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Short Communication

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Utilization of two Bacterial Strains *(Ochrobactrum Intermedium* BC1 and *Cupriavidus Taiwanensis* LA) to Biodegrade Anthracene, Fluorene, and Naphthalene.

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Abstract

Polycyclic aromatic hydrocarbons, known as PAHs, typically persist in the environment, exposing humans to a considerable health hazard because of the toxins they contain and their capability of triggering cancer. Anthropogenic activities have introduced high levels of PAHs into Arabian Gulf countries' soil and coastal waters. Numerous studies have indicated that diverse bacterial strains can successfully break down PAHs. The deduction made is that biodegradation stands as the top choice in terms of safety, effectiveness, and affordability when it comes to handling PAH-contaminated sites and locations. The efficiency of degrading three PAHs was analyzed in this study with the use of two novel bacterial strains, considering the optimal temperature and pH requirements. Coastal sediments from the Eastern Province of Saudi Arabia yielded pure cultures of *Ochrobactrum intermedium* BC1 and *Cupriavidus taiwanensis* LA, which were subjected to spiking with 100ppm concentrations each for anthracene, fluorene, and naphthalene. They were then incubated at 25°C in a shake incubator for 18 days. A solid-phase micro-extraction (SPME) device was used. The extraction of residual PAHs was the main objective of using the SPME device. Gas Chromatography/Mass Spectrometry (GC-MS) was utilized to quantify and analyze the residues at predefined time intervals. By the conclusion of the 18-day timeframe, *Ochrobactrum intermedium* BC1 degraded naphthalene completely. Additionally, anthracene experienced a reduction of approximately 87%, while fluorene underwent a decrease of about 67. *Cupriavidus taiwanensis* LA degraded anthracene, fluorene, and naphthalene by 88%, 53%, and 91% respectively. The degrading efficiency of these novel strains of bacteria is evidenced by these results. In closing, these strains can be considered potential members of a consortium of microbes capable of degrading PAHs that can be employed effectively in various cleanup endeavors.

Introduction

Polycyclic aromatic hydrocarbon (PAH) is the name given to hydrocarbons that have fused benzene rings [1]. There are several of these benzene rings, and they can range in size. The simplest examples are naphthalene (two rings), anthracene, fluorene, and phenanthrene (each with three rings) [2]. The quantities of PAHs in the environment have been considerably increased by anthropogenic activities. PAHs are primarily produced by human activities involving the combustion of fossil fuels, including the manufacture of coal and petroleum products, vehicle emissions, power generation, and industrial furnaces [3]. Manufacturing, waste incineration, and the synthesis of specific compounds like coal tar and pitch are examples of industrial processes that release PAHs into the environment as byproducts or through unintentional spills and leaks [4]. Saudi Arabia is one of the largest producers and exporters of oil in the world [5]. Despite being crucial for the Kingdom's economy, this thriving oil industry presents a significant threat in terms of oil pollution and hydrocarbon pollutants [6,7]. As a result, it is now of utmost importance to adopt methods to reduce the impact of PAH contaminants [8,9].

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PAHs are only partially soluble in water and tend to adhere strongly to organic debris, sediments, and soils. As a result, PAHs are typically found in higher concentrations in contaminated soils and sediments, particularly in areas closest to pollution sources [10]. Additionally, PAHs can bioaccumulate within organisms and move up the food chain, harming both aquatic and terrestrial ecosystems. Given that PAHs have been proven to cause cancer, their environmental persistence is of concern [11,12]. They turn into genotoxic substances when specific human enzymes convert them into DNA adducts. DNA adducts can lead to mutations that eventually result in cancerous tumors in human tissues [13]. The United States Environmental Protection Agency (USEPA) has designated sixteen (16) PAHs as major pollutants; benzo[a]pyrene, chrysene, pyrene, dibenz[a,h]anthracene, fluoranthene, fluorene, naphthalene, benzo[b]fluoranthene, acenaphthene, acenaphthylene, phenanthrene, indeno[1,2,3-ed]pyrene, benzo[ghi]perylene, anthracene, benz[a]anthracene, benzo[k]fluoranthene [14].

