

**BIODEGRADATION OF SPECIFIC POLYCYCLIC
AROMATIC HYDROCARBONS (PAHS) BY NOVEL
BACTERIAL STRAINS ISOLATED FROM SAUDI
ARABIAN SEDIMENTS**

BY

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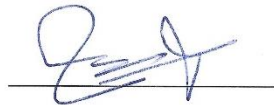
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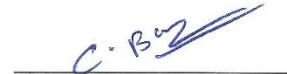
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Dedication

THIS WORK IS DEDICATED TO

My mum (Florence Mordecai), my sister (Sarah Balogun), and my fiancée (Blessing Akintola) for their unwavering and continuous support in my journey through life.

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LIST OF ABBREVIATIONS

GC	:	Gas Chromatography
MS	:	Mass Spectrometry
PAH	:	Polycyclic Aromatic Hydrocarbon
USEPA	:	United State Environmental Protection Agency
SEM	:	Scanning Electron Microscopy
SPME	:	Solid Phase Micro Extraction

ABSTRACT

Full Name : James Mordecai

Thesis Title : Biodegradation of Specific Polycyclic Aromatic Hydrocarbons (PAHs) by Novel Bacterial Strains Isolated from Saudi Arabian Sediments.

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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.

ملخص الرسالة

الاسم الكامل: جيمس مردخاي

عنوان الرسالة: التحلل الحيوي للهيدروكربونات العطرية متعددة الحلقات بواسطة سلالات بكتيرية جديدة معزولة من الرواسب في المملكة العربية السعودية

التخصص: علوم الحياة

تاريخ الدرجة العلمية: كانون الأول، 2023

عادة ما تبقى الهيدروكربونات العطرية متعددة الحلقات في البيئة مقاومة للتحلل أو التآكل، والتي يشار إليها باسم (PAHs) أختصاراً، مما يعرض البشر لخطر صحي كبير بسبب السموم وقدرتها على التسبب في السرطان. لقد أدت الأنشطة البشرية إلى إدخال مستويات عالية من الهيدروكربونات العطرية متعددة الحلقات إلى التربة والمياه الساحلية في دول الخليج العربي. أشارت العديد من الدراسات إلى أن سلالات بكتيرية متنوعة يمكنها تحطيم الهيدروكربونات العطرية متعددة الحلقات بنجاح. الاستنتاج المتوصل إليه هو أن التحلل الحيوي يمثل الخيار الأفضل من حيث السلامة والفعالية والأمثل من ناحية التكاليف عندما يتعلق الأمر بالتعامل مع المواقع والمواقع الملوثة.

في هذه الدراسة تم دراسة كفاءة تحليل ثلاثة مركبات PAHs وهي الأنثراسين والفورين والنفثالين، باستخدام سلالتين بكتيريتين جديدتين، مع الأخذ في الاعتبار متطلبات درجة الحرارة ودرجة الحموضة المثلى. أنتجت الرواسب الساحلية من المنطقة الشرقية بالمملكة العربية السعودية مزارع نقية من *Ochrobactrum intermedium* BC1 و *Cupriavidus taiwanensis* LA، والتي تم تعريضها لتركيزات قدرها 100 جزء في المليون لكل من المركبات الثلاثة. ثم تم تحضيرها عند درجة حرارة 25 درجة مئوية في حاضنة رج لمدة 18 يوماً. تم استخدام جهاز SPME، حيث كان الهدف من استخدامه استخراج PAHs المتبقية. كما تم استخدام جهاز كروماتوغرافيا الغاز / كتلة الطيف الضوئي لتحديد وتحليل هذه البقايا في فترات زمنية محددة.

وبنهاية الفترة الزمني وهي 18 يوماً، تم تحليل النفثالين بشكل تام بواسطة *Ochrobactrum intermedium* BC1. بالإضافة إلى ذلك، شهد الأنثراسين تحللاً بنسبة 87% تقريباً، بينما تحلل الفورين بنسبة حوالي 67%. كما عمل *Cupriavidus taiwanensis* LA على تفكيك الأنثراسين والفورين والنفثالين بنسبة 88% و 53% و 91% على التوالي. تتجلى الكفاءة المهيمنة والاستخدامات الواعدة لهذه السلالات البكتيرية الجديدة في هذه النتائج. ختاماً، يمكن اعتبار هذه السلالات أعضاء محتملين في اتحاد من الميكروبات القادرة على تحليل الهيدروكربونات العطرية متعددة الحلقات التي يمكن استخدامها بفعالية في مساعي الحلول البيئية المختلفة.

CHAPTER 1

INTRODUCTION

1.1 Polycyclic Aromatic Hydrocarbons (PAHs)

Hydrocarbons with fused benzene rings are referred to as "polycyclic aromatic hydrocarbons" (PAHs) [1]. These benzene rings are multiple and may be a variety of sizes. Naphthalene (with two rings), anthracene, fluorene, and phenanthrene (each with three rings) are considered the simplest examples [2]. PAHs are non-polar, planar, uncharged, lipophilic, mostly insoluble in water, and a lot of them are devoid of color [3]. They are contaminants that are widespread and persistent in the environment. PAHs are lipophilic, they often accrue and concentrate in the adipocytes of various animals resulting in bioconcentration and biomagnification in food chains. Thus, they pose significant environmental concern and human health risk due to having harmful biological effects, toxicity, mutagenicity, and carcinogenicity [4].

1.2 Sources of PAHs in the Environment

PAHs are introduced into the environment via natural and anthropogenic sources [5]. Natural sources of PAHs include geological processes like volcanic eruptions and forest fires. The combustion of organic material found in lava and volcanic ash causes significant amounts of PAHs to be discharged into the environment during volcanic activity. Burning of vegetation and organic materials during forest fires also produces and releases PAHs into the atmosphere [6,7]. PAHs can also be formed through the natural decomposition of plant and animal debris in soils and sediments [5].

But in recent decades, anthropogenic activities have dramatically elevated the levels of pollutants such as PAHs in the surroundings. Human activities associated with the burning of fossil fuels, such as the production of coal and petroleum products, vehicular emissions, power generation, and industrial furnaces, are major sources of PAHs [8]. Industrial activities, such as manufacturing, waste incineration, and the production of certain chemicals like coal tar and pitch, also release PAHs into the environment as byproducts or through accidental spills and leaks [9].

PAHs tend to bind tightly to organic matter, sediments, and soils and are only partially soluble in water [10]. Because of this, PAHs are frequently found in higher amounts in contaminated soils and sediments, especially in locations close to pollution sources. Water bodies, such as rivers, lakes, and oceans, can also serve as sinks for PAHs. PAHs enter these water bodies through surface runoff, leaching from contaminated soils, and direct discharge of industrial and municipal wastewater [11]. Areas with high anthropogenic activities tend to become hotspots of contamination, thus high levels of PAH contamination

are frequently found in urban areas, industrial locations, and regions close to highways and busy traffic [12].

1.3 Toxicity of PAHs

PAHs have been shown to be carcinogenic [13]. They become genotoxic when certain human enzymes metabolize them into DNA adducts. For example, cytochrome P450 monooxygenase oxidizes the aromatic rings of PAHs to epoxide and epoxide intermediates [14]. These intermediates are very reactive and through a variety of mechanisms attack the DNA, forming covalent adducts. DNA adducts can cause mutations, ultimately resulting in carcinogenic tumors in human tissues [14].

PAHs are highly chemically stable and long-term persistence of PAHs in the environment is a common occurrence. Because PAHs can bioaccumulate in species and travel up the food chain, endangering both aquatic and terrestrial ecosystems, their environmental persistence is a cause for concern [4].

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 [15].

1.4 Properties of PAHs

PAHs exhibit a range of physical and chemical properties that contribute to their behavior in the environment and their potential impacts on human health and ecosystems. The molecular structure of PAHs is responsible for most of their unique features. They generally possess multiple fused aromatic rings, the number of these rings can vary from two-ring structures, such as naphthalene, to complex compounds with several fused rings, such as benzo[a]pyrene [2]. The greater the number of rings, the larger the size and mass of the molecule. The solubility, volatility, and stability of PAHs are influenced by their molecular size and mass [16].

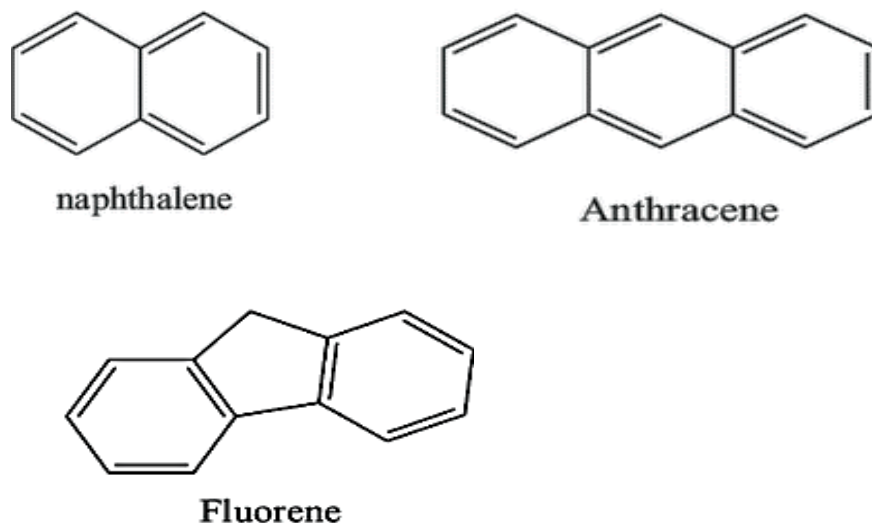


Figure 1: Structure of Specific PAHs [17].

Due to their hydrophobic nature, PAHs are poorly soluble in water. They thus tend to partition preferentially into organic phases like soils, sediments, and lipids, resulting in

their propensity to accumulate in sediment and bioaccumulate in animal tissues [18]. PAHs usually possess high values of octanol-water partition coefficient (LogKow). LogKow is a measure of the relative solubility of a chemical compound in octanol (a hydrophobic solvent) and water (a hydrophilic solvent). It quantifies the partitioning behavior of a compound between these two phases [19]. The high values of LogKow of PAHs indicate their hydrophobic nature and bioaccumulation potential. However, the solubility of PAHs in water generally improves with increases in the rings and alkyl substituents [16].

PAHs have great chemical stability, especially those with multiple rings. PAHs are resistant to hydrolysis, photolysis, and biodegradation under normal environmental conditions [20]. Their resilience to deterioration and ability to remain in the environment are both facilitated by this stability. In terms of volatility, PAHs vary with molecular weight. PAHs with lower molecular weights and fewer rings, like naphthalene, are more volatile and have higher vapor pressures, existing as gases under normal environmental conditions. Higher molecular weight PAHs, such as pyrene and benzo[a]pyrene, are more prevalent in the solid or adsorbed phase because they are less volatile [21].

PAHs can undergo various chemical reactions, including oxidation, reduction, and nitration [22]. In chemical processes involving PAHs, reactive intermediates and transformation products may be produced, some of which may be more potentially hazardous than the parent molecules [23].

