BACTERIAL DEGRADATION OF FOSSIL FUEL WASTE IN AQUEOUS AND SOLID MEDIA

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Abstract

The generation of environmental pollutants worldwide is mainly due to over reliance on fossil fuels as a source of energy. As a result of the negative impacts of these pollutants on the health of humans, animals, plants and microorganisms, global attention has been directed towards ways of containing this problem. Biodegradation of fossil fuel is one of the most effective methods used to remediate contaminated systems. However with regard to coal waste, much of what is known is based on the ability of fungal species to biosolubilize this material under enrichment conditions in a laboratory setting. For effective biodegradation as a remediation technique, there is an immediate need to source, isolate, enrich and incorporate other microorganisms such as bacteria into bioremediation technologies. The goal of this dissertation was to isolate bacteria from fossil fuel contaminated environments and to demonstrate competence for petroleum hydrocarbon degradation which was achieved using a combination of analytical methods such as spectrophotometry, FT-IR, SEM and GC-MS. Screening for biodegradation of coal and petroleum hydrocarbon waste resulted in the isolation of 75 bacterial strains of which 15 showed good potential for use in developing remedial biotechnologies.

Spectrophotometric analysis of bacteria both in coal and petroleum hydrocarbons (all in aqueous media) revealed a high proliferation of bacteria in these media suggesting that these microbes can effectively utilize the various substrates as a source of carbon.

The isolated bacteria effectively degraded and converted waste coal to humic and fulvic acids; products required to enrich coal mine dumps to support re-vegetation. Scanning electron microscopy showed the attachment of bacteria to waste coal surfaces and the disintegration of coal structures while FT-IR analysis of extracted humic-like substances from biodegraded waste coal revealed these to have the same functional groups as commercial humic acid.

Specific consortia which were established using the isolated bacterial strains, showed greater potential to biodegrade coal than did individual isolates. This was evident in experiments carried out on coal and hydrocarbons where the efficient colonization and utilization of these substrates by each bacterial consortium was observed due to the effect of added nutrients such as algae.

The biodegradation of liquid petroleum hydrocarbons (diesel and BTEX) was also achieved using the 15 bacterial isolates. GC-MS analysis of extracted residual PHC from aqueous and solid media revealed rapid breakdown of these contaminants by bacteria. Different bacterial consortia established from the individual isolates were shown to be more efficient than single isolates indicating that formulated consortia are the biocatalysts of choice for fossil fuel biodegradation.

This study represents one of the most detailed screenings for bacteria from fossil fuel contaminated sites and the isolation of strains with potential to biodegrade coal and petroleum hydrocarbon wastes. Several consortia have been developed and these show potential for further

development as biocatalysts for use in bioremediation technology development. An evaluation of efficiency of each established bacterial consortium for biodegradation in a commercial and/or industrial setting at pilot scale is now needed.

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List of Abbreviations

>	Greater than
<	Less than
%	Percentage
n-	Normal
µg	Microgram
µL	Microlitre
µm	Micrometre
A	Absorbance
ATR	Attenuated total reflectance
BHM	Bushnell Hass medium
BLAST	Basic Local Alignment Search Tool
BTEX	Benzene, toluene, ethylbenzene, xylene
CEC	Cation-exchange capacity
CFU	Colony forming unit
cm	Centimetre
CoA	Coenzyme A
d	Days
DNA	Deoxyribonucleic acid
EBRU	Environmental Biotechnology Research Unit
ECCN	EBRU Culture Collection Number
EDTA	Ethylenediamine tetraacetic acid
EC	Electrical conductivity
FA	Fulvic acid

FAD	Flavin adenine dinucleotide
FT-IR	Fourier transform infrared
g	Gram
GC-MS	Gas chromatography–mass spectrometry
GHG	Greenhouse gases
GMOs	Genetically modified microorganisms
h	Hour
HA	Humic acid
HMW	High molecular weight
HSs	Humic substances
HU	Humin
Kg	Kilogram
L	Litre
LB	Luria broth
LiP	Lignin peroxidase
LMW	Low molecular weight
M	Molarity
MAH	Monocyclic aromatic hydrocarbons
Me	Metal ion carboxylate interaction
mEq	Milliequivalent
min	Minute
mL	Millilitre
MSM	Mineral salts medium
NA	Nutrient agar
NAD	Nicotinamide adenine dinucleotide

NADP	Nicotinamide adenine dinucleotide phosphate
NCBI	National Centre for Biotechnology Information
nm	Nanometer
NOM	Natural organic matter
O_2	Oxygen
OM	Organic matter
PAH	Polycyclic aromatic hydrocarbon
PBS	Phosphate buffered saline
PCB	Polychlorinated biphenyl
PCR	Polymerase chain reaction
PHCs	Petroleum hydrocarbons
rpm	Revolution per minute
TCE	Trichloroethylene
UV-vis	Ultarviolet-visible
v	Volume
WAT	Wax appearance temperature
WC	Waste coal
WHC	Water holding capacity
wt	Weight

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To my loving mother

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First and foremost, I want to give all thanks and praises to GOD for seeing me through these past three years. He’s been so faithful.

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Overview

This thesis is comprised of a comtemporary discussion on the background of fossil fuel waste. Included, is a detailed discussion on fossil fuel usage and its effect on the environment followed by an indept elaboration of coal mining in South Africa and coal formation and classification. In addition, details of liquid fossil fuels in terms of composition and biodegradation are provided. Below is a summary of each chapter that makes up this thesis. The objectives of this work include (1) To isolate and enrich bacteria from fossil fuel contaminated sites which have degradation potentials and (2) To integrate the isolated bacteria with already known fungal strain that has biodegradation ability in order to provide new insight and knowledge into the function of microbial communities in these environments.

Paper 1 deals with an overview of the different approaches to bioremediation of fossil fuel contaminated soil with greater emphasis on PHC degradation pathways.

Paper 2 assesses the ability of various isolated bacterial strains to degrade waste coal in aqueous media in comparison to established bacterial consortia. During this experiment, the production of value added products such as humic acid- and fulvic acid-like substances was analyzed. Shifts in functional groups as a result of the action of different bacterial consortia on waste coal were also analyzed using FT-IR. Results obtained showed the ability of these microbial communities to degrade waste coal and produce HA- and HA-like substances with the established bacterial consortia showing the highest degradation when compared to single bacterial isolates.

Paper 3 is a continuation of Paper 2 and it deals with the degradation of waste coal in solid media. This experiment comprised of pots containing waste coal to which was added bacterial consortia, bacterial consortia/fungal strain and fungal strain alone. Initial co-habitation studies were carried out to assess the ability of the fungal strain to grow in the presence of each bacterial strain. Various parameters such as water retention, cation exchange capacity, electrical conductivity, bulk density and ash content of the waste coal substrate were analyzed and results showed that the bacterial consortia/fungus mix was able to adequately degrade waste coal.

Paper 4 deals with the degradation of liquid fossil fuels such as BTEX in aqueous media using various single bacterial isolates and established consortia. Attempts were made to stabilize various parameters to achieve optimum degradation conditions and results showed that both single and multiple bacterial strains were able to utilize BTEX as substrates for growth with bacterial consortia showing greater degradation efficiency.

Paper 5 addresses various questions raised in Paper 4 regarding the ability of bacterial isolates and established consortia to degrade more complex PHC both in aqueous and solid media. Two commercial biocatalysts known for their PHC degradation ability were used as positive controls. Diesel was used as the sole carbon source and the experiment carried out under greenhouse

conditions where moisture content was controlled for effective degradation to take place. Extraction of residual diesel in soil and aqueous media was carried out and analyzed by GC-MS. Results obtained showed effective degradation of diesel both in aqueous and solid media by formulated bacterial consortia.

Introduction

The quality of life on earth is linked to the overall quality of the environment. Unfortunately, the growing demand and supply of fuel and new chemicals by industrialized societies of the 21st century has placed increasing higher stress on the environment (Jaffe, 1991; Kaimi *et al*., 2006; Chemlal *et al*., 2012) thereby posing a serious problem for survival of mankind itself on earth (Karigar and Rao, 2011). Ogbonnaya and Semple (2013) however explained that the persistent nature of exploration and exploitation of mineral and hydrocarbon resources, inappropriate agrochemical use and uncontrolled combustion have also led to the introduction or displacement of chemicals to the environment. One of the largest concerns to science and technology in the last years has been the problem of environmental pollution (Pinedo-Rivilla *et al*., 2009). According to Megharaj *et al*. (2011), any unwanted substance introduced into the environment is referred to as a contaminant, while the damages done by the contaminants lead to pollution; a process by which a resource is rendered unfit for use. These contaminants are made up of varying amounts of organic compounds ranging from volatile to non-volatile (Hidayat and Tachibana, 2012) and they form the major back bone of fossil fuels (Mann *et al*., 2003). They may be a complex mixture of organic compounds consisting of aliphatic hydrocarbons, aromatic (monocyclic and polycyclic aromatic hydrocarbons (MAH and PAH)), BTEX (benzene, toluene, ethylbenzene and xylenes), chlorinated hydrocarbons {e.g., polychlorinated biphenyls (PCB), trichloroethylene (TCE)}, nitroaromatic compounds and organophosphorus compounds (Rogoff and Wender, 1957; Megharaj *et al*., 2011; Mustafa, 2011). However, most researchers have highlighted the importance of aromatic compounds such as BTEX in the environment. Contaminants which are introduced in the environment may be in the form of solids or liquids. Be it solid or liquid contaminants, the devastating effects these have on the environment have increased the awareness of the need to protect ecosystems and evaluate the damages caused by these contaminants. For instance, documented records have shown an estimate of the amount of natural crude oil seepage to be 600,000 metric tons per year with a range of uncertainty of 200,000 metric tons per year (Das and Chandran, 2011).

Fossil fuels usage and effects on the environment

The reliance on fossil fuels for different purposes over the years has increased as a result of increase in world population. Fossil fuels (coal, crude oil and natural gas) as an important energy source play vital roles in the energy system and economies of African countries. South Africa which is one of the world's largest producers (5th) and consumers (7th) of fossil fuels (coal) (BP, 2012), has experienced a boost in her economy due to her involvement in the production, consumption and exportation of coal (UNECA, 2011). There has been an increase in its production over the last 30 years due to the revenue accrued from it and over reliance on it as a source of energy (ERC, 2004). Another reason behind the increase in demand for coal is due to the various products derived from coal during its conversion processes. For instance, in South Africa where liquid and gaseous fossil fuels are not readily available, coal liquefaction is one

option available for obtaining these products. Statistics have shown that Africa has enormous potential with fossil fuels accounting for about 9.5%, 8% and 4% of the total proven reserves of crude oil, natural gas and coal in the world, respectively (BP, 2011). The generation of electricity from fossil fuels cannot be neglected because over 80% of electricity generated across the continent of Africa is from fossil fuels (IEA, 2011). The generation and supply of energy from fossil fuels has also been documented. IEA in their 2011 annual report stated that fossil fuels accounts for about 50% of total energy supply and one third of energy consumption (IEA, 2011). Over reliance on fossil fuels to produce energy has generated huge problems from both environmental and social perspectives, affecting our societies locally, regionally and globally (UNECA, 2011). Some of these problems include ozone depletion, global warming, and acidification and depletion of non-renewable resources. According to Höök and Tang (2013), mankind's energy production is the principal contributor to mankind's release of greenhouse gases (GHG) in particular, CO_2 to the atmosphere with fossil fuel combustion as the key factor. Among the 3 categories of fossil fuels, solids (coal) and liquids (petroleum) are the major contaminants in the environment.

Solid fossil fuels: Coal mining in South Africa

Coal mining in South Africa plays a crucial role in the development of the economy by generating energy and yielding foreign income and this has been on-going since 1870 (Vermeulen and Usher, 2006). South Africa is one of the world's largest producers and consumers of coal; generating annually approximately 224 million tonnes of coal. Deposits of coal in South Africa occur in three geologically separate but closely related environments within the Karoo Sequence. One is the Lower Beaufort Group with coalfields such as Springbok Flats, Waterberg, Limpopo, Soutpansberg and the Vryheid formation (Moolman and Fourie, 2000) with the province of Mpumalanga having the highest percentage of the country's total output (Bullock and Bell, 1997). The extraction of coal from either above or below ground is called mining and this is done by either surface or underground mining methods (Tiwary, 2001). Surface mining is by strip mining whereby draglines are used to remove the overburden which is later replaced in the mined-out area, or by open-casting, with the overburden being removed and dumped elsewhere (Rai *et al*., 2010). Surface mining technique is most effective when the mineral deposit is close to the surface. Blasting and removal of surface layers of soil and rock is applied when this method is carried out. The reason for blasting and soil/rock removal is for easy access to coal seams. Before raw coal is extracted from the seam, separation techniques are applied to eliminate waste by-products. Some of the methods used in separating raw coal from the by-products include the use of gravity, heavy liquids, electrostatic and magnetic processes. When the coal seam becomes exposed, it is then drilled, fractured and mined. Underground mining on the other hand, is different from opencast mining as it involves the creation of tunnels extending from the surface into the mineral seam where machines are used to extract the mineral. Figure 1, shows the processes involved in extracting coal using the different techniques.

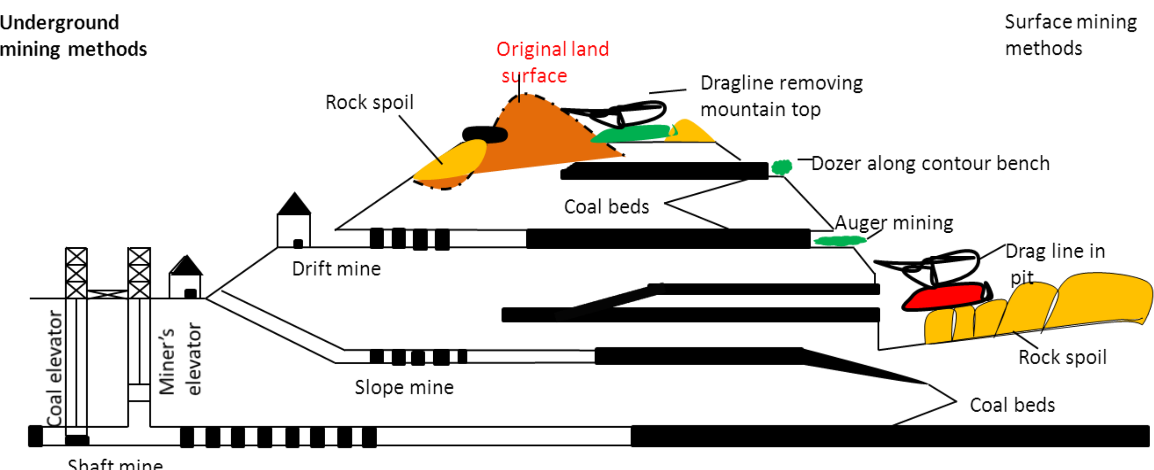

Figure 1. Extraction methods involved in coal mining

Be it surface or underground mining technique, these activities have considerable impact on the environment (Dhar, 1993). In the case of open cast mining, huge overburden dumps which are mixed with coal, spread across the horizon (Tiwary and Dhar, 1994) and spontaneous combustion of coal in such a case is inevitable. When this happens, deadly gases such as sulphurous fumes and smoke are emitted into the atmosphere. Soils meant for agricultural purposes are rendered useless because the spoils from coal cannot support plant growth (Chaubey *et al*., 2012). The spoils created during and after mining are characterised by high temperatures that leads to spontaneous combustion (Pone *et al*., 2007), low water holding capacity (Chaubey *et al*., 2012), high bulk density (Singh *et al*. 2002), with low cation exchange capacity and electrical conductivity (Juwarkar and Jambhulkar, 2007).

Because a large area of land cannot support plant growth due to mining activities, the lands are left without vegetation cover resulting to wind erosion. Acid rain is another effect caused by (most especially) open cast mining technique (Tiwary, 2001). In many cases, the top soil is excavated before surface mining takes place. After the mining activities, the soil is not returned to its former place, resulting to the formation of artificial lakes (Ghose, 2004). Underground waters have been at the receiving end of open cast mining system (Sheoran *et al*., 2010). Rain that falls after mining activities, penetrate through soils to underground aquifers and contaminate the water with acidic pyrites (McCarthy, 2011). A collapse of overlying rock strata when mining terminates is the major problem associated with underground mining. Acid mine drainage (AMD) is another effect of both surface and underground mining systems. Rain water seeps through strata created during mining activities and carries acidic contents in coal along with it (Fig. 2). Underground method damages water regimes and thus causes a reduction in the overall availability of water in and around the mining areas. In the sedimentary deposit of mining areas,

the water table and aquifers are damaged and thus the availability of water from these sources reduces.

Figure 2. Acid mine drainage as a result of surface run-off after mining activities have taken place

Coal formation and classification

Coal has been defined as a combustible, sedimentary, organic rock which is composed mainly of carbon, hydrogen and oxygen and comprises biological materials that are chemically and physically heterogeneous (Stout *et al*., 1988; Polman *et al*., 1994; Fakoussa and Hofrichter, 1999; Scott, 2002; ACF, 2005) formed from ancient vegetation. This ancient vegetation has been consolidated between other rock strata and transformed by the combined effects of microbial action, pressure and heat over a considerable time period. Because of its heterogeneous nature, it is mostly described by the term “macerals”. According to Neavel (1981), macerals are organic substances derived from plant tissues, cell contents, and exudates that were variably subjected to decay, incorporated into sedimentary strata, and then altered physically and chemically by natural (geological) processes. Coal as a fossil fuel is made up of aggregates of physically distinctive and microscopically distinguishable chemical macerals and minerals. The elemental content in coal is not uniformly mixed. Instead, coal is made up of a mixture of non-uniformly mixed carbon, hydrogen, oxygen, sulphur and some other minor elements.

The formation of coal involves two phases; biochemical and geochemical phases. In the biochemical phase of coal formation, plant materials were swallowed into the earth (usually in swampy areas). The dead plant materials were acted upon by bacteria in the absence of oxygen (anaerobic conditions) at very high temperature conditions forming a dense material known as peat and this phase is called peatification (Stout *et al*., 1989). As pressure increased underneath the surface of the earth, the water content in peat reduced giving rise to other forms of higher coals (geochemical phase). The term "coalification" has been used indiscriminately by many authors. For instance, Igbinigie (2007) explained coalification as the geochemical (second) phase in the transformation of peat to coal while KGS (2012) in their report, argued that the coalification process starts from the initial phase of coal formation (biochemical phase) through the geochemical phase. Other authors have affirmed the later claim by stating that the coalification process is essentially an initial biochemical phase followed by a geochemical or metamorphic phase (Chilingar and Yen, 1980; Thomas, 2012). Further explanations were made with regard to the coalification process using two terms; diagenesis and metamorphic changes. Diagenesis of coal is often referred to as biochemical coalification while metamorphism of coal is called geochemical coalification. Coalification in a nutshell is the conversion of dead plant materials by microorganisms through all the ranks to finally form anthracite coal which is the hardest of all coals (Fig. 3). Humification on the other hand has been defined as the process that leads to the formation of humic acid (Guggenberger, 2005) and it is considered to be the most important chemical process in geochemical transformation which is mainly involved in the early stages of coal formation (Francioso, 2003). As peat undergoes coalification, there is a subsequent formation of other ranks of coal. The different ranks of coal formed from peat include (in ascending order of hardness) lignite, sub bituminous (which is also known as low rank or brown coal) (Thomas, 2012), bituminous and anthracite coal. The classification of coal is based on its formation i.e., the degree at which the coal has undergone coalification (Fig. 3).

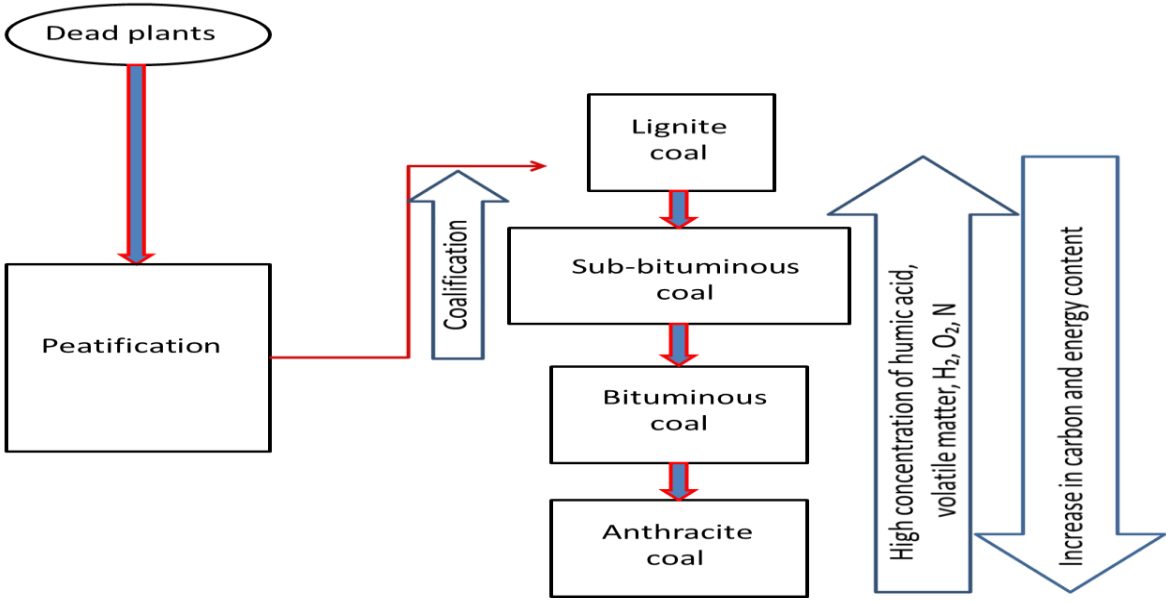

Figure 3. Formation and classification of coal based on moisture and carbon/energy contents

Such varying degrees of coalification are called coal ranks or categories and generally, four are recognised as stated above. Some authors have drawn attention to the fact that there are other categories of coal that are not usually grouped among coal during classification. These categories include peat which preceeds lignite and graphite which succeeds anthracite (Haenel, 1991). Increasing carbon content or decreasing moisture content, are some of the criteria to be considered when classifying coal into the different ranks. Low rank coals are generally identified by high moisture content compared to high rank coals. On the contrary, the lower the moisture content in coal, the higher it's rank. However, elemental composition and atomic distribution have also been considered when classifying coals into the various ranks (Table 1) (Hodek, 1994).

Table 1. Classification of coal based on moisture content, percentage elemental composition and atomic ratio of the component elements (Modified after Hodek, 1994)

		Elemental analysis (%)					Atomic ratio				
Coal ranks	Notes	C	H	O	N	S	C	H	O	N	S
Lignite	Mainly used for steam/electric production in power plants Has a high moisture content with heat energy ranging 8 –10 MJ/Kg	68.60	5.00	24.50	1.56	0.31	100	87.00	27.00	1.90	0.20
Sub-bituminous	A non-coking coal with less sulphur but more moisture (approximately 10 to 45 per cent)	82.70	5.10	9.40	1.59	0.95	100	74.00	8.50	1.70	0.40
Bituminous	This is the type most commonly used for electric power generation It has a heat generating value of 28MJ/Kg with less than 3% moisture content.	89.70	4.50	2.70	1.59	1.20	100	61.00	2.30	1.50	0.50
Anthracite	Frequently used in heating homes because it burns with little or no smoke. It appears as a shiny solid which has almost no moisture content with energy content of about 32MJ/Kg .	92.00	3.80	1.30	1.95	0.87	100	49.00	1.10	1.80	0.40

Structures of coal

According to Fakoussa and Hofrichter (1999), coal does not have a specific chemical structure because this changes with rank. However some literatures argue that the reason why there are different structures of coal is because of insufficient research in this field especially in the field of lignite (Stefanova *et al*., 1993). So many structures have been published by different authors in different literatures but unfortunately, they are all hypothetical models (Yağmur *et al*., 2000; van Krevelen, 1993; Haenel, 1991; Fakoussa, 1981). Lignite on the other hand, has an even more complex structure than hard coal. The complexity of lignite could be due to the presence of several distinct compound classes made up of the hydrophobic bitumen, the alkali-soluble humic and fulvic acids (humic substances) and an insoluble residue, the matrix or humin (Fakoussa and Hofrichter, 1999). Figure 4 gives a summary of different coal structure models.