The best, safest, and most affordable method for removing PAH pollutants is biodegradation [15]. It has been reported that many microorganisms, including bacteria, fungus, and actinomycetes, are capable of degrading PAHs [16]. The principal degraders of PAHs are bacteria, bacterial genera like *Bacillus, Paenibacillus, Rhodococcus, Pseudomonas, Mycobacterium*, and *Burkholderia* have been thoroughly investigated for their potential to break down different types of PAHs [17-20]. In addition, studies from various part of the world have used other species of bacteria to degrade a wide range of PAHs [21-25].

This study seeks to contribute to the body of knowledge on the use of indigenous bacterial species within the Kingdom of Saudi Arabia to degrade PAHs. It investigates the efficacy of degradation of anthracene, fluorene and naphthalene by two (2) novel bacterial strains; *Ochrobactrum intermedium* BC1 and *Cupriavidus taiwanensis* LA, at optimum environmental conditions.

Materials and Method

Chemicals

Analytical-grade anthracene, fluorene, and naphthalene were acquired from SigmaAldrich (St Louis, MO, USA), stock solutions of each PAH were made and kept for subsequent use. Bushnell Haas minerals medium (BH) was made in accordance with the protocol, it consists of $CaCl_2$, 0.02g/L; KH_2PO_4, 1.0g/L; $MgSO_4$, 0.2g/L; NH_4NO_3, 1.0g/L and $FeCl_3$, 0.05g/L [26]. All the chemicals were of high-quality scientific standards (99% purity).

Microorganisms

The bacterial strains *Ochrobactrum intermedium* BC1 and *Cupriavidus taiwanensis* LA that were employed in this study were isolated and cryopreserved from an earlier work [27]. These strains were activated following a pre-culture in nutrient broth.

Biodegradation Experiment

BH media containing phosphate buffer solution which maintain the pH at 7.0 ± 0.2 were sterilized via autoclaving at 121°C for 15 minutes to eliminate all biota with the potential to degrade PAHs. 2ml of a specific PAH (from a 5000ppm stock solution) was introduced to an Erlenmeyer flask along with 2ml of bacterial inoculum and 96ml of BH medium, resulting in an initial concentration of 100ppm of each PAH in every flask. Multiple flasks were prepared this way, so that there were flasks for anthracene, fluorene, and naphthalene. Additionally, control flasks were made without a bacterial cell inoculum. This was done to account for the loss of PAHs brought on by abiotic causes. Flasks were incubated for 18 days at pH 7 and 25°C while being continuously shaken at 120 rpm in a WiseCube Fuzzy System (model WIS-20) shake-incubator. A control and two replicates were included in the experimental design.

Sample Extraction

Over the course of the 18-day experiment, residual PAHs were extracted from the degradation experiments at intervals of 3 days. A solid-phase micro-extraction (SPME) technology was used for the extraction. With the help of SPME, target analytes can be extracted from aqueous samples effectively and without the need for traditional solvents [28]. The residual PAHs were removed by placing the SPME fiber into the flask containing the samples and agitating the sample with a magnetic stirrer for 20 minutes [29].

GC-MS Analysis

Residual PAHs were determined and measured using GC-MS. The GC equipment used in this study has the following specifications: Injector unit (series 7683B), MS unit (inert XL EI/CI MSD), and Agilent Technologies (series 6890N). The GC-MS was used under the following conditions: 250°C was specified as the inlet temperature. Initially set at 50°C, the oven's temperature gradually rose to 280°C over the course of 20 minutes.

Injecting the SPME fiber into the GC-MS system's injection port caused the extracted residues to desorb into the GC column. A chromatogram was produced after the GC-MS instrument had been running for 20 minutes. The peak area of this chromatogram was examined and integrated, and the data obtained were used to calculate the amount of residual PAHs.

Statistical Analysis

The experimental data gathered from biodegradation experiment during the course of the 18-day incubation period were analyzed using Microsoft Excel and Sigma Plot software 11.1.

Results and Discussion

Morphology of Bacterial Strains

Ochrobactrum intermedium BC1 and *Cupriavidus taiwanensis* LA grew abundantly on nutrient agar and form large colonies (Figure 1).

