BIOCHEMICAL AND MOLECULAR STUDIES ON BACTERIA DEGRADATION OF HIGH MOLECULAR WEIGHT POLYCYCLIC AROMATIC HYDROCARBONS

BY

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Matric No. 999009060

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DEPARTMENT OF BIOCHEMISTRY,
COLLEGE OF MEDICINE,
UNIVERSITY OF LAGOS, NIGERIA

UNIVERSITY OF LAGOS
SCHOOL OF POSTGRADUATE STUDIES

CERTIFICATION

This is to certify that the thesis:

"Biochemical and Molecular Studies on Bacteria Degradation of High Molecular Weight Polycyclic Aromatic Hydrocarbons"

Submitted to the School of Postgraduate Studies, University of Lagos

For the award of the degree of

DOCTOR OF PHILOSOPHY (Ph.D)

is a record of original research work carried out

by

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DECLARATION

The Research work contained in this Thesis was undertaken entirely by me. No part of this study has been presented either in part or as a whole to any other institution or organization for the purpose of obtaining any degree or qualification.

IGWO-EZIKPE, MIRIAM NWANNA

DEDICATION

This research work is dedicated first and foremost to God Almighty in whom I derived the strength to pursue this study.

To:

My dearly beloved husband, Mr. Igwo Ezikpe Anagha for his unreserved and ever-willing support of my academic pursuit.

My Dad, Chief Johnson Kalu Okoronkwo who laid this solid foundation.

All my family members.

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ABBREVIATIONS

S. paucimobilis	- *Sphingomonas paucimobilis*
Ps. arvilla	- *Pseudomonas arvilla*
Ps. putida	- *Pseudomonas putida*
A. anitratus	- *Acinetobacter anitratus*
A. mallei	- *Acinetobacter mallei*
A. faecalis	- *Alcaligenes faecalis*
S. p -1	- cured *S. paucimobilis*
Ps. -1	- cured *Ps. arvilla*
Ps. putida-1	- cured *Ps. putida*
A. anitratus-1	- cured *A. anitratus*
MS	- Mineral salt
Cfu/ml	- Colony forming unit per milliliter
LMW PAH	- Low molecular weight polycyclic aromatic hydrocarbon
HMW PAH	- High molecular weight polycyclic aromatic hydrocarbon
PAH	- Polycyclic aromatic hydrocarbon
OD_{600nm}	. Optical density measured spectrophotometrically at 600nm.
Consortium	- Mixture of *S. paucimobilis*, *Ps. putida* and *Ps. arvilla*
TVC	- Total viable count
Log TVC	- Logarithm of TVC
'E'	- Experimental culture media
C1	- Non-PAH MS culture media inoculated with organism

C2	- MS PAH culture media with no microbial inoculation
CHY	- Chrysene
FLU	- Fluoranthene
PYR	- Pyrene
USEPA	- United States Environmental Protection Agency
EDTA	- Ethylene diamino tetraacetic
Kbp	- Kilo base pair
LB	- Lauria Bertani
mAU	- Milli Area Unit
Da	- Daltons

DEFINITION OF TERMS

Anthropogenic: are sources that are derived from human activities, as opposed to those occurring in natural environment without human influences.

Autochthonous: refers to indigenous organisms or originating in the place where it is formed.

Bioaugmentation: can be defined as the addition of pregrown microbial cultures or a genetically engineered variant to treat contaminated soil or water in order to improve contaminant clean up and reduce clean up time and cost.

Biodegradation: is the breakdown of organic contaminants by microorganisms into smaller compounds. The microorganisms transform the contaminants through metabolic or enzymatic processes.

Microbial bioremediation: can be defined as any process that uses microorganisms or their enzymes to return the natural environment altered by contaminants to its original condition.

Spray-plate technique: is a solid phase coating method whereby a compound is aseptically sprayed on an agar plate before microbial inoculation.

Enrichment culture technique: is a primary isolation technique designed to make conditions of growth very favorable for organisms of interest

while having an unfavorable environment for any competing organisms.

Plasmid curing: this is the excision of plasmid from a plasmid harbouring organism.

Biotransformation: is the transfer of genetic material between bacteria from a donor cell to a recipient through direct cell-to-cell contact.

CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND OF STUDY

Polycyclic aromatic hydrocarbons (PAHs) and PAHs-containing compounds such as crude oil and refined petroleum products form an important class of pollutants on a global scale (Dong *et al.*, 1999; Rey-Salgueiro *et al.*, 2008). PAHs have been included in several priority pollutant lists which include the Agency of Toxic Substances and Disease Register, the International Agency for Research on Cancer, the European Community and the Environmental Protection Agency (Rey-Salgueiro *et al.*, 2008).

PAHs are a group of over 100 different fused aromatic compounds which consist of two or more fused aromatic rings in various structural configurations. Those with two to three fused aromatic rings are considered as low molecular weight PAHs (LMW PAHs). These include naphthalene, anthracene and phenanthrene, while those with four and more fused rings are high molecular weight PAHs (HMW PAHs) which include chrysene, fluoranthene and pyrene. The chemical properties and environmental fate of a PAH molecule are dependent in part upon both molecular size, i.e., the number of aromatic rings and molecule topology or the pattern of ring linkage (Kanaly and Harayama, 2000).

Generally, an increase in the size and angularity of a PAH molecule results in a concomitant increase in its hydrophobicity and electrochemical stability, thus making them less amenable to biodegradation. PAHs stability and hydrophobicity are two primary factors which contribute to their persistence in the environment (Kanaly and Harayama, 2000). Consequently, they have been detected in numerous aquatic and terrestrial ecosystems at concentrations high enough to warrant concern about their bioaccumulation (Masih and

Taneja, 2006; Johnson-Restrepo *et al.*, 2007). Studies have shown bioaccumulation of PAHs in fishes from the Niger Delta region of Nigeria (Anyakora and Coker, 2007; Anyakora *et al.*, 2008).

PAHs are produced during fossil fuel combustion, waste incineration or as by-products of industrial processes such as petroleum refining and coal gasification. They are also component of crude oil, wood preservatives, smoke houses, wood stoves and emissions from power generators and motor vehicles (Christensen and Bzdusek, 2005; Wilcke, 2007). PAHs generally occur as complex mixtures, found throughout the environment in the air, water, soil and sediment.

Discharged PAHs in the environment disperse over wide areas and are of public health and ecological concern because of their poor solubility, persistence, ability to be bioaccumulated and toxicity to biological systems (Cerniglia, 1992; Meador *et al.*, 1995).

PAHs especially the HMW PAHs are known to exert acutely toxic effects and/or possess mutagenic, teratogenic, cytotoxic and carcinogenic properties even at low concentrations. These properties have been found to increase with PAH molecular weight (Cerniglia, 1992; Xue and Warshawsky, 2005). PAHs may be transferred to humans through inhalation, body contacts, PAHs-contaminated plants and seafood consumption. These thus warrant the need to remediate PAHs-polluted environment and formulate remediation protocols that would be safe and cost effective (Dipple and Bigger, 1991; Wilson and Jones, 1993).

Although substantial progress has been made in reducing industrial releases of PAHs in Nigeria in recent years, major releases still occur. In addition, a considerable number of polluted sites have been identified and new ones are continually being generated. Many of these sites threaten to become sources of contamination to drinking water supplies, agricultural land use, quality air and food consumption thereby constituting a substantial

health hazard for current and future generations (Tao *et al.*, 2004; Meudec *et al.*, 2006; Anyakora and Coker, 2007; Nganje *et al.*, 2007).

Natural loss mechanism of PAHs in soils include volatilization, leaching, irreversible adsorption, photolytic and/or biological modifications. Treatment methods for PAHs-contaminated soils include: solvent extraction wet oxidation, landfilling and bioremediation. Several studies have shown that microbial biotransformation is a major environmental process affecting the fate of PAHs and petroleum products in both terrestrial and aquatic ecosystems (Mahmood and Rama, 1993; Kästner and Mahro, 1996; Palmroth *et al.*, 2005). The use of microbial bioremediation technologies for removing hydrocarbon pollutants provide a safe and economic alternative to physical-chemical treatment (Leahy and Colwell, 1990; Cerniglia, 1992; Kelley *et al.*, 1993). Bioremediation of PAHs contaminated soils is also a promising alternative remedial strategy (Cerniglia 1992; Antizar-Ladislao *et al.*, 2006; Rehmann *et al.*, 2008).

Studies have shown that the low molecular weight PAHs (LMW PAHs) are readily degraded (Ilori and Amund, 2000; Santos *et al.*, 2008, Seo *et al.*, 2009). However, the high molecular weight PAHs (HMW PAHs) are generally recalcitrant to microbial attack, this causes their persistence in the environment (Kanaly and Harayama, 2000; Johnsen *et al.*, 2005, Tian *et al.*, 2008). Nevertheless, bacterial isolates that can attack HMW PAHs have been reported (Walter *et al.*, 1991; Daugulis and McCracken 2003; Lin and Cai 2008). In addition, many HMW PAHs are also susceptible to at least partial degradation using LMW PAHs for carbon and energy source (Boonchang *et al.*, 2000; Supaka *et al.*, 2000).

Bacteria are particularly suitable for biodegradation application because of the wide variety of carbon sources or electron acceptors used by various strains (Koyama *et al.*, 2004; Sabaté

et al., 2004). In most hydrocarbon polluted sites, the concentration of the pollutant is usually lethal leaving the sites sterile.

Nevertheless, bacterial strains have been isolated and identified that survived in the presence of such pollutant and have degradation ability (Boopathy 2000; Ilori and Amund, 2000). Isolates from non-polluted sites have been found not to biodegrade or require more time to initiate degradation. Moreso, single strains of bacteria are often insufficient to degrade certain pollutants such that complete catabolism may require consortia or communities composed of two or more taxa (Piehler *et al.*, 1999; Juhasz and Naidu 2000). Therefore, efforts are directed at isolating various strains of bacteria from indigenous polluted sites with desirable degradation abilities.

The initial step in the aerobic catabolism of a PAH molecule by bacteria occur via oxidation of the PAH to a dihydrodiol by a dioxygenase; a multicomponent enzyme system. This is the rate limiting step (Kanaly and Harayama, 2000; Riegert *et al.*, 2001; Johnsen *et al.*, 2005). The dihydroxylated intermediates may then be processed through either an *ortho* cleavage or a *meta* cleavage type of pathway leading to central intermediates such as catechol and protocatechol. Oxidation of catechol is known as the lower metabolic pathway of PAHs degradation. The enzyme that catalyse this reaction is called catechol dioxygenase (Briganti *et al.*, 2000). Further oxidation of catechol via either *ortho* or *meta* cleavage type of pathway leads to tricarboxylic acid cycle intermediates, ensuring complete degradation of PAHs (Juhasz and Naidu, 2000; Jiang *et al.*, 2004).

Catabolic pathways which encode different PAHs degradation are reported to be located on plasmids (Cho and Kim, 2001; Coral and Karagöz, 2005). It has also been correlated that catechol dioxygenase involved in catabolism of PAHs are mediated by plasmids (Credan *et al.*, 1994).

The prolonged persistence of PAHs in contaminated environment is also contributed by their low water solubility which increases their sorption to soil particles and limits their availability to biodegrading microorganisms. Thus, approaches to enhancing biodegradation hydrophobic PAHs by treatments such as addition of synthetic surfactants or biosurfactants which increases their solubility have been investigated (Makkar and Rockne, 2003; Woo *et al.*, 2004).

Therefore, main factors that influence the extent of PAHs degradation are the PAH in question, availability of PAH degraders, range of hydrocarbons utilization, activity of the PAH degraders enzyme systems, potential transferability of degradation genes and bioavailability of the PAHs to the degraders (Makkar and Rockne, 2003; Jiang *et al.*, 2004; Bathe *et al.*, 2005; Johnsen *et al.*, 2005).

1.2 STATEMENT OF PROBLEM

Nigerian environs are continually faced with environmental pollution challenges contributed by anthropogenic processes especially from oil and gas exploration, development and usage. These activities have led to the deposition of refractory compounds such as crude oil, refined petroleum products and polycyclic aromatic hydrocarbons (PAHs) into the environment.

The reuse of crude oil-impacted land for agricultural purposes for food production has become paramount. Poor yields of agricultural produce caused by planting in oil-impacted land and regaining of soil fertility upon pollution cannot be over emphasized (Nwachukwu *et. al.*, 2001).

Petroleum hydrocarbon contamination is also of great concern due to the toxicity and recalcitrance of many fuel components such as PAHs (Saeed and Al-Mutairi, 2000). The high molecular weight PAHs (HMW PAHs) are of principal concern due to their recalcitrance,

persistence, bioaccumulation, carcinogenicity, genotoxicity and mutagenicity (Kewley *et al.*, 2004; Da Silva *et al.*, 2006; Castorena-Torres *et al.*, 2008; Topinka *et al.*, 2008).

Remediation of PAHs-contaminated system could be achieved either by physicochemical or biological methods. Microbial bioremediation has been explored, which represents the major route responsible for the ecological recovery of PAH-contaminated sites. It is also considered environmentally friendly, technologically feasible and cost effective (Samanta *et al.*, 2002). However, measuring the success of bioremediation of crude oil spills is based on several parameters, among them the degradation of PAHs in the crude oil.

In addition, the success of PAHs bioremediation projects has been limited by the failure to remove the HMW PAHs. This is compounded by the existence of complex mixtures of PAHs at contaminated sites (Guha *et al.*, 1999; Leblond *et al.*, 2001). Furthermore, there is dearth of studies on tropical microbial culture degradation of HMW PAHs, their degradation enzymes system and molecular diversity.

Therefore, for microbial bioremediation to be successfully implemented as a HMW PAH remediation technology, it is essential to employ microorganisms with a wide range of HMW PAH degradation potential and understand the biodegradation of mixtures of PAHs. The efficiency of such microorganisms in complete elimination of HMW PAHs in terms of their catechol dioxygenase activity needs to be examined, including the involvement of plasmid and biosurfactant in HMW PAHs degradations.

1.3 SIGNIFICANCE OF THE PROJECT

Polycyclic aromatic hydrocarbons (PAHs) are one class of toxic environmental pollutants that have accumulated in the environment due to a variety of anthropogenic activities (Masih and Taneja, 2006; Wilcke, 2007).

Bacterial bioremediation has been shown to be effective in ameliorating soils contaminated with low molecular weight PAHs (Kastner and Mahro, 1996; Woo *et al.*, 2004). However, the PAHs are generally recalcitrant to microbial attack (Johnsen and Karlson 2004).

Interest in the biodegradation of the HMW PAHs was motivated by their ubiquitous distribution, low bioavailability, persistence and potential deleterious effect on human health. HMW PAHs are carcinogenic, tetratogenic and mutagenic. They bioaccumulate in marine organisms and plants which could indirectly cause harm to exposed humans through food consumption ((Weinstein *et al.*, 2003; Yu *et al.*, 2005; Anyakora and Coker, 2007).

Studies have shown biodegradation of individual HMW PAHs especially in co-metabolism (Boonchang *et al.*, 2000, Supaka *et al.*, 2000). Moreover, at contaminated sites PAHs typically occur as mixture of compounds (Leblond *et al.*, 2001).

Interactions between HMW PAHs are possible, this can alter the rate and extent of biodegradation within a mixture (Beckles *et al.*, 1998). The effect of a single HMW PAH compound on the biodegradation of another will be crucial in determining the efficacy and metabolic versatility of the microorganisms competent to remediate a contaminated media. Most studies concerning the biodegradation of PAHs were conducted by pure cultures of microorganisms but less is known about the degradation of these compounds by mixed populations as found in nature.

Therefore, for bioremediation to be an effective tool for the clean-up of HMW PAH contaminated soils, a greater understanding of the processes involved and those that limit the degradation of HMW PAHs are required.

1.4 OBJECTIVES OF RESEARCH

The overall objective is to generate indigenous bacteria capable of degrading HMW PAHs. The specific aims of this research include to;

1. Isolate and identify indigenous bacteria with potential to degrade high molecular weight polycyclic aromatic hydrocarbons (chrysene, fluoranthene and Pyrene) and evaluate the range of hydrocarbons utilization by the isolated HMW PAHs degraders.
2. Evaluate the rate of degradation of individual HMW PAH and mixture of HMW PAHs by the degraders using single isolate, consortium with and without cosubstrate; phenanthrene a low molecular weight PAH.
3. Partially purify and characterize catechol dioxygenases of the HMW PAHs degraders.
4. Evaluate plasmid mediation in HMW PAHs degradation by the isolates and ascertain possible transfer of HMW PAHs degradation gene to other isolates.
5. Determine biosurfactant production potential of the HMW PAHs degraders.

In order to achieve the objectives, the research was divided into three major phases

PHASE 1: ISOLATION AND IDENTIFICATION OF INDIGENOUS HMW PAHS DEGRADERS

This involved the isolation of HMW PAHs degrading bacteria from PAHs polluted soils, screening and selection of the best HMW PAHs degrading bacteria, identification of the selected degraders and evaluation the range of hydrocarbons utilization by the HMW PAHs degrading bacteria.

PHASE 2: BIODEGRADATION STUDIES

This stage involved the evaluation of the rate of degradation of HMW PAHs using the degraders as single isolate or as a consortium with and without phenanthrene. The degradations were also carried out using the HMW PAHs as single substrate and as mixture.

PHASE 3: MECHANISM OF DEGRADATION

This phase involved partial purification and characterization of catechol dioxygenases of the HMW PAHs degrading bacteria, evaluation of plasmid involvement in HMW PAHs degradation, biotransformation of the degradation gene of the HMW PAHs degrading bacteria and ascertaining potential of the degraders to produce biosurfactant.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 PHYSIO-CHEMICAL PROPERTIES OF POLYCYCLIC AROMATIC HYDROCARBONS

The term PAH generally refers to a group of hydrocarbons containing two or more fused aromatic rings in linear, angular or clustered arrangements (Johnsen *et al.*, 2005). Beside this, they are neutral, nonpolar and hydrophobic. Each PAH exhibits a unique set of physical and chemical properties (Table 2.1) in terms of molecular weight, ring arrangement, aqueous solubility, adsorption-desorption properties, stability and volatility (Boopathy, 2000, Anyakora 2007). These properties stand against their ready microbial utilization and promote their accumulation in the solid phases of the terrestrial environment. The aqueous solubility and thus bioavailability of PAHs decreases almost logarithmically with increasing molecular mass (Cerniglia, 1992). In addition, volatility decreases with increasing number of fused rings. Hundreds of PAHs have been identified but sixteen of them are classified as priority pollutants by the United States Environmental Protection Agency, USEPA (Anyakora 2007; Kobayashi *et al.*, 2008) because of three main reasons. First, these sixteen are proposed to be more harmful than the other PAHs. Secondly, there is more information on them and lastly, there are greater possibilities of people being exposed to them. These PAHs include: acenaphthene, acenaphthylene, anthracene, benz(a)anthracene, benzo(a)pyrene, benzo(b)fluoranthene, benzo(g,h,i)perylene, benzo(k)fluoranthene, chrysene, dibenz(a,h)anthracene, fluoranthene, fluorene, indeno(1,2,3-c,d)pyrene, naphthalene, phenanthrene and pyrene (Fig. 2.1).

Table 2.1 Physical–chemical properties of 16 USEPA priority PAHs

PAH	No. of rings	Molecular weight	Aqueous solubility (mg/l)	Log K_{ow}	Vapour press. (Pa)
Naphthalene	2	128	31	3.37	$1.0x10^{2}$
Acenaphthylene	3	152	16	4.00	$9.0x10^{-1}$
Acenaphthene	3	154	3.8	3.92	$3.0x10^{-1}$
Fluorene	3	166	1.9	4.18	$9.0x10^{-2}$
Phenanthrene	3	178	1.1	4.57	$2.0x10^{-2}$
Anthracene	3	178	0.045	4.54	$1.0x10^{-3}$
Pyrene	4	202	0.13	5.18	$6.0x10^{-4}$
Fluoranthene	4	202	0.26	5.22	$1.2x10^{-3}$
Benzo[*a*]anthracene	4	228	0.011	5.91	$2.8x10^{-5}$
Chrysene	4	228	0.006	5.91	$5.7x10^{-7}$
Benzo[*b*]fluoranthene	5	252	0.0015	5.80	-
Benzo[*k*]fluoranthene	5	252	0.0008	6.00	$5.2x10^{-8}$
Benzo[*a*]pyrene	5	252	0.0038	5.91	$7.0x10^{-7}$
Dibenzo[*a,h*]anthracene	5	278	0.0006	6.75	$3.7x10^{-10}$
Indeno[1,2,3-cd]pyrene	6	276	0.00019	6.50	-
Benzo[*ghi*]perylene	6	276	0.00026	6.50	$1.4x10^{-8}$

Source: Anyakora (2007).

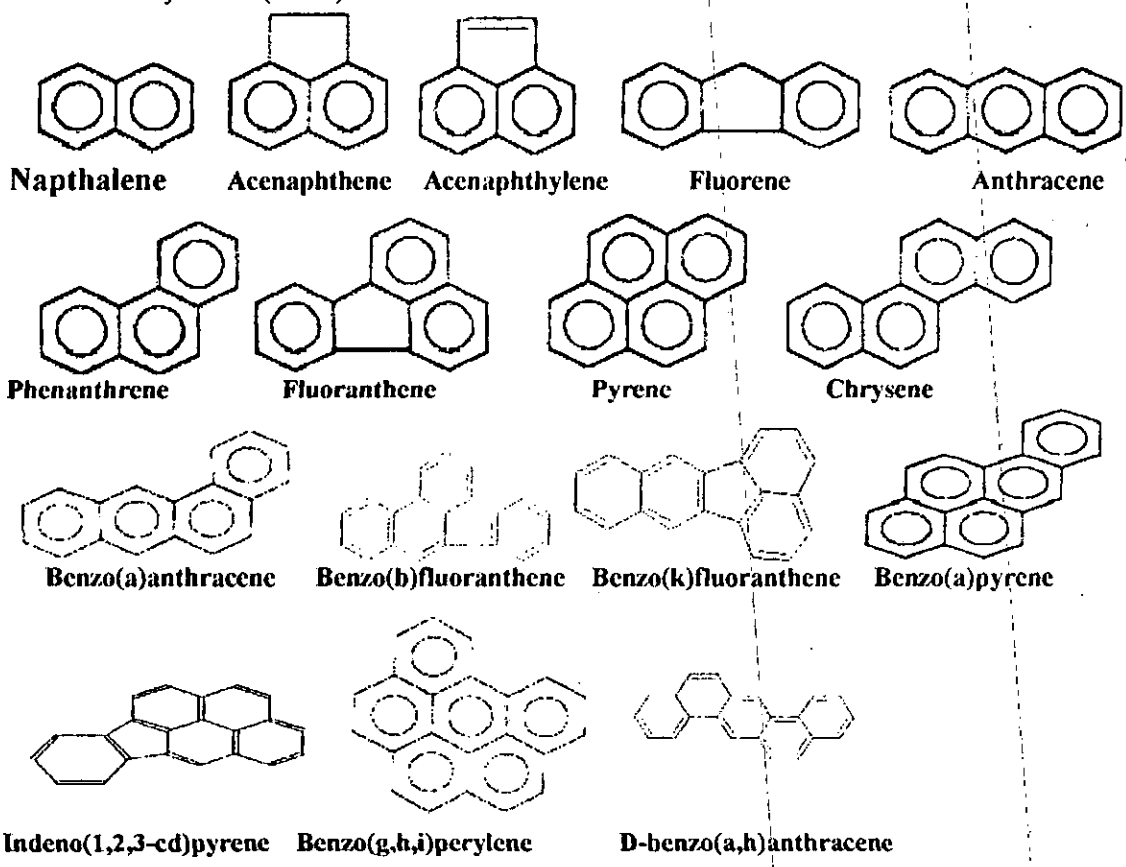


Fig. 2.1 Structure of the 16 USEPA priority PAHs

2.2 OCCURRENCE OF PAHS IN THE ENVIRONMENT

PAHs have been detected in a wide variety of environmental samples, including air, soil, sediments and foodstuffs. Several studies have been carried out to determine the levels of PAHs in different countries (Koyama *et al.*, 2004; Masih and Taneja, 2006; Cai *et al.*, 2007).

2.2.1 PAHS IN THE AIR

The main route for PAH transport is through the atmosphere. Results from ambient air monitoring programmes have shown that PAH concentrations are usually in the order of a few nanograms per cubic metre of air. Of the major air emission sources is the incomplete combustion of fuel by motor vehicles contributing significantly to air PAHs in urban areas particularly near busy streets (Mar *et al.*, 1999). PAHs concentrations in workplace air may be several orders of magnitude greater than concentration observed in ambient air.

The residents in urban districts of Tianjin, China were found to be exposed to ambient air that exceeded the national standard of 10 ng m^{-3} of benzo[a]pyrene equivalent concentration (Tao *et al.*, 2006). Motor vehicles, including diesel automobiles, trucks and buses also contribute to atmospheric PAH pollution through exhaust condensate and particulates, tyre particles, lubricating oils and greases. Once these compounds are released into the atmosphere, they can be transported away from their emission sources over long distances and/or deposited to the terrestrial and aquatic environment through dry and wet deposition.

2.2.2 PAHS IN SOILS

PAHs remain in the environment for long periods if the soil autochthonous microbial populations do not have metabolic ability to degrade these compounds. In such cases, soil inoculation with microorganisms that degrade PAHs (bioaugmentation) is a recommended

practice (Yu *et al.*, 2005; Johnsen *et al.*, 2007). The LMW PAHs are mostly found in the gas phase while the HMW PAHs are mainly associated with airborne particles. HMW PAHs rapidly attach to existing particles, usually soot particles, by adsorption or condensation upon cooling of fuel gas. Once they enter into the soil, they accumulate in horizons rich in organic matter where they are likely to be retained for many years due to their persistence and hydrophobicity (Krauss *et al.*, 2000). Consequently, soils are an important reservoir for these compounds (Ockenden *et al.*, 2003).

Major PAH emission sources are concentrated in urban areas, where PAH concentrations can exceed those in rural areas by as much as one or two orders of magnitude (Wagrowski and Hites, 1997; Zhang *et al.*, 2006). Investigation on the level of PAHs in surface soil from petroleum handling facilities in Calabar metropolis, southeastern Nigeria showed that total PAHs varied from 1.80 to 334.43 mg/kg with a mean of 50.31 mg/kg. It was also considered that the major source of soil contamination originated from petroleum products (Nganje *et al.*, 2007).

2.2.3 PAHS IN MARINE SEDIMENTS

PAHs are characterized by low water solubility and high octanol-water partitioning coefficients; hence their concentrations in water are extremely low. Therefore, due to their hydrophobic nature, PAHs accumulate in fine grain sediments partitioning to organic carbon-coated particles. As such, sediments may be considered as a reservoir for PAH accumulation. PAH concentrations in sediments may accumulate due to a number of sources including atmospheric deposition, marine seeps of petroleum hydrocarbons, off-shore production or petroleum transportation, sewage disposal or boating.

Investigation into the distribution of PAHs in sediments of Lagos, Oshogho and Ile-Ife in Western areas of Nigeria respectively showed an average of 228.57 mg kg^{-1} Σ PAH in Lagos and 91.13 mg kg^{-1} average Σ PAH in Osogho and Ile-Ife (Ogunfowokan *et al.*, 2004). This result correlates higher industrial activities and traffic density to PAHs exposure. Fluoranthene is one of the most common PAHs found in the environment. Sediment concentrations of fluoranthene in South Carolina tidal creeks ranged from 5.69 μg/g dry weight in creeks that drained urban watersheds to 0.003 μg/g dry weight in creeks that drained forested or reference watersheds (Sanger *et al.*, 1999). Organisms such as *Monopylephorus rubroniveus* has been found to bioaccumulate large amounts of sediment-associated fluoranthene in its tissues with mean tissue concentrations of fluoranthene as high as 6431 μg/g dry weight (Weinstein *et al.*, 2003).

2.2.4 PAHS IN MARINE ORGANISMS

In marine environments, most PAHs do not dissolve well in water and so they tend to accumulate in sediments (Means *et al.*, 1980). In coastal marine environments, sources of PAHs include atmospheric deposition, petrochemical industries, domestic and industrial wastewaters, rivers and spillage of petroleum products from ships (Sauer *et al.*, 1998). PAHs presence in coastal environments may pose a potential threat to public health and marine life. In addition to air, sediment and soil, PAHs may accumulate in marine organisms. Studies have reported the occurrence of PAHs in marine benthic invertebrates such as mussels and clams, marine mammals, fishes, sea otters and coastal birds (Meador *et al.*, 1995; Rey-Salgueiro *et al.*, 2008). Four species of fish, namely *Parahanna obscura, Pseudolithus elongatus, Lizza dumerillii* and *Clarais gariepinnus* widely consumed in the Niger Delta region of Nigeria have

been shown to bioaccumulate PAHs (Anyakora and Coker, 2007). The PAHs content of these four fishes showed the presence of chrysene, fluoranthene and pyrene (Fig. 2.2).

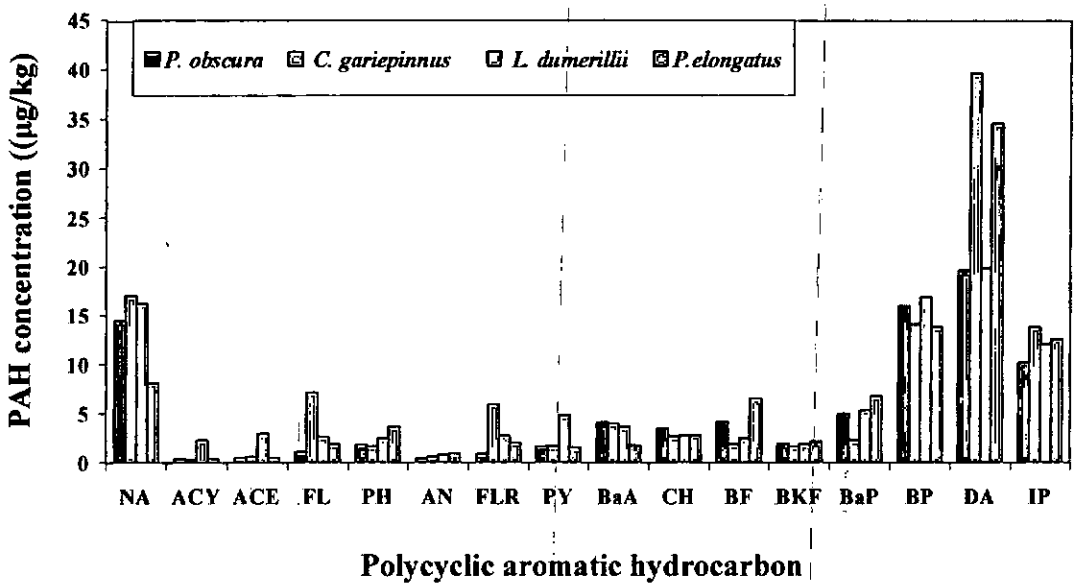


Fig 2.2. The PAHs content of four fishes widely consumed in the Niger Delta region of Nigeria (Adopted from Anyakora and Coker, 2007). NA= Naphthalene, ACY=Acenaphthylene, ACE=Acenaphthene, FL=Flourene, PH=Phenanthrene, AN=Anthracene, FLR=Flouranthene, PY=Pyrene, BaA=Benz[a]anthracene, CH=Chrysene , BF=Benzo[b]fluoranthene, BKF=Benzo[k]fluoranthene, BaP=Benzo[a]pyrene, BP=Benzo[ghi]perylene, DA=Dibenz[a,h]anthracene, IP=Indeno[1,2,3-cd]pyrene

2.2.5 PAHS IN PLANTS

PAHs may accumulate in vegetations, such accumulations determine the fate of PAHs in the environment (Barber *et al.,* 2003; Kluska, 2003). Although the PAHs stored in vegetation account for only a small proportion of the total burden in the environment, they are active in exchange with other media. This directly influence local air PAHs concentrations and can also be used as a passive sampler to indicate contamination levels of PAHs in local air.

Studies have investigated uptake of PAHs by plants and vegetables (Voutsa and Samara, 1998; Kipopoulou *et al.,* 1999; Tao *et al.,* 2004). Gaseous deposition is the principal pathway for the accumulation of PAHs in vegetable. PAHs emissions from fossil fuel combustion were shown to influence the PAH levels and profiles in vegetables grown in an industrial area (Kipopoulou

et al., 1999). In some cases, however, direct relationship between soil and plant PAH concentrations have been observed suggesting a possible pathway from contaminated soil to plant roots (Wild *et al.*, 1992). It has also been estimated that the PAH burden in rural vegetation is up to 10 times lower than in urban samples which correlated to the atmospheric PAH concentration gradient away from urban areas (Wagrowski and Hites, 1997). Meudec *et al.*, (2006) found phenanthrene, pyrene and chrysene to be major PAHs bioaccumulated in plants grown on oiled sediment.

2.3 PAHS TOXICITY

PAHs are ubiquitous environmental pollutants generated mainly from anthropogenic processes which pose serious concern on human health through bioaccumulation and toxicity (Yu *et al.*, 2005). PAHs are highly lipid-soluble, rapidly distributed in a wide variety of tissues with a marked tendency for localization in body fat.

Human exposure to PAHs is 88–98% connected to food through consumption of PAHs contaminated drinking water, sea foods, atmospherically PAHs-deposited crops, crops grown on PAHs polluted sites and flow of contaminated water to agricultural soil (Dipple and Bigger, 1991; Wilson and Jones, 1993; Kluska 2003; Tao *et al.*, 2004).

PAHs human exposure could also occur during intense thermal processing such as toasting, roasting, frying, wood smoke use through inhalation and body contact (Kuljukka *et al.*, 1996; Rey-Salgueiro *et al.*, 2008). PAHs have also been detected in occupational populations such as aluminum smelter workers (Levin, *et al.*, 1995), graphite-electrode plant workers (Angerer *et al.*, 1997), road pavers (Vaananen *et al.*, 2003), coal-tar distillation workers (Preuss *et al.*, 2005), bus-garage and waste-collection workers (Kuusimaki *et al.*, 2004) and traffic police officers (Merlo *et al.*, 1998).

PAHs metabolism in human body produces epoxide compounds with mutagenic and carcinogenic properties. Cases of lung, intestinal, liver, pancreas and skin cancers have been reported (Samanta *et al.*, 2002). Human exposure is often demonstrated by increased internal levels of PAH metabolites which are markers for early biological effects, like DNA adducts and cytogenetic aberrations (Xue and Warshawsky, 2005). PAHs act as ligands to the aryl hydrocarbon receptor (AhR) which is a ligand-activated member of the basic helix–loop–helix/Per-ARNT-Sim (bHLH/PAS) family of transcription factors (Kewley *et al.*, 2004). Activation of AhR may initiate a cascade of secondary and tertiary changes in gene expression leading to carcinogenicity, wasting syndrome, teratogenicity, hepatotoxicity, immunosuppression and enzyme induction (Denison and Heath-Paglius, 1998).

Marine organisms take-up PAHs depending on the bioavailability of the PAHs as well as the physiology of the organism. They metabolize PAHs to active and potent carcinogenic forms in the liver (Da Silva *et al.*, 2006). Metabolites of PAHs found in benthic fish are associated with hepatic lesions and liver neoplasm (Baumann and Harshbarger, 1995). *In vitro* study on effect of PAHs on ovarian steroidogenesis of the flounder (*Platichthys flesus* L.) showed the potential of PAHs to disrupt the reproductive cycle of fish living in polluted environments due to impairment of steroid biosynthesis (Monteiro *et al.*, 2000), PAHs have also been found to disrupt the immune system of fishes (Reynaud and Deschaux, 2006) and induced oxidative stress in *Perna viridis* (Cheung *et al.*, 2001).

PAH stress response studies in *Arabidopsis thaliana* exposed to phenanthrene showed morphological symptoms of growth reduction of the root and shoot, deformed trichomes, reduced root hairs, chlorosis, late flowering and the appearance of white spots which later developed into necrotic lesions. PAHs-contaminated plants also experience oxidative stress at the tissue and cellular levels (Alkio *et al.*, 2005).

2.4 BIODEGRADATION OF PAHS BY BACTERIA

Bacterial degradation of LMW PAH is well documented (Ilori and Amund, 2000; Woo *et al.*, 2004; Santos *et al.*, 2008). In the last decade, significant advance has been made in research pertaining specifically to bacterial biodegradation of HMW PAHs which are recalcitrant.

In natural environments, the LMW PAHs (consisting of 2–3 aromatic rings) are readily degraded, while the HMW PAHs (containing 4 or more aromatic rings) are persistent (Juhasz and Naidu, 2000). Studies suggested that many bacteria prefer LMW PAHs to HMW PAHs in pure culture conditions (Lotfabad and Gray, 2002). It has also been found that PAHs with different aromatic rings are degraded by different microbial groups (Singleton, 2005). In addition, HMW PAHs may be degraded via co-metabolism using LMW PAHs as carbon and energy source (Boldrin *et al.*, 1993).

However, a number of bacterial isolates capable of HMW PAH degradation have been described; *Alcaligenes denitrificans* strain WW1 has been identified to degrade fluoranthene at a rate of 0.3 mg/ml per day and also co-metabolized other PAHs, including pyrene and benz[*a*]anthracene (Weissenfels *et al.*, 1991). *Rhodococcus* sp. strain UW1 isolated from PAHs contaminated soil was found capable of utilizing pyrene and chrysene as sole sources of carbon and energy (Walter *et al.*, 1991).

Pseudomonas organism, strain HL7b, isolated by enrichment cultures derived from the aromatic fraction of crude oil was reported to degrade fluoranthene, but not as a sole carbon and energy source (Foght and Westlake, 1988). *Sphingomonas sp.* Strain P2 isolated from a lubricant-contaminated garage soil by phenanthrene enrichment was capable of degrading fluoranthene and pyrene via cometabolism (Supaka *et al.*, 2000). In contrast to bacteria, fungi do not usually utilize PAHs as their sole carbon and energy source but transform these compounds co-metabolically to detoxified metabolites (Boonchang *et al.*, 2000).

The mechanism of bacterial catabolism of PAHs involves initial oxidation to *cis*-dihydrodiols by dioxygenases, which incorporates two oxygen atoms into the aromatic nucleus; this is the rate-limiting step (Cerniglia, 1992). The *cis*-dihydrodiols are re-aromatised through a *cis*-dihydrodiol dehydrogenase to yield dihydroxylated derivatives which is further oxidized to form catechols. Catechols can then be oxidized via two pathways (Fig 2.3).

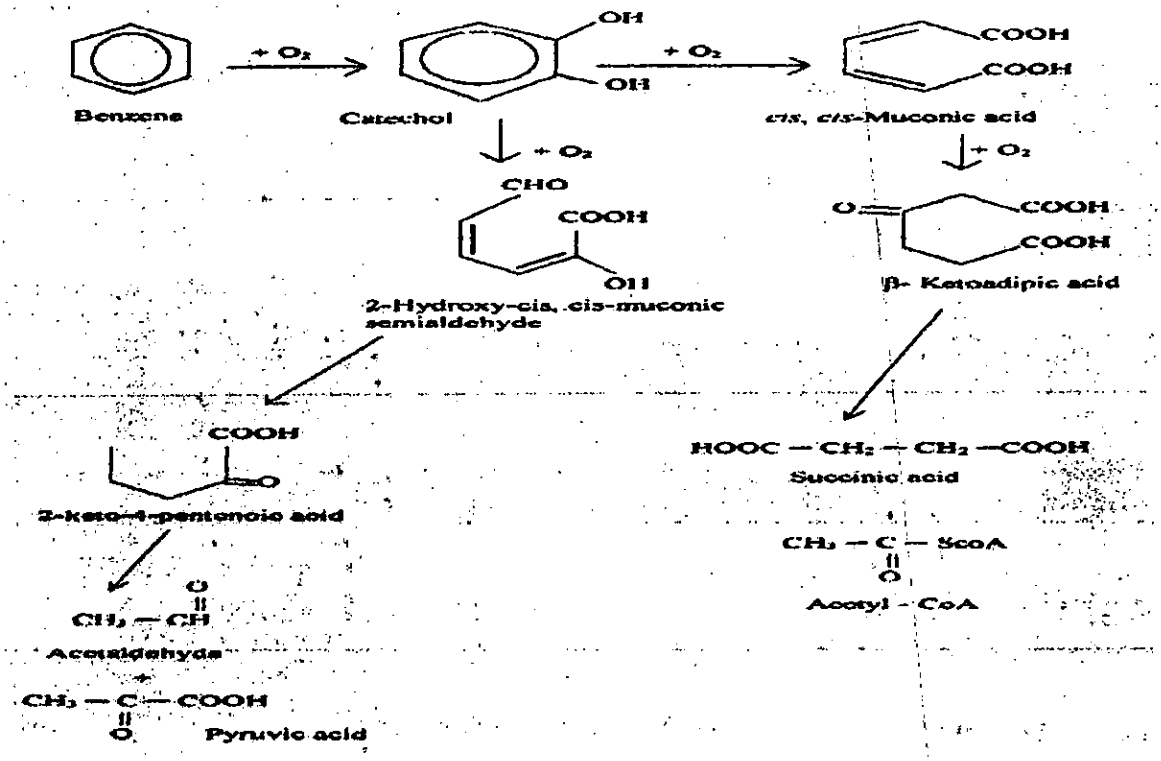


Fig. 2.3. Pathway for microbial metabolism of aromatic ring by *ortho* or *meta* cleavage

The *ortho* pathway involves cleavage of the bond between carbon atoms with hydroxyl groups to yield *cis,cis*-muconic acid. On the other hand the *meta* pathway involves cleavage of the bond between a carbon atom with a hydroxyl group and the adjacent carbon atom without a hydroxyl group to yield 2-hydroxymucaldehyde acid. The enzyme that catalyse both the *ortho* and *meta* cleavage pathway of catechol is called catechol dioxygenase.

Catechol dioxygenases are divided into two different classes based upon the position of bond cleavage. These are the intradiol cleavage by catechol 1,2-dioxygenases (EC 1.13.11.1) and the extradiol cleavage by catechol 2,3-dioxygenases (EC 1.13.11.2). Catechol 1,2-dioxygenase catalyzes the incorporation of dioxygen into catechol and the intradiol ring cleavage to form *cis cis* muconic acid (Strachan *et al.*, 1998) while catechol 2,3-dioxygenase catalyzes the incorporation of dioxygen into catechol and the extradiol ring cleavage to form 2-hydroxymucaldehyde acid (Credan *et al.*, 1994).

There are many differences between the active sites of the two different classes of the enzyme. These differences have a profound effect on the reaction mechanism, causing the reaction pathway to be very different for each enzyme.

The intradiol cleavage active site consists of an iron (III) metal center coordinated by two histidine moieties, two tyrosine moieties and a hydroxide ion in a trigonal bipyramidal geometry (Fig 2.4). In the presence of catechol substrate, one of the tyrosine moieties and the hydroxide ion ligands are protonated and dissociate away from the iron centre. The catechol substrate binds as a bidentate dianion to the iron centre. Once bound, the catechol reacts with dioxygen forming peroxide intermediate (Hitomi *et al.*, 2004). The peroxide intermediate undergoes a reaction with iron centre, forming cyclic peroxide, which after rearrangement, results in *cis cis* muconic acid.

The extradiol cleavage active site consists of an iron (II) metal centre coordinated by two histidine moieties, one glutamate moiety and two water molecules in a square pyramidal geometry (Kita *et al.*, 1999; Lin *et al.*, 2000). This difference in the oxidation state of the metal and the difference in the ligand environment as compared to the intradiol cleavage active site, causes the catechol to bind in a different mode. In the extradiol cleavage mechanism, the catechol is proposed to displace two water molecules, and bind as a bidentate, monoanion (Sato

et al., 2002). For extradiol cleavage, dioxygen interacts directly with the iron centre, resulting in a superoxide intermediate. After rearrangement of the cyclic peroxide, 2-hydroxymucaldehyde acid results (Fig. 2.5).

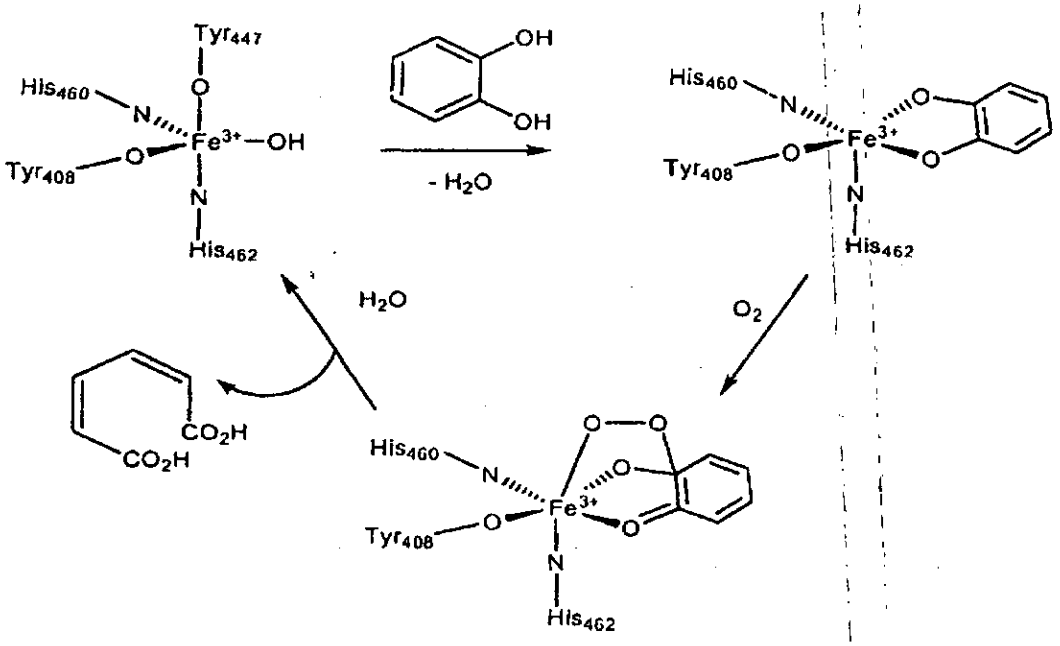


Fig. 2.4. Catalytic cycle for catechol 1,2- dioxygenase cleavage

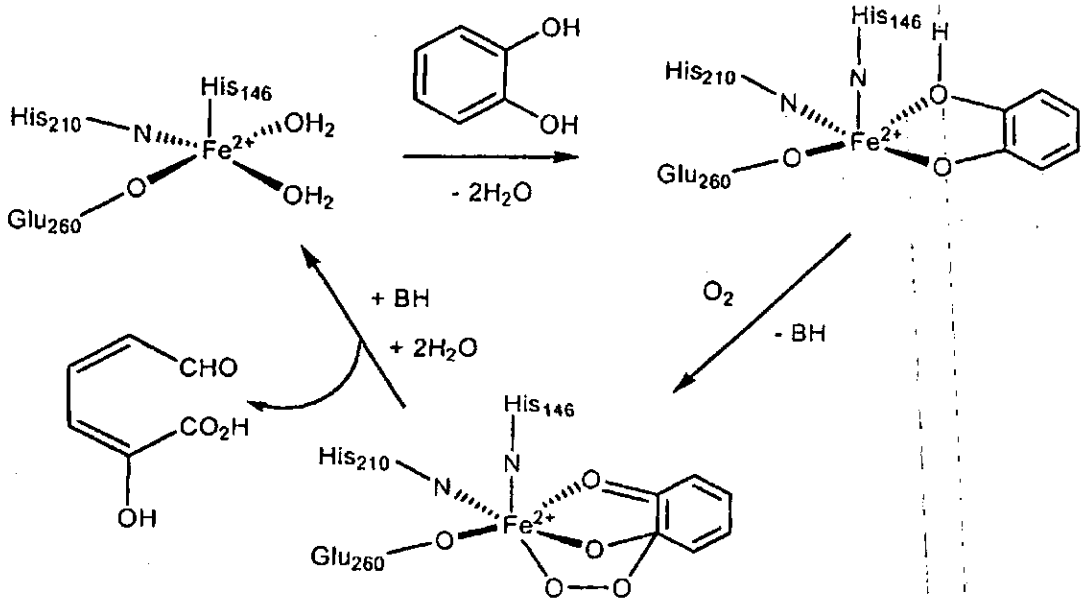


Fig. 2.5. Catalytic cycle for catechol 2,3- dioxygenase cleavage

Catechol ring cleavage results in the production of pyruvate and tricarboxylic acid intermediates which are utilized by the microorganism for synthesis of cellular constituents and energy (Wilson and Jones, 1993). A by-product of these reactions is the production of carbondioxide and water. Once the initial hydroxylated aromatic ring of the PAH is degraded (to pyruvic acid and carbondioxide), the second ring is then attacked in the same manner (Juhasz and Naidu, 2000).

2.5 ROLE OF PLASMIDS IN PAHS DEGRADATION

Plasmids are extrachromosomal circular deoxyribonucleic acid (DNA) molecules that replicate independently of their host chromosome. They are usually found in bacteria and eukaryotes. They carry genes that confer selective phenotypic advantages such as antibiotic resistance, degradation or catabolic gene, pathogenicity and conjugation potential. The size of plasmids varies from less than 1 to over 400 kilobase pairs (kbp).

Plasmids may be one copy, for large plasmids, to hundreds of copies of the same plasmid in a single cell, or even thousands of copies. The catabolic pathways which encode different PAHs degradation have been reported to be located on plasmids (Cho and Kim, 2001; Coral and Karagöz, 2005).

Pseudomonas strains harboring plasmids metabolizing naphthalene have been shown to contain silent genes of meta-pathway that may 'switch-on' when the strains are grown on methylated naphthalene and salicylate derivatives (Filonov *et al.*, 2000). Catchol dioxygenase involved in catabolism of PAHs has been found to be encoded by plasmids (Credan *et al.*, 1994). The presence of PAH-degradation genes on mobile, genetic elements may indicate the easy spreading of PAH-catabolic abilities among bacteria in polluted soils as a result of conjugative gene-transfer. This was evident in the observed transfer of plasmid-encoded NAH-genes

between phylogenetically different members of a bacterial community in tar-contaminated soil (Herrick *et al.*, 1997; Stuart-Keil *et al.*, 1998).

2.6 ROLE OF BIOSURFACTANTS IN PAHS DEGRADATION

Biosurfactants are amphiphilic surface-active molecules produced on living surfaces, mostly microbial cell surfaces, or excreted extracellularly. A biosurfactant may have one of the following structures: mycolic acid, glycolipids, polysaccharide–lipid complex, lipoprotein or lipopeptide, phospholipid, or the microbial cell surface itself.

They consist of hydrophilic and hydrophobic domains which tend to partition preferably at the interface between fluids of different degrees of polarity and hydrogen bonding (Ron and Rosenberg, 2001; Johnsen *et al.*, 2005).

Biosurfactants are involved in cell adhesion, emulsification, solubilization, dispersion, flocculation, surface tension reduction, cell aggregation, and desorption phenomena.

The relative toxicity, low biodegradability and limited efficiency at low concentrations have reduced the potential use of synthetic surfactants in contaminated sites (Desai and Banat, 1997). This purpose may be better served by biosurfactants whose primary function is to facilitate microbial life in environments dominated by hydrophilic-hydrophobic interfaces (Barkay *et al.*, 1999; Maneerat, 2005).

Biosynthesis and excretion of biosurfactants into medium are considered to be cell mechanisms aimed at an adaptation of the microorganism to using external lipophilic compounds as carbon and energy sources. Hydrophobic compounds such as hydrocarbons require solubilization before being degraded by microbial cells. Mineralization is governed by desorption of hydrocarbons in soil or water environment into the hydrophobic core of the biosurfactants. This

increases the surface area of hydrophobic materials thereby increasing their water solubility. Hence, the presence of biosurfactants may increase microbial degradation of hydrocarbons. Many bacteria growing on hydrocarbon produce biosurfactant to increase the bioavailability of these poorly available substrates (Ilori and Amund 2001, Nweke and Okpokwasili, 2003; Tabatabaee *et al.*, 2005).

A similar strategy was suggested for PAH degrading bacteria (Déziel *et al.*, 1996). *Pseudomonas aeurogenosa* grown on phenanthrene or naphthalene was found to produce biosurfactant which increased the apparent solubility of these PAHs, suggesting that the microorganism promoted the availability of its growth substrates (Déziel *et al.*, 1996).

In another study, 22 PAH degraders were found not to produce significant biosurfactant but reduced the surface tension of water by only 0-4mNm^{-1} (Johnsen and Karlson, 2004).

However in other studies, no correlation was found between biosurfactant production and PAH mineralization or dissolution rates (Volkering *et al.*, 1993; Willumsen and Karlson, 1997). It has thus become imperative to study the mechanisms of the biodegradation of hydrocarbon pollutants by individual microorganisms.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 MATERIALS

EQUIPMENT AND GLASS WARE

Major equipment used include:

- Autoclave (Portable SWP 15 Psi; H.T.30 Psi, Dixon's Ltd., England)
- Incubator (Gallenkamp economy Incubator with fan, USA)
- Centrifuge (Uniscope Sm112, Springfriend Medicals, England)
- Electrophoretic tanks and Kits, Vertical type (Shandom Disc electrophic kit,USA),
- Freeze Drier (Thermoservant, Microduodyo, USA)
- High Performance Liquid Chromatography (HPLC) Model No. 1100 series
    - HPLC Thermostated column compartment (Germany)
    - Variable wavelength detector (UV) Japan
    - Quaternary pump, (Germany)
    - Degasser (Japan)
    - Rheodyme manual injector (200µL)
    - Chemostation software revision
    - VYDAC Reverse Phase column (USA)
- Microscopes (Light microscope, Carl Zeiss, Germany; Swift Instruments International, South Africa)
- pH Meter (Mettler, Switzerland)

- Spectrophotometer (Thermospectronic Genesys 4001/1, USA; Spectonic Genesys TMS, USA)
- Ultra Cenrifuge (Superspeed RC-B, Sorvall Inc., Newton Connecticut, USA)
- Eppendorf machine (Minispain, Germany)
- Water bath (Gallenkamp, England; Equitron, Mumbai, India)
- Weighing Balance (Mettler Toledo Ab204, Switzerland)
- Vortex (Fisons scientific equipment)

Glass ware and other materials include:

Mc Cartney bottles, Erlenmeyer flasks, Separating funnel, Bunsen burners, Spirit lamps, Glass rods, Glass spreader, Inoculating (Wire) loops, Petri Dishes (disposable plastic types), Test tubes, Racks, Beakers, Pipette, Conical flasks, membrane filter, Eppendorf tubes and tips, Retort stand, Measuring cylinders, Pipettes, Microscopic slides, Cover slips, Calibrated Meter rule, Aluminum foils, Whatman No.1 filter paper, Cotton wool, Hand gloves and Face masks.

CHEMICALS AND REAGENTS

All chemicals used in this research work were of analytical grade. They were sourced as follows: LWM PAHs and HMW PAHs (anthracene, chrysene, fluoranthene, naphthalene, phenanthrene and pyrene) from Sigma chemical Co. (Germany). Dodecane, hexane, xylene and hexadecane (BDH, England). Crude oil (Shell, Nigeria), diesel oil, kerosene and engine oil from African Petroleum (Nigeria). Nutrient agar and Nutrient broth from Fluka (Germany). DEAE-Sephadex A-50-120 and Sephadex G-25-300 from Sigma chemical Co. (Germany). Bacteriophage - Hind III digested (0.12-23.1kbp) from Roche, (Germany). All other chemicals and reagents were supplied by Sigma chemical Co. (Germany).