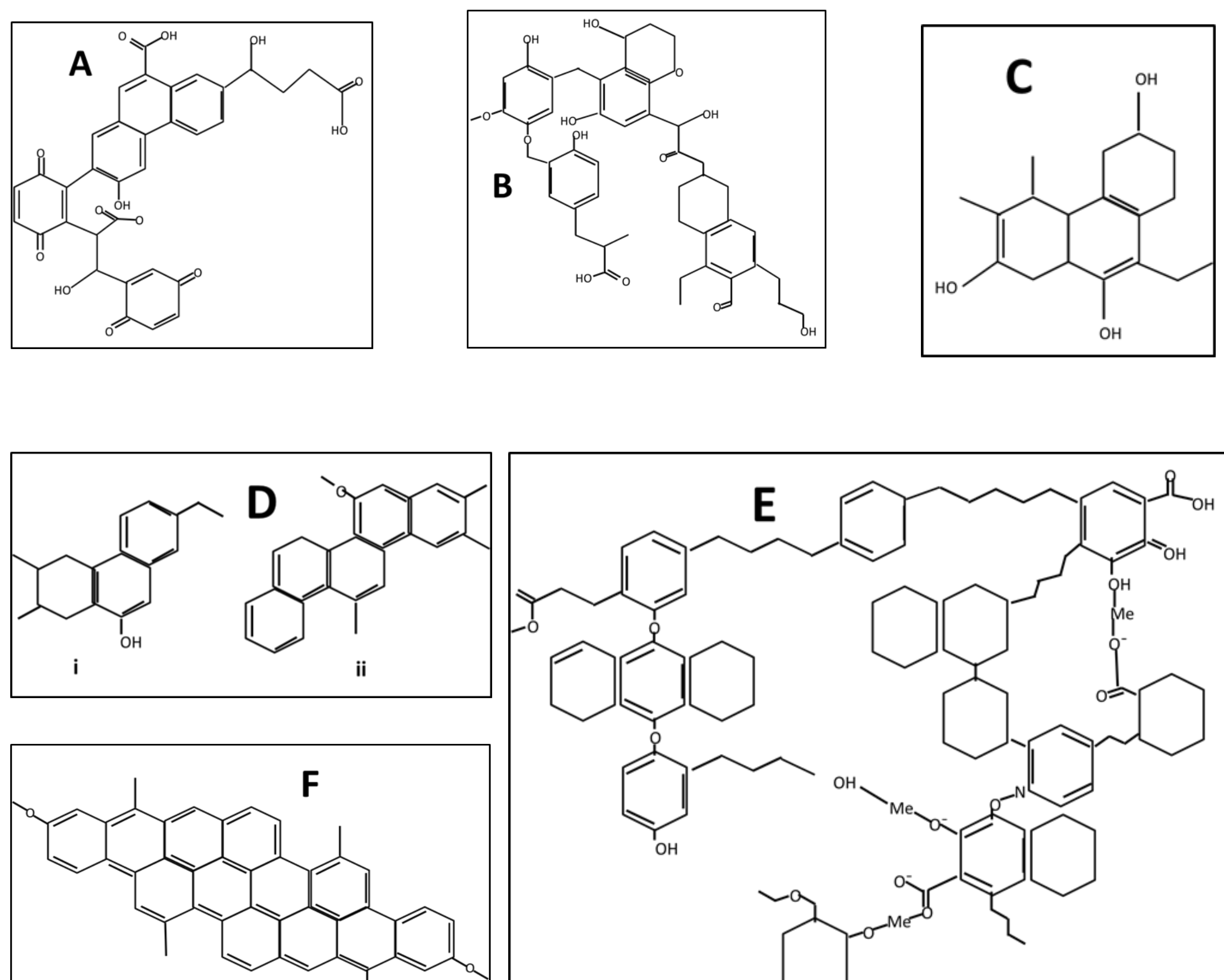

Figure 4. Typical structure models for coals (substances) of different rank. A, humic acid; B, low rank lignite; C, sub-bituminous coal (high rank coal); Di, high volatile and Dii, low volatile bituminous (hard) coal; E, lignite; F, anthracite hard coal modified from Fakoussa and Hofrichter (1999). *Me* in lignite molecule is a representation of possible metal ion carboxylate interactions.

Humic substances (HSs) have been defined as ubiquitous natural materials occurring in huge amounts in soils, sediments and waters as a product of the chemical and biological transformation of animal and plant residues (Kochany and Smith, 2001; Piccolo, 2002; Janoš, 2003; Tripathi *et al*., 2009). A clear definition of HSs has not been put in place but for the purpose of understanding, they have been defined by combining all known aspects of their properties, including the process of their isolation (Burlakovs *et al*., 2013). According to Aiken *et al*. (1985) and Burlakovs *et al*. (2013), HSs are a general category of naturally occurring heterogeneous organic substances that can generally be characterized as being yellow to black in colour, of high molecular weight and refractory. They are found in sediments, peat, lignites, brown coal, sewage, composts and other deposits (Adani *et al*., 2006; Erdogon *et al*., 2007; Grinhut *et al*., 2007). However low rank coals such as peat and lignite have the highest concentration of humic substances (Piccolo *et al*., 1997). HSs constitute a physically and chemically heterogeneous mixture of macromolecular organic compounds of aliphatic and aromatic nature (Steffen *et al*., 2002: Zavarzina *et al*., 2004; Burlakovs *et al*., 2013). The importance of HSs in the environment has been stressed due to their crucial role in oxidative and reductive reactions (Grinhut *et al*., 2007; Scapini *et al*., 2013) and sustaining plant growth (Stevenson, 1994; Nardi *et al*., 2002, 2007; Amir *et al*., 2007; Liu *et al*., 2010). The composition of HSs are mainly of aromatic, aliphatic, phenolic, quinonic and N-derived components, which are covalently bound through C-C, C-O-C and N-C bonds (Grinhut et al., 2007). Humic substances have been divided into three main fractions based on their solubility properties in acids and alkalis. They are humic acid (HA), fulvic acid (FA) and humin (HU) (Grinhut *et al*., 2007; Peña-Méndez *et al*., 2005; Penru *et al*., 2012; Scapini *et al*., 2013). Humic acid (HA) is the fraction soluble in an alkaline solution while fulvic acid (FA) is the fraction soluble in an aqueous solution regardless of pH, and humin (HU) is the fraction insoluble at any pH (Badis *et al*., 2010). The characteristic that remains associated with each humic fraction after separation from natural organic matter (NOM) is the high degree of heterogeneity. Researchers have found the HA fraction of natural organic matter (NOM) to be problematic in drinking water because it readily reacts with chlorine to form carcinogenic compounds (Gomes *et al*., 2009; Badis *et al*., 2010; Ghouasa *et al*., 2012). Analysis carried out on the various fractions of HSs all over the world suggests that their elemental compositions are essentially consistent. The range is usually from ~40 to 50 % C and ~40 to 50 % O for FA; ~50 to 65 % C and ~30 to 40 % O for HA with H, N and S contents ranging from ~3 to 7%, ~0.8 to 4.3 % and ~0.1 to 3.6 % respectively, in all of the fractions (Stevenson, 1994). A summary of the chemical properties of humic substances is presented in Figure 5. For a proper understanding of the structure of coal, humic acid derived from coal has been used as a standard.

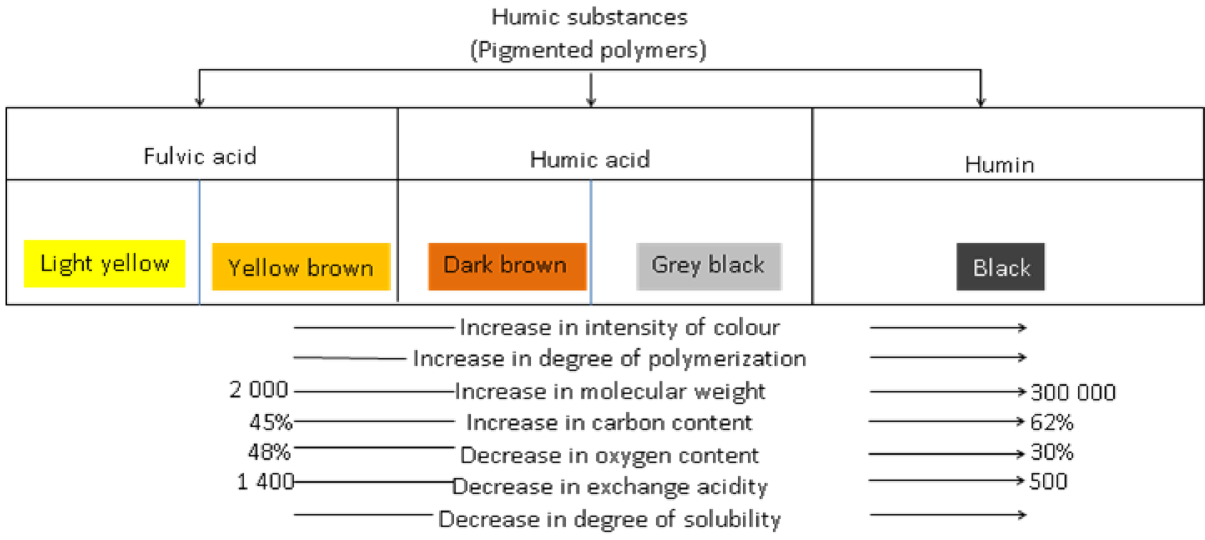

Figure 5. Chemical properties of humic substances (Modified after Stevenson, 1994).

Liquid fossil fuels: Petroleum hydrocarbons (PHCs)

Petroleum hydrocarbons otherwise called crude oil are fossil fuels formed from the remains of marine microorganisms deposited on the sea floor. The deposits end up in rock and sediment where oil is trapped in small spaces after millions of years (Enzler, 2011). Crude oil is the most widely used fossil fuel and it is composed of hundreds of compounds which are transformed to various products during the process of refining (Sathishkumar *et al*., 2008; Kok, 2011). Reports have shown that global demand for crude oil has been intensifying steadily over the past 20 years (Hasan *et al*., 2010). The demand for crude oil has grown from 60 million barrels per day to 84 million barrels per day (Hasan *et al*., 2010) because it is the most important hydrocarbon resource in the world (Chilingar and Yen, 1980; Langevin *et al*., 2004). Petroleum hydrocarbons are a common group of environmental contaminants, though they are not usually classified as hazardous wastes. Modern day society makes use of many petroleum products including those that are fundamental to our lives (i.e., transportation fuels, heating and power-generating fuels). Although different chemicals that are of environmental concern are being used in industries for the manufacturing of various products which are of benefit to man, the large volumes of crude oil being used on a daily basis has made the general public focus more attention on it. For instance, many chemical compounds are found to have serious ecological and human health effects (Carson, 1962; Thakker *et al*., 1985; Harvey, 1996; Lamb and Hentz, 2006). Due to the numbers of facilities, individuals, and processes and the various ways the products are stored and handled, environmental contamination is potentially widespread (Kaimi et al. 2006; Barrutia *et al*., 2011; Liu *et al*., 2011; Woolfenden *et al*., 2011; Pasumarthi *et al*., 2013). These contaminants according to Yanez *et al*. (2002), Prüss-Ustün *et al*. (2011) and Blacksmith Institute and Greencross (2012) have placed the lives of at least 125 million people at risk of death or disease

with a majority of these people residing in poor countries who don't have the technologies in place to combat pollution through remediation.

Composition of crude oil

Crude oil is a complex mixture of organic compounds, 75% of which consists of short and long chain hydrocarbons (Kok, 2011). Crude oil also consists of hetero atom groups that contain nitrogen, oxygen and sulphur and species which incorporate metals such as vanadium, nickel and iron (Ward *et al*., 2009). The composition and properties of crude oil are dependent on the origin, age, and conditions of the source geologic formation. The composition of light hydrocarbons (C_1-C_9) which constitute about 50% of the carbon in crude oil has been used by geochemists to identify its origin. They also use it to determine how, why and where petroleum exists as well as its migration pattern (Lin and Tjeerdema, 2008). Petroleum hydrocarbons (e.g., aliphatic and aromatic hydrocarbons) are also classified according to their structures. They usually are divided into paraffins, naphthenes, aromatics (Widdel and Rabus, 2001; Mirdamadian *et al*., 2010) and NSO (nitrogen, oxygen, and sulfur-containing compounds) (Baek et al., 2006).

Paraffins (also called wax) are saturated hydrocarbons (alkanes) made up of straight or branched chains and without ring structure. Paraffin is a generic name used synonymously with alkanes indicating hydrocarbons with the general formula of $C_nH_{2n+2.}$ It is a complex mixture containing *n*-alkane, iso-alkane, and cycloalkane (Fig. 6) (Xiao *et al*., 2012) and it can also be referred to as high molecular weight alkane hydrocarbons (Sood and Lal, 2008). The simplest paraffin molecule is that of methane (CH_4) and it appears in a tetrahedral form hence called *n*-alkanes. Alkanes that have branched structures are called isoparaffins. Examples of paraffins with straight chains are methane, ethane, propane, and butane (all gaseous), and pentane and hexane (which are liquids). Branched-chain paraffins are usually associated with higher octane rating because they are present in the heavier fractions. Paraffin accounts for 2–50% of the composition of crude oils and they tend to precipitate when the temperature is lower than wax appearance temperature (WAT) during the production and the transportation of crude oil (Sood and Lal, 2008; Wang *et al*., 2012; Xiao *et al*., 2012). Because of its high composition in crude oil and its precipitate forming characteristics, it is considered a serious problem in oil industries (Wang *et al*., 2012).

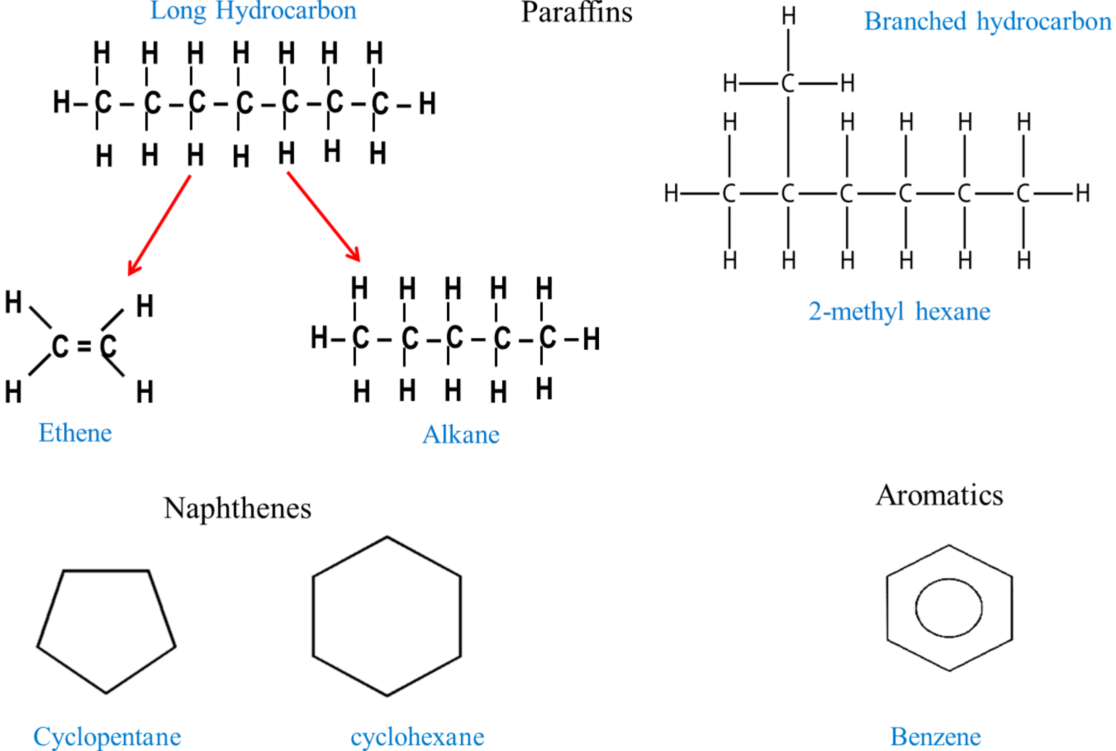

Figure 6. Representative structures of the three classes of petroleum hydrocarbons (paraffins, naphthenes and aromatics)

Naphthenes on the other hand are a class of cyclic aliphatic hydrocarbons obtained from petroleum and they may feature a paraffinic side chain. Naphthenes have the general formula C_nH_{2n}. Naphthenic hydrocarbons are the most abundant class present in most crude oils accounting for 25–75% (Lin and Tjeerdema, 2008). As the amount of naphthenic hydrocarbon increases, there is a proportionate increase in their boiling points with the exception of heavy oil fractions. Naphthenes are broadly divided into two; monocyclic and polycyclic naphthenes. Monocyclic naphthenes are distributed in the light fractions of crude oil, while polycyclic naphthenes are mainly in the heavier oil fractions. Hydrocarbons with alternating double and single bonds between carbon atoms forming rings are referred to as aromatic hydrocarbons. They contain single or multiple benzene rings in their structures (Table 2) with many important compounds in this class containing aliphatic hydrocarbon chains (e.g., alkylbenzenes) (Widel and Rabus, 2001). The name "aromatic" is derived from the word "aroma" which suggests that most of the compounds found in this group, have strong aromas. An aromatic hydrocarbon with single ring structure is called a mono aromatic or monocyclic aromatic hydrocarbon (MAH) with benzene as a typical example (Widdel and Rabus, 2001). Polycyclic aromatic hydrocarbons (PAHs) are compounds composed of two or more fused benzene rings. Although PAHs occur as common constituents of petroleum and coal tar, the main inputs to the environment is through

anthropogenic activities. These activities include incomplete combustion of fossil fuels, wood, and solid wastes (Baek *et al*., 1991; Kaushik and Haritash, 2006) or by-products of industrial processes (Jones *et at*., 1989a and 1989b; Lijinsky, 1991; Pothuluri and Cerniglia, 1994). Reports have also shown that some natural activities can lead to the production of PAHs in the environment. These natural activities include forest fires, diagenesis of organic matter, and biochemical synthesis (Wilcke, 2000, 2007).

Table 2. Different aromatic hydrocarbons and their representative structures

Chemical compound	**Structure**	**Chemical compound**	**Structure**
Benzne		Anthracene	
Ovalene		Coronene	
M-xylene	CH_3 CH_3	Toluene	CH_3
Chrysene		Corannulene	
Ethylbenzene	CH_3	P-xylene	H_3C CH_3
Tryphenylene		Benzo[a]pyrene	
Naphthacene		Naphthalene	
Pentacene		Pyrene	

The biodegradation of fossil fuels

Negative impacts of fossil fuels on the environment have been reported by various authors (Geerdink *et al*., 1996; Boopathy, 2004; Pone *et al*., 2007; Mariano *et al*., 2008; Svobodova *et al*., 2012; Wagner and Tlotleng, 2012; Chaubey *et al*., 2012). Due to the negative effects fossil fuels have on the environment, a lot of attention has been drawn towards developing cost effective strategies that can be used to clean these contaminated environments. Techniques such as incineration, disposal in landfills (which is very common) (Sarkar *et al*., 2005), thermal desorption (Yao *et al*., 2012), volatilization and weathering are some physical methods used in combating contaminants in the environment. The use of incineration and landfills has been discouraged because they are very expensive and result in air pollution (Ting *et al*., 1999). Chemical methods on the other hand are more effective than any other method but the disadvantage is that they are expensive and require high energy demand and consumption of many chemical reagents (Mrozik and Piotrowska-Seget, 2010). The use of biological methods in degrading fossil fuel contaminated sites has been widely accepted due to the fact that there is no deteriorating effect on the environment, it is also less expensive, efficient and versatile compared to other techniques (Cerniglia, 1993; Margesin and Schinner, 2001; Haghighat *et al*., 2008; Nwuche and Ugoji, 2008; Atlas and Bragg, 2009; Agbozu and Opuene, 2009; Balachandran *et al*., 2012; Chemlal *et al*., 2012; Pasumarthi *et al*., 2013). This method has been practiced from the early stages of petroleum exploration (Mesle *et al*., 2013). Isolation of microbes that are capable of degrading fossil fuels was first carried out from an oil reservoir as early as 1926 (Bastin *et al*., 1926). According to Mesle *et al*. (2013), it is only recently that the ecological significance and extent of microbial activity in the deep coal deposits and oil reservoirs has been recognized. Biological techniques employed in cleaning up fossil fuel contaminated sites include phytoremediation, bioreactors, biostimulation and bioaugmentation.

Phytoremediation is an emerging technology which uses various plants to extract, contain, degrade, and/or immobilize contaminants (Wang *et al*., 2011). The mechanism behind this action has to do with plant roots having affinity for contaminants in soils. When plants grow in contaminated soils, they tend to draw moisture from beneath the earth to the surface of the earth thereby, making the contaminants available for microorganisms to degrade. Microorganisms such as fungi and bacteria in the rhizosphere of particular plants also carry out degradation. Some plants are able to concentrate certain elements from the environment, thereby offering a permanent means of remediation. Harvesting of plant tissues rich in accumulated contaminants is carried out and safely processed. There are advantages and disadvantages that go with this technology. A major advantage is that phytoremediation avoids dramatic landscape disruption and preserves the ecosystem since it is done in-situ (Lasat, 2002). Phytoremediation processes include phytoextraction, rhizofiltration, phytostabilization, rhizodegradation, phytodegradation and phytovolatilization (Wang *et al*., 2011). Rhizodegradation is the breakdown of organic contaminants in soil through microbial activity which is enhanced by the presence of the root zone (EPA, 2003). While the use of higher plants to remove inorganic contaminants primarily

metals, from polluted soil is termed phytoextraction (Lasat, 2002). Rhizofiltration refers to the use of plant roots to absorb, concentrate, and precipitate toxic metals from contaminated groundwater (Khilji and Bareen, 2009). Phytovolatilization has been tagged "controversial" because it makes use of naturally occurring or genetically modified plants that are capable of absorbing elemental forms of metals from the soil, biologically converting them into gaseous species within the plant, and releasing them into the atmosphere (Sakakibara *et al*., 2007).

Bioreactors are vessels which carry out chemical processes involving organisms or biochemically active substances derived from such organisms and they are normally employed in waste water remediation. The term biostimulation is an enhanced bioremediation technology that is often used to describe the addition of electron acceptors, electron donors, or nutrients to stimulate naturally occurring microbial populations (Kanissery and Sims, 2011) in a contaminated environment. Biostimulation of contaminated sites has been carried out by numerous researchers such as Atagana (2006) and Mancera-Lopez *et al*. (2008). In their experiments, they observed that the biostimulation method was more efficient than any other biological method because it is cost effective. However, other reports have shown that the reason why this method is preferred to other methods is due to the fact that contaminant-degrading microorganisms are not sourced elsewhere but stimulated right on site (Maki *et al*., 1999; Juteau *et al*., 2003).

Bioaugmentation technology has been a subject of interest based on the controversy surrounding it. So many authors have come up with their own definition of the subject matter. The term used to describe the addition of cultured microorganism to the subsurface that are capable of biodegrading or transforming specific groundwater/soil contaminants is known as bioaugmentation. However, Megharaj *et al*. (2011) defined bioaugmentation as the introduction of microorganisms into contaminated media to promote the degradation of contaminants. The above definition opens a lot of questions relating the specificity of the organisms to be introduced into the contaminated media. In some cases certain microorganisms are more specialized at degrading specific target contaminants, i.e some microbes can degrade crude oil but not coal or vice-versa. A second school of thought sees bioaugmentation in a different way. They defined bioaugmentation as the applications of indigenous or allochthonous wild-type or genetically modified microorganisms to polluted hazardous waste sites in order to accelerate the removal of undesired compounds (Mrozik and Piotrowska-Seget, 2010). This definition also raises many questions relating to the microorganisms. With reference to bioaugmentation for the degradation of petroleum hydrocarbons or any aerobically degradable contaminant in any contaminated environment, it is rare if ever that the addition or augmentation of aerobic degraders is required to facilitate aerobic biodegradation processes. As a result, remediation industry practices have shifted toward a more prescriptive approach to bioaugmentation to achieve cost-savings and accelerate site remediation.

Different microorganisms such as bacteria and fungi have been used successfully in carrying out bioaugmentation strategies on different contaminated sites. Fungi have been the organism of choice for most bioaugmentation studies (D'Annibale *et al*., 2006). The reason why fungi are used is due to their ability in synthesizing relatively unspecific enzymes involved in cellulose and lignin decay that can degrade high molecular weight, complex and more recalcitrant toxic compounds, including aromatic structures (Mancera-Lopez *et al*., 2008). However, Sutherland (1992) went further to explain how fungi degrade hydrocarbons indirectly by co-metabolism stating that fungi generally do not utilize PAHs as their sole carbon and energy source but transform these compounds co-metabolically to detoxified metabolites. Different fungal species have been implicated in bioaugmentation studies involving both LMW and HMW PAHs in soils. For instance, Mancera-Lopez *et al*. (2008) carried out studies on petroleum hydrocarbon contaminated soils using *Rhizopus sp*., *Penicillium funiculosum* and *Aspergillus sydowii* isolated from two aged soils contaminated with petroleum hydrocarbons. Their end result was that each of the fungus was able to degrade PAHs effectively when compared to biostimulated soils. Published reports on the other hand have shown the inability or difficulty of bacteria to degrade HMW PAHs but are effective in degrading LMW PAHs (Boonchan *et al*., 2000).

Addition of nutrients to environments that are contaminated with hydrocarbons to enhance biodegradation has been reported (Xu and Obbard, 2003; Igbinigie *et al*., 2010; Rocchetti *et al*., 2011; Das and Chandran, 2011; Towell *et al*., 2011; Muter *et al*., 2012; Abioye *et al*., 2012; Chang *et al*., 2013). According to Das and Chandran (2011), the importance of nutrients with regard to biodegradation of hydrocarbon pollutants especially nitrogen, phosphorus and in some cases iron, cannot be overlooked. Different types of nutrient amendment such as poultry manure (Okolo *et al*., 2005), low molecular weight organic (LMO) fertilizers (Igbinigie *et al*., 2010), yeast extract (Wu *et al*., 2011), algal biomass (Hong *et al*., 2008; Jin *et al*., 2011) and plant extracts (Muter *et al*., 2012) have been used over the years.

Coal biodegradation

The degradation of coal has been described as a complex process which involves chemical, physical and biological processes (Cimadevilla *et al*., 2003). For the purpose of this work, biological degradation of coal will be discussed extensively. For so many years, several researchers have shown interest in using microorganisms to degrade coal (Strandberg and Lewis, 1987) but only a few have actually considered the possibility of microorganisms being able to modify the structure of coal (Laborda *et al*., 1997). Hard coal and lignite may seem to be resistant to microbial attack from the first view due to the complex chemical and physical structure of coal (Laborda *et al*., 1997; Klein *et al*., 2001). It was however not until Cohen and Gabriele (1982) reported a breakthrough in an experiment carried out on degradation of coal by the fungi *Polyporus versicolor* and *Poria monticola* that researchers started carrying out a series of studies to understand coal degradation mechanisms. Different classes of microbial and enzymatic transformation of coal (especially low ranked coal) have been reported which include

solubilisation, depolymerisation, polymerisation and decolourisation (Ralph and Catcheside, 1997) with the bulk of the reports on solubilization (Strandberg and Lewis, 1987; Yin *et al*., 2009; Yin *et al*., 2011) and depolymerization (Selvi *et al*., 1997; Fakoussa and Hofrichter, 1999).

According to Yin *et al*. (2011), three generally acceptable mechanisms are involved in each class of coal bio-transformation. These mechanisms are attributed to attack by enzymes (Willmann and Fakoussa, 1997), alkaline chemicals (Quigley *et al*., 1988) and surfactants (Singh and Tripathi, 2013). A wide range of enzymes which are secreted by microorganisms that have the ability to degrade coal have been highlighted by Sekhohola *et al.* (2013).

