to target species based on similarity indices. Polyphasic phenotypic metrics combined with chemotaxonomic markers thus generate a metabolic fingerprint for deducing isolate identity, while highlighting adaptable strains for contaminated site conditions (Samanta *et al.* 2002, Dean-Ross *et al.* 2002). Moreover, detecting enzymes produced by PAHs-degrading bacteria directly profiles catabolic pathways involved in PAH biotransformation. Linking this degradative capacity to taxonomic position focuses strain selection efforts on characterized species with proven pollutant-mineralization ability, aiding deployment at field sites requiring bioaugmentation intervention.

Based on these findings, it can be inferred that the bacterial isolate (Isolate N^{o} 1) shares similarities with those recognized as *Enterobacter sp.*, as evidenced by the comparison of data presented in tables V and VI with information reported elsewhere (Krieg & Holt 1984, Zhang *et al.* 2013, Guo *et al.* 2012, Bahamdain *et al.* 2015). The 16S rRNA analysis (Boye *et al.* 1999) further supports this conclusion, as depicted in Fig. 8, illustrating the phylogenetic tree of the PAH-degrading bacterial isolate (Isolate N^{o} 1) and its relationship to other bacterial species based on the 16S rRNA sequence.

Notably, (Isolate N^{o} 1) appears closely related to the strain *Enterobacter asburiae*. This polyphasic appraisal combining phenotypic, chemotaxonomic and genotypic analyses provides consistent evidence that Isolate N^{o} 1 belongs to the *Enterobacter asburiae* species, which includes strains proven to possess PAH biodegradation capabilities.

Nucleotide sequence alignment between a Query sequence and a Subject sequence (*Enterobacter asburiae* strain JM-458 (NR_145647.1) 16S ribosomal RNA, partial sequence):

```
Query  1     TCCACCGCCGGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATCGACGGGGGCCCGC  60
             || |||||| |||||||||||||||||||||||||||||||| |||||||||||||
Sbjct  832   TCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGC  891

Query  61    ACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTACTCTTGA  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  892   ACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTACTCTTGA  951

Query  121   CATCCAGAGAACTTTCCAGAGATGGATTGGTGCCTTCGGGAACTCTGAGACAGGTGCTGC  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  952   CATCCAGAGAACTTTCCAGAGATGGATTGGTGCCTTCGGGAACTCTGAGACAGGTGCTGC  1011

Query  181   ATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCC  240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1012  ATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCC  1071

Query  241   TTATCCTTTGTTGCCAGCGGTTAGGCCGGGAACTCAAAGGAGACTGCCAGTGATAAACTG  300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1072  TTATCCTTTGTTGCCAGCGGTTAGGCCGGGAACTCAAAGGAGACTGCCAGTGATAAACTG  1131

Query  301   GAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGAGTAGGGCTACACACGTGC  360
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1132  GAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGAGTAGGGCTACACACGTGC  1191

Query  361   TACAATGGCGCATACAAAGAGAAGCGACCTCGCGAGAGCAAGCGGACCTCATAAAGTGCG  420
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1192  TACAATGGCGCATACAAAGAGAAGCGACCTCGCGAGAGCAAGCGGACCTCATAAAGTGCG  1251

Query  421   TCGTAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGT  480
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1252  TCGTAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGT  1311

Query  481   AGATCAG  487
             |||||||
Sbjct  1312  AGATCAG  1318
```

Biodegradation Assay of Polycyclic Aromatic Hydrocarbons

An experiment was conducted to assess the biodegradation of three polycyclic aromatic hydrocarbons (PAHs) – Naphthalene, Fluorene, and Anthracene – by *Enterobacter asburiae* strain N^{o} 1. The results from the biodegradation assay indicated residual concentrations of 1.05 ppb for Naphthalene, 0.516 ppb for Fluorene, and 0.862 ppb for Anthracene after inoculation with 1 ml of bacterial suspension (12*10^{7} cfu/ml) of *Enterobacter asburiae* strain N^{o}1.