MICROORGANISMS

HMW PAHs degrading bacteria used in this study were isolated from PAHs polluted soils in Nigeria. Soil *E. coli* and *Klebsiella* used in molecular studies were obtained from Department of Botany and Microbiology, University of Lagos.

3.2 METHODS

3.2.1 SOIL SAMPLING

(i) Site selection: Four sampling locations were explored in Nigeria. PAH contaminated soil samples for microbial seeding were collected from (1) Eleme refinery, PortHarcourt; (2) coal mining site, Onyeama mine, Enugu; (3) wood processing site, Oshodi, Lagos and (4) diesel-power-generator site, Lagos. Accessibility, availability of open space and evidence of PAH soil pollution were the main criteria for the choice of sampling site.

(ii) Soil sample collection and PAHs analysis: Soil samples were collected using sterile spatula at a tillage depth of about 2 cm from 20 core points, mixed thoroughly in a clean plastic container and properly labelled. A subsample of the soil mixture was removed and placed in a soil sample bag, labelled and stored in coolers at the site, transported immediately to the laboratory for further work. The soil samples were analysed for PAHs content by high performace liquid chromatography (HPLC).

One gram (1g) of soil sample was extracted with ethyl acetate twice and filtered through a 0.45 µm membrane. The HPLC analyses were performed with VYDAC RP C18 reverse phase column (250 x 0.4 mm). Separation was achieved by gradient elution in acetonitrile: water (60%, 50%, 40%, 30%, 20%, 10%, 0% water), temperature 25^{0}C, with a flow rate of 0.8 ml/min and UV absorbance detector set at 254nm (Clemente *et al.*, 2001).

3.2.2 ISOLATION OF HMW PAHS DEGRADERS

HMW PAHs microbial degraders were isolated from soil samples by enrichment culture technique on mineral salt (MS) as described by Kastner *et al.*, (1994) using respective HMW PAHs (chrysene, fluoranthene and pyrene) as sole carbon and energy source. The MS contained per liter: Na_2HPO_4, 2.13 g; KH_2PO_4, 1.3 g; NH_4Cl, 0.5g and $MgSO_4.7H_2O$, 0.2 g (pH 7.2) and trace elements solution (1 ml per liter) as described by Ilori and Amund (2000), both sterilized separately by autoclaving at 121^{0}C for 20 min.

Microbial inocula were obtained by serially diluting one gram (1 g) of each soil sample to 10^{-5}. Aliquots (1 ml) of 10^{-4} and 10^{-5} of the soil samples were respectively transferred into sterile 250 ml Erlenmeyer flasks containing 20 ml MS (pH 7.2) and the respective HMW PAHs; chrysene, pyrene and fluoranthene (100 µg/ml HMW PAH in ethyl acetate) as sole carbon and energy source. The ethyl acetate was previously allowed to evaporate under sterilized condition before addition of MS. The flasks were labeled and wrapped with foil paper then incubated in the dark at 30±2.0°C for 7 days. Control MS media inoculated with the serially diluted soil samples without the test HMW PAHs were included to check the increase in turbidity of the experimental culture media as a measure of microbial growth. Turbidity of media were measured spectrophometerically as optical density at wavelength 600nm (OD_{600nm}) using MS as blank.

3.2.3 HARVEST AND ADAPTATION OF ISOLATED HMW PAHS DEGRADERS

Microbial cells were cold harvested from the various culture broths after 7 days of incubation by centrifugation at 4,000xg, 4^{0}C for 20 min and then washed twice in 10 ml sodium phosphate buffer (50 mM, pH 7.2). These served as the stock isolates. The microbial stocks (3 ml) were suspended in 20 ml MS containing the respective previously used carbon sources. The culture

flasks were wrapped with foil and incubated for 7 days with shaking at intervals. This ensured maximum adaptation of the microbes to the carbon source used. At the end of the adaptation period, the cells were cold harvested again, washed twice in sodium phosphate buffer (50 mM, pH 7.2, 4^0C) and resuspended in 10 ml of sodium phosphate buffer. These were labeled "adapted stock".

Bacteria were isolated from the "adapted stock" by culturing on nutrient agar plates for 48 h. This medium supports the growth of a wide range of bacteria. Isolates were picked from the plates and purified by sub-culturing on nutrient agar plates by streaking. Purified isolates were stored on nutrient agar slants properly labeled as "Purified isolate".

3.2.4 SCREENING AND SELECTION OF BEST HMW PAHS DEGRADING BACTERIA

In order to screen the isolates for the best HMW PAHs degrading bacteria, purified isolates were plated on solid MS agar containing the respective HMW PAHs (chrysene, fluoranthene and pyrene) as the sole carbon and energy source by spray-plate technique (Survery *et al.*, 2004). Culture plates were wrapped in foil paper and black polyethylene bag, and then incubated in the dark at 30±2.0°C for 14 days. Control MS plates free of HMW PAHs were included. Bacterial colonies which formed cleared zones on the HMW PAHs coated plates were selected as HMW PAHs degraders. They were further screened by subjecting the selected isolates to growth on respective HMW PAH MS broth, evaluating the turbidity of media (OD_{600nm}) and subsequent growth on replica HMW PAH MS agar plate (Supaka *et al.*, 2001). These were compared to control MS broth without HMW PAH.

3.2.5 IDENTIFICATION OF THE SELECTED HMW PAHS DEGRADING BACTERIA

Biochemical and morphological studies were undertaken to partially identify the selected HMW PAHs degraders according to Holt *et al.*, (1994) and Barrow and Felthanm (2003).

Gram Reaction

A thin smear of each of the isolates was prepared on a clean slide. It was air dried and heat fixed by passing it horizontally through a Bunsen flame. The smear was flooded with crystal violet stain for one minute and rinsed with sterile distilled water. Lugol's iodine was then poured on the smear for one minute before washing gently with sterile distilled water. The iodine is a mordant; it fixes the crystal violet stain more firmly into the cell. The smear was then decolourized with absolute alcohol continuously until no more crystal violet was left and immediately rinsed with water. The slide was counter stained with safranin for 30 sec. The slide was drained, washed, blotted dry with a piece of filter paper and finally allowed to air dry. It was then examined under oil immersion lens of a light microscope at x100 magnification. Gram positive organisms were characterized by retention of the purple colour of the basic stain while Gram negative organisms showed reddish pink colour.

Motility Test

This was carried out by hanging-drop method. Each of the bacterial isolate was grown in nutrient broth at room temperature for 24 hr. A drop of distilled water was placed in the center of a slide using loop. A small portion of the bacterial culture was transferred to the distilled water and mixed thoroughly. A circular wall was made around the depressed of a cavity slide using vaseline. A loopful of the mixture from the ordinary slide was transferred to the coverslip and pressed down gently to make an air tight seal. The slide was carefully re-inverted and

examined under oil immersion lens of the light microscope. Motility was observed as movement of cells in various directions.

Catalase Test

Catalase test is dependant on the presence of an enzyme catalase which breaks down hydrogen peroxide releasing oxygen. A suspension of the organism was made on a clean glass slide and a few drops of hydrogen peroxide added. Production of gas bubbles indicated a positive result.

Oxidase Test

Small pieces of the filter paper were soaked in 1% aqueous tetramethyl phenylenediamine dihydrochloride. Fresh culture of the isolates were picked with a sterile platinum wire and rubbed on the filter paper. A blue colour within 10 sec indicated a positive result.

Indole Test

This test was a demonstration of the ability of isolates to decompose an amino acid, tryptophan to indole. The bacteria cultures were grown in peptone water for 48 hr at room temperature. Kovac's reagent (0.5 ml) was added to the culture. Development of a rose pink colour was an indication of indole production.

Methyl Red Test

This test showed the production of an acid during the fermentation of glucose such that the pH of the culture was sustained below the value of 4.5 as shown by the colour change of the methyl red indicator added at the end of incubation period. Each isolate was inoculated in 5 ml Methyl-Red Voges Proskauer medium (MRVP) and incubated for 3 days at room temperature. Five drops of methyl-red (0.4%) were added. Red colour indicated a positive reaction while yellow colour indicated a negative reaction.

Voges-Proskauer Test

This reaction was dependant on the production of acetoin from glucose, which was then oxidized by the addition of alkali to diacetyl, resulting in a pinkish colour. Isolates were inoculated into MacCartney bottles containing MRVP medium and incubated for 2 days at room temperature. 1 ml potassium hydroxide (10%) was added, each bottle left at room temperature for 1 hr. Pink colour indicated a positive reaction and negative reaction was indicted by no colour change.

Gelatin liquefaction

Tubes of sterile nutrient gelatin were inoculated with the cultures and incubated at room temperature for 48 hrs. The tubes were then put in the refrigerator for 24 hrs at 5^0C and failure of the medium to solidify indicated production of gelatinase.

Citrate utilization

Slopes of Simmon's citrate were inoculated by streaking the isolates on the surface. The agar slopes were incubated at room temperature for 24 hrs. Positive reaction was shown by a change in colour of the agar from green to blue. Organisms unable to utilize citrate as their source of carbon did not grow.

Urease activity

Slants of Christensen's urea were inoculated with isolates and incubated at room temperature for 5 days. The production of red colour showed a positive urease.

Sugar fermentation (Bromo cresol purple indicator)

Sugar-indicator peptone water base (5 ml) in test tubes were tyndallised for 3 days at 100^0C for 30 min per day. The test tubes were inoculated with 24 hrs old bacterial cultures and incubated at room temperature for 48 hrs. Positive reaction was shown by colour change from purple to yellow. Durham tubes placed in the medium were also observed for presence of gas.

3.2.6 DETERMINATION OF THE GROWTH OPTIMAL pH AND TEMPERATURE OF THE SELECTED HMW PAHS DEGRADING BACTERIA

Growth of the isolates at different pH was determined by adjusting the pH values of different nutrient broth media to pH 2.0 - pH 9.6 using 0.1N HCL and 0.1 N NaOH solution. The medium (10 ml) were dispersed into test tubes before autoclaving. After autoclaving, the pH values were rechecked. The test tubes were inoculated with the test cultures and incubated at 30±2.0^{0}C for 48 - 120 hrs in water bath. Tubes containing sterilized 10 ml of nutrient broth were inoculated with respective isolates and incubated in water bath set at temperature range of 20^{0}C - 65^{0}C to determine growth of isolates at different temperatures. Turbidity (OD_{600nm}) of test broths were compared with the non-inoculated broth controls. Values for test culture media were recorded.

3.2.7 GROWTH POTENTIAL OF THE SELECTED ISOLATES ON LIQUID HYDROCARBONS

Growth of the respective isolates on liquid hydrocarbons were evaluated by growing each in sterile 250 ml Erlenmeyer flasks containing 99 ml MS and various sterile substrates (1% v/v) which included hexane, xylene, toluene, phenol, benzene, diesel, kerosene, crude oil and engine oil (Ilori and Amund, 2000). Incubation was carried out at 30±2.0^{0}C for 7 days. Turbidity (OD_{600nm}) of test broths were compared with the non-inoculated broth controls. Cultures without increase in turbidity over initial optical density and optical density of the non-inoculated control were scored as no growth (-) while cultures with increased turbidity significantly greater than the control were scored as growth (+). The growth rate was expressed as percentage hydrocarbon degradation.

3.2.8 GROWTH POTENTIAL OF THE SELECTED ISOLATES ON DIFFERENT PAHS

Spray-plate technique was followed for this purpose as described by Kiyohara *et al.*, (1983) and Survery *et al.*, (2004). The PAHs used are naphthalene, anthracene, phenanthrene, chrysene, pyrene and fluoranthene. PAHs solution (0.1% w/v PAH ethyl acetate) was sprayed onto the surface of MS agar plate aseptically. The ethyl acetate was evaporated leaving behind a thin film of PAH on the agar surface. Overnight culture of respective isolates was spotted on the MS agar plates, incubated in the dark at 30±2.0^{0}C for 14 days. The appearance of growth on the plates indicated positive test, these were scored and compared to control non-PAH MS agar plates. Growth rate on the solid PAHs was evaluated as zone clearance on MS PAH agar plate and colony formed as compared to control expressed as percentage PAH degradation.

3.2.9 BIODEGRADATION OF HMW PAHS

Biodegradation of single HMW PAH (chrysene, fluoranthene and pyrene) were set up (Nwachukwu *et al.*, 2001; Supaka *et al.*, 2001) using the isolates individually and as consortium. Biodegradation of mixture of the HMW PAH and mixture of the HMW PAH in the presence of phenanthrene were also studied using the degraders also individually and as consortium.

PREPARATION OF STARTER CULTURE

Bacterial cells selected as best HMW PAHs degraders were inoculated into 10ml nutrient broth respectively and incubated for 48 hrs. Growth cultures from nutrient broth were readapted to HMW PAH utilization by growing in sterilized 20 ml of respective MS HMW PAH broths and incubated for 7 days at 30±2.0^{0}C. The cells were cold harvested, washed twice by

centrifugation at 4000× *g*, 4^0C for 15 min and suspended in 10 ml sodium phosphate buffer (50 mM, pH 7.2). The harvested cells were used for the HMW PAHs degradation experiment.

BIODEGRADATION OF SINGLE HMW PAH BY INDIVIDUAL ISOLATES

The isolates were used individually to biodegrade single HMW PAH (chrysene, fluoranthene or pyrene) using it as sole carbon and energy source. MS media (20 ml, pH 7.2) were put into clean 250 ml Erlenmeyer flasks containing evaporated HMW PAH (100 µg/ml). The media were sterilized and inoculated with 3 ml (10^4 cells) of the starter culture. These were foiled and incubated in the dark at 30±2.0^0C, shaking at intervals. This set up was designated "Experimental" (E).

Two controls (C1 and C2) were also set up to evaluate the role played by the test isolate in biodegrading the HMW PAHs. C1 consisted of the same materials present in E but without HMW PAH while C2 contained all the materials in E with no test bacteria inoculated and was designed to evaluate the role played by contaminants picked from the laboratory environment where the study was carried out.

Biodegradation test were set up in duplicate for 8 days, with samples taken at 48 hrs interval. Biodegradation of the HMW PAHs was assayed by determining the microbial population density measured spectrophometerically as optical density at wavelength 600nm (OD_{600nm}), total viable count (TVC) of the organisms by spread plate technique on nutrient agar plate, pH of the media and residual HMW PAHs using HPLC (Clemente *et al.*, 2001). All samples for HPLC were extracted with 5 ml of ethyl acetate and filtered before chromatographic separation.

BIODEGRADATION OF SINGLE HMW PAH BY BACTERIAL CONSORTIUM

The same procedure in biodegradation of single HMW PAH by individual isolates was undertaken but the HMW PAHs degrading bacteria were used as a consortium to degrade the single HMW PAH using it as the sole carbon and energy source.

BIODEGRADATION OF SINGLE HMW PAH BY BACTERIAL CONSORTIUM IN THE PRESENCE OF PHENANTHRENE

The same procedure in biodegradation of single HMW PAH by individual isolates was undertaken but the HMW PAHs degrading bacteria were used as a consortium to degrade the single HMW PAH using it as the sole carbon and energy source in the presence of phenanthrene as co-substrate.

BIODEGRADATION OF MIXTURE OF THE HMW PAHS BY INDIVIDUAL ISOLATES

The same procedure in biodegradation of single HMW PAH by individual isolates was undertaken but the HMW PAHs degrading bacteria were used individually to degrade mixture of the HMW PAHs using them as carbon and energy sources.

BIODEGRADATION OF MIXTURE HMW PAHS BY BACTERIAL CONSORTIUM

The same procedure in biodegradation of single HMW PAH by individual isolates was undertaken but the HMW PAHs degrading bacteria were used as a consortium to degrade mixture of the HMW PAH using them as carbon and energy sources.

BIODEGRADATION OF MIXTURE HMW PAHS BY BACTERIAL CONSORTIUM IN THE PRESENCE OF PHENANTHRENE

The same procedure in biodegradation of single HMW PAH by individual isolates was undertaken but the HMW PAHs degrading bacteria were used as a consortium to degrade mixture of the HMW PAHs using them as carbon and energy sources in the presence of phenanthrene as co-substrate.

3.2.10 ISOLATION OF CATECHOL DIOXYGENASE

3.2.10.1 PREPARATION OF CELL EXTRACT

Isolates were grown at 30±2.0°C in five separate 200 ml aliquots of MS containing fluoranthene as the growth substrate. After the cultures had grown to an optical density (OD_{600nm}) of 1.6, the cells were harvested. Cells were removed by centrifugation at 4,000× *g*, 4°C for 20 min. The pellets were resuspended in Tris-HCl buffer (50 mM; pH 7.5). After another centrifugation at 4,000× *g*, 4°C for 20 min, the cells were suspended in 9 ml of the same buffer. Disruption took place in cold homogenization with glass beads. Cells debris was removed by centrifugation at 10,000× *g*, 4°C for 20 min and the supernatant removed by decanting. The supernatant was used as the crude enzyme extract.

3.2.10.2 ENZYME ASSAY

The HMW PAHs degraders were evaluated for catechol dioxygenase activity. Catechol 1,2-dioxygenase was determined (Briganti *et al.*, 1997) as the increase in absorbance at 260nm (corresponding to the formation of *cis,cis*-muconate at ε of 26,000 liters/mol · cm) measured in a silica cuvette with a 1.0-cm light path. One unit of activity was defined as the amount of enzyme required to form 1 µmol of product per min under the conditions of the assay. Catechol

2,3-dioxygenase was measured by a modification of the method of Nozaki (Kaschabek *et al.*, 1998) as the increase in absorbance at 375nm (corresponding to the formation of 2-hydroxymuconic semialdehyde at ϵ of 36,000 liters/mol · cm) measured in a silica cuvette with a 1.0cm light path. One unit of activity was defined as the amount of enzyme required to form 1 µmol of product per min under the conditions of the assay.

The reaction mixture contained 50 µmol of phosphate buffer (pH 7.5) and 0.1 µmol of catechol in a total volume of 1 ml.

3.2.10.3 PARTIAL PURIFICATION OF CATECHOL DIOXYGENASE

All enzyme purification steps were carried out at 4°C in duplicate. Protein concentrations were determined by the Bradford method using crystalline bovine serum albumin as protein standard as described by Jayaraman (1992).

AMMONIUM SULFATE PRECIPITATION

Ammonium sulfate $(NH_4)_2SO_4$ was added to crude enzyme extract (10 ml) with constant stirring to give 40% saturation. After 30 min of equilibration, the precipitate was collected by centrifugation at 8,000× *g*, 4°C for 20 min. The pellet was dissolved in Tris-HCl buffer (50 mM; pH 7.5) to give a total volume of 3 ml and the resulting protein solution was desalted by dialysis.

DIALYSIS

The crude enzyme extract (3 ml) from ammonium sulfate precipitation process was placed in dialysis bag. The extract was dialyzed against Tris-HCl buffer (50 mM; pH 7.5). The set-up was allowed to stand in the refrigerator for 5 hrs after which the buffer solution was replaced

and kept in the refrigerator for further 24 hrs. The dialyzed crude enzyme extract was made up to 3 ml with Tris-HCl buffer (50 mM; pH 7.5).

DEAE-SEPHADEX A-50-120 ION-EXCHANGE CHROMATOGRAPHY

Dialysed sample (0.5 ml) was applied to anionic-exchange column DEAE-Sephadex A-50-120 (1.4 x 33 cm). The column was pre-equilibrated with buffer A and flow rate adjusted to 3.0 ml/min. Catechol dioxygenase was eluted with buffer A containing NaCl in a linear gradient from 0 to 1M. Absorbance at 280nm was read and fractions containing the highest level of enzyme activity were pooled.

GEL FILTRATION SEPHADEX G-25-300

The pooled supernatants from DEAE-Sephadex A-50-120 ion-exchange chromatography were subjected to gel filtration using Sephadex G-25-300 (1.5 x 34 cm), pre-equilibrated with buffer A containing 200 mM NaCl and flow rate adjusted to 1.0 ml/min. Fractions of 1 ml were collected for the determination of the catechol dioxygenase activity and absorbance at 280nm.

3.2.10.4 PARTIAL CHARACTERIZATION OF CATECHOL DIOXYGENASE

The molecular weight of the catechol dioxygenase was determined. In addition, the effects of temperature, pH, metal ions and catechol concentration on the isolates catechol dioxygenase were evaluated.

MOLECULAR WEIGHT OF THE CATECHOL DIOXYGENASE ENZYME

The molecular weight of the catechol dioxygenase of the isolates was determined through gel filtration using sephadex G-25-300. This was achieved by subjecting a mixture (10 µg each) of

lyophilised catechol dioxygenase fractions from gel filtration (sephadex G-25-300) and known molecular mass proteins to gel filtration using sephadex G-25-300. The protein markers used were chicken egg lysozyme (*Mr* 14,000), chicken egg albumin (*Mr* 43,000), bovine serum albumin (*Mr* 67,000) and catalase (*Mr* 240, 000). Elution volume for each protein standard plotted against its relative molecular mass was used to extrapolate the molecular mass of the catechol dioxygenase enzymes.

EFFECT OF TEMPERATURE ON ENZYME ACTIVITY

Effect of temperature on catechol dioxygenase activity was estimated at temperature range 20^0C - 80^0C. The enzyme (10 µg) was incubated with catechol (0.1 µM) in sodium phosphate buffer in adjusted water bath for 30 min after which enzyme activity was evaluated.

EFFECT OF pH ON ENZYME ACTIVITY

The optimum pH of catechol dioxygenase of isolates were determined by measuring enzyme activity after 10 µg of enzyme was incubated with catechol (0.1 µM) at different pH values of the reaction mixtures for 30 min. The pH was adjusted with 2 N NaOH or 2 N HCl. The various buffers used for observing the effect of pH on the enzyme activity were 50 mM citrate buffer (pH 3 - 6), sodium phosphate buffer (pH 7 - 8) and glycine-NaOH buffer (pH 8.5 to 10).

EFFECT OF METAL IONS ON ENZYME ACTIVITY

To assign the metal of the catechol dioxygenase, reconstitution studies were carried out. The enzyme (200 µg) was dialyzed for 5 hr at 4°C against 1liter of Tris-HCl buffer (100 mM; pH 7.5). Aliquots of 10 µg of protein were incubated with catechol (0.1 µM) for 15 min at room temperature in the presence of 0.1 mM metal ion such as Mg^{2+}, Co^{2+}, Ni^{2+}, Mn^{2+}, Fe^{2+}, Fe^{3+} and

also ethylene diamino tetraacetic (EDTA). Thereafter, the enzyme activity was determined and compared with enzyme activity of a sample without an added metal ion.

KINETIC CONSTANTS DETERMINATION

Kinetic constants for the isolates catechol dioxygenase were determined using concentrations varying between 0.1 μM and 10 μM of catechol in buffer A at optimum temperature and pressure. The Michaelis-Menten constant (K_m) and maximal velocity (V_{max}) were determined using the method of double reciprocals (Jayaraman, 1992). All catechol dioxygenase activity values are provided as the means of three replications.

3.2.11 PLASMID DETECTION, CURING AND BIOTRANSFORMATION

In order to evaluate the role played by plasmid in HMW PAHs degradation, the isolates were evaluated for possession of plasmid. Thereafter, their plasmids were cured. In addition, the potential of the HMW PAHs degrading bacteria to transfer their HMW PAHs degradation plasmid to non-plasmid habouring organisms were evaluated by biotransformation process.

All isolates used (original isolates, cured and biotransformed) were evaluated for HMW PAHs degradation and tetracycline selectivity by streaking on tetracycline selective medium.

EXTRACTION OF PLASMID

The method adopted for plasmid isolation and detection was as described by Kado and Liu (1981). Bacteria cells were cultured in Lauria Bertani (LB) broth for 18 hrs, the medium centrifuged at 9,000× *g* for 5 min and the supernatant removed. The pellets were then suspended in 200 μl of buffer E (40 mM Tris and 2 mM EDTA), the eppendorf tubes equilibrated on ice for 10 min, then centrifuged and supernatants collected.

Lysing buffer (400 µl, 4% SDS and 100 mM Tris) was added to the supernatant mixed by inverting the tube until the solution became slimy. Buffer F (300 µl, 3M sodium acetate at pH 5.5) was then added to the mixture, vortexed for 10 sec and incubated on ice for 30 min then centrifuged at 10,000× *g* for 15 min and the supernatant transferred into another eppendorf tube. Chloroform (700 µl) was added to the supernatant and vortexed to remove impurities. This was done twice to ensure complete removal of all the impurities. The supernatant was later separated by gentle aspiration; this was done with the use of a micropipette. The supernatant obtained was the solution containing the bacterial DNA.

Absolute ethanol (1 ml) was then added to the supernatant to precipitate the DNA after which incubation was carried out for 1 hr on ice to ensure complete precipitation. The solution was centrifuged at 4000× *g* for 30 min and decanted to retain the DNA pellets. Ethanol (1 ml, 70%) was then added to the pellets to wash away the effect of the buffers that were previously added and then centrifuged at 4000× *g* for 5 min. The solution containing the DNA pellets and the ethanol was removed from the centrifuge, decanted and rinsed again with 70% ethanol to completely wash away all the buffers. The supernatant was rinsed off and the DNA pellets were air dried by ensuring that the eppendorf tubes attained a slanted position inclined at an angle of 45^0C to the horizontal.

The pellets after air drying were then dissolved in 60 µl of Tris EDTA buffer (TE Buffer) at room temperature for 5 min after which they were stored at -20^0C.

AGAROSE GEL ELECTROPHORESIS

Agarose gel (0.8%) was prepared and 2 drops of ethidium bromide was added to it and swerved gently on the bench for some minutes. The agarose gel mixture was then loaded onto electrophoretic slab till it gelled properly. Subsequently, a comb was inserted into the

electrophoretic slab and removed after the agarose solidified creating wells on the gel. The samples and DNA molecular markers were mixed with bromophenol blue and then loaded into the wells. The electrophoretic tank was plugged to the electrophoresis power pack (Thermo EC machine) and the samples in the wells were allowed to run for about an hour or until the samples migrated fully from the negative pole (since DNA is negatively charged) to the positive pole. The gel was then removed from the tank and placed on a UV-transilluminator and photographed with polaroid film.

PLASMID CURING

To ascertain whether degradation of the HMW PAHs was mediated by chromosomal or extra chromosomal DNA, the isolates plasmids were cured by chemical agent, Sodium deodecyl sulfate (SDS) (El-Mansi *et al.*, 2000). An SDS solution (10% w/v, pH 7.4) was prepared with LB (double strength), this was further diluted to 10 ml in MacCartney bottles with distilled H_2O to give 5, 4, 3, 2, 1 and 0.5% SDS in the LB. An inoculum of 100 µl of the isolate was then used to seed the SDS-containing LB and the cultures were incubated overnight with shaking at 37^0C until growth became visible, as evidenced by turbidity.

Aliquots from the highest concentration of SDS that still allowed bacterial growth were serially diluted and spread onto nutrient agar plates and incubated at 37^0C for 48 hrs. Individual colonies that developed were thereafter evaluated for HMW PAHs degradation and tetracycline sensitivity. Colonies that proved unable to utilize the HMW PAHs as sole carbon and energy source were evaluated for the presence of plasmid and tetracycline sensitivity.

BIOTRANSFORMATION

Biotransformation (*in vivo* direct gene transfer) was performed by method of Sabeen *et al.*, 2004). The donor organisms were the original HMW PAHs degraders while the recipients were the cured isolates and two other soil organisms cured of their plasmids which were *E. coli* and *Klebsiella*. Donor and recipient cultures were grown separately in LB broth at 37^{0}C for 24 hrs. Next day both cultures were inoculated in fresh LB broth separately and incubated with shaking at 37^{0}C for 24 hrs. Donor and recipient cultures were mixed in1:10 ratio and incubated again at 37^{0}C for 24 hrs. Tubes contents were then centrifuged at 4,000× *g*, 20 min, supernatant discarded and loopfull of pellet were evaluated for HMW PAHs degradation potential and streaked onto tetracycline selective medium.

3.2.12 BIOSURFACTANT PRODUCTION

A bacterial strategy which influences hydrocarbon transfer between cell and medium is the release of biosurfactant. The HMW PAHs degrading bacteria were evaluated for production of biosurfactant by two methods; emulsification index (E24) and haemolytic assay (Ilori and Amund, 2001; Moraes *et al.*, 2002) by growing isolates on varying quantities of chrysene and diesel oil respectively.

GROWTH CONDITIONS UTILIZING VARYING QUANTITIES OF CHRYSENE AND DIESEL OIL RESPECTIVELY AS THE SOLE CARBON AND ENERGY SOURCE

The growth on varying quantities of chrysene and diesel oil respectively and concomitant production of biosurfactant by the HMW PAHs degraders were evaluated over a time course. The MS media contained per liter (pH 7.2) NH_4NO_3, 4.0 g; Na_2HPO_4, 2.0 g; KH_2P0_4, 0.53 g;

K_2SO_4, 0.17 g; $MgSO_4.7H_2O$, 0.10 g and trace element (Ilori and Amund, 2001). Varying quantities of chrysene (1.5 mg, 2.5 mg and 5 mg dissolved in ethyl acetate) and diesel oil (3%, 5%, 15% and 30% v/v) respectively as sole carbon source were added in duplicate into 250 ml Erlenmeyer flasks containing sterilized 50 ml MS. The ethyl acetate was allowed to evaporate before the MS was added. Starter cultures (3 ml) were inoculated into the media. Chrysene culture flasks were foiled to prevent photolysis. The inoculated flasks were incubated at 30±2.0°C and shaken at interval. Growth of isolates was determined at 24 hrs interval for 7 days by monitoring total viable counts (TVC) and the optical density (OD_{600nm}). Non-carbon source media were also incubated and analyzed. Measurements were carried out in triplicate.

EMULSIFYING ACTIVITY MEASUREMENT

After 7 days of incubation, culture broths were made cell free by centrifugation at 10,000× *g*, 10 min, 4°C and heat sterilized. Cell-free extracts (2.0 ml) were vortex with 2.0 ml n-hexadecane in a test tube and left undisturbed for 24 hrs. The emulsification index (E24) was determined by measuring the volume occupied by the emulsion formed in the test tubes. Results are expressed as the percentage volume occupied by the emulsion (Ilori and Amund, 2001; Moraes *et al.*, 2002). Emulsifying activity was also measured against different hydrophobic sources (kerosene, diesel oil, engine oil, dodecane, xylene and hexane). Emulsifying activity measurement was repeated using the bacterial cells.

HAEMOLYTIC ACTIVITY

Zones of clearance on blood agar plates have been used as a rapid method for screening microorganisms for potential biosurfactant production (Lin, 1996). This was undertaken to confirm the production of biosurfactant by the HMW PAHs degrading bacteria. The bacterial

cells from chrysene and diesel oil growth media respectively were plated onto blood agar plate and incubated at 30±2.0^{0}C for 48 hrs (Bicca *et al.*, 1999, Tabatabaee *et al.*, 2005).

DATA ANALYSIS

The experimental assays were done in triplicates, unless otherwise stated. Statistically significant difference ($p<0.05$) was determined using Student's t-test. Results are expressed as MEAN ± SEM (Standard Error of Mean). These statistical analyses were done using Statistical Package for the Social Science11.0 for windows (SPSS 11.0).

CHAPTER FOUR

4.0 RESULTS

4.1 SOIL SAMPLE SITES AND DISTRIBUTION OF PAHS IN SOILS

In this study, soil samples from locations of potential sources of polycyclic aromatic hydrocarbons (PAHs) were selected for seeding HMW PAHs degraders. These are Eleme refinery, Portharcourt and coal mining site (Onyeama mine), Enugu, located on the eastern part of Nigeria while wood processing site, Oshodi and diesel-power-generator site in Lagos are located on the Western part of Nigeria.

The soil samples were found to contain both high molecular weight PAHs (HMW PAHs) and low molecular weight PAHs LMW PAHs which include chrysene, fluoranthrene, pyrene, anthracene and phenanthrene (Fig. 4.1 A and B). The concentrations of chrysene, fluoranthene, pyrene and phenanthrene obtained were as follows: For Eleme refinery, PortHarcourt: chrysene (45.21 μg/g), fluoranthene (57.01 μg/g), pyrene (46.35 μg/g) and phenanthrene (50.37 μg/g). Coal mining site (Onyeama mine); chrysene (157.21 μg/g), fluoranthene (10.83 μg/g), pyrene (0.0 μg/g) and phenanthrene (0.0 μg/g). Wood processing site; chrysene (58.23 μg/g), fluoranthene (15.82 μg/g), pyrene (7.57 μg/g) and phenanthrene (14.01 μg/g) while for diesel-power-generator site; chrysene (70.97 μg/g), fluoranthene (96.78 μg/g), pyrene (50.29 μg/g) and phenanthrene (43.69 μg/g).

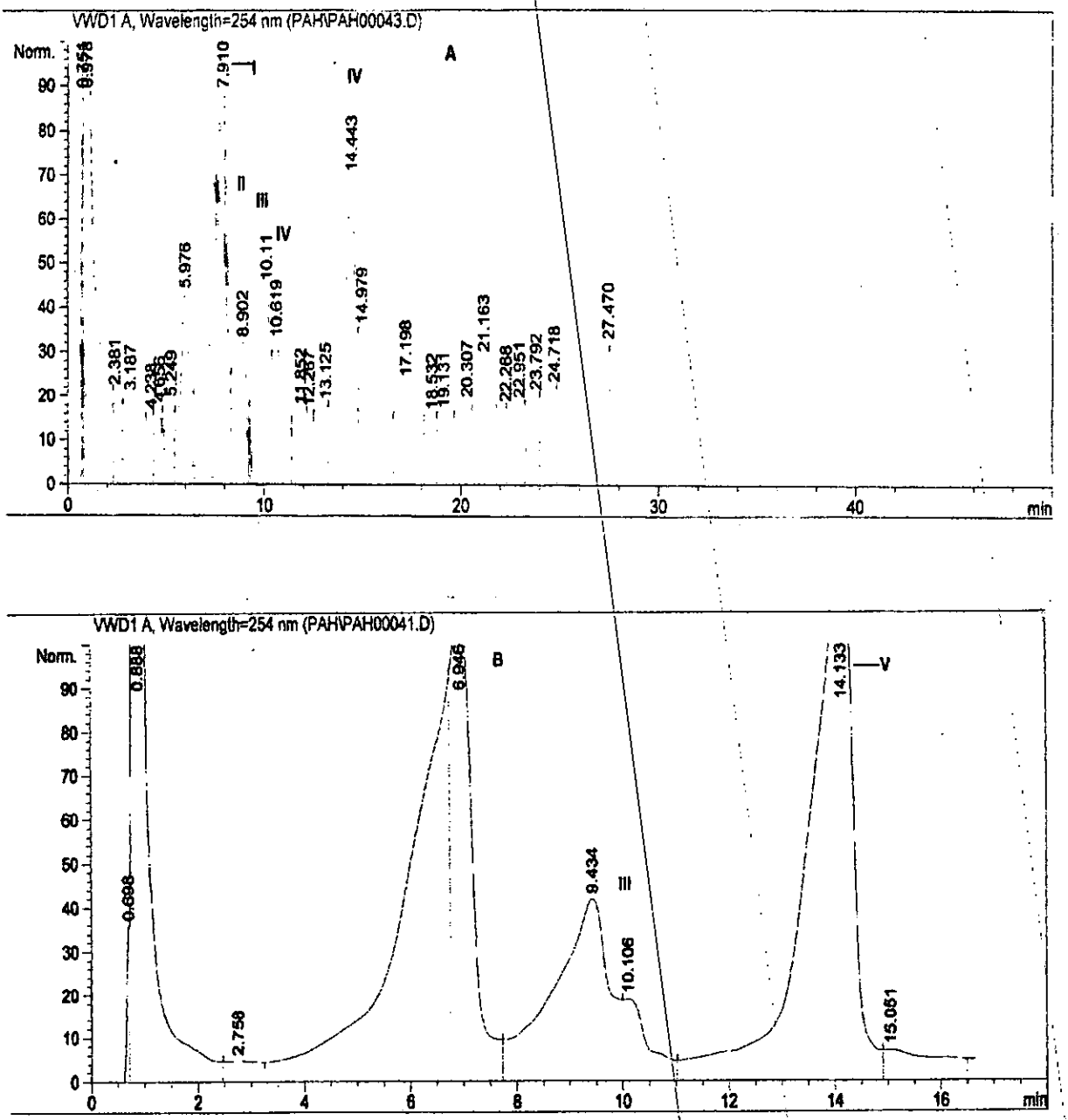


Fig. 4.1A. HPLC chromatograms showing the distribution of polycyclic aromatic hydrocarbons in soil samples. A = Refinery soil sample, B= coal mining soil sample.

Key:

I = Phenanthrene **II = Anthracene** **III = Fluoranthene**

IV = Pyrene **V = Chrysene**

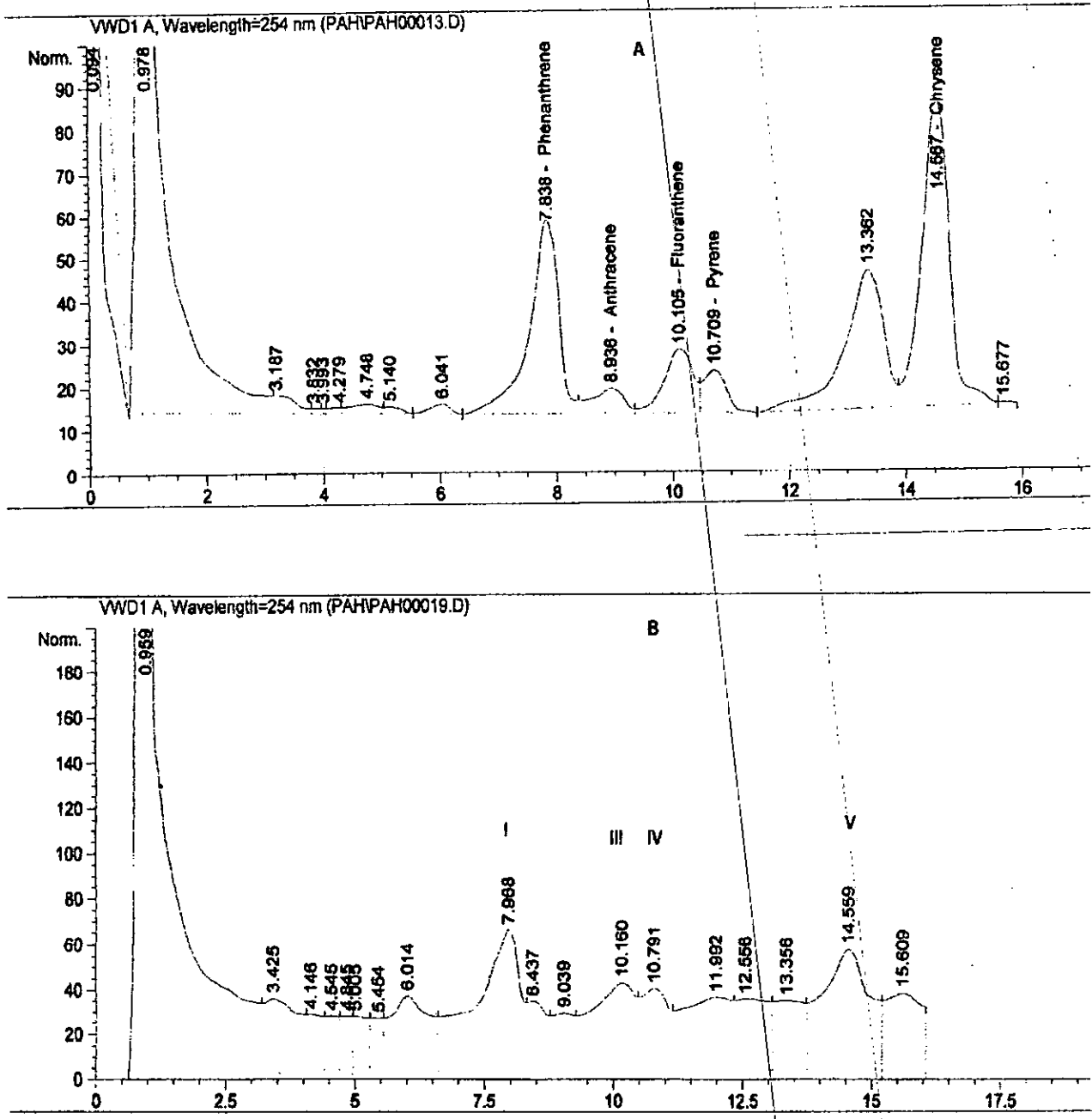


Fig. 4.1B. HPLC chromatograms showing the distribution of polycyclic aromatic hydrocarbons in soil samples. A= wood processing soil sample, B= diesel oil -power-generator soil sample.

I = Phenanthrene **II = Anthracene** **III = Fluoranthene**

IV = Pyrene **V = Chrysene**

4.2 ISOLATION AND ADAPTATION OF HMW PAHS DEGRADERS

Serially diluted soil samples inoculated into MS HMW PAHs (chrysene, pyrene and fluoranthene) media showed microbial growth and utilization of the HMW PAHs as sole carbon and energy source as indicated by increases in turbidity of the test growth media compared to controls. Twenty seven isolates were isolated as HMW PAHs degraders. These are the "purified isolates" and a representation of the four sampling sites.

4.3 SCREENING AND SELECTION OF BEST HMW PAHS DEGRADING BACTERIA

Six bacterial isolates (6) were selected out of the 27 isolates as the best HMW PAHs degraders. They were able to grow appreciably on the three HMW PAHs (chrysene, fluoranthene and pyrene) respectively. They were selected based on cleared zones on MS HMW PAHs agar and increased turbidity of MS HMW PAHs media compared to control and other isolates.

4.4 IDENTIFICATION OF THE SELECTED HMW PAHS DEGRADING BACTERIA

Based on morphological and biochemical identification studies on the selected best HMW PAHs degraders such as Gram reaction, cellular morphology, catalase, oxidase, indole, motility, methyl red, voges-proskauer, citrate, urease, starch hydrolysis, gelatin hydrolysis, NO_3 reduction, coagulase test, spore test and sugar utilization, the 6 bacteria isolates were identified as *Acinetobacter anitratus*, *Acinetobacter mallei*, *Alcaligenes faecalis*, *Sphingomonas paucimobilis*, *Pseudomonas arvilla* and *Pseudomonas putida* (Tables 4.1 a and b).

Table 4.1a. Biochemical and morphological properties of the selected HMW PAHs degrading bacteria

PARAMETER TESTED	RESULTS OBTAINED					
Gram reaction	-	-	-	-	-	-
Cellular morphology	Coccoid rods	Rods	Rods	Rods	Rods	Rods
Catalase	+	+	+	+	+	+
Oxidase	-	-	+	+	+	-
Indole	-	-	-	-	-	-
Motility	-	-	+	+	-	+
Methyl red	-	-	-	-	-	-
Voges-Proskauer	-	-	-	-	-	-
Citrate	+	+	+	+	+	+
Urease	+	-	-	+	-	-
Starch hydrolysis	-	-	-	-	-	-
Gelatin hydrolysis	-	-	-	-	-	-
Probable Identity	***Acinetobacter anitratus***	***Acinetobacter mallei***	***Alcaligenes faecalis***	***Sphingomonas paucimobilis***	***Pseudomonas arvilla***	***Pseudomonas putida***

Key - = Negative reaction + = Positive reaction

Table 4.1b. Biochemical and morphological properties of the selected HMW PAHs degrading bacteria Contd.

PARAMETER TESTED	RESULTS OBTAINED					
NO_3 reduction	-	+	+	+	+	+
Coagulase test	-	-	-	-	-	-
Spore test	-	-	-	-	-	-
Mannitol	-	+	-	-	+	+
Glucose	+	+	+	+	+	+
Xylose	+	-	-	-	+	+
Lactose	+	-	-	-	-	-
Sucrose	-	-	+	-	-	-
Raffinose	-	-	-	-	-	-
Arabinose	-	-	-	-	-	-
Maltose	-	-	-	-	-	-
Galactose	-	-	+	-	-	-
Salicin	-	-	-	-	-	-
Probable Identity	*Acinetobacter anitratus*	*Acinetobacter mallei*	*Alcaligenes faecalis*	*Sphingomonas paucimobilis*	*Pseudomonas arvilla*	*Pseudomonas putida*

Key **- = Negative reaction** **+ = Positive reaction**

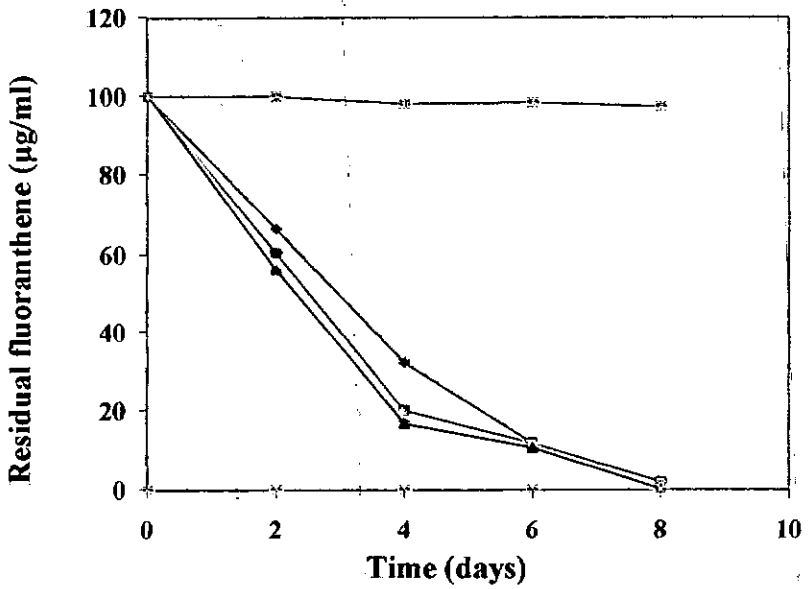


Fig 4.8. Residual fluoranthene during degradation of fluoranthene by individual isolates

- *S. paucimobilis*
- *Ps. arvilla*
- *Ps. putida*
- C1 (no fluoranthene + isolate)
- C2 (fluoranthene + no isolate)

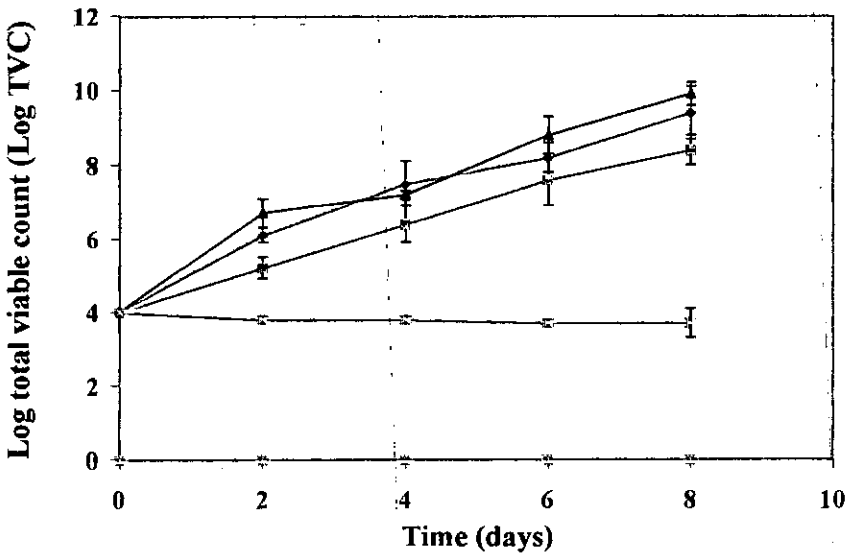


Fig. 4.9. Growth of isolates during degradation of fluoranthene by individual isolates

- *S. paucimobilis*
- *Ps. arvilla*
- *Ps. putida*
- C1 (no fluoranthene + isolate
- C2 (fluoranthene + no isolate

4.5 DETERMINATION OF THE GROWTH OPTIMAL pH AND TEMPERATURE OF THE SELECTED HMW PAHS DEGRADING BACTERIA

Isolates were incubated at different pH and temperature range to determine the appropriate pH and temperature that best supports the isolates growth.

4.5.1 GROWTH OF THE HMW PAHS DEGRADING BACTERIA AT DIFFERENT pH

Growth of isolates at various pH (Fig. 4.2) showed that the isolates were found not to grow at pH range of 2.0 – 4.9. They all had growth above 0.2 (OD_{600nm}) at pH range of 5.0 – 9.6 and growth at these pH were significantly ($P<0.05$) higher than at pH 2.0 – 4.9. At pH range 8.1 – 9.6, *Ps. putida* showed significantly greater growth than the other isolates. However, pH range of 7.1 – 7.5 best supported the isolates growth.

4.5.2 GROWTH OF THE HMW PAHS DEGRADING BACTERIA AT DIFFERENT TEMPERATURE

A. anitratus, A. mallei, A. faecalis, S. paucimobilis, Ps. arvilla and *Ps. putida* grew significantly ($P<0.05$) at temperature range of 20^0C - 40^0C (Fig. 4.3). At 45^0C, A. *faecalis, Ps. arvilla* and *S. paucimobilis* showed lowered growth compared to *A. anitratus, A. mallei* and *Ps. putida* which grew significantly ($P<0.05$) as observed by the turbidity of the culture media. *Ps. putida* was the only degrader that grew significantly ($P<0.05$) at 55^0C. At 65^0C, none of the degraders showed significant growth.

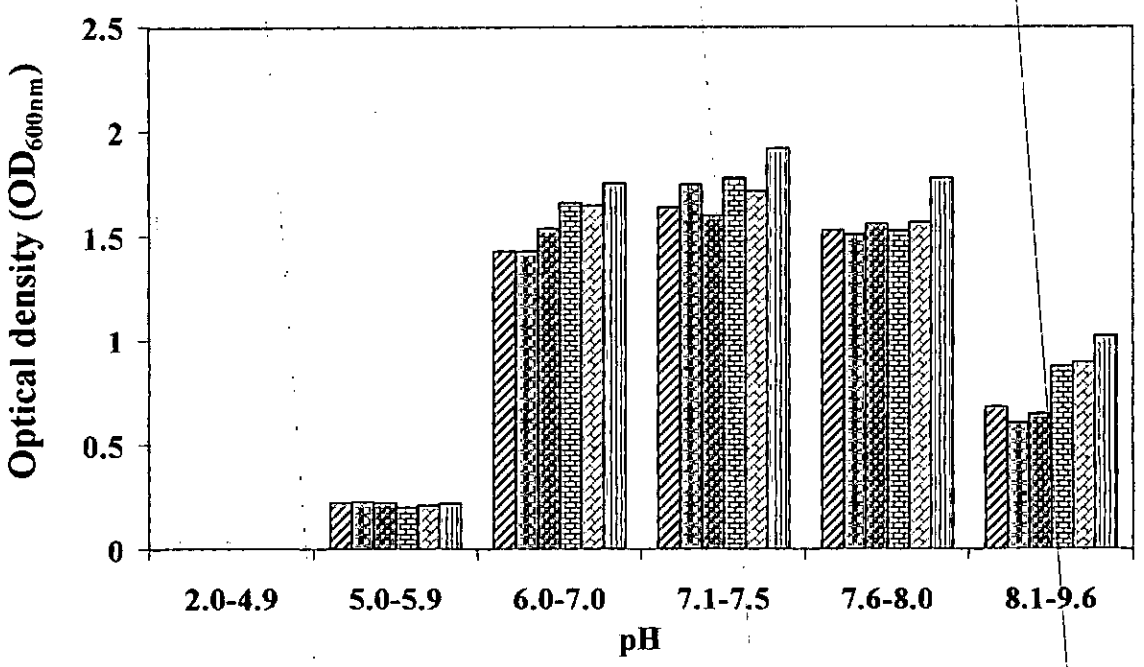


Fig. 4.2. Growth of the HMW PAHs degrading bacteria at different pH

A. anitratus *A.mallei* *A.faecalis* *S.paucimobilis* *Ps.arvilla* *Ps.putida*

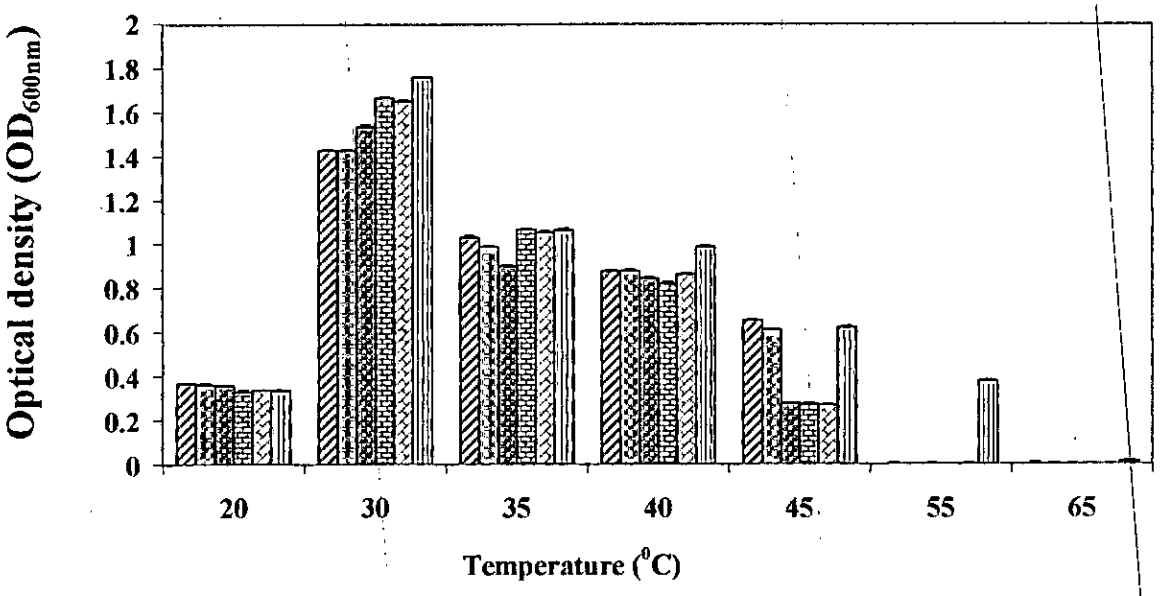


Fig. 4.3. Growth of the HMW PAHs degrading bacteria at different temperature

A.anitratus *A.mallei* *A.faecalis* *S.paucimobilis* *Ps.arvilla* *Ps.putida*

4.6 GROWTH POTENTIAL OF THE SELECTED ISOLATES ON LIQUID HYDROCARBONS AND DIFFERENT PAHS

The selected HMW PAHs degrading bacteria were evaluated for potential utilization of various hydrocarbons as growth substrates. The isolates growth rate was expressed as percentage degradation (Table 4.2). Under the experimental conditions, rate of degradation of crude oil and engine oil by *A. anitratus*, *A. mallei*, *A. faecalis*, *S. paucimobilis*, *Ps. arvilla* and *Ps putida* was 100%. Diesel oil was degraded by the six isolates at 80%, while 70% degradation of kerosene was observed for *A. anitratus*, *A. mallei*, *S. paucimobilis*, *Ps. arvilla* and *Ps. putida* whereas for *A. faecalis* it was 60%.

None of the isolates degraded hexane, xylene and toluene. Benzene was degraded by *Ps. putida* (70%), *Ps. arvilla* (60%) and *S. paucimobilis* (60%). Percentage degradation of phenol *S. paucimobilis*, *Ps. arvilla* and *Ps. putida* were 70%, 60% and 70% respectively.

The low molecular weight PAHs (anthracene, naphthalene and phenanthrene) were degraded 100% by all the isolates.