Due to the similarities in chemical structure between coal and lignin (Fakoussa and Hofrichter, 1999), most reports on enzymatic degradation of low rank coals have been on lignin-degrading enzymes such as lignin peroxidase, manganese peroxidase and laccase (Sekhohola *et al*., 2013). These enzymes are mostly secreted by fungi, which makes fungi the most implicated of microorganisms capable of degrading coal (Ralph and Catcheside, 1997). Different fungal strains such as *Poria vaporaria, Pholiota aurivella, Piptoporus betulinus, Fusarium culmorum, Marasmius scorodonius* (Osipowicz *et al*., 1996), *Coprinus sclerotigenis* (Hofrichter *et al*., 1997), *Trichoderma* sp (Pokorný *et al*., 2005), *Neosartorya fischeri* (Igbinigie *et al*., 2008) and *Penicillium chrysogenum* (Haider *et al*., 2012) have been reported to have coal degrading ability (for details on historical overview of advances in coal bioconversion, refer to Edeki and Cowan, 2014, Paper 1 of this thesis). Chemicals of alkaline origin/nature also cause coal degradation because under alkaline conditions, the humic components of low rank-coal can be released (Quigley *et al*., 1988) and when this takes place, it is referred to as bio-solubilization (Yuan *et al*., 2006a). Surfactants on the other hand, always have a role to play during the process of solubilization of low rank coals (Yuan *et al*., 2006b). During degradation of coal, microorganisms tend to release surfactants into the medium thereby decreasing the coal surface tension and then increasing the solubility of coal in aqueous medium (Fakoussa, 1988). Some microorganisms reported in literatures to have surfactant producing abilities include *Bacillus licheniformis* (Polman *et al*., 1994a), *Candida bombicola* (Breckenridge and Polman, 1994) and *Pseudomonas aeruginosa* (Patel and Desai, 1997). It has recently been reported that coal bio-solubilization by surfactant producing microorganisms is as a result of the mutual interaction between coal, enzymes produced by the microorganisms and the surfactants and not just the coal and surfactants alone (Yin *et al*., 2011).

Biodegradation of petroleum hydrocarbons

Products derived from petroleum are the major source of energy for industries and our daily lives. These products are mostly acquired through the fractional distillation of crude oil using a range of temperatures. In countries like South Africa where crude oil is not readily available, these petroleum-based products are derived from coal using the Fischer-Tropsch process. Pollution by petroleum based products has been one of the largest concerns to science and the general public in the last years (Pinedo-Rivilla *et al*., 2009). The products according to Das and

Chandran (2011) find their way into soils, water and air through leaks and accidental spills during exploration, production, refining, transport, and storage of petroleum. Another source of pollution in the environment as highlighted by Chandra *et al*. (2013) is the improper discharge of industrial wastes on lands and into water bodies. Death or mutations through the accumulation of pollutants by plants and animals in PHC contaminated environments has been highlighted (Alvarez and Vogel, 1991). Reports have shown that oil spillage is not restricted to a particular continent but occurs globally (Chandra *et al*., 2013) and because of this, a lot of attention is drawn towards preventing or combating the negative effects of oil spillages. Different remediation strategies used in treating contaminated sites have been highlighted. For instance, incineration and landfill which are currently the acceptable methods for disposing of wastes have been termed 'prohibitively expensive' because of the amount of energy required in the case of incineration and the size of land needed with regard to landfill (Taiwo, 2011). Other physical method used in treating contaminated sites include; evaporation, dispersion, and washing etc., (Sonawdekar, 2012). Unfortunately, these technologies are expensive (Kumari *et al*., 2013) and may lead to incomplete decomposition of contaminants (Yang *et al*., 2000; Sonawdekar, 2012).

Based on this, alternative remediation strategies have been developed which are cheap and do not require energy. Bioremediation, the use of biological entities such as microorganisms which include bacteria, fungi and algae in treating contaminated sites, is now preferred to other methods. Bioaugmentation, biostimulation, phytoremediation and bioreactors are some of the bioremediation strategies employed today in combating contaminants in water, soil and air. Biological remediation technologyfor the treatment of petroleum hydrocarbon contaminated sites has been intensively studied both in controlled conditions and field experiments (Okoh, 2006). It is a natural process that converts/transforms harmful compounds through metabolic or enzymatic activities of microorganisms into carbon dioxide, water and cellular biomass and these contaminants can be degraded into smaller products that can undergo successive degradation until the compound is fully mineralized (Kissin, 1987; Mango, 1997). It involves the use of biological entities such as microorganisms that are capable of using these contaminants as carbon sources to detoxify or remove pollutants from contaminated environment (Medina-Bellver *et al*., 2005; Kumari *et al*., 2013). This process appears to be the most environmentally friendly method for removal of hydrocarbon pollutants (Barathi and Vasudevan, 2001; Balba *et al*., 2002; Urum *et al*., 2003; Liu, 2008; Das and Chandran, 2011). The ranking of PHCs according to susceptibility to microbial degradation can be generally presented as follows: linear alkanes > branched alkanes > small aromatics > cyclic alkanes (Ulrici 2000) while some compounds such as the high PAHs may not be degraded at all (Atlas and Bragg, 2009). Different microorganisms such as bacteria, fungi and algae have been used over the years to detoxify contaminants in the environment (Maletić *et al*., 2013). According to published articles, bacteria are the most active agents in petroleum degradation, and they work as primary degraders of spilled oil in contaminated environments (Das and Chandran, 2011). A comprehensive list of microorganisms used in degrading petroleum hydrocarbons is presented in Paper 1 (Edeki and Cowan, 2014) of this thesis.

Hydrocarbon biodegradation mechanisms and products

The two major mechanisms for petroleum hydrocarbon degradation are aerobic and anaerobic. The process of degrading petroleum hydrocarbons in the presence of oxygen was highlighted as the most rapid process where complete detoxification is achieved (Das and Chandran, 2011; Chandra *et al*., 2013). During aerobic degradation, oxidation is achieved when oxygen is incorporated enzymatically typically catalyzed by oxygenases and peroxidases (Chandra *et al*., 2013; Maletic *et al*., 2013). The conversion of organic pollutants into intermediates of central metabolism via the tricarboxylic acid cycle is achieved as a result of peripheral degradation pathways. Cell growth and biomass accumulation follows through biosynthesis from the central precursor metabolites such as acetyl-CoA, succinate, and pyruvate which is the next step involved in the aerobic degradation mechanism. Gluconeogenesis also helps in the synthesis of sugars required for various biosynthetic pathways and microbial growth. Attachment of microbial cells to the substrates and production of biosurfactants are some other mechanisms involved in aerobic degradation. According to Das and Chandran (2011), the uptake mechanism linked to the attachment of cells to oil droplets is still unknown but the production of biosurfactants has been well studied. Different studies have shown the importance of biosurfactants in the degradation of PHCs in contaminated environments (Cameotra and Singh 2008; Diab and Gamal El Din, 2013). A detailed description of the aerobic degradation pathway is presented in Paper 1 (Edeki and Cowan, 2014) of this thesis.

Unlike the aerobic degradation mechanism, anaerobic degradation is much slower with little understanding of the biochemical mechanisms involved (Holliger and Zehndert, 1996; Haritash and Kaushik, 2009) although these are carried out by anaerobic microorganisms (Maletic *et al*., 2013). Although aerobic processes are rapid, there are associated problems such as the production of overwhelming amounts of biomass which may cause clogging in *in situ* treatment systems with insufficient supply of oxygen. Based on this, alternatives for the bioremediation of hydrocarbon contaminated sites with nitrate, Fe (III), sulphate, and carbon dioxide as electron acceptors was adopted (Holliger and Zehndert, 1996) and this was first conducted and reported by Battermann and Werner (1984). Studies have shown that aliphatic hydrocarbons (Aeckersberg *et al*., 1991) and two- and three-ring PAHs can be degraded anaerobically (Lagenhoff *et al*., 1996; Coates *et al*., 1996; Bregnard *et al*., 1996), but there is lack of evidence for the anaerobic degradation of PAHs with more than three rings (Haritash and Kaushik, 2009) except a few published reports (Foght, 2008).

Mechanisms involves in anaerobic degradation of aliphatic (alkanes) and aromatic (benzene) hydrocarbons

Over the years, it was thought that anaerobic microorganisms contributed marginally to the overall biodegradation of contaminants but recently, anaerobic biodegradation mechanisms have been gaining more attention due to increased information regarding contaminant site conditions and rapid oxygen depletion (Burland and Edwards, 1999). Reports have shown that different

microorganisms such as bacteria, filamentous fungi and yeasts can degrade alkanes, as carbon sources (Wentzel *et al*., 2007). The most implicated microorganisms capable of degrading alkanes are bacteria that have very versatile metabolism which enables them utilize these and other compounds as carbon sources (Margesin *et al*., 2003; Harayama et al., 2004). According to Rojo (2009), alkanes are not preferred growth substrates for the bacteria, which will rather utilize other compounds before turning to alkanes. Alkanes are activated under strict anaerobic conditions through a mechanism that does not rely on O_2 (Widdel and Rabus, 2001) and the metabolic pathways used have been investigated for some strains. During the degradation of alkanes by bacteria, two general mechanisms are used (Callaghan *et al*., 2006). One involves activation of the alkane at a sub-terminal position by addition of fumarate to the alkane, yielding an alkylsuccinate derivative (Fig. 7).

Figure 7. Anaerobic degradation of alkanes. Alkanes are normally activated initially by the addition of fumarate molecule (in red), which is later regenerated. The addition (arrow) of this molecule usually occurs at a sub-terminal position with the products being processed by β-oxidation.

The product of this reaction is subsequently linked to CoA and converted into an acyl-CoA that can be further metabolized by β-oxidation and this reaction is believed to occur through generation of an organic radical intermediate, most likely a glycyl radical (Rabus *et al*., 2001). In the second reaction mechanism, which has been described only for propane, the fumarate molecule is added to one of the terminal carbon atoms of the alkane (Kniemeyer *et al*., 2007). This second mechanism for alkane anaerobic degradation is the carboxylation, mainly developed from the growth pattern of the sulfate-reducing strain Hxd3 (So *et al*., 2003).

With regard to aromatic compounds, the biochemistry of some anaerobic degradation pathways has been studied to some extent; however, the genetic determinants of all these processes and the mechanisms involved in their regulation are much less studied (Sierra-Garcia and de Oliveira, 2013). The pathway for anaerobic benzene degradation is still a subject of considerable debate, with five mechanisms having been proposed for initiating anaerobic attack on benzene (Foght, 2008). Among the five proposed pathways, two have little support in the literature compared to the other three (Fig. 8).

Figure 8. Degradation pathways (a, b and d) proposed for benzene. a, Hydroxylation to form phenol, cyclohexanone, or *p* –hydroxybenzoate and benzoyl-CoA. b, Alkylation to form toluene, followed by fumarate addition to form benzylsuccinate and benzoyl- CoA. The methyl donor may be methyltetrahydrofolate, S-adenosylmethionine or a cobalamin protein. c, Carboxylation to form benzoate (possibly through more than one enzymatic step) and benzoyl- CoA. The carboxyl donor is not likely bicarbonate but may be derived from benzene. Square brackets indicate a postulated intermediate; broken arrows indicate multiple enzymatic steps; open arrows indicate further metabolism.

The most common mode of initial activation of methyl substituted aromatics is fumarate addition. This reaction has been recognized for the activation of several alkyl-substituted benzenes and for *n*-alkanes (Sierra-Garcia and de Oliveira, 2013). According to Coates *et al.* (2002) there is little supporting evidence to show that the large activation energy required to

remove hydrogen from the benzene ring precludes this mechanism for initiating benzene metabolism and proposed a pathway involving initial attack by ring saturation.

Microbial enzymes in bioremediation of pollutants

Any biological catalyst that facilitates the conversion of substrates into products by providing favorable conditions that lowers the activation energy of the reaction is called an enzyme. Enzymes may be proteins or glycoproteins which consist of at least one polypeptide moiety (Karigar and Rao, 2011). Enzyme technology has received increased attention over the years due to improvements in biological remediation techniques (Whiteley and Lee, 2006). The use of microbial enzymes in bioremediation has the same function as many other harsh chemicals such as solvents, with varying pH, moderate temperatures and without production of hazardous wastes (Ruggaber and Talley, 2006). Enzymes are naturally produced by almost every known organism in order to aid processes such as digestion, metabolism and cell synthesis (Ruggaber and Talley, 2006). They may act intracellularly, i.e. in the presence of or inside their originating cells, extracellularly i.e., both in the presence or absence of their originating cells, free i.e., soluble in solution and the catalysis will be homogenous, or immobilized i.e., linked through different conjugates to a solid matrix and catalysis will be heterogeneous (Gianfreda and Rao, 2004). Published reports have shown that there are six main categories of enzymes including; oxidoreductases, transferases, hydrolases, lyases, isomerases, and ligases (synthetases) (Whiteley and Lee 2006; Karigar and Rao, 2011). Oxidoreductases comprise a large class of enzymes that catalyse biological oxidation/reduction reactions (May, 1999). This category of enzyme is mostly secreted by bacteria, fungi (Gianfreda *et al*., 1999) and higher plants with fungi producing a significant fraction that is of importance to the environment during remediation of pollutants (Bumpous, 1993; Reddy, 1995; Pointing, 2001; Asgher *et al*., 2008; Rubilar *et al*., 2008). They participate in the humification of various phenolic substances that are produced from the decomposition of lignin in a soil environment (Park *et al*., 2006). Some practical examples of oxidoreductases are oxygenases (monooxygenases and dioxygenases) and they participate in the oxidation of reduced substrates by transferring oxygen from molecular oxygen (O_2) utilizing FAD/NADH/NADPH as a co-substrate e.g., cytochrome P450 (Das and Chandran, 2011). Other examples of oxidoreductases are laccase and peroxidase (Karigar and Rao, 2011). Transferases catalyze the transfer of a functional group from a donor to an acceptor (Harms *et al*., 1995). Hydrolases on the other hand facilitate the cleavage of C–C, C–O, C–N, and other bonds by water (Lee and Huang, 2013) and examples from this group of enzyme include lipases, phosphatases, proteases and sulphatases (Whiteley and Lee, 2006). The formation of double bonds by removing chemical groups from a substrate without hydrolysis (or catalysing the addition of chemical groups to double bonds) is being carried out by the group of enzymes called lyases. Isomerases facilitate geometric or structural rearrangements or isomerizations. Finally, ligases catalyze the joining of two molecules.

Biotechnological implication of bioremediation as a tool for treating fossil fuel contaminated sites

Due to man's daily interaction with the environment and the quest for better standards of living, the environment has greatly been altered to an extent that pollution becomes inevitable. The accumulation of pollutants in the tissues of plants and animals which causes death or mutation has been documented (Alvarez and Vogel, 1991). Various types of environmental contaminants such as salts, organic alcohols, phenols, acids, heavy metals such as lead, chromium, copper, cadmium, zinc, mercury etc., PAH, and trace elements have been documented (Pathak *et al*., 2010).

Biotechnological aspects of bioremediation involve the use of enriched microorganisms that have potential in the treatment of pollutants (Singh and Chandran, 2014). According to Halden *et al*. (1999) enrichment techniques offer an opportunity to equip microorganisms known for their ability to survive and be active when they are introduced into contaminated environments with the desired catabolic potential. The biological conversion of solid fossil fuels into value added products such as methane and humic substances has been widely studied (Yin *et al*., 2009) and many literatures have cited the advantages with regard to bioremediation over every other remediation techniques with little or no evidence on the cost and time taken for these conversions to get to their final stages. Some very important aspects of biotechnological conversion or breakdown of fossil fuels which must be considered before and during biodegradation/remediation include;

I. Sources of the microorganisms used for this process

II. Time taken for each microorganism when cultured to get to active phase

III. The source and cost of nutrient additives such as fertilizers applied during the process

IV. The amount (grams or volumes) of microorganism and nutrient needed for effective bioremediation

V. Concentration of contaminants that can be degraded per unit gram/volume of microorganism.

VI. The time it will take for effective bioremediation to be achieved

One of the problems faced by researchers trying to develop biocatalysts with potential degradation ability especially coal, is gaining access to coal mines. This has discouraged many researchers from going into this field due to laws set up by mining authorities in this regard. In this present study, bacteria were isolated from environmental samples sourced from contaminated sites (Sathishkumar *et al*., 2008) of which, only 15 out of 72 bacterial isolates showed metabolic potential after screening and continuous enrichments in both waste coal and

diesel media. An average of 21.50 $g.l^{-1}$ of dried bacterial biomass was produced using this enrichment technique for the purpose of remediation.

Nutrient addition to a contaminated site which is one of the basic criterions for microorganisms to carry out effective remediation has been well documented (Selvi *et al*., 2009). These nutrients may come in the form of organic or inorganic fertilizers. Some examples of inorganic fertilizers which have been used for bioremediation purposes include ammonium nitrate, potassium nitrate, calcium nitrate and urea while organic fertilizers include compost, manure, marine byproducts, meals, minerals and mulch. The nutrient supplement used in the present study was algae biomass which was sourced from an integrated algae pond system (IAPS) treating municipal sewage and located in our institute (Mambo *et al*. 2014).

Harvested algal biomass was freeze dried and mixed with the different bacterial consortia before application (Un-published Papers 3-6). During bioremediation studies, the optimum concentration of algae nutrient needed by the various bacterial consortia for effective bioremediation was determined. In experiments on waste coal biodegradation using bacterial consortia and *Neosartorya fischeri* ECCN 84 (Paper 3), dried algal biomass (4.2 g) was mixed with 2 kg of waste coal and inoculated with 20 mL bacterial cultures and parameters such as concentrations of HA- and FA-like substances, increase in WHC, EC, CEC and reduction in bulk density were monitored for nine months. Obtained results showed the steady increase in HA-and FA-like substances, WHC, EC, CEC and decompaction of waste coal. With the above results obtained, it could be said that for a waste coal dump site of one hectare, 210 kg of dried algal biomass mixed with 1000 L (3.20×10^6 - 3.86×10^6 CFU/mL) bacterial consortium (Paper 3), will be used to effectively carry out rehabilitation under a one year period while it will take a mixture of 125 kg of dried algal biomass and 500 L of bacterial consortium (3.20×10^6 - 3.86×10^6 CFU/mL) (Paper 5) to degrade petroleum hydrocarbon (1500 $g.kg^{-1}$ of dried soil) in one hectare of land within 45 days.

Research objectives

For a proper understanding and acceptance of biodegradation as a cost effective method for remediating fossil fuel contaminated sites, there is a need to study the relationships and assess the mechanisms by which various bacterial communities carry out degradation under different conditions. The objective of this doctoral dissertation was:

- To isolate and enrich bacteria from fossil fuel contaminated sites which have degradation potentials.
- To integrate the isolated bacteria with already known fungal strain that has biodegradation ability in order to provide new insight and knowledge into the function of microbial communities in these environments.

Specific goals were set up within these overall objectives and these goals include:

- The formulation of bacterial consortia that can effectively degrade both liquid and solid fossil fuels.
- To develop different bacterial consortia that have mutualistic co-habitation relationships with an already isolated fungal strain which can effectively biosolubilze waste coal.
- The determination of optimum conditions required by these bacteria to effectively degrade fossil fuels.

In order to address these goals, experiments were set up which ranged from flasks studies containing liquid petroleum hydrocarbons (BTEX and diesel) and waste coal as the sole carbon sources to greenhouse studies which comprised soil containing diesel and/or waste coal to simulate contaminated sites in the natural environment. Different techniques were used in this study with regard to waste coal degradation to monitor and determine the attachment of bacteria to coal particles and these techniques include scanning electron microscopy, FT-IR, alkaline extraction of HA- and FA-like substances while GC-MS analysis was used in the case of PHC. However, molecular characterization of isolated microbes bioprospected from both waste coal and PHC contaminated sites was the first step undertaken in this study.

Hypothesis

The efficient colonization and utilization of coal and petroleum hydrocarbon wastes as substrates for bacterial growth is enhanced in the presence nutrients such as algae, thereby promoting biodegradation of fossil fuel wastes to increase the rate of coal dump decompaction through formation of value added products such as humic-like substances for use as soil conditioners in agriculture.

Results and discussion

Bacterial degradation of waste coal

In the present study, a total of 72 bacterial strains were isolated from environmental samples which were collected from the root zones of grasses growing on coal dumps in the Witbank coal mining area and diesel contaminated soils sourced from a motor mechanic workshop in Grahamstown all in South Africa and initial screening test was carried out to determine the potential of each bacterial isolate to utilize waste coal, BTEX and diesel as carbon sources. Bacteria known for their ability to degrade HMW PAH such as *P.aeruginosa, P.putida* and *B.subtilis* (Grishchenkov et al., 2000; Rahman et al., 2002; Mrozik and Piotrowska-Seget, 2010; Hemalatha and Veeramanikandan, 2011; Jiang et al., 2013; Pasumarthi et al., 2013; Diab and Gamal El Din, 2013) were used as positive controls. Results obtained from this screening showed that 15 of the 72 isolates and the positive controls were able to proliferate in minimal medium with waste coal, BTEX and diesel as carbon sources.

Experiments on waste coal biodegradation were carried out using each bacterial isolate with the aim of establishing bacterial consortia that can convert waste coal into soluble products such as humic substances (humic- and fulvic-like substances). Waste coal Experiments were carried out in aqueous media and incubated for 21 days during which, analysis were carried to determine the concentrations of humic acid- and fulvic acid-like substances produced as a result of the action of bacteria on waste coal. Results obtained at the end of experiment showed that the concentrations of humic-like and fulvic-like substances produced from waste coal (WC) as a result of the action of single bacterial cultures on coal was insignificant when compared to that obtained from un-inoculated (negative) controls and this result was confirmed by analyzing each extract (already freeze dried humic acid-like and fulvic acid-like substances) and waste coal pellets using an FTIR PerkinElmer Spectrum 100 instrument (PerkinElmer, Waltham, MA).

With the above result, different bacterial consortia were established to enhance the biodegradability of coal and each consortium was made of 2 or 3 bacterial isolates mixed together in the ratio of either 1:1 (g/g) or 1:1:1 (g/g/g). A bacterial consortium (positive control) was also established by carefully and aseptically mixing the 3 bacterial isolates *P.aeruginosa, P.putida* and *B.subtilis* in the ratio of 1:1:1 (g/g/g).

Pre-treatment of coal prior to inoculation using nitric acid has been reported by various authors. For instance, Xiu-xiang *et al*. (2009) used three different pre-treatment steps during an experiment on bio-solubilization of Chinese lignite which was carried out to analyze extra-cellular protein produced by inoculated microorganisms. Most experiments conducted in this regard were carried out on agar plates and dark liquids (soluble products) were extracted from the plate over time for analysis (Stewart *et al*., 1990; Basüaran *et al*., 2003). According to literatures, the reason for pre-treating coal was for easy oxidation of coal surfaces prior to attack by microorganisms (Kai-yia *et al*., 2009). However, other researchers have carried out

experiments on the biosolubilzation of low rank coals without pre-treating the coal (Haider *et al*., 2012) and this method was what was adopted in this study because the minimal medium used already contained oxygen which will be supplied to the coal surface and be used in turn by the microorganisms to degrade coal. Another reason why the waste coal used in this study was not pre-treated, was to simulate the natural environment knowing fully well that these organisms will be used on a commercial scale in treating waste coal dumps. At the end of the experiment, coal pellets were separated from liquid culture media and humic-like substances were extracted from the liquid culture as described in Paper 3. Results obtained from FT-IR analysis of waste coal from un-inoculated control, revealed no substantial change in functional groups but in the presence of bacterial consortia, significant shifts in functional groups were observed for waste coal exposed to either a consortium of *P. aeruginosa*, *P. putida* and *B. subtilis* or ECCN 13b. Similar shifts in the spectra of waste coal substrates were also observed after inoculation with ECCN 7b and ECCN 10b and at the end of the study, it was observed that waste coal was effectively degraded by different bacterial consortia due to the formation of new functional groups within the waste coal.

The mutual relationship between various bacterial isolates and *Neosartorya fischeri* was ascertained by inoculating spores of the fungus; ECCN 84 on agar agar plates made up of extracts from various aqueous solutions containing waste coal which had already been degraded by different bacterial consortia while un-inoculated controls from aqueous media were also used on agar plates. After 7 days post inoculation, fungal growth was observed in all the plates that were enriched with waste coal extracts which had already been degraded by bacteria. No fungal growth was observed in the plates that had waste coal extracts used as control.

Furthermore, studies on the biodegradation of waste coal as a solid substrate using bacterial consortia with or without the fungus; *Neosartorya fischeri* (ECCN 84) was carried out and during this experiment, water holding capacity (WHC) was determined and initial result before inoculation suggested that the WHC (%) of waste coal was 33.55 ± 3.4. However at the end of experiment, it was observed that the bacterial consortia; ECCN 13b in combination with the fungal strain; ECCN 84 had the highest value for WHC. Similar results were observed by Juwarkar *et al*. (2009) in an experiment carried out on developmental strategies for sustainable ecosystem on mine spoil dumps. WHC of waste coal inoculated with bacterial consortia alone did not increase significantly when compared to that of bacterial consortia/fungal mix.

Prior to this experiment, high bulk density was recorded and this was due to compaction, a characteristic of low levels of aeration on mine spoils (Juwarkar and Jambhulkar, 2008). However with the inoculation of different microorganisms into waste coal, an observed reduction in bulk density was recorded. Researchers such as Juwarkar and Jambhulkar (2007) and Chaubey *et al*. (2012) have observed same reduction in bulk density in their experiments and they concluded that the reduction was mainly due to the action of the inoculated microorganisms which were stimulated by the addition of nutrients.

Increase in electrical conductivity (EC) on the other hand was recorded in waste coal inoculated with bacterial consortia with or without the fungal strain ECCN 84 with consortium ECCN 13b/84 having the highest EC value. According to Chaubey *et al.* (2012), there is a direct relationship between EC and WHC of mine spoils. As the EC values increase, there is a proportionate increase in WHC and this was observed in this study. However, when there is high percentage of water on the dumps, leaching of minerals from overburdens/mine spoils becomes inevitable and this reduces the EC value thereby having negative effects on the fertility levels of the mine spoils (Sadhu *et al*., 2012).