Table 1: Selected Properties of Specific PAHs [24]

PAH	MW (g/mol)	SW (gm ⁻³)	LogKow	VP (Pa)	H (Pa.m ³ /mol)
Naphthalene	128.17	31	3.37	10.4	43.01
Anthracene	178.23	0.045	4.57	0.02	3.24
Fluorene	166.22	0.001992	4.18	1332	9.71

Note: Molecular Weight (MW), Solubility in Water (SW), Octanol-Water Partition Coefficient (Kow), Vapor Pressure (VP) and Henry's Constant (H).

1.5 Degradation of PAHs

Degradation of PAHs can generally be achieved through various biological, chemical, and physical methods. The chemical and physical methods available include Coker, thermal desorption, incineration, landfill and solvent extraction. These methods are either not effective, very expensive or environmentally toxic [25][26]. For instance, solvent extraction is a very effective chemical approach to PAH degradation, however it is capital intensive and there are health and environmental safety concerns associated with the various solvents employed during the extraction [27].

Thus, biodegradation is considered the best, safest and cheapest technique for treating PAH contaminants [28]. Microbial degradation of PAHs involves the enzymatic breakdown of the aromatic rings, leading to the mineralization of these compounds. The enzymes responsible for PAH degradation are typically oxygenases, which catalyze the initial reaction [29]. It has been demonstrated that a wide variety of microorganisms may break

down PAHs. Bacteria are the primary degraders of PAHs, bacterial genera like *Pseudomonas*, *Mycobacterium*, and *Burkholderia* have been thoroughly investigated for their potential to break down different types of PAHs [30].

1.6 Properties influencing Bacterial Degradation of PAHs

Microbial degradation of PAHs is influenced by a variety of environmental factors that can either enhance or inhibit the degradation processes [1]. Some key factors include oxygen availability, availability of nutrients, temperature, pH, and physical properties of the PAHs. Oxygen is a critical component in the break down of PAHs. Most microbial degradation pathways of PAHs require oxygen as an electron acceptor. As a result, in aerobic environments with plenty of oxygen, PAH breakdown is more effective than anaerobic conditions where PAH degradation rates are significantly slower [31].

The availability of essential nutrients, specifically nitrogen and phosphorus, influences bacterial degradation of PAHs. Bacterial cells require these nutrients for growth and various metabolic processes, including the degradation of PAHs. Thus, plentiful supply of nutrients promotes the growth and activity of PAH-degrading microorganisms [32].

Temperature is a pivotal factor in PAH-degradation processes [1]. Higher temperatures typically enhance the rates of microbial activity and enzymatic reactions, which speeds up the pace of breakdown. The optimal temperature range for PAH degradation varies depending on the microbial community involved and the specific PAH compound [33].

Variations in pH is another factor that influences biodegradation of PAHs. The solubility and ionization state of PAHs can be influenced by the prevailing pH, which can then have

an impact on their bioavailability and capacity for degradation. In addition, different bacterial species have specific pH optima for their growth and enzyme activity, and by extension their PAH-degrading activities [34].

The physical features of PAHs, such as weight, solubility, and volatility, can influence their degradation rates and pathways [35].

1.7 Objective

The objective of this study is to test the efficacy of *Ochrobactrum intermedium* BC1. and *Cupriavidus taiwanensis* LA. in degrading the following PAHs;

- Anthracene
- Fluorene
- Naphthalene

At pH 7 and 25°C.

1.8 Problem Statement

Saudi Arabia ranks among the top producers and exporters of oil worldwide [36]. This robust oil industry, while vital for the Kingdom's economy, poses challenges in terms of oil pollution and hydrocarbon contaminants. Oil pollution can occur via oil spills during offshore exploration, transportation accidents, leaks from pipelines, and operational discharges from oil refineries and petrochemical plants [37]. In addition, industrial activities, transportation, waste incineration, and wastewater discharges also release

significant number of pollutants into Saudi Arabian soils and coast waters. PAHs are one group of contaminants that have garnered tremendous attention.

The PAH contamination in Saudi Arabia has been a cause for concern due to the potential adverse effects on human health and the environment [38][39]. As a result, the government and the appropriate regulatory bodies have taken action to address PAH contamination in Saudi Arabia [40]. For instance, the Kingdom has been investigating and adopting bioremediation techniques to mitigate the effect of PAH pollutants on the quality of soil, water, and air [41]. Contaminated sites have been subjected to bio-remedial treatments, such as the introduction of microorganisms, capable of degrading hydrocarbons, to contaminated soil or water to degrade the pollutants into less harmful substances [42]. In addition, substantial investments have been made into research aimed at identifying native hydrocarbon-degrading microorganisms that are well-adapted to local environmental conditions [43].

This research seeks to contribute to the body of knowledge on the use of indigenous bacterial species to degrade PAHs. It investigates the efficacy of degradation of three (3) PAHs by two (2) novel bacterial strains at optimum environmental conditions. This study will be unique because there is no available literature describing the biodegradation of anthracene, fluorene and naphthalene by these specific bacterial strains isolated from sediments in Saudi Arabia.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

Biodegradation is achieved by utilizing diverse microorganisms to transform PAHs to H₂O, CO₂ or CH₄. The primary microorganisms that degrade PAHs in the environment are bacteria. *Bacillus*, *Rhodococcus*, *Pseudomonas*, and *Paenibacillus* species are some of the most researched bacteria for this process [44][45]. A few studies that used bacterial species in the breakdown of various PAHs are highlighted in this literature review, organized according to geographical regions.

2.2 North America

In the U.S., a study reported isolating strains of *Rhodococcus* species from contaminated sediments of the Grand Calumet River, U.S.A. They examined the strain's capacity to break down PAHs. As its only source of carbon, the *Rhodococcus* sp. grew well on fluoranthene (5%), pyrene (13%), phenanthrene (31%), and anthracene (53%) [46]. A recent study isolated six (6) strains of bacterial species from PAH-contaminated estuarine sediments near former creosoting facilities in Virginia, U.S.A. The species belonged to *Azoarcus*, *Hydrogenophaga*, and *Croceicoccus* genera, and are potential targets for biostimulation and bioaugmentation [47].

Researchers in Mexico evaluated the efficacy of a strain of *Pseudomonas putida* for the removal of PAHs from an aged oil-polluted soil of Veracruz, Mexico. They reported rapid degradation of various PAHs from the soil within 10 days [48]. A similar study showed that a strain of *Amycolatopsis nivea* metabolized pyrene and naphthalene, the study highlighted the metabolic pathways and enzymes responsible for this degradation [49].

2.3 South America

Activated sludge samples gotten from a Petrochemical Wastewater Treatment Plant in Triunfo, Rio Grande do Sul, Brazil, were enriched with benzo(a)pyrene or naphthalene. Bacterial species isolated from the enriched sludge were tested for their ability to degrade PAHs. The study reported that four strains, belonging to the *Burkholderia cepacia* complex, degraded benzo(a)pyrene by 30%, demonstrating their biotechnological potential for wastewater treatment and bioremediation [50]. In a similar vein, a *Serratia* sp. strain isolated from a peatland in Brazil, were immobilized and deployed to degrade fluoranthene. The strain successfully degraded 56% of fluoranthene per day [51].

Researchers from Argentina examined the genome of *Streptomyces* sp. MC1 for the potential to degrade phenanthrene. The genomic analysis indicates that the bacterial strain has the enzymes and metabolic complex to efficiently degrade phenanthrene [52].

2.4 Europe

Nair *et. al.*, 2008 characterized and isolated PAH-metabolizing bacteria from the Spanish shoreline of Cadiz. A total of 16 bacterial strains, capable of breaking down naphthalene, were isolated from these sample soils, and 16S rRNA homology analysis found these strains to be predominately *Bacillus* spp. [53]. A similar study in France, demonstrated the PAH-degrading ability of *Cycloclasticus* spp. and *Pseudomonas* spp. Bacterial communities containing these species, degraded 43% naphthalene and 46% phenanthrene within 9 weeks [54].

A more recent study hypothesized that the heavily polluted Sarno River, Gulf of Naples, Italy is home to unique bacterial species capable of eliminating heavy metals and PAHs. *Alcanivorax xenomutans* strain-SRM1 and *Halomonas alkaliantarctica* strain-SRM2) showed the highest PAH-degrading ability with a 60-100% efficiency rate. The researchers propose using these strains for the remediation of environments contaminated with PAHs and heavy metals [55]. Another study isolated a microbial community from subsurface soil collected at a former wood impregnation plant in northern Poland. *Acinetobacter* was the most abundant genus in the enrichment media that efficiently degraded PAHs and heavy metals [56].

2.5 Australia

Soil samples collected from manufactured gas plants in Newcastle, Australia, were mixed with pyrene, phenanthrene and benzo(a)pyrene. Screening for PAH-degrading bacteria indicated a mixed enrichment culture, dominated by an uncultured *Rhizobiaceae*. The bacterial family; *Rhizobiaceae* was shown to degrade pyrene and benzo(a)pyrene by 80-90% after 28 days [57].

2.6 Africa

A study in Egypt recorded 76% degradation of pyrene by a bacterial strain. The strain was isolated from a soil contaminated with oil. 16S rRNA gene analysis identified it as *Achromobacter denitrificans*. The study found that *Achromobacter denitrificans* degraded 76.5 mg/L of pyrene in just 18 days after being tested to see if it could use it as its sole carbon source [58].

Four bacterial strains identified as *Bacillus anthracis*, *B. cereus*, *B. mojavensis* and *B. subtilis* were isolated from oil-contaminated sludge, soil and water samples collected from Borg Al Arab City, Alexandria Governorate, Egypt. These strains showed potential for degrading PAHs, however *B. subtilis* showed the most potential, degrading 99% of anthracene in five (5) days [59].

2.7 Asia

Anthracene and pyrene decomposition was investigated by Indonesian researchers using a bacterial consortium formed from bacterium symbionts of marine sponges. Sponge samples were collected from waters around Kodingareng Keke Island, Makassar Metro City, Indonesia. Three bacteria strains which were symbionts to the sponges, were isolated, co-cultured as a consortium and tested for PAH-degrading capacity. The strains were identified as *Bacillus pumilus*, *Pseudomonas stutzeri* and *Acinetobacter calcoaceticus*. They efficiently decreased the abundance of anthracene and pyrene after 25 days of incubation [60]. A similar genus of bacteria isolated from oil contaminated soil of Rawalpindi, Pakistan, has been reported to decompose PAHs. In the study, a strain of *Bacillus marsiflavi* degraded selected PAHs by up to 46% [61].

In India, two strains of *Pseudomonas* isolated from effluent of a petroleum refinery were reported to be very efficient in metabolizing benzo(a)pyrene [62]. Another study from India investigated the degradation of a mixture of PAHs by *Kocuria flava* and *Rhodococcus pyridinivorans*. The study not only demonstrated the efficient break down of PAHs by the bacterial strains, but significant increases in the expression of genes associated with PAH-metabolism were reported [63]. A similar study examined the ability of strains of *Vibrio algiolyticus* isolated from marine sediments collected from Erwadi, Ramanathapuram, India, in degrading a combination of low molecular weight and high molecular weight PAHs. The researchers reported an 89.5% degradation rate [64].