The high molecular weight PAHs; chrysene was degraded at 100%, 80%, 80%, 70%, 60% and 70% by *A. anitratus*, *A. mallei*, *A. faecalis*, *S. paucimobilis*, *Ps. arvilla* and *Ps. Putida* respectively. Fluoranthene was degraded 100% by *A. anitratus*, *A. mallei*, *S. paucimobilis*, *Ps. arvilla* and *Ps. putida* whereas 80% degradation was obtained for A. *faecalis*. Percentage degradation of pyrene was 90% for *S. paucimobilis* and *Ps. putida*, 80% for *Ps. arvilla*, 70% for *A. anitratus* and 60% for *A. mallei* and *A. faecalis*.

Table 4.2. Percentage Hydrocarbon Degradation by the selected HMW PAHs Degrading Bacteria (%)

Substrate	*A. anitratus*	*A. mallei*	*A. faecalis*	*S. paucimobilis*	*Ps. arvilla*	*Ps. putida*
Crude oil	100	100	100	100	100	100
Kerosene	70	70	60	70	70	70
Diesel oil	80	80	80	80	80	80
Engine oil	100	100	100	100	100	100
Hexane	0	0	0	0	0	0
Xylene	0	0	0	0	0	0
Phenol	0	0	0	70	60	70
Toluene	0	0	0	0	0	0
Benzene	0	0	0	70	60	60
Anthracene	100	100	100	100	100	100
Naphthalene	100	100	100	100	100	100
Phenanthrene	100	100	100	100	100	100
Chrysene	100	80	80	70	60	70
Fluoranthene	100	100	80	100	100	100
Pyrene	70	60	60	90	80	90

4.7 BIODEGRADATION OF HMW PAHS

Of the six bacteria isolates selected as best HMW PAHs degraders, it was observed that *A. anitratus*, *A. mallei* and *A. faecalis* easily lost their HMW PAHs degradation potential and as such, they were excluded from use in the biodegradation studies.

Therefore *S. paucimobilis*, *Ps. arvilla* and *Ps. putida* were used to study the biodegradation of chrysene, fluoranthene and pyrene as single substrates, mixture and in the presence of phenanthrene, a LMW PAH.

4.7.1 BIODEGRADATION OF SINGLE HMW PAH BY INDIVIDUAL ISOLATES

Biodegradation of the single HMW PAH (chrysene, fluoranthene and pyrene) was studied using *S. paucimobilis*, *Ps. arvilla* and *Ps. putida* individually.

4.7.1.1 RESIDUAL CHRYSENE DURING DEGRADATION OF CHRYSENE BY INDIVIDUAL ISOLATES

Degradation of 100 µg/ml chrysene by *S. paucimobilis*, *Ps. arvilla* and *Ps. putida* respectively resulted to a decrease in concentration to 30.5±0.3, 40.6±0.7 and 17.2±0.2 (µg/ml) respectively after 8 days indicating individual isolates utilization and thus degradation of chrysene (Fig. 4.4). There was significant ($P<0.05$) difference in the isolates rate of degradation even within same *Pseudomonas* genera. However, no significant change in chrysene concentration was observed in the control cultures.

4.7.1.2 GROWTH OF ISOLATES DURING DEGRADATION OF CHRYSENE BY INDIVIDUAL ISOLATES

The isolates were found to increase in cell mass significantly ($P<0.05$) in the experimental culture media (E) as the degradation of chrysene proceeded (Fig. 4.5). Total viable count (TVC) of $1.3x10^6$, $6.3x10^5$ and $3.2x1\ 0^6$ cfu/ml were obtained with *S. paucimobilis*, *Ps.*

arvilla and *Ps. putida* respectively after 8 days of degradation. There was difference in cell mass generation of the different isolates indicating that their rate of utilization of chrysene varied. The non-chrysene control (C1) inoculated with the organism did not show significant ($P<0.05$) growth increase but there was gradual decrease in cell mass. The control (C2) which had chrysene but without organism inoculated showed no significant microbial growth during the experimental period.

4.7.1.3 POPULATION DENSITY AND pH OF MEDIA DURING DEGRADATION OF CHRYSENE BY INDIVIDUAL ISOLATES

The population density of the experimental culture (E) increased as degradation of chrysene proceeded (Fig. 4.6). Population density (OD_{600nm}) of *S. paucimobilis*, *Ps. arvilla* and *Ps. putida* increased from 0.0018±0.0002 to 0.2715±0.0014, 0.2540±0.0045 and 0.3182±0.0038 respectively. This was a reflection of the increase in cell densities observed as the isolates utilized chrysene as sole source of carbon and energy. Turbidity of both control cultures C1 and C2 did not significantly ($P<0.05$) change during the experimental period.

During degradation of chrysene by the isolates, pH of 'E' changed compared to C1 and C2 where there was no change in pH (Fig. 4.7). The pH of *S. paucimobilis* and *Ps. arvilla* experimental culture media dropped from 7.2±0 to 6.8±0.1 indicating the breakdown of chrysene to acidic metabolites whereas for *Ps. putida*, pH dropped from 7.2±0.0 to 6.9±0.2 at day 4 and then rose to 7.3 at day 8.

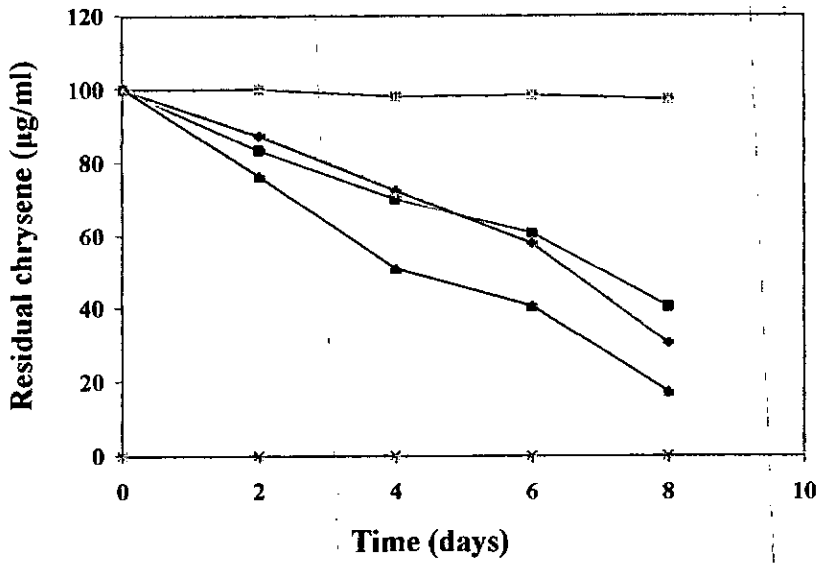


Fig 4.4. Residual chrysene during degradation of chrysene by individual isolates

- *S. paucimobilis*
- *Ps. arvilla*
- *Ps. putida*
- C1 (no chrysene + isolate)
- C2 (chrysene + no isolate)

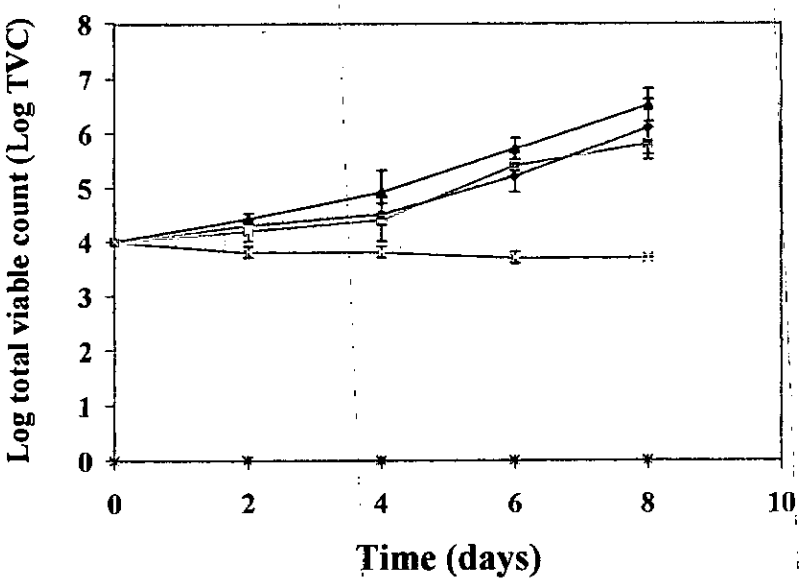


Fig. 4.5. Growth of isolates during degradation of chrysene by individual isolates

- *S. paucimobilis*
- *Ps. arvilla*
- *Ps. putida*
- C1 (no chrysene + isolate)
- C2 (chrysene + no isolate)

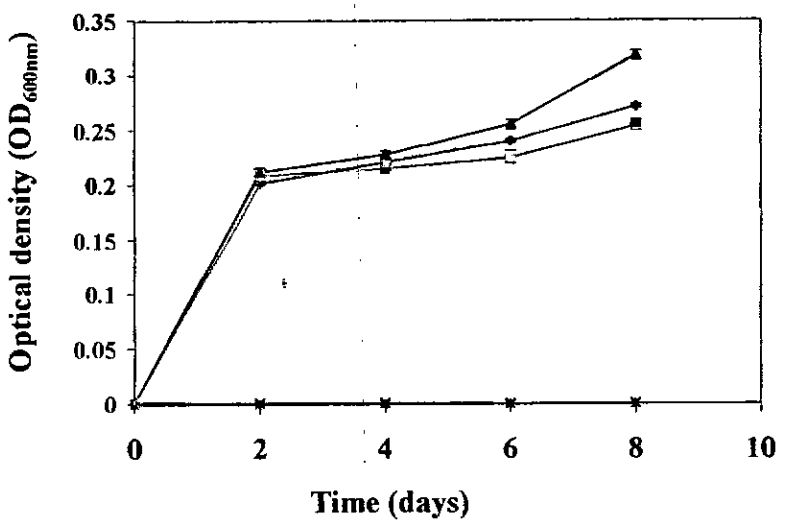


Fig. 4.6. Population dynamics of isolates during degradation of chrysene by individual isolates

- *S. paucimobilis*
- *Ps. arvilla*
- *Ps. putida*
- C1 (no chrysene + isolate)
- C2 (no chrysene + isolate)

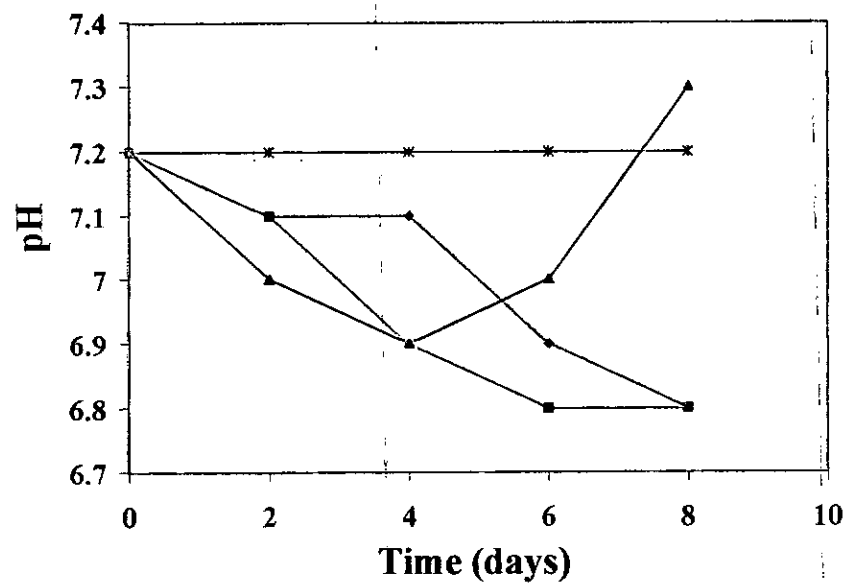


Fig. 4.7. pH of media during degradation of chrysene by individual isolates

- *S. paucimobilis*
- *Ps. arvilla*
- *Ps. putida*
- C1 (no chrysene + isolate)
- C2 (chrysene + no isolate)

4.7.1.4 RESIDUAL FLUORANTHENE DURING DEGRADATION OF FLUORANTHENE BY INDIVIDUAL ISOLATES

Residual fluoranthene concentrations after 8 days of 100 µg/ml degradation by *S. paucimobilis*, *Ps. arvilla* and *Ps. putida* was 2.0±0.1, 2.0±0.4 and 0.02±0.7 (µg/ml) respectively. There was significant ($P<0.05$) difference in the individual isolates degradation of fluoranthene (Fig. 4.8). The residual fluoranthene of 'E' was also significantly ($P<0.05$) different compared to the controls.

4.7.1.5 GROWTH OF ISOLATES DURING DEGRADATION OF FLUORANTHENE BY INDIVIDUAL ISOLATES

Significant ($P<0.05$) increase in cell densities of the isolates was observed when each of the isolates was cultivated on 100 µg/ml fluoranthene as the sole carbon source (Fig. 4.9). *Ps. putida* was better supported for growth with peak cell density of $7.9x10^9$ cfu/ml while *S. paucimobilis* and *Ps. arvilla* had cell densities of $2.5x10^9$ and $2.5x10^8$ cfu/ml respectively. No significant growth was observed in the C1 control, rather there was degeneration of the inocula.

4.7.1.6 POPULATION DENSITY AND pH OF MEDIA DURING DEGRADATION OF FLUORANTHENE BY INDIVIDUAL ISOLATES

The Population density (OD_{600nm}) of isolates in 'E' increased compared to controls as degradation of fluoranthene proceeded indicating utilization of fluoranthene as growth substrate (Fig. 4.10). Population density (OD_{600nm}) of *S. paucimobilis*, *Ps. arvilla* and *Ps. putida* increased from 0.0018±0.0002 to 0.3584±0.0012, 0.2520±0.0042 and 0.3804±0.0012 respectively after 8 days of incubation. The pH of 'E' changed compared to the controls during the experimental period (Fig. 4.11).

Degradation of fluoranthene resulted to a shift of pH from neutrality to alkalinity. Degradation by *S. paucimobilis* and *Ps. arvilla* resulted in a drop of pH from 7.2±0.0 to 6.8 at day 4 which rose to 7.7 while with *Ps. putida*, pH dropped from 7.2±0.0 to 7.0±0.1 at day 2 which rose to 8.0 at day 8.

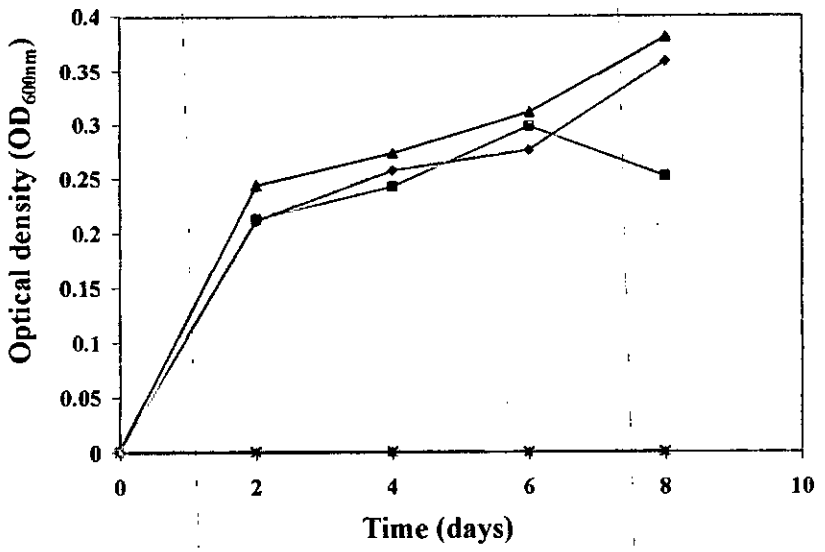


Fig. 4.10. Population dynamics of isolates during degradation of fluoranthene by individual isolates

- *S. paucimobilis*
- *Ps. arvilla*
- *Ps. putida*
- C1 (no fluoranthene + isolate)
- C2 (fluoranthene + no isolate)

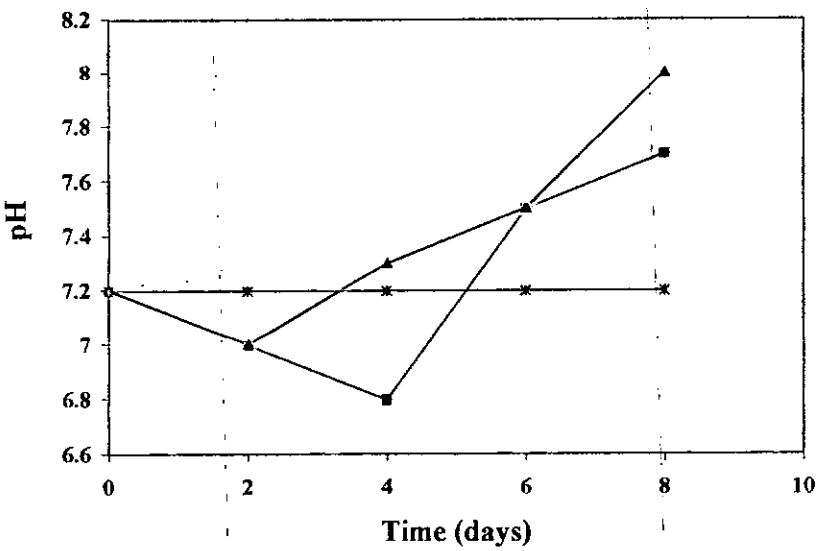


Fig. 4.11. pH of media during degradation of fluoranthene by individual isolates

- *S. paucimobilis*
- *Ps. arvilla*
- *Ps. putida*
- C1 (no fluoranthene + isolate)
- C2 (fluoranthene + no isolate)

4.7.1.7 RESIDUAL PYRENE DURING DEGRADATION OF PYRENE BY INDIVIDUAL ISOLATES

S. paucimobilis, *Ps. arvilla* and *Ps. putida* cultivated on MS containing 100 µg/ml pyrene as sole carbon and energy source resulted to a decrease in concentration to 0.06±0.2, 6.5±0.3and 6.6±0.4 (µg/ml) respectively after 8 days (Fig. 4.12). *S. paucimobilis* degraded pyrene better than the other isolates. There was no significant ($P<0.05$) difference in the degradation of pyrene by the two *Pseudomonas*. Furthermore, there was no significant change in pyrene concentration in C2 control cultures.

4.7.1.8 GROWTH OF ISOLATES DURING DEGRADATION OF PYRENE BY INDIVIDUAL ISOLATES

Optimal cell densities of $8.9x10^4$, $4.7x10^4$ and $7.0x10^4$ cfu/ml were obtained for *S. paucimobilis*, *Ps. arvilla* and *Ps. putida* respectively after 8 days of pyrene degradation (Fig. 4.13). The isolates were found to increase in cell mass significantly ($P<0.05$) in the experimental culture media (E) as the degradation of pyrene proceeded. Control C1 did not show significant ($P<0.05$) growth increase rather there was gradual decrease in cell mass. Control C2 showed no significant microbial presence during the experimental period.

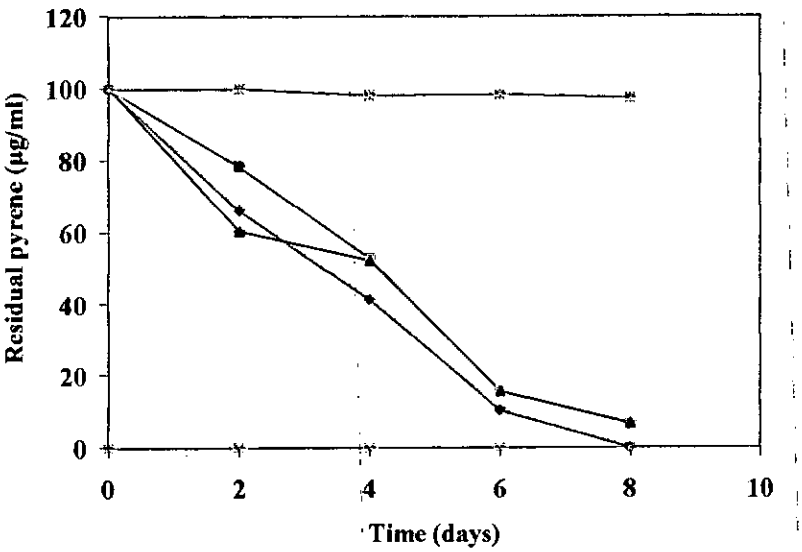


Fig 4.12. Residual pyrene during degradation of pyrene by individual isolates

S. paucimobilis *Ps. arvilla*
Ps. putida C1 (no pyrene)
C2 (pyrene + no organism)

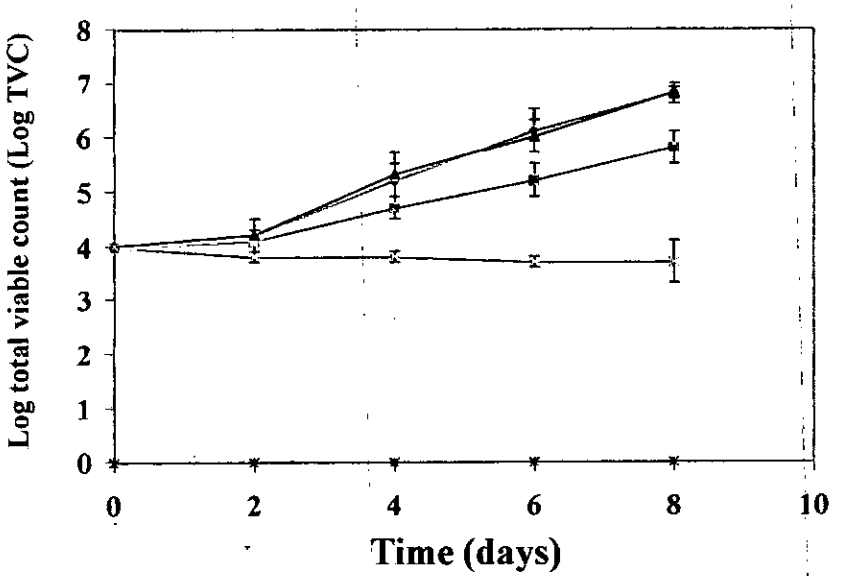


Fig. 4.13. Growth of isolates during degradation of pyrene by individual isolates

S. paucimobilis *Ps. arvilla* *Ps. putida*
C1 (no pyrene + isolate) C2 (pyrene + no isolate)

4.7.1.9 POPULATION DENSITY AND pH OF MEDIA DURING DEGRADATION OF PYRENE BY INDIVIDUAL ISOLATES

Population density (OD_{600nm}) of *S. paucimobilis*, *Ps. arvilla* and *Ps. putida* experimental media during pyrene degradation increased from 0.0018±0.0002 to 0.3480±0.0053, 0.2546± 0.0036 and 0.2741±0.0068 respectively (Fig. 4.14). The Population density (OD_{600nm}) of 'E' increased as degradation proceeded. There was significant ($p<0.05$) increase in the turbidity of the culture media compared to the controls.

The pH of 'E' dropped from 7.2±0.0 to 6.9 at day 4, after which there was rapid increase to 7.9, 7.5 and 7.5 respectively for *S. paucimobilis*, *Ps. arvilla* and *Ps. putida* (Fig. 4.15).

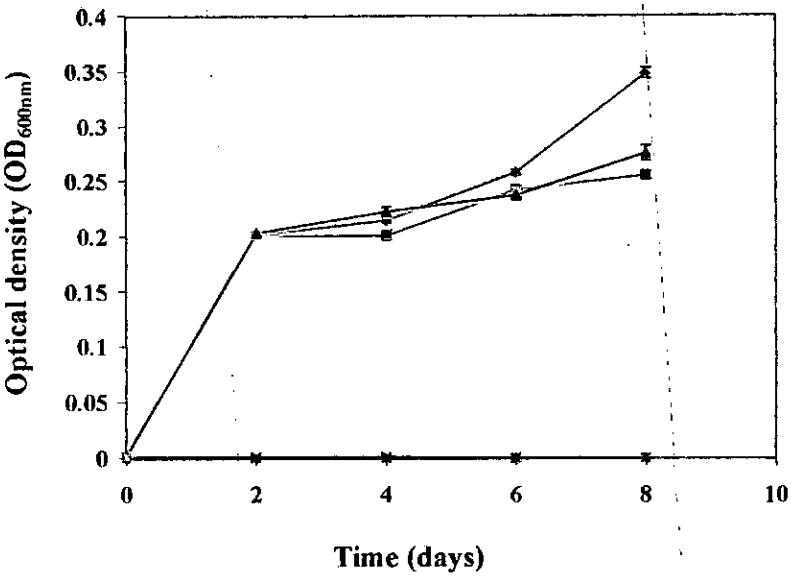


Fig. 4.14. Population dynamics of isolates during degradation of pyrene by individual isolates

S. paucimobilis *Ps. arvilla* *Ps. putida*
C1 (no pyrene + isolate) C2 (pyrene + no isolate)

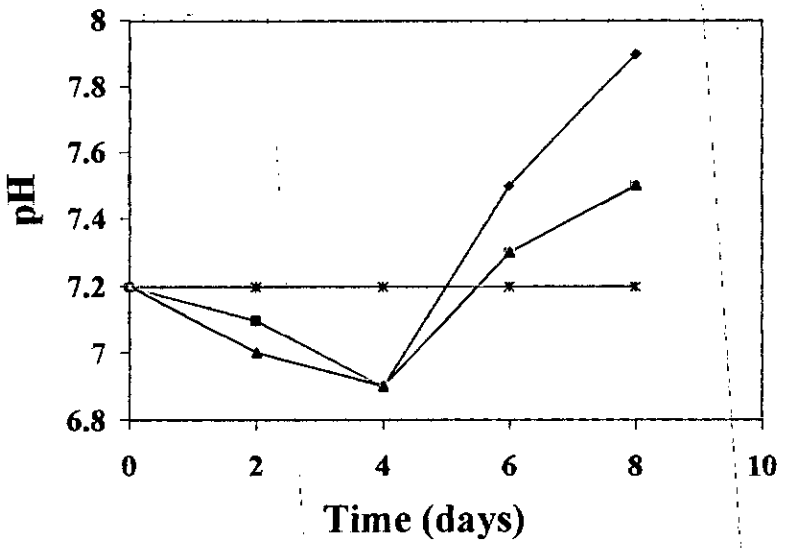


Fig. 4.15. pH of media during degradation of pyrene by individual isolates

S. paucimobilis *Ps. arvilla* *Ps. putida*
C1 (no pyrene + isolate) C2 (pyrene + no isolate)

4.7.2 BIODEGRADATION OF SINGLE HMW PAH BY BACTERIAL CONSORTIUM

In nature, microbes exist as consortia as such biodegradation of single HMW PAH (chrysene, fluoranthene and pyrene) was studied using *S. paucimobilis*, *Ps. arvilla* and *Ps. putida* together as consortium.

4.7.2.1 RESIDUAL HMW PAH DURING DEGRADATION OF SINGLE HMW PAH BY BACTERIAL CONSORTIUM

The consortium cultivated on MS containing 100 µg/ml of chrysene, fluoranthene and pyrene respectively as sole carbon and energy source, resulted to decrease in concentration to 21.3±0.2 (chrysene), 2.2±0.8 (fluoranthene) and 10.6±0.8 (pyrene) (µg/ml) after 8 days. As consortium, fluoranthene was better degraded than the other HMW PAHs with 97.8% degradation while 78.7% chrysene and 89.4% pyrene was degraded (Fig. 4.16). In control C2, there was no significant ($p<0.05$) loss of the HMW PAHs.

4.7.2.2 GROWTH OF BACTERIAL CONSORTIUM DURING DEGRADATION OF RESPECTIVE HMW PAH BY BACTERIAL CONSORTIUM

Cell densities of $8.9x10^4$, $2.3x10^6$ and $2.5x10^5$ cfu/ml respectively were obtained when the consortium degraded chrysene, fluoranthene and pyrene respectively (Fig. 4.17). Control C1 did not show significant ($P<0.05$) growth increase but there was gradual decrease in cell mass. Control C2 also showed no significant ($p<0.05$) microbial presence during the experimental period.

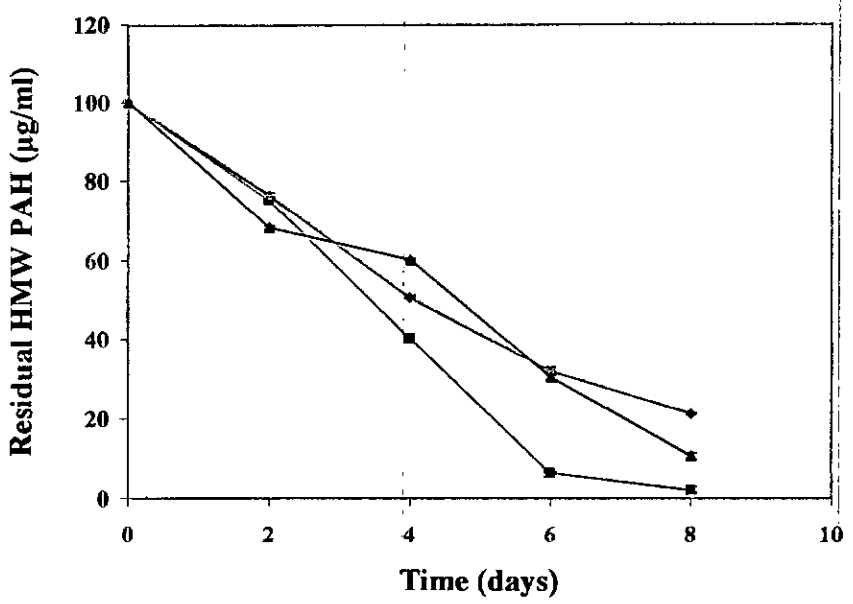


Fig 4.16. Residual HMW PAH during degradation of respective HMW PAH by bacterial consortium

Chrysene (µg/ml) Fluoranthene (µg/ml) Pyrene (µg/ml)

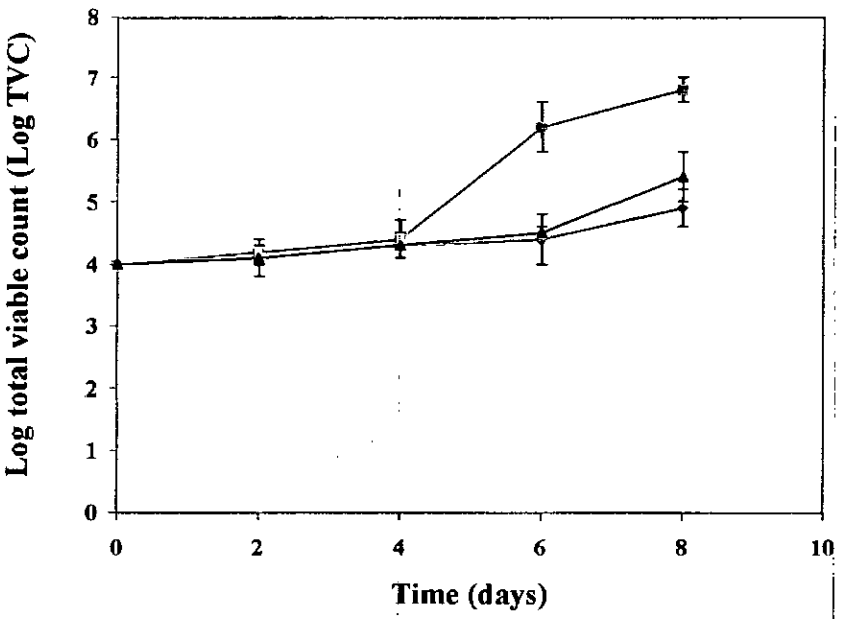


Fig. 4.17. Growth of bacterial consortium during degradation of respective HMW PAH by bacterial consortium

Chrysene Fluoranthene Pyrene

4.7.2.3 POPULATION DENSITY AND pH OF MEDIA DURING RESPECTIVE HMW PAH DEGRADATION BY BACTERIAL CONSORTIUM

Population density (OD_{600nm}) of consortium in the experimental media 'E' increased significantly ($p<0.05$) compared to the controls as degradation of respective HMW PAH by consortium proceeded (Fig. 4.18).

The pH of 'E' increased from 7.2±0.0 to 7.3±0.1, 7.7±0.1 and 7.5±0.2 respectively during chrysene, fluoranthene and pyrene degradation (Fig. 4.19). There were no significant changes in pH of the controls.

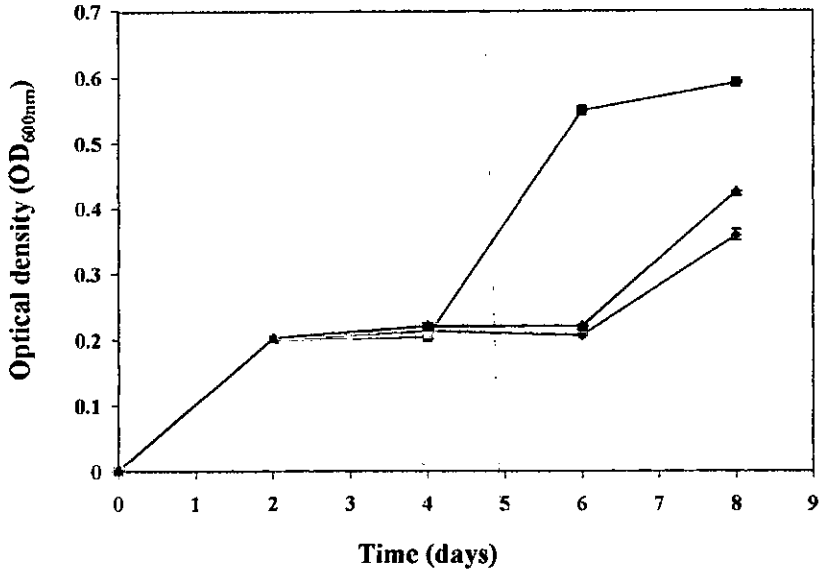


Fig. 4.18. Population dynamics of consortium during degradation of respective HMW PAH by consortium

Chrysene Fluoranthene Pyrene

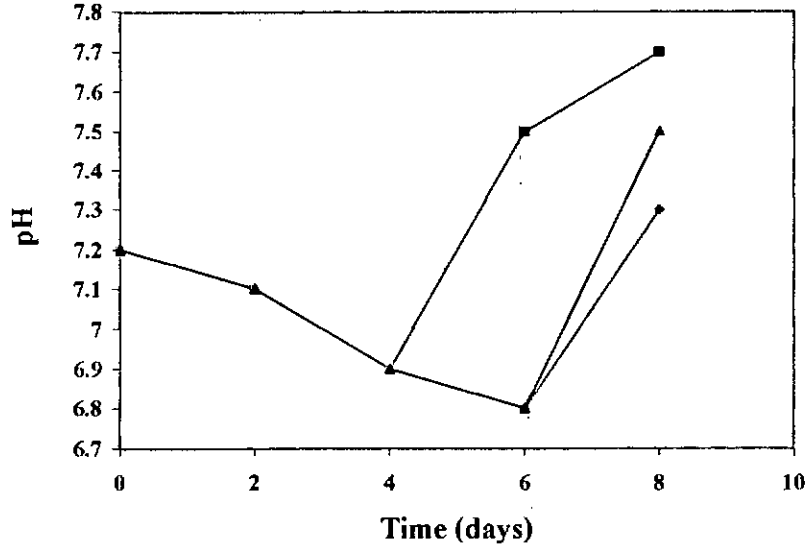


Fig. 4.19. pH of media during degradation of respective HMW PAH by consortium

Chrysene Fluoranthene Pyrene

4.7.3 BIODEGRADATION OF SINGLE HMW PAH BY BACTERIAL CONSORTIUM IN THE PRESENCE OF PHENANTHRENE

This study was to determine the role of phenanthrene in HMW PAHs degradation as observed in nature that PAH exists as complex mixture of LMW PAHs and HMW PAHs.

4.7.3.1 RESIDUAL CHRYSENE DURING DEGRADATION OF CHRYSENE BY BACTERIAL CONSORTIUM IN THE PRESENCE OF PHENANTHRENE

During the degradation of chrysene in the presence of phenanthrene, it was observed that chrysene degradation was delayed until 80.7% of phenanthrene had been utilized at day 4 (Fig. 4.20). Phenanthrene was not detectable after day 4 of degradation. Chrysene (100 µg/ml) was degraded to 12.4±0.1 µg/m after 8 days of incubation. No significant loss in chrysene was observed in C2.

4.7.3.2 GROWTH OF BACTERIAL CONSORTIUM DURING DEGRADATION OF CHRYSENE BY BACTERIAL CONSORTIUM IN THE PRESENCE OF PHENANTHRENE

Cell density of 2.3×10^6 cfu/ml was obtained when consortium degraded chrysene in the presence of phenanthrene. There was significant ($P<0.05$) change in cell densities at day 6 from Log TVC of 4.0 to 6.1 (Fig. 4.21). Presence of phenanthrene contributed to the immersed growth of the consortium. Control C1 and C2 did not show significant ($P<0.05$) microbial growth increase during the experimental period.

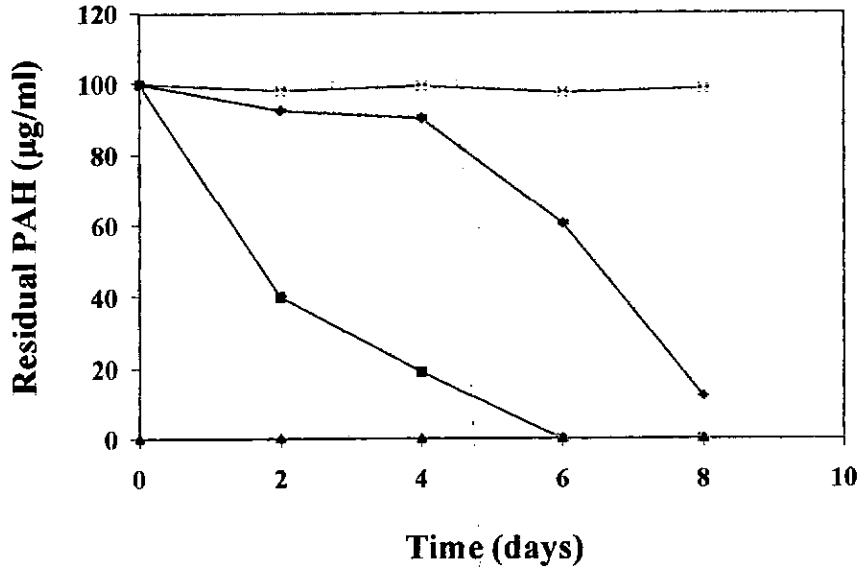


Fig 4.20. Residual chrysene during degradation of chrysene by consortium in the presence of phenanthrene

Chrysene
Phenanthrene
C1 (PAHs + no consortium)
C2 (no PAHs + consortium)

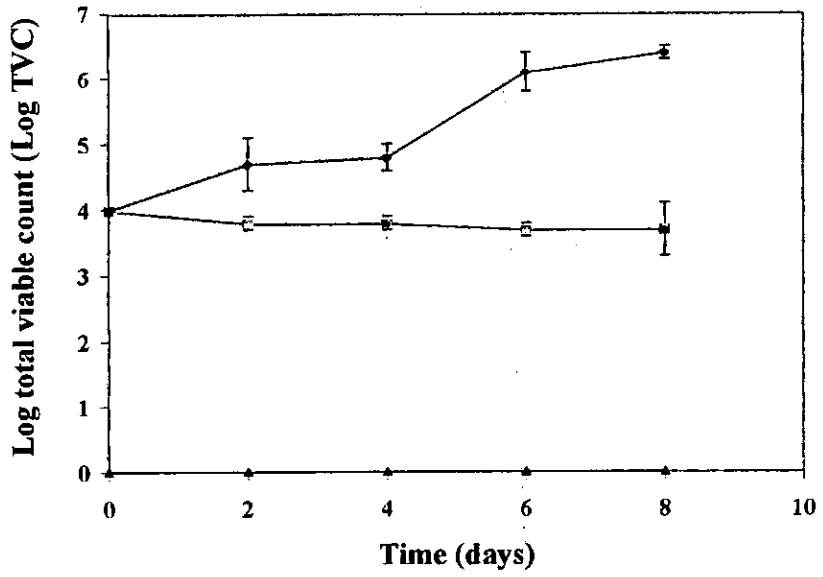


Fig 4.21. Growth of consortium during degradation of chrysene by consortium in the presence of phenanthrene

E (PAHs + consortium)
C1 (no PAHs + consortium)
C2 (PAHs + no consortium)

4.7.3.3 POPULATION DENSITY AND pH OF MEDIA DURING DEGRADATION OF CHRYSENE BY BACTERIAL CONSORTIUM IN THE PRESENCE OF PHENANTHRENE

The Population density (OD_{600nm}) of consortium in 'E' increased as degradation of chrysene proceeded (Fig. 4.22). There was significant ($P<0.05$) increase in the turbidity of 'E' compared to the controls wherein turbidity of 'E' increased from 0.0018±0.0002 to 0.5972±0.0037. The pH of 'E' dropped from 7.2 to 7.1 on day 2 which later rose to 7.5 on day 8. The pH of the control culture media remained unchanged (Fig. 4.23).

4.7.3.4 RESIDUAL FLUORANTHENE DURING DEGRADATION OF FLUORANTHENE BY BACTERIAL CONSORTIUM IN THE PRESENCE OF PHENANTHRENE

During the degradation of fluoranthene in the presence of phenanthrene, it was observed that fluoranthene degradation was delayed until 63.8% of phenanthrene had been utilized on day 2. Fluoranthene (100 µg/ml) was degraded to 0.2±0.3 µg/ml after 8 days of incubation. No significant loss in fluoranthene was observed in C2 (Fig. 4.24).

4.7.3.5 GROWTH OF BACTERIAL CONSORTIUM DURING DEGRADATION OF FLUORANTHENE BY BACTERIAL CONSORTIUM IN THE PRESENCE OF PHENANTHRENE

Cell density of $2.3x10^{11}$ cfu/ml was obtained when consortium degraded fluoranthene in the presence of phenanthrene after 8 days. There was significant ($P<0.05$) change in cell density at day 2 from Log TVC of 4.0 to 6.7 and later to Log TVC of 11.4 on day 8 (Fig. 4.25).

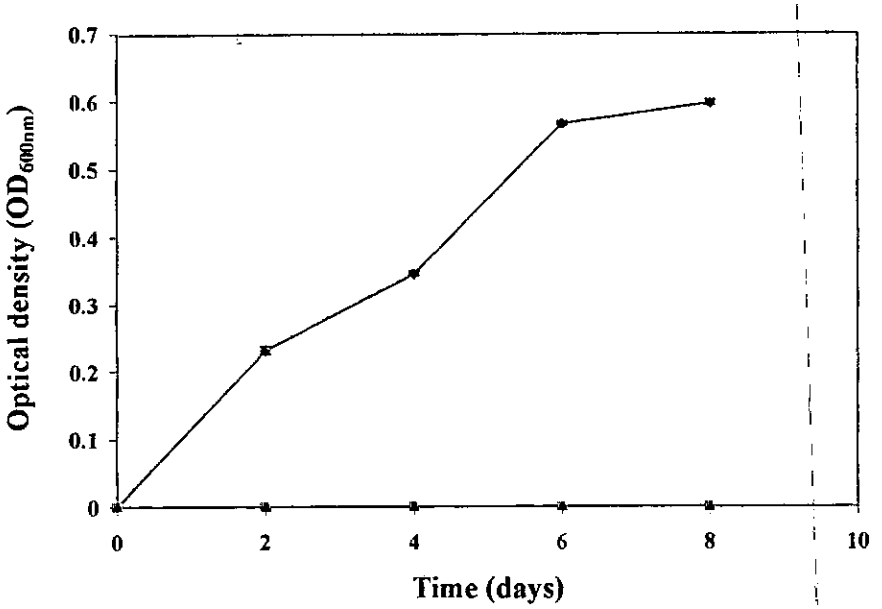


Fig 4.22. Population dynamics of consortium during degradation of chrysene by consortium in the presence of phenanthrene

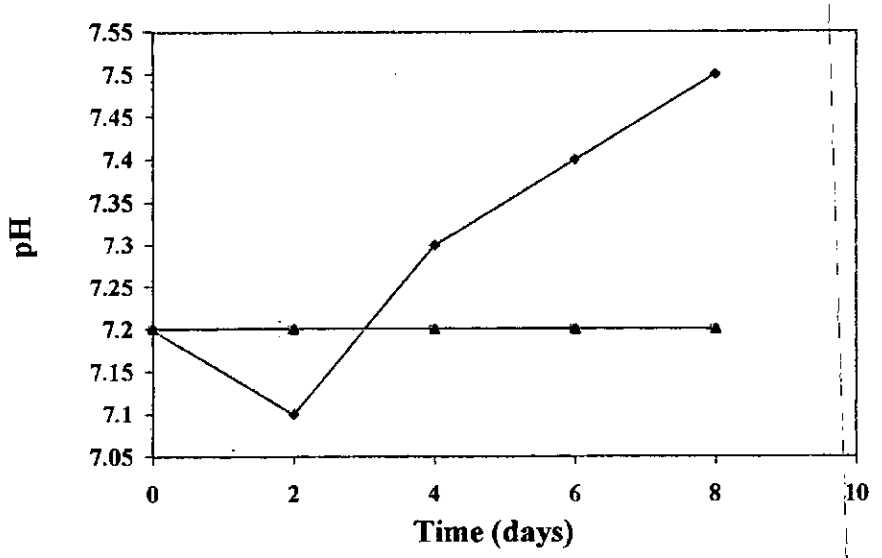


Fig 4.23. pH of media during degradation of chrysene by consortium in the presence of phenanthrene

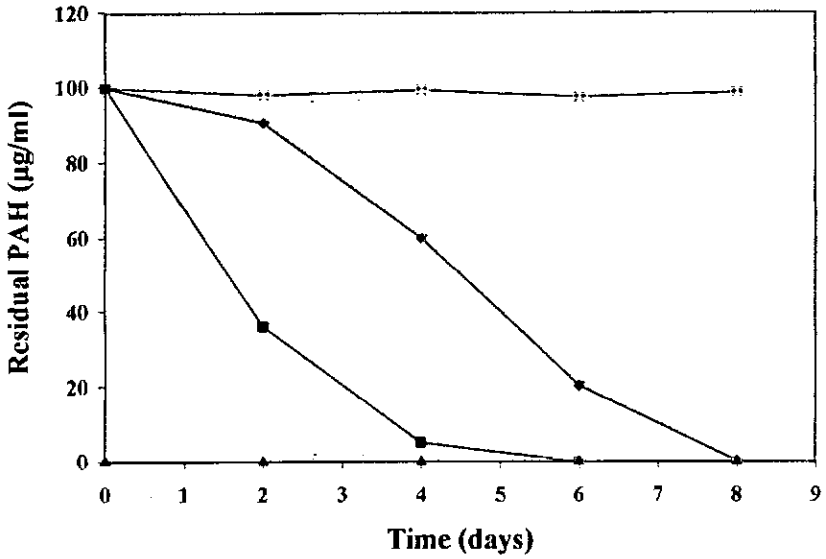


Fig. 4.24. Residual fluoranthene during degradation of fluoranthene by consortium in the presence of phenanthrene

Fluoranthene **Phenanthrene**
C1 (no PAHs + consortium) **C2 (PAHs + no consortium)**

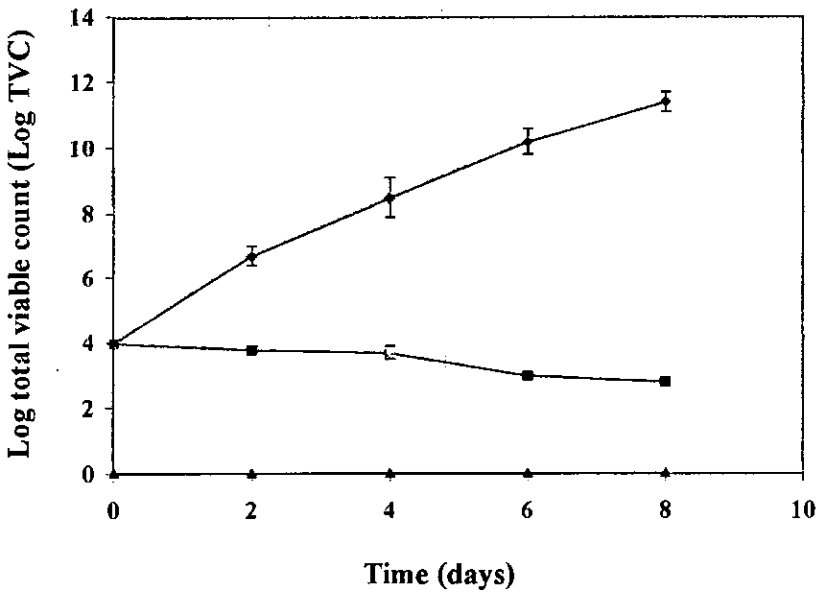


Fig. 4.25. Growth of consortium during degradation of fluoranthene by consortium in the presence of phenanthrene

E (PAHs + consortium) **C1 (no PAHs + consortium)**
C2 (PAHs + no consortium)

4.7.3.6 POPULATION DENSITY AND pH OF MEDIA DURING DEGRADATION OF FLUORANTHENE BY BACTERIAL CONSORTIUM IN THE PRESENCE OF PHENANTHRENE

The Population density (OD_{600nm}) of consortium in 'E' increased as degradation of fluoranthene by consortium in the presence of phenanthrene proceeded. Population density (OD_{600nm}) of 'E' increased from 0.0018±0.0002 to 1.2308±0.0076 after 8 days of incubation. There was significant ($P<0.05$) increase in the population density of 'E' compared to the controls (Fig. 4.26).

The pH of 'E' was increased as degradation proceeded compared to the controls where there was no significant ($P<0.05$) change (Fig. 4.27).

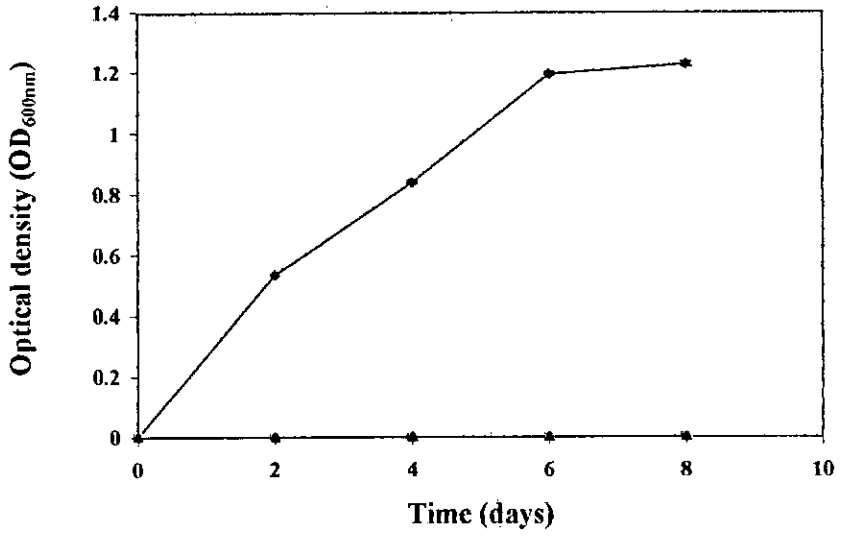


Fig. 4.26. Population dynamics of consortium during degradation of fluoranthene by consortium in the presence of phenanthrene

E (PAHs + consortium)
C1 (no PAHs + consortium)
C2 (PAHs + no consortium)

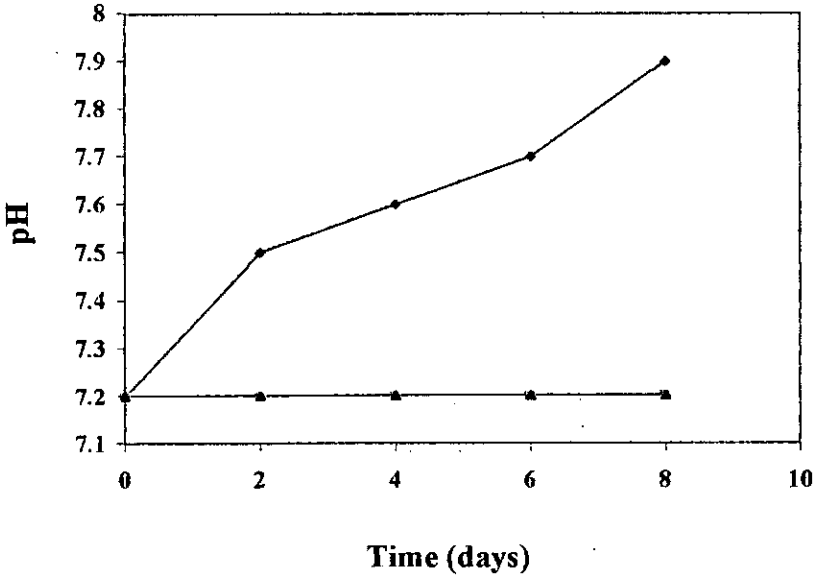


Fig. 4.27. pH of media during degradation of fluoranthene by consortium in the presence of phenanthrene

E (PAHs + consortium)
C1 (no PAHs + consortium)
C2 (PAHs + no consortium)

4.7.3.7 RESIDUAL PYRENE DURING DEGRADATION OF PYRENE BY BACTERIAL CONSORTIUM IN THE PRESENCE OF PHENANTHRENE

Degradation of pyrene in the presence of phenanthrene proceeded concomitantly. Pyrene and phenanthrene were degraded from 100 µg/ml to 40.3±0.5 and 25.3±0.6 µg/ml respectively at day 4. No significant concentration of phenanthrene was obtained after day 6 while 0.7±0.2 µg/ml pyrene was obtained on day 8. No significant loss in pyrene was observed in C2 (Fig. 4.28).

4.7.3.8 GROWTH OF BACTERIAL CONSORTIUM DURING DEGRADATION OF PYRENE BY BACTERIAL CONSORTIUM IN THE PRESENCE OF PHENANTHRENE

Highest cell density of $8.9x10^9$ cfu/ml was obtained when consortium degraded pyrene in the presence of phenanthrene. There was significant ($P<0.05$) change in cell density at day 4 from Log TVC of 4.0 to 6.4 (Fig. 4.29). The non-PAH control (C1) inoculated with the consortium did not show significant ($P<0.05$) growth increase but there was gradual decrease in cell mass. The control C2 which had PAHs but without consortium inoculated showed no significant ($P<0.05$) microbial presence during the experimental period.

4.7.3.9 POPULATION DENSITY AND pH OF MEDIA DURING DEGRADATION OF PYRENE BY BACTERIAL CONSORTIUM IN THE PRESENCE OF PHENANTHRENE

The population density (OD_{600nm}) of consortium in 'E' increased as degradation of the PAHs proceeded (Fig. 4.30). There was significant increase in population density (OD_{600nm}) of 'E' compared controls. The pH of 'E' was increased towards alkalinity as degradation proceeded compared to the controls where there was no significant change (Fig. 4.31).