The bioconversion of coal into value added products such as humic acid- and fulvic acid-like substances was also monitored in this study (Paper 3) and results obtained showed that the bacterial consortia; ECCN 10b and ECCN 13b in combination with the fungal strain; ECCN 84, had the highest concentrations of these substances when compared to bacterial consortia or the fungal strain alone. Mukasa-Mugerwa *et at.* (2011) observed the formation of both humic acid- and fulvic acid-like substances over time in an experiment that was carried out to determine the role of a plant/fungal consortium in the degradation of bituminous hard coal. An increase in the formation of humic and fulvic acids over time has also been documented in an experiment carried out by Gonzalez-sangregorio *et al*. (1991) on the early stages of lignite mine soil genesis. A positive correlation between WHC, CEC and HA has been documented (Amir *et al*., 2006) and this positive correlation was also observed in this study (Paper 3) which showed the various increases in WHC and CEC of waste coal. FA-like substances on the other hand were extracted from the formed HA-like substances in waste coal as a result of the action of both bacteria and fungus and results showed that FA-like substances were formed in all the waste coal inoculated with bacterial consortia with or without the fungal strain; ECCN 84.

Bacterial degradation of petroleum hydrocarbons

Several studies have shown that during the bioconversion of coal to liquid petroleum, several products such as diesel, petrol, paraffin, etc., are derived and these products can also cause serious problems when introduced into the environment either through accidental spills and leaks (Kobayashi and Rittman, 1982; Smith and Dragun, 1984; Pena-Castro *et al*., 2006; Xia *et al*., 2006: Sharma and Rehman, 2009; Padayachee and Lin 2011) or human activities (Das and Chandran, 2011).

In this present work (Paper 4 and 5), bacterial consortia capable of degrading waste coal were examined for their ability to degrade petroleum hydrocarbons both in aqueous media and solid media. Screening of bacterial strains in BHM containing benzene, toluene, ethylbenzene, xylene (BTEX) was carried out and obtained results revealed that only 6 strains successfully increased medium turbidity suggesting hydrocarbon breakdown.

Tolerance of each strain to different concentrations of BTEX was monitored and results obtained showed that BTEX metabolism by each strain peaked at concentrations between 1000 and 2000

mg.l^{-1} suggesting that the optimum concentration of BTEX that each bacterium can tolerate is ~1500 mg.l^{-1}. Inhibition of bacterial metabolic activity at higher concentrations of BTEX has also been reported when biodegradation experiment was carried on BTEX using a bacterial consortium made up of *Pseudomonas putida* and *Pseudomonas fluorescens* under hypoxic conditions (Shim *et al*., 2005). A lower optimum concentration of 100 mg.l^{-1} has also been reported (Shim *et al*., 2002; Otenio *et al*., 2005) and as a consequence, it was concluded that the rate of of biodegradation was dependent on the concentrations of benzene, toluene, and *m*-xylene in the culture medium at the end of this study. Similar conclusion was also made by Mukherjee and Bordoloi (2012) when they carried out an experiment on the biodegradation of benzene, toluene, and xylene (BTX) in liquid culture and in soil by *Bacillus subtilis* and *Pseudomonas aeruginosa* strains and a formulated bacterial consortium. With the establishment of different bacterial consortia, effective BTEX degradation was observed and chromatographic results showed a constant and rapid reduction in BTEX within 6 days although this result was only observed in BTEX media that were inoculated with three different bacterial consortia.

Based on the observed results above, each bacterial isolate and consortia that were able to degrade BTEX were used as biocatalysts in an effort to demonstrate degradation of a more complex substrate (diesel) both in aqueous and solid media (Paper 5). For the purpose of achieving efficient biodegradation of diesel in aqueous media, bacterial strains and established consortia used in Paper 4 were inoculated in MSM with diesel as the sole carbon source. The establishment of different consortia used in this study, was in line with earlier experiments carried out by Cohen *et al*. (2002), Sharma and Rehman (2009), and Moliterni *et al*. (2012). Two commercial biocatalysts (termed C-1 and C-2) commonly used in rehabilitation exercises to degrade petroleum hydrocarbons in aqueous and solid media were also used in this study as positive controls. Based on comprehensive physicochemical analysis of the organic composition of diesel fuel (Liang *et al*., 2005), various compounds such as toluene which represents the alkylbenzene, and pentadecane and hexadecane which represent the *n*-alkanes were selected as the 'organic fingerprints' with which to evaluate biodegradation. Results from experiments using diesel in aqueous media showed that the two commercial products, C-1 and C-2 behaved similarly and that consumption of pentadecane, hexadecane and toluene by these products was not significantly different ($p>0.05$)) from the un-inoculated control while the bacterial consortium, ECCN 13b, was able to completely degrade diesel.

During the course of this study, it was observed that when bacterial strains or consortia were inoculated into different substrates without the addition of nutrients, the degradation process was slow and incomplete. However with the addition of nutrients such as algae, complete degradation was achieved. Different researchers have emphasised the importance of nutrients during biodegradation of contaminants (Chaineau *et al*., 2005; Wrenn *et al*., 2006: Östberg *et al*., 2007). Moreover, during microbial degradation of organic pollutants such as coal and PHC, a combination of two or more microorganisms in consortium, appears to be more efficient than the individual strains. For instance, Sathishkumar *et al.* (2008) conducted an experiment on

biodegradation of crude oil by individual bacterial strains and a mixed bacterial consortium isolated from hydrocarbon contaminated areas and concluded that the mixed bacterial consortium showed more growth and degradation than did individual strains. Researchers such as Ghazali *et al.* (2004), Arulazhagan *et al*. (2010) and Malik and Ahmed (2012) have also recorded 95% degradation rates when microbial consortia were used.

Conclusions and future work

The main objective of this study was to demonstrate the efficiency of different bacterial strains/consortia in degrading fossil fuel wastes such as waste coal and diesel. This objective was met based on positive results obtained during the course of this study. In this regard, the hypothesis on which this study was based that the efficient colonization and utilization of coal and petroleum hydrocarbon wastes as substrates for bacterial growth is enhanced in the presence nutrients such as algae, thereby promoting biodegradation of fossil fuel wastes to increase the rate of coal dump decompaction through formation of value added products such as humic-like substances for use as soil conditioners in agriculture, was effectively demonstrated. Based on this, the various techniques/methods such as the sterilization of coal using freeze thawing method in this study were carefully chosen and adopted to curb the rigorous and complex approaches such as the oxidation/pre-treatment of coal outlined in the literature. Although the above demonstrations were successfully carried out during this study, many questions were raised with regard to the different mechanisms involved in the degradation of these substrates and the enzymes secreted by the different bacteria during this process. Although it can be argued that FT-IR spectra and SEM analysis of coal during this study showed the effective degradation of waste coal, various metabolic pathways during degradation needs to be established for proper understanding of the process. Based on this, it is suggested that future work needs to be considered in which the bacterial consortia used in this study will be applied on a commercial scale whereby the various mechanisms involved in the biodegradation process can be monitored coupled with the enzymes involved.

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Paper 1

Approaches to Bioremediation of Fossil Fuel Contaminated Soil: An Overview

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Approaches to bioremediation of fossil fuel contaminated soil: an overview

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A reliance on fossil fuels as a source of energy has resulted in the generation of pollutants which have entered the environment. Health of humans, animals, plants and microorganisms has been compromised due to activities linked to fossil fuel extraction, processing and use. Coal conversion to value added products has been investigated in an effort to reduce the cumulative effects of waste generated during mining. Clean coal technology, developed to convert coal into value added products with reduced pollution, has been a major source of liquid petroleum in South Africa. Although the conversion process, neither generates waste nor pollutes the soil environment, the final products either through accidental or deliberate spillage can have a severe and protracted impact. Biological methods for combating pollutants generated within the fossil fuels sector are preferred to mechanical or physicochemical practices. This is due to the production of non- or less toxic by-products, cost effectiveness and safety. In this manuscript, an overview of the approaches adopted and factors influencing microbial metabolism of fossil fuel contaminants in soil and water bodies is presented. In particular, emphasis is placed on bacteria as biocatalysts of choice and their ability to degrade waste coal and liquid petroleum hydrocarbons.

Key words: Fossil fuels, coal, petroleum hydrocarbons, biodegradation, pollutants.

INTRODUCTION

Fossil fuels are natural substances formed from the remains of ancient plants and animals. Over time, heat and pressure converted these remains into fuels which release energy when burned. The term fossil fuel also includes hydrocarbon-containing natural resources that are not derived from animal or plant sources. These are sometimes called mineral fuels. For the purpose of this review, the hydrocarbons derived from decayed plants and animals will be referred to as fossil fuels. The age of these ancient plant and animal fossil fuels is typically millions of years, and in some cases, in excess of 650 million years (Mann et al., 2003). Different types of fossil fuels are formed depending on the combination of animal and plant debris present. However, the length of time for which the material was buried and the temperature and pressure during decomposition also contributed to the type of fossil fuel formed. Fossil fuel has been broadly divided into 3 categories based on the mode of its formation. These are solid,

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liquid and gaseous fossil fuels and each is characterized by a high carbon and hydrogen content. Within these categories are volatile materials with low carbon:hydrogen ratios such as methane, liquid petroleum and the non-volatile materials composed almost of pure carbon, like anthracite coal. Fossil fuels have played an important role in providing energy for transportation, power generation, industrial growth, agricultural production and other basic human needs (Basha et al., 2009). Irrespective of the major roles that fossil fuels have played in sustaining the global economy, combustion of these fuels is a major source of anthropogenic CO_2 emissions (Muradov, 2001). For the purpose of this review, only the solid and liquid fossil fuels will be discussed.

Fossil fuel resources are generally a major source of revenue for the main oil and gas producing countries in Africa (Zalik and Watts, 2006). South Africa, which is one of the world's largest producers (5th) and consumers (7th) of fossil fuels (BP, 2012), has experienced a boost in her economy due to the production, consumption and exportation of coal (UNECA, 2011). Increased production over the last 30 years and an over reliance on coal as a source of energy has stimulated revenue accrual (ERC, 2004). BP statistics in 2011 showed that Africa has enormous potential in the fossil fuels sector with proven reserves accounting for about 9.5, 8 and 4% of the crude oil, natural gas and coal in the world, respectively (BP, 2011). The generation of electricity from fossil fuels cannot be neglected as more than 80% of electricity generated across the continent of Africa is from fossil fuels (IEA, 2011). The generation and supply of energy from fossil fuels has also been documented. IEA, in their 2011 annual report, stated that fossil fuels account for about 50% of the total energy supply and one-third of the energy consumed (IEA, 2011).

Huge problems have emerged due to an over reliance on fossil fuels and when viewed from an environmental and social perspective, it affects societies locally, regionally and globally (UNECA, 2011). Some of these problems include ozone depletion, global warming, acidification, and depletion of non-renewable resources. According to Höök and Tang (2013), energy production is the principal contributor to release of greenhouse gases, in particular CO_2, with fossil fuel combustion the major source. Of the 3 categories of fossil fuels, liquids (petroleum) and solids (coal) are the major contaminants in the environment. Any unwanted substance introduced into the environment is referred to as a 'contaminant' and the deleterious effects of these contaminants leads to 'pollution', a process in which a resource (natural or man-made) is rendered unfit for use, more often than not, by humans (Megharaj et al., 2011).

The drastic increase in the demand for coal has led to an increase in mining of this natural resource in countries like South Africa with subsequent generation of wastes and an increase in the level of pollution. One of the basic reasons behind the increase in demand for coal is due to the various products derived from coal during its conversion processes. For instance, in South Africa where liquid and gaseous fossil fuels are not

readily available, coal liquefaction is one option available for obtaining these products. However, the utilization of coal and coal derived products is associated with serious environmental problems from the mining stage through to its final utilization by consumers (Geo-4, 2007). To reduce environmental damage by this energy source, new conversion technologies are urgently needed. One of the strategies adopted in reducing environmental damage is clean coal technology. Clean coal technologies which make use of biological processes to effect pollutant biodegradation have received considerable attention in recent years (Klein et al., 2008; Sekhohola et al., 2013). Similarly, the use of biocatalysts to remediate liquid petroleum hydrocarbon and diesel contaminated sites has been the subject of much recent attention (Sander et al., 2010; Diya'uddeen et al., 2011; Vaidehi and Kulkarni, 2012; Elazhari-Ali et al., 2013; Kang, 2014). In this paper, we present an overview of some of the approaches used in the biodegradation of coal, coal related contaminants, and liquid hydrocarbon pollutants in an effort to stimulate the search for and emergence of successful bioremediation strategies.

BIODEGRADATION OF COAL AND COAL-RELATED PRODUCTS

Studies on the breakdown of coal by bacteria and fungi started as far back as 1920 (Olson and Brinckman, 1986). Although, it was accepted that microorganisms are capable of degrading coal, significant research effort occurred only after demonstration of the successful breakdown of coal by bacteria (Fakoussa, 1981). One year later, Cohen and Gabriele (1982) demonstrated the breakdown of low rank leonardite using wood rot fungi. Following these breakthroughs, intensive study by various research groups was carried out with the aim of establishing a better understanding of the mechanisms involved in the biological transformation of coal and in combination with the production of value-added products ((Polman et al., 1994; Fakoussa and Frost, 1999; Fakoussa and Hofrichter, 1999; Gotz and Fakoussa, 1999; Ralph and Catcheside, 1999; Machnikowska et al., 2002; Igbinigie et al., 2008; Jiang et al., 2013).

The complexity and recalcitrance of coal suggested initially that microorganisms might not be able to modify the physicochemical structure of this substrate. Thus, and according to Klein et al. (2008), the colonization and breakdown of coal by microorganisms was not possible unless certain necessary conditions such as moisture content, mineral salt availability, additional nitrogen sources and a stable pH were met. To date, a number of microorganisms have been identified as being able to modify the structure of coal (Yuan et al., 2006). Different mechanisms as suggested by various authors appear to be used to achieve modification of the coal structure and these include enzymatic changes (Cohen et al., 1987; Pyne et al., 1987; Fakoussa and Hofrichter, 1999), alkaline solubilisation (Strandberg and Lewis, 1987; Quigley et al.,

1989a), metal ion chelation, and the action of surfactants (Fakoussa, 1988; Quigley et al., 1989b; Fredrickson et al., 1990).

Enzymatic modification of coal structure

A large number of biological molecules responsible for many chemical interconversions have been linked to the structural modification of coal otherwise called depolymerization (Hofrichter and Fakoussa, 2001). The depolymerization of brown coal occurs at low pH values (pH 3-6) resulting in the cleavage of bonds inside the coal molecular structure which leads to the formation of yellowish, fulvic-like substances with low molecular mass (Hofrichter and Fakoussa, 2001). Although a wide range of enzymes with coal degrading ability have been identified the majority appear to be from fungi. For instance, Sekhohola et al. (2013) provided a detailed list of the purported catalysts used in coal biodegradation which shows that nearly all of the enzymes that have been linked to coal biodegradation are of fungal origin. Even so, contradictory reports have been published with regard to fungal activity and breakdown of coal (Torzilli and Isbister, 1994). For instance, studies by Cohen et al. (1987) initially suggested that the ability of fungi to degrade coal was the result of enzymatic activity. However, in a subsequent report, these authors identified the coal solubilizing agent from *T. versicolor* by infrared spectroscopy and x-ray studies as ammonium oxalate monohydrate (Cohen et al., 1990) while Fredrickson et al. (1990) argued that the coal solubilizing activity of *T. versicolor* was not ammonium oxalate monohydrate but a siderophore-like compound. In addition to fungi, several gram positive and negative bacteria have been implicated in the biodegradation of coal. Studies by Crawford and Gupta (1991) demonstrated that extracellular bacterial enzymes were capable of depolymerizing a soluble coal polymer although the enzymes involved were neither specified nor identified. Nevertheless, the depolymerisation process appears to be non-oxidative which may indicate that non-oxidative, enzymatic depolymerization of coal is possible. Reports on the utilization of low rank coal as a source of carbon by several bacteria including *Pseudomonas oleovorans, Rhodococcus ruber* and *Bacillus* sp. Y7 have also been published (Fuchtenbusch and Steinbuchel, 1999; Jiang et al., 2013). The ability of *Bacillus* sp. Y7 to degrade lignite was attributed to the production of extracellular substances (Jiang et al., 2013) while oxidized lignin solubilisation was ~90% in the presence of *Pseudomonas putida* (Machnikowska, 2002). For the latter example however, it was stated that pre-treatment of lignite with nitric acid was essentially responsible for the enhanced rate of biodegradation. In an experiment carried out by Tripathi at al. (2010) on the fungal biosolubilisation of lignite and the subsequent production of humic acid, these authors concluded that the likely mechanism of lignin breakdown by fungi was somehow linked to action of oxidative (peroxidases and laccases) and hydrolytic enzymes (esterases) initially secreted by bacteria confirming an earlier observation based on a comparative study of coal solubilisation by both bacteria and fungi (Torzilli

and Isbister, 1994). Some of the enzymes secreted by fungi which are believed to play a major role in the biodegradation of coal include lignin peroxidase (Hofrichter and Fritsche, 1997b; Laborda et al., 1999), laccase (Fakoussa and Frost, 1999), esterase (Laborda et al., 1999) and phenol oxidase (Laborda et al., 1999) and although their precise role in coal biodegradation remains unclear, a model for the phyto-biodegradation of low rank coal by mutualistic interaction between ligninolytic microorganisms and higher plants has recently been proposed (Sekhohola et al., 2013).

Alkaline substance modification of coal structure

A different mechanism of coal biodegradation has been suggested based upon results which indicate microbial secretion of alkaline substances that facilitate the breakdown of coal (Quigley et al., 1988). During this non-enzymatic process, often the formation of black liquids is observed coincident with higher pH (pH 7-10). The increase in pH has been attributed to the release of alkaline substances by bacteria which aid in coal solubilisation (Hofrichter and Fakoussa, 2001). The actual mechanism of coal biodegradation by bacteria due to alkaline substances is not well defined and as a consequence, not fully understood. Thus, Machnikowska et al. (2002), in an experiment on the microbial degradation of low rank coals, reported an increase in pH of medium containing sub-bituminous coal and suggested that the pH change arose as a result of the production of alkaline substances. Details of the alkaline substances involved however, in this and other studies and the effect of these on coal biodegradation remain obscure. As highlighted by Sekhohola et al. (2013) many different bacteria appear capable of secreting alkaline substances when inoculated into coal media including; *Pseudomonas putida, Arthrobacter sp., Streptomyces viridosporous, Streptomyces setonii, Bacillus pumilus,* and *Bacillus cereus.*

Metal ion chelation and the action of surfactants on the modification of coal

Experiments on coal bio-solubilisation carried out by Yin et al. (2011) pointed to the importance of surfactants in the synthesis of enzymes responsible for coal breakdown. These authors went further and showed that in the absence of surfactants; limited enzymes were adsorbed onto the coal surface while the reverse was the case in the presence of surfactants. Thus, interaction between enzyme and coal is possibly due to the presence of surfactants which modify the charge and the hydrophilic properties of the coal surface (Yin et al., 2011). Nonetheless, studies on the biological breakdown of coal have concentrated on fungi as the biocatalysts of choice and very few reports have examined the contribution by bacteria. A summary of the historical progress made so far in the field of coal biodegradation is presented in Table 1.

BIODEGRADATION OF PETROLEUM HYDROCARBONS

The biosolubilisation of coal and the serial production of liquid fuels has been investigated (Ackerson et al., 1990). In this report, bio-extracts from solubilized coal were converted to liquid alcohols, one of the earliest clean coal technologies for petroleum production. In South Africa many petroleum products are derived from coal using Fischer-Tropsch synthesis including fuels, plastics, oils, synthetic rubbers etc. Globally, there is high demand for petroleum products (Hasan et al., 2010) and during transportation of these from point of production to point of consumption spillage is inevitable (Das and Chandran, 2011). It has been estimated that natural crude oil seepage exceeds 600000 metric tons per year with a range of uncertainty of 200000 metric tons per year (Kvenvolden and Cooper, 2003). Accidental or deliberate release of crude oil into the environment has also led to serious pollution which affects both water and soil resources (Atlas, 1981; Okoh, 2006). Just like coal, different strategies including mechanical, chemical and biological have been developed and used to remediate sites contaminated with these petroleum hydrocarbons (Lohi et al., 2008).

A common mechanical means of remediating petroleum contaminated waters includes floating booms, skimmers, and oil-water separators (Ventikos, 2004; Yang et al., 2000). Unfortunately, removal of spilled oils from contaminated sites by these means is usually incomplete leading to progressive accumulation of residual hydrocarbons (Yang et al., 2000). Chemical remediation of oil contaminated sites on the other hand has been associated with increased dissolution of oil in seawater, which affects both water bodies and benthic biota (Doerffer, 1992). The reason why this technology is associated with increased dissolution of oil in water is because it makes use of chemical dispersants such as surfactants (Lohi et al., 2008). In contrast to the above, biological remediation technologies which have been intensively studied both in controlled conditions and field experiments (Okoh, 2006), appear to be the most environmentally friendly methods for removal of hydrocarbon pollutants (Barathi and Vasudevan, 2001; Balba et al., 2002; Urum et al., 2003; Liu, 2008; Das and Chandran, 2011). Bioremediation, which is one example of a biological remediation process, has been defined as the use of microorganisms to detoxify or remove pollutants from contaminated water and soil bodies (Medina-Bellver et al., 2005; Mukherjee, 2012) and a comparison of treatment costs for South Africa reveals that it is by far the most economical technology (Table 2).

Different microorganisms including bacteria and fungi have been used to remediate hydrocarbon contaminated sites. Addition of nutrients to an oil spilled site to stimulate the growth of resident microorganisms in degrading contaminants is known as biostimulation while isolation, growth and introduction of microorganisms (that can degrade contaminants) from a different environment into oil spilled sites to remediate those sites is known as bioaugmentation. It has been argued that biostimulation is a

superior technique to bioaugmentation (Alexander, 1999; Van Hamme et al., 2003; Philp and Atlas, 2005; Lohi et al., 2008) based on the outcome of field experiments (Abdulsalam et al., 2011). Studies by Devinny et al. (2000) and Bento et al. (2005) seem to support the above conjecture and show that augmented microorganisms easily lose their intrinsic degradation ability during the time it takes for acclimatisation to the new environment. Different amendments have been used to stimulate resident microorganism populations in oil spilled environments and a summary of these is presented in Table 3.

According to D'Annibale et al, (2006) and Yi et al. (2011), fungi are the organisms of choice with regards to bioaugmentation as these synthesize relatively unspecific enzymes involved in cellulose and lignin decay. Fungal enzymes degrade high molecular weight, complex and more recalcitrant toxic compounds, including aromatic structures (Grinhut et al., 2007; Mancera-Lopez et al., 2008). However, Sutherland (1992) explained how fungi degrade hydrocarbons indirectly by co-metabolism and stated that fungi generally do not utilize petroleum hydrocarbons (PHC) as their sole carbon and energy source but transform these compounds co-metabolically to detoxified metabolites. Different fungal species have been implicated in bio-augmentation studies involving both low and high molecular weight polyaromatic hydrocarbons (PAHs) in soils. For instance, Mancera-Lopez et al. (2008) carried out studies on petroleum hydrocarbon contaminated soils using *Rhizopus sp.*, *Penicillium funiculosum* and *Aspergillus sydowii* isolated from two aged soils contaminated with petroleum hydrocarbons and showed that each fungus was able to degrade PAHs effectively when compared to biostimulated soils. Bacteria on the other hand, though able to degrade aromatic hydrocarbons, only degrade low molecular weight PAHs. Many pure cultures of bacteria, including various strains of *Pseudomonas putida*, have been evaluated for their benzene, toluene and xylene (BTX) biodegradation potential (Jean et al., 2002, 2008). The highest PAHs that bacteria have been recorded to degrade are the PAHs containing four benzene rings such as pyrene and chrysene (Boonchan et al., 2000).

Mukherjee and Bordoloi (2011) reported that remediation of oil spilled sites usually requires the cooperation of more than a single species of microorganism because individual microorganisms can metabolize only a limited range of hydrocarbon substrates. Therefore, assemblages of mixed populations with overall broad enzymatic capabilities are required to energize the rate and extent of petroleum hydrocarbon degradation. Thus, various researchers have shown that consortia comprising bacteria and fungi are better bioaugmentation agents than individual bacterial and fungal isolates (Boonchan et al., 2000; Jacques et al., 2008). Table 4 presents a brief summary of single isolates of bacteria and fungi that are known to degrade aromatic hydrocarbons using bioaugmnetation as a strategy and various consortia of bacteria and fungi that successfully carry out this process.

Aliphatic hydrocarbons on the other hand which are basically made up of straight, branched and cyclic structures are more readily degraded by microorganisms than aromatic hydrocarbons (Das and Chandran, 2011). For instance, Colombo et al. (1996) investigated the biodegradation of aliphatic and aromatic hydrocarbons by natural soil microflora and pure cultures of imperfect and ligninolytic fungi. In their experiments, they discovered that the natural microbial soil assemblage isolated from an urban forest area was unable to significantly degrade crude oil, whereas pure fungi cultures effectively reduced the residues by 26–35% in 90 days. They also reported that normal alkanes were almost completely degraded in the first 15 days, whereas degradation of aromatic compounds (e.g. phenanthrene and methylphenanthrene) exhibited slower kinetics. Another experiment conducted on the kinetics of the degradation of aliphatic hydrocarbons by the bacteria *Rhodococcus ruber* and *Rhodococcus erythropolis,* showed that the growth of these bacterial isolates on *n*-alkanes was intense when compared to growth in diesel medium (Zhukov et al., 2007). A comparative study on the degradation of both aliphatic and aromatic hydrocarbons by *Nocardia* sp. H17-1 was conducted and the results obtained showed a 99.0±0.1% and 23.8±0.8% reduction of both classes of hydrocarbons (Baek et al., 2006).