Bacosa and Inuoe 2015 studied the capacity of bacterial communities to degrade PAHs in samples collected from tsunami sediments in Miyagi, Japan. Ten microbial consortia were

identified. The consortia were dominated by *Sphingomonas*, *Pseudomonas*, *Sphingobium*, *Dokdonella*, *Luteimonas*, and *Dokdonella*. Among these consortia, seven mineralized 100% fluorene and 95% phenanthrene in 10 days, and four only partially decompose pyrene [65]. Recently, another study by the same researchers described a bacterial consortium that was isolated from paddy rice soil in northern Japan and is highly efficient in degrading PAHs. The consortium was composed of *Achromobacter*, *Castelaniella*, *Rhodanobacter*, and *Hypomicrobium*. This consortium degraded fluorene by 100%, phenanthrene by 95%, and pyrene by 52% in 5 days [66].

Researchers from northern China, described a strain of *Achromobacter xylosoxidans* found in polluted soil at a coal chemical facility with excellent PAHs degradation capacity. The degradation efficiency rate ranged from 29.86% to 98.47% [67]. A different study from China, designed a mixed bacterial community (MBC) using the activated sludge from a coking wastewater facility. Testing this MBC against various PAHs, they found degradation rates of 99% for phenanthrene and pyrene [68]. Moreso, a strain of *Pontibacillus* sp. isolated from mangrove sediments in Dongzhai Port, Hainan Province, China, has been reported to degrade benzo(a)pyrene by 42.15% even at high salinity [69]. Another bacterial strain with the capacity to break down the aromatic rings of PAHs was extracted from a paddy soil in China and identified as *Klebsiella pneumoniae*. The strain could metabolize 55.8 % of benzo [a] pyrene and 63.4 % of pyrene in 10 days [70].

2.8 Kingdom of Saudi Arabia

Oyehan and Al-Thukair 2017 reported isolating PAH-degrading bacterial species from contaminated sediments in the Eastern Province of Saudi Arabia. 16S rRNA analysis identified the strains as *Cupriavidus taiwanensis*, *Pseudomonas aeruginosa*, *Ochrobactrum intermedium* and *Pseudomonas citronellolis*. *P. citronellolis*_LB was reported to have efficiently removed phenanthrene, it mineralized 94% of 100ppm in 15days [71]. In a similar study, three unique bacterial strains that were discovered in oil-contaminated Arabian Gulf sediment were examined for their ability to decompose PAHs. These isolates, (*Proteus mirabilis* T2A12001, *Brevibacillus brevis* T2C2008, and *Rhodococcus quinshengi* TA13008) efficiently metabolized naphthalene and pyrene [72]. Nzila *et. al.*, 2021 identified a bacterial strain from a sediment polluted with oil, collected from a petrol station, located at KFUPM, Dhahran, Saudi Arabia. 16S rRNA analysis, identified the strain as *Staphylococcus haemoliticus*. The strain had the ability to mineralize 80% Benzo(a)pyrene (BaP) [73]. The same group of researchers examined the capacity of three bacterial species to break down benzo(a)pyrene, these strains were defined as *Micrococcus luteus*, *Brad rhizobium japonicum* and *Bacillus cereus*. Each of these strains were shown to mineralize benzo(a)pyrene and using these microorganisms in a consortium made this deterioration even more evident. [74].

Another interesting study aimed to ascertain how the halophilic bacteria *Cryptococcus* sp. MR22 and *Halomonas* sp. BR04 degraded and transformed phenanthrene and anthracene. The halotolerant bacteria were isolated from a high salinity soil that had been tainted with crude oil in Saudi Arabia. *Cryptococcus* sp. MR22 completely metabolized anthracene and

phenanthrene, while *Halomonas* sp. BR04 reached a 95% degradation rate [75]. In addition, a recent study reported the isolation of a bacterial strain from petroleum-contaminated soil that demonstrated significant growth when given pyrene as its only carbon source. The strain was identified as *Klebsiella oxytoca*, and it is capable of completely metabolizing 70% of the pyrene in 12 days [76].

2.9 Studies involving Species Related to the Two Novel Strains used in this Study.

In the study by Oyehan and Al-Thukair 2017 [71], the sample sediments contained several bacterial species, among which are strains of *Ochrobactrum intermedium* and *Cupriavidus taiwanensis*. The present study seeks to investigate the PAH-degrading ability of these two strains. A few studies have suggested that these bacterial species may be able to break down PAHs.

Kuppusamy *et. al.*, 2016 demonstrated that a strain of *Cupriavidus* had the potential to degrade PAHs in the presence of heavy metals. This strain completely degraded phenanthrene, benzo(a)pyrene and pyrene within 30 days. It metabolized 90-100% of PAHs in 15 days across a range of pH and even with the addition of heavy metals [77]. A similar study identified *Ochrobactrum intermedium* along with two other bacterial strains isolated from soil samples collected from Poza Rica, Veracruz, Mexico. The study demonstrated that *O. intermedium* biodegraded fluoranthene (100%), benzo(b)fluoranthene (81.8%), and benzo(a)pyrene (87.0%) [78].

Tao *et. al.*, 2020 sort to determine if some microorganisms, assisted by plants, could remediate soils co-contaminated by cadmium and benzo(a)pyrene. Among other things, the results of the study indicated that *Ochrobactrum intermedium* is a B[a]P-degrading bacterium [79].

Li *et. al.*, 2021 found that phenanthrene-degrading bacteria that are bioaugmented with fungi have an enhanced efficiency of removing phenanthrene. Seven genera of bacteria were used in this study, one of which is *Cupriavidus* [80].

CHAPTER 3

MATERIALS AND METHODS

3.1 Microorganisms

The bacterial strains *Ochrobactrum intermedium* BC1. and *Cupriavidus taiwanensis* LA. that were used in this study, were isolated and cryopreserved from an earlier study conducted in the Life Science Department Laboratory at King Fahd University of Petroleum and Minerals [71].

These strains were activated by incubating nutrient broth containing inoculums of each strain for 48 hours until it visibly became very turbid. The turbid nutrient broth was spined at 5000 rpm for 5 min. The bacterial pellets were collected and resuspended in 10ml of normal saline and monitored until its optical density (OD 600nm) equals 1.0. This bacterial suspension was retained and used in biodegradation experiments.

3.2 Materials

Anthracene, fluorene, and naphthalene of analytical grade were purchased from SigmaAldrich (St Louis, MO, USA). The PAHs were dissolved in DMSO and stock solution of each PAH, at a concentration of 5000ppm, were prepared and utilized throughout the duration of the experiment.

Bushnell Haas minerals medium (BH) was prepared following the protocol [81]. The BH medium 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. Phosphate buffer solution was used to maintain a pH of 7.0 ± 0.2 . The media was autoclaved at 121°C for 15 minutes.

3.3 Biodegradation Experiment

The biodegradation experiments were conducted as follows. 96ml of BH media was placed in an Erlenmeyer flask (250ml), 2ml of bacterial inoculum was added to the flask, and 2ml of a specific PAH (from the 5000ppm stock solution) was also added to the flask. Multiple flasks were prepared this way, so that there were flasks for each PAH of interest. This resulted in an initial concentration of 100ppm of each PAH (anthracene, fluorene, and naphthalene) in each flask.

A control flask containing 98ml of BH media and 2ml of PAHs was prepared. In order to make provision for potential PAH depletion brought on by abiotic factors, this flask was devoid of bacterial organisms.

Flasks were shaken continuously at 120 rpm for 18 days while being incubated at pH 7 and temperature of 25°C in a WiseCube Fuzzy System (model WIS-20) shake-incubator. The experimental setup included a control, two replicates.

3.4 Analytical Techniques

3.4.1 Extraction of Sample

Extraction of residual PAHs from the degradation experiments was done at intervals of 3 days within the 18-days duration of the experiment. Solid-phase micro-extraction (SPME) device was used to extract residual PAHs from the flask containing the mixture of BH media, bacterial inoculum, and PAHs. SPME is an efficient technique for extracting target analytes from aqueous samples without the use of conventional solvents [82]. It involves submerging SPME fibers in the sample and monitoring the partitioning of analytes between the aqueous matrix and the fiber coating [83]. Thus, the residual PAHs were extracted by inserting the SPME fiber into the flask holding the samples and stirring the sample with a magnetic stirrer for 20 minutes.

3.4.2 GC-MS Analysis of Residual PAHs

GC-MS was used to detect and quantify residual PAHs. Agilent Technologies (series 6890N), Injector unit (series 7683B), and MS unit (inert XL EI/CI MSD) were the specifications for the GC system that was utilized in this study. The operating conditions of the GC-MS were as follows; Inlet temperature was set at 250°C. Initial oven temperature was 50°C, which gradually increased to 280°C, and a runtime of 20 minutes. The SPME fiber was introduced into the GC-MS system's injection port, and extracted residues desorbed into the GC column. The GC-MS system was allowed to run for 20 minutes, and

the chromatogram was obtained and analyzed. This data was used to compute the quantity of residual PAHs.

CHAPTER 4

RESULTS

4.1 Morphology

The two strains of bacteria that were utilized in this study were grown in nutrient agar and plated in petri dishes. *Ochrobactrum intermedium* BC1 and *Cupriavidus taiwanensis* LA grew abundantly on nutrient agar and form large colonies. Both strains appear to have similar morphology. Figure 2 is a photograph of the strains growing on nutrient agar plates. Scanning Electron Microscopy (SEM) was also carried out to confirm the growth of the strains on nutrient agar plate and to determine the shape of the bacteria. The results of SEM experiment indicates that both bacterial strains were rod-shape (Figure 3).

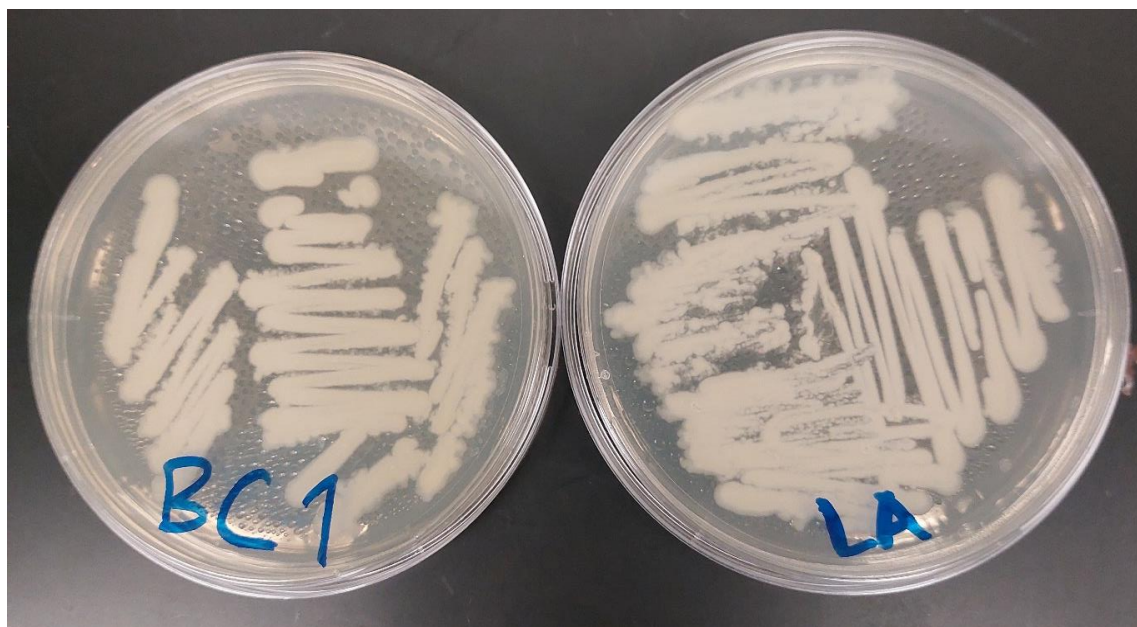


Figure 2: Photograph of plates of *Ochrobactrum intermedium* BC1 and *Cupriavidus taiwanensis* LA.