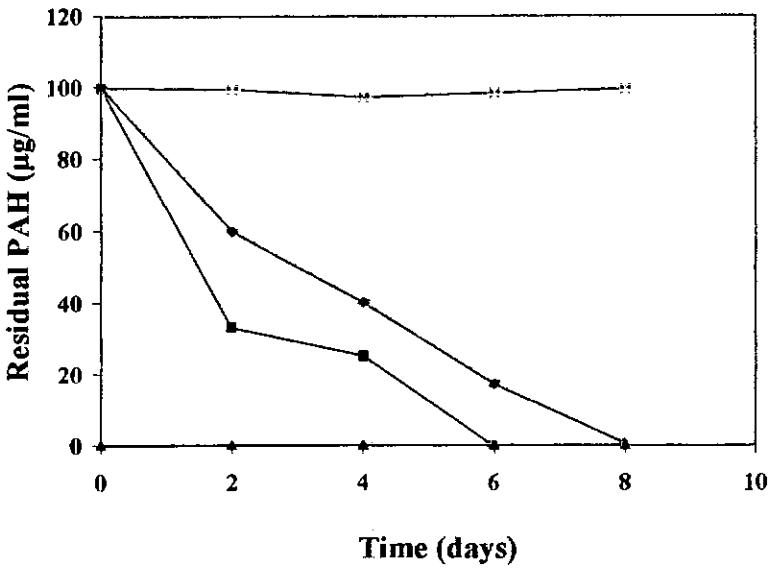


Fig. 4.28. Residual pyrene during degradation of pyrene by consortium in the presence of phenanthrene

Pyrene — Phenathrene
C1 (no PAHs + consortium) — C2 (PAHs + no consortium)

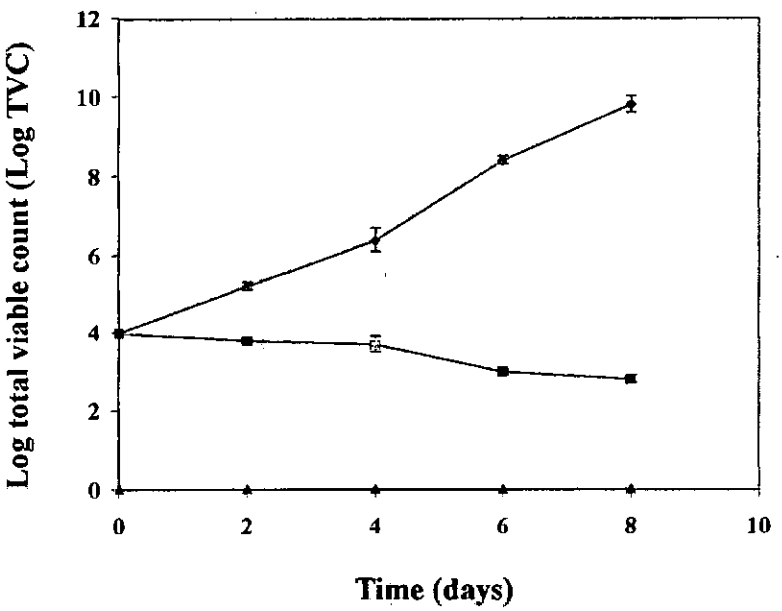


Fig. 4.29. Growth of consortium during degradation of pyrene by consortium in the presence of phenanthrene

E (PAHs + consortium) — C1 (no PAHs + consortium)
C2 (PAHs + no consortium)

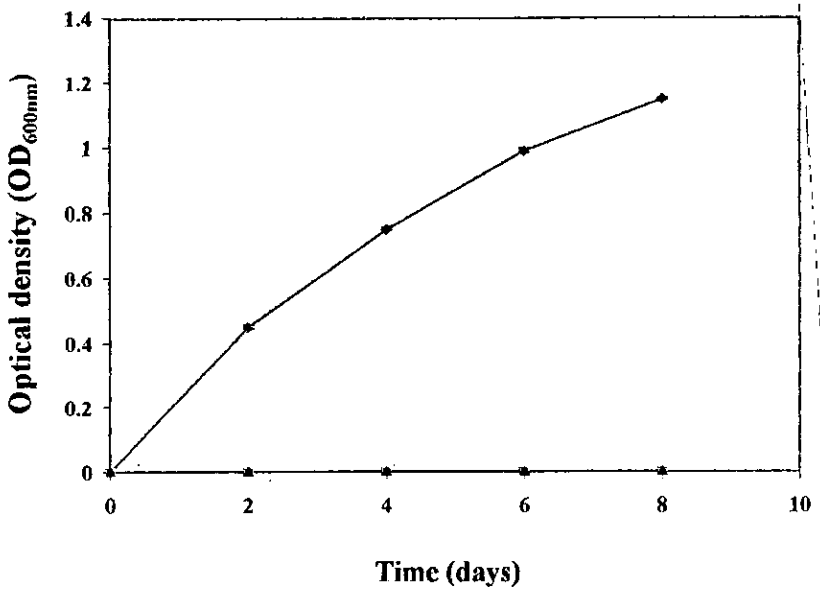


Fig. 4.30. Population dynamics of consortium during degradation of pyrene by consortium in the presence of phenanthrene

E (PAHs + consortium) **C1 (no PAHs + consortium)**

C2 (PAHs + no consortium)

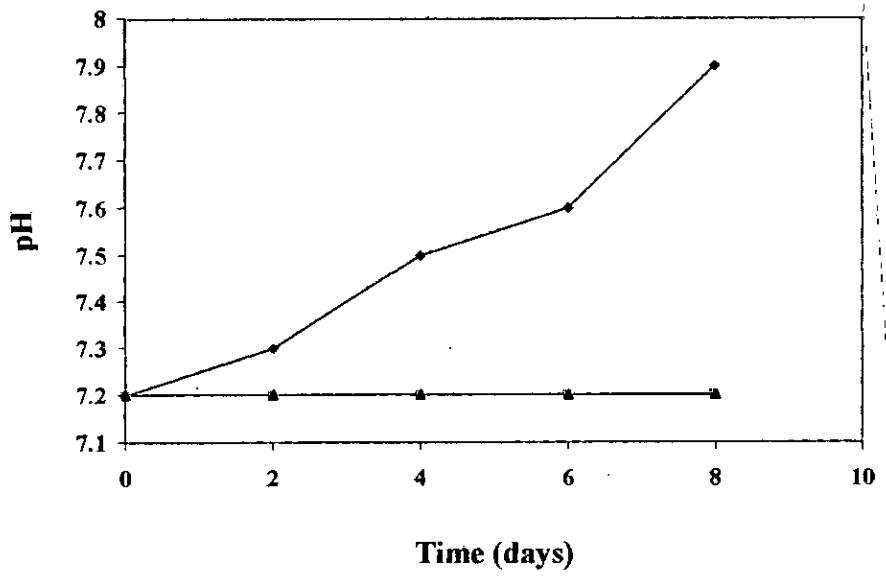


Fig. 4.31. pH of media during degradation of pyrene by consortium in the presence of phenanthrene

E (PAHs + consortium) **C1 (no PAHs + consortium)**

C2 (PAHs + no consortium)

4.7.4 BIODEGRADATION OF MIXTURE OF THE HMW PAHS BY INDIVIDUAL ISOLATES

Biodegradation of the mixture HMW PAHs was studied using *S. paucimobilis*, *Ps. arvilla* and *Ps. putida* individually.

4.7.4.1 RESIDUAL HMW PAHS DURING DEGRADATION OF MIXTURE OF HMW PAHS BY INDIVIDUAL ISOLATES

S. paucimobilis, *Ps. arvilla* and *Ps. putida* degradation of mixture of the HMW PAHs (100 µg/ml each), resulted to residual concentration of chrysene, fluoranthene and pyrene respectively for *S. paucimobilis* as 40.2±0.4, 32.5±0.3 and 37.5±0.2 (µg/ml), with *Ps. arvilla* it was 40.3±0.2, 35.4±0.2 and 34.2±0.4 (µg/ml) while with *Ps. putida* it was 27.4±0.8, 10.1±0.5 and 32.0±0.2 (µg/ml) (Fig. 4.32 a, b and c). Fluoranthene was relatively degraded quicker than the other HMW PAHs.

4.7.4.2 GROWTH OF ISOLATES DURING DEGRADATION OF MIXTURE OF HMW PAHS BY INDIVIDUAL ISOLATES

During degradation of mixture of the HMW PAHs by the individual isolates, highest cell densities of $3.5x10^4$, $3.6x10^4$ and $4.7x10^4$ cfu/ml respectively were observed for *S. paucimobilis*, *Ps. arvilla* and *Ps. putida*. No significant ($P<0.05$) microbial cell increase was observed in C1 and C2 rather cell count of C2 decreased during the experimental period (Fig. 4.33).

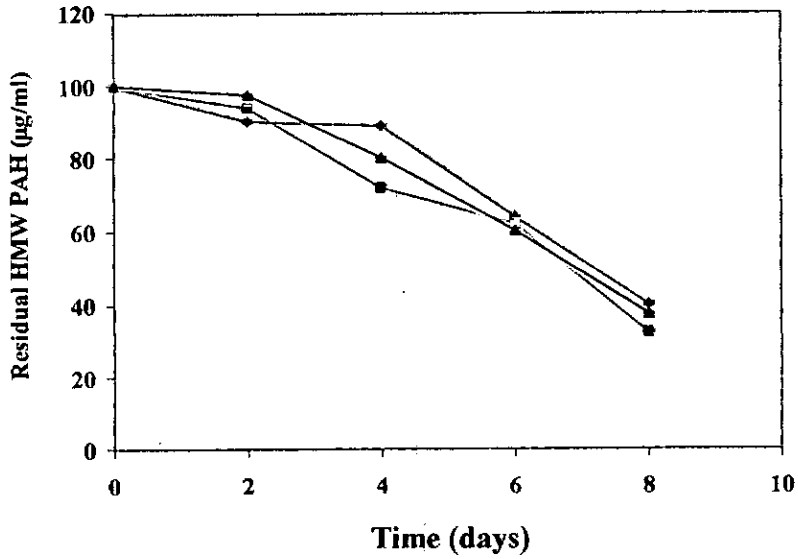


Fig 4.32a. Residual HMW PAHs during degradation of mixture of HMW PAHs by *S. paucimobilis*

Chrysene Fluoranthene Pyrene

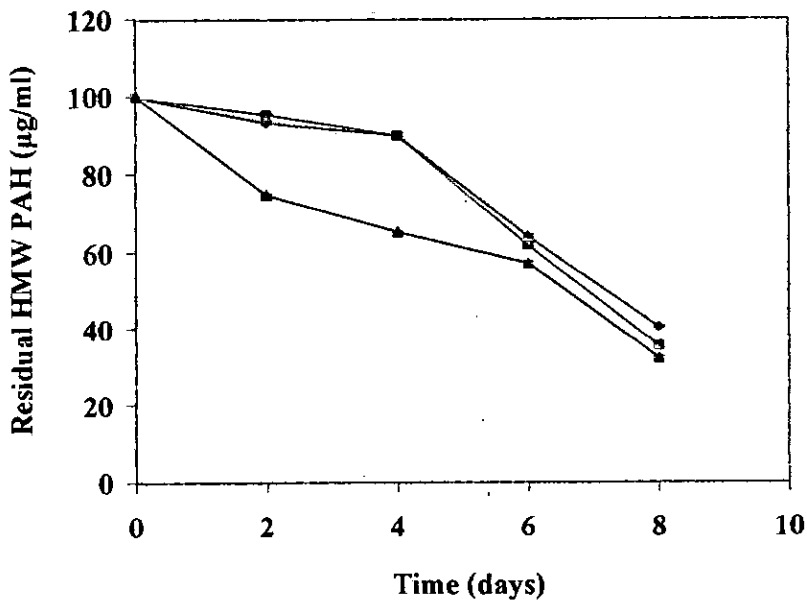


Fig 4.32b. Residual HMW PAH during degradation of mixture of HMW PAHs by *Ps. arvilla*

Chrysene Fluoranthene Pyrene

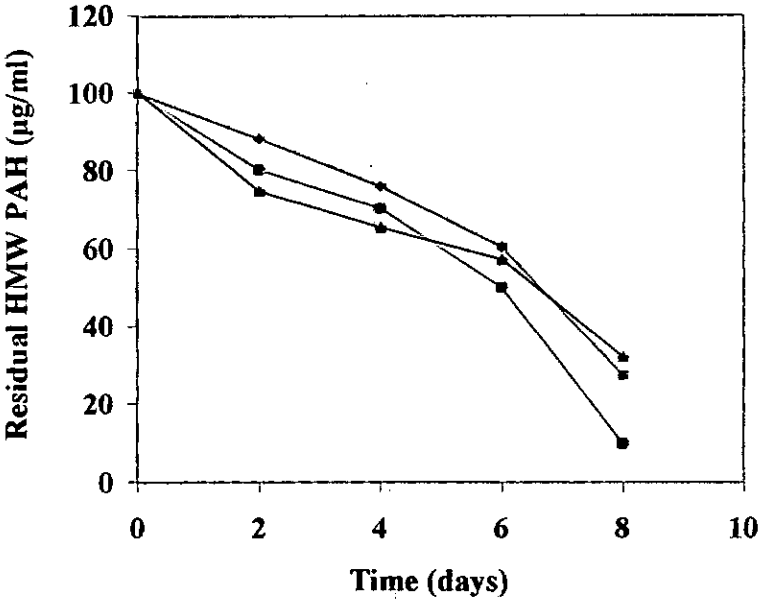


Fig 4.32c. Residual HMW PAH during degradation of mixture of HMW PAHs by *Ps. putida*

Chrysene Fluoranthene Pyrene

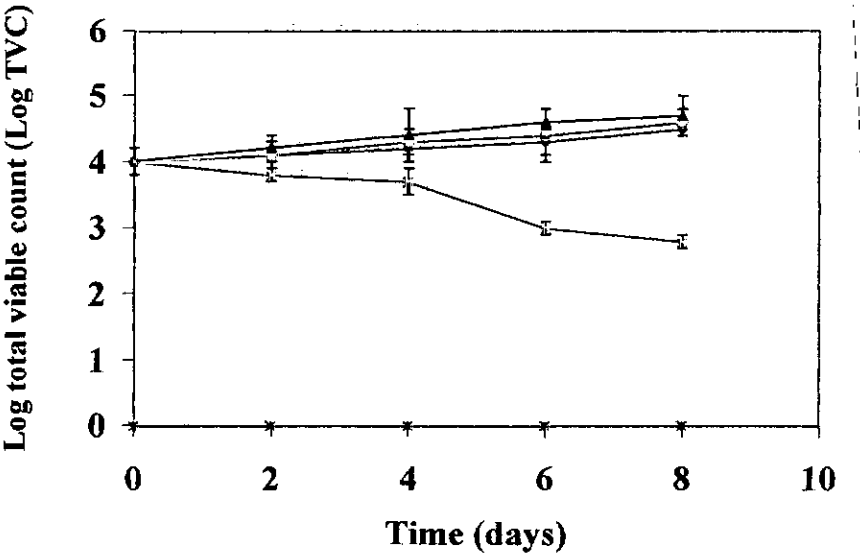


Fig 4.33. Growth of isolates during degradation of mixture of HMW PAHs by individual isolates

S. paucimobilis *Ps. arvilla*
Ps. putida C1 (no HMW PAHs + isolate)
C2 (HMW PAHs + no isolate)

4.7.4.3 POPULATION DENSITY AND pH OF MEDIA DURING DEGRADATION OF MIXTURE OF HMW PAHS BY INDIVIDUAL ISOLATES

Population density (OD_{600nm}) of 'E' increased as degradation proceeded. There was significant increase in the population density (OD_{600nm}) of 'E' compared to the controls (Fig. 4.34).

The pH of 'E' for all the isolates dropped from 7.2±0.0 to 6.9±0.1 during the experimental period (Fig. 4.35). In both controls, there was no change in pH of 'E'.

4.7.5 BIODEGRADATION OF MIXTURE OF HMW PAHS BY BACTERIAL CONSORTIUM

Biodegradation of the mixture of HMW PAH was studied using the isolates as consortium as a representation of natural scenario.

4.7.5.1 RESIDUAL HMW PAHS DURING DEGRADATION OF MIXTURE OF HMW PAHS BY BACTERIAL CONSORTIUM

When consortium of the isolates was cultivated on MS containing mixture of 100 µg/ml each of chrysene, fluoranthene and pyrene as the sole carbon and energy source, degradation resulted to a decrease to 11.5±0.4 (chrysene), 6.2±0.3 (fluoranthene) and 6.0±0.8 (pyrene) (µg/ml) after 8 days. In mixture, fluoranthene and pyrene were better degraded, however, the three HMW PAHs were concomitantly degraded (Fig. 4.36).

4.7.5.2 GROWTH OF CONSORTIUM DURING DEGRADATION OF MIXTURE OF HMW PAHS BY CONSORTIUM

Highest cell density of $2.1x10^5$ cfu/ml was obtained when consortium degraded mixture of the HMW PAHs (Fig. 4.37). Mixture of the HMW PAHs did not inhibit microbial growth as there was significant ($P<0.05$) growth difference between the 'E' and the controls.

4.7.5.3 POPULATION DENSITY AND pH OF MEDIA DURING DEGRADATION OF MIXTURE OF HMW PAHS BY CONSORTIUM

Population density (OD_{600nm}) of consortium in 'E' increased as degradation of mixture of the HMW PAHs proceeded compared to control media (Fig. 4.38).

The pH of 'E' increased from 7.2±0.0 to 7.5±0.1 compared to control where there was no change during the experimental period (Fig. 4.38).

4.7.6 BIODEGRADATION OF MIXTURE OF THE HMW PAHS BY CONSORTIUM IN THE PRESENCE OF PHENANTHRENE

PAHs exist as mixture hence biodegradation of mixture of HMW PAHs was studied using consortium in the presence of phenanthrene as co-substrate.

4.7.6.1 RESIDUAL HMW PAHS DURING DEGRADATION OF MIXTURE OF HMW PAHS BY CONSORTIUM IN THE PRESENCE OF PHENANTHRENE

During consortium degradation of mixture of each of the HMW PAH (100 µg/ml) in the presence of phenanthrene, residual chrysene, fluoranthene, pyrene and phenanthrene after 8 days of incubation was 0.4±0.3, 0.02±0.02, 0.2±0.04 and 0.000±0.00 (µg/ml) respective (Fig. 4.39). Phenanthrene was rapidly degraded to 20.2±0.2 (µg/ml) at day 2 and was undetectable after day 4. Likewise the HMW PAHs were degraded at a rapid rate and their degradation was concomitant with phenanthrene degradation.

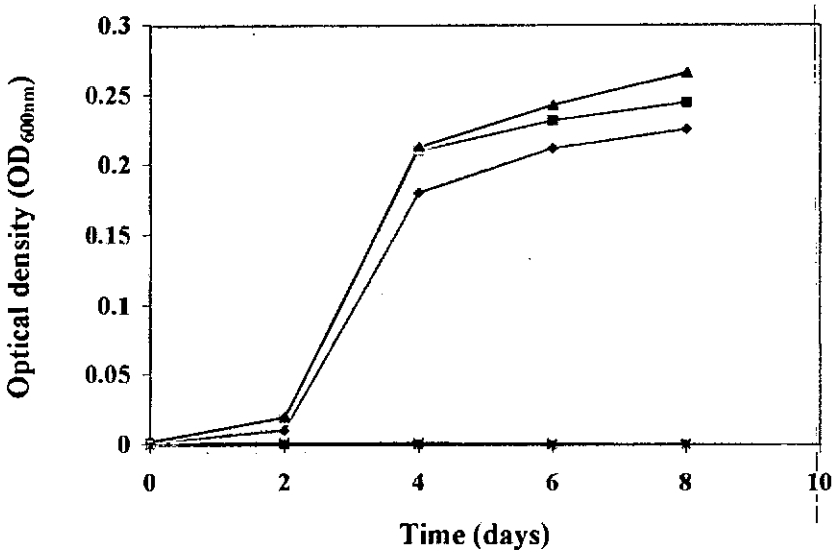


Fig 4.34. Population dynamics of media during degradation of mixture of HMW PAHs by individual isolates

- *S. paucimobilis*
- *Ps. arvilla*
- *Ps. putida*
- C1 (no HMW PAHS + isolate)
- C2 (HMW PAHS + no isolate)

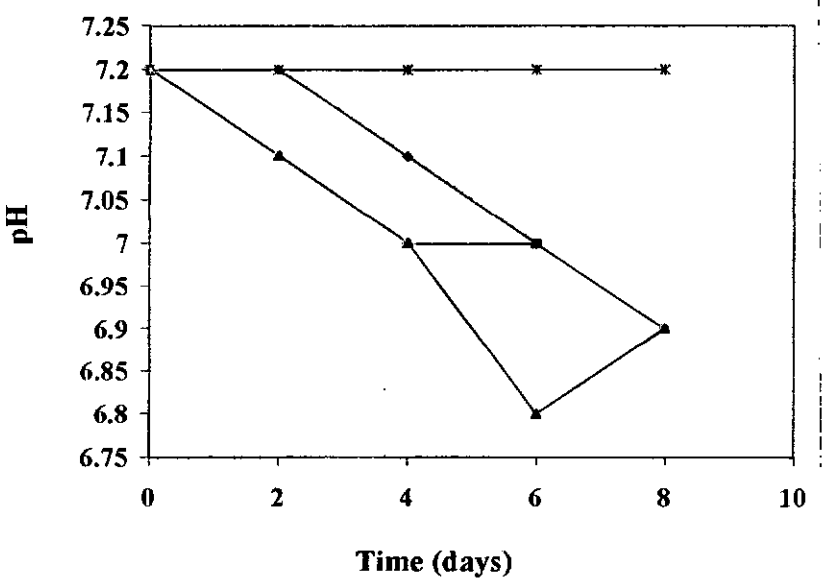


Fig 4.35. pH of media during degradation of mixture of HMW PAHs by individual isolates

- *S. paucimobilis*
- *Ps. arvilla*
- *Ps. putida*
- C1 (no HMW PAHS + isolate)
- C2 (HMW PAHS + no isolate)

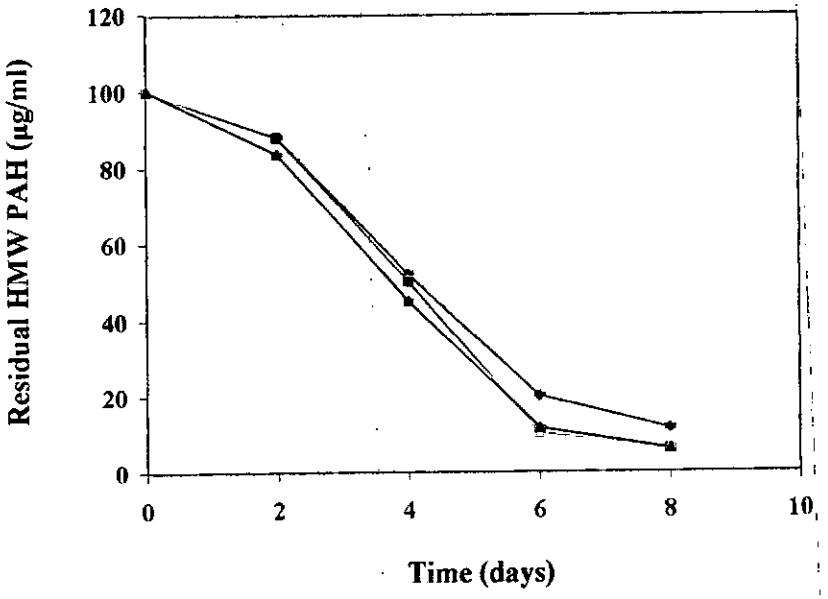
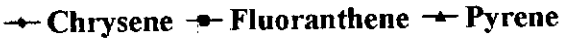


Fig 4.36. Residual HMW PAHs during degradation of mixture of HMW PAHs by consortium



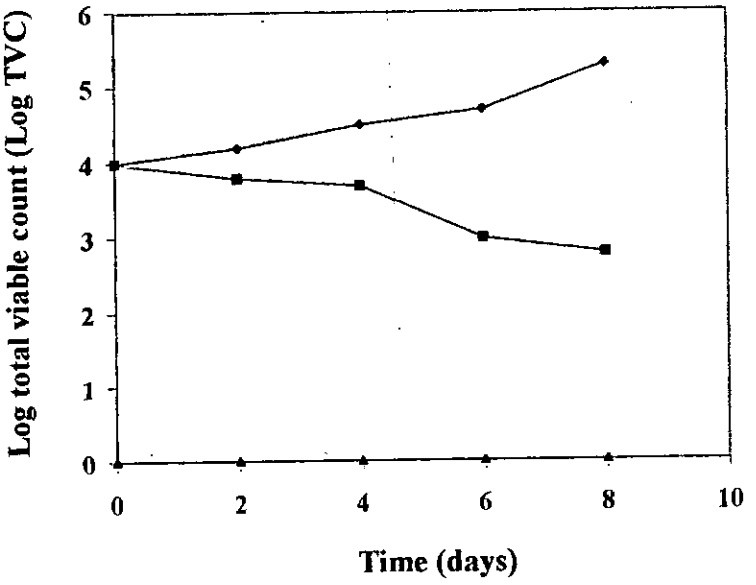
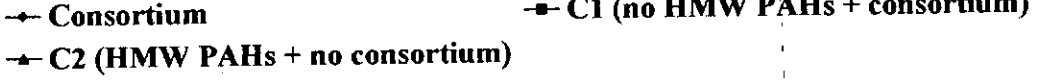


Fig 4.37. Growth of consortium during degradation of mixture of HMW PAHs by consortium



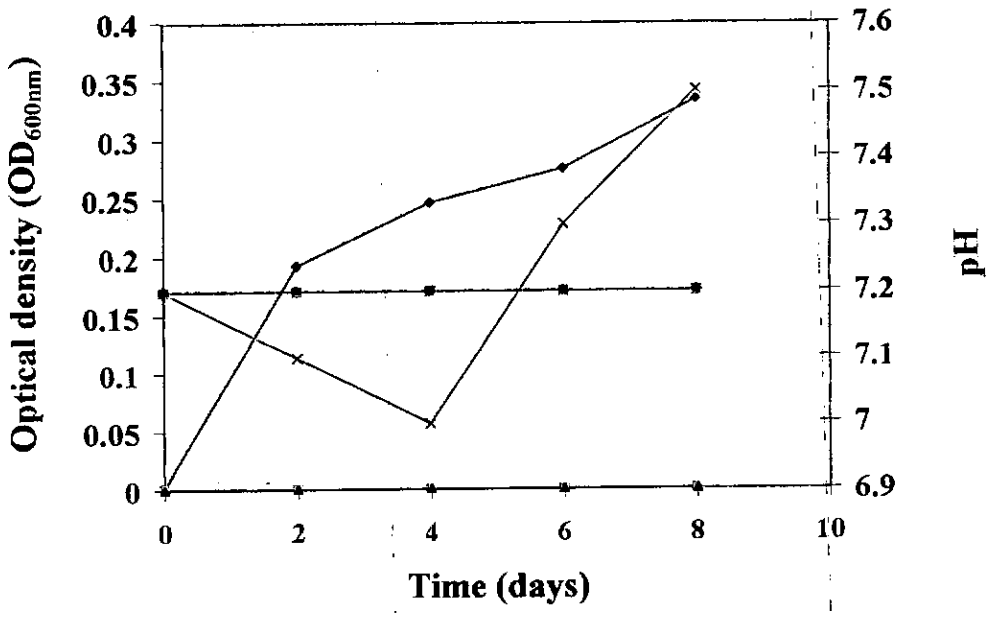


Fig 4.38. Population dynamics and pH of media during degradation of mixture of HMW PAHs by consortium

Consortium (OD) C1 C2 Consortium (pH) C1 (pH) C2 (pH)

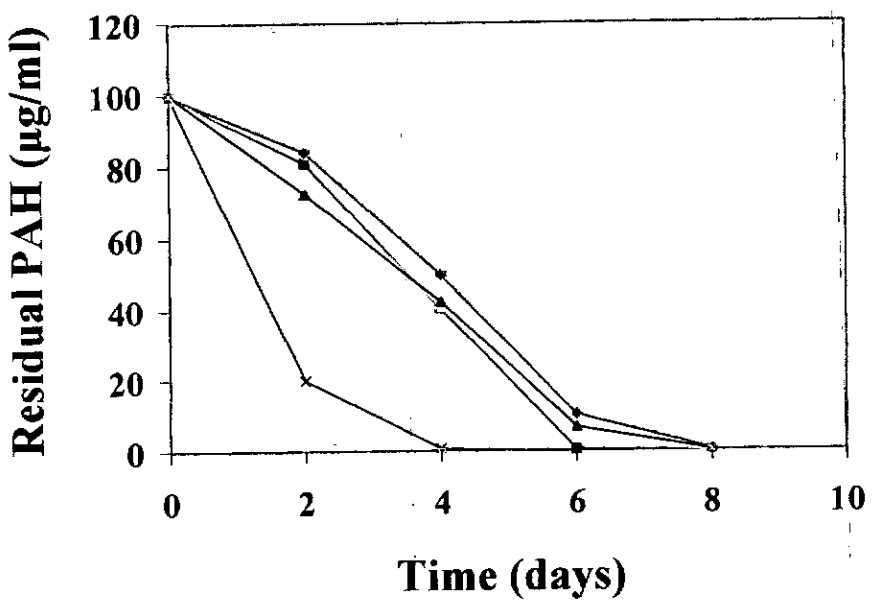


Fig 4.39. Residual HMW PAHs during degradation of mixture of HMW PAHs by consortium in the presence of phenanthrene

Chrysene Fluoranthene Pyrene Phenanthrene

4.7.6.2 GROWTH OF BACTERIAL CONSORTIUM DURING DEGRADATION OF MIXTURE OF HMW PAHS BY BACTERIAL CONSORTIUM IN THE PRESENCE OF PHENANTHRENE

Highest cell densities of $12.4x10^{12}$ cfu/ml was obtained when consortium degraded mixture of the HMW PAHs in the presence of phenanthrene. There was significant ($P<0.05$) change in cell densities at day 2 from Log TVC of 4.0 to 12.1±0.2 (Fig. 4.40). Control C1 did not show significant ($P<0.05$) growth increase but there was gradual decrease in cell mass. Control C2 also showed no significant microbial growth during the experimental period.

4.7.6.3 POPULATION DENSITY AND pH OF MEDIA DURING DEGRADATION OF MIXTURE OF HMW PAHS BY BACTERIAL CONSORTIUM IN THE PRESENCE OF PHENANTHRENE

Degradation of mixture of the HMW PAHs in the presence of phenanthrene by consortium resulted to a rapid increase in population density (OD_{600nm}) of consortium in the experimental media. The population density (OD_{600nm}) of 'E' was significantly ($P<0.05$) different from the controls where there was decreased cell mass during the experimental period. The pH of 'E' was increased as degradation proceeded compared to the controls (Fig. 4.41).

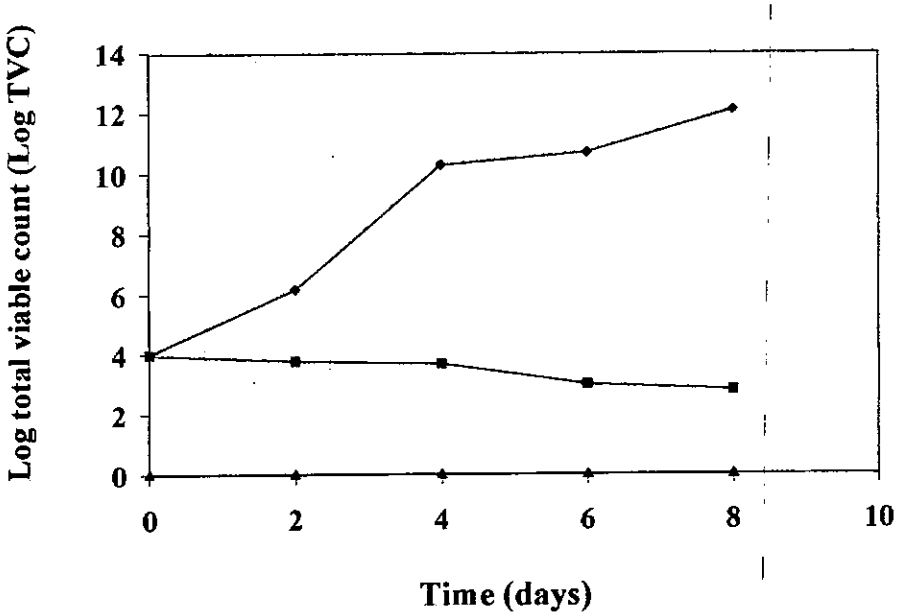
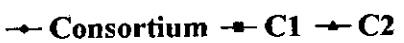


Fig 4.40. Growth of consortium during degradation of mixture of HMW PAHs by consortium in the presence of phenanthrene



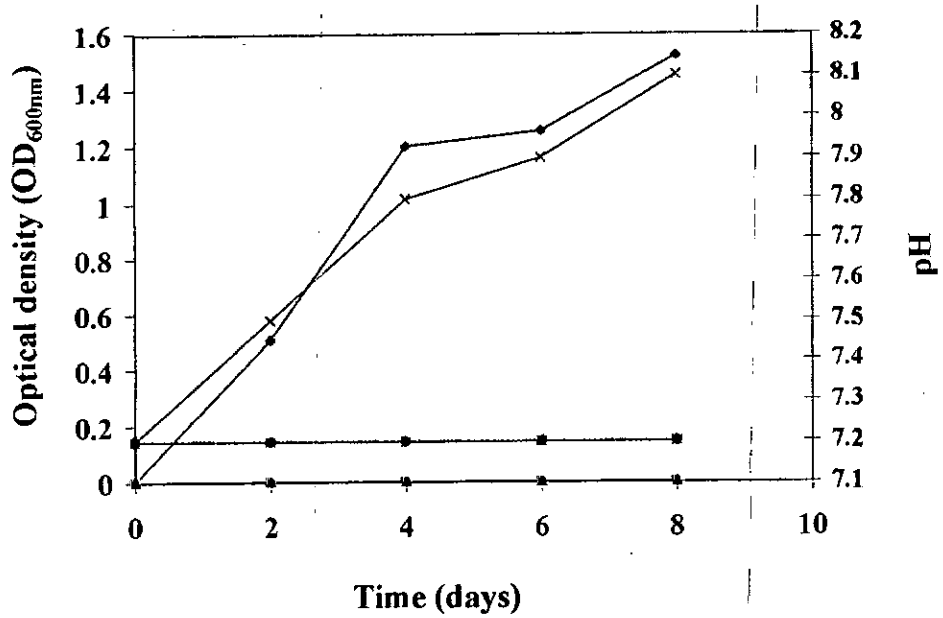
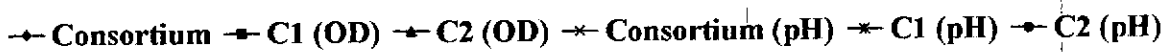


Fig 4.41. Population dynamics and pH of media during degradation of mixture of HMW PAHs by consortium in the presence of phenanthrene



4.8 ENZYME STUDIES

The catechol dioxygenases involved in the lower metabolic pathway of PAHs degradation was studied. This was to evaluate the potential of the isolates in metabolizing the HMW PAHs to carbon dioxide and water and/or intermediates of tricarboxylic acid cycle for energy generation as such remediating the HMW PAHs to non-toxic compounds and ensuring complete elimination of the HMW PAHs. The study showed that *S. paucimobilis* had catechol 1,2-dioxygenase (EC 1.13.11.1) activity while the *Pseudomonas* strains had catechol 2,3-dioxygenase (EC 1.13.11.2) activity.

4.8.1 PARTIAL PURIFICATION OF CATECHOL DIOXYGENASE ENZYMES

Crude catechol dioxygenase extract from *S. paucimobilis*, *Ps. arvilla* and *Ps. putida* culture media respectively were partially purified by ammonium sulphate precipitation (at 40% saturation), dialysis, gel filtration using DEAE-sephadex A-50-120 and Sephadex G25-300 (Table 4.3). Catechol 1,2-dioxygenase enzyme of *S. paucimobilis* was purified by 23.0 folds with specific activity 7.14 Umg Protein^{-1}. Catechol 2,3-dioxygenase enzymes of *Ps. arvilla* and *Ps. putida* respectively were purified by17.5 and 18.3 folds with specific activity 5.24 and 5.66 Umg Protein^{-1} respectively.

Elution profile of catechol dioxygenase enzyme of *S. paucimobilis*, *Ps. arvilla* and *Ps. putida* during DEAE-sephadex gel filtration showed two active enzyme peaks (Fig. 4.42 – 4.44). Catechol 1,2-dioxygenase enzyme of *S. paucimobilis* was eluted with buffer A containing 200 mM NaCl, same for catechol 2,3-dioxygenase enzymes of *Ps. arvilla*, however, 150 mM NaCl eluted Catechol 1,2-dioxygenase enzyme of *Ps. putida*.

Table 4.3 **Partial purification of catechol dioxygenase of *S. paucimobilis*, *Ps. arvilla* and *Ps. putida***

Organism	Purification step	Total Protein (mg)	Total activity (U)	Specific activity (Umg^{-1})	Recovery of activity (%)	Purification Factor
S. paucimobilis	Crude extract	350	108.5	0.31	100	1
	Ammonium sulfate precipitation	168	92.4	0.55	85.2	1.8
	Dialysis	80	73.1	0.91	67.4	2.9
	DEAE-sephadex	21.3	57.2	2.69	52.7	8.7
	Gel Filtration	5.0	35.7	7.14	32.9	23.0
Ps. arvilla	Crude extract	295	105	0.3	100	1
	Ammonium sulfate precipitation	175	86	0.5	82.0	1.6
	Dialysis	80	61.5	0.7	58.6	2.3
	DEAE-sephadex	10.5	40.6	3.9	38.7	12.9
	Gel Filtration	6.2	32.5	5.24	23.1	17.5
Ps. putida	Crude extract	320	98.2	0.31	100	1
	Ammonium sulfate precipitation	155	76.5	0.49	77.9	1.6
	Dialysis	71	62.7	0.88	63.8	2.8
	DEAE-sephadex	19.5	54.8	2.8	55.8	9.0
	Gel Filtration	6.1	34.5	5.66	35.1	18.3

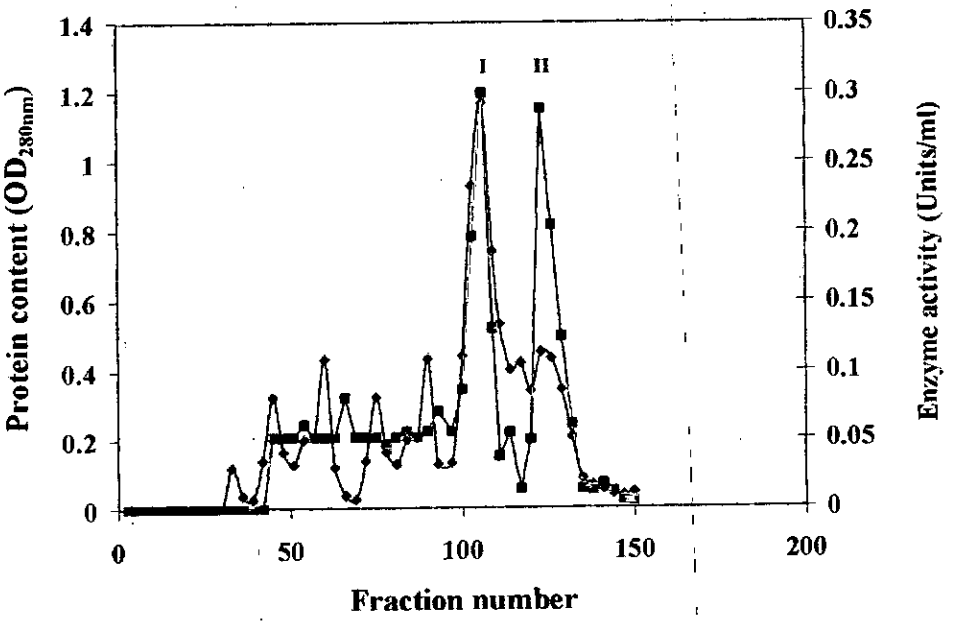


Fig 4.42. Elution profile of catechol 1,2-dioxygenase of *S. paucimobilis*

Protein content — Enzyme activity

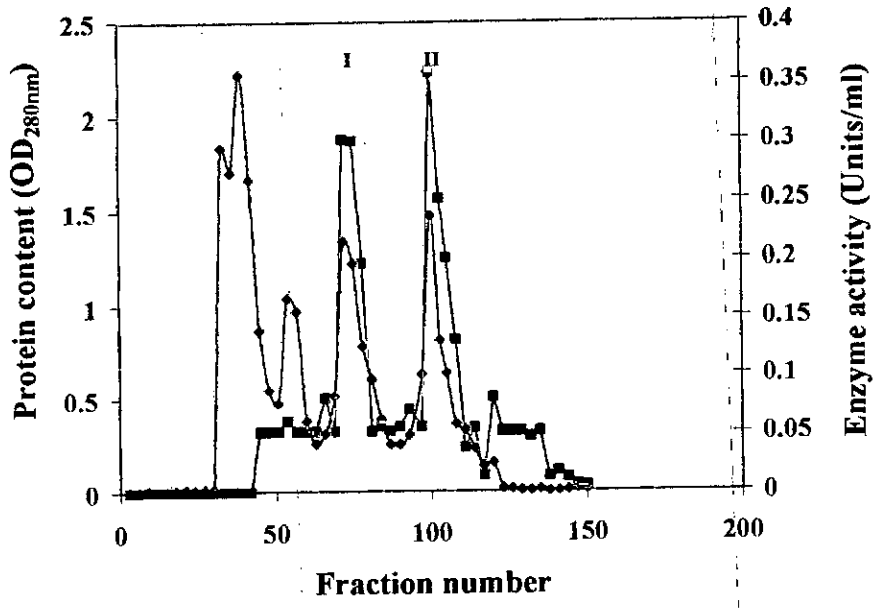


Fig 4.43. Elution profile of catechol 2,3-dioxygenase of *Ps. arvilla*

Protein content — Enzyme activity

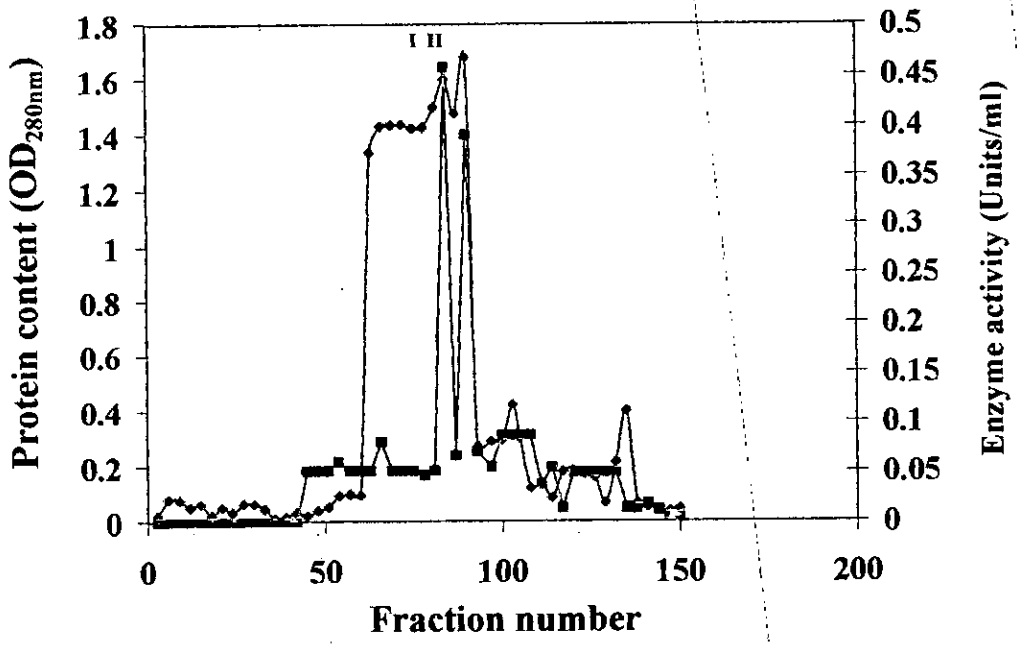


Fig 4.44. Elution profile of catechol 2,3-dioxygenase of *Ps. putida*

Protein content **Enzyme activity**

4.8.2 CHARACTERIZATION OF CATECHOL DIOXYGENASE ENZYMES

The molecular weight and the effect of temperature, pH, metal ions, ethylene diamino tetraacetic acid (EDTA) and varying substrate concentrations on the partially purified catechol dioxygenase of *S. paucimobilis*, *Ps. arvilla* and *Ps. putida* were determined.

4.8.2.1 MOLECULAR WEIGHT OF THE CATECHOL DIOXYGENASE ENZYMES

The molecular weight of two peaks of the catechol dioxygenase were determined. Molecular weight of catechol 1,2-dioxygenase enzyme (C1,2O) of *S. paucimobilis* was 86.4 kDa (Isoenzyme I) and 98.4 kDa (Isoenyme II). Catechol 2,3-dioxygenase (C2,3O) of *Ps. arvilla* molecular weight was 55.2 kDa (Isoenzyme I) and 62.0 kDa (Isoenzyme II) whereas that of catechol 2,3-dioxygenase of *Ps. putida* was 116.0 kDa (Isoenzyme I) and 118.4 kDa (Isoenzyme II) (Fig. 4. 45).

4.8.2.2 TEMPERATURE PROFILE OF CATECHOL DIOXYGENASE ENZYMES

The effects of temperature range of 20^0C - 60^0C on the catechol dioxygenase activities showed that catechol 1,2-dioxygenase of *S. paucimobilis* had optimum temperature at 45^0C with 83.2% activity while catechol 2,3-dioxygenase of *Ps. arvilla* and *Ps. putida* respectively had optimum temperature at 45^0C and 50^0C respectively with 92.5% and 99.8% activity correspondingly (Fig. 4. 46).

4.8.2.3 pH PROFILE OF CATECHOL DIOXYGENASE ENZYMES

pH profile study on catechol dioxygenase of *S. paucimobilis*, *Ps. arvilla* and *Ps. putida* showed optimum at pH 8.0, 8.0 and 9.6 respectively with enzyme activity of 99.4%, 98.2% and 99.4% correspondingly (Figures 4.47).

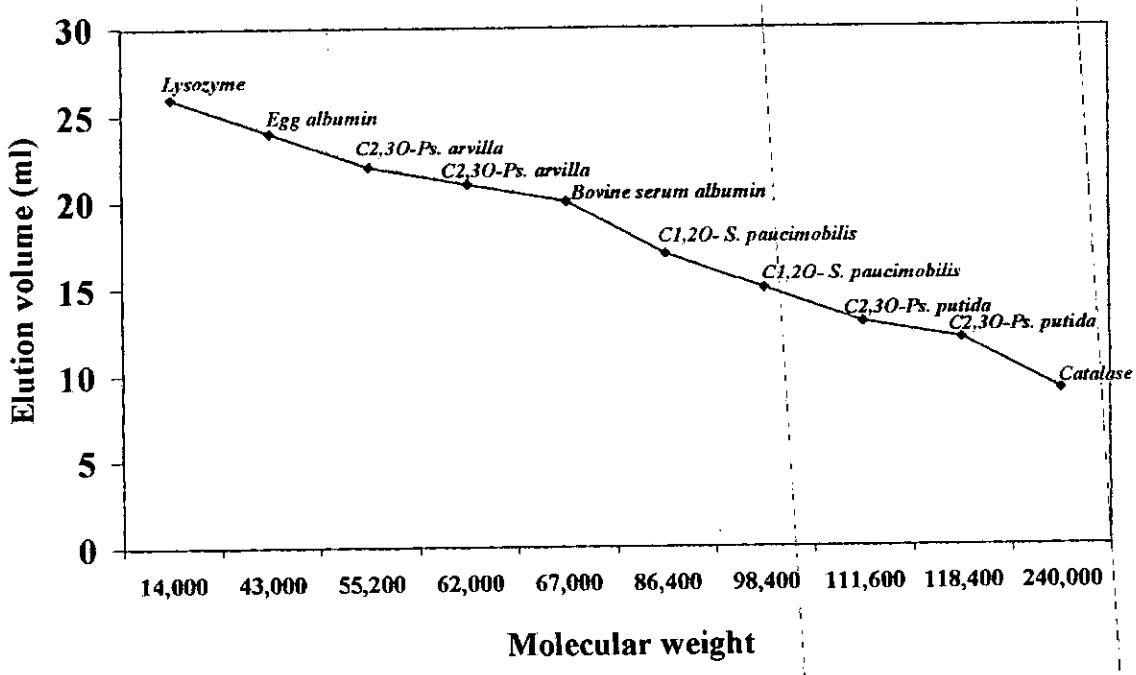


Fig 4.45. Molecular weight of catechol dioxgyenase of the isolates

→ Elution point

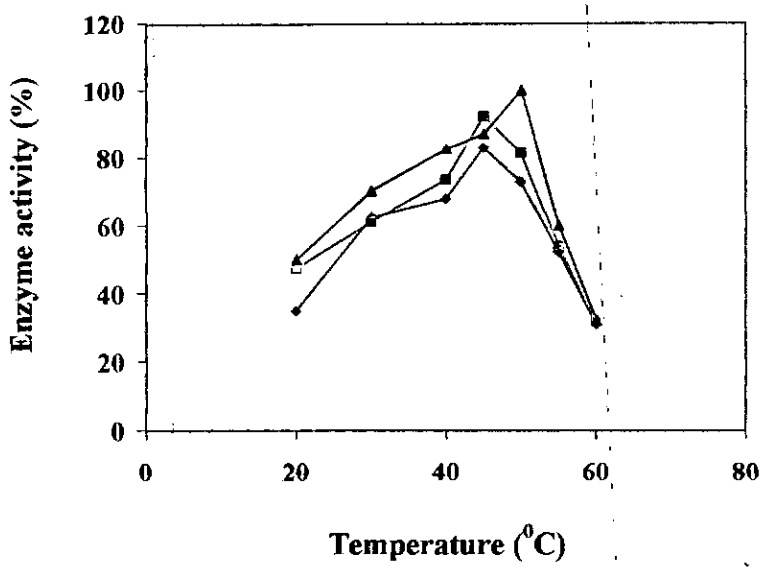


Fig. 4.46. Effect of temperature on catechol dioxygenase of isolates

—◆— *S. paucimobilis* —■— *Ps. arvilla* —▲— *Ps. putida*

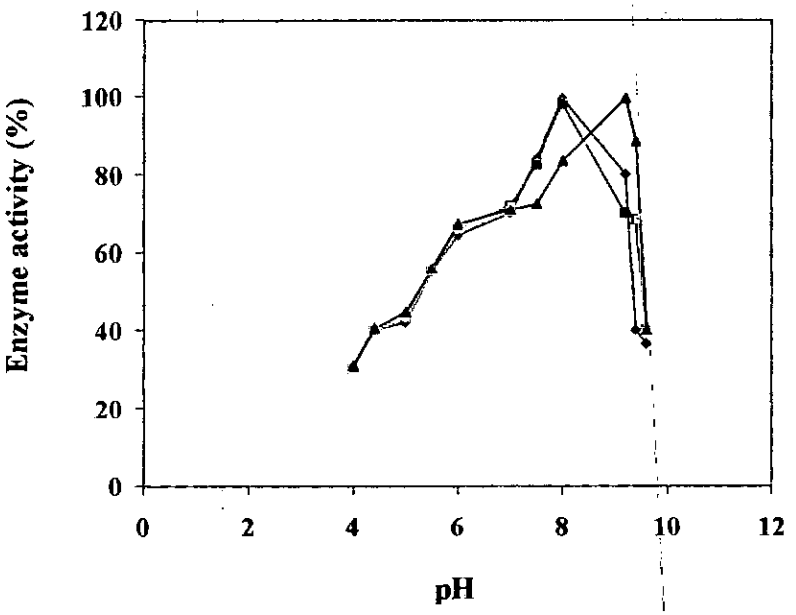


Fig. 4.47. Effect of pH on catechol dioxygenase of isolates

—◆— *S. paucimobilis* —■— *Ps. arvilla* —▲— *Ps. putida*

4.8.2.4 EFFECT OF METAL IONS AND ETHYLENE DIAMINO TETRAACETIC ACID (EDTA) ON CATECHOL DIOXYGENASES ACTIVITY

The bacterial isolates catechol dioxygenase were incubated with catechol in the presence of 0.1mM concentrations of different metal ions and EDTA, the effects of these compounds on the enzymes activity were evaluated (Table 4.4). Ferrous ion (Fe^{2+}) was found to increase catechol 2,3- dioxygenase activity of *Ps. arvilla and Ps. putida* respectively by 115% and 117% respectively while 63% activity was obtained for catechol 1,2-dioxygenase of *S. paucimobilis*. Ferric ion (Fe^{3+}) increased catechol 1,2-dioxygenase activity of *S. paucimobilis* to 120% while 60% and 62% catechol 2,3- dioxygenase activity were obtained for *Ps. arvilla and Ps. putida* respectively. Copper ion (Cu^{2+}) inhibited catechol dioxygenase activity of *S. paucimobilis, Ps. arvilla and Ps. putida* respectively by 25%, 25% and 26% respectively. Silver ion (Ag^{2+}) inhibited enzymes activity by 85% while manganese ion (Mn^{2+}) and cobalt ion (Co^{2+}) inhibited the enzymes activity by 66.1% - 79.7%. EDTA and magnesium ion (Mg^{2+}) gave a 70% - 80% activity of catechol dioxygenase of the isolates.

4.8.2.5 EFFECT OF SUBSTRATE CONCENTRATION ON CATECHOL DIOXYGENASE ACTIVITY

Lineweaver-Burk plot was used to determine the maximum velocities (Vmax) and Michaelis-Menten constants (Km) of the catechol dioxygenases (Table 4.5). Maximum velocity (Vmax) of 53.7±0.9, 57.5±0.3 and 58.5±1.2 μmol^{-1} min^{-1} mg $Protein^{-1}$ respectively were obtained for catechol dioxygenase of *S. paucimobilis, Ps. arvilla and Ps. putida* respectively. The Km values obtained were 2.1±0.2 µM, 2.0±0.5 µM and 1.9±0.3 µM respectively for catechol dioxygenase of *S. paucimobilis, Ps. arvilla and Ps. putida* respectively.

Table 4.4. Effect of metal ions and Ethylene Diamino Tetraacetic acid (EDTA) on catechol dioxygenase activity of the HMW PAHs degrading bacteria

Compound	Concentration (mM)	*S. paucimobilis* Remaining activity (%)	*Ps. arvilla* Remaining activity (%)	*Ps. putida* Remaining activity (%)
None	-	100	100	100
$CuSO_4$	0.1	75	75	74
$FeSO_4$	0.1	63	115	117
$FeCl_3$	0.1	120	60	62
$AgNO_3$	0.1	15	15	15
$CoCl_2.6H_2O$	0.1	24	24.3	23.2
$MgCl_2$	0.1	94.6	96.2	95.4
$MnSO_4$	0.1	33.9	20.3	22.1
EDTA	0.1	70.1	70.2	71.0
$CaCl_2$	0.1	77.2	78.2	79.2

Table 4.5. Kinetic parameters (Vmax and Km) of catechol dioxygenase of the isolates

Parameter	*S. paucimobilis*	*Ps. arvilla*	*Ps. putida*
Vmax (Units mg Protein^{-1})	53.7±0.9	57.2±0.3	58.5±1.2
Km (µM)	2.1±0.2	2.0±0.5	1.9±0.3

4.9 PLASMID EXTRACTION, CURING AND BIOTRANSFORMATION

In order to find out the location (chromosomal or extra chromosomal) of gene(s) responsible for the HMW PAHs degradation, plasmid extraction, curing and biotransformation experiments were performed.