Mechanisms involved in petroleum hydrocarbon degradation

Various mechanisms of biodegradation of pollutants in the environment have been proposed by different researchers. For effective biodegradation of pollutants in environment, the chemicals must be accessible to the biological catalyst (Fritsche and Hofrichter, 2000). The first mechanism for degradation of petroleum hydrocarbons involves enzymes and was proposed by Fritsche and Hofrichter, (2000). These authors stated that for complete degradation of the majority of organic pollutants to be accomplished, aerobic conditions are a requirement. Thus, introduction of oxygen into the environment is vital as a co-substrate in reactions catalysed by oxygenases and peroxidases (Kariga and Rao, 2011) which are the main enzymes responsible for the aerobic degradation of most pollutants. The conversion of organic pollutants step by step through peripheral pathways such as the tricarboxylic acid cycle into intermediates of central intermediary metabolism is one of the results achieved during the microbial degradation process (Fritsche and Hofrichter, 2000; Das and Chandran, 2011). Biosynthesis of cell biomass occurs from the central precursor metabolites acetyl-CoA, succinate, and pyruvate derived from sugars via gluconeogenesis. Different pathways for the aerobic degradation of the various components of petroleum hydrocarbons have been proposed. For instance *n*-alkanes, a major group in crude oil contamination have several pathways through which it is biodegraded.

Pathways for degradation of *n*-alkanes

Aerobic degradation of *n*-alkanes begins with the oxidation of a terminal methyl group which renders a primary alcohol to be oxidized to the corresponding aldehyde, and finally conversion into a fatty acid (van Hamme et al., 2003; Wentzel et al., 2007). The fatty acids which are formed are subsequently transformed to acyl-CoA by aldehyde dehydrogenase and acyl-CoA synthetase respectively (Wentzel et al., 2007). Figure 1 shows the general degradation pathways for *n*-alkanes by two types of oxidation systems. Different enzymes are involved in the initial terminal hydroxylation of *n*-alkanes by bacteria (van Beilen et al., 2003; van Beilen and Funhoff, 2007). Methane monooxygenases are the major group of enzymes that carry out the hydroxylation of short chain-length alkanes (C_2-C_4) (Hamamura et al., 1999) while the non-heme iron monooxygenases and soluble cytochrome P450 (CYP153) are known to degrade medium chain alkanes (C_5-C_{11}) (Maier et al., 2001; van Beilen et al., 2005). The long chain alkanes (C_{10}-C_{30}) are easily degraded by alkane hydroxylases such as LadA, the thermophilic flavin-dependent monooxygenase (Wentzel et al., 2007).

Anaerobic biodegradation of petroleum hydrocarbons has been achieved using different bacterial strains (Widdel et al., 2006; Foght, 2008; Salehi et al., 2008) and reports show that these bacteria activate hydrocarbons by unprecedented biochemical mechanisms that differ completely from those employed in aerobic hydrocarbon metabolism. These unprecedented biochemical mechanisms may be initiated by bacteria through metabolic pathways by oxygen-independent hydrocarbon-activating reactions (Heider and Schuhle, 2013).

Pathways for degradation of aromatic hydrocarbons

The biodegradation of aromatic compounds has been extensively studied due to its importance in the biogeochemical carbon cycle. Since many aromatic compounds such as benzene, toluene, ethylbenzene and xylene (BTEX) are major environmental pollutants; their detection and removal from contaminated sites are of great biotechnological interest (Dıaz et al., 2013). Different catabolic pathways for the degradation of aromatic compounds have been described. According to Harayama et al. (1999), toluene is degraded by bacteria along five different pathways and for the purposes of this review, only two of these pathways are highlighted.

Different enzymes are involved in the degradation of toluene and they include toluene monooxygenase, benzyl alcohol dehydrogenase, benzaldehyde dehydrogenase and catechol-2, 3-dioxygenase. These enzymes are organized into two different pathways with the upper pathway, coding for enzymes that convert aromatic alcohols to acids, while the lower pathway is involved in aromatic acid metabolism via an *ortho* and *meta* pathway (Hamzah et al., 2011). Ring hydroxylation, which is involved in the second pathway, yields methyl catechol as the metabolic intermediate with toluene dioxygenase

as the key enzyme. A second mechanism involved in petroleum hydrocarbon degradation involves attachment of microbes to the substrates while a third mechanism involves the production of bio-surfactants.

Factors influencing rate of petroleum hydrocarbon biodegradation

For a successful bioremediation technology to be achieved, a full knowledge of the characteristics of the contaminated site and the parameters that affect the biodegradation of the pollutants must be accounted for. Different abiotic factors have been highlighted in various studies which influence the rate of hydrocarbon degradation in any environment. These factors include temperature, pH, nutrient availability, moisture content, and chemical composition of the contaminant, salinity of the environment, concentration and physical state of the contaminant (Leahy and Colwell, 1990; Salleh et al., 2003; Okoh, 2006).

The effect of temperature on the degradation of pollutants is very important as it affects solubility of the contaminants in the environment (Foght et al., 1996). Degradation of hydrocarbons occurs over a very wide range of temperatures. However, the biodegradability of a contaminant decreases with a decrease in temperature (Das and Chandran, 2011). Researchers have isolated a number of hydrocarbon utilizing bacteria which include psychrotrophic, mesophilic, and thermophilic bacteria. Psychrotrophic bacteria such as *Rhodococcus* sp. were reported by Whyte et al. (1998 and 1999) to have successfully degraded short chain alkanes at 0 ^{0}C. However in a report by Atlas (1981), a direct correlation between increased microbial degradation with an increase in temperature was recorded. This means that when microorganisms that are isolated from a cold region are introduced into an environment that has an elevated temperature, their metabolic activities tend to be faster (Atlas, 1981). According to Okoh (2006), highest degradation rates generally occur in the range 30–40 ^{0}C in soil environments, 20–30 ^{0}C in some fresh water environments, and 15–20 ^{0}C in marine environments.

Biodegradation rates have also been measured in relation to pH. Outcomes from various experiments conducted show that biodegradation is effectively carried out at an optimum of pH 7.0 (Zaidi and Imam, 1999). In a contaminated environment such as soil that is acidic in nature, the dominant microbial species that are capable of metabolising the contaminants in a short space of time appear to be fungi (Jones et al., 1970). The isolation of bacteria from an alkaline medium that were able to degrade phenol at pH 7.0-10.6 has also been reported (Kanekar at al., 1999).

The importance of nutrients in the degradation of hydrocarbons has also been stressed (Cooney, 1984). During biodegradation of hydrocarbons, lack of nutrients such as nitrogen, phosphorus, potassium, and iron may either hinder the breakdown process or result in an incomplete breakdown of contaminants. In fresh water environments, nutrients are particularly deficient. The supply of carbon significantly increases during

major oil spills in marine and fresh waters with nitrogen and phosphorus serving as limiting factors (Atlas, 1985). A deficiency in these nutrients in fresh water is due to demand by plants, and photosynthetic and non-photosynthetic microorganisms. Enhancement of biodegradation in different experiments has been achieved through the addition of nutrient supplements (Breedveld and Sparrevik, 2000; Li et al., 2006; Xia et al., 2006; Vyas and Dave, 2010). It should be noted however, that excessive nutrient concentration can impact the microbial degradation of hydrocarbons negatively (Oudot et a., 1998; Chaîneau et al., 2005).

The stability of water activity in aquatic environments has caused researchers to focus more attention on soils. For instance, Bossert and Bartha (1984) stated that the water activity of an aquatic environment is 0.98 while that of soil has a range between 0.0 and 0.99. The wide range of water activity in soils has made biodegradation of petroleum hydrocarbons very difficult. For effective biodegradation in soils, water activity must be kept constant and at an optimum level.

The chemical composition of contaminants in any environment is another factor that influences microbial degradation of such contaminants. Petroleum hydrocarbons which is made up of four classes; saturates, aromatics, asphaltenes (phenols, fatty acids, ketones, esters, and porphyrins), and resins, differ in their susceptibility to microbial attack. Biodegradation of hydrocarbons in decreasing order of susceptibility is ranked in the following order: *n*-alkanes > branched alkanes > low-molecular-weight aromatics > cyclic alkanes (Leahy and Colwell, 1990). According to Okoh (2006) the biodegradation of heavier crude oils is generally much more difficult than lighter ones. However, a report contrary to that of Okoh (2006) published by Cooney et al. (1985) stated that the degradation of more complex compounds such as naphthalene was faster than that of hexadecane in water-sediment mixtures from a freshwater lake. This observed result could be as a result of the action of co-metabolism by the organisms acting on the substrates.

Metabolic rate of microorganisms in mineralising contaminants in different environments tends to decline with increasing salinity (Ward and Brock, 1978; Minai-Tehrani et al., 2006). The ability of different microorganisms to degrade hydrocarbons in contaminated environments in the presence of elevated concentration of salts has been tested. Results showed that almost 100% of initial phenanthrene and dibenzothiophene were degraded at a salt concentration of 35 g/L (Díaz et al., 2002) while Abed et al. (2006) reported that at salinities ranging from 60 to 140 g/L, alkane biodegradation rates were 50 to 60% with a lesser degradation rate of less than 30% at 180 g/L. Contrary to these reports, Bertrand et al. (1990) isolated an Achaeon from a water-sediment interface with salinity of 310 g/L which was able to degrade eicosane more efficiently at a rate of 64% in a medium that contained sodium chloride at a concentration of 146g/L.

Due to the dispersion of oil in water during spillage, a slick typically forms which gives rise to emulsions (mousse) (Leahy and Colwell, 1990). The formation of an emulsion in

water increases the surface tension of the oil thereby making it available for microorganisms to degrade (Salleh et al., 2003). Emulsion formation through microbial production and release of biosurfactants has been documented (Kosaric, 2001; Kumar et al., 2008; Aparna et al., 2011; Mnif et al., 2011). Kumar et al. (2008) reported that a hydrocarbon degrading and biosurfactant producing strain of *Pseudomonas*, DHT2, which was isolated from oil contaminated soil was able to degrade crude oil, fuels, alkanes and PAHs. These authors also established that the biosurfactants which were produced by the organism lowered the surface tension of the medium from 54.9 to 30.2 dN/cm and formed a stable emulsion.

CONCLUSION

Bacterial degradation of fossil fuels (solids and liquids) is an important and emerging aspect of biotechnology which is neither fully described nor understood and as a consequence, technologies for implementation as commercial remediation strategies are few. While fungal biodegradation/biosolubilisation of coal and coal related products has been widely reported, it appears that work with bacteria has lagged and in some cases it has been completely ignored. In contrast, the use of bacteria and bacterial consortia for the remediation of petroleum hydrocarbon contamination is well established (Pinedo-Rivilla et al., 2009; Basha et al., 2010; Zhang et al., 2013; Ma et al., 2013; Martin et al., 2013) and as a consequence, commercial remediation protocols and the associated biocatalysts are widely available. Even so, there is a growing realisation that a mutualistic relationship between microorganisms and higher plants is necessary for complete remediation of contaminated sites (Ndimele 2010; Sekhohola et al., 2013). Thus, further study is needed to enhance our understanding of the processes involved in the bacterial bioconversion of coal and petroleum hydrocarbon contaminants in order to facilitate both a reduction in pollutant levels and to explore the potential for generating products of value. While the use of single strains to degrade coal and liquid hydrocarbon contaminants has been widely reported, consortia of bacteria or bacteria together with fungi appear to be the biocatalysts of choice as biodegradation agents.

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FIGURE HEADINGS

Figure 1. Simplified pathways for the degradation of *n*-alkanes by terminal and sub-terminal oxidation.

Figure 2. Mechanisms of toluene metabolism.

Table 1. Historical overview of advances in coal bioconversion.

Year	Progress	Reference
1927	Ability of micro-organisms to grow on LRC and modify its physicochemical properties	Fischer and Fuchs, 1927a; Fischer and Fuchs, 1927b
1981	Effects on hard coals by *Pseudomonas* strains, simultaneous biotenside-excretion	Fakoussa, 1981
1982	Solubilization of lignite to droplets on agar plates by fungal action	Cohen and Gabriele, 1982
1986	Acceleration of solubilisation by pre-treatment of coal	Scott, 1986; Grethlein, 1990
1986	Solubilisation of coal by an extracellular component produced by *Streptomyces setonii* 75Vi2 in submerged culture	Strandberg and Lewis, 1987
1987	First solubilisation mechanism elucidated: production of alkaline substances (fungi + bacteria)	Quigley et al., 1988; Quigley et al., 1989a; Quigley et al., 1991
1988	Second mechanism elucidated: production of chelating agents	Quigley et al., 1988; Quigley et al., 1989; Cohen et al., 1990; Quigley et al., 1991
1989	First product on market: Solubilized lignite as fertilizer	Arctech Inc., 2007
1991	Evidence that chelators alone are not responsible for all effects	Fakoussa, 1994
1994	Decolourisation and reduction of molecular weight of soluble lignite-derived humic acids proves catalytic enzymatic attack	Ralph and Catcheside, 1994; Hofrichter and Fritsche, 1997a and b
1994	Analysis of low-molecular mass products from bio-solubilised coal	Toth-Allen et al., 1994
1997	In vitro systems shown to degrade humic acids and attack matrix and coal particles	Hofrichter and Fritsche, 1997a and b
1997	First fine chemical produced successfully from heterogeneous humic acid mixtures to polyhydroxyalkanoates (PHA, "Bioplastic'') by pure cultures	Steinbüchel and Fuchtenbusch, 1997; Fuchtenbusch and Steinbuchel, 1999
1999	Involvement of laccase in depolymerization of coal implied by conversion of coal humic acid to fulvic acids *in vivo* by *Trametes versicolor* (basidiomycetous fungi)	Fakoussa and Frost, 1999
2001	Microbial solubilisation of lignites. Preliminary gasification tests with solubilized coal yielding 21% energy recovery from methane	Gokcay et al., 2001
2006	Mechanisms of coal solubilisation in *Penicillium decumbens* P6 combination of production of alkaline materials, peroxidase and esterase. First report on involvement of biosurfactant in coal solubilisation by fungi	Yuan et al., 2006
2007	Degradation of LRC by *Trichoderma atrovide* (ES 11)	Silva-Stenico et al., 2007
2007	Phytoremediation of coal mine spoil dump through integrated biotechnological approach	Juwarkar and Jambhulkar, 2008
2008	The effect of the particulate phase on coal biosolubilisation mediated by *Trichoderma atrovide* in a slurry bioreactor	Oboirien et al., 2008
2008	Fungal biodegradation of hard coal by a newly reported isolate, *Neosartorya fischeri*	Igbinigie et al., 2008
2013	Formation of biosolubilised humic acid from lignite using *Bacillus* sp. Y7	Jiang et al., 2013
2013	Fungal degradation of coal as a pretreatment for methane production	Haider et al., 2013

Table 2. A comparison of soil remediation treatment technology costs in South Africa.

Method of treatment	Approximate cost (ZAR/tonne soil)
Biological	70 - 2 395
Chemical	169 - 8 455
Physical	282 - 2 395
Solidification/stabilization	239 - 2 409
Thermal	423 - 10 569

Table 3. Examples of various biostimulation methods used to treat hydrocarbon contaminated sites.

Amendment types	Reference
Chelating agents	Da silver et al., 2005
Activated sludge from wastewater treatment	Juteau, et al., 2003; Maki et al., 1999
Bio-solids and maize	Sarkar, 2005; Rivera-Espinoza & Dendooven, 2004.
Immobilized-cell systems	Chen et al., 2009
Nitrogen and phosphorous	Jiménez et al., 2006; Bento et al., 2005; Evans et al., 2004.
Surfactants or bio-surfactants	Rahman et al., 2002.
Bulking agents e.g. wheat straw, hay and sawdust	Namkoong et al., 2002; Rahman et al., 2002; Rhykerd et al., 1999.
Biocompatible hydrophobic solvents	Zawierucha et al., 2011.

Table 4. A summary of microorganisms involved in the degradation of aromatic hydrocarbons using bioaugmentation as a strategy.

Microorganism	Contaminants treated	References
Single strains		
Mycobacterium sp.	Pyrene (PAH)	Heitkamp et al., 1988
Pseudomonas paucimobilis	Fluoranthene (PAH)	Weissenfels & Beyer, 1990
Pseudomonas cepacia	HMW PAHs	Juhasz et al., 1996
Sphingomonas paucimobilis	PAHs	Ye at al., 1996
Burkholderia cepacia	fluoranthene, pyrene, benz[a]anthracene and dibenz[a,h]anthracene	Boonchan et al., 1998
Comamonas testosteroni BR60	Crude oil, PAHs	Gentry et al., 2001
Arthrobacter chlorophenolicus A6L	4-Chlorophenol	Jernberg & Jansso, 2002
Absidia cylindrospora	Fluorene	Garon et al., 2004
Pseudomonas sp. ST41	Marine gas oil	Stallwood et al., 2005
Pseudomonas aeruginosa WatG	Diesel oil	Ueno et al., 2006
Sphingobium chlorophenolicum ATCC 39723	Pentachlorophenol	Dams et al., 2007
Burkholderia sp. FDS-1	Fenitrothion	Hong et al., 2008
Aspergillus sp. LEBM2	Phenol	Santos et al., 2008
Gordonia sp. BS29	Aliphatic/aromatic hydrocarbons	Franzetti et al., 2009
Pseudomonas putida ZWL73	4-Chloronitrobenzene	Niu et al., 2009
Aspergillus sp.	LMW-PAHs (2–3 rings)	Silva et al., 2009a
Trichocladium canadense, Fusarium oxysporum, Aspergillus sp.*, Verticillium* sp., *Achremonium* sp.	HMW-PAHs (4–7 rings)	Silva et al., 2009a
Neosartorya sp. BL4	Total petroleum hydrocarbons	Yi et al., 2011
Consortia		
Rhodococcus sp.*, Acinetobacter* sp., *Pseudomonas* sp.	PAHs (fluorene, phenanthrene, pyrene)	Yu et al., 2005
Bacillus subtilis DM-04, *Pseudomonas aeruginosa* M and NM	Crude petroleum-oil hydrocarbons	Das & Mukherjee, 2007
Mycobacterium fortuitum, *Bacillus cereus*, *Microbacterium* sp., *Gordonia polyisoprenivorans, Microbacteriaceae bacterium*, *Fusarium oxysporum*	PAHs (anthracene, phenanthrene, pyrene)	Jacques et al., 2008
Rhizopus sp., *Penicillium funiculosum*, *Aspergillus sydowii*	Petroleum hydrocarbons	Mancera-Lopez et al., 2008
Bacillus strains B1F, B5A and B3G, *Chromobacterium* sp. 4015, *Enterobacter aglomerans* sp. B1A, *Achremonium* sp., *Aspergillus* sp., *Verticillium* sp.	Mixture of PAHs (naphthalene, phenanthrene, anthracene, pyrene, dibenzo[a]anthracene, benzo[a]pyrene)	Silva et al., 2009b

FIGURE 1

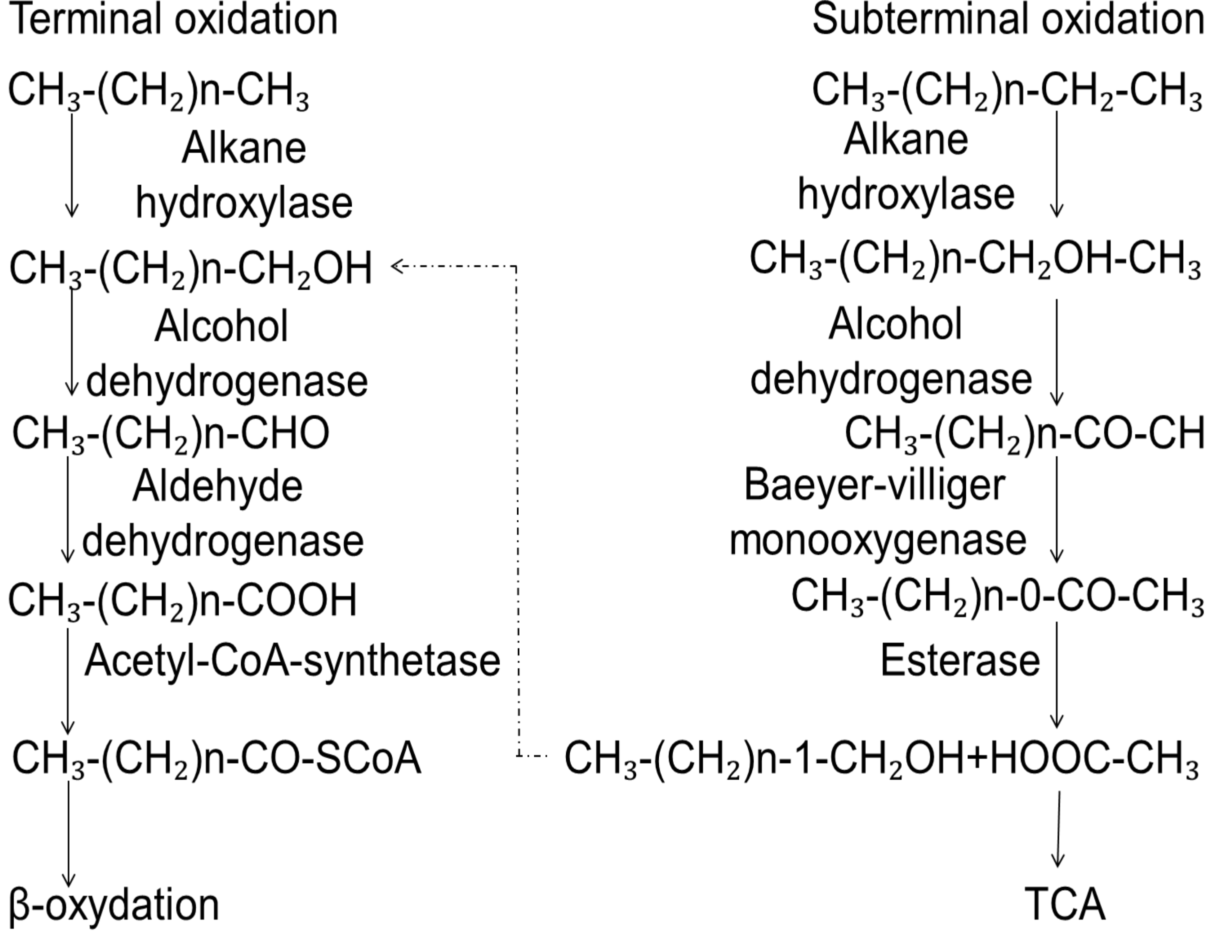

FIGURE 2

CH_3 CH_3 HO CH_3 HO COOH COOH

HOOC

HOOC

HO HO HO

Toluene Cresol

CO_2 CO_2

CH_2OH CHO COOH HO

HOOC

CO_2 HOOC

HO

Benzyl alcohol Benzaldehyde Benzoic Catechol

$CO_2 + H_2O$ + energy + biomass + other organics

Paper 2

Bacterial Degradation of Waste Coal and Production of Humic-like Substances

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Abstract Various strategies for beneficiation of discard and waste coal and related products have been the subject of study in South Africa in an effort to mitigate pollution by opencast mining and for successful rehabilitation post mine closure. Biodegradation, or the use of microorganisms to degrade and/or solubilize coal, is currently the most acceptable strategy of rehabilitating waste coal dumps. Bacteria were isolated from coal slurry, screened for coal biodegradation competence, characterized, formulated into consortia, and the colonization and degradation of waste coal in aqueous medium investigated in an effort to increase the suite of microorganisms available for use in rehabilitation practices. Nine novel coal degrading strains of bacteria were isolated and characterized and the gene sequences deposited with GenBank. Studies described in the present work showed that waste coal was colonized by and broken down by bacterial consortia formulated using these isolated strains and with the release of soluble substances which displayed characteristics similar to those of humic and fulvic substances. Total losses in waste coal substrate of 43 and 37% were observed for the consortia ECCN 10b and ECCN 13b respectively while a significant reduction in pH of the media was observed for consortia ECCN 7b, ECCN 10b and ECCN 13b. Reduced pH and associated decolourization occurred concomitant with formation of humic acid- and fulvic acid-like substances which was partially confirmed following analysis of these products using FTIR spectroscopy.

Keywords Bacteria · Biodegradation · Waste coal · Humic acid · Fulvic acid · FTIR

Introduction

Microbial treatment has been reported as an economical, effective and environmentally safe method for degrading the macromolecular network of coal (Couch 1987; Fakoussa et al. 1999; Machinikowska et al. 2002). Hard coal, which is one of the most abundant fossil fuels and has worldwide deposits that are larger than those of oil and gas, will be available for most if not all of this century (Hofritcher et al. 1997a). Due to its organic origin and structural similarity to lignin, it has been proposed that coal is susceptible to transformation by both bacteria and fungi (Hayatsu et al. 1979; Catcheside and Ralph, 1998). The idea that coal might be a substrate for microorganisms is not new. Several articles on the subject were published during the period 1908-1927. However, there was little further interest in this field until bacteria were shown to solubilize part of the organic phase of hard coal. At about the same time it was reported that wood-rot fungi quantitatively solubilized the low-rank coal Leonardite. This led to a series of studies which showed that a wide range of microorganisms was capable of catalysing the degradation of coal (Cohen and Gabriele 1982; Ralph and Catcheside 1994; Achi 1994;

Osipowicz 1996; Hofrichter et al. 1997b; Catcheside and Ralph 1998; Laborda et al. 1999; Fakoussa et al. 1999).

Waste coals are the low-energy-value discards from coal mining (Livingston et al. 1983; Ryu et al.2008) and typically comprise mixed coal, soil, and rock (mine waste or spoil). When left in piles over a period of time, these tend to leach iron, manganese and aluminium into waterways which may lead to acid rock drainage that pollutes recipient water courses and streams (Claassens 2006). Land devastation in regions where coal mining activities have taken place has also been recorded (Sheoran et al. 2010). These, and other consequences of coal mining, emphasize the need for the continued development and implementation of sound rehabilitation process technologies. It is thus not surprising that a number of different technologies are available for the rehabilitation of waste coal dumps and these include natural (Cimadevilla et al. 2003), chemical (Mukherjee and Borthakur 2001), thermal (Matsuoka et al. 2005) and biological (Hayatsu et al. 1979; Fakoussa 1981). Biological methods for rehabilitation of waste coal dumps are preferred over other methods as these are generally more economical, safer, energy conserving, and provide a way of converting waste coal to value added products (Machnikowska et al. 2002).