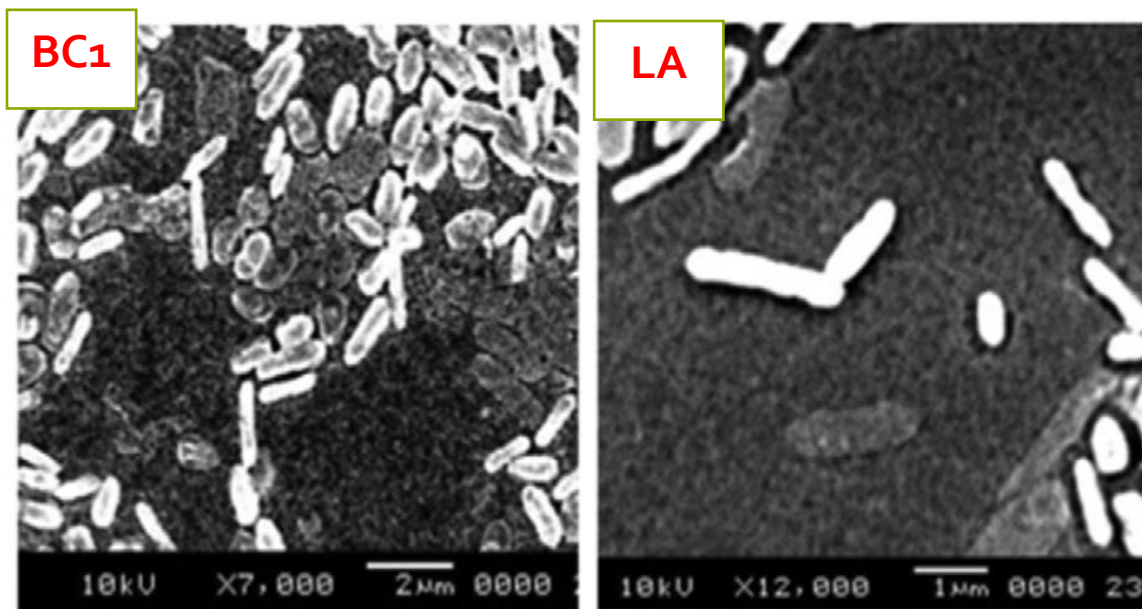


Figure 3: SEM images of *O. intermedium* BC1 and *C. taiwanensis* LA.

4.2 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 Figures 4, 5 and 6, respectively.

The GC retention time of anthracene was 15 minutes with molecular ion (M^+) at m/z of 178.1 and major fragments ions at m/z 89.1 and 150 (Figure 4). Fluorene's retention time was 12 minutes, its molecular ion at m/z was 166 with major fragments ions at m/z 82.4-126.1 (Figure 5). Retention time of 7 minutes was found for naphthalene. The molecular ion was at m/z 128.0, whereas major fragment ions at m/z were 64 and 100 (Figure 6).

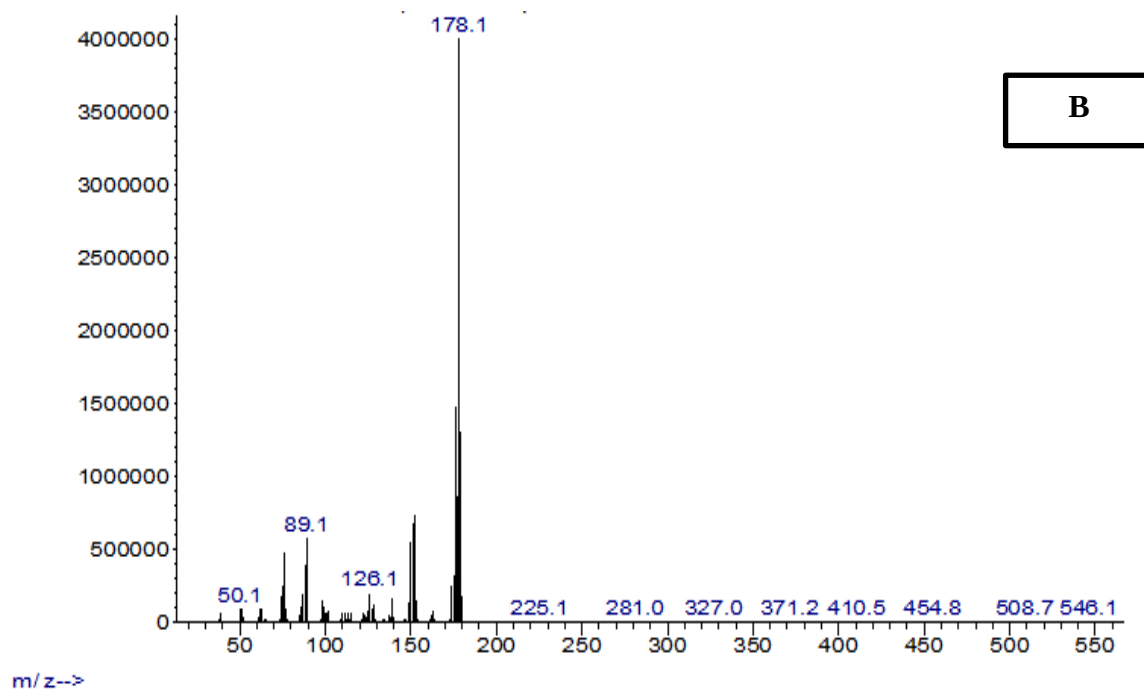
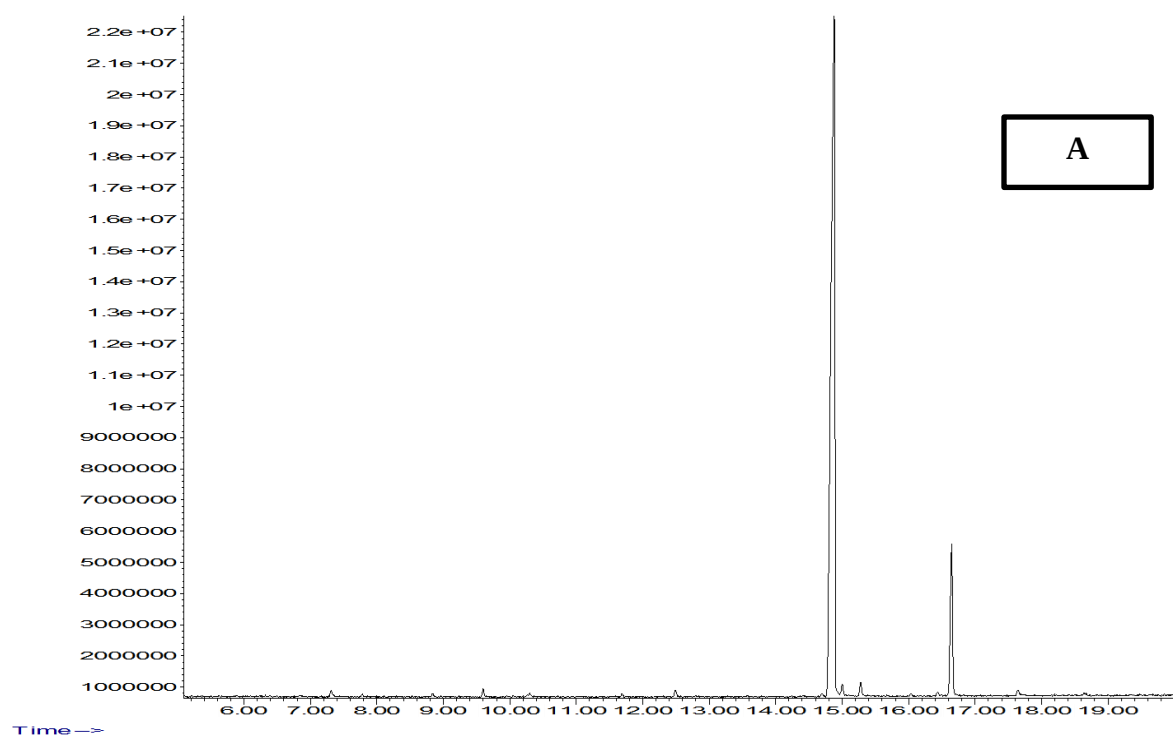


Figure 4: Chromatogram (A) and Mass Spectrum (B) of Anthracene.