All the six bacteria isolated as HMW PAHs degraders; *A. anitratus*, *A. mallei*, *A. faecalis*, *S. paucimobilis*, *Ps. arvilla* and *Ps. putida* evaluated for plasmid possession were all found to harbor multiple plasmids (Plate 4.1). *S. paucimobilis*, *Ps. putida*, *A. anitratus* and *A. mallei* possessed 12.8 kb (kilo base) plasmid. 2.5 kb plasmid was found in *Ps. putida*, *Ps. arvilla*, *A. anitratus*, *A. mallei* and *A. faecalis* but not in *S. paucimobilis*. The isolates also had 2.0 kb plasmid except *A. anitratus* and *A. mallei*.

These six HMW PAHs degrading bacteria were found to be tetracycline sensitive, but after curing of their plasmids (Plate 4.2), they became resistant to tetracycline and also lost their HMW PAHs degradation potential.

Biotransformation of the isolates habouring plasmid (original isolates) with their cured isolates resulted in the acquisition of plasmids of the original plasmid habouring isolates by the cured isolates (Plates 4.3). These biotransformations gave rise to new mutants with varied HMW PAHs degradation potential (Table 4.6). The cured isolates used as recipient of the plasmids were designated as *S. p -1*(cured *S. paucimobilis*), *Ps.* -1 (cured *Ps. arvilla*), *Ps. putida*-1 (cured *Ps. putida*), *A. anitratus*-1 (cured *A.* anitratus), *E. coli* and *Klebsiella*.

S. paucimobilis before curing degradation of chrysene, fluoranthene and pyrene was 70%, 100% and 90% respectively but after biotransformation with *S. p -1*, the new isolate retained same activity except in pyrene degradation which reduced to 80%. Mutant isolate from biotransformation of *Ps. arvilla* and *Ps.* -1 retained same activity as *Ps. arvilla* except

in chrysene degradation which increased from 60% to 70%. Likewise *Ps. putida* biotransformation with *Ps. putida*-1 gave a mutant with improved chrysene and pyrene degradation which increased from 70% and 90% respectively to 80% and 100%. Mutants from biotransformation of *A. anitratus* and *A. anitratus*-1 had a reduced fluoranthene degradation activity from 100% to 80%. However cured *E. coli* and *Klebsiella* lacking HMW PAHs degradation activity biotransformed with *Ps. putida* resulted to a mutant *E. coli* having degradation potential of 80%, 100% and 70% for chrysene, fluoranthene and pyrene respectively while the mutant of *Klebsiella* had 70%, 80% and 70% respectively.

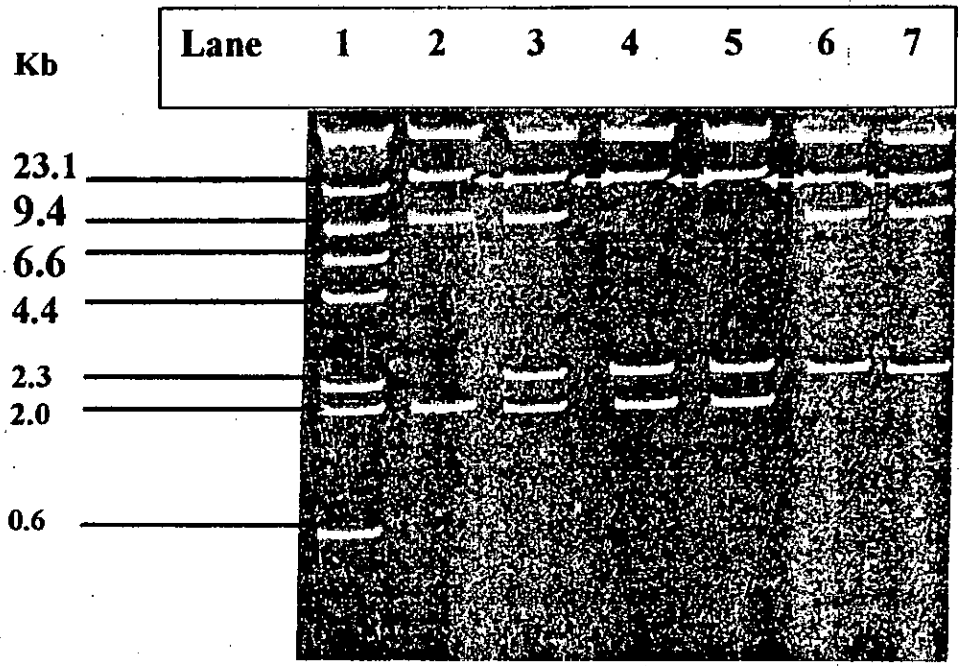


Plate 4.1 Electrophoregram of chromosomal and plasmid DNA of HMW PAHs degrading bacteria.

Key: 1 = DNA molecular weight marker (0.12-23.1kbp), 2 = *S. paucimobilis*, 3 = *Ps. putida*, 4 = *Ps. arvilla*, 5 = *A. faecalis*, 6 = *A. mallei*, 7 = *A. anitratus*.

↔ = Arrow pointing at chromosomal DNA

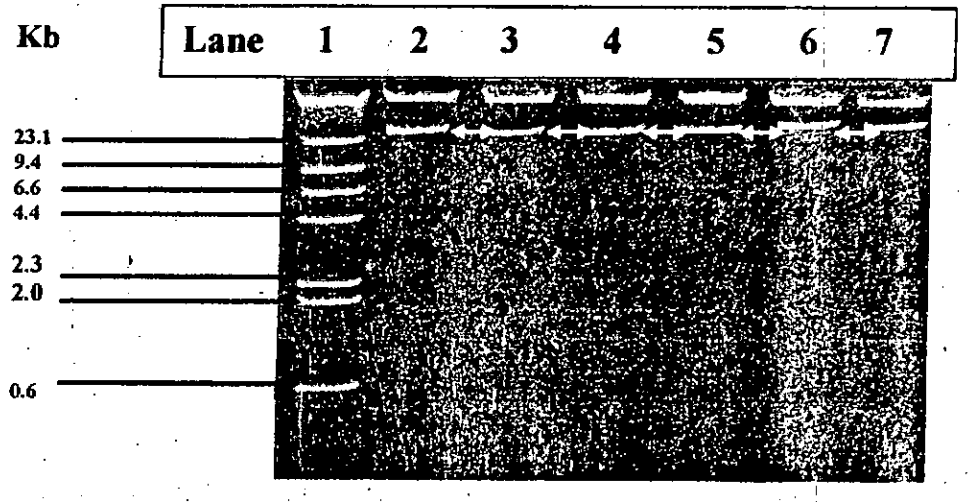


Plate 4.2 Electrophoregram of chromosomal DNA and cured HMW PAHs degrading bacteria. 1 = DNA molecular weight marker, 2 = *S. paucimobilis* (*S. p -1*), 3 = *Ps. putida* (*Ps. putida-1*), 4 = *Ps. arvilla* (*Ps-1*), 5 = *A. anitratus*, 6 = *E.coli* and 7 = *Klebsiella*.

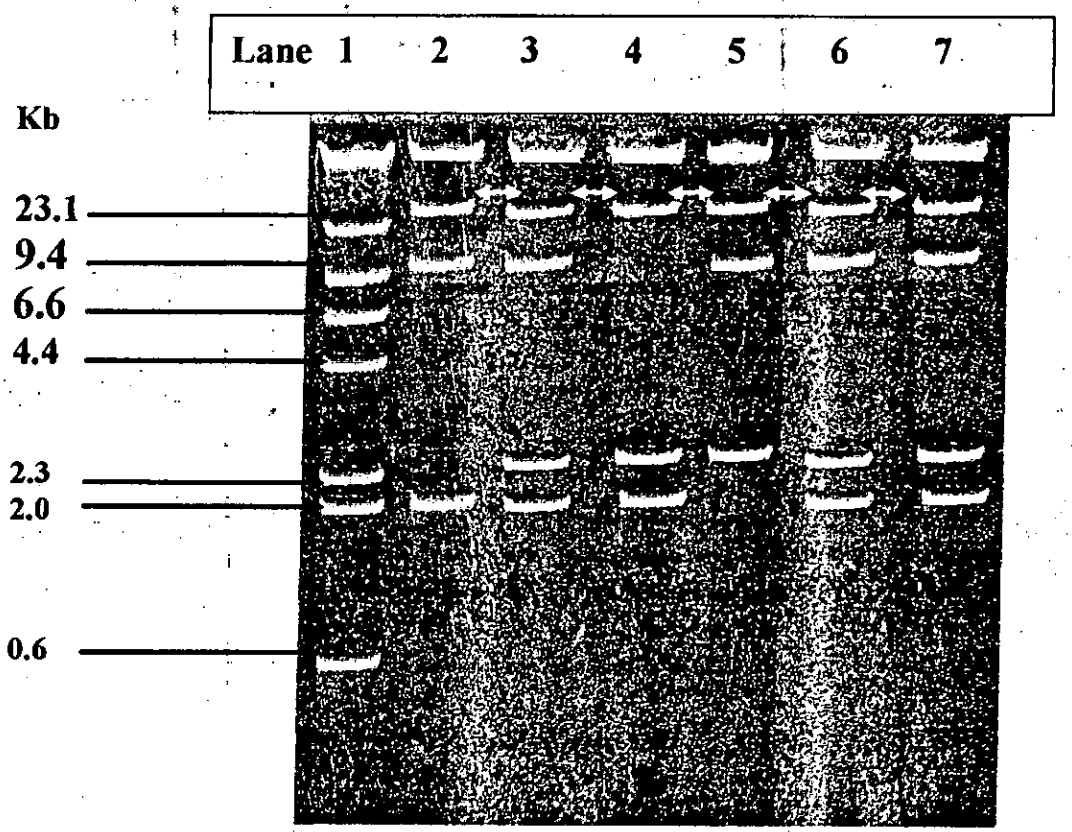


Plate 4.3. Electrophoregram of chromosomal and plasmid DNA of biotransformed HMW PAHs degrading bacteria.

Key:

1 = DNA molecular weight marker, 2 = *S. paucimobilis* (*S. p -1*), 3 = *Ps. putida* (*Ps. putida-1*), 4 = *Ps. arvilla* (*Ps-1*), 5 = *A. anitratus*, 6 = *E.coli* and 7 = *Klebsiella*.

↔ = Arrow pointing at chromosomal DNA

Table 4.6 ***In vivo*** **gene transfer between HMW PAHs degrading bacteria (donor) and cured non-HMW PAHs degrading bacteria (recipient)**

Group	Isolates	Antibiotic tetracycline selective marker (500µg/ml)	Chrysene (% degradation)	Fluoranthene (% degradation)	Pyrene (% degradation)
DONOR	*S. paucimobilis*	S	70	100	90
	Ps. arvilla	S	60	100	80
	Ps. putida	S	70	100	90
	A. anitratus	S	100	100	70
RECIPIENT	*S. p -1*	R	0	0	0
	Ps. –1	R	0	0	0
	Ps. putida-1	R	0	0	0
	A. anitratus-1	R	0	0	0
	E. coli	R	0	0	0
	Klebsiella	R	0	0	0
TRANSCONJUGANT (Mutants)	*S. paucimobilis /S. p-1*	R	70	100	80
	Ps putida/Ps. putida-1	R	80	100	100
	Ps. arvilla./Ps-1	R	70	100	80
	Ps putida/E.coli	R	80	100	70
	Ps putida/ Klebsiella	R	70	80	70
	A.anitratus/A.anitratus-1	R	100	80	70

Key:

R = **resistance**

S = **sensitive**

S. p -1 = **cured *S. paucimobilis***

Ps. -1 = **cured *Ps. arvilla***

Ps. putida-1 = **cured *Ps. putida***

A. anitratus-1 = **cured *A.* anitratus**

4.10 BIOSURFACTANT PRODUCTION

Biosynthesis and excretion of biosurfactants into media are considered to be one of cell mechanism aiming at adaptation of microorganism in using external lipophilic compounds as carbon and energy sources.

All the six HMW PAH degrading bacteria; *A. anitratus*, *A. mallei*, *A. faecalis*, *S. paucimobilis*, *Ps. putida* and *Ps. arvilla* were evaluated for biosurfactant production by growing respective isolates on varying quantities of chrysene and diesel oil respectively using them as sole carbon and energy source. Isolates growth on the substrates were measured as a function of total viable count of cell and population density (OD_{600nm}) of the media. Microbial cells and extracellular fluid of the media were thereafter evaluated for emulsifying activity against different hydrocarbons.

4.10.1 GROWTH PROFILE OF BACTERIA ISOLATES GROWN ON VARYING QUANTITIES OF CHRYSENE

All the six bacteria isolates utilized varying quantities of chrysene as the sole carbon and energy source as observed in their growth in MS chrysene media compared to control non-chrysene MS media for which there was significant ($P<0.05$) growth in the chrysene media. Highest cell densities of $1.6x10^7$, $2.58x10^8$ and $2.58x10^7$ cfu/ml respectively were obtained during *A. anitratus* growth in 1.5 mg, 2.5 mg and 5 mg/50ml MS chrysene media (Fig. 4.48). *A. mallei* had highest cell densities of $1.6x10^7$, $2.58x10^7$ and $6.8x10^6$ cfu/ml respectively (Fig. 4.49) while *A. faecalis* had $3.2x10^6$, $6.8x10^6$ and $6.8x10^6$ cfu/ml correspondingly (Fig. 4.50). When *S. paucimobilis* was grown in 1.5 mg, 2.5 mg and 5 mg/50 ml MS chrysene media respectively, highest cell densities of $2.5x10^5$, $5.9x10^5$ and $6.2x10^5$ (cfu/ml) was obtained (Fig. 4.51), with *Ps. putida*, highest cell densities of $1.5x10^5$, $7.5x10^5$ and $1.5x10^6$ (cfu/ml) was obtained (Fig. 4.52) while with *Ps. arvilla* highest cell

densities of $1.5x10^5$, $3.2x10^5$ and $4.3x10^5$ (cfu/ml) was obtained correspondingly (Fig. 4.53). Population density (OD_{600nm}) of the media increased significantly ($P<0.05$) compared to the controls.

4.10.2 GROWTH PROFILE OF BACTERIA ISOLATES GROWN ON VARYING QUANTITIES OF DIESEL OIL

All the isolates were found to utilize varying concentrations of diesel oil as the sole source of carbon and energy as observed in the increase in cell mass multiplication. Highest cell densities of $3.9x10^{14}$, $5.0x10^{12}$, $1.6x10^{11}$ and $7.9x10^8$ cfu/ml respectively were obtained during *A. anitratus* growth in 3%, 5%, 15% and 30% diesel oil (Fig. 4.54). When *A. mallei* was grown in the respective diesel oil, highest cell densities of $1.6x10^{14}$, $5.0x10^{12}$, $1.6x10^{12}$ and $5.0x10^6$ cfu/ml were obtained respectively ((Fig. 4.55) while with *A. faecalis*, $3.2x10^{14}$, $2.5x10^{12}$, $3.2x10^6$ and $1.3x10^6$ cfu/ml were obtained respectively (Fig. 4.56).

Moreover, when *S. paucimobilis* was grown in 3%, 5%, 15% and 30% diesel oil respectively, highest cell densities of $3.6x10^{13}$, $3.6x10^{13}$, $1.6x10^{12}$ and $3.2x10^9$ cfu/ml were obtained (Fig. 4.57), with *Ps. putida*, highest cell densities of $1.3x10^{13}$, $3.6x10^{14}$, $1.6x10^{11}$ and $2.5x10^9$ cfu/ml were obtained respectively (Fig. 4.58) while with *Ps. arvilla*, highest cell densities of $3.2x10^{12}$, $3.2x10^{13}$, $3.9x10^{11}$ and $1.6x10^9$ cfu/ml were obtained respectively (Fig. 4.59). Population density (OD_{600nm}) of the media increased significantly ($P<0.05$) compared to the controls. Increasing the concentration of diesel oil resulted to a decrease in cell mass proliferation.

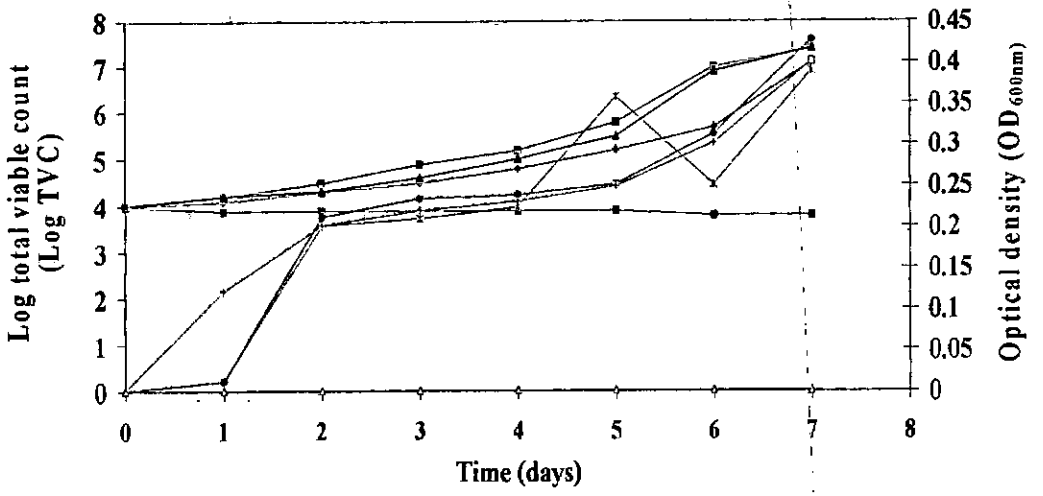
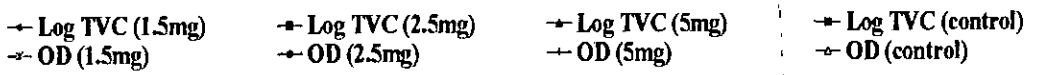


Fig. 4.48. Growth profile of *A. anitratus* on varying quantities of chrysene



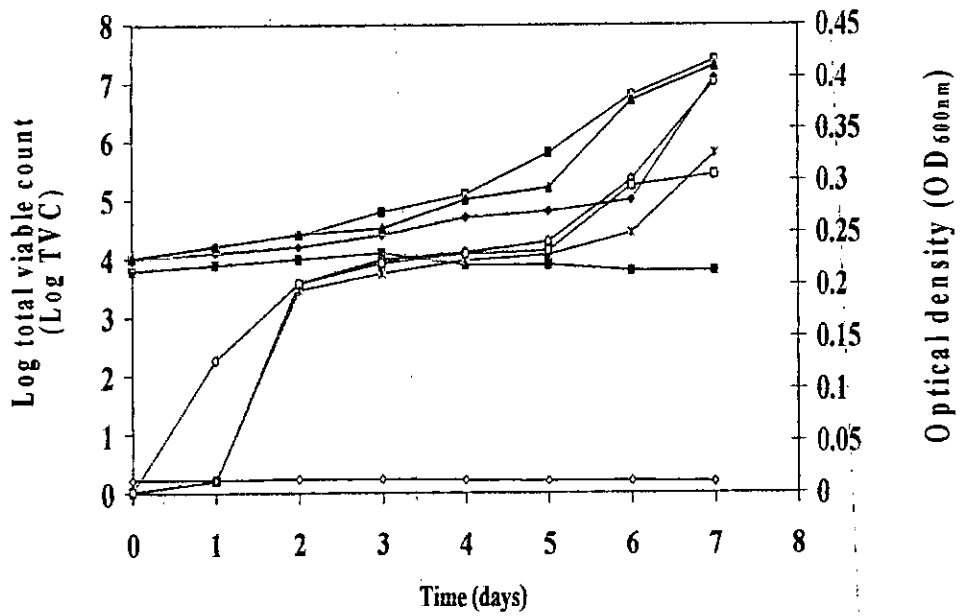
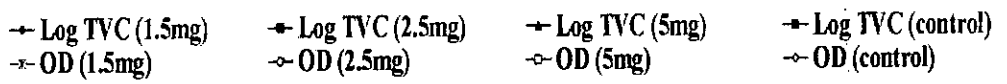


Fig. 4.49. Growth profile of *A. mallei* on varying quantities of chrysene



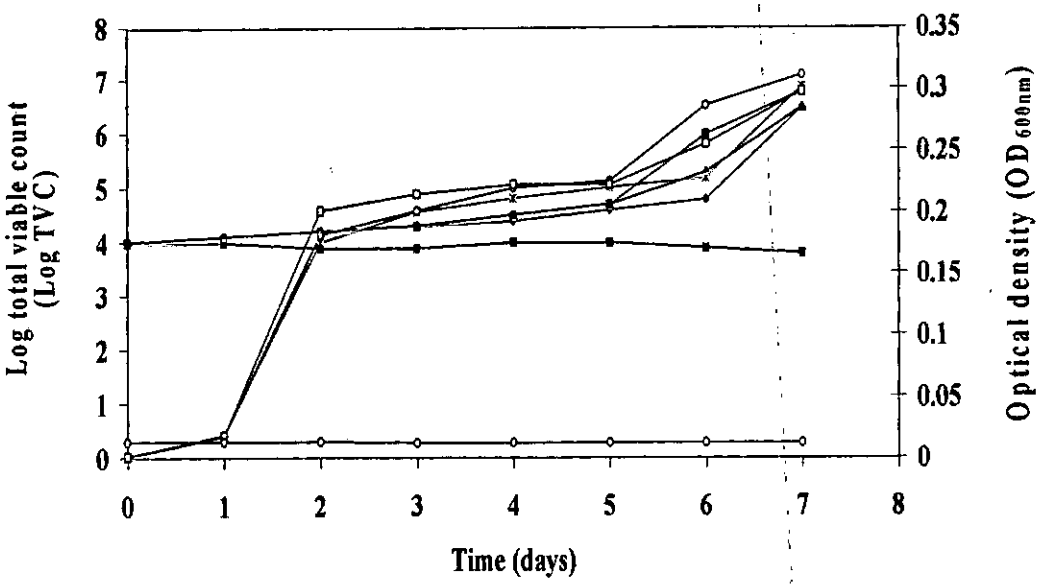


Fig. 4.50. Growth profile of *A. faecalis* on varying quantities of chrysene

Log TVC (1.5mg) Log TVC (2.5mg) Log TVC (5mg) Log TVC (control)
OD (1.5mg) OD (2.5mg) OD (5mg) OD (control)

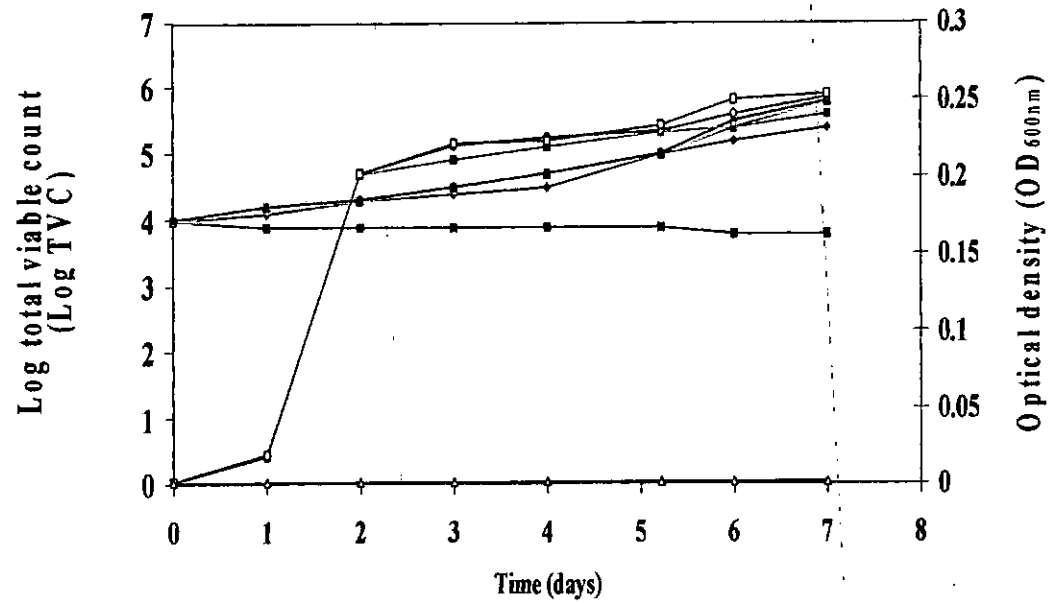


Fig. 4.51. Growth profile of *S. paucimobilis* on varying quantities of chrysene

Log TVC (1.5mg) Log TVC (2.5mg) Log TVC (5mg) Log TVC (control)
OD (1.5mg) OD (2.5mg) OD (5mg) OD (control)

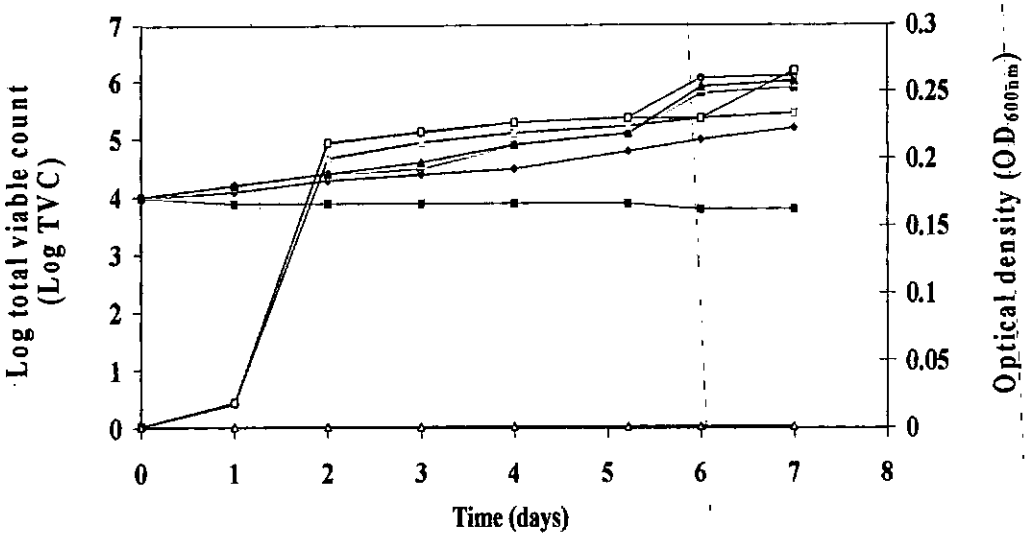


Fig. 4.52. Growth profile of *Ps. putida* on varying quantities of chrysene

Log TVC (1.5mg) Log TVC (2.5mg) Log TVC (5mg) Log TVC (control)
OD (1.5mg) OD (2.5mg) OD (5mg) OD (control)

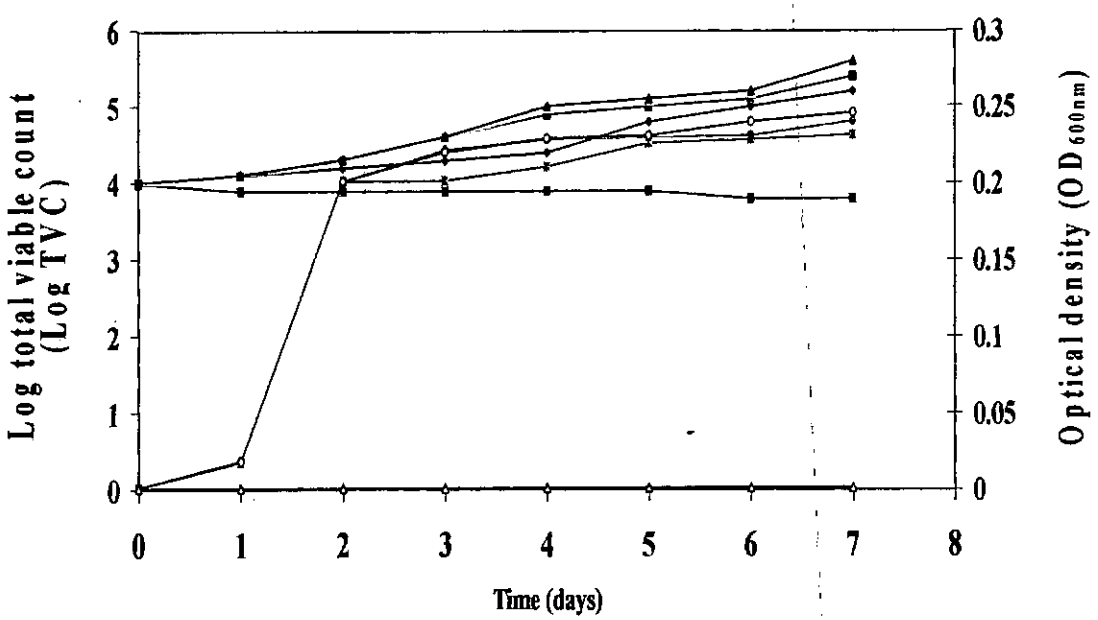


Fig. 4.53. Growth profile of *Ps. arvilla* on varying quantities of chrysene

Log TVC(1.5mg) Log TVC (2.5mg) Log TVC (5mg) Log TVC (control)
OD (1.5mg) OD (2.5mg) OD (5mg) OD (control)

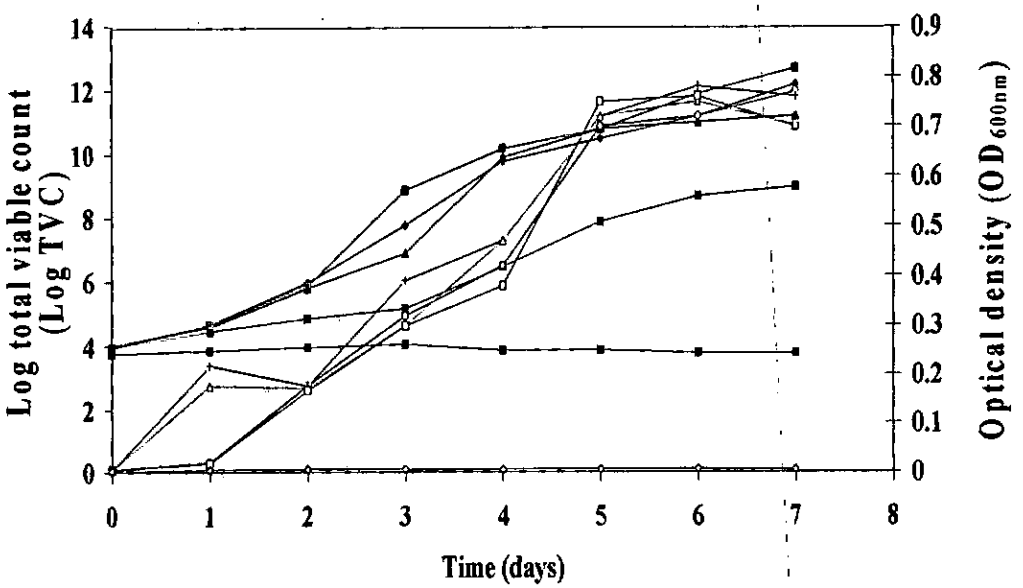


Fig. 4.54. Growth profile of *A. anitratus* on varying quanties of diesel oil

Log TVC (3%) Log TVC (5%) Log TVC (15%) Log TVC (30%) Log TVC (control)
OD (3%) OD (5%) OD (15%) OD (30%) OD (control)

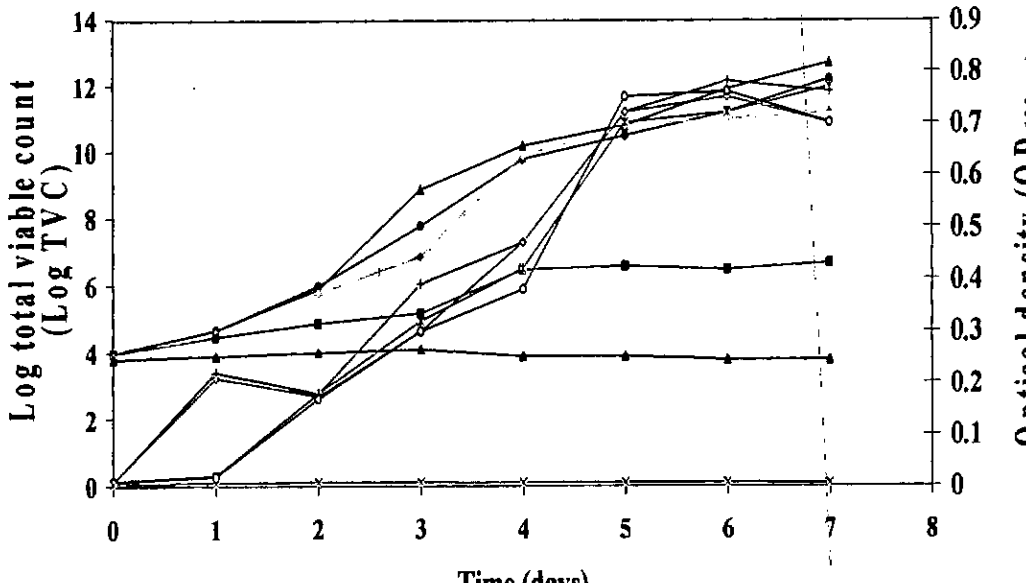


Fig. 4.55. Growth profile of *A. mallei* grown on varying quantities of diesel oil

Log TVC (3%) Log TVC (5%) Log TVC (15%) Log TVC (30%) LogTVC (control)
OD (3%) OD (5%) OD (15%) OD (30%) OD (control)

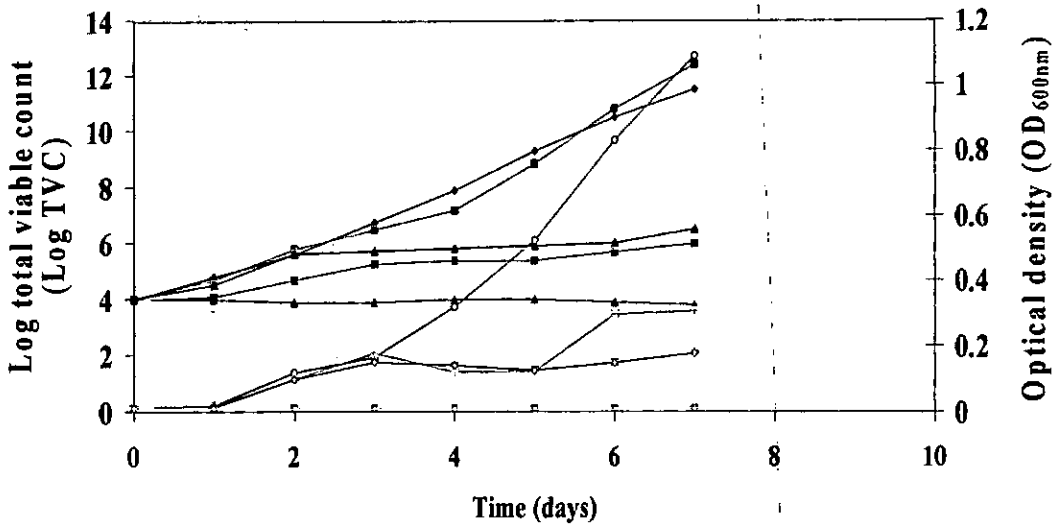


Fig. 4.56. Growth profile of *A. faecalis* on varying quantities of diesel oil

Log TVC (3%) Log TVC (5%) Log TVC (15%) Log TVC (30%) OD (3%)
Log TVC (Control) OD (5%) OD (15%) OD (30%) OD (Control)

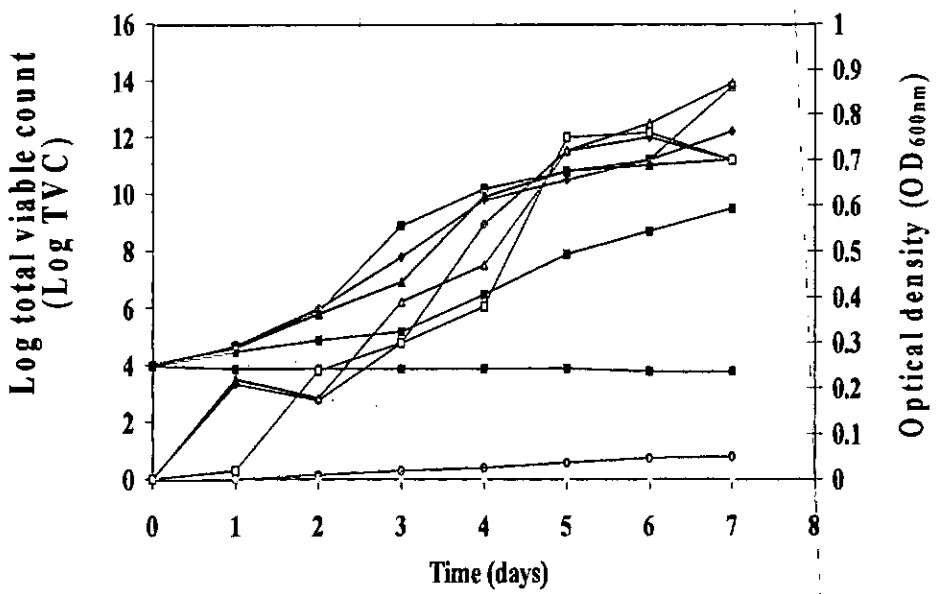


Fig. 4.57. Growth profile of *S. paucimobilis* on varying quantities of diesel oil

Log TVC (3%) Log TVC (5%) Log TVC (15%) Log TVC (30%) Log TVC (control)
OD (3%) OD (5%) OD(15%) OD (30%) OD (control)

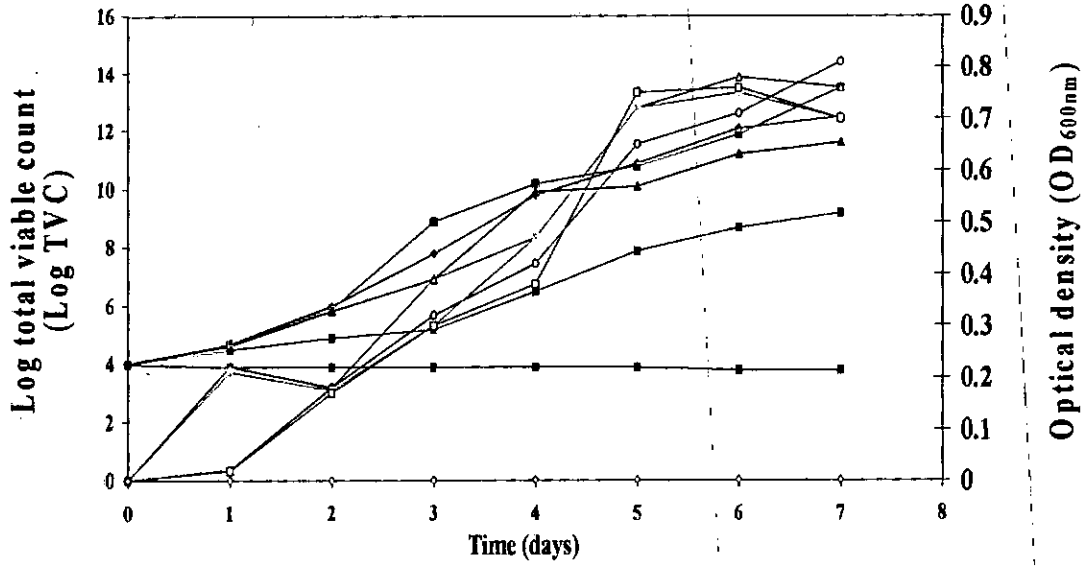


Fig. 4.58. Growth profile of *Ps. arvilla* on varying quantities of diesel oil

Log TVC (3%) Log TVC (5%) Log TVC (15%) Log TVC (30%) Log TVC (control)
OD (3%) OD (5%) OD(15%) OD (30%) OD (control)

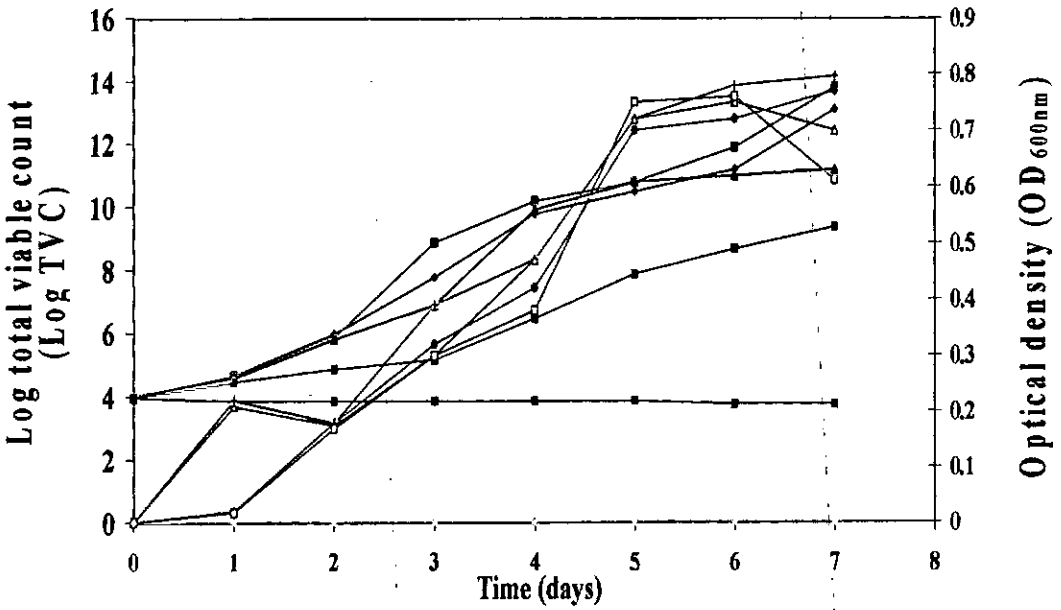


Fig. 4.59. Growth profile of *Ps. putida* on varying quantities of diesel oil

Log TVC (3%) Log TVC (5%) Log TVC (15%) Log TVC (30%) Log TVC (control)
OD (3%) OD (5%) OD(15%) OD (30%) OD (control)

4.10.3 EMULSIFYING ACTIVITY

Emulsifying activity (E24%) measured against different hydrocarbons (kerosene, diesel oil, engine oil, hexadecane, dodecane and xylene) using both the microbial cells and cell-free extracts of the isolates grown in varying quantities of chrysene and diesel oil respectively were evaluated.

Microbial cells and cell-free extracts of the isolates grown on chrysene did not show any emulsification activity against the various hydrocarbons tested. However cell-free extracts from diesel oil media showed varied emulsifying activity (Fig. 4.60 - 4.64). Cell-free extracts from 3% and 5% diesel oil had the highest emulsifying activity. Microbial cells of *A. faecalis* grown on varying quantities of diesel oil also showed emulsifying activity unlike microbial cell of the other isolates.

4.10.4 HAEMOLYTIC ACTIVITY

Microbial cells of all the isolates cleared zones on blood agar plates when they were cultivated in diesel oil media. This further confirmed biosurfactant production using diesel oil as the sole carbon source. However, haemolytic activity was not observed for cells and cell-free extract from chrysene growth media. Haemolysis on blood agar plate by *A. anitratus* from chrysene and diesel oil growth media respectively is illustrated in (Plate 4.4).

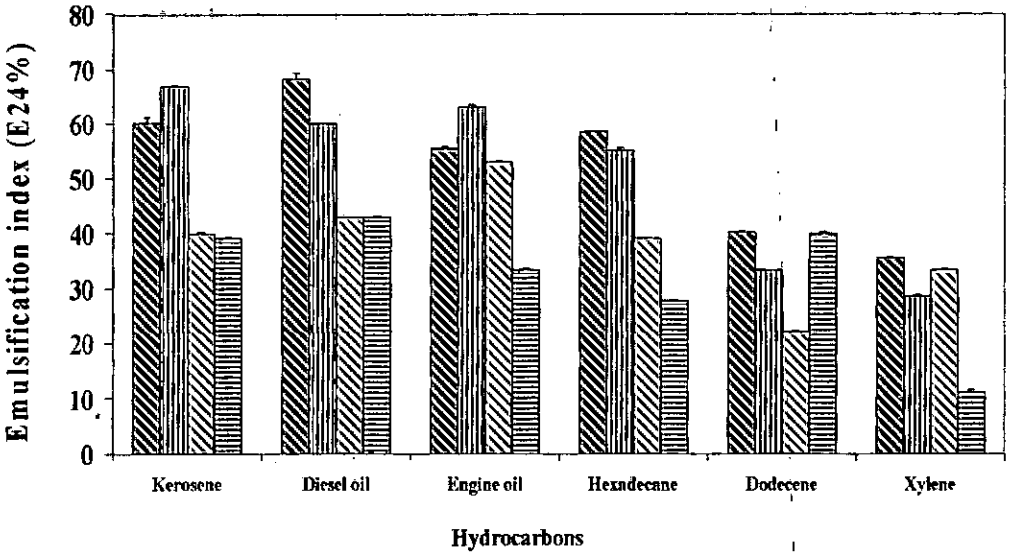


Fig. 4.60. Emulsify activities of cell-free extract of *A. anitratus* grown on diesel oil

3% diesel oil 5% diesel oil 15% diesel oil 30% diesel oil

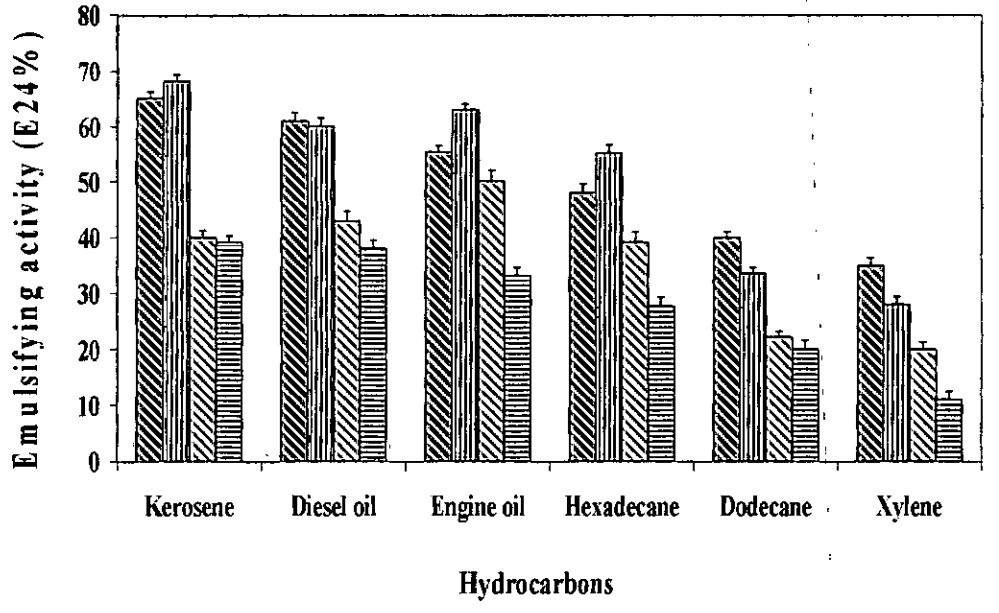


Fig. 4.61. Emulsifying activities of cell-free extract of *A. mallei* grown on diesel oil

3% diesel oil 5% diesel oil 15% diese oil 30% diesel oil

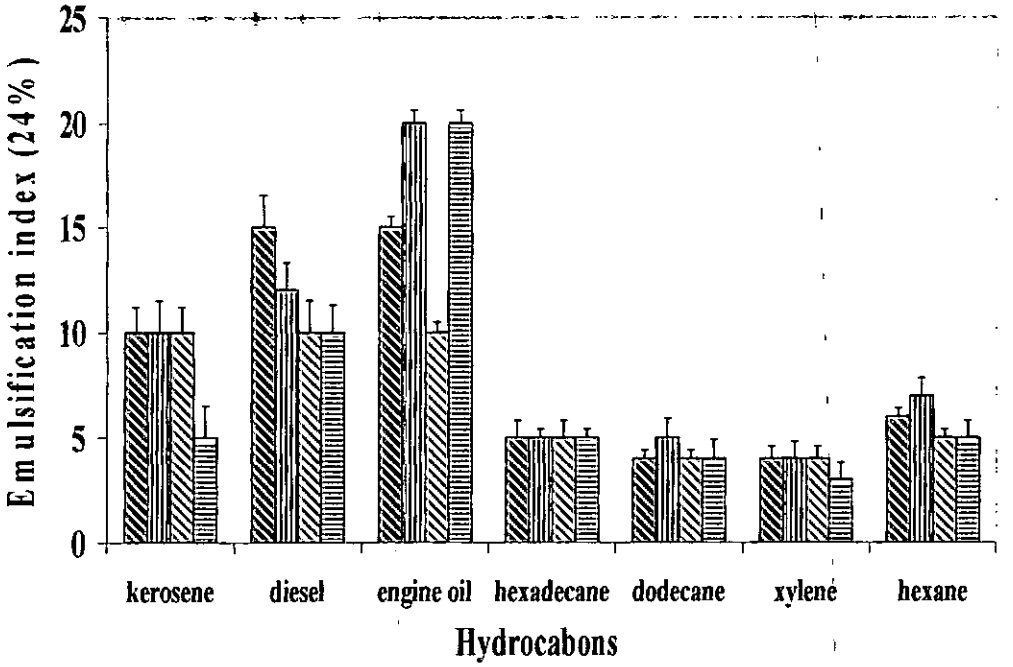


Fig. 4.62. Emulsifying activities of cell-free extract of *A. faecalis* grown on diesel oil

3% diesel oil 5% diesel oil 15% diesel oil 30% diesel oil

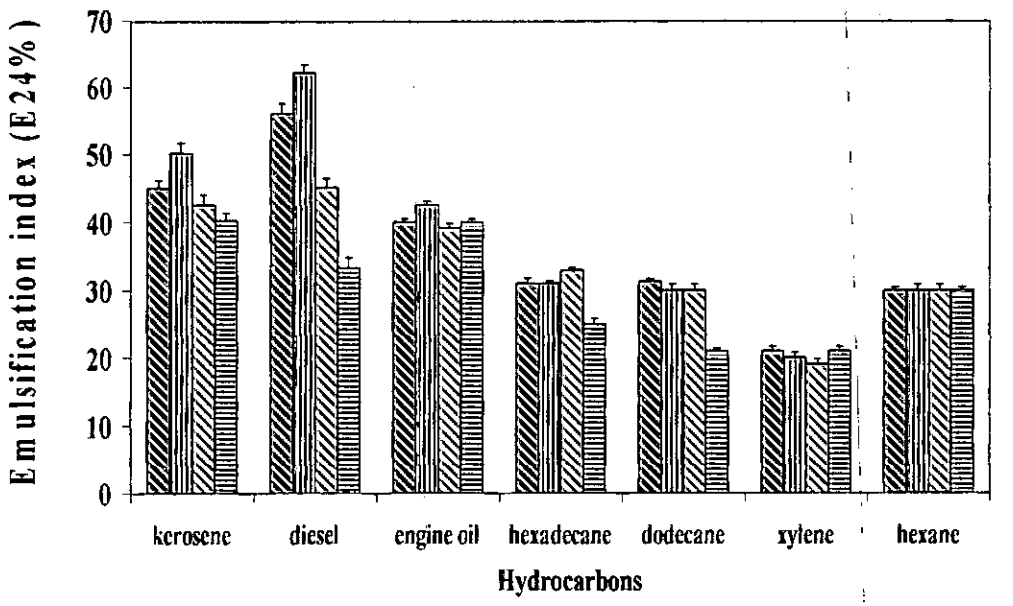


Fig. 4.63. Emulsifying activities of microbial cells of *A. faecalis* grown on diesel oil

3% diesel oil 5% diesel oil 15% diesel oil 30% diesel oil

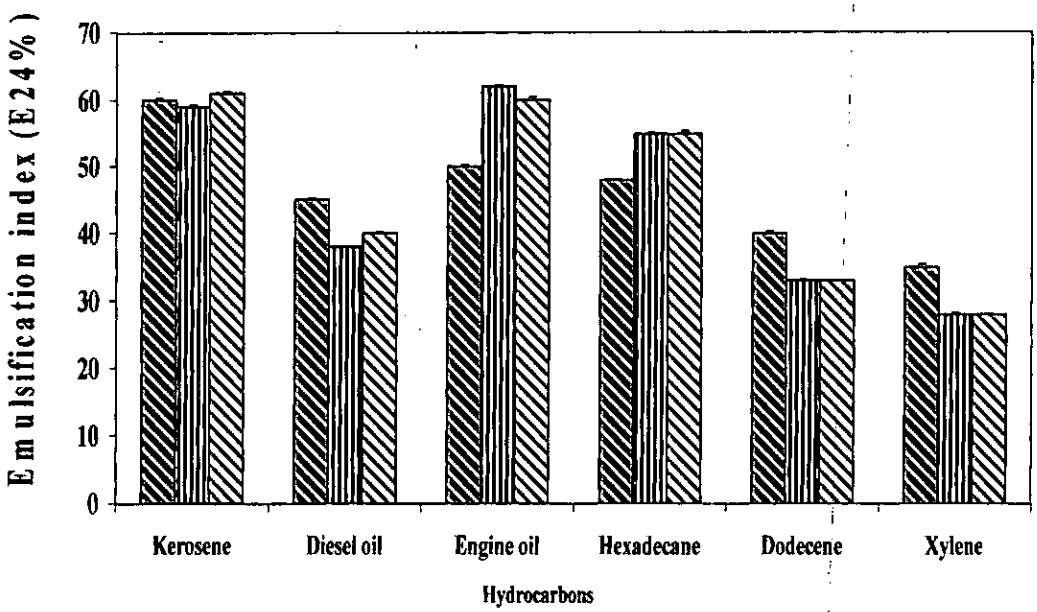


Fig. 4.64. Emulsify activities of cell-free extract of *S. paucimobilis*, *Ps. arvilla* and *Ps. putida* grown on 5% diesel oil

▨ *S. paucimobilis* ▥ *Ps. arvilla* ▨ *Ps. putida*

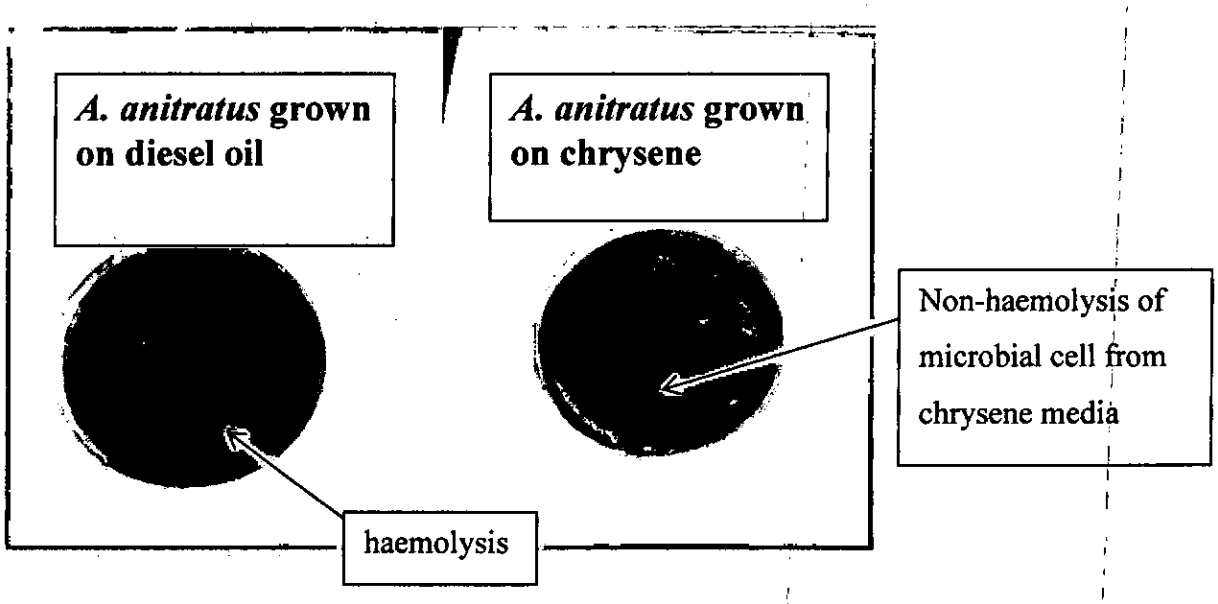


Plate 4.4. Screening of *A. anitratus* for biosurfactant production by haemolytic assay

CHAPTER FIVE

DISCUSSION

SOIL SAMPLE SITES AND DISTRIBUTION OF PAHS IN SOILS

Polycyclic aromatic hydrocarbons (PAHs) released into the environment have been found to originate from many sources which include petroleum refining and transport activities, gasoline and diesel fuel combustion, wood treatment and coal mining. PAHs impacted soil by crude petroleum or its products spillages and deposition usually lead to soil sterility. This is because crude oil, particularly the aromatic fractions are acutely lethal in concentration of a few ppm (parts per million) and chronically lethal in sub lethal concentrations in ppb (parts per billion) (Nwachukwu *et al.*, 2001).