Both physiological and nutritional aspects affect coal biodegradation (Moolick et al. 1988; Achi and Emeruwa 1993) and while well accepted, the basic biochemical mechanisms involved are poorly understood (Achi 1994). Both non-enzymatic (Kevin et al. 1994; Klein et al. 1999) and enzymatic (Sivan 1984; Chahal 1985; Cohen et al. 1987; Kurzatkowski et al. 1996; Park et al. 2002; Khan et al. 2007; Hao 2007; Song et al. 2008) processes have been shown to be responsible for the biological breakdown of coal. Non-enzymatic biodegradation of coal may arise as a consequence of the presence of various metabolites including alkaline substances, surfactants, and chelators (Strandberg and Lewis 1987; Faison and Lewis 1990). Even so, it has been reported that oxidative and non-oxidative enzymes are major contributing factors in the biodegradation of coal (Wondrack et al. 1989; Cohen et al. 1990; Crowford and Gupta 1991).

A large number of fungi and some bacteria capable of coal degradation/solubilisation have been identified (Sekhohola et al. 2013). Included are *Streptomyces viridosporous* (Strandberg and Lewis 1987), *Bacillus cereus* (Maka et al. 1989), *Arthrobacter* sp. (Torzilli and Isbister 1994), *Nematoloma frowardii* (Hofrichter and Fritsche 1997), *Aspergillus* sp. (Laborda et al. 1999), *Penicillium* sp. (Laborda et al. 1999), *Trichoderma* sp. (Laborda et al. 1999), *Phanerochaete chrysporium* (Ralph and Catcheside 1999), *Trametes versicola* (Gotz and Fakoussa 1999), *Pseudomonas putida* (Machnikowska et al. 2002), *Pseudomona stutzeri* (Singh andTripathi 2011) and *Neosartorya fischeri* (Igbinigie et al. 2008). It is evident that the bulk of the work on coal biodegradation is based on fungi (Moolick et al. 1988; Basaran et al. 2003; Kai-yi et al. 2009). By comparison, relatively little is known about bacterial degradation of coal (Machnikowska et al. 2002). In the present work bacteria obtained by bio-prospecting were isolated from coal slurry and screened for coal biodegradation potential in liquid medium. Analysis by scanning electron microscopy, Fourier transform-infrared spectroscopy and ultra violet spectrophotometry confirmed the ability of the selected bacterial consortia to degrade

waste coal. Biodegradation of waste coal by these bacterial consortia occurred concomitant with accumulation of humic- and fulvic acid-like substances.

Materials and methods

Preparation of waste coal substrate

Waste coal obtained from coal discard dumps on coal mines in eMalahleni (Witbank), Mpumalanga Province, South Africa was powdered using a HP-M 100 Pulverizer (HERZOG Maschinenfabrik GmbH Co., Osnabrück, Germany) to yield particles of approximately 0.2-0.5 mm in diameter and sterilised by freeze thawing using liquid nitrogen (three cycles) to eliminate any *in situ* bacterial activity. Confirmation of sterilization was achieved by monitoring microbial growth after plating on nutrient agar which was incubated at 30°C for 48 h.

Bacterial strains, media, and culture conditions

Bacterial strains investigated in this study were sourced by bio-prospecting and isolated from coal slurry collected at coal mines in eMalahleni (Witbank), Mpumalanga Province, South Africa. Coal slurry was serially diluted and each dilution plated on nutrient agar. After incubation for 24 h at 30°C, pure colonies were obtained and used for enrichment by inoculating each strain in sterile mineral salts medium (MSM) containing waste coal as sole carbon source.

Control strains: *Pseudomonas aeruginosa, Pseudomonas putida* and *Bacillus subtilis* were from The Department of Biochemistry, Microbiology and Biotechnology, Rhodes University, Grahamstown, South Africa. Each strain was grown in nutrient broth overnight and harvested by centrifugation at 4000×*g* for 10 min. Pellets were washed in sterile 0.1 M phosphate buffered saline (PBS) and re-centrifuged, a process that was repeated three times, in order to ensure removal of residual carbon (Igbinigie et al. 2008). Mass of each culture was determined and a consortium established by carefully and aseptically mixing the three bacteria in the ratio of 1:1:1 (g/g/g). This consortium was used as the positive control and was maintained in stock culture in 30% glycerol and stored at -20°C. Consortia of strains isolated from coal slurry were formulated similarly and stock cultures likewise maintained in 30% glycerol and stored at -20°C.

Experiments were carried out in MSM containing, per litre of distilled water, K_2HPO_4 (1.71 g), KH_2PO_4 (1.32 g), $NHCl_4$ (1.26 g), $MgCl_2.6H_2O$ (0.01 g), $CaCl_2$ (0.02 g) and enriched with 4 ml of a trace element solution (Banga and Tripathi, 2009; Roy et al., 2014) and the pH adjusted to 7.0. 1g of sterile powdered waste coal was added to 150 ml aliquots of MSM. Bacterial inoculum was prepared by harvesting cells in log phase by gentle centrifugation (2000 × g for 10 min), rinsing with sterile PBS to remove nutrient medium and 1 ml containing 3.20×10^6 - 3.86×10^6 CFU added. Un-inoculated flasks containing waste coal and MSM were used as controls while a positive control contained equal mass of *P. aeruginosa, P. putida* and *B. subtilis*, strains known for their ability to degrade polycyclic aromatic hydrocarbons (Yu et al. 2001:

Abutor and Ball 2009; Mukherjee and Bordoloi 2012). Flasks were incubated on a rotary shaker (150 rpm) at 30^{0}C for 21 d and analysed at the intervals specified in Results.

Scanning electron microscopy

Bacterial consortia were incubated at 30°C in MSM containing sterile coal particles (0.2-0.5 mm) as the only carbon source for 21 d on a rotary shaker at 100 rpm. Coal particles colonized by the microorganism were collected and fixed in 2.5% buffered glutaraldehyde at 4°C overnight. The buffered glutaraldehyde was decanted and the samples washed twice with phosphate buffer (0.1 M, pH 7.3) and then dehydrated using a graded alcohol series. The alcohol concentrations used were 30% through to absolute ethanol in increments of 10% and incubated for 10 min at each step. Thereafter, samples were placed into a Critical Point Dryer apparatus basket and immersed in absolute ethanol and dried at 15 °C for 2 h. After critical point drying the desired part of the sample was mounted onto clean specimen stubs with double-sided conductive tape and gold-coated in order to improve the secondary electron emission and to reduce charge build up. Samples were examined using a Vega 3 LMU (TESCAN, Brno, Czech Republic) analytical scanning electron microscope at 30 kV.

Analysis of residual waste coal and formation of humic/fulvic acid-like substances

Residual waste coal was recovered by centrifugation, washed in distilled water and the pellets freeze dried to a constant weight and placed in a desiccator with activated silica gel to remove excess moisture (Cimadevilla et al. 2003; Elbeyli et al. 2006). FTIR spectra were recorded using a PerkinElmer Spectrum 100 instrument (PerkinElmer, Waltham, MA) with attenuated total reflectance (ATR) accessory eliminating the need for mixing of samples with KBr. The ATR accessory, fitted with a diamond top-plate, has spectral range of 25 000-100 cm^{-1} and refractive index of 2.4 and 2.01 µ depth of penetration. FTIR spectra were recorded in the range of 4000-700 cm^{-1}.

Extraction of HA- and FA-like substances from the supernatant was as previously described (Igbinigie et al. 2008). Briefly, humics were precipitated by adjusting the pH of the supernatant, after removal of residual waste coal substrate, to 1 using concentrated HCl followed by centrifugation (4000×g for 90 min) at 10°C, desiccation, and analysis by FTIR spectroscopy as described above. Fulvic acid-like substances were precipitated in the presence of NaOH and after centrifugation (4000×g for 90 min) the pellet dried and likewise analysed by FTIR spectroscopy. The HA-like substance containing pellet was re-suspended in an aliquot of 0.1 M NaOH and the FA-like substances in the remaining supernatant were quantified spectrophotometrically by interpolation from standard curves for leonardite-derived HAs and peat-derived FAs (purchased from the International Humic Substance Society, St. Paul, MN) after determining the absorbance (Thermo Spectronic Aquamate, ThermoFisher Scientific, Waltham, MA) at 370 nm and 450 nm respectively.

Identification and molecular characterization of bacterial strains

Total genomic DNA was extracted from bacteria grown on plates in pure culture using the methods outlined by Head et al. (1998) and Bond *et al*. (2002). Briefly, lysozyme stock solution (6 µl; 50 mg/ml) was added to a bacteria suspension in 500 µl of TE buffer and incubated for 3 h at 37°C with occasional mixing. Extracts were then subjected to five heating (100 °C for 1 min) and freeze-thaw (liquid nitrogen-80 °C water bath) cycles, cooled to room temperature, 50 µl sodium dodecyl sulphate (10%) and 2.5 µl proteinase K stock solutions (50 µg/ml) added, thoroughly mixed, and incubated overnight at 37 °C. Then, 100 µl of cyltrimethylammonium bromide (10%) and 200 µl of NaCl (5 M) was added and the extracts incubated at 55 ºC for 1 h. An equal amount of buffer saturated phenol, pH 8 (Sigma-Aldrich St Louis, MO) was then added to 500 µl aliquots of each extract which were mixed for 30 min and centrifuged (17950×g, 2 min). The upper aqueous layer was partitioned against an equal volume of phenol:chloroform:isoamyl alcohol (24:24:1, v/v/v), mixed and the layers separated by centrifugation as above. Upper aqueous layers were then partitioned against equal volumes of chloroform:isoamyl alcohol (24:1, v/v) until clear. To the final clear upper aqueous layers 2.5 volumes of ice-cold redistilled ethanol (96%) was added and DNA precipitated at -20°C for 12 h and recovered by centrifugation (17950×g; 20 min). Pellets were air dried and re-suspended in triple distilled water.

For molecular typing of strains, PCR was carried out by amplifying 16S ribosomal DNA using the universal primer 907R (5'- CCCCGTCAATTCCTTTGAGTTT-3') and the bacterial primer GMF5 (5'-CCTACGGGAGGCAGCAG-3') manufactured by Integrated DNA Technologies, IO. The PCR conditions were as follows: 95°C for 2 min, 40 cycles of 94°C for 30 s, 68°C for 45 s, with a final extension step of 2 min at 72 °C. PCR product was purified and subsequently sequenced using an ABI 3130XL sequencer (Inqaba Biotec, Pretoria, South Africa). Chromatograms were converted to text format using Chromas, entered into the NCBI BLAST database and the resultant sequences submitted to GenBank using BankIT and the accession numbers assigned to each strain are shown in Table 1.

Statistical analysis

All data were processed using Microsoft Office Excel 2010 and line diagrams constructed using Sigma Plot version 11.2 (SPSS Inc., Chicago, IL). Data are presented as the mean of at least three determinations ± standard deviation (SD).

Results and discussion

Bacterial colonisation of coal particles

Consortia of bacteria isolated from coal slurry were formulated as indicated (Table 1) and inoculated into MSM containing waste coal as substrate. After 21 d, a marked discoloration of the coal-containing MSM was evident in the presence of the consortia ECCN 7b, ECCN 10b and

ECCN 13b relative to the uninoculated control (Fig. 1A). Less dramatic but still pronounced discoloration was observed for the consortia ECCN 1b, ECCN 2b, ECCN 5b, and ECCN 6b. Surprisingly, very slight discoloration of waste coal containing MSM was observed after inoculation with a consortium comprising a mixture of the strains *P. aeruginosa, P. putida* and *B. subtilis*, as positive control and designated +0 (Fig. 1A). Decolourisation of waste coal in MSM by these bacterial consortia and the formation of a precipitate were accompanied by a decline in media pH. For clarity of presentation, only those consortia responsible for a dramatic change in coal colouration are shown (Fig. 1B). Thus, consortia ECCN 7b, ECCN 10b and ECCN 13b caused the most pronounced change in pH. Although an initial decrease in pH of the media can be attributed to the acidic composition of waste coal (Hayatsu et al. 1978) the decline observed in the present work appeared to be reflected the intensity of coal decolourisation. For the un-inoculated control, pH remained fairly constant throughout the incubation period confirming observations from similar studies on the bacterial solubilisation of coal (Jiang et al. 2013).

Decolourisation, the formation of a precipitate and a decline in pH of the incubation media suggested that the bacterial consortia formulated from strains isolated from coal slurry were and in particular the consortia ECCN 7b, ECCN 10b, and ECCN 13b were capable both of colonising and degrading the waste coal substrate.

It has been argued that the recalcitrance of coal to biodegradation stems in part from an inability to bring metabolically active biocatalysts into direct contact with the substrate (Sekhohola et al. 2013). Scanning electron microscope examination of coal particles abstracted from the MSM 21 d after inoculation either with consortium ECCN 7b, ECCN 10b or ECCN 13b revealed bacterial colonization of the substrate (Fig. 2). Attachment to and colonization of the coal surface by bacteria was observed for the consortia ECCN 7b (Fig. 2 A), ECCN 10b (Fig. 2B), and ECCN 13b (Fig. 2C) with filaments ramifying the coal structure. Fracturing and partial disintegration of the coal particles presumably occurred as a consequence of filamentation as has been observed in other studies (Fakoussa 1988; Laborda et al. 1997; Igbinigie et al. 2008). Earlier work showed that bacterial and fungal strains grow extensively in a media containing either hard coal, subbituminous coal or lignite and that a fibrilar extracellular polymer produced by the microorganisms appeared to facilitate not only adhesion to the coal particle but microbial attack (Laborda et al. 1997).

Bacterial filamentation is regarded as a survival tactic employed by diverse bacteria under a variety of conditions and particularly in stressful environments (Justice et al. 2008). The process is initiated by amongst other mechanisms, both quorum sensing and antimicrobial cues such as β-lactam-containing antibiotics. It is unknown which mechanism prevails in a coal environment but the recent isolation and molecular characterization of *Penicillium chrysogenum*, which produces the hydrophobic β-lactam compound penicillin, from a core sample of coal (Haider et al. 2013) strongly suggests that coal slurry may contain chemical cues that initiate bacterial filamentation.

Gravimetric determination of coal biodegradation by bacterial consortia

Biodegradation of waste coal by the various bacterial consortia formulated from coal slurry isolates was determined gravimetrically throughout the course of incubation and after careful recovery of residual substrate. As illustrated in Figure 3, a drastic reduction in coal mass was observed in the presence of the consortia ECCN 7b, ECCN 10b and ECCN 13b which, based on decolourization observations (Fig. 1A) was not unexpected. Average losses of 43 and 37 wt% were determined for ECCN 10b and ECCN 13b respectively. Less dramatic losses were measured for the consortia, ECCN 6b and ECCN 11b while the remaining consortia, ECCN 1b, ECCN 2b and ECCN 5b showed responses similar to that of the positive control (i.e. a consortium of *P. aeruginosa, P. putita* and *B. subtilis*). Although loss in waste coal weight was measured after inoculation with this consortium, the response appeared delayed and substantially less than that of consortia ECCN 7b, ECCN 10b and ECCN 13b. No change in weight of waste coal substrate was detected for the un-inoculated control.

Confirmation of the biodegradation of waste coal was obtained by detailed FTIR analysis of the waste coal substrate both prior to and following inoculation and incubation with the bacterial consortia ECCN 13b and the combination of *P. aeruginosa, P. putita* and *B. subtilis* (positive control) and by comparing FTIR spectra from inoculated and un-inoculated samples and the results are shown in Figure 4. FTIR spectra of residual coal from un-inoculated treatments after 21 d incubation (Fig. 4A) and spectra of the raw waste coal substrate (Fig. 4B) revealed no substantial change in functional groups. In the presence of bacterial consortia however, significant shifts in functional groups were observed for waste coal exposed to either a consortium of *P. aeruginosa*, *P. putida* and *B. subtilis* (Fig 4C; positive control) or the ECCN 13b consortium (Fig. 4D). Similar chemical shifts in the spectra from waste coal substrate were also observed after inoculation with ECCN 7b and ECCN 10b (data not shown). Together, these data indicated not only the formation of new functional groups within the waste coal but biodegradation of the substrate.

Thus, strong absorbance in the 1600 cm^{-1} region consistent with alkene (C=C) stretching frequencies (Coates 1996) was observed for all the treatments including the un-inoculated control. The bands at 1660-1820 cm^{-1} denoting C=O and –COO groups (Yuan et al. 2006) were most prominent in substrate remaining after treatment with consortium ECCN 13b (and ECCN 7b and ECCN 10b; not shown). With the formation of C=O functional groups, Coates (1996) suggested that hydroxyl (O-H) groups will be evident in the 3400-2400 cm^{-1} region as observed for waste coal exposed to the consortium ECCN 13b (Fig. 4D). This suggests that the various bacterial consortia formulated using isolates from coal slurry were capable of hydroxylating waste coal. The principal hydroxylation agent in biological systems is cytochrome P-450 and many variants of this enzyme have been isolated and characterized (Isin and Guengerich 2007) Embedded under the O-H stretch is the C-H group (Coates 1996). This group consists of alkane stretches at frequency (3000-2850 cm^{-1}), alkene stretches (3100-3000 cm^{-1}), aromatic stretches (3150-3050 cm^{-1}) and alkyne stretches (3300 cm^{-1}). The formation of C-H groups denotes the

addition of methyl moieties to the substrate or the substitution of an atom or group by a methyl moiety.

Formation of humic acid- and fulvic acid-like substances

Decolourisation coupled with the observed changes in functional groups in the presence of various consortia indicated bacterial conversion of waste coal substrate when inoculated with the various consortia formulated from isolates derived from coal slurry. The most likely products of microbial catalysed coal biodegradation appear to be HA-like substances (Sekhohola et al. 2013) and results in Figure 5 would seem to support this thesis. Thus, spectrophotometric analysis of the soluble fractions remaining after incubation of waste coal in the presence of the various bacterial consortia revealed a sustained accumulation of HA-like substances with greatest activity observed for the consortia ECCN 2b, ECCN 7b, ECCN 10b and ECCN 13b (Fig. 5A). The rate of HA-like substance accumulation slowed 10-14 d after inoculation and was associated with an increase in FA-like substances (Fig. 5B). Several studies have confirmed the ability of microorganisms (bacteria, fungi and algae) to utilise HA and HA-like substances as the sole carbon source (Steffen et al. 2002; Grinhut et al. 2007). Furthermore, microbial breakdown of HA and production of FA-like substances has been documented (Grinhut et al. 2007). Results from the present study appear to support these observations and were in part confirmed by accumulation of FA-like substances which was initially slow but increased substantially and coincidentally with a reduction in accumulation of HA-like substances. Thus, a reduction in accumulation of HA-like substances after 14 d probably arose as a consequence of both direct utilization and the further bio-conversion of these substances. Confirmation of bacterial conversion of waste coal to HA-like substances and the subsequent formation of FA-like substances was by FTIR spectroscopy and the results are shown in Figure 6. Spectra for HA-like substances extracted from the soluble fraction after inoculation and incubation of waste coal with consortium ECCN 13b revealed no substantial differences in the structural composition of functional groups when compared to the spectrum generated by that of commercial HA (Fig. 6A). This observation clearly indicates that the bacteria used in the present work were in deed capable of biodegrading coal to form HA.

FA extracted from the soluble HA-like substance fraction and formed from waste coal after inoculation and incubation with the various bacterial consortia was likewise analyzed by FTIR and showed that the FA-like substances produced by ECCN 13b from waste coal (Fig. 6D) was strikingly similar to the FA-like substances extracted from commercially available HA (Fig. 6C).

Conclusion

The present study investigated bacterial degradation of waste coal and production of HA- and FA-like substances with potential commercial value. Different bacterial consortia were formulated from isolates obtained from coal slurry and when provided waste coal in liquid

medium these consortia utilised the substrate for growth with the resultant formation of both HA- and FA-like substances. The results contrast with previous studies which reported an inability or recalcitrance by bacteria to degrade coal (Fakoussa and Hofrichter 1999; Machnikowska et al. 2002). Furthermore, a significant decrease in the mass of waste coal substrate was recorded following exposure to bacterial consortia; typically between 10 and 40%, with the most effective biodegradation observed for consortia ECCN 13b and ECCN 10b. Thus, bacterial consortia are capable of converting waste coal into products with potential commercial application and in particular, as soil conditioners to enhance revegetation and rehabilitation post mining and for use in arable agriculture. Studies are now underway to unequivocally identify of the products of bacterial degradation of waste coal and to elucidate the mechanism(s) employed by these bacteria in the biodegradation of coal.

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Table 1 GenBank accession numbers and composition of bacterial consortia

Bacterial Consortium by Accession Number	ECCN[A]
KC620473+KC620475	ECCN 1b
KC620473+KC620474	ECCN 2b
KC620473+KC620474+KC620475	ECCN 5b
KC620473+KC620476+KC620474	ECCN 6b
KC620473+KC620475+KC620476	ECCN 7b
KC620474+KC620476+KC700329	ECCN 10b
KC620475+KC620478+KC700330	ECCN 11b
KC620474+KC700328+KC700329	ECCN 13b

[A]ECCN = EBRU Culture Collection Number

FIGURE HEADINGS

Fig. 1 Decolourisation of waste coal by consortia of various bacterial strains isolated from coal slurry obtained from Witbank coal mines in the Mpumalanga Province of South Africa. Bacterial consortia were incubated in mineral salts media with and without waste coal as substrate on a rotary shaker at 30 °C for 21 d. A) Shows decolourization of the coal-containing media after incubation; and B) change in pH during the incubation period for media inoculated with consortia ECCN 7b, ECCN 10b, ECCN 13b, un-inoculated control; and the positive control (+0=consortium comprising *P. aeruginosa*, *P. putida* and *B. subtilis*).

Fig. 2 Scanning electron micrographs showing colonization of coal particles by bacteria from the various consortia prepared as described in Materials and Methods compared to an un-inoculated control. Arrows indicate attachment to and colonization of the coal surface by bacteria. A) Un-inoculated control; B) ECCN 7b; C) ECCN 10b; and, D) ECCN 13b.

Fig. 3 Time course of the change in weight of waste coal after treatment with different bacterial consortia. Mineral salts medium containing waste coal as a substrate was inoculated either with a consortium of known bacteria (positive control) or consortia of unknown strains (ECCN 1b, 2b, 5b, 6b, 7b, 10b, 11b and 13b) prepared as described in Materials and Methods and incubated at 30°C for 21 d on a rotary shaker. Residual waste coal was recovered, oven dried at 50°C to a constant weight and the mass determined gravimetrically. All data are the mean ± SD of three determinations and are representatives of duplicate experiments.

Fig. 4 FTIR spectra of waste coal before and after bacterial degradation. Mineral salts medium containing waste coal as substrate was inoculated either with the bacteria consortium, ECCN 13b, and incubated at 30°C for 21 d. Residual waste coal was oven dried at 50°C to a constant weight and analyzed by FT-IR. (A) Waste coal substrate, (B) waste coal residue from un-inoculated control after 21 d, (C) waste coal residue after exposure to a consortium of *P. aeruginosa*, *P. putida* and *B. subtilis* (positive control) after 21 d, (D) waste coal residue after exposure to ECCN 13b for 21 d.

Fig. 5 Time course of humic acid- and fulvic acid-like substance production from waste coal after treatment with different bacterial consortia. Mineral salts medium containing waste coal as the substrate was inoculated either with a consortium of known bacteria (positive control) or consortia comprising unknown strains (ECCN 1b, 2b, 5b, 6b, 7b, 10b, 11b and 13b) and incubated at 30°C for 21 d. Humic and fulvic acid-like substances were extracted and analyzed spectrophotometrically as described in the Materials and Methods. All date are the mean ± SD of three determinations and are representative of duplicate experiments.

Fig. 6 FTIR spectra of humic acid- and fulvic acid-like products produced by bacterial degradation of waste coal. Mineral salts medium containing waste coal as substrate was inoculated with the consortium, ECCN 13b, and incubated at 30°C for 21 d on a rotary shaker. Humic and fulvic acid-like substances were extracted, dried and analyzed by FT-IR. (A) Commercial HA, (B) humic acid-like substances produced from waste coal by ECCN 13b, (C) fulvic acids extracted from commercial humic acid (i.e. extracted from A), and (D) fulvic acid-like substances extracted from the humic acid-like substances produced from waste coal substrate by ECCN 13b (i.e. extracted from B).