Figure 1 Photograph of plates of *Ochrobactrum intermedium* BC1 and *Cupriavidus taiwanensis* LA

Analysis of Residual PAHs

A GC/MS analysis was conducted on samples that had been spiked with anthracene, fluorene, and naphthalene at the conclusion of the biodegradation experiments. The mass spectra of anthracene, fluorene, and naphthalene, as well as GC chromatograms, are displayed in Figure 2,3 and Figure 4 respectively.

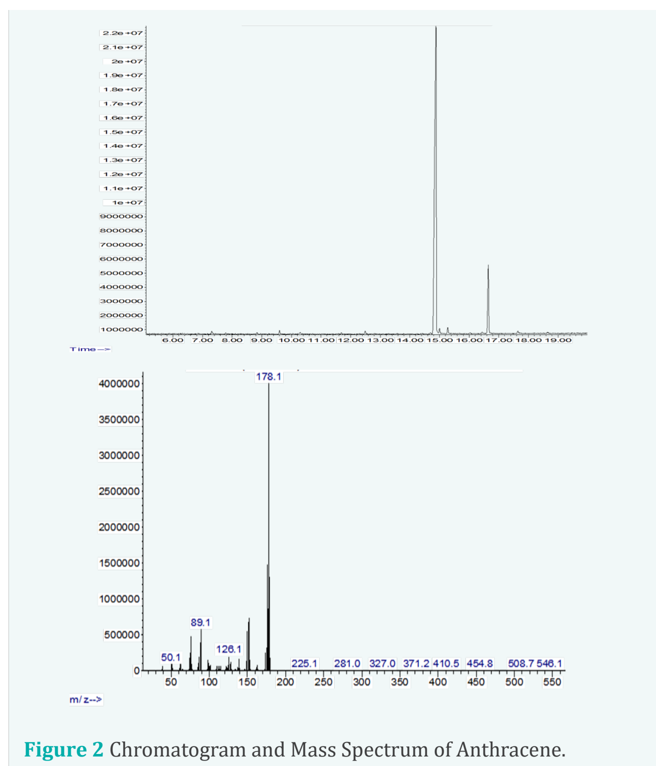

Figure 2 Chromatogram and Mass Spectrum of Anthracene.

Anthracene Biodegradation

At the end of the 18-day period, both strains of bacteria degraded anthracene at a similar rate. Compared to the 88% decomposition rate of *Cupriavidus taiwanensis* LA, *Ochrobactrum intermedium* BC1 degraded 100ppm of anthracene by 87% Figure 5 and Table 1. This result is comparable to reports from similar studies. For instance, *Ochrobactrum anthropi* has been shown to grow rapidly in high concentration of anthracene [30], and consortia containing a strain of *Ochrobactrum,* have

been reported to completely degrade anthracene in 8-10 days [31,32]. Other studies have reported efficient degradation of anthracene using strains of *Sphingomonas* sp. and *Stenotrophomonas maltophilia* [33,34].

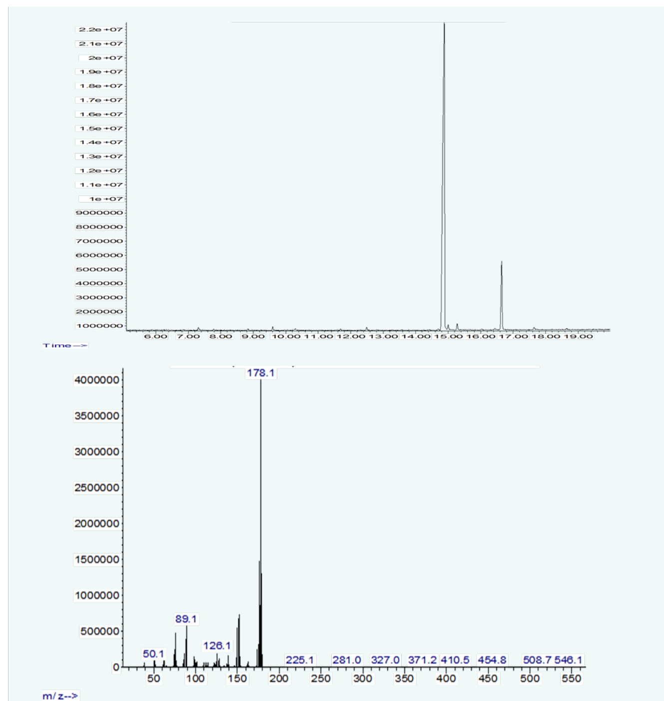

Figure 3 Chromatogram and Mass Spectrum of Fluorene..