However, bacterial strains have been isolated and identified that survived in the presence of the pollutants and have degradability potential (Ilori and Amund, 2000; Woo *et al.*, 2004; Santos *et al.*, 2008). Bacterial cells tend to detect temporal changes in the concentrations of specific chemicals and respond behaviourally to these changes, thereby adapting to the new concentration of the chemical stimuli through chemotaxis. Chemotaxis could be positive (the microorganism migrates towards a compound) or negative (microorganisms migrate away from a compound). Both cases require concentration gradients of the attractant or repellent for a chemotactic response to occur. This explains the presence of selective microbial consortia in a specific pollutant ecosystem. Some toxic organic compounds are chemoattractant for different bacterial species, such as *Pseudomonas* sp. and *Ralstonia* sp. (Grimm and Harwood, 1999; Bhushan *et al.*, 2000), which could lead to improved degradation. Studies have shown improved microbial degradation of pollutants by isolates cultivated from samples of pollution sites (Johnsen *et al.*, 2007).

Therefore, microbial seeding from PAH-contaminated soils was essential for isolating HMW PAHs degrading bacteria in this project. Degradation of high molecular weight polycyclic aromatic hydrocarbons (HMW PAHs) by the degraders could be accelerated due to chemically induced selection or adaptation of the microorganisms as a result of previous exposure to the pollutants.

In this study, PAHs contaminated soils were collected from four locations in Nigeria that were potential sources of PAHs. These were Eleme refinery; PortHarcourt, coal mining site (Onyeama mine); Enugu, wood processing site, Oshodi and diesel-power generator site; Lagos. These sites were observed to have PAHs deposition which could have resulted from the locations activities. The presence of PAHs at the polluted sites ensured that the isolated bacteria would have had previous exposure to PAHs.

Previous studies have detected PAHs at refinery sites, petroleum handling facilities, urban areas with increased vehicular activities (Kluska 2003; Coral and Karagöz 2005; Zhang *et al.*, 2006; Nganje *et al.*, 2007).

ISOLATION, ADAPTATION, SCREENING AND SELECTION OF THE BEST HMW PAHS DEGRADING BACTERIA

Enrichment culture technique used in this research ensured the selective isolation of HMW PAHs degrading bacteria from the soil samples.

In order to select the best HMW PAHs degrading bacteria, the bacterial isolates were screened by zone clearance on HMW PAH coated MS agar plates and subsequent growth in MS HMW PAHs broth. This was because, Supaka *et al.*, (2001) found that despite clearance of zone on phenanthrene-coated agar plates, some strains of *Sphingomonas* was not able to degrade phenanthrene in liquid medium.

The six bacteria isolates were selected as the best HMW PAHs degraders because they utilized the HMW PAHs (chrysene, fluoranthene and pyrene) as sole carbon and energy source for growth indicated by clearing zones on MS HMW PAH agar plates. They also showed increased turbidity of the MS HMW PAH media compared to controls. This was in accordance with previous study where clearing of zones on MS PAH agar plates and increase in turbidity of media has been used to isolate PAHs degraders (Ilori and Amund, 2000; Supaka *et al.*, 2001; Haimou *et al.*, 2004).

Based on the procedure of the isolation, adaptation, screening and selection, the isolated HMW PAHs degrading bacteria could therefore have application in both solid and liquid phase HMW PAHs decontamination. This protocol also ensured that the isolates could utilize and degrade the HMW PAHs concomitantly.

IDENTIFICATION OF THE SELECTED BEST HMW PAHS DEGRADING BACTERIA

The six bacteria isolates selected as the best HMW PAHs degrading bacteria identified based on biochemical and morphological characteristics showed that they were *Acinetobacter anitratus*, *Acinetobacter mallei*, *Alcaligenes faecalis*, *Sphingomonas paucimobilis*, *Pseudomonas arvilla* and *Pseudomonas putida*. These six Gram negative rods were able to utilize chrysene, fluoranthene and pyrene as their sole carbon and energy source. It is hitherto unreported of the identification of these isolates as concomitant utilizers of the three HMW PAHs (chrysene, fluoranthene and pyrene) as sole carbon and energy sources.

GROWTH OPTIMAL pH AND TEMPERATURE OF THE SELECTED HMW PAHS DEGRADING BACTERIA

The six isolates had wide range of pH tolerance of 5.0 - 9.6 exhibiting best growth at pH 7.1 - 7.5. They also showed growth at temperature range of 20^0C - 45^0C. This result was similar to those reported by Kim *et al.*, (2005) and Zhao *et al.*, (2008) on microbial physiological growth condition, pH and temperature values observed in Nigerian environs (Nweke and Okpokwasili 2003). These indicated their possible use in Nigerian terrestrial polluted soils and aquatic waters

GROWTH POTENTIAL OF THE SELECTED ISOLATES ON LIQUID HYDROCARBONS AND DIFFERENT PAHS

Microorganisms for potential use in bioremediation of PAHs polluted sites must have the ability to biodegrade a wide range of hydrocarbons since in most instances PAHs exist as mixtures in the presence of other hydrocarbons.

In this study, the isolates degraded at varying degrees both tested liquid and solid hydrocarbons such as crude oil, kerosene, diesel oil, engine oil, anthracene, naphthalene, phenanthrene, chrysene, fluoranthene and pyrene, utilizing them as sole carbon and energy source. Analogous results were reported by other workers showing the degradation of solid hydrocarbons by microbial populations in oil contaminated soil (Margesin, *et al.*, 2003). The present results are also in agreement with the findings of bacteria growth on media containing liquid and solid hydrocarbons as the sole source of carbon (Siddiqui and Adams, 2002; Survey *et al.*,2004).

The isolates degradation of crude oil and its petroleum products; diesel oil, engine oil and kerosene indicated the potential use of the isolates in bioremediation of oil polluted sites in Nigeria.

In terms of low molecular weight PAHs (LMW PAHs) degradation by the isolates, the observed 100% degradation of anthracene, naphthalene and phenanthrene confirmed that LMW PAHs are readily biodegradable by microorganisms. This is in agreement with earlier findings (Haimou *et al.*, 2004; Kim *et al.*, 2005). *Sphingomonas aromaticivorans* B0695 has been identified as LMW PAHs degrader (Janikowski *et al.*, 2002). *Pseudomonas aeruginosa*, *Alcaligenes eutrophus*, *Bacillus subtilis* and *Micrococcus lutetus* were isolated from crude oil polluted soil as anthracene degrader (Ilori and Amund, 2000).

Regarding HMW PAHs utilization as the sole source of carbon and energy and possibly degradation, previous studies have shown that HMW PAHs are recalcitrant, persistent and barely degraded (Kanaly and Harayama, 2000; Samanta *et al.*, 2002). Some bacteria have been isolated that degraded HMW PAHs via co-metabolism or in the presence of surfactants (Boonchang *et al.*, 2000; Juhasz and Naidu, 2000; Makkar and Rockne 2003). A strain of *Sphingomonas* P2 was observed as phenanthrene degrader and co-metabolised fluoranthene and pyrene (Supaka *et al.*, 2001).

However, present study results showed degradation of HMW PAHs (chrysene, fluoranthene and pyrene) by bacteria that utilize them as sole source of carbon and energy.

BIODEGRADATION OF HIGH MOLECULAR WEIGHT POLYCYCLIC AROMATIC HYDROCARBONS

Bioaugmentation efficiency is a function of the degradation potential of the inoculated microbial degrader. Thus, bioaugmentation of contaminated soils should be preceded by series of laboratory evaluations aimed at analyzing selected microorganisms capacity to colonize and degrade the pollutant (Sabaté *et al.*, 2004).

PAHs especially the HMW PAHs occur in various ecosystems and are priority pollutants due to their potential toxicity (Castorena-Torres *et al.*, 2008; Topinka *et al.*, 2008). There is therefore the need to study ways of remediating PAHs polluted sites.

Moreover, PAHs usually occur as a mixture of both LMW and HMW PAHs (Guha *et al.*, 1999; Leblond *et al.*, 2001). The success of most PAHs bioremediation projects has been limited by failure to remove the HMW PAHs, this is further compounded by the existence of HMW PAHs in complex mixture (Leblond *et al.*, 2001; Lotfabad and Gray, 2002). The recalcitrance of HMW PAHs to microbial degradation has led to research focused on evaluating a wide phylogenetic spectrum of microorganisms possessing HMW PAHs degradation potential.

In this study, of the six bacteria isolates; *A. anitratus*, *A. mallei*, *A. faecalis*, *S. paucimobilis*, *Ps. arvilla* and *Ps. putida* isolated and identified as HMW PAHs degraders, *A. anitratus*, *A. mallei* and *A. faecalis* easily lost their HMW PAHs degradation potential as such they were excluded from the detailed biodegradation of HMW PAHs studies.

These further buttress the need to search for stable and suitable bacteria degraders of HMW PAHs. It also gave insight to the recalcitrance of HMW PAHs in the environment due probably to the ecotoxicity of HMW PAHs to possible degraders. *S. paucimobilis*, *Ps. arvilla* and *Ps. putida* were subjected to degradation of individual HMW PAHs (chrysene, fluoranthene and pyrene), mixture of the HMW PAHs and in the presence of phenanthrene. HMW PAHs degradation was evaluated in terms of residual HMW PAHs, total viable count of isolates, population density (OD_{600nm}) and pH of media during degradation.

RESIDUAL PAHS DURING PAHS DEGRADATION

Residual chrysene after 8 days of degradation of 100 µg/ml was 30.5±0.3, 40.6±0.7 and 17.2±0.2 (µg/ml) respectively using *S. paucimobilis*, *Ps. arvilla* and *Ps. putida* while 21.3±0.2 and 12.4±0.1 (µg/ml) respectively was obtained using consortium and consortium in the presence of phenanthrene correspondingly.

Residual fluoranthene after 8 days of degradation of 100 µg/ml was 2.0±0.1, 2.0±0.4 and 0.02±0.07 (µg/ml) respectively using *S. paucimobilis*, *Ps. arvilla* and *Ps. putida* while 2.2±0.8 and 0.2±0.3 (µg/ml) respectively was obtained using consortium and consortium in the presence of phenanthrene correspondingly.

Furthermore, residual pyrene after 8 days of degradation of 100 µg/ml was 0.06±0.2, 6.5±0.6 and 6.6±0.4 (µg/ml) respectively using *S. paucimobilis*, *Ps. arvilla* and *Ps. putida* while 10.6±0.8 and 0.7±0.2 (µg/ml) respectively was obtained using consortium and consortium in the presence of phenanthrene correspondingly.

The above residual HMW PAHs showed that fluoranthene was better preferred as the sole carbon and energy source and thus better degraded by the isolates. Amongst the bacteria isolates, *Ps. putida* may be the better isolate for field trials in degrading the HMW PAHs, however, pyrene was better degraded by *S. paucimobilis*.

Individual isolates degradation of mixture of the HMW PAHs (100 µg/ml each), resulted to residual chrysene of 40.2±0.4, 40.3±0.2 and 27.4±0.8 (µg/ml) respectively after 8 days of degradation by *S. paucimobilis*, *Ps. arvilla* and *Ps. putida*. Moreso, consortium degradation of a mixture (100 µg/ml each) of the HMW PAHs and mixture in the presence of phenanthrene respectively, resulted to residual chrysene of 11.5±0.4 and 0.45±0.03 (µg/ml).

Degradation of the HMW PAHs mixture by the individual isolates resulted to residual fluoranthene of 32.5±0.3, 35.4±0.2 and 10.1±0.5 (µg/ml) respectively by S. *paucimobilis*, *Ps.*

arvilla and *Ps. putida* whereas as a consortium, degradation of mixture of HMW PAHs and mixture in the presence of phenanthrene gave residual fluoranthene of 6.2±0.3 and 0.02±0.02 (µg/ml) respectively.

Nevertheless, when the HMW PAHs were degraded as a mixture (100 µg/ml each) by S. *paucimobilis*, *Ps. arvilla* and *Ps. putida* respectively, residual pyrene obtained was 37.5±0.2, 34.2±0.4 and 32.0±0.2 (µg/ml). Consortium degradation of a mixture of the HMW PAHs and mixture of the HMW PAHs in the presence of phenanthrene respectively resulted to residual pyrene of 6.0±0.8 and 0.2±0.04 (µg/ml).

These results showed the potential of the isolates in concomitant degradation of chrysene, fluoranthene and pyrene. Previous studies showed the potential of a strain of *Sphingomonas sp.* Strain P2 capable of co-metabolism of HMW PAHs such fluoranthene and pyrene in liquid medium supplemented with phenanthrene (Supaka *et al.*, 2000). In another study, it observed that HMW PAHs could be mineralized by fungi-bacterial cocultures (Boonchang *et al.*, 2000). However, *mycobacterium* species have been found to biodegrade PAHs such as fluoranthene, fluorine, phenanthrene and pyrene (Bastiaens *et al.*, 2000; Pagnout *et al.*, 2006).

In this study, the observed relatively higher residual HMW PAH when the organisms were used as individual isolates compared to when they were used as a consortium to degrade mixture of the HMW PAHs may be as a result of the constitutive toxicity of the HMW PAHs to the individual isolates affecting their degradation. This may account for the persistence of HMW PAHs in the environment colonized by specific bacteria consortia. The collective metabolism by the mixed culture of the isolates may have resulted in enhanced HMW PAHs utilization since intermediary biotransformation products from one isolate may serve as substrate for catabolism and growth others (Johnsen *et al.*, 2005). Bouchez *et al.*, (1995)

suggested that degradation of PAH-mixture may be as a result of a co-operative process involving a consortium of strains with complementary capacities.

It was also observed that there was delayed chrysene and fluoranthene degradation respectively in the presence of phenanthrene. This may be as a result of preferential utilization of phenanthrene as carbon source and may account also for the persistence of HMW PAHs in the environment. This is in accordance with previous research that microbial strains could degrade several PAHs but preferred one (Tadros and Hughes, 1997).

However, degradation of the HMW PAHs in the presence of phenanthrene lead to increased degradation of the PAHs in both individual and consortium participation. One of the strategies proposed to enhance the degradation of specific PAHs is to offer bacteria metabolic or pathway inducers to stimulate both selective growth of PAHs degraders and induce PAHs metabolism. The potential pathway inducers which may be LMW PAH or intermediates produced during PAH degradation includes phenanthrene, salicycate, salicylaldehyde, 1-hydroxyl-2-naphthoate and catechol. Previous studies have shown that some pathway inducers could stimulate PAHs degradation (Chen and Aitken, 1999; Ogunseitan and Olson, 1995, Woo *et al.*, 2004). Thus, the presence of phenanthrene may have resulted in the production of metabolic intermediates which aided in the higher rate of degradation of the HMW PAHs compared to degradation in the absence of phenanthrene.

Therefore, in terms of achieving a quicker and efficient bioremediation of HMW PAHs polluted sites, using a consortium in the presence of a supportive growth substrate may be ideal due to synergistic effect.

From the results, it was observed that the trend of degradability of the PAHs was Phenanthrene>Fluoranthene>Pyrene>Chrysene. The physio-chemical properties of PAHs have been found to affect their degradability and thus their persistence in the environment.

Phenanthrene is a tricyclic aromatic hydrocarbon with molecular weight of 178 and water solubility of 1.29 mgl^{-1}. Fluoranthene is a tetra aromatic hydrocarbon with molecular weight of 202 and water solubility of 0.26 mgl^{-1}, pyrene is a tetra aromatic hydrocarbon with molecular weight of 202 and water solubility of 0.13 mgl^{-1} while Chrysene is also a tetra aromatic hydrocarbon, molecular weight of 228 and water solubility of 0.002 mgl^{-1} (Boopathy, 2000). From these, it could be seen that the number of rings, molecular weight and water solubility may have accounted for the trend in the biodegradation of the PAHs.

GROWTH OF THE ISOLATES DURING PAHS DEGRADATION

Primary metabolism of an organic compound has been defined as the use of the substrate as a source of carbon and energy (Johnsen *et al.*, 2005). Degradation of the HMW PAHs by the individual isolates and consortium led to increase in total viable cell. This was an indication that the HMW PAHs supported microbial biomass production even as sole source of carbon and energy. This is in accordance with previous studies (Yolcubal *et. al.*, 2002).

It was observed that the presence of phenanthrene greatly increased biomass production. This may reflect the enhanced degradation of HMW PAHs in the presence of phenanthrene and the microbial growth supportive role of phenanthrene. Fluoranthene better supported biomass generation than the other HMW PAHs, this may reflect its better degradation than the other HMW PAHs which may also be as a result of its physio-chemical properties.

In degrading mixture of the HMW PAHs, it was observed that individual isolates biomass generation were lower compared to the consortium. This reflected the observed lower degradation of mixture of HMW PAHs by individual isolates and may indicate the toxicity of mixture of HMW PAHs to the individual isolates and may account for HMW PAHs persistence in the environment.

POPULATION DENSITY OF ISOLATES DURING PAHS DEGRADATION

The population density (OD_{600nm}) of the isolates increased compared to the controls as degradation of the HMW PAHs proceeded. This was an indication of metabolic activity reflected in the increased cell mass. Researchers have shown that population density (OD_{600nm}) increase in media is a measure of degradation, proliferation of cell mass and secretion of extracellular products (Khalid *et al.*, 2004; Kyung-Su *et al.*, 2005). The presence of phenanthrene in the degradation of HMW PAHs enhanced the population density (OD_{600nm}) of the isolates reflecting its role in biomass generation for degradation.

pH OF MEDIA DURING PAHS DEGRADATION

There were changes in the pH of the experimental media either towards acidity or alkalinity compared to controls during the degradation of the HMW PAHs. These changes signified metabolic activity leading to production of acidic or alkaline metabolites which indicated breakdown of HMW PAHs.

Production of acidic metabolites during PAHs degradation has been reported to occur at the initial ring cleavage while metabolism of catechol, the lower metabolic pathway intermediate of PAHs, leads to the production of metabolites that shifts pH of media towards alkalinity (Rehmann *et al.*, 2001).

In this study, the observed pH shift of the experimental media from 7.2 to 6.8 during chrysene degradation by the individual isolates of *S. paucimobilis* and *Ps. arvilla* may indicate that the degradation was predominantly at the initial ring cleavage stage. However, with *Ps. putida*, the pH tended towards alkalinity. Furthermore, the pH shift towards alkalinity observed during individual isolates degradation of fluoranthene and pyrene respectively, may also indicate the

production of metabolites of the lower metabolic pathway. This thus may imply that the isolates had degraded the HMW PAHs towards completion.

It could also be inferred that degradation of mixture of the HMW PAHs by the individual isolates during the experimental period was predominated by initial ring cleavage reactions, thus the observed higher residual HMW PAHs compared to individual isolates degradation of single HMW PAHs.

Moreover, during degradation of single and mixture of HMW PAHs by the consortium, the pH also shifted towards alkalinity. This may indicate that degradation proceeded towards complete elimination of the HMW PAHs. This was also reflected in the lower residual HMW PAHs obtained during consortium degradation, a similar result was observed in the presence of phenanthrene. It can therefore be inferred that monitoring pH of media may be used to monitor the progress of HMW PAHs degradation.

ENZYME STUDIES

Bacterial PAH degradation occurs by successive oxidations catalyzed by dioxgyenase that require iron as co-fator (Juhasz and Naidu, 2000; Jiang *et al.*, 2004). PAHs are degraded to catechol by catechol dioxygenases which catalyzes the ring cleavage of catechol and its derivatives in either an intradiol or extradiol manner at the lower metabolic PAHs degradation pathway leading to tricarboxylic acid intermediates, carbondioxide and water.

S. paucimobilis exhibited catechol 1,2-dioxygenase activity (EC 1.13.11.1) while the *Ps. arvilla* and *Ps. putida* exhibited catechol 2,3-dioxygenase activity (EC 1.13.11.2). These enzymes were partially purified and characterized in this study. Catechol 1,2-dioxygenase of *S. paucimobilis* was purified to 23.0 purification fold with specific activity of 7.14 Umg Protein^{-1} while purification fold of 17.5 and specific activity of 5.24 Umg Protein^{-1} were obtained for

catechol 2,3-dioxygenase of *Ps. arvilla* whereas for *Ps. putida* catechol 2,3-dioxygenase, purification fold of 18.3 with specific activity of 5.66 Umg Protein^{-1} were obtained.

Catechol dioxygenase of *S. paucimobilis, Ps. arvilla* and *Ps. putida* showed two different peaks from DEAE-Sephadex A-50-120 ion-exchange chromatography. The two enzyme fractions peaks may represent two isoenzymic forms of the catechol dioxygenase enzyme. There was no significant difference between the activities of the two peak enzyme fractions of each organism. Molecular weight estimate of the two enzyme fractions peaks by subjecting each peak fractions to Sephadex G-25-300 gel filtration showed that catechol 1,2-dioxygenase of *S. paucimobilis* had 86.4 kDa (Isoenzyme I) and 98.4 kDa (Isoenyme II). Catechol 2,3-dioxygenase of *Ps. arvilla* showed molecular mass of 55.2 kDa (Isoenzyme I) and 62.0 kDa (Isoenzyme II) whereas catechol 2,3-dioxygenase of *Ps. putida* showed molecular weight of 116.0 kDa (Isoenzyme I) and 118.4 kDa (Isoenzyme II). Previous studies have shown the existence of catechol dioxygenase enzyme in isoenzyme forms. Catechol 1,2-dioxygenase of *Acinetobacter radioresistens* was isolated as an isoenzyme with molecular weight of 38.6 and 37.7 kDa respectively (Briganti *et al.*, 2000). Catechol 2,3-dioxygenase of *Pseudomonase putida* has been found to have molecular weight of 135.0 kDa (Kaschabek *et al.*, 1998). In this study, the two isoenzymes exhibited about the same activity towards catechol and were pooled for subsequent analyses.

Catechol dioxygenase of *S. paucimobilis, Ps. arvilla* and *Ps. putida* exhibited activity at temperature range of 30^{0}C - 45^{0}C with the enzyme of *Ps putida* extending to 50^{0}C. The optimum temperature obtained was at 45^{0}C, 45^{0}C and 50^{0}C respectively for *S. paucimobilis, Ps. arvilla* and *Ps. putida*. The enzymes had activity at pH range of 5.0 - 8.0, with the enzyme of *Ps putida* extending to 9.6. The optimum pH obtained was at 8.0, 8.0 and 9.6 respectively

for *S. paucimobilis, Ps. arvilla* and *Ps. putida*. These are in accordance with previous work of kaschabek *et al.*, (1998) and Mars *et al.*, (1999).

In this research. it was observed that Fe^{2+} led to increase in activity of catechol 2,3-dioxygenase of the *Pseudomonas* sp. whereas Fe^{3+} increased catechol 1,2-dioxygenase activity of *S. paucimobilis*. This is in accordance with previous studies which indicated that both enzymes use non-haem iron as the sole cofactor (Briganti *et al.*, 1997; Lin *et al.*, 2000; Riegert *et al.*, 2001; Hitomi *et al.*, 2004). The catechol 1,2-dioxygenase intradiol cleavage enzyme cofactor is in the ferric state whereas the 2,3-dioxygenase extradiol cleavage enzymes is in the ferrous state (Strachan *et al.*, 1998). Heavy metals Co^{2+}, Cu^{2+} and Ag^{2+} resulted to significant decrease in catechol dioxygenase activity. This showed that some part of the enzyme was accessible to inhibitors.

Present study also showed that catechol 2,3-dioxygenase of *Ps. putida* exhibited stronger affinity for catechol than the other isolates with Km and Vmax of 1.9±0.3 µM and 58.5±1.2 Umg $Protein^{-1}$ respectively. Catechol 1,2-dioxygenase of *S. paucimobilis* had Km of 2.1±0.2 µM and Vmax of 53.7±0.9 Umg $Protein^{-1}$ while catechol 2,3-dioxygenase of *Ps. arvilla* had Km of 2.0±0.5µM and V max of 57.5±0.3Umg $Protein^{-1}$. Similar results have been obtained for other catechol dioxygenase enzymes (Mars *et al.*, 1999; Briganti *et al.*, 2000).

MOLECULAR STUDIES

This study has revealed the possession of plasmid by the six HMW PAHs bacteria degraders which mediated their degradation of the HMW PAHs. All the six HMW PAHs degraders were found to harbour multiple plasmids, molecular weight range of 2.0 - 12.8kb with similarity amongst the isolates. Similar studies have shown the possession on plasmids by PAHs degrading bacteria (Filonov *et al.*, 2000, Coral and Karagöz, 2005).

The plasmids of the isolates were transferable to their cured isolate and other organisms generating new mutant isolates with differed degradation rate. This is an important factor for a potential candidate in bioremediation studies in that a potential candidate should be able to survive in the environment and transfer its degradation gene to surrounding organisms. Genetic exchange by horizontal gene transfer contribute to acquiring new catabolic pathways in microbial communities present in diverse environments (Christensen *et al.*, 1998). Studies have shown transfer of plasmid-encoded NAH-genes between phlogenetically different members of the bacteria community (Herrick *et al.*, 1997; Stuart-Keil *et al.*, 1998).

Apart from plasmid involvement in degradation, plasmids may also be involved in chemotaxis whereby microorganisms migrate towards specific carbon source site. Studies have shown that a 5.9 kb *Eco*RI fragment encoding a chemoreceptor Nah Y adjacent to the catabolic gene in NAH7 was involved in naphthalene chemotaxis (Grimm and Harwood, 1999). Another naphthalene and salicylate-degrading plasmid, pRKJ1, transferred into plasmid-free *P. putida* KT2442 resulted in the acquisition of chemotaxis and degradation properties (Samanta and Jain, 2000), as such plasmids of the HMW PAHs degraders could also have been involved in chemotaxis activity.

Catechol dioxygenase activity has been found to be encoded by plasmid of organisms involved in aromatic hydrocarbon degradation (Cerdan *et al.*, 1994; Cho and Kim, 2001).

Therefore, because of the enormous practical importance of conjugative plasmid transfer, it could be exploited for enhancing degradation capabilities in bioreactors or contaminated soils (Bathe *et al.*, 2005). This factor is also important in commercial production of novel HMW PAHs degraders.

BIOSURFACTANT PRODUCTION

In this study, the HMW PAHs degraders were evaluated for biosurfactant production when cultivated in varying quantities of chrysene and diesel oil. One of the main reasons for the prolonged persistence of HMW PAHs in contaminated sites is their low water solubility which increases their sorption to soil particles and limit their availability to biodegrading microorganisms. Thus, approaches to enhancing PAHs biodegradation often attempt to increase their apparent solubility by treatments such as addition of synthetic surfactants or biosurfactants (Barkay *et al.*, 1999).

In this research, *A. anitratus*, *A. mallei*, *A. faecalis*, *S. paucimobilis*, *Ps. arvilla* and *Ps. putida* were able to utilize various tested quantities of chrysene and diesel oil as sole source of carbon and energy indicated by microbial growth and turbidity of culture media. Increasing the quantity of chrysene from 1.5mg/50ml to 5mg/50ml did not inhibit microbial growth rather the organisms adapted by utilizing it for growth multiplying in cell density and increasing in turbidity of the culture media, but growth rate decreased with increasing chrysene quantity. A Similar report has been given by Yuan *et al.*, (2001).

The lowered microbial growth of *A. anitratus*, *A. mallei* and no difference observed for *A. faecalis* at 5.0mg/50ml chrysene compared to their growth on 2.5mg/50ml may indicate that at 5.0/mglml chrysene, these isolates degradation potential was affected. This justified their exclusion in the biodegradation studies carried out in this research.

This further indicated that *S. paucimobilis*, *Ps. arvilla* and *Ps. putida* perhaps adapted better compared to *A. anitratus*, *A. mallei* and *A. faecalis* to toxicity of the HMW PAHs even though all six isolates are good degraders of HMW PAHs.

All the six isolates were found to utilize 3%, 5%, 15% and 30% diesel oil as the sole carbon and energy but as the quantity of diesel oil increased, microbial biomass generation decreased.

15% and 30% diesel oil decreased isolates biomass proliferation compared to 3% and 5% diesel oil, but 5% diesel oil best supported cell mass generation.

This research work showed that extracellular fluid of all six isolates growth media containing diesel oil had emulsification activity on various hydrocarbons such as kerosene, diesel oil, engine oil, hexadecane, dodecane and xylene. This may indicate the production of biosurfactant by the isolates when grown on diesel oil.

The emulsification activities were found to be dependent on the test hydrocarbons and the source of extracellular fluid. Extracellular fluid from 3% and 5% diesel oil showed better emulsification activities than those from 15% and 30% suggesting toxicity and inhibition of microbial growth, increased constrain of microbial assess and poor solubilization of 15% and 30% diesel oil. This may also reflect why crude oil and its petrochemical products cause sterility of agricultural soils and persist in the environment. The bioavailability of a contaminant is controlled by a number of physico-chemical processes such as sorption and desorption, diffusion and dissolution (Iqbal *et al.*, 1995; Johnsen *et al.*, 2005), as such 15% and 30% diesel oil may have not been sufficiently available to the isolates for biosurfactant production.

In the case of *A. faecalis*, its microbial cells and cell-free extracts from diesel oil growth media showed emulsification of the various hydrocarbons tested. However, the microbial cells emulsification activities were higher than the cell-free extracts, this suggested that *A. faecalis* biosurfactant may be cell mediated and partially extracellular. Microbial cells of the other isolates harvested from the various quantities of diesel oil growth media showed no significant emulsification of the hydrocarbons tested. This suggested that *A. anitratus*, *A. mallei*, *S. paucimobilis*, *Ps. arvilla* and *Ps. putida* biosurfactant production in the presence of diesel oil may be wholly extracellular.

However, microbial cells and extracellular fluid of all six isolates grown on the varying quantities of chrysene showed no emulsification of the different hydrocarbons tested. This may indicate that the organisms do not produce emulsifier using chrysene as the sole carbon and energy source. Previous studies have shown that biosurfactant production is carbon source dependent (Maneerat, 2005). Nevertheless, non-emulsification does not totally rule out biosurfactant production, other parameters such as surface tension and adhesion may be investigated. Some biosurfactants have be found to enhance biodegradation of low-solubility hydrocarbons by mechanisms other than micelle solubilization (Barkay *et al.*, 1999).

All six isolates were tested for haemolytic activity, which is regarded as an indicative of biosurfactant production and used as a rapid method for bacterial screening (Lin, 1996). Cleared zones on blood agar plates were observed for all six isolates grown on diesel oil while isolates grown on chrysene showed no haemolytic activity. Again, non-haemolysis does not totally suggest non-biosurfactant production using chrysene as the sole carbon and energy source, this is because there are studies where organisms showed no haemolytic activity yet produced biosurfactant (Hsueh *et al.*, 2007). Therefore, this study has been able to show that the six isolates could be used to produce biosurfactant which was shown to be carbon source dependent.

CHAPTER SIX

SUMMARY

1. This research has isolated and identified indigenous bacteria strains; *Sphingomonas paucimobilis*, *Pseudomonas arvilla* and *Pseudomonas putida* capable of concomitant degradation of high molecular weight polycyclic aromatic hydrocarbons (HMW PAHs) such as chrysene, fluoranthene and pyrene which are classified as priority pollutants due to their recalcitrance, persistence, toxicity and ubiquity. The HMW PAHs degrading bacteria showed potential use in remediating crude oil and petrochemical products polluted sites as such could be employed in Nigerian petroleum polluted sites.

2. The isolates were competent to degrade the HMW PAHs either as individual isolates or as a consortium however, consortium degradation of both individual and mixture of the HMW PAH resulted to a lower residual HMW PAHs during the degradation period. It could therefore be that the use of consortium may be ideal in field trials.

3. This study also showed that constitutive toxicity of mixture of HMW PAHs to microbes may be a contributory factor to their persistence in the environment. This may be compounded by the competitive interactions of mixture of PAHs with respect to microbial enzyme system and the preferential utilization of certain PAHs as source of carbon.

4. The HMW PAHs degrading bacteria from this research showed active catechol dioxygenase enzyme activity involved in the catabolism of HMW PAHs to tricarboxylic acid intermediate thus tending towards complete elimination of the pollutants. In this study, catechol dioxygenase enzyme of the isolates were partially purified and characterized. Enzyme activity could be enhanced in the presence of iron as cofactor.

5. HMW PAHs degradation genes of the isolates were found to be plasmid mediated. The isolates showed potential transferability of their degradation genes essential for horizontal degradation gene transfer in field application.

6. Evidence was shown that the isolates could be used to produce biosurfactant which is of industrial importance.

In conclusion, this study has been able to isolate and identify indigenous bacterial isolates which could be used in clean-up of HMW PAHs polluted sites, crude oil and petrochemical polluted sites in Nigeria. Efficiency of the isolates HMW PAHs degradation potential may be characterized by their intrinsic tendency to degrade HMW PAHs especial in complex mixture, possession of efficient catechol dioxygenase activity and the ability to acquire or transfer and express degradation genes.

CONTRIBUTIONS TO KNOWLEDGE

1. This study has isolated and identified indigenous bacteria; *S. paucimobilis*, *Ps. arvilla* and *Ps. putida* capable of degrading recalcitrant high molecular weight polycyclic aromatic hydrocarbons, crude oil and petrochemical products for which they could be employed in remediating PAHs and oil spill sites.

2. The HMW PAHs degrading bacteria either as individual isolate or consortium were hitherto unreported as concomitant degraders of chrysene, fluoranthene and pyrene.

3. The bacteria isolates possessed efficient catechol dioxygenase enzyme which was partially purified and characterized in this study. Their activity could be enhanced in the presence iron as cofactor.

4. This study showed that catabolic degradation of HMW PAHs by the isolates was plasmid mediated. The plasmids were transferable; this led to generation of novel genetically modified organisms with HMW PAHs degradation potential.

REFERENCES

Alkio M., Tabuchi M.T., Wang X. and Colón-Carmona A. (2005). Stress responses to polycyclic aromatic hydrocarbons in *Arabidopsis* include growth inhibition and hypersensitive response-like symptoms. J. Exp. Bot. 56 (421): 2983-2994.

Angerer J., Mannschreck C. and Gundel J. (1997). Occupational exposure to polycyclic aromatic hydrocarbons in a graphite-electrode producing plant: biological monitoring of 1-hydroxypyrene and monohydroxylated metabolites of phenanthrene. Int. Arch. Occup. Environ. Health. 69: 323-331.

Antizar-Ladislao B., Lopez-Real J. and Angus James Beck A.J. (2006). Bioremediation of polycyclic aromatic hydrocarbons (PAH) in an aged coal-tar-contaminated soil using different in-vessel composting approaches. J. Hazardous Materials. 137 (3): 1583-1588.

Anyakora C., Arbabi M. and Coker H. (2008). A screen for Benzo(a)pyrene in Fish Samples From Crude Oil Polluted Environments. A. J. Environ. Sc. 4 (2): 145-150.

Anyakora C. and Coker H. (2007). Assessment of polynuclear aromatic hydrocarbon content in four species of fish in the Niger Delta by gas chromatography/mass spectrometry. Afr. J. Biotechnol. 6(6): 737-743.

Anyakora C. (2007). Environmental impact of polynuclear aromatic hydrocarbons. Kerala, India: Research Sgnpost. Pg. 71-79.

Banat I.M. (1995). Biosurfactant production and possible use in microbial enhanced oil recovery and oil pollution remediation: Review. Biores. Tech. 51: 1-12.

Barber J.L., Thomas G.O., Kerstiens G. and Jones K.C. (2003). A study of plant–air transfer of PCBs: from an evergreen shrub. Environ. Sci. Technol. 37: 3838-3844.

Barkay T., Navon-Venezia S., Ron E. and Rosenberg E. (1999). Enhancement of solubilisation and biodegradation of polycyclic aromatic hydrocarbon by the bioemulsifier Alasan. Appl. Environ. Microbiol. 65:2697-2702.

Barrow I.G and Felthanm K.A.R (2003). Cowan and Steel's manual for the identification of medical bacteria. Edinburgh: Cambridge University Press. PP 21-178.

Bastiaens, L., Springael, D., Wattiau, P., Harms, H., deWatcher, R., Verachtert, H. and Diels, L. (2000). Isolation of adherent polycyclic aromatic hydrocarbon (PAH)-degrading bacteria using PAH-sorbing carriers. Appl. Environ. Microbiol. 66: 1834–1843.

Bathe S., Schwarzenbeck N. and Hausner M. (2005). Plasmid-mediated bioaugmentation of activated sludge bacteria in a sequencing batch moving bed reactor using pNB2. Lett. Appl. Microbiol. 41: 242–247.

Baumann P. and Harshbarger J. (1995). Decline in liver neoplasms in wild brown bullhead catfish after coking plant closes and environmental PAHs plummet. Environ. Health Perspect. 103: 168-170.

Beckles, D.M., Ward, C.H. and Hughes, J.B. (1998). Effects of mixtures of PAHs and sediments on fluoranthene biodegradation patterns. *Environ. Toxicol. Chem.* 17, 1246-1251.

Bhushan B. (2000). Chemotaxis and biodegradation of 3-methyl-4-nitrophenol by *Ralstonia* sp. SJ98. Biochem. Biophy. Res. Commun. 275: 129–133.

Bicca F.C., Fleck L.C. and Ayub M.A.Z. (1999). Production of biosurfactant by hydrocarbon degrading *Rhodococcus rubber* and *Rhodococcus erythropolis*. Rev. Microbiol. 30:231-236.

Boonchang S., Britz L.M and Stanley A.G. (2000). Degradation and mineralization of high molecular–weight polycyclic aromatic hydrocarbons by defined fungal-bacterial cocultures. Appl. Environ. Microbiol. 66(3): 1007-1019.

Boldrin B., Tiehm A. and Friasche C. (1993). Degradation of phenanthrene, fluorine, fluoranthene, and pyrene by a *Mycobacterium* sp. Appl. Environ. Microbiol. 59: 1927-1930.

Boopathy R. (2000). Factors limiting bioremediation technologies. Biores. Technol. 75: 63–67.

Bouchez M., Blanchet D. and Vandecasteele, J.P. (1995). Degradation of polycyclic aromatic hydrocarbons by pure strains and by defined strain associations: inhibition phenomena and cometabolism. Appl. Microbiol. Biotechnol. 43: 154-156.

Briganti F., Pessione E., Giunta C., Mazzoli R. and Scozzafava A. (2000). Purification and catalytic properties of two catechol 1,2-dioxygenase isoenzymes from benzoate-grown cells of *Acinetobacter radioresistens*. Protein Chem. 19(8): 709-716.

Briganti F., Pessione E., Giunta C. and Scozzafava A. (1997). Purification, biochemical properties and substrate specificity of a catechol 1,2-dioxygenase from a phenol degrading Acinetobacter radioresistens. FEBS Letters. 416: 61-64.

Brown M. (1991). Biosurfactants in cosmetics applications. Int. J. Cosmet. Sci. 13: 61-64.

Cai Q., Mo C., Li Y. and Zeng Q, Katsoyiannis A., Wu Q. and Fe´rard J. (2007). Occurrence and assessment of polycyclic aromatic hydrocarbons in soils from vegetable fields of the Pearl River Delta, South China. Chemosphere 68: 159–168.

Castorena-Torres F., de León B.M., Cisneros B., Zapata-Pérez O., Salinas E.J. and Albores A. (2008). Changes in gene expression induced by polycyclic aromatic hydrocarbons in the human cell lines HepG2 and A549. Toxicol. Vitro. 22(2): 411-421.

Cerniglia C.E. (1993). Biodegradation of PAHs. Curr. Opin. Biotechnol. 4: 331-338.

Cerniglia C.E. (1992). Biodegradation of polycyclic aromatic hydrocarbons. Biodegradation. 3: 351–368.

Chen S.H. and Aitken M.D. (1999). Salicylate stimulates the degradation of high-molecular weight polycyclic aromatic hydrocarbons by *Pseudomonas saccarophila* P15. Environ. Sci. Technol. 33: 435-443.

Cheung C.C.C., Zheng G.J., Li A.M.Y., Richardson B.J. and Lam P.K.S. (2001). Relationships between tissue concentrations of polycyclic aromatic hydrocarbons and antioxidative responses of marine mussels, *Perna viridis*. Aquatic Toxicol. 52(3-4): 189-203.

Cho J. and Kim S. (2001). Detection of mega plasmid from polycyclic aromatic hydrocarbon-degrading Sphingomonas sp. Strain KS14. J. Mol. Microl. Biotechnol. 3(4): 503-506.

Christensen E.R. and Bzdusek P.A. (2005). PAHs in sediments of the Black River and the Ashtabula River, Ohio: source apportionment by factor analysis. Wat. Res. 39:511–524.

Christensen B.B., Sternberg C., Andersen J.B., Eberl L., Moller S., Givskov M. and Molin S. (1998). Establishment of new genetic traits in a microbial biofilm community. Appl. Environ. Microbiol. 64: 2247-2255.

Clemente A.R., Anazawa T.A and Durrant L.R. (2001). Biodegradation of polycyclic aromatic hydrocarbons by soil fungi. Braz. J. Microbiol. 32(4): 255-261.

Coral G. and Karagöz S. (2005). Isolation and characterization of phenanthrene-degrading bacteria from a petrochemical refinery soil. Annals of Microbiol. 55(4): 255-259.

Credan P., Wasserfallen A., Rekik M., Timmis N.K and Harayama S. (1994). Substrate specificity of catechol 2,3-dioxygenase encoded by TOL plasmid pWW0 of *Pseudomonas putida* and its relationship to cell growth. J. Bacteriol. 176(19): 6074-6081.

Da Silva D., Buzitis J., Krahn M.M., Bicego M.C. and Pires-Vanin A.M.S. (2006). Metabolites in bile of fish from Sao Sebastiao Channel, Sao Paulo, Brazil as biomarkers of exposure to petrogenic polycyclic aromatic compounds. Mar. Pollut. Bull. 52: 175-183.

Daugulis J.A. and McCracken M.C. (2003). Microbial degradation of high and low molecular weight polyaromatic hydrocarbons in a two-phase portioning bioreactor by two strains of *Sphinogomonas* sp. Biotechnol. Letts. 25: 1441-1444.

Denison M.S. and Heath-Pagliuso S. (1998). The Ah receptor: a regulator of the biochemical and toxicological actions of structurally diverse chemicals. Bull. Environ. Contam. Toxicol. 61: 557–568.

Desai J. and Banat I. (1997). Microbial production of surfactants and their commercial potential. Microbiol. Mol. Rev. 61:47-64.

Déziel E., Paquette A., Villemur R., Lepine F. and Bisaillon J. G. (1996). Biosurfactant production by a soil *Pseudomonas* strain growing on polycyclic aromatic hydrocarbons. Appl. Environ. Microbiol. 62:1908-1912.

Dipple A. and Bigger C.A.H. (1991). Mechanism of action of food associated polycyclic aromatic hydrocarbon carcinogens. Mutation Research 259: 263-276.

Dong R.B., Xu D.F. and Liu L.D. (1999). Behavior of PAHs in the environment. Environ. Dev. 14: 10–11.

El-Mansi M., Anderson J.A., Inche A.C., Knowles K.L. and Platt J.D. (2000). Isolation and curing of the *Klebsiella pneumoniae* large indigenous plasmid using sodium dodecyl sulphate. Res. Microbiol. 151(3): 201-208.

Filonov A.E., Karpov A.V., Kosheleva A.I., Puntus F.I., Balashova V.N. and Boronin M.A. (2000). The efficiency of salicylate utilization by *Pseudomonas putida* strains catabolizing naphthalene via different biochemical pathways. Process Biochem. 35(9): 983-987.

Foght J.M. and Westlake D.W.S. (1988). Degradation of polycyclic aromatic hydrocarbons and aromatic heterocycles by a *Pseudomonas* species. Can J Microbiol. 34:1135-1141.

Grimm A.C. and Harwood C.S. (1999). NahY, a catabolic plasmid-encoded receptor required for chemotaxis of *Pseudomonas putida* to the aromatic hydrocarbon naphthalene. J. Bacteriol. 181: 3310–3316.

Guha S., Peters C.A. and Jaffé P.R. (1999). Multisubstrate biodegradation kinetics of naphthalene, phenanthrene, and pyrene mixtures. Biotech. Bioengr. 65: 491-499.

Haimou Z., Aristeidis K. and Anna I.K. (2004). Isolation and characterization of novel bacteria degrading polycyclic aromatic hydrocarbons from polluted Greek soils, Appl. Microbiol. Biotechnol. 65: 124-31.

Herrick J.B., Stuart-Keil K.G., Ghiorse W.G. and Madsen E.L., (1997). Natural horizontal transfer of naphthalene dioxygenase gene between bacteria native to a coal tar-contaminated field site. Appl. Environ. Microbiol. 63: 2330-2337.

Holt J.G., Krieg N.R., Sneath P.H.A., Stanley J.T. and William S.T. (1994). Bergey's Manual of Determinative Bacteriology. Baltimore: Williams and Wilkins. PP 1256-2352.

Hitomi Y., Tase Y., Higuchi M., Tanaka T. and Funabiki T. (2004). A reaction intermediate involved in oxygenation of catacholatiron (III) complexes with molecular oxygen-relevance to catechol dioxygenases. Chem. Lett. 33: 316-317.

Hsueh Y., Somers B. E., Lereclus D., Ghelardi E., Amy C.L. and Wong L.C.A. (2007). Biosurfactant Production and Surface Translocation Are Regulated by PlcR in *Bacillus cereus* ATCC 14579 under Low-Nutrient Conditions. Appl. Environ. Microbiol. 73(22): 7225-7231.

Ilori M.O. and Amund D.I. (2001). Production of peptidoglycolipid bioemulsifier by *Pseudomonas aeruginosa* grown on hydrocarbons. Z. Naturforsch. 56c: 547-552.

Ilori M. O.N. and Amund D. L. (2000). Degradation of anthracene by bacteria isolated from soil polluted tropical soils. Z. Naturforsch. 55c: 890-897.

Iqbal S, Khalid ZM, Malik KA (1995). Enhanced biodegradation and emulsification of crude oil and hyperproduction of biosurfactants by gamma ray-induced mutant of *Pseudomonas aeruginosa*. Lett. App. Microbiol. 21: 176-179.

Janikowski T.B., Velicogna D., Punt M. and Daugulis A.J. (2002). Use of Two-phase partitioning bioreactor for degrading polycyclic aromatic hydrocarbons by a *Sphingomonas* sp. Appl. Microbiol. Biotechnol. 59: 368-376.

Jayaraman J. (1992). Laboratory Manual in Biochemistry. India, Wiley Eastern limited PP 55.

Jiang Y., Yang X., Liu B., Zkao H. Cheng Q. and Cai B. (2004). Catechol 2,3-dioxygenase from Pseudomonas sp. Strain ND6: Gene sequence and enzyme characterization. Biosci. Biotechnol. Biochem. 68(8): 1798-1800.

Johnsen A.R. and Karlson U. (2004). Evaluation of bacterial strategies to promote the bioavailability of polycyclic aromatic hydrocarbons (PAHs). Appl. Microbiol. Biotech. 63: 452-459.

Johnsen A.R., Schmidt S., Hybholt K.T, Henriksen S., Jacobsen S.C. and Andersen O. (2007). Strong Impact on the Polycyclic Aromatic Hydrocarbon (PAH)-Degrading Community of a PAH-Polluted Soil but Marginal Effect on PAH Degradation when Priming with Bioremediated Soil Dominated by *Mycobacteria*. Appl. Environ. Microbiol. 73(5): 1474-1480.

Johnsen A.R., Wick L.Y. and Harms H. (2005). Principles of microbial PAH-degradation in soil, *Environ.* Pollut. 133: 71–84.

Johnson-Restrepo B., Olivero-Verbel J, Lu S., Guette-Fernández J., Baldiris-Avila R., O'Byrne-Hoyos I., Aldous K.M., Addink R. and Kannan K. (2008). Polycyclic aromatic hydrocarbons and their hydroxylated metabolites in fish bile and sediments from coastal waters of Colombia. Environ. Poll. 151(3): 452-459.

Juhasz A.L. and Naidu R. (2000). Bioremediation of high molecular weight polycyclic aromatic hydrocarbons: a review of the microbial degradation of benzo[*a*]pyrene. Int. Biodeter. Biodegr. 45: 57-88.

Kado C.I. and Liu S.T. (1981). Rapid procedure for detection and isolation of large and small plasmids. J. Bacteriol. 145(3): 1365-1373.

Kanaly R.A. and Harayama S. (2000). Biodegradation of High-Molecular-Weight Polycyclic Aromatic Hydrocarbons by Bacteria. Bacteriol. 182(8): 2059–2067.

Kaschabek R.S., Kasberg T., Muller D., Mars A., Janssen B.D and Reineke W. (1998). Degradation of chloroaromatics: Purification and characterization of a novel type of chlorocatecol 2,3 – dioxygenase of pseudomonas putida GJ31. J. Bacteriol. 180(2):296-302.

Kästner M. and Mahro B. (1996). Microbial degradation of polycyclic aromatic hydrocarbons in soils affected by the organic matrix of compost. Appl. Microbiol. Biotechnol. 44(5): 668-675.

Kästner M., Breuer-Jammlia M. and Mahroe B. (1994). Enumeration and characterization of the soil microflora from hydrocarbon contaminated soil sites able to mineralize polycyclic aromatic hydrocarbon (PAH). Appl. Microbiol. Biotechnol. 41: 267- 273.

Kita A., Kita S-I., Fujisawa I., Inaka K., Ishida T., Horiike K., Nozaki M. and Miki K. (1999). An archetypical extradiol-cleaving catecholic dioxygenase: the crystal structure of catechol 2,3-dioxygenase (metapyrocatechae) from *Pseudomonas putida* mt-2. Structure. 7: 25-34.

Kelley I., Freeman J.P., Evans F.E. and Cerniglia C.E. (1993). Identification of metabolites from the degradation of fluoranthene by *Mycobacterium* sp. Strain PYR-1. Appl. Environ. Microbiol. (59): 3: 800-806.

Kewley R.J., Whitelaw M.L. and Chapman-Smith A. (2004). The mammalian basic helix–loop–helix/PAS family of transcriptional regulators. Int. J. Biochem. Cell Biol. 36: 189–204.

Khalid M., Tianling Z., Huasheng H., Zhiming Y., Jianjun Y. and Zhong H. (2004). Preliminary study on PAH degradation by bacteria from contami-nated sediments in Xiamen Western Sea, Fujian, China. Chinese J. Oceanol. Limnol. 22(4): 431-435.

Kluska, M. (2003). Soil contamination with polycyclic aromatic hydrocarbons in the vicinity of the Ring road in Siedlce City. Polish J. Environ. Stud. 12(3): 309-313.

Kim J.D., Shim S.H. and Lee C.G. (2005). Degradation of phenanthrene by bacterial strains isolated from soil in oil refinery fields in Korea, J. Microbiol. Biotechnol. 15(2): 337-345.

Kiyohara H. Sugiyama M., Mondello F.J., Gison D.T and Yano K. (1983). Plasmid involvement in the degradation of poly aromatic hydrocarbons by a Beijerinekia species. BBRC., 111:939-945.

Kipopoulou A.M., Manoli E. and Samara C. (1999). Bioconcentration of polycyclic aromatic hydrocarbons in vegetables grown in an industrial area. Environ. Pollut. 106: 369-380.

Kobayashi T., Morimoto K. and Tatsumi K. (2008). Removal of carotene-like colored compounds by liquid–liquid extraction during polycyclic aromatic hydrocarbons analysis of plant tissue. Chemosphere. 70(11): 2002-2008.

Koyama J., Uno S. and Kohno K. (2004). Polycyclic aromatic hydrocarbon contamination and recovery characteristics in some organisms after the *Nakhodka* oil spill. Marine Pollut. Bullet. 49(11-12): 1054-1061.

Krauss M., Wilcse W. and Zech W. (2000). Availability of polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs) to earthworms in urban soils. Environ. Sci. Technol. 34: 4335-4340.

Kuljukka T., Vaaranrinta R., Veidebaum T., Sorsa M., and Peltonen K. (1996). Exposure to PAH compounds among cokery workers in the oil shale industry. Environ Health Perspect. 104(Suppl 3): 539-541.

Kuusimaki L., Peltonen Y., Mutanen P., Peltonen K. and Savela K. (2004). Urinary hydroxy-metabolites of naphthalene, phenanthrene and pyrene as markers of exposure to diesel exhaust. Int. Arch. Occup. Environ. Health.77: 23-30.

Kyung-SU N.A., Kuruoda A., Takiguchi N., Ikeda T., Ohtake H. and Kato J. (2005). Isolation and characterization of benzene-tolerant *Rhodococcus opacus* strains. J. Biosc. Bioeng. 99(4): 378-382.

Leahy J.G. and Colwell R.R. (1990). Microbial degradation of hydrocarbons in the environment. Microbiol. Rev. 51: 305-315.

Leblond J.D., Schultz T.W. and Sayler G.S. (2001). Observations on the preferential biodegradation of selected components of polyaromatic hydrocarbon mixtures. Chemosphere. 42: 333-343.

Levin J.O., Rhen M. and Sikstrom E. (1995). Occupational PAH exposure: urinary 1-hydroxypyrene levels of coke oven workers, aluminum smelter pot-room workers, road

pavers, and occupationally non-exposed persons in Sweden. Sci. Total Environ. 163: 169-177.

Lloyd-Jones G. and Hunter D.W. (1997). Characterization of fluoranthene- and pyrene-degrading mycobacterium-like strains by RAPD and SSU sequencing. FEMS Microbiol. Lett. 153: 51-56.

Lin Y. and Cai L. (2008). PAH-degrading microbial consortium and its pyrene-degrading plasmids from mangrove sediment samples in Huian, China. Marine Pollut. Bullet. 57 (6-12): 703-706.

Lin G., Reid G. and Bugg T.D.H. (2000). A biomimetic model reaction for the extradiol catechol dioxygenases. Chem. Commun., 1119–1120.

Lin S.C. (1996). Biosurfactants: Recent advances. J. Chem. Technol. Biotechnol. 66:109-120.

Lotfabad S.K. and Gray M.R. (2002). Kinetics of biodegradation of mixtures of polycyclic aromatic hydrocarbons. Appl. Microbiol. Biotechnol. 60: 361-365.

Mahmood S.K. and Rama R.P. (1993). Microbial abundance and degradation of polycyclic aromatic hydrocarbons in soil. Bullet. Environ. Contam. Toxicol. 50(4): 486-491.

Makkar S.R. and Rockne J.K. (2003). Comparison of synthetic surfactants and biosurfactants in enhancing biodegradation of polycyclic aromatic hydrocarbons. Environ. Toxicol. Chem. 22(10):2280-2292.