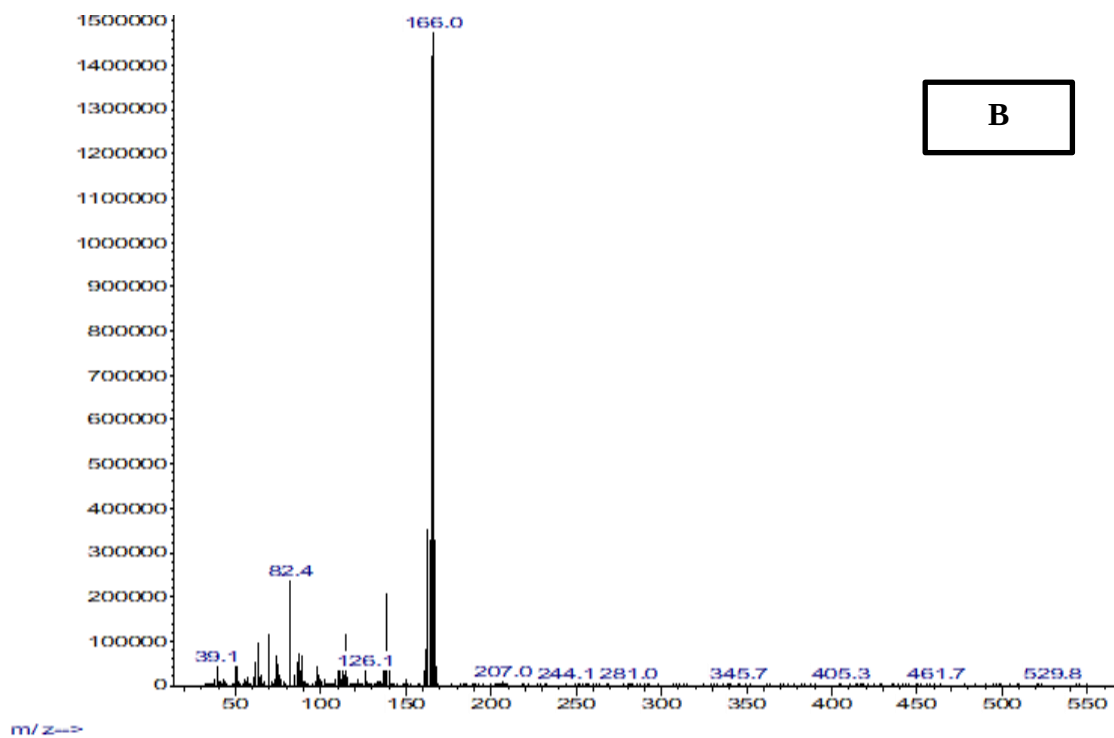
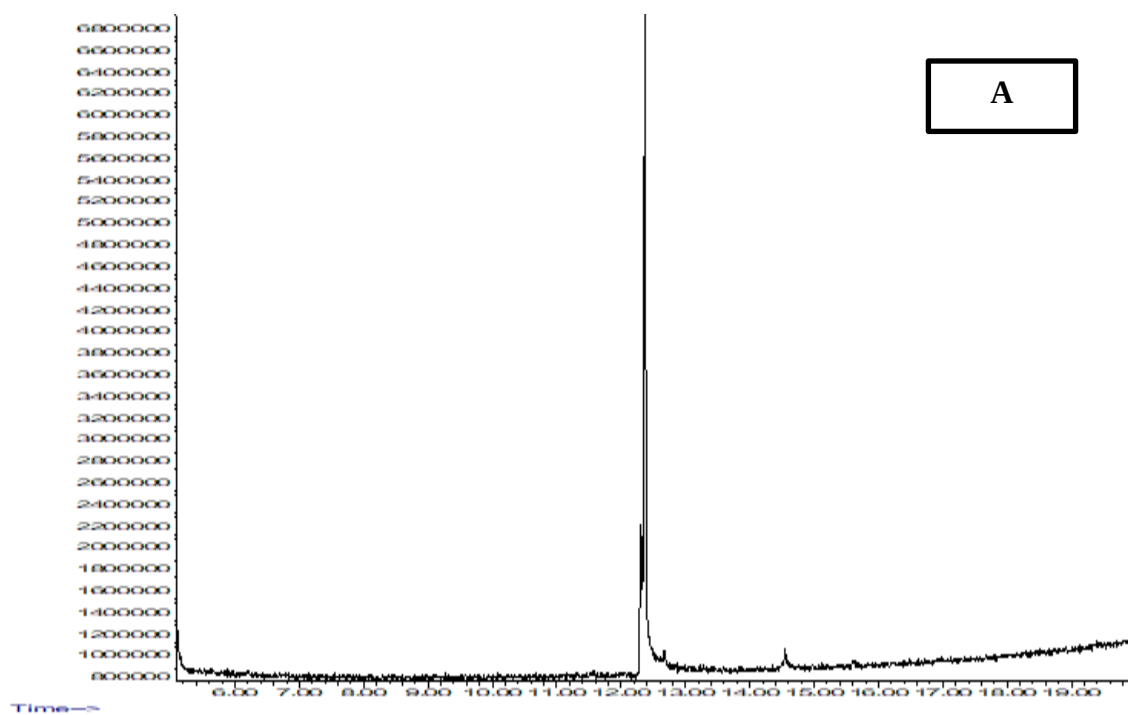


Figure 5: Chromatogram (A) and Mass Spectrum (B) of Fluorene.

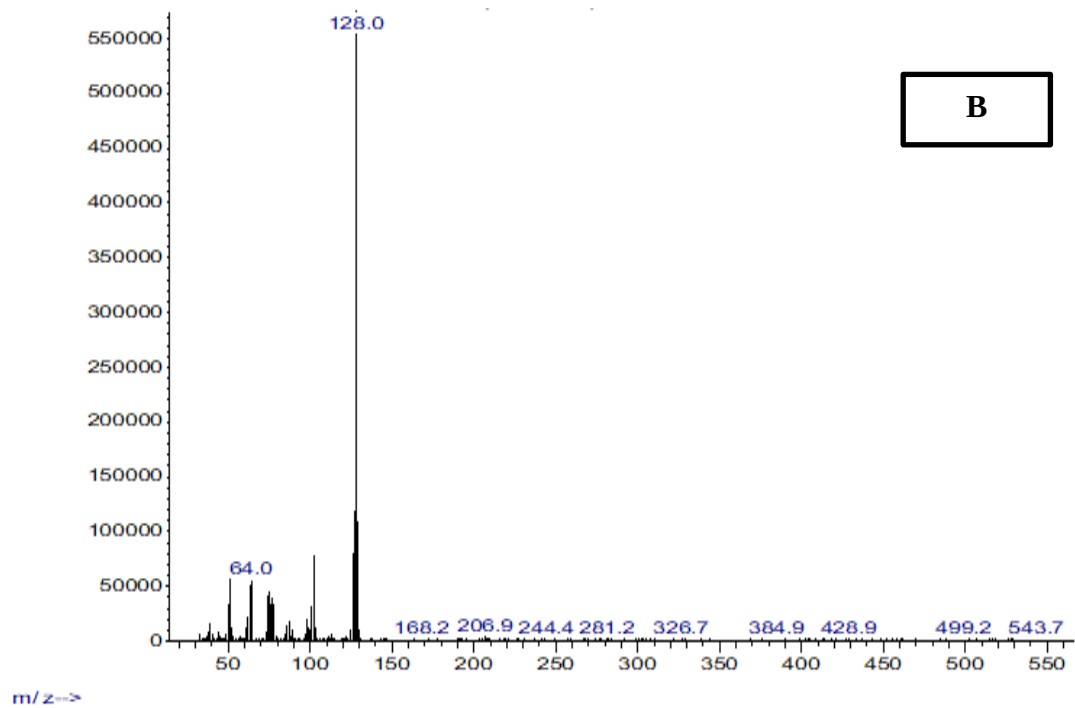
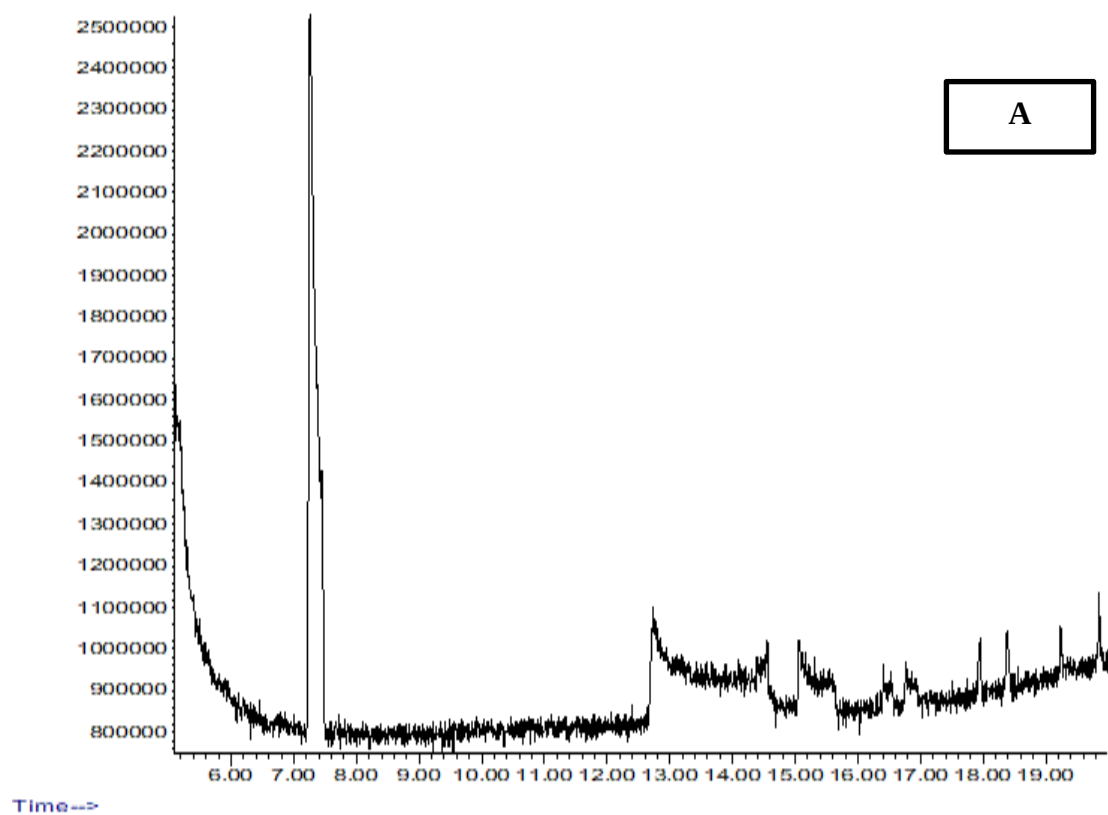


Figure 6: Chromatogram (A) and Mass Spectrum (B) of Naphthalene.

4.3 Biodegradation of Anthracene

At the end of the 18-day period, both strains of bacteria degraded anthracene at a similar rate. *Cupriavidus taiwanensis* LA decomposed 100ppm of anthracene by 88%, while *Ochrobactrum intermedium* BC1 degraded 100ppm of anthracene by 87% (Figure 7 and Table 2). The following intermediates were observed; dihydroxyanthracene, anthraquinone, and catechol.

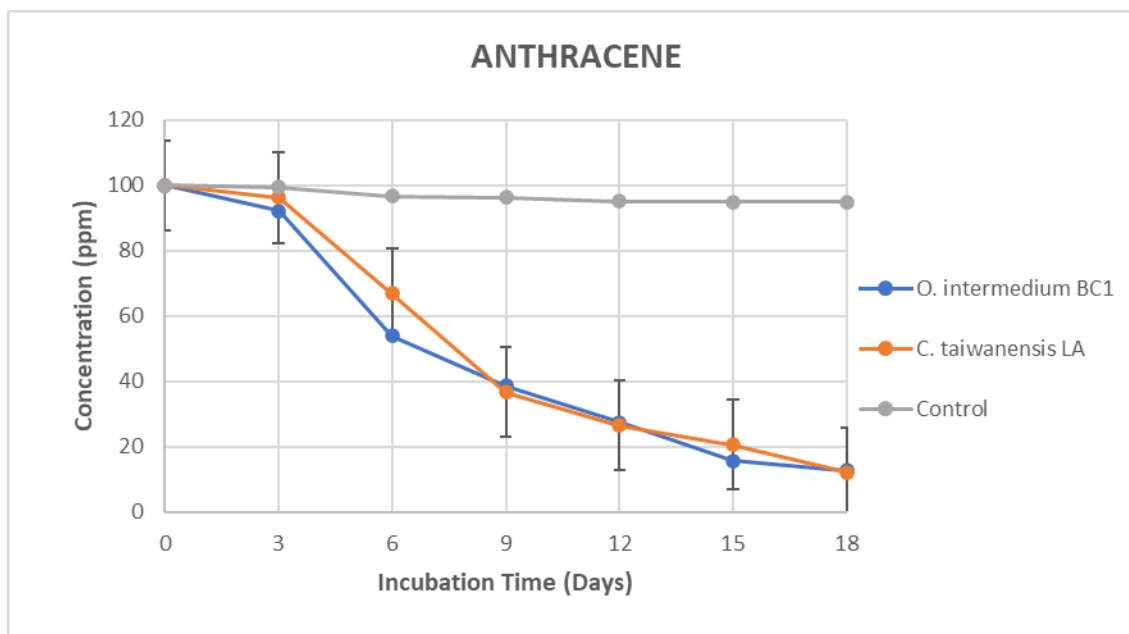


Figure 7: Anthracene Degradation Curve.