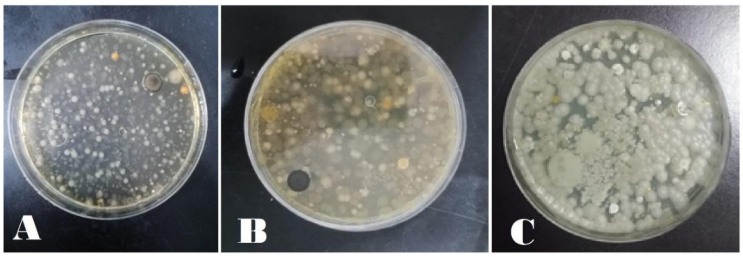

Fig. 7.- Images depicting the complex growth of PAHs-degrading bacteria on minimal salt medium plates supplemented with PAHs (1 µg/L). Specifically, (A) represents the complex growth culture derived from the Kitchener Drain sample, (B) depicts the complex growth culture from the Total gas station sample, and (C) illustrates the complex growth culture originating from the Neamaa restaurant sample

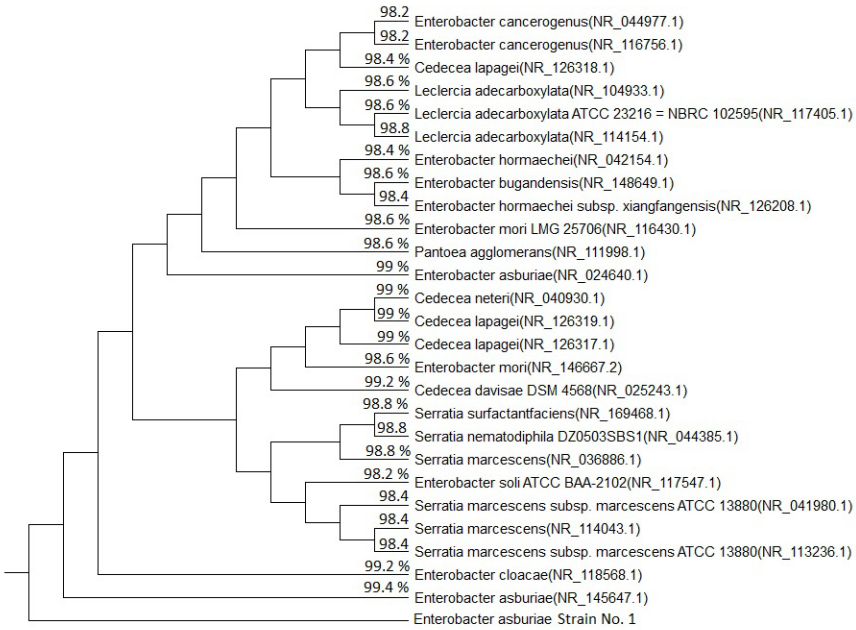

Fig. 8.-. Phylogenetic dendrogram obtained by distance matrix analysis of 16S rRNA sequences, showing the position of strain *Enterobacter asburiae* strain No. 1 among phylogenetic neighbors

The experiment extended over 14 days at 35 °C with a pH of 7.0. Considering the initial concentration of PAHs as 5 ppb, the removal percentages for the three PAH compounds were determined as 79 %, 89.7 %, and 82.8 %. The experiment was repeated twice, and averages along with standard deviations were calculated. Table VII presents the duplicate experiments, showcasing residual concentrations of PAHs after a 14-day incubation period, including calculated means and standard deviations.

In the control experiment, where no bacterial presence was introduced, the residual concentrations after the incubation period were measured as 4.92 ppb for Naphthalene, 4.85 ppb for Fluorene, and 4.95 ppb for Anthracene. Fig. 9 illustrates the removal percentages for the three PAH compounds, taking into account abiotic loss. The abiotic loss percentages for Naphthalene, Fluorene, and Anthracene were

recorded as 1.6 %, 3.1 %, and 1 %, respectively.