Fig. 1

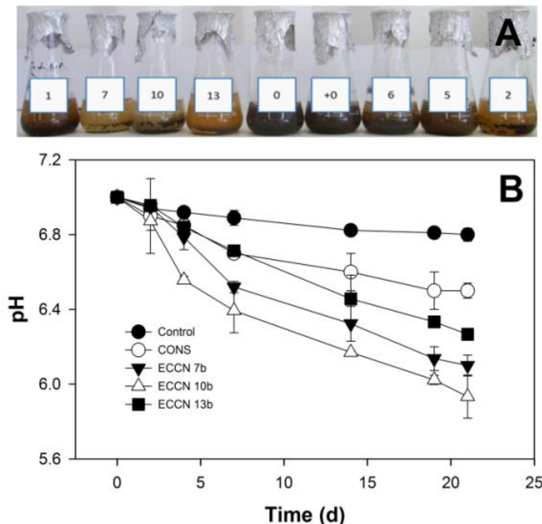
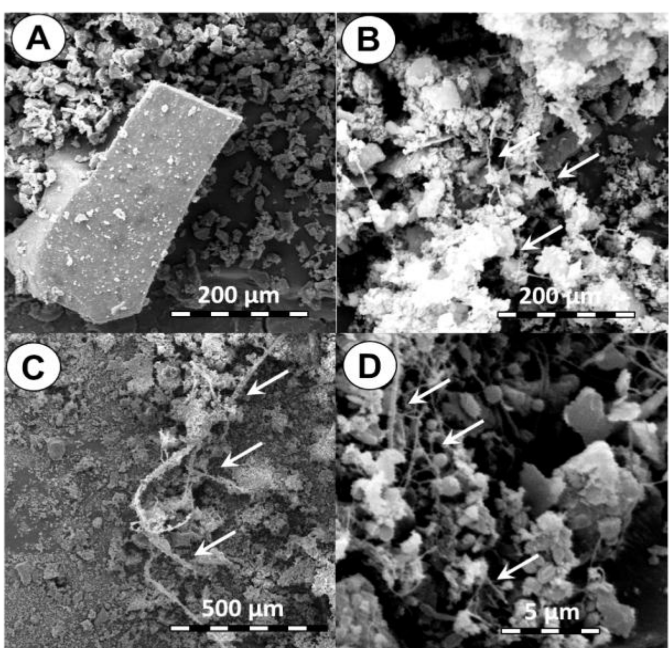


Fig. 2



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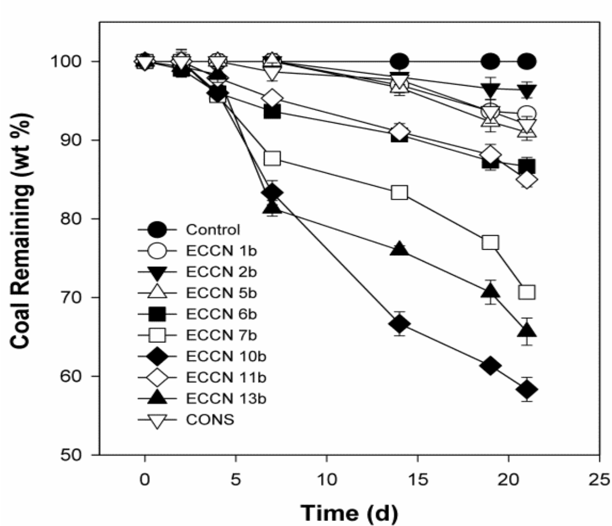

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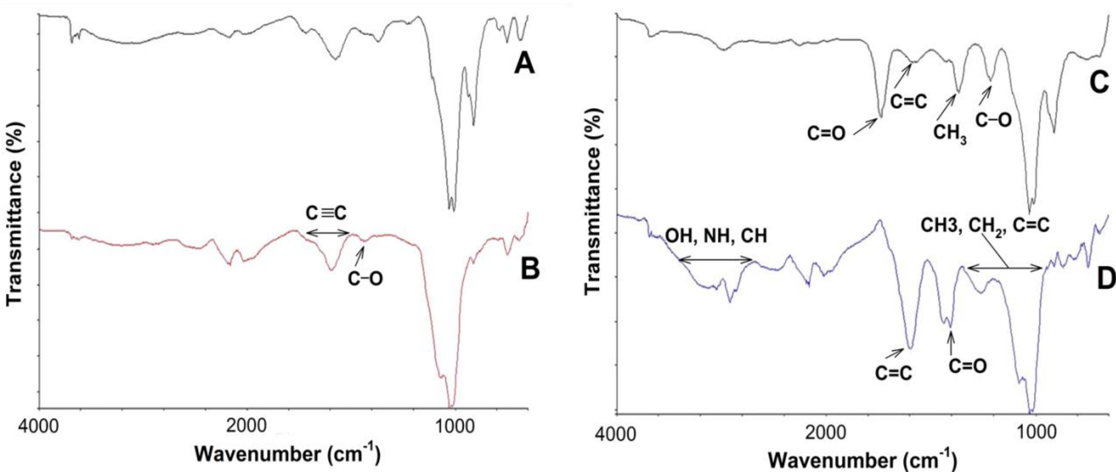


Fig. 5

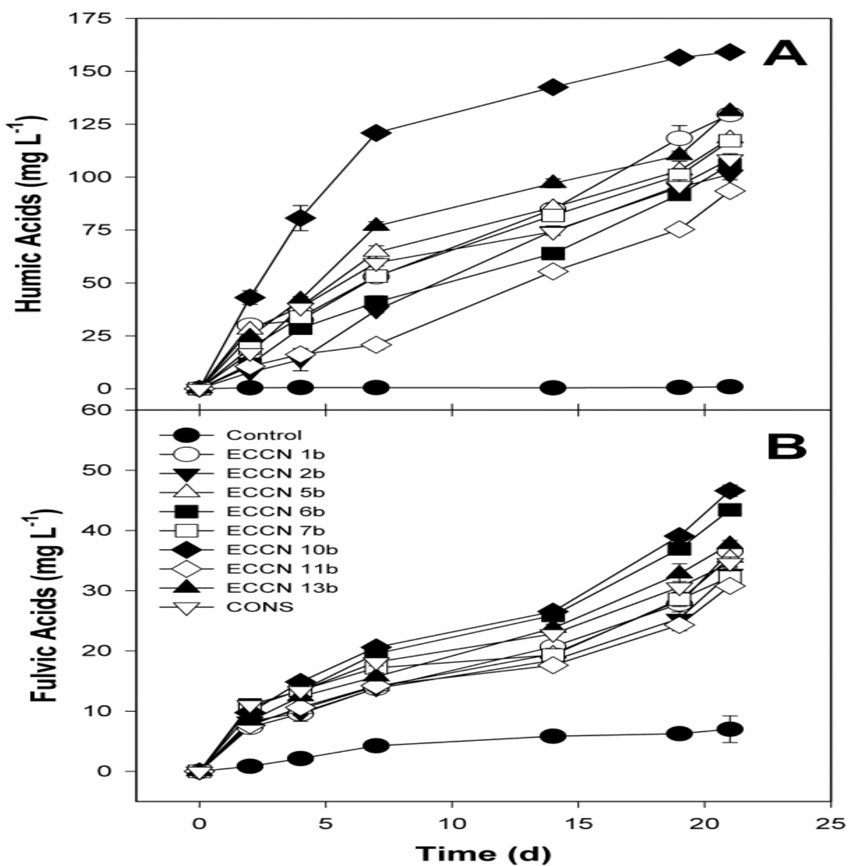
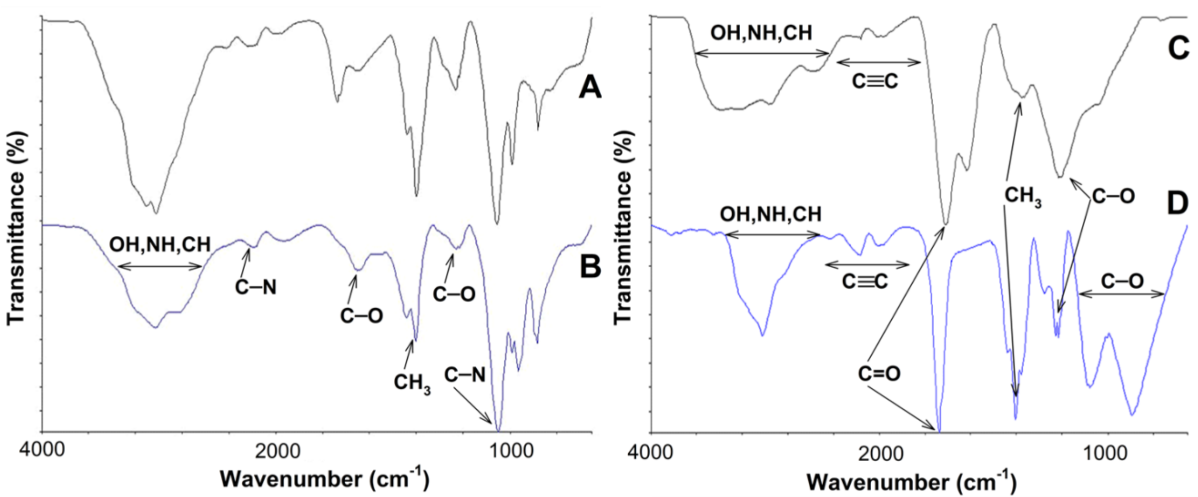


Fig. 6



Paper 3

Waste Coal Biodegradation using Bacterial Consortia and *Neosartorya fischeri* ECCN 84

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Waste Coal Biodegradation using Bacterial Consortia and *Neosartorya fischeri* ECCN 84

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Abstract

Waste coal biodegradation using bacteria in combination with Neosartorya fischeri was carried out in a greenhouse and the formation of useful products such as humic substances was monitored. Physico-chemical properties of waste coal were determined during the period of this study using already established bacterial consortia with or without Neosartorya fischeri; ECCN 84 with algal biomass as a source of nutrient with an aim of establishing a microbial consortium with coal degradation potentials. Obtained results showed an increase in humic-like and fulvic-like substances for bacterial consortia ECCN 10b and 13b in combination with the fungal strain ECCN 84. The rate of humic acid prodion by bacteria/fungal consortia (ECCN 10b/84 and ECCN 13/84) at the end of the experiment were 62 and 65 mgL^{-1} respectively. Increases in fulvic acid for same consortia above were 46 and 49 mg.l^{-1} while water holding capacities were observed to be 45 and 47 %. Decompaction of the substrate due to a reduction in bulk density and subsequemt increases in moisture content, electrical conductivity and cation exchange capacity were also recorded. Based on the results obtained in this study, the bacterial consortia; ECCN 10b and ECCN 13b in combination with the fungal isolate ECCN 84 can be used for the bioconversion of waste coal to obtain useful products and the rehabilitation of waste coal dumps for commercial purposes.

Keywords: *Waste coal dumps · Bacterial consortia · Rehabilitation · Fungus · Humic acid · Fulvic acid*

1. Introduction

Studies on the rehabilitation of waste coal by microorganisms have been ongoing since the early 1920s [1, 2, 3, 4, 5, 6, 7, 8] and most published reports show the ability of fungal strains to degrade coal [9, 10, 11, 12]. In contrast to studies using fungi, there are only a few reports on coal bioconversion using bacteria. Maka and colleagues [13] reported the solubilization of untreated North Dakota lignite in 2 weeks using a mix of bacteria which included *Bacillus cereus, Bacillus pumilus* and *Bacillus subtilis*. Other reports on the use of bacteria in the degradation of coal have shown the ability of some bacterial species either as single isolates or as a consortium to degrade coal (most especially low rank coals) into value added products such as fertilizers [14, 15, 8]. Even so, research on biosolubilization of coal using bacteria has not attracted as much attention presumably as these organisms are less capable of degrading coal due to its complex structure [16, 17, 18]. Orem and colleagues on the other hand [19] recently showed that bacteria can degrade sub-bituminous coal anaerobically and produce organic intermediates such as acetate that can be transformed to methane in the headspace of a bioreactor which was designed for this purpose. Other organic intermediates produced during this experiment include; long chain fatty acids, alkanes (C_{19}-C_{36}) and various low molecular weight aromatics such as phenols. These organic intermediates are products by the breakdown of coal geopolymers by anaerobic microbial activity and

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accumulate in bioreactor fluids where they serve as precursors to low molecular weight (LMW) compounds such as acetate and H_2 utilized by methanogens [20, 21].

Previous research into the biodegradation of coal in our laboratories using *Neosartorya fischeri* strain ECCN 84, showed effective breakdown of coal [6]. In this study, biodegradation of coal in liquid culture and in a perfusion fixed-bed bioreactor was observed and the degradation products analysed by pyrolysis gas chromatography-mass spectrometry (pyGC-MS) and Fourier transform infra-red (FT-IR) spectrometry and compared to products from raw waste coal that had not been exposed to microbial catalysts. Results showed oxidation of the coal surface and nitration of the condensed aromatic structures. The above experiment led to the establishment of the FungCoal process [22] which is now being tested at full commercial scale by Anglo American Thermal Coal at coal mines in Witbank, South Africa for rehabilitation of waste coal dumps. This process makes use of *Neosartorya fischeri* in combination with mycorrhizal fungi and appears to depend on the presence of certain grass species and in particular *Cynodon dactylon* for success [6, 23, 24]. Even so, a review on coal biodegradation has emphasised the role of bacteria and their potential contribution to the overall process [25]. As stated by the authors, "Coal biodegradation is a naturally complex process and appears to be driven by an arsenal of extracellular enzymes in the presence of various chelators and supporting enzymes released by different microorganisms that co-inhabit the coal environment." Perhaps different microorganisms are indeed required to improve the efficiency of processes like FungCoal for large scale commercial implementation.

The biodegradation of coal using coal degrading microorganisms such as bacteria and fungi is currently the most acceptable strategy of rehabilitating waste coal dumps and this technology has been used to obtain specific useful products such as humic substances from coal. Spoils (waste coal) generated during mining are characterised by low water holding capacity, are devoid of nutrients and physico-chemically unstable. Based on this observation, attempts were made to explore the rehabilitation potential of *Neosartorya fischeri*, ECCN 84 in the presence of bacterial consortia (ECCN 10b and ECCN 13b) using algal biomass as a source of nutrient with the aim of establishing a microbial consortium with coal degradation potential to be used for commercial purposes. Results are discussed in terms of utilising coal degrading bacteria in combination with *Neosartorya fischeri* for commercial rehabilitation purposes.

2. Materials and Methods

2.1. Bacterial consortia, fungal strain and culture conditions

Bacterial consortia (ECCN 10b and ECCN 13b) and the fungus *Neosartorya fischeri* strain ECCN 84 used for this study were sourced from the EBRU culture collection (Institute for Environmental Biotechnology, Rhodes University, and Grahamstown, South Africa). Initial experiment on the biodegradation of waste coal in aqueous media using single bacterial strains and established bacterial consortia, showed that these bacterial consortia (above), were able to effectively degrade waste coal in aqueous media (Edeki et al., 2014, unpublished, Paper 2) and these consortia were stored as stocks in 30% glycerol medium at -20 °C for further study. In this study, bacteria stock cultures were resuscitated in nutrient broth at 30 °C for 18 h and bacterial biomass was harvested by washing with phosphate buffered saline solution (PBS) in 3 cycles as described by Edeki et al. (2014, unpublished manuscript, Paper 2).

A stock culture of ECCN 84 was resuscitated in potato dextrose broth which was incubated at room temperature for 48 h. Fungal biomass was harvested by washing as described above. For temporary storage, inoculated PDA slants were prepared in triplicate and refrigerated at 4°C.

2.2. Preparation of algae-derived nutrient solution

Algal biomass was used as a source of nutrients and was obtained from the Integrated Algal Pond System (IAPS) at EBRU, Rhodes University in Grahamstown, South Africa where municipal waste water is treated daily. Suspended algae from the high rate algae oxidation ponds (HRAOPs) was concentrated by allowing cells to settle before centrifugation (10 000 × g, 20 min) and the biomass sterilized by autoclaving at 121 ^{0}C for 15 min. Optimum algal biomass concentration needed for effective degradation of coal was determined by inoculating bacterial consortia into sterile phosphate buffered saline containing 1 g of waste coal with different masses (g) of dried algal biomass. Waste coal media was incubated in a controlled environment at 30 ºC for 21 d during which, bacterial growth was monitored daily. Serial dilutions from each flask were inoculated onto agar and plate counts were used to determine bacterial growth in coal media and the highest bacterial counts on agar were used to score the optimum algal biomass (g) needed by bacteria for efficient biodegradation of waste coal. Studies on the fertilizer potentials of algae used for this experiment revealed an empirical formula of C (1.0), H (1.91), N (0.084), S (0.003) and O (0.36) respectively.

2.3. Experimental design

In an earlier study, it was shown that consortia of bacterial isolates from coal mine wastes were more efficient degraders of waste coal than individual strains (Edeki et al., 2014, unpublished manuscript, Paper 2). Waste coal (2 kg) obtained from coal discard dumps on coal mines in eMalahleni (Witbank), Mpumalanga Province, South Africa was placed into aluminium pots (10 × 20 cm) and inoculated with a 20 mL suspension of either bacterial (3.20×10^6 - 3.86×10^6 CFU/mL) consortia, bacteria consortia plus *Neosartorya fischeri* ECCN 84 (1.640×10^9 spores/mL), or *Neosartorya fischeri* ECCN 84 alone while uninoculated waste coal was used as control. To the waste coal was added 20 mL of algal biomass which had already been sterilized as described above. Inoculated waste coal and uninoculated control were arranged in a complete randomized design in a polycarbonate-covered tunnel (Ulma Agricola, Spain) and spray irrigated (50 mL) twice a week.

2.4. Extraction and analysis of humic acid- (HA) and fulvic acid- (FA) like substance

Humic acid (HA)-like and fulvic acid (FA)-like substance were extracted using the well documented alkaline extraction method [26, 27, 6, 28]. Waste coal (1 g) was pulverised and extracted in 100 mL 0.1 M NaOH for 24 h on a rotary shaker at 120 rpm. Extracts were centrifuged (Eppendorf 5415D desktop centrifuge) at 3220×g for 90 min at 10°C, and the supernatant filtered through a 0.45 µm glass fibre filter to remove particulates and the pH adjusted to <1 and the HA-like substances precipitated by centrifugation as above. The acidic supernatant was considered the HA-like substance containing fraction. Pellets were re-suspended in 100 mL 0.1 M NaOH and left to stand for 4 h before centrifugation as above and the alkaline supernatant carefully decanted and this was considered the FA-like substances containing fraction. The amount of HA-like and FA-like substance was determined spectrophotometrically at 450 and 380 nm and values interpolated from standard curves prepared using authentic HA and FA obtained from leonardite-derived HAs and peat-derived FAs (purchased from the International Humic Substance Society, St. Paul, MN).

2.5. Determination of water holding capacity (WHC)

Water retention of waste coal was determined using the methods described by Juwarkar and Jambhulkar [29] and Viji and Rajesh [30]. Whatman No. 42 filter paper was used to cover the perforated bottom of a metal container. Then the weight of container plus filter paper was determined (W_i). Waste

coal samples were dried, crushed and transferred into the metal container. Gentle tapping (20-30 times) was required to ensure uniform distribution of waste coal within the container before the weight (W_{ii}) was determined. The container plus coal was placed in a petri dish containing water and e water allowed to enter the metal container and saturate the coal overnight. Thereafter, the Petri dish was covered to prevent evaporation of water from the coal surface confirmed by the presence of shining film. After 24 h, the container was removed from the Petri dish, blotted dry and the final weight recorded (W_{iii}).
Water holding capacity was determined using the following:

$$\text{Water holding capacity in coal sample (\%)} = \frac{W_{iii} - W_{ii}}{W_{ii} - W_{i}} \times 100$$

Where W_i = weight of box + filter paper
W_{ii} = weight of box + filter paper + dried coal
W_{iii} = weight of box + filter paper + wet coal

2.6. Gravimetric determination of moisture content

Moisture content was determined using the method described by Viji and Rajesh [30]. Waste coal (1 g) was dried to a constant weight at 50 °C and the weight of dried waste coal sample was recorded. The weight difference between the initial and that of the oven dried sample was considered to be the moisture content.

2.7. Measurement of bulk density (laboratory method)

The bulk density or apparent gravity of coal is the mass of a unit volume including pore space. Briefly, 80 g of coal from each pot was placed in dishes and oven dried for 2 h at 50 ºC to allow for easy sieving of coal particles. Dried coal was sieved to a particle size of 0.2–0.5 mm diameter and each weighing bottle filled with sieved coal and weighed. After weighing, the coal was removed and the bottle was filled with water using a burette and the volume of water was recorded. The bulk density of the waste coal sample was determined by dividing the weight of the waste coal by the volume of water and calculated as:
Weight of coal = W_2-W_1
Volume of water = V

$$\text{Bulk Density} = \frac{W_2 - W_1}{V} \ (\text{g/cm}^3)$$

Where W_1= weight of empty bottle (g)
W_2= weight of empty bottle + coal (g)
V= volume of water needed to fill the empty bottle (mL)

2.8. Measurement of electrical conductivity (EC)

Waste coal samples were air dried and passed through a 2 mm sieve. After sieving, 10 g of the dried sample was added to 50 mL of deionized water. The sample was shaken at 150 rpm for 1 h. After settling for 4 h, the solution was filtered through a Whatman No. 42 (12.5 cm) paper and the filtrate collected. An already prepared standard solution of 0.005 *N* KCl was used to calibrate the instrument and electrical conductivity (EC Tester 11, Thermo Fisher Scientific, Singapore, 68X546501) determined according to the manufacturer’s instructions.

2.9. Determination of cation exchange capacity (CEC)

The method used to determine CEC of waste coal was modified from that outlined by Gillman and Sumpter [31]. A solution of 0.1 M $BaCl_2.2H_2$ was prepared and 20 mL added to pre-weighed centrifuge tubes. Waste coal (2 g) was added to the tubes which were capped and shaken for 2 h. After centrifugation (10,000 rpm for 30 min), the supernatant was carefully decanted. The pellet was resuspended in 20 mL $BaCl_2.2H_2O$ (2 mM) and shaken vigorously for 1 h followed by centrifugation as above and the supernatant discarded. This process was repeated 5 times. Finally, 10 mL of 0.1 M $MgSO_4.7H_2O$ was added to each tube with shaking for 1 h and the conductivity determined using a conductivity meter (EC Tester 11, Thermo Fisher Scientific, Singapore, 68X546501). A solution of $MgSO_4$ (1.5 mM) was prepared and its conductivity was determined (it should be ~300 μS or μmhos) and compared to the conductivity of test sample (waste coal) which must be 1.5 times the conductivity of 1.5 m*M* $MgSO_4$. Whenever the conductivity of sample solution was not 1.5 times the value of 1.5 m*M* $MgSO_4$, 0.100 mL of 0.1 *M* $MgSO_4$ was added to the test sample until it was 1.5 times that of 1.5 m*M* $MgSO_4$. Addition of distilled water to sample solution was carried out and mixed thoroughly until the conductivity of solution was the same with that of 1.5 m*M* $MgSO_4$.
The final weight of tube was determined and the CEC was calculated using the equation: (meq/100g) = (TM - MS) × 50
Where TM = Total Magnesium added
MS = Magnesium in final solution
50 is to convert from 2 g of coal to 100 g
And TM was calculated as
0.1 meq [meq in 10 mLs of 5 m*M* MgSO4] + meq added in 0.1 *M* MgSO4
[mLs of 0.1 *M* $MgSO_4$ × 0.2 meq/mL (0.1 *M* $MgSO_4$ has 0.2 meq/mL)]
and MS was calculated as:
total solution (mLs) × 0.003 (meq/mL) [1.5 m*M* MgSO4 has 0.003 meq/mL]
but total solution = final tube weight (g) - tube tare weight (g) - 2 g [weight of coal used]

3. Statistical analysis

IBM-SPSS statistics 20 software was used to analyze all data via one way analysis of variance (ANOVA) with a 95% degree of confidence to detect significant differences between the treated groups and controls with level of statistical significance at $p < 0.05$ and line diagrams were constructed using Microsoft Office Excel 2010. Data are presented as the mean of at least three determinations ± standard deviation (SD).

4. Results

For the formation/production of value added products such humic substances, extraction of HA-like substances was carried using the alkaline extraction method. Results obtained during and at the end of the 9 months experiment, showed that each inoculum was able to adequately breakdown waste coal and covert it to HA-like materials (Fig. 1A). The fungal strain ECCN 84 was able to effectively degrade waste coal over the 9 months period of experiment with a gradual but steady increase in the formation of HA-like substances. The formation of HA-like substances was also observed in waste coal treated with bacterial consortia; ECCN 10b and ECCN 13b but when the concentrations were compared to that produced by ECCN 84, they were lower (Fig. 1A). A combination of each bacterial consortium with the fungal strain ECCN 84 in waste coal showed the formation of higher concentrations of HA in waste coal treated with consortium ECCN 13b/84 having the highest concentration of this substance. Although there

was formation of HA-like substances in the controlled experiment, the concentration of was not significant in comparison to every other test experiment.

In contrast to the formation of HA-like substances from waste coal, the formation of fulvic acid-like substances from HA was very fast. With very low concentrations of FA-like substances obtained at the beginning of experiment, its formation sharply increased over time (Fig. 1B). A high concentration of FA-like substances was observed at the sixth month of experiment in waste coal inoculated with ECCN 84 as compared to ECCN 10b and ECCN 13b.

The steady increase in the concentrations of FA-like substances extracted from waste coal using different inoculums was similar to that obtained in Figure 1A. Bacterial and fungal mix produced higher concentrations of this substance (Fig. 1B) and this result suggests a possible co-habitation between the bacteria used in this study and the fungal strain ECCN 84.

Increased water holding capacity (WHC) of the substrate was observed during this study with a sharp increase recorded in the sixth month. ECCN 84 had a 45.3 % WHC compared to the un-inoculated control (Fig. 2A) while the highest percentages recorded in waste coal inoculated with ECCN 10b and ECCN 13b at the end of 9 months experiment were not as high as that recorded for ECCN 84 (Fig. 2A). However, with the bacterial/fungal consortia, there was an observed difference in WHC of the waste coal inoculated with ECCN 84, ECCN 10b/84 and ECCN 13b/84. The bacterial/fungal mix; ECCN 13b/84 had the highest percentage of WHC at the end of experiment and this result, further confirmed the trend observed in Figure 1A and 1B respectively.

Moisture content was also monitored throughout the experiment and with increases observed in all the waste coal inoculated with bacterial consortia with or without strain ECCN 84. Waste coal inoculated with fungal strain; ECCN 84 also had an increased moisture content which was higher than that of ECCN 10b and ECCN 13b (Fig. 2B). This process was greatly enhanced when strain ECCN 84 was mixed with each bacterial consortium. Observed results showed that both ECCN 10b/84 and ECCN 13b/84 were able to effectively utilise waste coal as a substrate thereby increasing its soil-like properties (Fig. 2B) and this increase was mostly prominent between the fourth and fifth months.

Cation exchange capacity (CEC) of waste coal was measured and recorded during this study and an initial value of 7.683 ± 3.13 meq.100g^{-1} for waste coal substrate was measured while during the experiment, the value increased drastically in each month throughout the nine months period for each inoculum. Figure 2C shows the increase in CEC for all treatments including un-inoculated control with ECCN 10b having the lowest value of CEC on the sixth month and at the end of experiment (Fig. 2C). This was not the case with the fungal strain ECCN 84 which had a high CEC value when compared to each bacterial consortium. However, CEC values increase drastically when these consortia were mixed with ECCN 84 and inoculated into waste coal. The highest CEC value obtained during the sixth month and at the end of experiment was with waste coal inoculated with ECCN 13b/84 (Fig. 2C). When ECCN 84 was used as an inoculum, an observed CEC value of 32.33 meq.100g^{-1} was recorded at the end of experiment but when this fungal strain was mixed with bacterial consortium; ECCN 13b, a 100 % increase was recorded (Fig. 2C).

A slow increase in electrical conductivity (EC) was observed during the first 4 months of experiment for each inoculum but the values doubled on the sixth month and got to its peak at the end of experiment. Waste coal inoculated with ECCN 84 and ECCN 10b had the same EC values of 2.3 mS.cm^{-1} on the sixth month while ECCN 13b had a slightly higher EC value but at the end of experiment, EC value of waste coal inoculated with ECCN 84 became higher than the bacterial consortia; ECCN 10b and ECCN 13b respectively (Fig. 2D). A 25 % increase in EC was observed when bacterial consortium; ECCN 13b was mixed with the fungal strain; ECCN 84while an 11 % increase was recorded for consortium ECCN 10b/84 in comparison to ECCN 84.