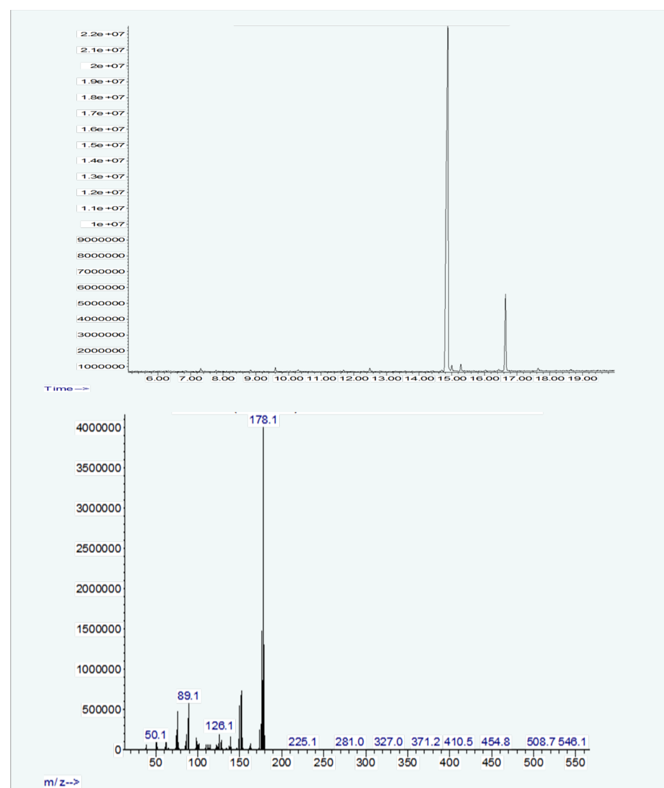

Figure 4 Chromatogram and Mass Spectrum of Naphthalene.

Table 1: Levels of unmetabolized anthracene (ppm) in bacterial cultures at day 18. (SD = standard deviation).

Bacterial Strain	Mean ± SD
Ochrobactrum intermedium **BC1**	12.72 ± 4.58
Cupriavidus taiwanensis **LA**	12.26 ± 3.73
Abiotic Control	94.82 ± 3.09

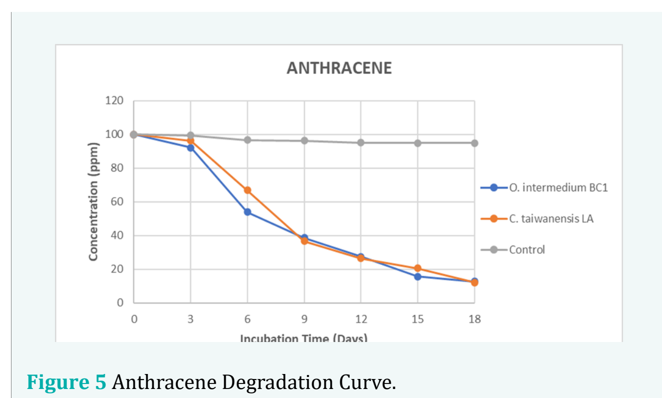

Figure 5 Anthracene Degradation Curve.

Fluorene Biodegradation

The metabolism of fluorene by both strains was relatively slow compared to the other two PAHs examined in this study. *Ochrobactrum intermedium* BC1 decreased 100ppm of fluorene by 67% in 18 days, while *Cupriavidus taiwanensis* LA degraded the PAH by 53% Figure 6 and Table 2. This is not surprising considering that fluorene has been shown to be resistant to degradation when a single bacterial species is used [35,36]. However, when a consortium is utilized for the biodegradation tests, degradation of fluorene has been demonstrated to be much more efficient [37,38].