Maneerat S. (2005). Biosurfactants from marine microorganisms. Songklanakarin J. Sci. Technol. 27(6):1263-1272.

Margesin R., Labbe D., Schinner F., Gracer C.W. and Whyte L.G. (2003). Characterization of hydrocarbon degrading microbial population in contaminated and pristine alpine soil. Appl. Environ. Microbiol, 69: 3085-3095.

Marr L.C., Kirchstetter T.W., Harley R.A., Miguel A.H., Hering S.V. and Hammond S.K. (1999). Characterization of polycyclic aromatic hydrocarbons in motor vehicle fuels and exhaust emissions. Environ. Sci. Technol. 33: 3091–3099.

Mars A.E., Kingama J., Kaschabek S.R., Reineke W. and Janssen D.B. (1999). Conversion of 3-chlorocatechol dioxygenase region of *Pseudomonas putida* GJ31. J. bacterial. 181: 1309-1318.

Masih A. and Taneja A. Polycyclic aromatic hydrocarbons (PAHs) concentrations and related carcinogenic potencies in soil at a semi-arid region of India. Chemosphere. 65(3): 449-456.

Meador J.P., stein J.E., Reichert W.L. and Varanasi U (1995). Bioaccumulation of polycyclic aromatic hydrocarbons by marine organisms. Rev. Environ. Contam. Toxicol. 143:79-165.

Means J.C., Wood S.G., Hassett, J.J. and Banwart W.L. (1980). Sorption of polynuclear aromatic hydrocarbons by sediments and soils. Environmental Science and Technology 14: 1524-1528.

Merlo F., Andreassen A., Weston A., Pan C.F., Haugen A., Valerio F., Reggiardo G., Fontana V., Garte S., Puntoni R. and Abbondandolo A. (1998). Urinary excretion of 1-hydroxypyrene as a marker for exposure to urban air levels of polycyclic aromatic hydrocarbons. Cancer Epidemiol. Biomarkers Prev. 7: 147-155.

Meudec A., Dussauze J., Deslandes E. and Poupart N. (2006). Evidence for bioaccumulation of PAHs within internal shoot tissues by a halophytic plant artificially exposed to petroleum-polluted sediments. Chemosphere. 65(3): 474-481.

Monteiro P.R., Reis-Henriques M.A. and Coimbra J. (2000). Polycyclic aromatic hydrocarbons inhibit in vitro ovarian steroidogenesis in the flounder (*Platichthys flesus* L.) Aquatic Toxicol. 48(4): 549-559.

Moraes I.D., Benincass M. and Alegre R.M. (2002). Production and characterization of rhamnolipids produced by a newly isolated strain of *Pseudomonas aeruginosa*. Braz. J. Food Technol. 5:145-149.

Ngabje T.N., Edet A.E. and Ekwere S.J (2007). Distribution of PAHs in surface soils from petroleum handling facilities in Calabar. Environ. Monit. Assess. 130(1-3): 27-34.

Nwachukwu S.C.U., James P. and Gurney T.R. (2001). Impact of crude oil on the germination and growth of crees seeds (Lepidium sp.) after bioremediation of agricultural soil polluted with crude petroleum using "adapted" *Pseudomonas putida*. J. Environ. Biol. 22(1): 29-36.

Nweke C.O and Okpokwasili G.C (2003). Drilling fluid base oil biodegradation potential of a soil *Staphylococcus species*. Afri. J. Biotech. 2(9): 293-295.

Oberbremer A. and Miiller-Hurtig R. (1989). Aerobic stepwise hydrocarbon degradation and formation of biosurfactants by an original soil population in a stirred reactor. Appl. Microbiol. Biotechnol. 31:582-586.

Ockenden W.A., Breivik K., Meijer S.N., Steinnes E., Sweetman A.J. and Jones K.C. (2003). The global re-cycling of persistent organic pollutants is strongly retarded by soils. Environ. Pollut. 121: 75-80.

Ogunfowokan O.A., Asubiojo I.O. and Fatoki S.O. (2003). Isolation and Determination of Polycyclic Aromatic Hydrocarbons in Surface Runoff and Sediments. Wat. Air & Soil Pollut. 147(1-4): 245-261.

Ogunseitan O.A. and Olson B.H. (1995). Effect of 2-hydroxybenzoate on the rate of naphthalene mineralization in soil. Appl. Microbiol. Biotechnol. 38: 799-806.

Pagnout C., Rast C., Veber A., Poupin P. and Fe´rard J. (2006). Ecotoxicological assessment of PAHs and their dead-end metabolites after degradation by Mycobacterium sp. strain SNP11. Ecotoxicol. Environ. Safety. 65: 151–158.

Palmroth M.R.T, Münster U, Pichtel J. and Puhakka J.A. (2005). Metabolic responses of microbiota to diesel fuel addition in vegetated soil. Biodegradation. 16: 91-101.

Piehler M.F., Swistak J.G., Pinckney J.L. and Paerl H.W. (1999). Stimulation of diesel fuel biodegradation by indigenous nitrogen fixing bacterial consortia. Microb. Ecol. 38: 69-78.

Providenti M., Lee H. and Trevors J. (1993). Selected factors limiting the microbial degradation of recalcitrant compounds. J. Ind. Microbiol. Biotechnol. 12: 379-393.

Rehmann L., George P. Prpich G.O. and Andrew J. Daugulis A.J. (2008). Remediation of PAH contaminated soils: Application of a solid–liquid two-phase partitioning bioreactor. Chemosphere. 73 (5): 798-804.

Rehmann K., Hertkorn N. and Kettrup A.A. (2001). Fluoranthene metabolism in *Mycobacterium* sp. strain KR20: identity of pathway intermediates during degradation and growth. Microbiol. 147: 2783-2794.

Rey-Salgueiro L., Martínez-Carballo E., arcía-Falcón S.M. and Simal-Gándara J. (2008). Effects of a chemical company fire on the occurrence of polycyclic aromatic hydrocarbons in plant foods. Food chemistry.108(1): 347-353.

Reynaud S. and Deschaux P. (2006). The effects of polycyclic aromatic hydrocarbons on the immune system of fish: A review. Aquatic Toxicol. 77(2): 229-238.

Riegert U., Burger S. and Stolz A. (2001). Altering catalytic properties of 3-chlorocatechol-oxidizing extradiol dioxygenase from Sphingomonas xenophaga BN6 by random mutagenesis. J. Bacteriol. 183:2322-2330.

Ron E.Z. and Rosenberg E. (2001). Natural roles of biosurfactants. Environ. Micribiol. 3:229-236.

Sabaté J., Vinas M. and Solanas A.M. (2004). Laboratory-scale bioremediation experiments on hydrocarbon-contaminated soils. Int. Biodeterior. Biodegrad. 54: 19-25.

Sabeen S., Ahmad S., Subhan A.S., Ajaz M. and Rasool A.S. (2004). Hydrocarbon degrading bacteria from Pakistani soil: isolation, identification, screening and genetic studies. Pakistan J. Biol. Sci. 7(9): 1518-1522.

Saeed T. and Al-Mutairi M. (2000). Comparative composition of polycyclic aromatic hydrocarbons (PAHs) in the sea water-soluble fractions of different Kuwaiti crude oils. Ad. Environ. Res. 4(2): 141-145.

Samanta S.K. and Jain R.K. (2000). Evidence for plasmid mediated chemotaxis of *Pseudomonas putida* towards naphthalene and salicylate. Can. J. Microbiol. 46: 1-6.

Samanta S.K., Singh O.V. and Jain R.K. (2002). Polycyclic aromatic hydrocarbons: environmental pollution and bioremediation. Trends Biotechno. 20: 243-248.

Sanger D.M., Holland A.F. and Scott G.I. (1999). Tidal creek and salt marsh sediments in South Carolina coastal estuaries: I. Distribution of trace metals. Archives of Environmental Contamination and Toxicology. 37: 445-457.

Santos E.C., Jacques J.S.R., Bento M.F., Peralba R.M.C., Selbach P.A., Sá L.S.E., Flavio A.O. and Camargo O.A.F. (2008). Anthracene biodegradation and surface activity by an iron-stimulated *Pseudomonas* sp. Bioresource Technol. 99(7): 2644-2649.

Sato N., Uragami Y., Nishizaki T., Takahashi Y., Sazaki G., Sugimoto K., Nonaka T., masai E., Fukuda M. and Senda T. (2002). Crystal structures of the reaction intermediate and its homologue of an extradiol-cleaving catecholic dioxygenase. J. Mol. Biol. 321: 621-636.

Sauer T.C., Michel J., Hayes M.O. and Aurand D.V. (1998). Hydrocarbon characterization and weathering of oiled intertidal sediments along the Saudi Arabian Coast two years after the Gulf War oil spill. Environ. Intl. 24: 43-60.

Seo J., Keum Y. and Li Q.X. (2009). Bacterial Degradation of Aromatic Compounds. Int. J. Environ. Review. Res. Public Health. 6: 278- 309.

Siddiqui A. and Adams W.A. (2002). Phytotoxicity and degradation of diesel hydrocarbons in the soil. Environ. Toxicol 1: 49-62.

Singleton D.R., Powell S.N., Sangaiah R., Gold A., Ball L.M. and Aitken M.D. (2005). Stable-isotope probing of bacteria capable of degrading salicylate, naphthalene, or phenanthrene in a bioreactor treating contaminated soil. Appl. Environ. Microbiol. 71: 1202-1209.

Strachan D.P., Freer A.A and Fenson A.C (1998). Purification and characterization of catecol 1,2 – dioxygenase from Rhodococcus rhodochrons NCIMB 13259 ,cloning and sequencing of it's CatA gene. Biochem. J. 333: 741-747.

Stuart-Keil K.G., Hohnstock A.M., Drees K.P., Herrick J.B. and Madsen E.L. (1998). Plasmids responsible for horizontal transfer of naphthalene catabolism genes between bacteria at a coal tar-contaminated site are homologous to pDTG1 from *Pseudomonas putida* NCIB 9816-4. Appl. Environ. Microbiol. 64: 3633-3640.

Supaka N., Pinphanichakarn P., Pattaragulwanit K., Thaniyavarn S., Omori T and Juntongjin K. (2001). Isolation and characterization of a phenanthrene degrading *Sphingomonas sp.* Strain P2 and it's ability to degrade fluoranthene and pyrene via cometabolism. ScienceAsia. 27: 21-28.

Survey S., Ahmad S., Subhan S.A., Ajaz M. and Rasool S.A (2004). Hydrocarbon degrading bacteria from Pakistani soil: Isolation, Identification, Screening and genetical studies. Pakistan J. Biol. Sc. 7(a):1518-1522.

Tabatabaee A., Assadi M.M., Noohi A.A. and Sajadian V.A. (2005). Isolation of biosurfactant producing bacteria from oil reservoirs. Iranian J. Envirn. Health Sci. Eng. 2(1): 6-12.

Tadros M.G. and Hughes J.B. (1997). Degradation of polycyclic aromatic hydrocarbons (PAHs) by indigenous mixed and pure cultures isolated from coastal sediments. Appl. Biochem. Biotechol. 865: 63-65 .

Tao S. Li X.R., Yang Y., Lu X.X., Chen H.T. and Shen W.R. (2006). Dispersion modeling of polycyclic aromatic hydrocarbons from various sources in Tianjin. China Environ. Sci. Technol. 40: 4586-4591.

Tao S., Cui Y.H., Xu F.L., Li B.G., Cao J., Liu W.X., Schmitt G., Wang X.J., Shen W.R., Qing B.P. and Sun R. (2004). Polycyclic aromatic hydrocarbons in agricultural soil and vegetables from Tianjin. Sci. Total Environ. 320: 11–24.

Tian Y., Liu H.J., Zheng T.L., Kwon K.K., Kim S.J. and Yan C.L. (2008). PAHs contamination and bacterial communities in mangrove surface sediments of the Jiulong River Estuary, China. Marine Pollut. Bullet. 57 (6-12): 707-715.

Topinka J., Marvanová S., Vondráček J., Sevastyanova O., Nováková Z., Krčmář P., Pěnčíková K. and Machala M. (2008). DNA adducts formation and induction of apoptosis in rat liver epithelial 'stem-like' cells exposed to carcinogenic polycyclic aromatic hydrocarbons. Mut. Res. Funda. Mol. Mech. Mutag. 638(1-2): 122-132.

Vaananen V., Hameila M., Kontsas H., Peltonen K. and Heikkila P. (2003). Air concentrations and urinary metabolites of polycyclic aromatic hydrocarbons among paving and remixing workers. J. Environ. Monit. 5: 739-746.

Volkering F., Breure A.M. and Van A.J.G. (1993). Effect of microorganisms on the bioavailibility and biodegradation of crystalline naphthalene. Appl. Microbiol. Biotechnol. 40:535-540.

Voutsa D. and Samara C. (1998). Dietary intake of trace elements and polycyclic aromatic hydrocarbons via vegetables grown in an industrial Greek area. Sci. Total Environ.218: 203–216.

Wagrowski D.M. and Hites R.A. (1997). Polycyclic aromatic hydrocarbon accumulation in urban, suburban, and rural vegetation. Environ. Sci. Technol. 31: 279-282.

Walter U., Beyer M., Klein J. and Rehm H.J. (1991). Degradation of pyrene by *Rhodococcus* sp. UW1. Appl Microbiol. Biotechnol. 34: 671-676.

Weinstein J.E., Sanger D.M. and Holland A.F. (2003). Bioaccumulation and toxicity of fluoranthene in the estuarine oligochaete *Monopylephorus rubroniveus*, Ecotoxicology and Environmental Safety. 55: 278–286.

Weissenfels W.D, Beyer M., Klein J. and Rehm H.J. (1991). Microbial metabolism of fluoranthene: isolation and identification of ring fission products. Appl Microbiol Biotechnol. 34: 528-535.

Wetzel D.L. and Vleet V.S.E. (2003). Persistence of petroleum hydrocarbon contamination in sediments of the canals in Venice, Italy: 1995 and 1998. Marine Pollut. Bullet. 46(8): 1015-1023.

Wilcke W (2007). Global patterns of polycyclic aromatic hydrocarbons (PAHs) in soil, Geoderma. 141:157–166.

Wild S.R., Berrow M.L., McGrath S.P. and Jones K.C. (1992). Polynuclear aromatic hydrocarbons in crops from long term sewage sludge amended field experiments. Environ. Pollut. 76: 23 -31.

Willumsen P.A. and Karlson U. (1997). Screening of bacteria isolated from PAH-contaminated soils for production of biosurfactants and bioemulsifier. Biodegrad. 7: 415-423.

Wilson S.C. and Jones K.C (1993). Bioremediation of soils contaminated with poly nuclear aromatic hydrocarbons (PAHs); a review. Environ. Pollut. 88: 229-249.

Woo H.S., Jeon O.C. and Park M.J. (2004). Phenanthrene biodegradation in soil slurry systems: influence of salicyclate and triton X-100. Korean J. Chem. Eng. 21(2): 412-418.

Xue W. and Warshawsky D. (2005). Metabolic activation of polycyclic and heterocyclic aromatic hydrocarbons and DNA damage: A review. Toxicol. Appl. Pharm. 206: 73-93.

Yolcubal I., Pierce A.S., Maier M.R. and Brusseau L.M. (2002). Biodegradation during Contaminant Transport in Porous Media V. The Influence of Growth and Cell Elution on Microbial Distribution. J. Environ. Quality. 31: 1824-1830.

Yu S.H., Ke L., Wong Y.S. and Tam N.F.Y. (2005). Degradation of polycyclic aromatic hydrocarbons by a bacterial consortium enriched from mangrove sediments. Environ. Int. 31: 149-154.

Yu K.S.H., Wong A.H.Y., Yau K.W.Y., Wong Y.S. and Tam N.F.Y. (2005). Natural attenuation, biostimulation and bioaugmentation on biodegradation of polycyclic aromatic hydrocarbons (PAHs) in mangrove sediments. Marine Pollut. Bull. 51(8-12): 1071-1077.

Yuan S.Y., Wei S.H. and Chang B.V. (2001). Biodegradation of phenanthrene in river sediment. Chemosphere. 43: 273-278.

Zhao H., Wang L., Ren J., Li Z., Li M. and Gao H. (2008). Isolation and characterization of phenanthrene-degrading strains *Sphingomonas* sp. ZP1 and *Tistrella* sp. ZP5 J. Hazardous Materials. 152(3): 1293-1300.

Zhang H.B., Luo Y.M., Wong M.H., Zhao Q.G. and Zhang G.L (2006). Distribution and concentrations of PAHs in Hong Kong soils. Environ. Pollut. 141: 107-114.

APPENDIX

PREPARATION OF NUTRIENT AGAR

Composition:

Meat extract	1g/l
Yeast extract	2g/l
Peptone	5g/l
Sodium chloride	5g/l
Agar	15g/l
Final	pH7.4+/-0.2 at 37C

28 g of Nutrient agar was weighed and dissolved in one litre of distilled water. It was boiled to dissolve completely and then sterilised by autoclaving at 121°C for 15 min and allowed to cool to 47°C.

PREPARATION OF NUTRIENT BROTH

Composition:

Peptone	15g/L
Yeast extract	3 g/L
Sodium chloride	6 g/L
D(+)-Glucose	1 g/L
Final pH	7.5+/-0.2(at 37C)

13 g of Nutrient broth was weighed and dissolved in one litre of distilled water. It was boiled and autoclaved as in the preparation of nutrient agar above.

PREPARATION OF LAURIA BERTANI BROTH

Composition:

Tryptone	10g/L
Yeast extract	5 g/L
Sodium chloride	5 g/L

Buffer A (Tris-HCl buffer, 50 mM; pH 7.5)

Mass = mole x molar mass

Mass of Tris =0.05 x 121.12

=6.0 g

6.0 g of Tris in 1 litre of distilled water, pH adjusted with 1N HCL

Buffer E (40 mM Tris and 2 mM EDTA)

Mass = mole x molar mass

Mass of Tris=0.04 x 121.12

= 4.84 g

Mass of EDTA =0.002 x 372.24

= 0.74 g

4.84 g of Tris + 0.74 g of EDTA dissolved in 1000ml of distilled water

Lysing buffer (4% SDS and 100 mM Tris)

4%= 4 g of SDS in 100ml of Distilled water + (0.1 x121.11/10) g of Tris in 100ml of Distilled water

=4g of SDS + 1.211 of Tris dissolved in 100ml of distilled water

Buffer F (3 M sodium acetate at pH 5.5)

41 g of CH_3COONa + 5.98 g 0f CH_3COOH in 1L of distilled water

CALCULATION OF ENZYME ACTIVITY

$$\textbf{Enzyme Activity} = \frac{OD/min}{\Sigma} \left[\frac{v}{V}\right] \left[\text{dilution factor}\right]$$

Where;

OD = absorbance value

v = volume of enzyme sample used

V = total volume of reagents used

Σ = extinction coefficient; for

Catechol 2,3-dioxygenase = 26,000 liters/mol · cm

Catechol 1,2-dioxygenase = 36,000 liters/mol · cm

PREPARATION OF AGAROSE GEL

0.4g of agarose was dissolved in 50 ml of TBE buffer to give a total concentration of 0.8%.The mixture was placed in a microwave for some minutes after which it was removed and allowed to cool.

PREPARATION OF BRADFORD REAGENT AND ASSAY

100 mg of coomassie brilliant blue G – 250 was dissolved in 50 ml of 95% ethanol to which 100 ml of 85% phosphoric acid has been added and the volume made up to 1 litre. The assay was carried out by adding 5 ml of coomassie brilliant blue G – 250 solution to 0.1 ml of protein solution, incubated at room temperature for 5 min and absorbance read at 595nm.

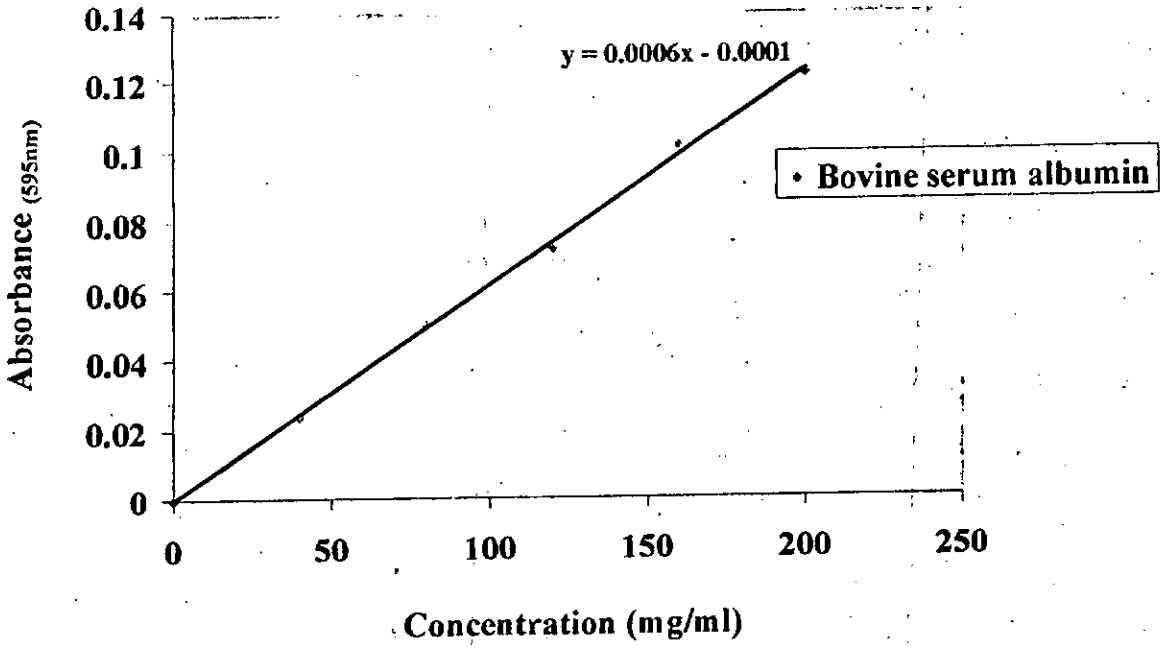


Fig. 1. Protein standard curve

Table 1. Residual Chrysene during individual isolates degradation

Day	*S. paucimobilis*	*Ps. arvilla*	*Ps. putida*	C1 (no chrysene)	C2 (chrysene+no organism)
	Residual chrysene (µg/ml)	Residual chrysene (µg/ml)	Residual chrysene (µg/ml)	Residual chrysene (µg/ml)	Residual chrysene (µg/ml)
0	100± 0.0	100±0.0	100±0.0	0.0±0.0	100±0.0
2	87.4±0.3	83.5±0.2	76.2±0.3	0.0±0.0	100±0.0
4	72.5±0.1	70.2±0.4	50.7±0.2	0.0±0.0	98.1±0.5
6	57.8±0.2	60.7±0.3	40.5±0.4	0.0±0.0	98.4±0.2
8	30.5±0.3	40.6±0.7	17.2±0.2	0.0±0.0	97.3±0.3

Table 2. Total viable count of isolates during chrysene degradation

Day	*S. paucimobilis*			*Ps. arvilla*			*Ps. putida*		
	Log TVC (E)	Log TVC (C1)	Log TVC (C2)	Log TVC (E)	Log TVC (C1)	Log TVC (C2)	Log TVC (E)	Log TVC (C1)	Log TVC (C2)
0	4.0±0.0	4.0±0.0	0.0	4.0±0.0	4.0±0.0	0.0	4.0±0.0	4.0±0.0	0.0
2	4.3±0.1	3.8±0.1	0.0	4.2±0.2	3.8±0.1	0.0	4.4±0.1	3.8±0.1	0.0
4	4.5±0.2	3.8±0.1	0.0	4.4±0.4	3.8±0.1	0.0	4.9±0.4	3.8±0.1	0.0
6	5.2±0.3	3.7±0.1	0.0	5.4±0.1	3.8±0.1	0.0	5.7±0.2	3.8±0.1	0.0
8	6.1±0.5	3.7±0.0	0.0	5.8±0.3	3.7±0.1	0.0	6.5±0.3	3.8±0.1	0.0

Table 3. Turbidity of culture media during chrysene degradation

Day	*S. paucimobilis*			*Ps. arvilla*			*Ps. Putida*		
	OD (E)	OD (C1)	OD (C2)	OD (E)	OD (C1)	OD (C2)	OD (E)	OD (C1)	OD (C2)
0	0.0018± 0.0002	0.0018± 0.0002	0.0001± 0.0001	0.0018± 0.0002	0.0018± 0.0001	0.0001± 0.0000	0.0018± 0.0001	0.0018± 0.0001	0.0001± 0.0000
2	0.2019± 0.0014	0.0015± 0.0001	0.0001± 0.0000	0.2090± 0.0059	0.0015± 0.00012	0.0001± 0.0000	0.2114± 0.0020	0.0015± 0.0001	0.0000± 0.0000
4	0.2207± 0.0012	0.0015± 0.0002	0.0001± 0.0000	0.2154± 0.0024	0.0010± 0.0004	0.0000± 0.0000	0.2270± 0.0031	0.0015± 0.0012	0.000± 0.0000
6	0.2402± 0.0012	0.0010± 0.0001	0.0000± 0.0000	0.2248± 0.0057	0.0007± 0.0002	0.0000± 0.0000	0.2551± 0.0042	0.00010 ± 0.0010	0.0000± 0.0000
8	0.2715± 0.0014	0.0007± 0.0002	0.0000± 0.0000	0.2540± 0.0045	0.0007± 0.0002	0.0000± 0.0000	0.3182± 0.0038	0.0007± 0.0002	0.0000± 0.0000

Table 4. pH change of culture media during chrysene degradation

Day	*S. paucimobilis*			*Ps. arvilla*			*Ps. putida*		
	pH (E)	pH (C1)	pH (C2)	pH (E)	pH (C1)	pH (C2)	pH (E)	pH (C1)	pH (C2)
0	7.2±0.0	7.2±0.0	7.2±0.0	7.2±0.0	7.2±0.0	7.2±0.0	7.2±0.0	7.2±0.0	7.2±0.0
2	7.1±0.1	7.2±0.0	7.2±0.0	7.1±0.1	7.2±0.0	7.2±0.0	7.0±0.1	7.2±0.0	7.2±0.0
4	7.1±0.1	7.2±0.0	7.2±0.0	6.9±0.1	7.2±0.0	7.2±0.0	6.9±0.2	7.2±0.0	7.2±0.0
6	6.9±0.2	7.2±0.0	7.2±0.0	6.8±0.1	7.2±0.0	7.2±0.0	7.0±0.1	7.2±0.0	7.2±0.0
8	6.8±0.1	7.2±0.0	7.2±0.0	6.8±0.1	7.2±0.0	7.2±0.0	7.3±0.1	7.2±0.0	7.2±0.0

Table 5. Residual fluoranthene during individual isolates degradation

Day	*S. paucimobilis*	*Ps. arvilla*	*Ps. putida*	Control C1 (no fluoranthene)	Control C2 (fluoranthene + no organism)
	Residual fluoranthene (µg/ml)	Residual fluoranthene (µg/ml)	Residual fluoranthene (µg/ml)	Residual fluoranthene (µg/ml)	Residual fluoranthene (µg/ml)
0	100± 0.0	100±0.0	100±0.0	0.0±0.0	100±0.1
2	66.5±0.1	60.5±0.2	55.8±0.3	0.0±0.0	100±0.0
4	32.2±0.4	20.1±0.8	16.5±0.6	0.0±0.0	98.1±0.4
6	11.8±0.3	11.8±0.6	10.5±0.2	0.0±0.0	98.4±0.2
8	2.0±0.1	2.0±0.4	0.02±0.07	0.0±0.0	97.3±0.1

Table 6. Total viable count of isolates during fluoranthene degradation

Day	*S. paucimobilis*			*Ps. arvilla*			*Ps. putida*		
	Log TVC (E)	Log TVC (C1)	Log TVC (C2)	Log TVC (E)	Log TVC (C1)	Log TVC (C2)	Log TVC (E)	Log TVC (C1)	Log TVC (C2)
0	4.0±0.0	4.0±0.0	0.0	4.0±0.0	4.0±0.0	0.0	4.0±0.0	4.0±0.0	0.0
2	6.1±0.2	3.8±0.1	0.0	5.2±0.3	3.8±0.1	0.0	6.7±0.4	3.9±0.1	0.0
4	7.5±0.6	3.8±0.1	0.0	6.4±0.5	3.8±0.1	0.0	7.2±0.1	3.8±0.1	0.0
6	8.2±0.4	3.7±0.1	0.0	7.6±0.7	3.8±0.1	0.0	8.8±0.5	3.8±0.1	0.0
8	9.4±0.7	3.7±0.4	0.0	8.4±0.4	3.7±0.1	0.0	9.9±0.3	3.8±0.1	0.0

Table 7. Turbidity of culture media during fluoranthene degradation

Day	*S. paucimobilis*			*Ps. arvilla*			*Ps. putida*		
	OD (E)	OD (C1)	OD (C2)	OD (E)	OD (C1)	OD (C2)	OD (E)	OD (C1)	OD (C2)
0	0.0018± 0.0002	0.0018± 0.0002	0.0001± 0.0001	0.0018± 0.0002	0.0018± 0.0001	0.0001± 0.0000	0.0018± 0.0002	0.0018± 0.0001	0.0001± 0.0000
2	0.2120± 0.0064	0.0015± 0.0001	0.0001± 0.0000	0.2135± 0.0145	0.0015± 0.00012	0.0001± 0.0000	0.2437± 0.0245	0.0015± 0.0001	0.0000± 0.0000
4	0.2575± 0.0045	0.0015± 0.0002	0.0001± 0.0000	0.243± 0.0500	0.0010± 0.0004	0.0000± 0.0000	0.2727± 0.0812	0.0015± 0.0012	0.000± 0.0000
6	0.2761± 0.0018	0.0010± 0.0001	0.0000± 0.0000	0.2982± 0.0411	0.0007± 0.0002	0.0000± 0.0000	0.3108± 0.0200	0.00010 ± 0.0010	0.0000± 0.0000
8	0.3584± 0.0012	0.0007± 0.0002	0.0000± 0.0000	0.2520± 0.0042	0.0007± 0.0002	0.0000± 0.0000	0.3804± 0.0012	0.0007± 0.0012	0.0000± 0.0000

Table 8. pH change of culture media during fluoranthene degradation

Day	*S. paucimobilis*			*Ps. arvilla*			*Ps. putida*		
	pH (E)	pH (C1)	pH (C2)	pH (E)	pH (C1)	pH (C2)	pH (E)	pH (C1)	pH (C2)
0	7.2±0.0	7.2±0.0	7.2±0.0	7.2±0.0	7.2±0.0	7.2±0.0	7.2±0.0	7.2±0.0	7.2±0.0
2	7.0±0.1	7.2±0.0	7.2±0.0	7.0±0.1	7.2±0.0	7.2±0.0	7.0±0.1	7.2±0.0	7.2±0.0
4	6.8±0.1	7.2±0.0	7.2±0.0	6.8±0.2	7.2±0.0	7.2±0.0	7.3±0.2	7.2±0.0	7.2±0.0
6	7.5±0.2	7.2±0.0	7.2±0.0	7.5±0.1	7.2±0.0	7.2±0.0	7.5±0.2	7.2±0.0	7.2±0.0
8	7.7±0.2	7.2±0.0	7.2±0.0	7.7±0.2	7.2±0.0	7.2±0.0	8.0±0.2	7.2±0.0	7.2±0.0

Table 9. Residual pyrene during individual isolates degradation

	S. paucimobilis	*Ps. arvilla*	*Ps. putida*	Control C1 (no pyrene)	Control C2 (pyrene + no organism)
Day	Residual pyrene (µg/ml)	Residual pyrene (µg/ml)	Residual pyrene (µg/ml)	Residual pyrene (µg/ml)	Residual pyrene (µg/ml)
0	100± 0.0	100±0.0	100±0.0	0.0±0.0	100±0.1
2	66.2±0.1	78.5±0.2	60.3±0.2	0.0±0.0	100±0.0
4	41.4±0.1	53.1±0.3	52.3±0.4	0.0±0.0	98.1±0.1
6	10.3±0.3	15.5±0.6	15.4±0.8	0.0±0.0	98.4±0.2
8	0.06±0.2	6.5±0.6	6.6±0.4	0.0±0.0	97.3±0.1

Table 10. Total viable count of isolates during pyrene degradation

Day	*S. paucimobilis*			*Ps. arvilla*			*Ps. putida*		
	Log TVC (E)	Log TVC (C1)	Log TVC (C2)	Log TVC (E)	Log TVC (C1)	Log TVC (C2)	Log TVC (E)	Log TVC (C1)	Log TVC (C2)
0	4.0±0.0	4.0±0.0	0.0	4.0±0.0	4.0±0.0	0.0	4.0±0.0	4.0±0.0	0.0
2	4.2±0.1	3.8±0.1	0.0	4.1±0.2	3.8±0.1	0.0	4.2±0.3	3.9±0.1	0.0
4	5.2±0.3	3.8±0.1	0.0	4.7±0.2	3.8±0.1	0.0	5.3±0.4	3.8±0.1	0.0
6	6.1±0.4	3.7±0.1	0.0	5.2±0.3	3.8±0.1	0.0	6.0±0.3	3.8±0.1	0.0
8	6.8±0.1	3.7±0.4	0.0	5.8±0.3	3.7±0.1	0.0	6.8±0.2	3.8±0.1	0.0

Table 11. Turbidity of culture media during individual isolates pyrene degradation

Day	*S. paucimobilis*			*Ps. arvilla*			*Ps. putida*		
	OD (E)	OD (C1)	OD (C2)	OD (E)	OD (C1)	OD (C2)	OD (E)	OD (C1)	OD (C2)
0	0.0018 ±0001	0.0018± 0.0002	0.0001± 0.0001	0.0018± 0.0001	0.0018± 0.0001	0.0001± 0.0000	0.00018 ±0.0001	0.0018± 0.0001	0.0001± 0.0000
2	0.2019± 0.0015	0.0015± 0.0001	0.0001± 0.0000	0.2014± 0.0023	0.0015± 0.00012	0.0001± 0.0000	0.2027± 0.0012	0.0015± 0.0001	0.0000± 0.0000
4	0.2145± 0.0022	0.0015± 0.0002	0.0001± 0.0000	0.2009± 0.0042	0.0010± 0.0004	0.0000± 0.0000	0.2215± 0.0041	0.0015± 0.0012	0.000± 0.0000
6	0.2570± 0.0024	0.0010± 0.0001	0.0000± 0.0000	0.2417± 0.0028	0.0007± 0.0002	0.0000± 0.0000	0.2364± 0.0018	0.00010 ± 0.0010	0.0000± 0.0000
8	0.3480± 0.0053	0.0007± 0.0002	0.0000± 0.0000	0.2546± 0.0036	0.0007± 0.0002	0.0000± 0.0000	0.2741± 0.0068	0.0007± 0.0002	0.0000± 0.0000

Table 12. pH change of culture media during pyrene degradation

Day	*S. paucimobilis*			*Ps. arvilla*			*Ps. putida*		
	pH (E)	pH (C1)	pH (C2)	pH (E)	pH (C1)	pH (C2)	pH (E)	pH (C1)	pH (C2)
0	7.2±0.0	7.2±0.0	7.2±0.0	7.2±0.0	7.2±0.0	7.2±0.0	7.2±0.0	7.2±0.0	7.2±0.0
2	7.1±0.0	7.2±0.0	7.2±0.0	7.1±0.0	7.2±0.0	7.2±0.0	7.0±0.1	7.2±0.0	7.2±0.0
4	6.9±0.1	7.2±0.0	7.2±0.0	6.9±0.1	7.2±0.0	7.2±0.0	6.9±0.1	7.2±0.0	7.2±0.0
6	7.5±0.1	7.2±0.0	7.2±0.0	7.3±0.1	7.2±0.0	7.2±0.0	7.3±0.2	7.2±0.0	7.2±0.0
8	7.9±0.2	7.2±0.0	7.2±0.0	7.5±0.1	7.2±0.0	7.2±0.0	7.5±0.2	7.2±0.0	7.2±0.0

Table 13. Residual HMW PAHs during consortium degradation

Day	Consortium	Consortium	Consortium
	Residual chrysene (µg/ml)	Residual fluoranthene (µg/ml)	Residual pyrene (µg/ml)
0	100±0.0	100±0.0	100±0.0
2	76.5±0.5	75.4±0.4	68.2±0.5
4	50.7±0.4	40.5±0.2	60.1±0.3
6	32.1±0.9	6.5±0.3	30.4±0.6
8	21.3±0.2	2.2±0.8	10.6±0.8

Table 14. Total viable count of consortium during respective HMW PAH degradation

Day	Chrysene			Fluoranthene			Pyrene		
	Log TVC (E)	Log TVC (C1)	Log TVC (C2)	Log TVC (E)	Log TVC (C1)	Log TVC (C2)	Log TVC (E)	Log TVC (C1)	Log TVC (C2)
0	4.0±0.0	4.0±0.0	0.0	4.0±0.0	4.0±0.0	0.0	4.0±0.0	4.0±0.0	0.0
2	4.1±0.1	3.8±0.1	0.0	4.2±0.1	3.8±0.1	0.0	4.1±0.3	3.8±0.1	0.0
4	4.3±0.2	3.8±0.1	0.0	4.4±0.3	3.8±0.1	0.0	4.3±0.2	3.8±0.1	0.0
6	4.4±0.4	3.7±0.1	0.0	6.2±0.4	3.8±0.1	0.0	4.5±0.1	3.8±0.1	0.0
8	4.9±0.3	3.7±0.0	0.0	6.8±0.2	3.7±0.1	0.0	5.4±0.4	3.8±0.1	0.0

Table 15. Turbidity of culture media during consortium degradation of respective HMW PAHs

Day	Chrysene			Fluoranthene			Pyrene		
	OD (E)	OD (C1)	OD (C2)	OD (E)	OD (C1)	OD (C2)	OD (E)	OD (C1)	OD (C2)
0	0.0018± 0.0001	0.0018± 0.0002	0.0001± 0.0001	0.0018± 0.0001	0.0018± 0.0001	0.0001± 0.0000	0.00018 ±0.0001	0.0018± 0.0001	0.0001± 0.0000
2	0.2019± 0.0015	0.0015± 0.0001	0.0001± 0.0000	0.2014± 0.0023	0.0015± 0.00012	0.0001± 0.0000	0.2027± 0.0012	0.0015± 0.0001	0.0000± 0.0000
4	0.2144± 0.0022	0.0015± 0.0002	0.0001± 0.0000	0.2053± 0.0042	0.0010± 0.0004	0.0000± 0.0000	0.2210± 0.0041	0.0015± 0.0012	0.000± 0.0000
6	0.2072± 0.0016	0.0010± 0.0001	0.0000± 0.0000	0.5507± 0.0008	0.0007± 0.0002	0.0000± 0.0000	0.2209± 0.0015	0.00010 ± 0.0010	0.0000± 0.0000
8	0.3584± 0.0084	0.0007± 0.0002	0.0000± 0.0000	0.5921± 0.0014	0.0007± 0.0002	0.0000± 0.0000	0.4253± 0.0002	0.0007± 0.0002	0.0000± 0.0000

Table 16. pH change of culture media during consortium degradation of respective HMW PAHs

Day	Chrysene			Fluoranthene			Pyrene		
	pH (E)	pH (C1)	pH (C2)	pH (E)	pH (C1)	pH (C2)	pH (E)	pH (C1)	pH (C2)
0	7.2±0.0	7.2±0.0	7.2±0.0	7.2±0.2	7.2±0.0	7.2±0.0	7.2±0.0	7.2±0.0	7.2±0.0
2	7.1±0.1	7.2±0.0	7.2±0.0	7.1±0.1	7.2±0.0	7.2±0.0	7.1±0.2	7.2±0.0	7.2±0.0
4	6.9±0.2	7.2±0.0	7.2±0.0	6.9±0.0	7.2±0.0	7.2±0.0	6.9±0.1	7.2±0.0	7.2±0.0
6	6.8±0.1	7.2±0.0	7.2±0.0	7.5±0.1	7.2±0.0	7.2±0.0	6.8±0.1	7.2±0.0	7.2±0.0
8	7.3±0.1	7.2±0.0	7.2±0.0	7.7±0.1	7.2±0.0	7.2±0.0	7.5±0.2	7.2±0.0	7.2±0.0

Table 17. Residual chrysene during consortium degradation in the presence of phenanthrene

Day	consortium	consortium	C 1	C 2
	Residual chrysene (µg/ml)	Residual phenanthrene (µg/ml)	Residual chrysene (µg/ml)	Residual chrysene (µg/ml)
0	100±0.0	100±0.0	0.0	100±0.0
2	92.5±0.2	40.2±0.1	0.0	98.1±0.3
4	90.4±0.5	19.3±0.2	0.0	99.4±0.2
6	60.7±0.4	0.0±0.00	0.0	97.5±0.1
8	12.4±0.1	0.0±0.00	0.0	98.6±0.2

Table 18. Total viable count of isolates during chrysene degradation by consortium in the presence of phenanthrene

Day	Consortium	Control	
	Log TVC (E)	Log TVC (C1)	Log TVC (C2)
0	4.0±0.0	4.0±0.0	0.0
2	4.7±0.4	3.8±0.1	0.0
4	4.8±0.2	3.8±0.1	0.0
6	6.1±0.3	3.7±0.1	0.0
8	6.4±0.1	3.7±0.4	0.0

Table 19. Turbidity of culture media during consortium degradation of chrysene in the presence of phenanthrene

Day	Consortium	Control	
	OD (E)	OD (C1)	OD (C2)
0	0.0018±0.0002	0.0016± 0.0002	0.0000± 0.0000
2	0.2320±0.0052	0.0015± 0.0001	0.0000± 0.0000
4	0.3473±0.0028	0.0015± 0.0002	0.0000± 0.0000
6	0.5671±0.0025	0.0010± 0.0001	0.0000± 0.0000
8	0.5972±0.0037	0.0007± 0.0002	0.0000± 0.0000

Table 20. **pH change of culture media during consortium degradation of chrysene in the presence of phenanthrene**

Day	Consortium	Control	
	pH (E)	pH (C1)	pH (C2)
0	7.2±0.0	7.2±0.0	7.2±0.0
2	7.1±0.1	7.2±0.0	7.2±0.0
4	7.3±0.1	7.2±0.0	7.2±0.0
6	7.4±0.2	7.2±0.0	7.2±0.0
8	7.5±0.1	7.2±0.0	7.2±0.0

Table 21. **Residual fluoranthene during consortium degradation in the presence of phenanthrene**

Day	consortium	consortium	C 1	C 2
	Residual fluoranthene (µg/ml)	Residual phenanthrene (µg/ml)	Residual fluoranthene (µg/ml)	Residual fluoranthene (µg/ml)
0	100±0.0	100±0.0	0.0	100±0.0
2	90.7±0.2	36.2±0.5	0.0	98.1±0.3
4	60.2±0.4	5.3±0.6	0.0	99.4±0.2
6	20.4±0.7	0.0±0.0	0.0	97.5±0.1
8	0.2±0.3	0.0±0.0	0.0	98.6±0.2

Table 22. Total viable count of isolates during fluoranthene in the presence of phenanthrene

Day	Consortium	Control	
	Log TVC (E)	Log TVC (C1)	Log TVC (C2)
0	4.0±0.0	4.0±0.0	0.0
2	6.7±0.3	3.8±0.1	0.0
4	8.5±0.6	3.7±0.2	0.0
6	10.2±0.4	3.0±0.1	0.0
8	11.4±0.3	2.8±0.1	0.0

Table 23. Turbidity of culture media during consortium degradation of fluoranthene in the presence of phenanthrene

Day	Consortium	Control	
	OD (E)	OD (C1)	OD (C2)
0	0.0018±0.0002	0.0016± 0.0002	0.0001± 0.0001
2	0.536±0.00035	0.0015± 0.0001	0.0001± 0.0000
4	0.841±0.0044	0.0015± 0.0002	0.0001± 0.0000
6	1.197±0.0053	0.0010± 0.0001	0.0000± 0.0000
8	1.230±0.0076	0.0007± 0.0002	0.0000± 0.0000

Table 24. pH change of culture media during consortium degradation of fluoranthene in the presence of phenanthrene

Day	Consortium	Control	
	pH (E)	pH (C1)	pH (C2)
0	7.2±0.0	7.2±0.0	7.2±0.0
2	7.5±0.2	7.2±0.0	7.2±0.0
4	7.6±0.1	7.2±0.0	7.2±0.0
6	7.7±0.1	7.2±0.0	7.2±0.0
8	7.9±0.2	7.2±0.0	7.2±0.0

Table 25. Residual pyrene during consortium degradation in the presence of phenanthrene

Day	consortium	consortium	C 1	C 2
	Residual pyrene (µg/ml)	Residual phenanthrene (µg/ml)	Residual pyrene (µg/ml)	Residual pyrene (µg/ml)
0	100±0.0	100±0.0	0.0	100±0.0
2	60.1±0.3	33.2±0.4	0.0	99.5±0.3
4	40.3±0.5	25.3±0.6	0.0	97.3±0.6
6	17.3±0.4	0.015±0.001	0.0	98.4±0.1
8	0.7±0.2	0.000±0.00	0.0	99.5±0.4

Table 26. Total viable count of isolates during pyrene in the presence of phenanthrene

Day	Consortium	Control	
	Log TVC (E)	Log TVC (C1)	Log TVC (C2)
0	4.0±0.0	4.0±0.0	0.0
2	5.2±0.1	3.8±0.1	0.0
4	6.4±0.3	3.7±0.2	0.0
6	8.4±0.1	3.0±0.1	0.0
8	9.8±0.2	2.8±0.1	0.0

Table 27. Turbidity of culture media during consortium degradation of pyrene in the presence of phenanthrene

Day	Consortium	Control	
	OD (E)	OD (C1)	OD (C2)
0	0.0018±0.0002	0.0016± 0.0002	0.0001± 0.0001
2	0.4523±0.0056	0.0015± 0.0001	0.0001± 0.0000
4	0.7515±0.0028	0.0015± 0.0002	0.0001± 0.0000
6	0.9910±0.0059	0.0010± 0.0001	0.0000± 0.0000
8	1.1478±0.0029	0.0007± 0.0002	0.0000± 0.0000

Table 28. pH change of culture media during consortium degradation of pyrene in the presence of phenanthrene

Day	*Consortium*	*Control*	
	pH (E)	pH (C1)	pH (C2)
0	7.2±0.0	7.2±0.0	7.2±0.0
2	7.3±0.1	7.2±0.0	7.2±0.0
4	7.5±0.2	7.2±0.0	7.2±0.0
6	7.6±0.2	7.2±0.0	7.2±0.0
8	7.9±0.1	7.2±0.0	7.2±0.0

Table 29. Residual HMW PAH during individual isolates degradation of mixture of HMW PAHs

Day	*S. paucimobilis*			*Ps. arvilla*			*Ps. putida*		
	CHY	FLU	PYR	CHY	FLU	PYR	CHY	FLU	PYR
0	100±0.0	100±0.0	100±0.0	100±0.0	100±0.0	100±0.0	100±0.0	100±0.0	100±0.0
2	90.4±0.3	94.2±0.2	97.5±0.2	93.3±0.4	95.5±0.2	90.2±0.3	88.2±0.3	80.3±0.2	74.6±0.3
4	89.2±0.3	72.1±0.5	80.1±0.1	90.1±0.5	90.1±0.3	90.2±0.1	76.0±0.3	70.4±0.2	65.2±0.3
6	64.1±0.2	62.3±0.4	60.2±0.5	64.2±0.4	62.0±0.5	60.1±0.5	60.4±0.6	50.1±0.3	57.1±0.2
8	40.2±0.4	32.5±0.3	37.5±0.2	40.3±0.2	35.4±0.2	34.2±0.4	27.4±0.8	10.1±0.5	32.0±0.2

Table 30. Total viable count of individual isolates during mixture of HMW PAHs degradation

	S. paucimobilis			*Ps. arvilla*			*Ps. Putida*		
Day	**Log TVC (E)**	**Log TVC (C1)**	**Log TVC (C2)**	**Log TVC (E)**	**Log TVC (C1)**	**Log TVC (C2)**	**Log TVC (E)**	**Log TVC (C1)**	**Log TVC (C2)**
0	4.0±0.2	4.0±0.0	0.0	4.0±0.2	4.0±0.0	0.0	4.0±0.2	4.0±0.0	0.0
2	4.1±0.1	3.8±0.1	0.0	4.1±0.3	3.8±0.1	0.0	4.2±0.1	3.8±0.1	0.0
4	4.2±0.1	3.7±0.2	0.0	4.3±0.2	3.7±0.2	0.0	4.4±0.4	3.7±0.2	0.0
6	4.3±0.2	3.0±0.1	0.0	4.4±0.4	3.0±0.1	0.0	4.6±0.2	3.0±0.1	0.0
8	4.5±0.1	2.8±0.1	0.0	4.6±0.2	2.8±0.1	0.0	4.7±0.3	2.8±0.1	0.0

Table 31. Turbidity of culture media during individual isolates degradation of mixture of HMW PAHs

Day	*S. paucimobilis*			*Ps. arvilla*			*Ps. Putida*		
	OD (E)	**OD (C1)**	**OD (C2)**	**OD (E)**	**OD (C1)**	**OD (C2)**	**OD (E)**	**OD (C1)**	**OD (C2)**
0	0.0018± 0.0015	0.0018± 0.0002	0.0001± 0.0001	0.0018± 0.0001	0.0018± 0.0002	0.0001± 0.0001	0.0018±0 .0001	0.0018 0.0002	0.0000± 0.0000
2	0.0105± 0.0044	0.0015± 0.0001	0.0001± 0.0000	0.0182± 0.0034	0.0015± 0.0001	0.0001± 0.0000	0.0191± 0.0015	0.0015± 0.0001	0.0001± 0.0000
4	0.1804± 0.0057	0.0015± 0.0002	0.0001± 0.0000	0.2106± 0.0055	0.0015± 0.0002	0.0001± 0.0000	0.2127± 0.0028	0.0015± 0.0002	0.0001± 0.0000
6	0.2119± 0.0037	0.0010± 0.0001	0.0000± 0.0000	0.2319± 0.0017	0.0010± 0.0001	0.0000± 0.0000	0.2425± 0.0016	0.0010± 0.0001	0.0000± 0.0000
8	0.2254± 0.0063	0.0007± 0.0002	0.0000± 0.0000	0.2447± 0.0029	0.0007± 0.0002	0.0000± 0.0000	0.2658± 0.0038	0.0007± 0.0002	0.0001± 0.0000

Table 32. pH change of culture media during individual isolates degradation of mixture of HMW PAHs

	S. paucimobilis			*Ps. arvilla*			*Ps. Putida*		
Day	**pH (E)**	**pH (C1)**	**pH (C2)**	**pH (E)**	**pH (C1)**	**pH (C2)**	**pH (E)**	**pH (C1)**	**pH (C2)**
0	7.2±0.0	7.2±0.0	7.2±0.0	7.2±0.0	7.2±0.0	7.2±0.0	7.2±0.0	7.2±0.0	7.2±0.0
2	7.2±0.0	7.2±0.0	7.2±0.0	7.1±0.1	7.2±0.0	7.2±0.0	7.1±0.1	7.2±0.0	7.2±0.0
4	7.1±0.1	7.2±0.0	7.2±0.0	7.0±0.1	7.2±0.0	7.2±0.0	7.0±0.1	7.2±0.0	7.2±0.0
6	7.0±0.1	7.2±0.0	7.2±0.0	7.0±0.1	7.2±0.0	7.2±0.0	6.8±0.1	7.2±0.0	7.2±0.0
8	6.9±0.1	7.2±0.0	7.2±0.0	6.9±0.1	7.2±0.0	7.2±0.0	6.9±0.1	7.2±0.0	7.2±0.0

Table 33. Residual HMW PAHs during consortium degradation of mixture of HMW PAHs

Day	**Consortium**		
	CHY	**FLU**	**PYR**
0	100±0.0	100±0.0	100±0.0
2	88.1±0.4	88.1±0.3	83.5±0.3
4	52.4±0.3	50.6±0.1	45.1±0.4
6	20.1±0.2	10.5±0.3	11.5±0.3
8	11.5±0.4	6.2±0.3	6.0±0.8

Table 34. **Total viable count of consortium during mixture of HMW PAHs degradation by consortium**

Day	Consortium	Control	
	Log TVC (E)	Log TVC (C1)	Log TVC (C2)
0	4.0±0.0	4.0±0.0	0.0
2	4.2±0.1	3.8±0.1	0 0
4	4.5±0.3	3.7±0.2	0.0
6	4.7±0.2	3.0±0.1	0.0
8	5.3±0.3	2.8±0.1	0.0

Table 35. **Turbidity of culture media during consortium degradation of mixture of HMW PAHs**

Day	Consortium	Control	
	OD (E)	OD (C1)	OD (C2)
0	0.0018±0.0002	0.0018±0.0002	0.0000±0.0000
2	0.1934±0.0013	0.0015±0.00001	0.0000±0.0000
4	0.2469±0.0014	0.0014±0.0001	0.0000±0.0000
6	0.2756±0.0022	0.0010±0.0001	0.0000±0.0000
8	0.3348±0.0015	0.0007±0.0001	0.0000±0.0000

Table 36. pH change of culture media during consortium degradation of mixture of HMW PAHs

Day	Consortium	Control	
	pH (E)	pH (C1)	pH (C2)
0	7.2±0.0	7.2±0.0	7.2±0.0
2	7.1±0.1	7.2±0.0	7.2±0.0
4	7.0±0.1	7.2±0.0	7.2±0.0
6	7.3±0.1	7.2±0.0	7.2±0.0
8	7.5±0.1	7.2±0.0	7.2±0.0

Table 37. Residual HMW PAH after mixture degradation by consortium in the presence of phenanthrene

Day	Consortium + PHE			
	CHY	FLU	PYR	PHE
0	100±0.0	100±0.0	100±0.0	100±0.0
2	84.2±0.3	81.2±0.3	72.4±0.2	20.2±0.2
4	50.1±0.5	40.5±0.1	42.2±0.4	0.82±0.1
6	10.2±0.1	0.4±0.3	6.5±0.20	0.000±0.2
8	0.45±0.03	0.02±0.02	0.2±0.04	0.000±0.00

Table 38. Total viable count of consortium during mixture of HMW PAHs degradation by consortium in the presence of phenanthrene

Day	Consortium	Control	
	Log TVC (E)	Log TVC (C1)	Log TVC (C2)
0	4.0±0.0	4.0±0.0	0.0
2	6.2±0.3	3.8±0.1	0.0
4	10.3±0.5	3.7±0.2	0.0
6	10.7±0.8	3.0±0.1	0.0
8	12.1±0.2	2.8±0.1	0.0

Table 39. Turbidity of culture media during mixture of HMW PAHs degradation by consortium in the presence of phenanthrene

Day	Consortium	Control	
	OD (E)	OD (C1)	OD (C2)
0	0.0018±0.0002	0.0016± 0.0002	0.0001± 0.0001
2	0.5109±0.0004	0.0015± 0.0001	0.0001± 0.0000
4	1.204±0.0003	0.0015± 0.0002	0.0001± 0.0000
6	1.257±0.0003	0.0010± 0.0001	0.0000± 0.0000
8	1.522±0.0015	0.0007± 0.0002	0.0000± 0.0000

Table 40. **pH of culture media during mixture of HMW PAHs degradation by consortium in the presence of phenanthrene**