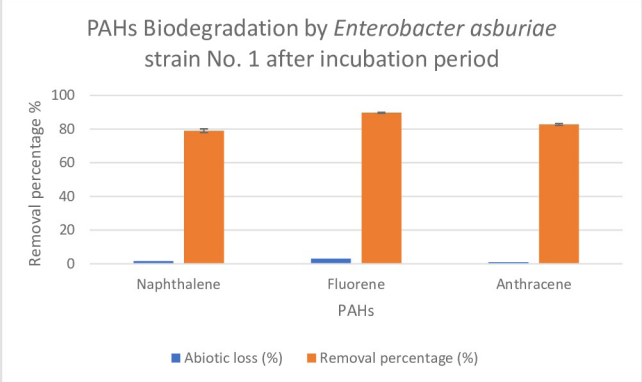

Fig. 9.- Removal percentages of three PAHs after incubation with *Enterobacter asburiae strain No. 1.* as well as abiotic loss percentages; (bar graphs represents the geometric mean of removal percentages and error bars represents the standard deviation)

Similar findings were reported by Belal *et al.* (2018) in a study using *Paenibacillus sp.* strain for testing the biodegradation of Organochlorine Pesticides in aqueous solutions. The study demonstrated efficient biodegradation ranging between 24.4 % and 98 % for different Organochlorine Pesticides after a two-week incubation period. Additionally, Sarma *et al.* (2019) developed a plant-microbes assisted remediation technology involving bacterial strains, including *Enterobacter asburiae*, for polyaromatic hydrocarbons (phenanthrene, anthracene, pyrene, and benzo[a]pyrene) degradation and heavy metals (Cr, Ni, and Pb) removal in a microcosmic experiment by bacterial strains used in two formulated consortia includes ─ Cpm1 (*Enterobacter cloacae* HS32, *Brevibacillus reuszeri* HS37, and *Stenotrophomonas sp.* HS16) and Cpm2 (*Acinetobacter junii* HS29, *Enterobacter aerogenes* HS39 and *Enterobacter asburiae* HS22); Both the consortia that were newly developed showed similar trends of metals removal and PAHs degradation.

Enterobacter asburiae's biodegradation capability was further emphasized by El Gendy *et al.* (2020), who highlighted its potential for the biodegradation and removal of Organochlorine pesticides residues in an aqueous system, affirming the reduction of toxicity in the tested organochlorine pesticides.

Numerous studies have explored various microorganisms for their ability to degrade PAHs. Ahmed *et al.* (2010) found that *Kocuria flava* and *Kocuria rosea* can grow on naphthalene, phenanthrene, and fluoranthene. Haritash & Kaushik (2016) identified two bacterial species, *Micrococcus luteus* and *Kocuria rosea*, capable of degrading low molecular weight PAHs, along with three fungal species

of *Aspergillus* showing potential for PAH remediation. Goswami *et al.* (2018) demonstrated that *Rhodococcus opacus* bacteria could degrade a mixture of PAHs, including Naphthalene, Phenanthrene, and Fluoranthene.

The biodegradation mechanism of *Enterobacter asburiae* strain 1 in PAHs degradation involves the utilization of PAHs as the sole carbon source. This perspective has been discussed in various studies, such as Igwo-Ezikpe *et al.* (2010), who isolated bacterial strains from engine-oil polluted sites in Lagos, Nigeria. These isolates, belonging to genera *Micrococcus*, *Staphylococcus*, *Kurthia sp.*, *Acinetobacter*, *Pseudomonas*, and *Corynebacterium*, exhibited varying rates of growth on PAHs (anthracene, fluoranthene, and pyrene) as sole sources of carbon and energy. The findings suggest that PAHs degradation may be plasmid and/or chromosomally mediated, depending on the bacterial isolate and the specific PAHs being degraded. Igwo-Ezikpe *et al.* (2010) also revealed that different compounds induce varied genetic changes in bacterial isolates in response to stimuli.

	initial	control Experiment conc.	Experiment No. 1 Concentration	Experiment No. 2 (Duplicate) Concentration	Average concentration	Standard deviation
Naphthalene	5	4.92	1.01	1.09	1.05	0.06
Fluorene	5	4.845	0.505	0.527	0.516	0.02
Anthracene	5	4.95	0.882	0.842	0.862	0.03