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

Bacterial Strain	Mean $\pm$ SD
<i>Ochrobactrum intermedium</i> BC1	12.72 $\pm$ 4.58
<i>Cupriavidus taiwanensis</i> LA	12.26 $\pm$ 3.73
Abiotic Control	94.82 $\pm$ 3.09

4.4 Biodegradation of Fluorene

The metabolism of fluorene by both strains was relatively slow compared to the other two PAHs examined in this study. *Ochrobactrum intermedium* BC1 used up 100ppm of fluorene by 67% in 18 days, while *Cupriavidus taiwanensis* LA degraded the PAH by 53% (Figure 8 and Table 3). Fluorene-1,2-diol, 2-Indanone, and 9-Fluorenol were the intermediates observed during the degradation experiment.

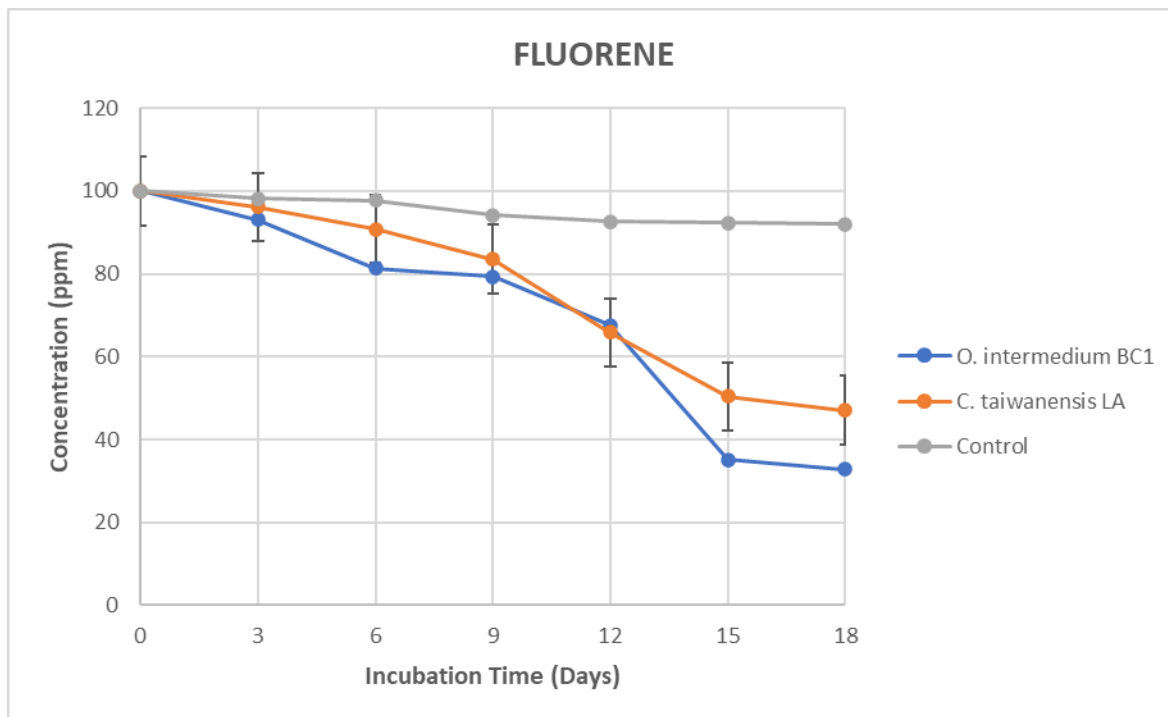


Figure 8: Fluorene Degradation Curve.

Table 3: Levels of unmetabolized fluorene (ppm) in bacterial cultures at day 18. (SD = standard deviation).

Bacterial Strain	Mean $\pm$ SD
<i>Ochrobactrum intermedium</i> BC1	32.85 $\pm$ 1.04
<i>Cupriavidus taiwanensis</i> LA	47.09 $\pm$ 1.31
Abiotic Control	91.96 $\pm$ 1.13

4.5 Biodegradation of Naphthalene

Ochrobactrum intermedium BC1 degraded naphthalene completely, and *Cupriavidus taiwanensis* LA achieved degradation at the rate of 91% (Fig. 9 and Table 4). Dihydroxynaphthalene, naphthoquinone, hydroxybenzaldehyde, and catechol were detected as intermediates.

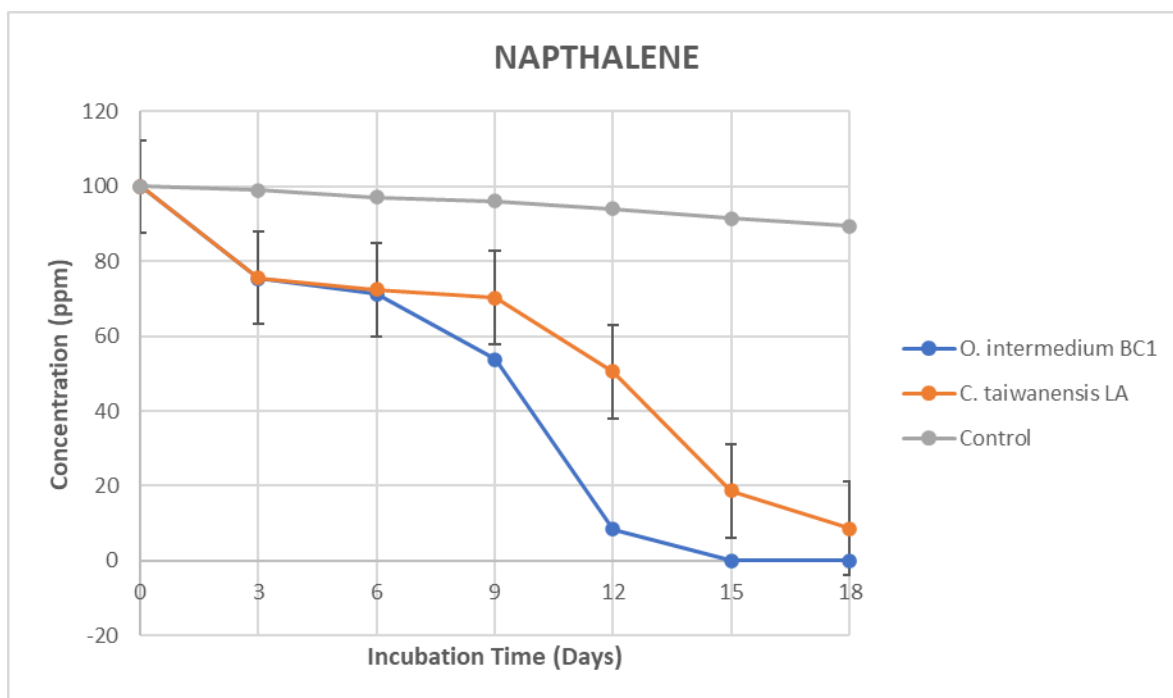


Figure 9: Naphthalene Degradation Curve.

Table 4: Levels of unmetabolized naphthalene (ppm) in bacterial cultures at day 18. (ND = not detected, SD = standard deviation).

Bacterial Strain	Mean $\pm$ SD
<i>Ochrobactrum intermedium</i> BC1	ND
<i>Cupriavidus taiwanensis</i> LA	8.63 $\pm$ 2.11
Abiotic Control	89.38 $\pm$ 1.92

CHAPTER 5

DISCUSSION

The results from this study were interesting and informative. The two bacterial strains investigated do have the capacity to degrade PAHs. The extent and efficiency of metabolism of PAHs by each individual bacterial strain is consistent with previously published literature.

5.1 Anthracene Degradation

In this study, both strains efficiently degraded anthracene at a similar rate (Figure 7 and Table 2). The efficiency of degradation of anthracene by both bacterial strains is comparable to reports from similar studies. For instance, *Ochrobactrum anthropic* has been shown to grow rapidly in high concentration of anthracene [84], and consortia containing a strain of *Ochrobactrum*, have been reported to completely degrade anthracene in 8-10 days [85][86]. Other studies have reported efficient degradation of anthracene using strains of *Sphingomonas* sp. and *Stenotrophomonas maltophilia* [87][88].

5.2 Fluorene Degradation

The strains did not degrade fluorene as efficiently as the other two PAHs utilized in this study (Figure 8 and Table 3). This is not surprising considering that fluorene has been shown to be resistant to degradation when a single bacterial species is used [89][90]. However, when a consortium is utilized for the biodegradation tests, degradation of fluorene has been demonstrated to be much more efficient [91][92].

5.3 Naphthalene Degradation

Naphthalene was completely degraded by *Ochrobactrum intermedium* BC1 and almost completely metabolized by *Cupriavidus taiwanensis* LA (Figure 9 and Table 4). 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* [93-97].

5.4 Proposed Mechanism of Degradation

The bacterial degradation of PAHs generally follows a multiple step process, beginning with the addition of oxygen to the ring catalyzed by dioxygenases to form a dihydroxy-PAH as the first intermediate. Dehydrogenases catalyze the removal of hydrogen atoms to form the second intermediate. The process continues until the ring fuses and disintegrates, and catechol or pyruvate is formed which the bacteria use as substrate for Krebs cycle (Figure 10).

In this study, the detection of dihydroxyanthracene as an intermediate for anthracene, fluorene-1,2-diol as an intermediate for fluorene, and dihydroxynaphthalene as an intermediate for naphthalene indicates that both bacterial strains may have degraded these PAHs following this generalized mechanism. Previous studies with various bacterial species using anthracene, fluorene and naphthalene as substrates have reported mechanisms like the generalized mechanism described above [98].