Naphthalene Degradation

Ochrobactrum intermedium BC1 degraded naphthalene completely, and *Cupriavidus taiwanensis* LA achieved degradation at the rate of 91% Figure 7 and Table 3. The high efficiency of naphthalene degradation demonstrated by these strains is likely due to the simple nature of naphthalene (two benzene rings) [2]. A few studies have reported similar results with a variety of bacterial species, such as, *Janthinobacterium, Paraburkholderia aromaticivorans, Polaromonas, Proteus mirabilis, Pseudomonas aeruginosa, Pseudomonas alcaligenes, Rhodococcus quinshengi, Sphingomonas paucimobilis* and *Stenotrophomonas rhizophila* [39-43].

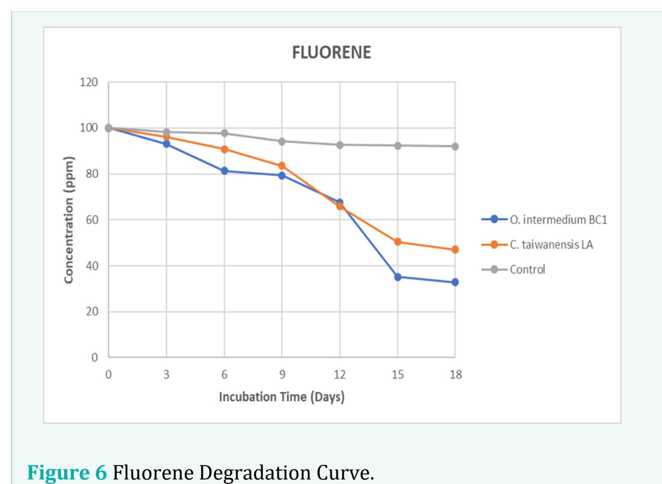

Figure 6 Fluorene Degradation Curve.

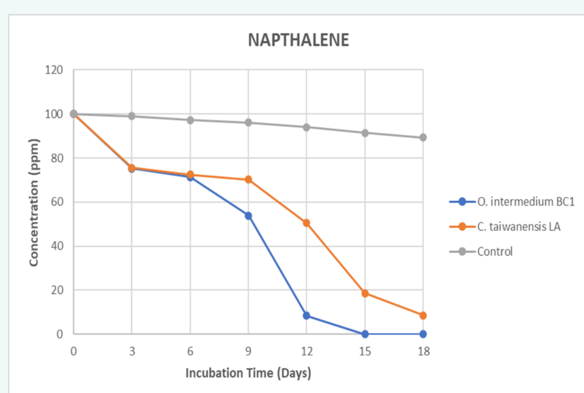

Figure 7 Naphthalene Degradation Curve.

Table 2: Levels of unmetabolized fluorene (ppm) in bacterial cultures at day 18. (SD = standard deviation).

Bacterial Strain	Mean ± SD
***Ochrobactrum intermedium* BC1**	32.85 ± 1.04
***Cupriavidus taiwanensis* LA**	47.09 ± 1.31
Abiotic Control	91.96 ± 1.13

Table 3: Levels of unmetabolized naphthalene (ppm) in bacterial cultures at day 18. (ND = not detected, SD = standard deviation).

Bacterial Strain	Mean ± SD
***Ochrobactrum intermedium* BC1**	ND
***Cupriavidus taiwanensis* LA**	8.63 ± 2.11
Abiotic Control	89.38 ± 1.92

Conclusion

The result of this study demonstrates the efficiency of the isolated bacterial strains in degrading anthracene, fluorene, and naphthalene. *Ochrobactrum intermedium* BC1 degraded naphthalene completely and reduced the concentration of anthracene and fluorene by 87% and 67% respectively. *Cupriavidus taiwenensis* LA decreased anthracene, fluorene, and naphthalene by 88%, 53%, and 91% respectively.

This high efficacy of degradation capacity of these two bacterial strains suggest that they can be optimized for bioremediation of contaminated environments and potential members of a consortium of microbes capable of degrading PAHs that can be employed effectively in various cleanup endeavors.

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