Day	Consortium	Control	
	Log TVC (E)	Log TVC (C1)	Log TVC (C2)
0	7.2±0.0	7.2±0.0	7.2±0.0
2	7.5±0.1	7.2±0.0	7.2±0.0
4	7.8±0.2	7.2±0.0	7.2±0.0
6	7.9±0.1	7.2±0.0	7.2±0.0
8	8.1±0.1	7.2±0.0	7.2±0.0

Table 41. **Summary of residual HMW PAHs after 8 days of degradation using the individual isolates**

	Individual HMW PAHs			Mixture of HMW PAHs		
	S. paucimobilis	*Ps. arvilla*	*Ps. putida*	*S. paucimobilis*	*Ps. arvilla*	*Ps. putida*
Chrysene	**30.5 ± 0.3**	**40.6 ± 0.7**	**17.2 ± 0.2**	**40.2 ± 0.4**	**40.3 ± 0.2**	**27.4 ± 0.8**
Fluoranthene	**2.0 ± 0.1**	**2.0 ± 0.4**	**0.02 ± 0.07**	**32.5 ± 0.3**	**35.4 ± 0.2**	**34.2 ± 0.5**
Pyrene	**0.06 ± 0.2**	**6.5 ± 0.6**	**6.6 ± 0.4**	**37.5 ± 0.2**	**34.2 ± 0.4**	**32.0 ± 0.2**

Table 42. Summary of residual HMW PAHs after 8 days of degradation using consortium of the isolates

PAHs	Individual PAH	Individual PAH + phenanthrene	Mixture of PAHs	Mixture of PAHs + phenanthrene
Chrysene	21.3 ± 0.2	12.4 ± 0.1	11.5 ± 0.4	0.45 ± 0.03
Fluoranthene	2.2 ± 0.8	0.2± 0.3	6.2 ± 0.3	0.02 ± 0.02
Pyrene	10.6 ± 0.8	0.7 ± 0.2	6.0 ± 0.8	0.2 ± 0.04
phenanthrene	-	-	-	-

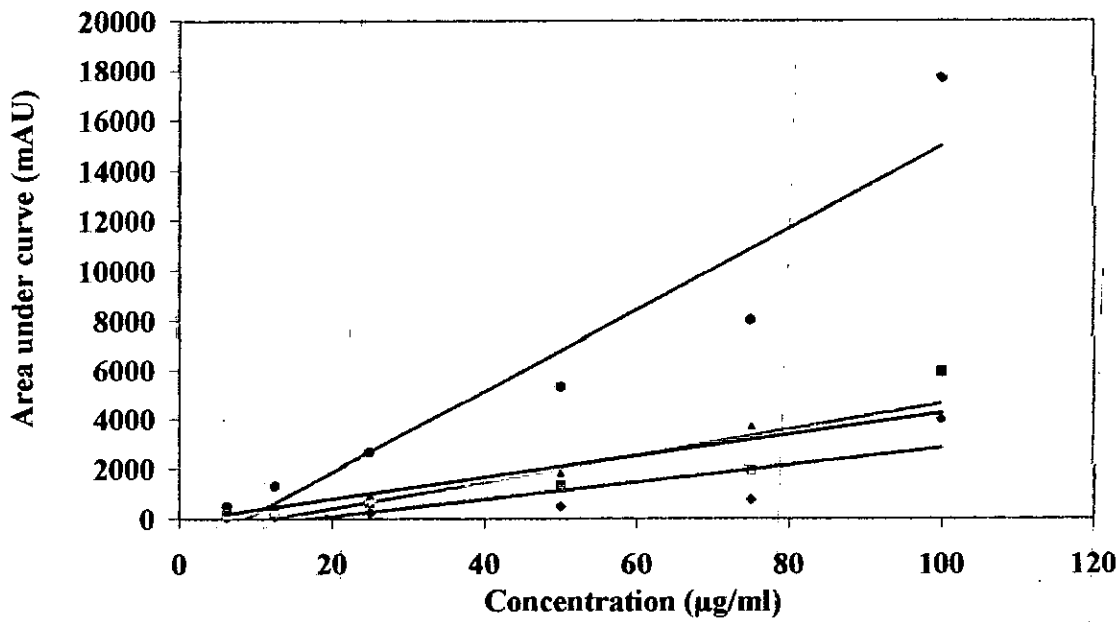


Fig. 2. Standard curve of chrysene, fluoranthene, pyrene and phenathrene

◆ Chrysene ■ Fluoranthene ▲ Pyrene ● Phenanthrene

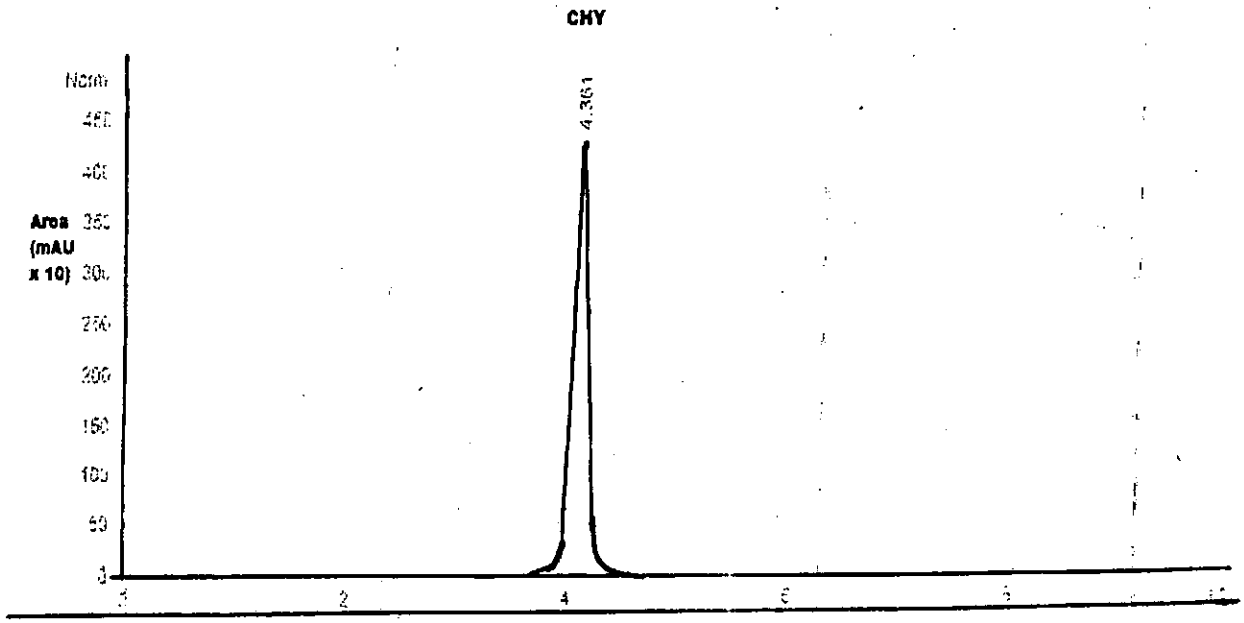


Fig. 3. Chromatogram of 100 µg/ml chrysene

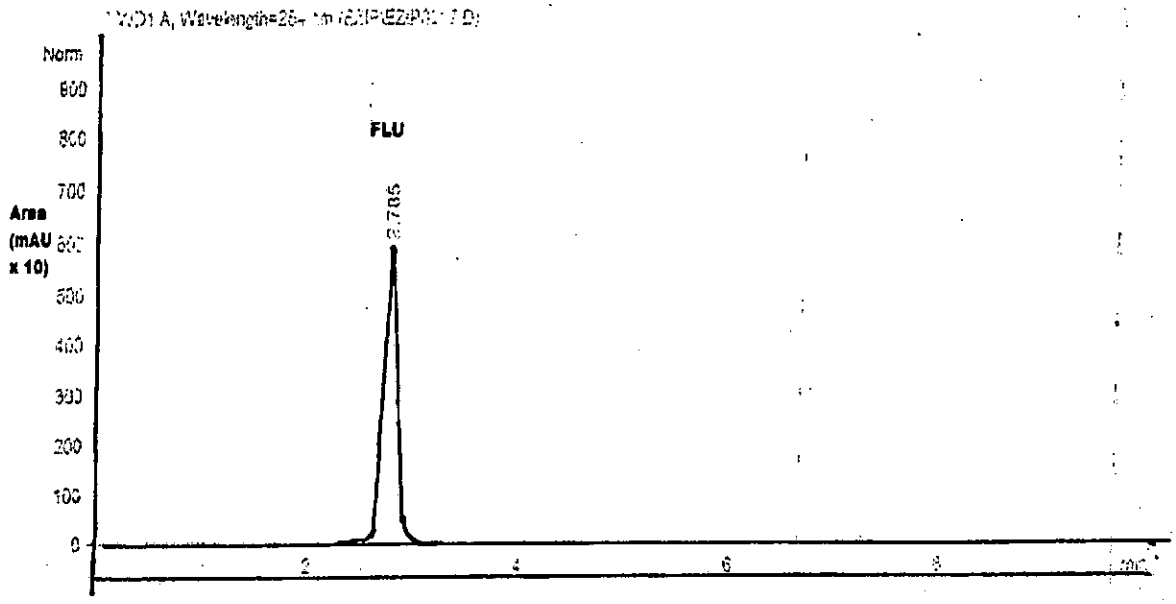


Fig. 4. Chromatogram of 100 µg/ml fluoranthene

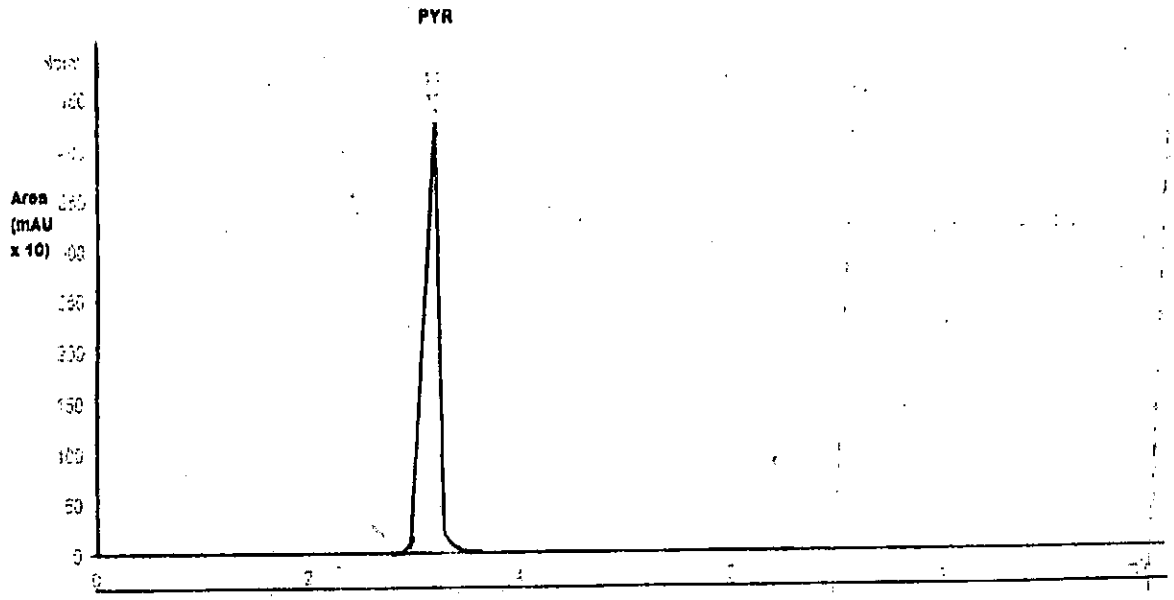


Fig. 5. Chromatogram of 100 µg/ml pyrene

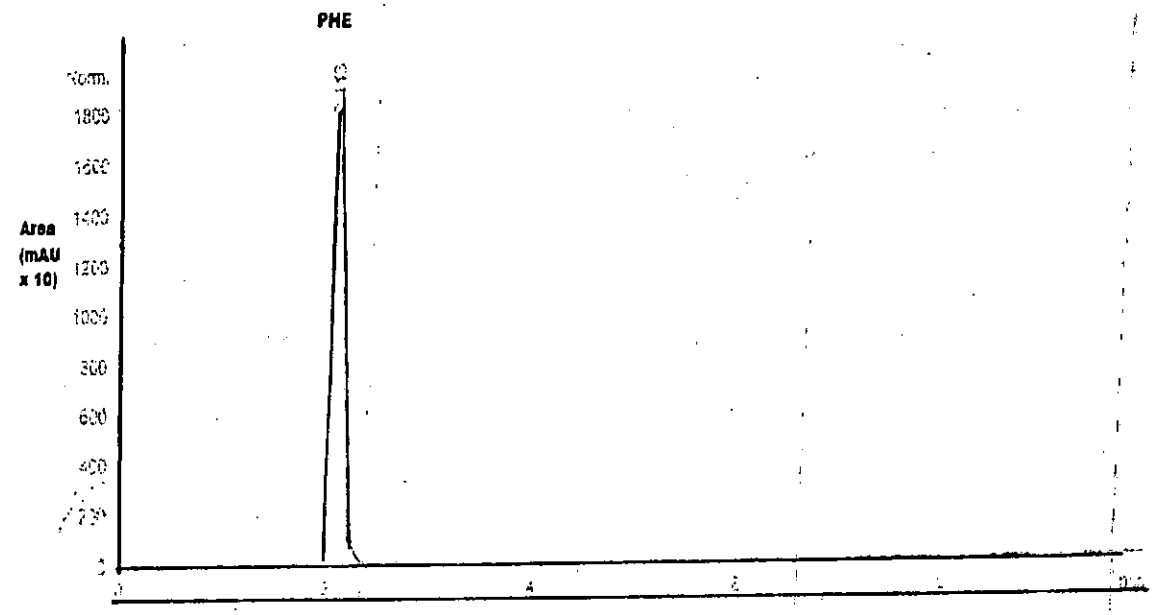


Fig. 6. Chromatogram of 100 µg/ml phenanthrene

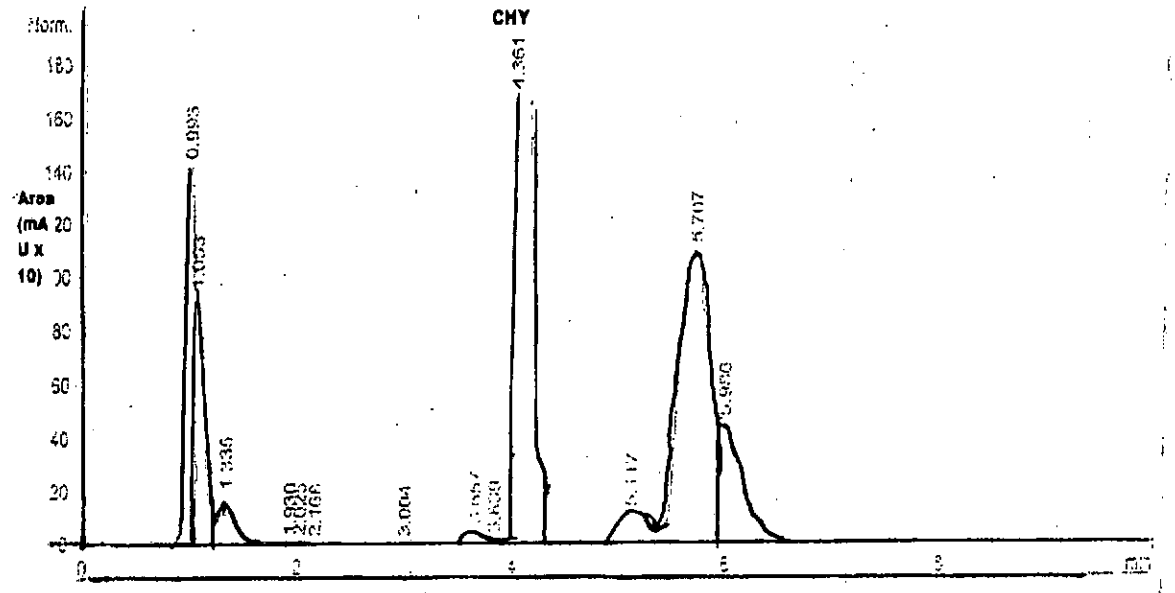


Fig. 7. Chromatogram of chrysene after 8 days degradation by *Ps. putida* (CHY = chrysene)

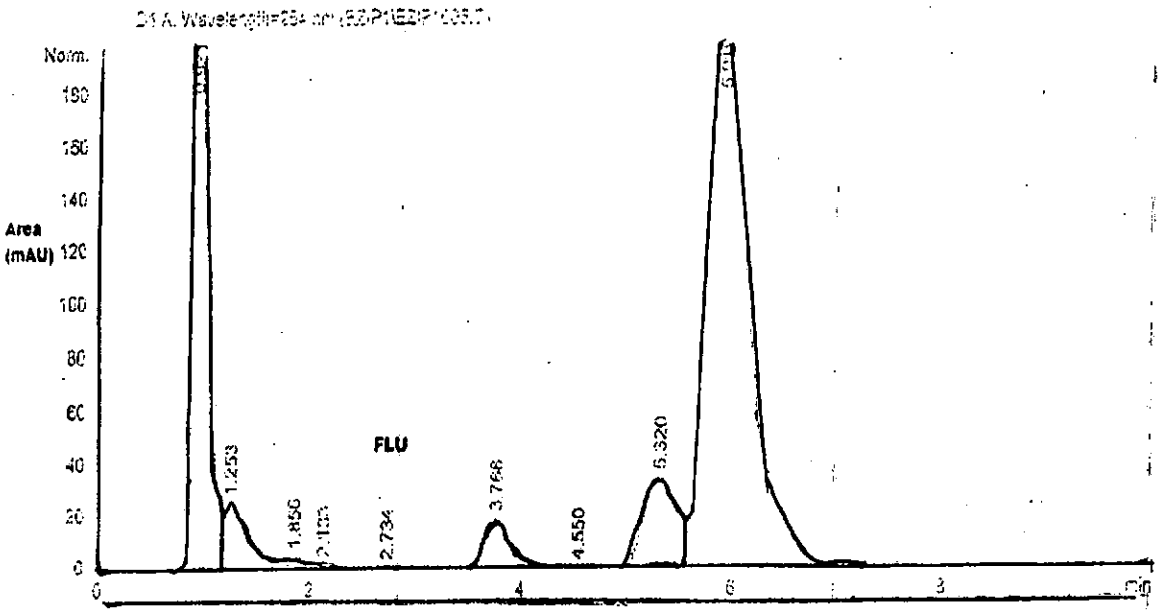


Fig. 8. Chromatogram of fluoranthene after 8 days degradation by *Ps. putida* (FLU = fluoranthene)

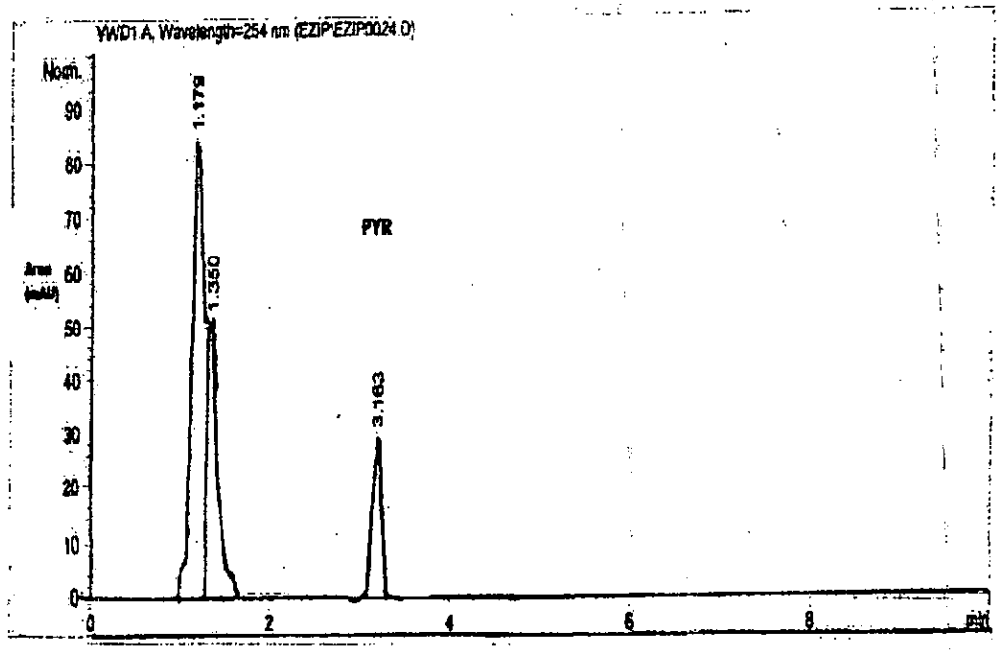


Fig. 9. Chromatogram of pyrene after 8 days degradation by *Ps. putida* (PYR = pyrene)

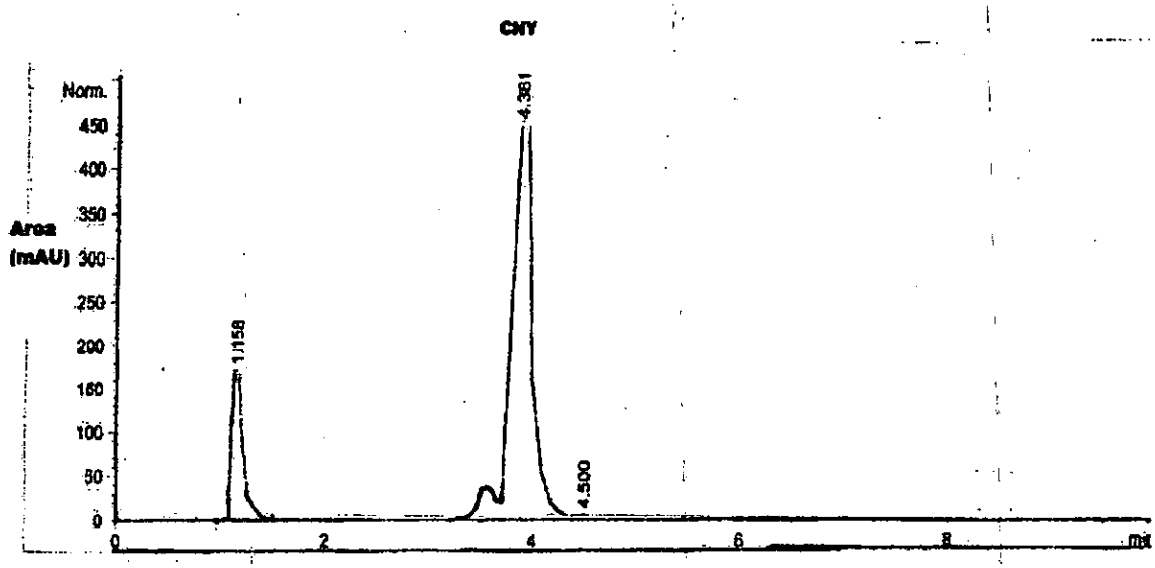


Fig. 10. Chromatogram of chrysene after 8 days degradation by *Ps. arvilla*

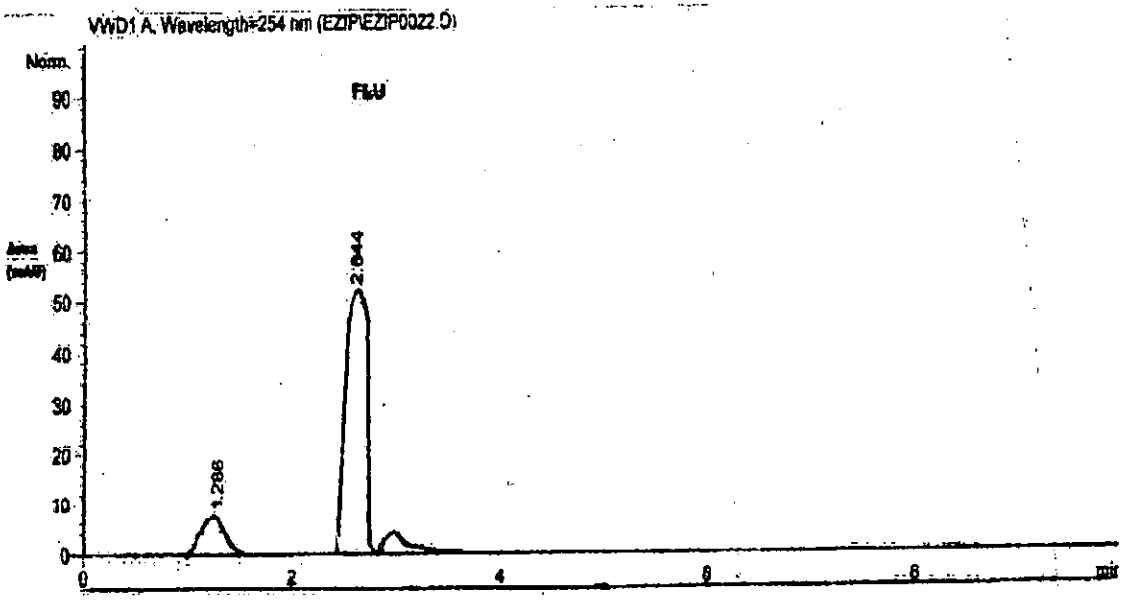


Fig. 11. Chromatogram of fluoranthene after 8 days degradation by *Ps. arvilla*

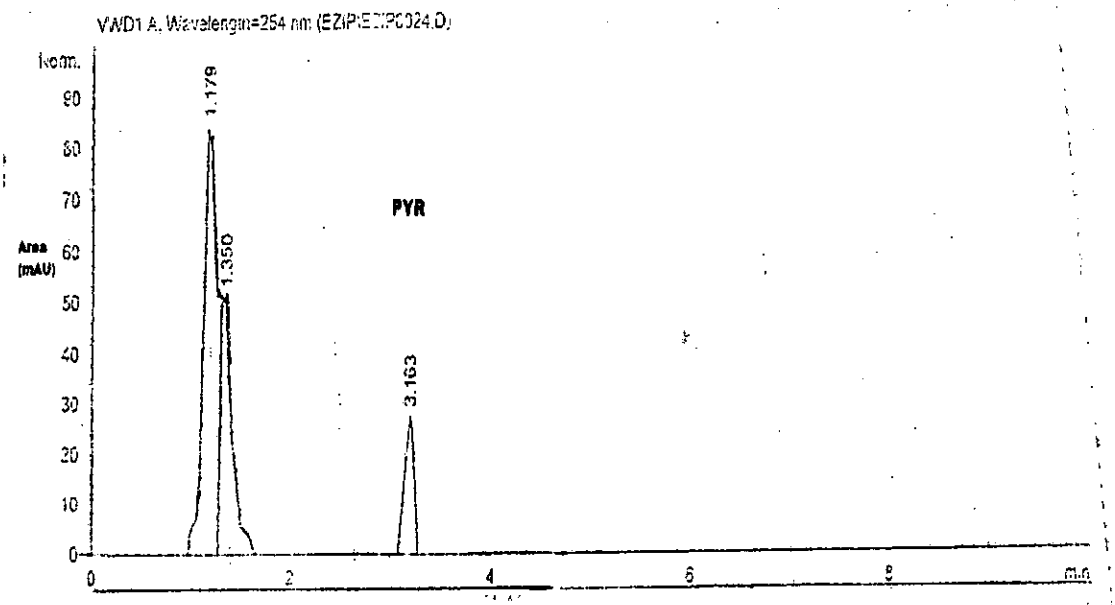


Fig. 12. Chromatogram of pyrene degradation by *Ps. arvilla* after 8 days

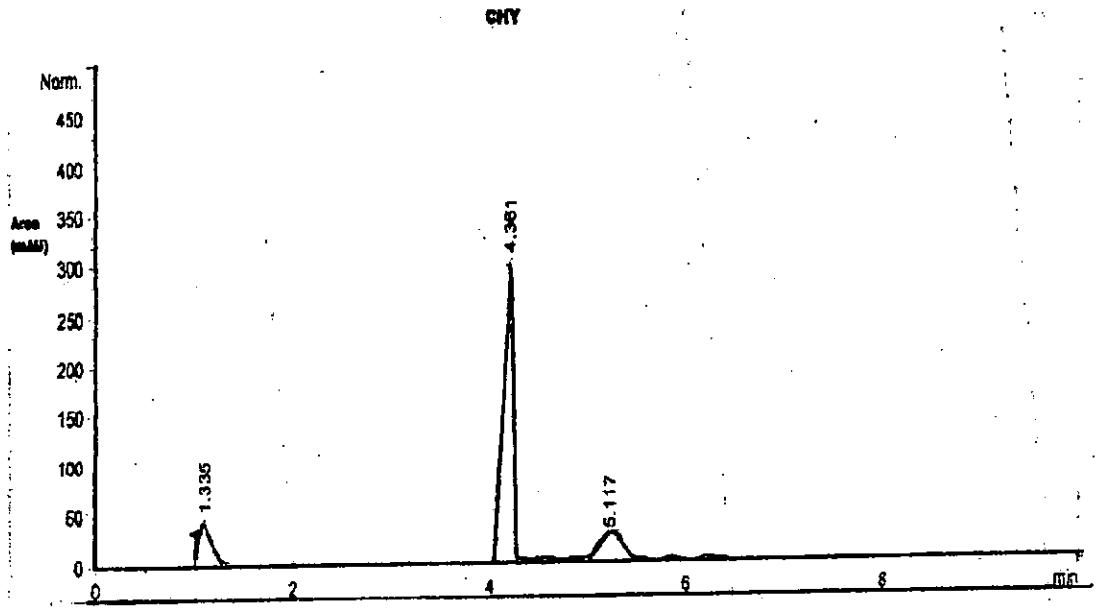


Fig. 13. Chromatogram of chrysene after 8 days degradation by *S. paucimobilis*

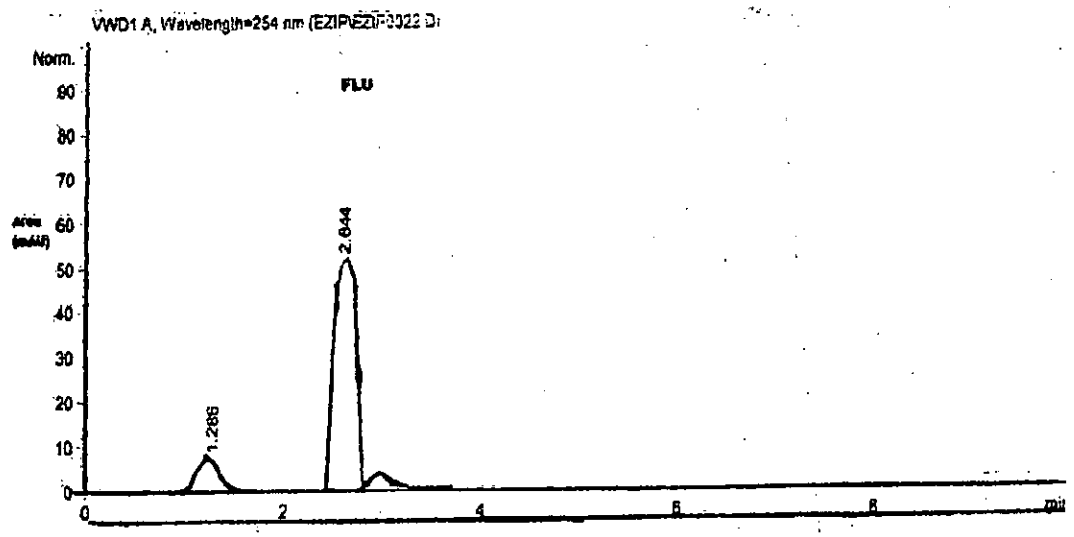


Fig. 14. Chromatogram of fluoranthene after 8 days degradation by *S. paucimobilis*

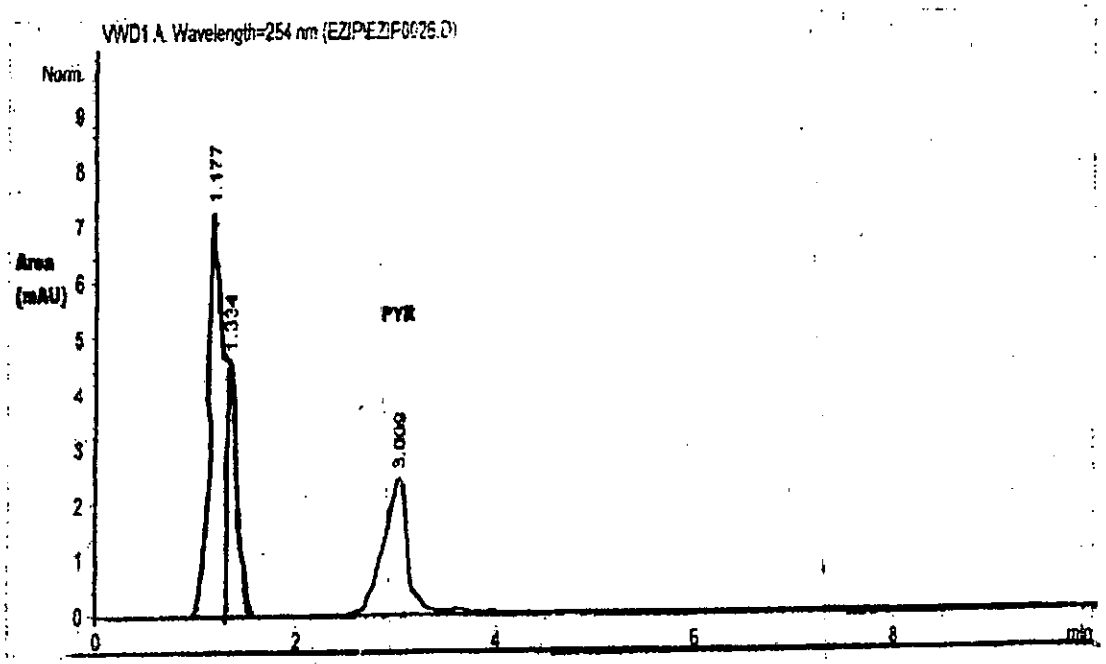


Fig. 15. Chromatogram of pyrene after 8 days degradation by *S. paucimobilis*

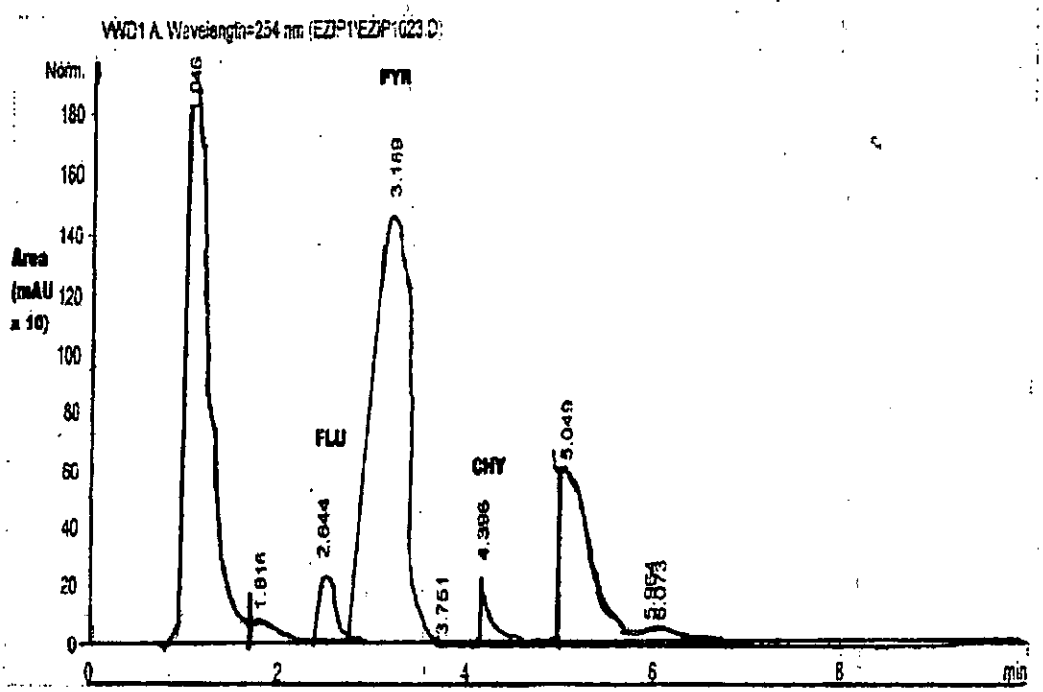


Fig. 16. Chromatogram of mixture of HMW PAHs after 8 days degradation by *Ps. putida*

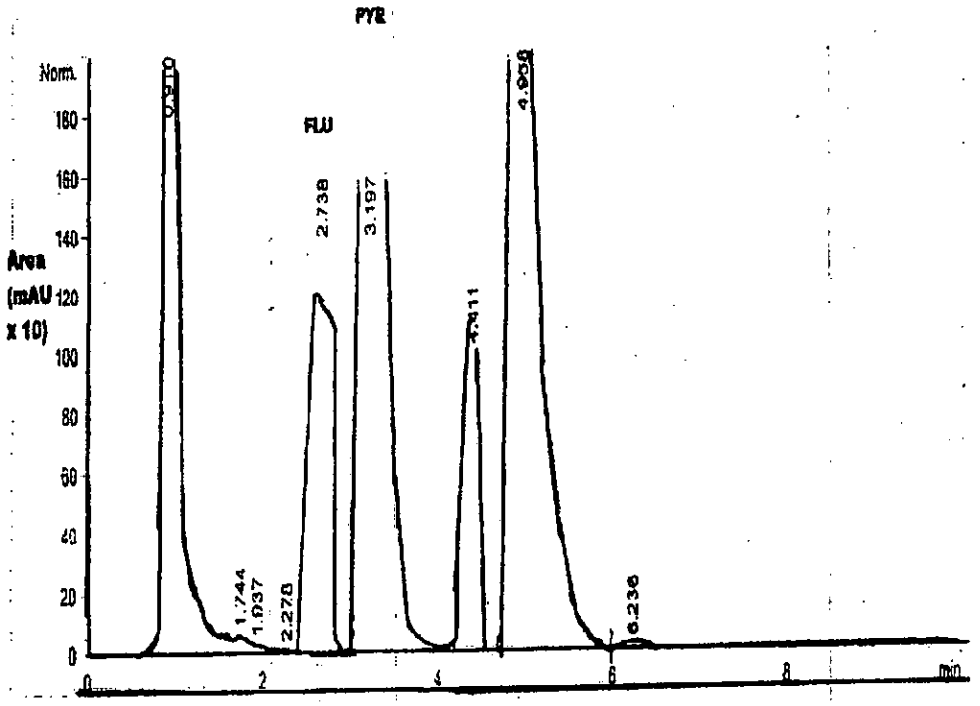


Fig. 17. Chromatogram of mixture of HMW PAHs after 8 days degradation by *Ps. arvilla*

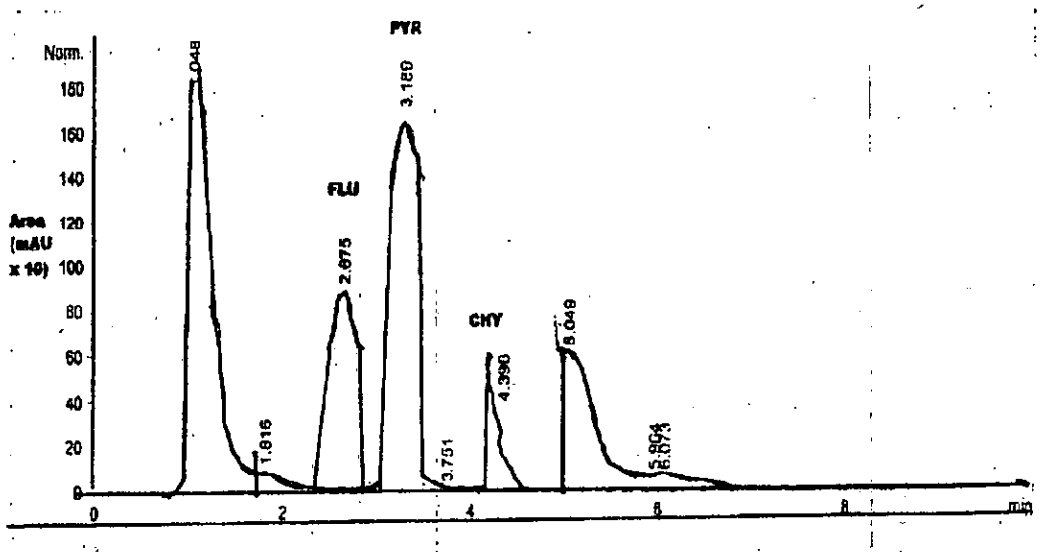


Fig. 18. Chromatogram of mixture of HMW PAHs after 8 days degradation by *S. paucimobilis*

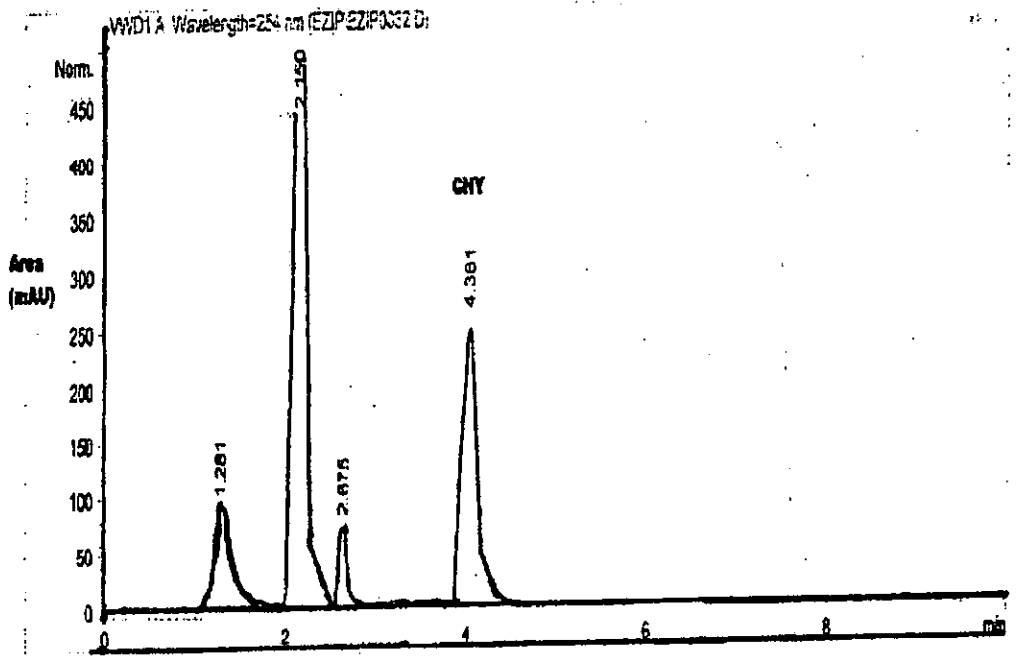


Fig. 19. Chromatogram of chrysene after 8 days degradation by consortium

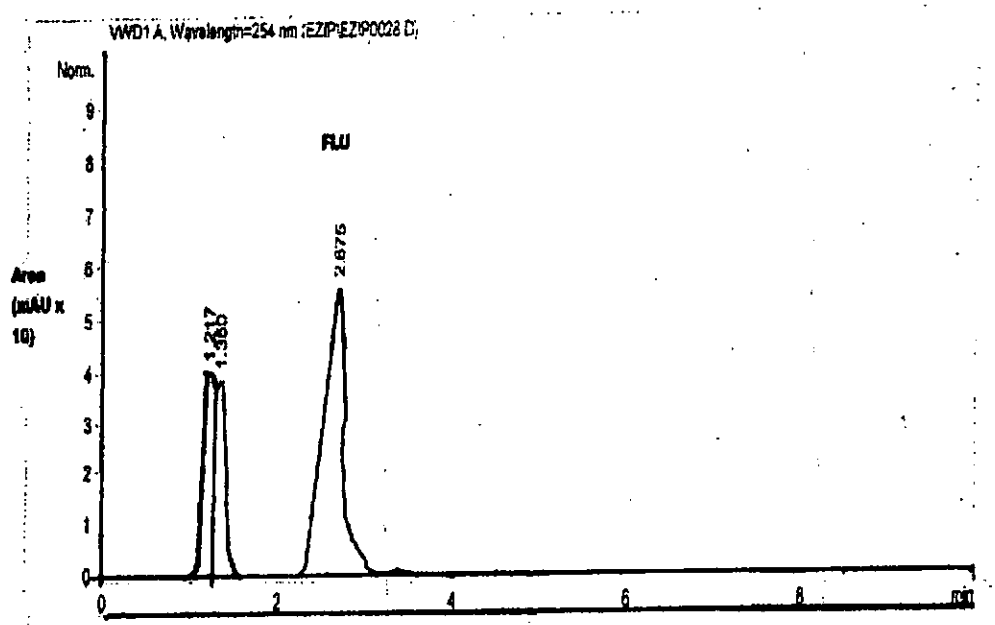


Fig. 20. Chromatogram of fluoranthene after 8 days degradation by consortium

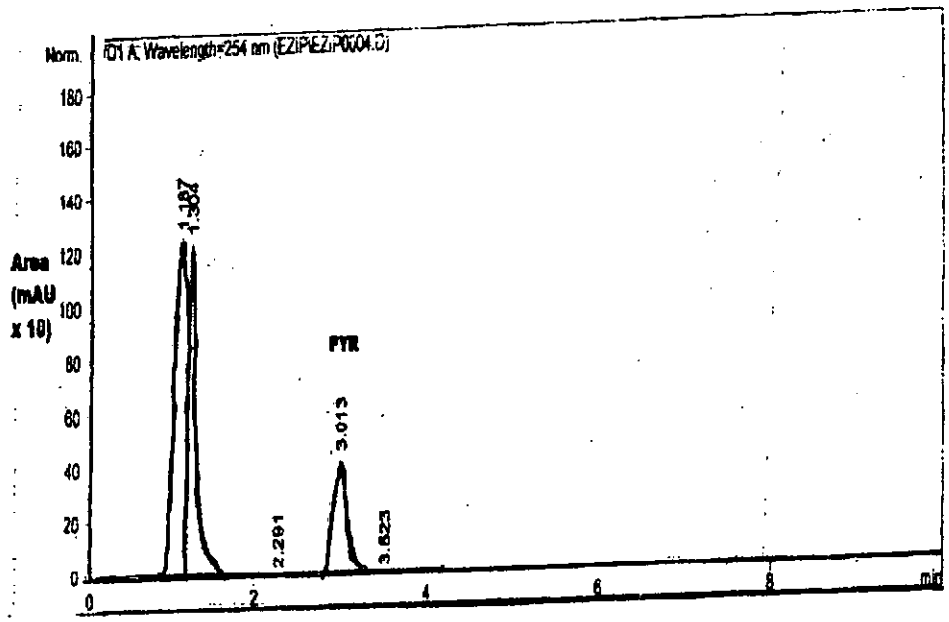


Fig. 21. Chromatogram of pyrene after 8 days degradation by consortium

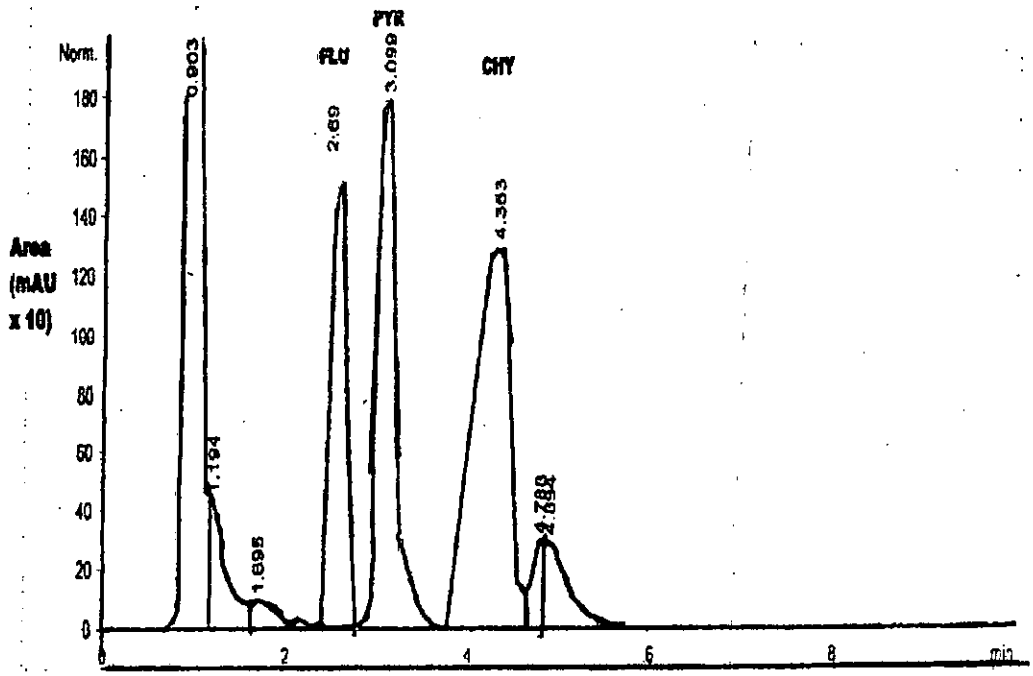


Fig. 22. Chromatogram of mixture after 8 days degradation by consortium

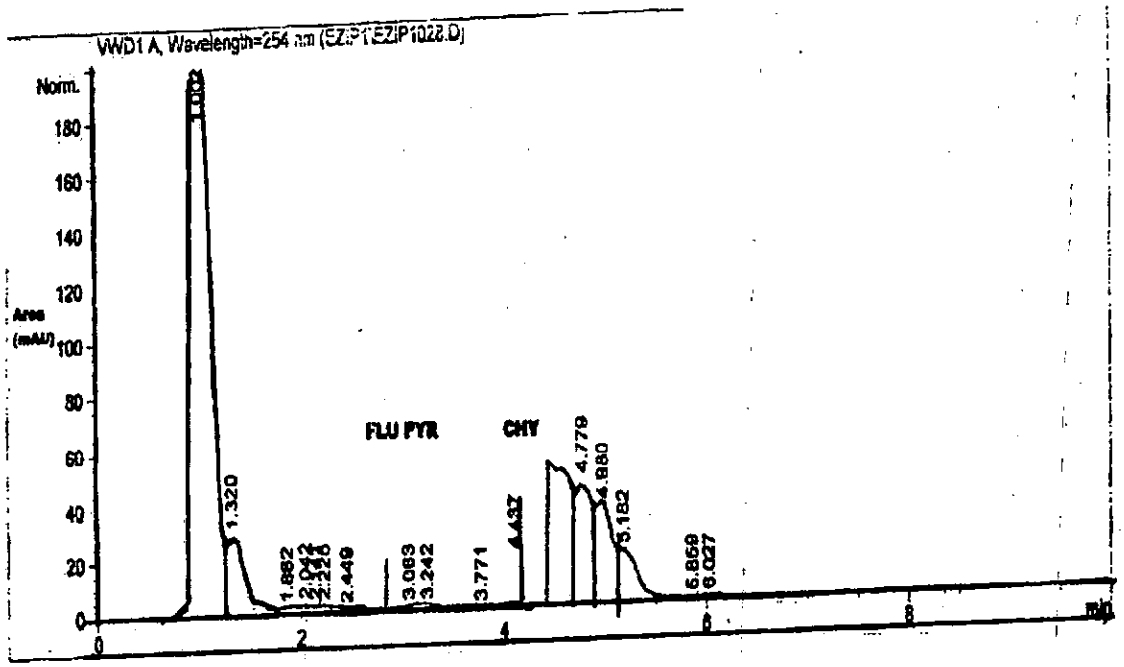


Fig. 23. Chromatogram of mixture of HMW PAHs after 8 days degradation by consortium in the presence of phenanthrene

Table 43. Molecular weight of plasmids

Group	Isolates	Molecular weight of plasmids		
DONOR	*S. paucimobilis*	12.8	-	2.0
	Ps. arvilla	-	2.5	2.0
	Ps. putida	12.8	2.5	2.0
	A. anitratus	12.8	2.5	-
RECIPIENT	*S. p -1*	-	-	-
	Ps. –1	-	-	-
	Ps. putida-1	-	-	-
	A. anitratus-1	-	-	-
	E. coli	-	-	-
	Klebsiella	-	-	-
BIOTRANSFORMED (Mutants)	*S. paucimobilis /S. p-1*	12.8	-	2.0
	Ps putida/Ps. putida-1	12.8	2.5	2.0
	Ps. arvilla./Ps-1	-	2.5	2.0
	Ps putida/E.coli	12.8	2.5	2.0
	Ps putida/ Klebsiella	12.8	2.5	2.0
	A.anitratus/A.anitratus-1	12.8	-	2.0

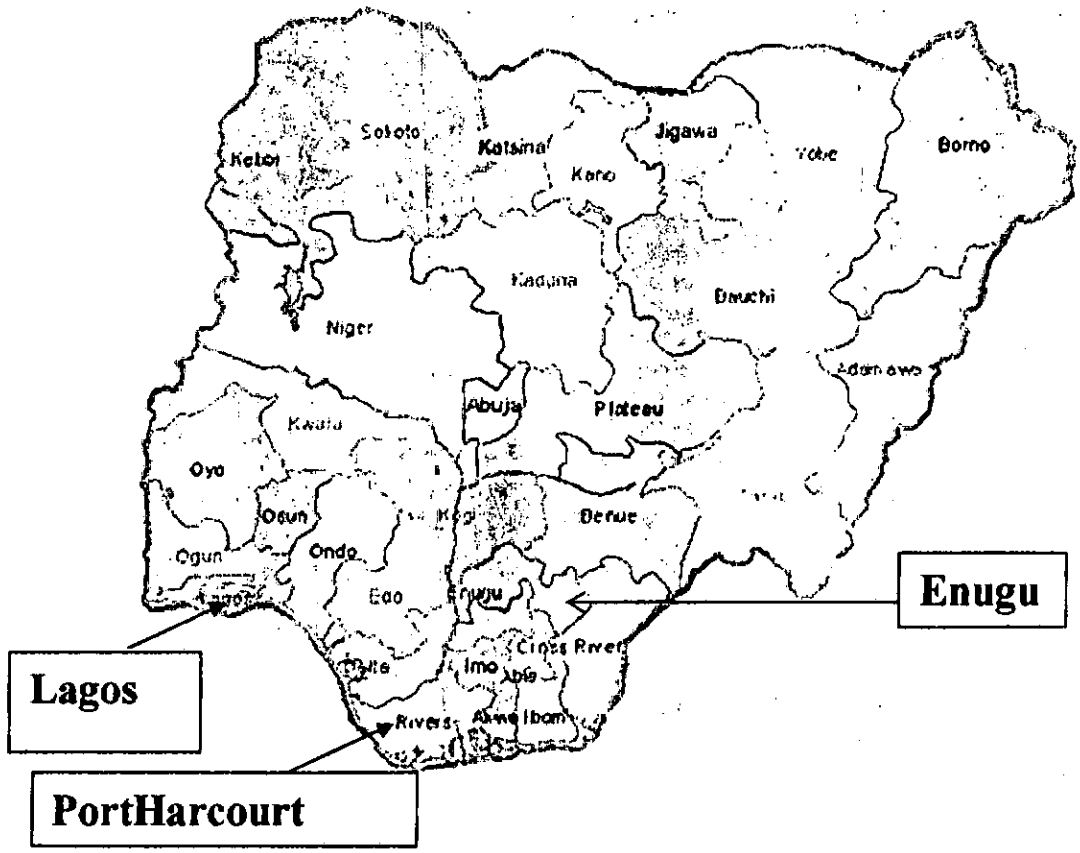


Fig. 24. Locations of polycyclic aromatic hydrocarbon polluted soil sample sites