Table VII.-. Residual concentrations of three PAHs after incubation with *Enterobacter asburiae strain N^{o} 1.*

Toxicity assessment of biodegradation byproducts

Due to the possibility of formation of toxic intermediate products during the biodegradation of Polycyclic Aromatic Hydrocarbons, the evaluation of biodegradation ratio was not sufficient to decide the effectiveness of the biodegradation process; thus, the possible toxic impact of intermediate products should be taken into account, and the toxicity of the degradation intermediate products of the tested PAHs should be confirmed by the experiment. Polycyclic Aromatic Hydrocarbons degrading bacterium Enterobacter asburiae (Isolate N^{o} 1) had the ability to biologically degrade approximately 79 %, 89.7 % and 82.8 % of Naphthalene, Fluorene and Anthracene active ingredients respectively with initial

concentration 5 ppb under optimum condition.

Toxicity of the remaining PAHs compounds in the aqueous solution after 14 days of incubation with *Enterobacter asburiae* (Isolate N^{o}1) was evaluated using Bacillus subtilis as a target organism. The results of the toxicity assessment showed that the PAHs compounds after 14 days of incubation had no toxicity; consequently, there was no antibacterial activity could be detected against *B. subtilis* as a test organism. The obtained result was compared with control treatment (active ingredients of PAHs without bacterial activity) which revealed 100 % inhibition against *B. subtilis* growth under the same conditions as showed in Fig. 10. This implied that the aqueous solutions spiked with three PAHs compounds separately were detoxified after 14 days of treatment with Enterobacter asburiae (Isolate N^{o}1). Table VIII represented the percentages % of the inhibition caused by supernatant of PAHs compounds treated with bacterial isolates against the target organism *Bacillus subtilis* in comparison to the inhibition in the control experiment. Fig. 10 represented the control experiment and showed the 100 % inhibition of Bacillus subtilis growth; and revealed the bioremediation of PAHs without any antibacterial activity against the target organism (*Bacillus subtilis*). Polycyclic Aromatic Hydrocarbons have serious toxic and even lethal effects on other microorganisms' growth and metabolism.

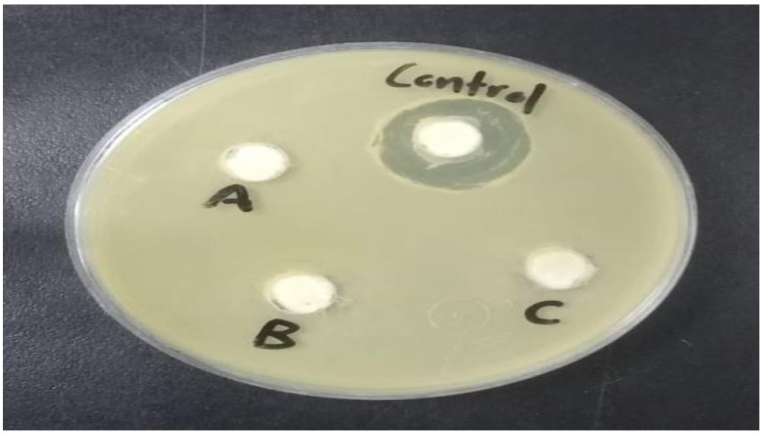

Fig. 10.- Toxicity bioassay experiment showing the growth of *Bacillus subtilis* without inhibition with supernatant of biologically treated PAHs compounds in aqueous solutions compared to the inhibited growth in the control experiment; A, control experiment showing the inhibition zone of *Bacillus subtilis* growth by supernatant of untreated PAHs compounds; B, supernatant of Naphthalene treated with *E. asburiae* (Isolate N^{o}1); C, supernatant of Fluorene treated with *E. asburiae* (Isolate N^{o} 1); D, supernatant of Anthracene treated with *E. asburiae* (Isolate N^{o}1)

The extensive toxicity assessment throughout the degradation process would yield valuable insights into the application of microbe-focused approaches for the bioremediation of polycyclic aromatic hydrocarbons (PAHs) and ensures the safety of organisms and the surrounding environment from potential hazardous secondary metabolites resulting from the degradation

process. Shuttleworth & Cerniglia (1995) indicated that degradation products of PAHs are, however, not necessarily less toxic than the parent compounds; and therefore, toxicity assays need to be incorporated into the procedures used to monitor the effectiveness of PAH bioremediation.