General Mechanism of Biodegradation of PAHs

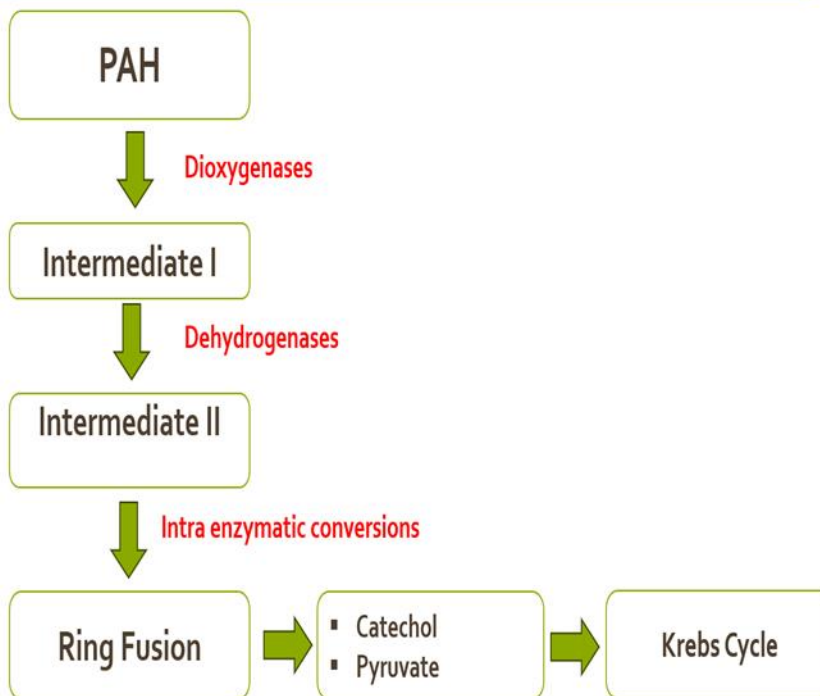


Figure 10: Proposed Mechanism for Bacterial Degradation of PAHs.

CHAPTER 6

CONCLUSION AND RECOMMENDATIONS

6.1 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.

6.2 Recommendations

This study only investigated the optimum pH and temperature (7 and 25°C) for bacterial degradation of PAHs. Further research should be undertaken to explore the rate of degradation at varying pHs and temperatures, such as in acidic or basic medium and low temperatures (5 to 15°C) or high temperatures (45 to 80°C).

The intermediates formed during the 18-days degradation process of each strain could be an area of research by a future study. The nature of the intermediates needs to be elucidated to determine their toxicity profile and potential for re-used in a different situation. In addition, determining the identity of the intermediates formed during the degradation will help accurately map out the mechanism of degradation by each individual bacterial strain.

It is also important to test for the capacity and efficiency of these strains in degrading complex and high molecular weight PAHs. This knowledge is important when designing a bacterial consortium for metabolism of PAHs, it will help determine how best the strains could fit into a consortium.

APPENDIX

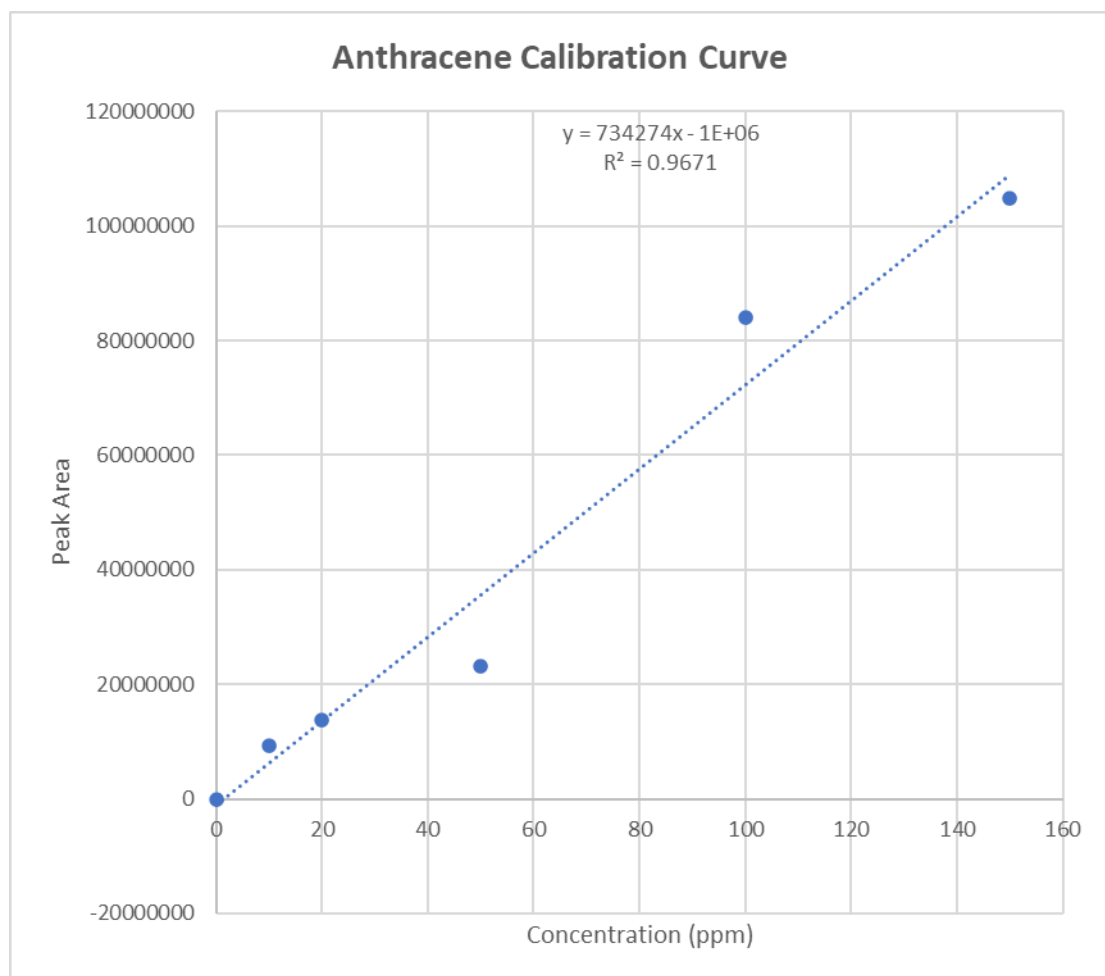


Figure 11: Anthracene Calibration Plot.

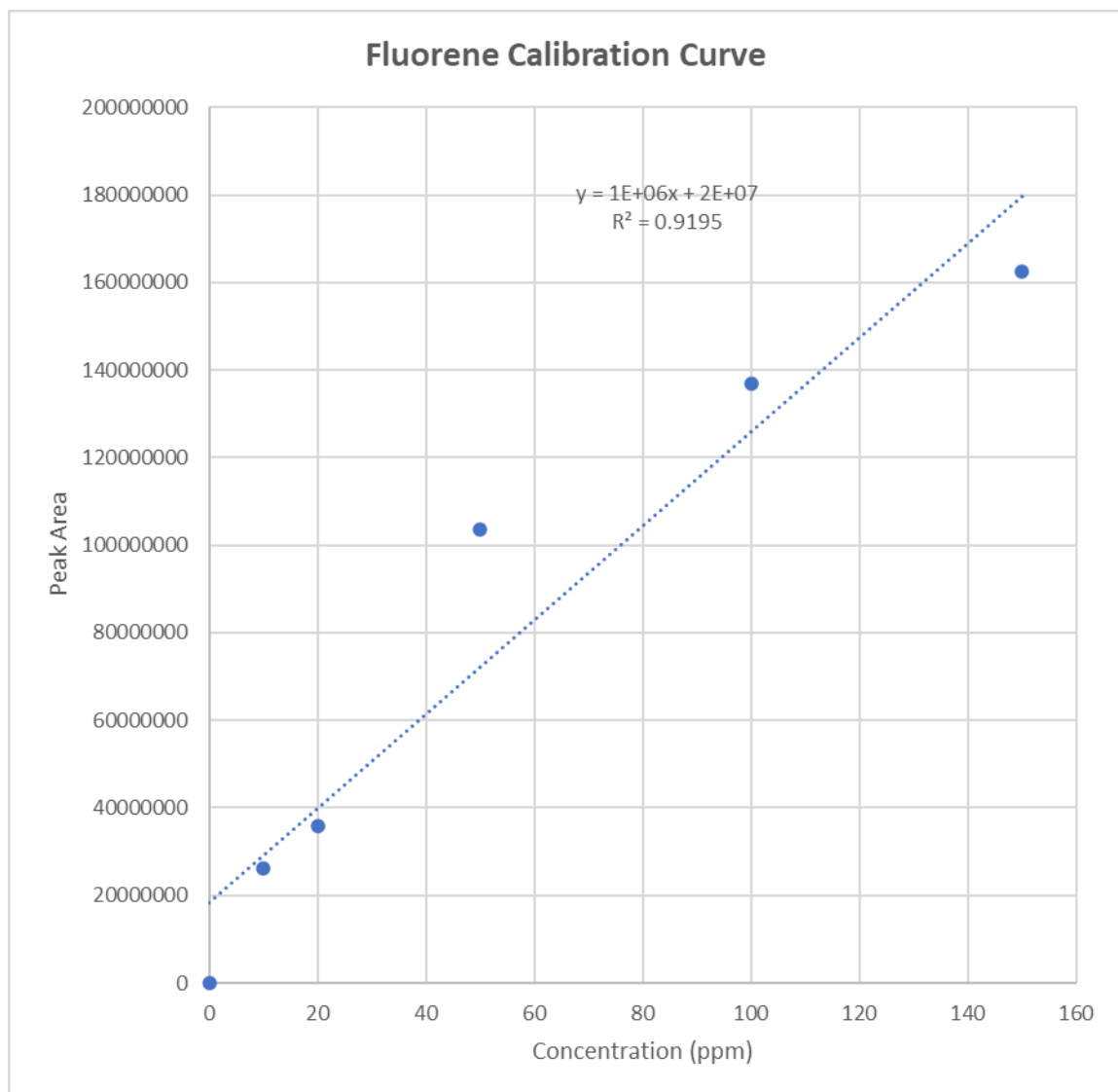


Figure 12: Fluorene Calibration Plot.