Experiment	Incubation period	*Bacillus subtilis* growth inhibition %
Naphthalene treated by E. *asburiae (*Isolate No. 1*)*	14 Days	0
Fluorene treated by E. *asburiae (*Isolate No. 1*)*	14 Days	0
Anthracene treated by E. *asburiae (*Isolate No. 1*)*	14 Days	0
Control treatment (untreated PAHs)	14 Days	100

Table VIII.- The percentages % of the inhibition caused by supernatant of treated/untreated PAHs against the target organism *Bacillus subtilis*

Wulandari *et al.* (2021) showed that the newly isolated *Trametes polyzona* PBURU 12 demonstrated a high tolerance and potential for the degradation of phenanthrene. The fungal isolate was able to tolerate 100 ppm of phenanthrene with 45 % relative growth and proved that the biodegradation metabolites showed the absence of toxic compounds as microbial viability tests using *E. coli* and *B. subtilis* revealed that the treated phenanthrene was less toxic than untreated phenanthrene; also phytotoxicity and genotoxicity tests, using *Vigna radiata* and *Allium cepa*, indicated that the treated phenanthrene was less toxic to the plants and no mutagenic activity was found in the Ames test. *In another study,* Shanker *et al.* (2017) examined the degradation of selected toxic PAHs (3-5 rings) using potassium zinc hexacyanoferrate (KZnHCF) nanocubes and proved higher proficiency of the catalyst regarding PAHs degradation into small and non-toxic by-products such as malealdehyde, 4-oxobut-2-enoic acid and o-xylene. *On the other hand,* Wang *et al.* (2021) investigated the accumulated pattern of the metabolites of phenanthrene biodegradation by *Rhodococcus qingshengii* strain FF and evaluated and their toxicity to *Vibrio fischeri*, effect on microbiota diversity of farmland soil and influence on seed of wheat, and indicated that the accumulated metabolites in later phase were more toxic to *Vibrio fischeri*, microbe and wheat seed response to the different stages of phenanthrene metabolites indicated pollution significantly decreased microbial richness and evenness of farmland soil and lower germinal length, root length or root number of wheat seed.

CONCLUSION

Three wastewater sources in Kafr El-Sheikh governorate, Egypt, underwent testing to determine the presence of Polycyclic Aromatic Hydrocarbons (PAHs) and were found to contain varying concentrations of PAHs (ranging from 0.42 to 253.1 ppb). The biodegradation of PAHs was distinctly observed using different bacterial isolates in aqueous media. Numerous bacterial isolates with the capability of degrading PAHs were obtained through an enrichment technique from wastewater samples. These isolates were systematically screened to select the most efficient ones, followed by characterization, identification based on morphological and biochemical characteristics, and 16S rRNA analysis. Subsequently, these isolates were subjected to testing for their ability to biodegrade PAHs. *Enterobacter asburiae* strain 1 demonstrated the capacity to biologically degrade approximately 79 %, 89.7 %, and 82.8 % of the active ingredients of Naphthalene, Fluorene, and Anthracene, respectively. Furthermore, the results of the toxicity assessment indicated that the PAHs compounds, after a 14-day incubation period, underwent biodegradation, and secondary metabolites were found to have no toxicity. Consequently, no antibacterial activity could be detected against *Bacillus subtilis*, employed as a test organism. This study advocates for the use of *Enterobacter asburiae* strain for the safe breakdown of PAHs found in industrial wastewater before reaching surface water. The biological degradation of PAHs compounds offers a more functional, cost-effective, and thermodynamically affordable process for the elimination of such hazardous residues.

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Received on November 23, 2023
Accepted on November 18, 2024
Associate editor: D. Stien