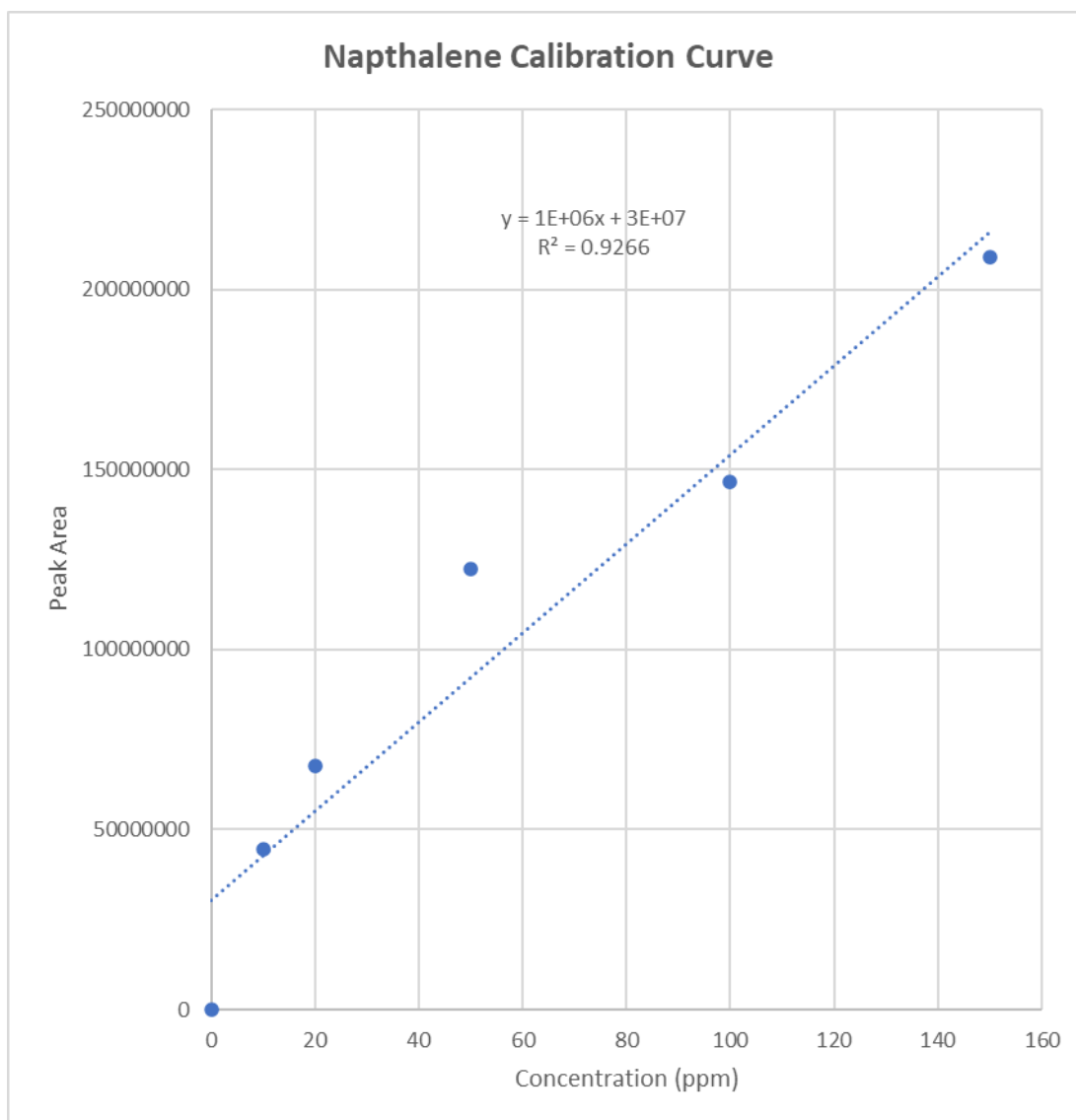


Figure 13: Calibration Plot for Naphthalene.

SPME Device

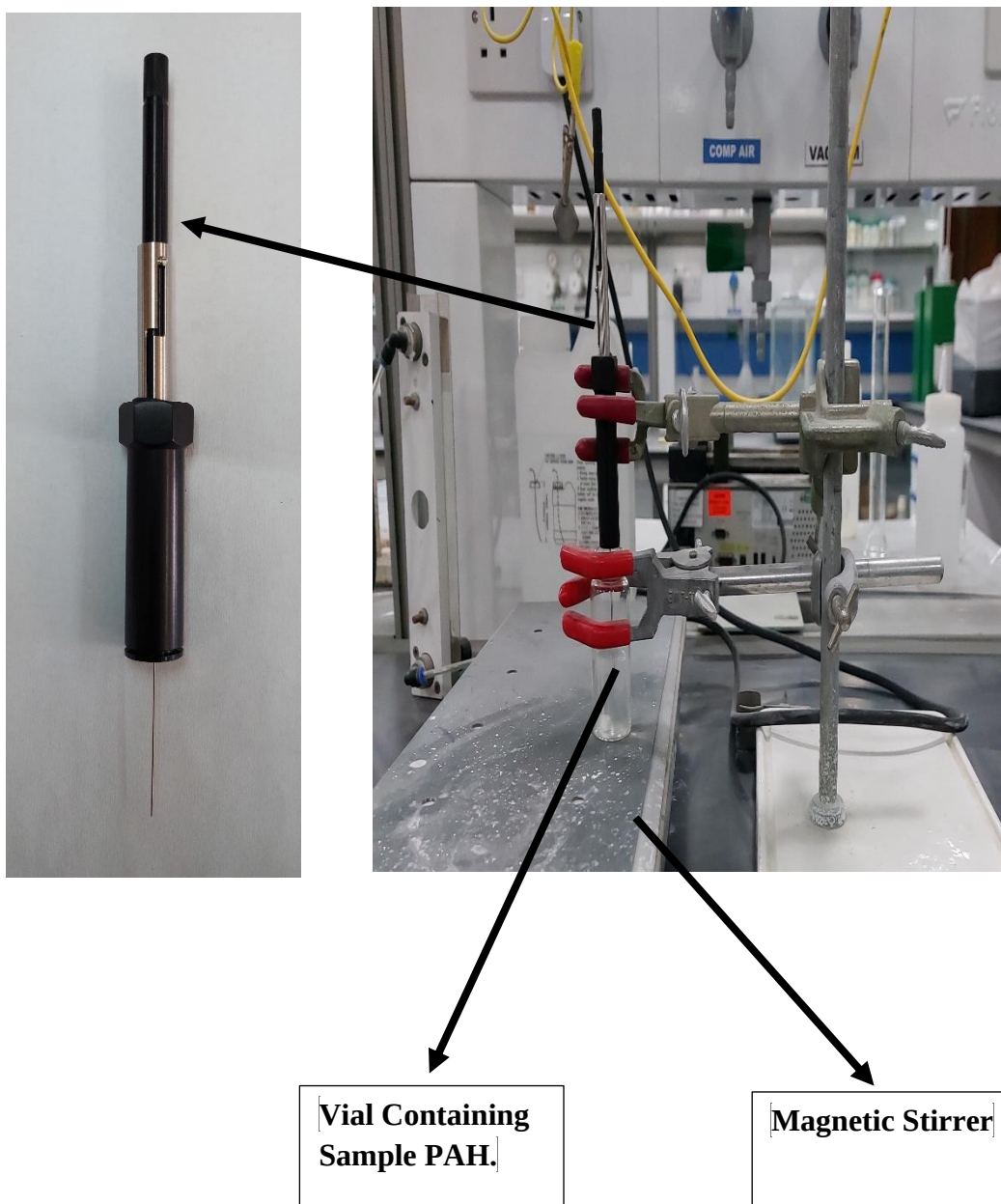


Figure 14: SPME set-up for PAH extraction.



Figure 15: GC/MS Instrument.

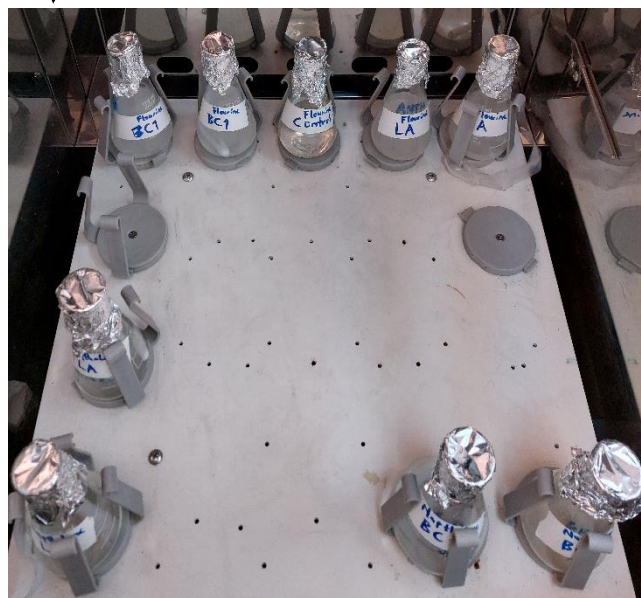
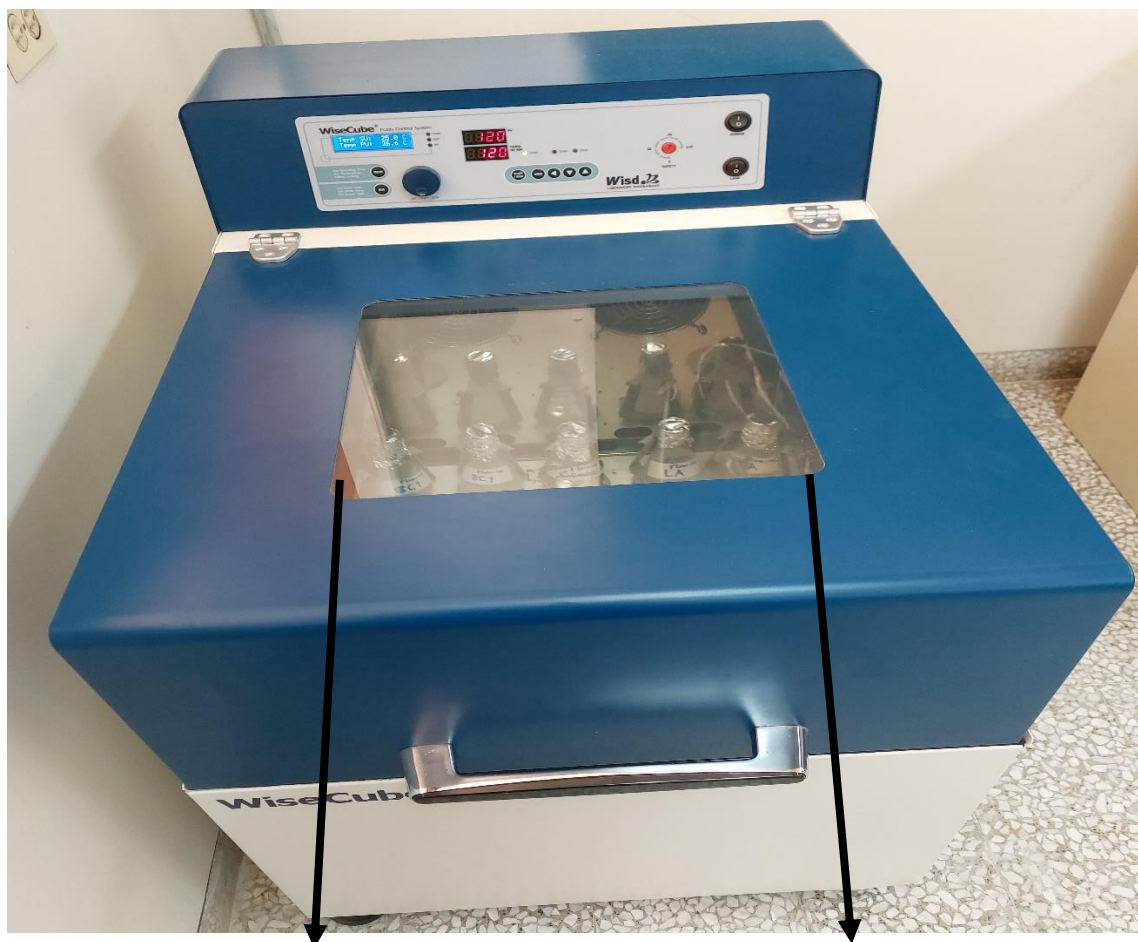


Figure 16: Samples in WiseCube Shake-Incubator.

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