Bulk density; a measure of compactness of the substrate (waste coal), was also monitored in this study. A significant reduction in bulk density from 1.45 ± 0.07 g.cm^{-3} to 1.13 ± 0.08 g.cm^{-3} was recorded and this value was lower than those recorded for waste coal treated with the bacterial consortia; ECCN 10b and ECCN 13b respectively (Fig. 3). Although the bulk density values of waste coal inoculated with bacterial consortia were lower than that of fungal strain ECCN 84, greater decompaction values were

observed and recorded when these bacteria consortia were mixed with strain ECCN 84 with waste coal treated with ECCN 13b/84 having the lowest bulk density value of 1.05 ± 0.03 $g.cm^{-3}$ (Fig. 3).

5. Discussion

5.1. Determination of HA- and FA-like substances in waste coal

The formation of HA-like substances from waste coal was monitored over time and inoculated and un-inoculated waste coal was collected and analysed monthly for the production of HA-like substances. A basic parameter for a healthy soil used in assessing the growth of plants is the concentration of humic acid contained in that soil [32, 33, 34]. The breakdown of waste coal inoculated with ECCN 84, ECCN10b, ECCN 13b, ECCN10B/84 and ECCN13b/84 was determined based on the production/formation of HA-like materials [24]. HA-like material was quantified using UV-Vis spectrophotometry and results obtained, showed a gradual but steady increase in the concentration of HA-like materials. With a slow start in the formation of HA-like substances, progressive changes/increases in concentration were observed with ECCN 13b/84 having the highest concentration while un-inoculated control had slight formation of HA-like substances at the end of experiment (Fig. 1A) and this could be as a result of the action of resident microorganisms which were stimulated by the addition of nutrients (algae). However, with the highest values of HA-like substances recorded in this study with waste coal inoculated with ECCN 10b/84 and ECCN 13b/84, it could be said that the increase in concentration of these substances was mainly due to biological activities of the various microbial consortia. Although high concentrations of this substance was observed when waste coal was inoculated with ECCN 84, ECCN 10b and ECCN 13b, the results became better when bacterial consortia were mixed with fungal strain.

Researchers such as Mukasa-Mugerwa and colleagues [24] have also observed the formation of these substances over time in an experiment that was carried out to determine the role of a plant/fungal consortium in the degradation of bituminous hard coal. Igbinigie et al. [6] also reported similar results in an experiment carried out on fungal biodegradation of hard coal by a newly reported isolate, *Neosartorya fischeri* ECCN 84.

In addition to the formation of HA-like substances, the formation of FA-like substances was also monitored and the same formation trend for HA-like substances was observed for FA-like substances (Fig. 1A). According to Salati et al. [34], they represent the organic matter (OM) fraction, soluble in both alkali and acid dilute solutions. Quantification results during this experiment showed that FA-like substances were formed in all the waste coal when inoculated with either bacterial consortia or fungal strain ECCN 84. Although the formation of these substances was observed in un-inoculated controls, there was a significant difference (IBM-SPSS statistics 20 software, ANOVA) in concentrations of FA-like substances produced by un-inoculated controls and the various treatments. A positive correlation between the various bacterial consortia and strain ECCN 84 was observed during this study. The low concentrations of FA-like substances extracted from its corresponding HA-like substances as a result of the action of ECCN 10b, ECCN 13b and ECCN 84 was given a major boost when these bacterial consortia were mixed with the fungal strain and an increase in the concentrations of FA-like substances was observed and recorded. Although reports on the antibacterial activities of *Neosartorya* sp. has been published [35, 36, 37], in this study, it was observed that when the fungal isolate was mixed with various bacterial consortia, waste coal degradation efficiency got increased.

5.2. Determination of physico-chemical parameters in waste coal

Due to the action of microorganisms and the added organic fertilizer (algal extract) on waste coal, significant changes in the physico-chemical properties of mine spoil (waste coal) were observed. Water holding capacity of waste coal was monitored alongside other physico-chemical parameters. An initial

water holding capacity of waste coal was observed to be 33.55 ± 3.4% and this reduced WHC according Singh et al. [38] is mainly due to mining activities. As t experiment progressed, a gradual increase in WHC was observed in all waste coal inoculated with various microorganisms with consortium ECCN 13b/84 showing the highest WHC value (Fig. 2A) and this steady increase was recorded between the first and the fifth months of experiment. Juwarkar et al. [39] also observed and reported a similar result in an experiment carried out on developmental strategies for sustainable ecosystem on mine spoil dumps. Waste coal inoculated with bacterial consortia alone had the lowest values of WHC and this observation was more pronounced with ECCN 10b at the end of experiment as compared to ECCN 13B/84. A significant difference (IBM-SPSS Statistics 20, ANOVA) in WHC was observed between the un-inoculated controls and treated waste coal samples at the end of experiment. The above observation was also reported by Chaubey et al [40] when they carried out a case study on the impact of bio-reclamation of coal mine spoil on nutritional and microbial characteristics. However, the treatments involving bacterial consortia alone could not significantly increase the WHC of waste coal when compared to that of ECCN 84 (fungal strain alone), ECCN 10b/84 and ECCN 13b/84 respectively.

Low values were recorded when CEC was determined during this study and according to Sadhu et al. [41], it is as a result of leaching of minerals from overburden/mine spoils. According to Aprile and Lorandi [42], when a low CEC is recorded in an environment such as coal or soil, there must be low levels of water holding capacity, soil organic carbon and nutrient properties. In our experiment, we observed that waste coal treated with ECCN 13b/84 had the highest value of CEC as compared to all other treatment at the end of the experiment. Increase in CEC was not significant in un-inoculated control as there was only a slight increase from 7.68 ± 1.03 meq.100g^{-1} at the start of experiment to 9.33 ± 0.58 meq.100g^{-1} at the end of experiment.

Electrical conductivity (EC) on the other hand was found to improve in all the waste coal treated with the various microorganisms and un-inoculated control. ECCN 13b/84 had the highest EC value when compared to every other treatment and this result was a reflection of the increase in WHC of waste coal which was determined during the experiment. According to Chaubey et al. [40] , increase in EC is directly proportional to increase in WHC. Similar increase in EC was observed by Juwarkar et al. [29, 39] but in their experiment, the increase that was observed was not high enough to support the growth of plants because plants generally need an optimum EC value between the range of 2.0-8.0 mS/cm for growth [41].

The inability of plants to grow on mine spoils is as a result of the high bulk densities recorded [44]. High bulk density which is a characteristic of compaction of mine spoils was recorded in the waste coal used for this experiment. However, with the inoculation of various microbial treatments and the addition of algal extracts as an organic fertilizer, a reduction in bulk density was observed over time. Juwarkar and Jambhulkar [44] and Chaubey et al. [40] have observed the same reduction in bulk density in experiments and concluded that the reduction was mainly facilitated by the addition of organic fertilizer amendments. According to Juwarkar and Jambhulkar [29] and Juwarkar et al [39], the decrease in bulk density will reduce compaction and facilitate aeration and better penetration and spreading of roots thereby making the rhizosphere favorable for massive root development. In our controlled experiment, there was a reduction in waste coal bulk density and according to Juwarkar et al. [39], this could be mainly due to the influence of algal extract on waste coal which was used as an organic fertilizer amendment. Although the above result was observed for un-inoculated controls, statistical analysis (IBM-SPSS Statistics 20, ANOVA) showed that there was a significant difference between the un-inoculated controls and inoculated waste coal. This observed result was also observed by Singh et al. [38] when an experiment on ecological study of re-vegetated coal mine spoil of an Indian dry tropical ecosystem along an age gradient was carried. They stated that mining activities brings about high compaction rate which in turn increases the bulk density of the mine spoil. In our experiment, the highest rate of decompaction (reduction in bulk density) was observed in waste coal treated with ECCN 13b/84 which was 1.05 g.cm^{-3} which is an ideal density for plant growth [45].

6. Conclusion

The present study explores the possible use of various bacterial consortia that were isolated from coal mines in combination with a previously isolated fungal species (*Neosartorya fischeri*) ECCN 84 in rehabilitating mine spoils such as waste coal dumps. The combination of different bacterial strains with the fungal isolate, proved to be the biocatalysts of choice with regard to rehabilitating waste coal dumps. However, more work has to be done to determine the ability of these organisms to support plant growth by secreting plant growth promoting hormones in a harsh environment such as waste coal dumps.

7. ACKNOWLEDGMENTS

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FIGURE HEADINGS

Figure 1. Formation of (A); humic acid- and (B); fulvic acid-like substance from waste coal after treatment with different microbial formulations. Waste coal in pots were inoculated with various microbial treatments (ECCN 84, ECCN 10b, ECCN 13b, ECCN 10b/84 and ECCN 13b/84) and a set of pots were left un-inoculated to serve as controls. These pots were kept in a greenhouse (to mimic the natural environment) for 9 months. Humic and fulvic acid-like substances were extracted monthly and analyzed spectrophotometrically as described in the Materials and Methods. All date are the mean ± SD of three determinations and are representative of duplicate experiments with T_0, T_6 and T_9 representing sampling months 0, 6 and 9 respectively.

Figure 2. Determination of physico-chemical properties of waste coal. A); Estimation of water holding capacity (WHC %) of waste coal for all treatments (ECCN 84, ECCN 10b, ECCN 13b, ECCN 10b/84 and ECCN 13b/84) including un-inoculated control, B); Gravimetric determination of moisture content in waste coal, C); Change in cation exchange capacity (meq/100g) of waste coal during experiment and D); Electrical conductivity (mS/cm) of waste coal over a period of 9 months using various microbial treatments with an un-inoculated control. All data are the mean ± SD of three determinations and are representatives of duplicate experiments with T_0, T_6 and T_9 representing sampling months 0, 6 and 9 respectively.

Figure 3. Determination of bulk density of waste coal treated with various microbial inocula (ECCN 84, ECCN 10b, ECCN 13b, ECCN 10b/84 and ECCN 13b/84) and un-inoculated control. 50 mL bottles were filled up with already sieved coal to the brim by taping and each bottle containing coal was weighed. After weighing, the coal was removed and the bottle was filled with water by means of burette and the volume of water needed to fill the bottle was recorded. The bulk density of the waste coal sample was determined by dividing the weight of the waste coal with the volume of water needed to fill the bottle. All data are the mean ± SD of three determinations and are representatives of duplicate experiments. All data are the mean ± SD of three determinations and are representatives of duplicate experiments with T_0, T_6 and T_9 representing sampling months 0, 6 and 9 respectively.

Figure 1

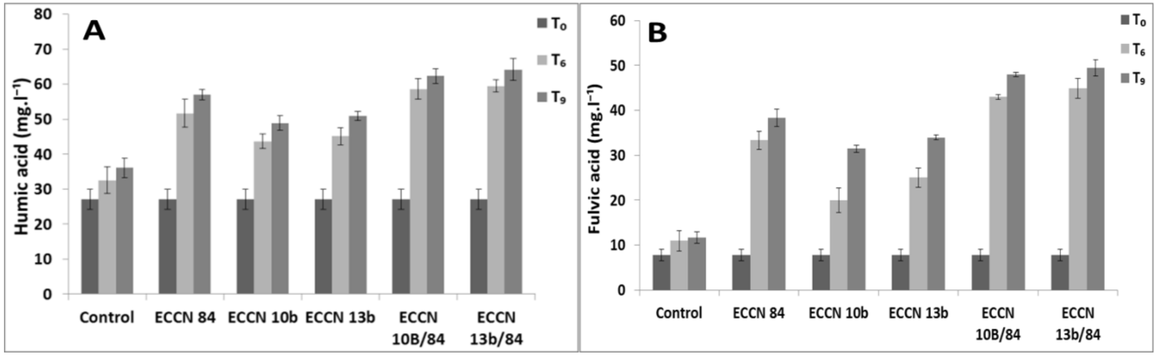


Figure 2

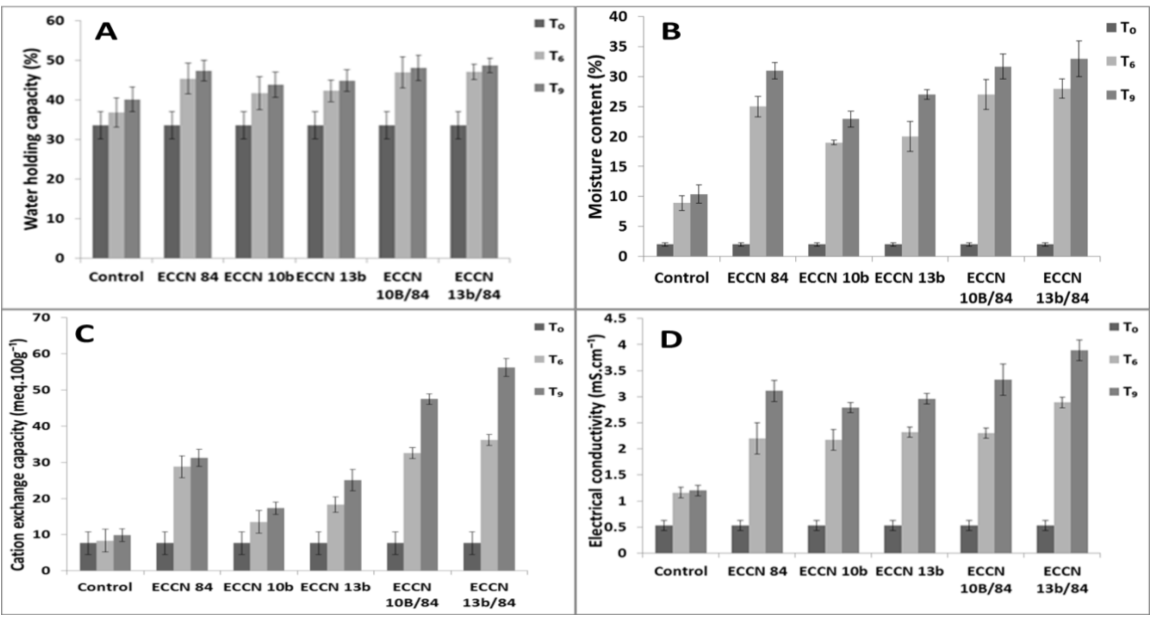
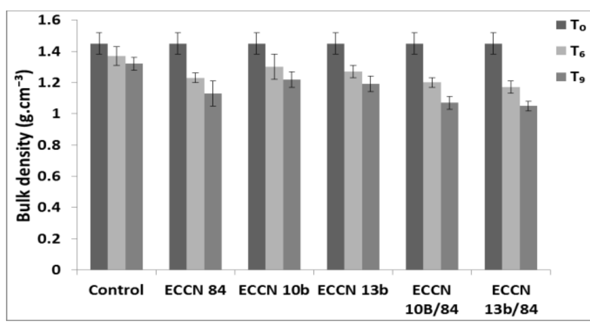


Figure 3



Paper 4

Biodegradation of Benzene, Toluene, Ethylbenzene and Xylene by Bacterial Consortia in Aqueous Medium

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Biodegradation of benzene, toluene, ethylbenzene and xylene by bacterial consortia in aqueous media

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ABSTRACT

Petroleum aromatic hydrocarbons (PHC) such as benzene, toluene, ethylbenzene and xylene (BTEX), represent a serious risk to groundwater. The biodegradation of benzene, toluene, ethylbenzene and xylene was carried out in this study using both single bacterial strains and consortia. These bacteria were sourced from Environmental Biotechnology Research Unit at Rhodes University in Grahamstown, South Africa and comparative biodegradation studies were carried out to derive an efficient treatment for the remediation of PHC contaminated sites. GC-MS analysis of residual BTEX in aqueous media showed that only two bacteria strains were able to degrade BTEX in pure culture with an efficiency of breakdown of 43-50% within 6 days. However, with the establishment and use of bacterial consortia, an increase in degradation efficiency was observed and consortia ECCN 2b and ECCN 7b were able to degrade BTEX in MSM routinely in the range of 80-100% within 6 days. Thus the application of pre-selected bacterial consortia in biodegradation processes can represent a reliable and effective tool in the treatment of BTEX contaminated environments.

Keywords: BTEX, Biodegradation, PHC, Bacterial consortia

1. Introduction

Petroleum hydrocarbon (PHC) contamination in soil, atmosphere, marine waters and sediment is a widespread problem (Balachandran et at., 2012), with many hotspots of pollution arising from individual spills (Whittaker et al., 1995; Martinho et al., 2010). During the fractional distillation of crude oil, different fractions such as fuel, diesel, gasoline and paraffin are obtained, which are major sources of heat and energy for industries (Das and Chandran, 2011). During exploration, production, refining, transport, and storage of these products, leaks and accidental spills occur (Thouand et al., 1999; Lohi et al., 2008). Although the use of PHC as an energy source has led to intensive economic development worldwide (Das and Chandran, 2011), the negative impacts have affected the lives of humans, animals, plants and the ecosystem in general (Alvarez and Vogel, 1991; Holliger et al., 1997). The negative impact of PHC in the environment is due to the toxic nature of its constituents which include non-aqueous (Pao-Wen et al., 2011) hydrophobic components like *n*-alkanes, resins, asphaltines and aromatics such as benzene, toluene, ethylbenzene and xylene isomers (Xiong et al., 2012). These aromatic compounds are widely distributed in fossil fuels such as coal and PHC, and are used in several industrial processes (Hutchins et al., 1991; Lin et al., 2010). BTEX constituents are considered among the most prevalent groundwater pollutants (Coates et al., 2002; Chakraborty and Coates, 2004) and have been highlighted as the most toxic set of compounds with mutagenic and carcinogenic potentials (Bogan and Sullivan, 2003; Kasai et al., 2006; Wolicka and Suszek, 2008; Mazzeo et al., 2010). These substances are hazardous and threaten the health of humans and the safety of the soil ecological system (Ting et al., 1999; Samanta et al., 2002; Sarkar et al., 2005; Zhou et al., 2005). Their relatively high solubility in water is directly proportional to the high risk they pose to ground water (Mazzeo et al., 2010). This persistence is influenced by the nature of the contaminant, the amount of the contaminant present and the interplay between chemical, geological, physical and biological characteristics of the contaminated site (Balachandran et al., 2012). However, the residence time of PHC in any environment has been highlighted as a factor which also leads to persistence (Erickson et al., 1993) i.e., the longer the contaminant remains in the environment; the harder it is for microorganisms to degrade it. Due to the adverse impact of these chemicals on human health and the environment, they are classified as priority environmental pollutants by the US Environmental Protection Agency (US EPA, 1986; Wu et al., 2008) and the US EPA has consequently established maximum permissible levels in water of 0.005, 1.0, 0.7 and 10 ppm for benzene, toluene, ethylbenzene and xylene, respectively.

Over the years, technologies have been developed to clean up petroleum hydrocarbon contaminated sites. Some of the methods used include mechanical, physico-chemical and biological technologies (Murygina et al., 2000; Okoh, 2006; Das and Chandran, 2011; Balachandran et al., 2012). These technologies have been used in conjunction with one another (Reddy et al., 1999), while other studies have demonstrated the use of these technologies in isolation (Long, 1993; USEPA 1999). The use of mechanical and physico-chemical technologies for the rehabilitation of hydrocarbon contaminated sites has been documented as prohibitively expensive and can lead to incomplete decomposition of contaminants (Yang et al., 2000; Das and Chandran, 2011) and is labour intensive (Salleh et al., 2003). Biological technology on the other hand, has proven to be the best and most cost effective method of rehabilitating PHC contaminated sites (April et al., 2000; Balachandran et at., 2012; Mao et al., 2012). Biodegradation, an example of a biological method of remediation, exploits the ability of microorganisms to degrade and/or detoxify organic contaminants in a given environment (Margesin and Schinner, 1997) and the implicated microorganisms in this regard include bacteria, yeasts and fungi (Korda et al., 1997; Das and Chandran, 2011). Several studies have shown that the hydrocarbon degradation efficiency of soil fungi ranges between 6 and 82% and is 0.13 - 50% for soil bacteria (Jones et al., 1970; Pinholt et al., 1979; Wolicka et al., 2009) while some marine bacteria show degradation efficiency up to 100% (Phillips and Stewart, 1974; Hollaway et al., 1980). According to Das and Chandran (2011), bacteria are the most active agents in PHC degradation, and these organisms work as primary degraders of spilled oil in the environment. Their high frequency, fast growth and utilization of PHC within a wide spectrum make them the organisms of choice with regard to biodegradation (Wolicka et al., 2009). The use of single strains of bacteria in the degradation of PHC has been widely documented and results show that these are generally not as effective when compared to consortia of multiple strains (Korda et al., 1997; Morlett-Chávez et al., 2010).

Although the literature has emphasised the crucial role of some microorganisms in the degradation of BTEX, the main objective of this study was to evaluate the potential of bacterial isolates capable of degrading coal in combination with isolates from diesel contaminated soils and established bacterial consortia (Edeki et al., 2014, unpublished Paper 2) on BTEX breakdown using bioaugmentation as a strategy. The study was carried out to establish biocatalysts that can contribute to the design of a simple and efficient and effective protocol for treatment of contaminated soils.

2. Materials and Methods

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2.1. Preparation of BTEX

BTEX was prepared by mixing equal weights (wt %) of benzene (99%; CAS Nº 71-43-2), toluene (99%; CAS Nº - 108-88-3), ethylbenzene (99.80%; CAS Nº 100-41-4) and xylene (99% mixed isomers; CAS Nº - 1330-20-7) in the ratio of 1:1:1:1 (g/g/g/g/g).

2.2. Isolation, selection and enrichment of bacteria strains with BTEX degrading abilities and determination of tolerance level to BTEX by each strain

Bacterial isolates and consortia used in this study were sourced from the culture collection at Rhodes University in the Institute for Environmental Biotechnology, Grahamstown, South Africa, These isolates and consortia were initially used in previous studies on the biodegradation of waste coal (Edeki et al., 2014, unpublished Paper 2). Strains and consortia stored in glycerol stock were resuscitated in Luria broth incubated at 30 °C for 18 h. Cells were harvested by centrifugation (Eppendorf 5415D desktop centrifuge) at 10.000 x g for 10 min and the supernatant discarded while the pellets were washed using sterile phosphate buffered saline (PBS). The washed bacterial cells were centrifuged as above and this process repeated 3 times to ensure removal of any residual carbon from the medium (Igbinigie et al., 2008). Concentration of washed bacterial cells was adjusted to 0.5 by determining the absorbance of PBS diluted bacterial cultures at 600 nm. Each strain was subjected to preliminary screening for their ability to grow in benzene, toluene, ethylbenzene, xylene or BTEX media by inoculating 1 mL (0.5 AU, 3.20 x 10^6 - 3.86 x 10^6 CFU/mL) of pre-washed cells into Bushnell Haas media (Youssef et al., 2010) at pH 7.0 (Xia et al., 2006) containing the above substrates incubated at 30 °C for 2 weeks during which, turbidity of each medium was monitored. Bacterial cultures that showed signs of increased turbidity indicative of growth were collected and used for the remaining part of this study.

For the determination of bacterial biomass in BHM containing BTEX as the sole carbon source, cells were harvested into pre weighed Eppendorf tubes and centrifuged at 10 000 x g for 10 min; the supernatant discarded and pellets dried (50 ºC) to a constant weight. The initial weights of tubes were subtracted from the final weights and the resultant biomass expressed in mg.

Tolerance of each strain to different concentrations of BTEX was monitored to determine the optimum concentration of BTEX needed by each bacterial strain for effective degradation. BHM medium was prepared as described above and different concentrations of BTEX (10, 100, 500, 1000, 1500, 2000, 2500 and 3000 $mg.l^{-1}$) were added to BHM containing pre-washed bacterial isolates (as above) and incubated at 30 °C for 2 weeks. Optimum concentration of BTEX was determined after 2 weeks by taking absorbance readings spectrophometrically at 600 nm (Thermo Spectronic Aquamate, ThermoFisher Scientific, Waltham, MA) of each medium and values obtained were used to derive a measure of tolerance to BTEX.

2.2.1. Cultivation and incubation procedures

Mineral salt medium (MSM) containing: K_2HPO_4 (1.71 $g.l^{-1}$), KH_2PO_4 (1.32 $g.l^{-1}$), $NHCl_4$ (1.26 $g.l^{-1}$), $MgCl_2$ $6H_2O$ (0.011 $g.l^{-1}$), $CaCl_2$ (0.02 $g.l^{-1}$) which was enriched with 4 ml trace mineral solution (TMS containing ($mg.l^{-1}$): $MnSO_4.H_2O$; 5.0, NaCl; 10.0, $FeSO_4.H_20$; 5.0, $CoCl_2$; 1.0, $CaCl_2$; 1.0, $ZnSO_4.7H_20$; 1.0, $CuSO_4.5H_20$; 0.1, $AlK(SO_4).12H_20$; 0.8, H_3BO_3; 0.1, $NaMoO_4.2H_20$; 0.9) at pH 7.0 was dispensed (50 mL) into 100 mL flasks and autoclaved at 121° C for 15 min. After cooling to ambient, 1 g of BTEX was added into each flask. Six of the bacterial isolates which showed signs of turbidity during preliminary screening, were inoculated into different flasks, the flasks sealed completely, and incubated at 30 °C and biodegradation monitored continually over a period of 6 d as indicated in the Results. In a second experiment flasks containing BTEX and MSM prepared as above were inoculated with 1 mL bacteria consortia suspension prepared by combining the individual bacterial isolates used above. Each consortium (3.20 x 10^6 - 3.86 x 10^6 CFU/mL) comprised either 2 or 3 bacterial isolates as described by Edeki et al. (2014, unpublished Paper 2). The degradation of BTEX by each bacterial isolate was monitored over time and compared to that of the bacterial consortia.

2.3. Extraction and analysis of petroleum hydrocarbons

Residual BTEX following inoculation and incubation was extracted and analysed by GC-MS. Briefly, aliquots of culture medium were centrifuged (Eppendorf 5415D desktop centrifuge) at 10 000 x g for 20 min at 4 ºC to remove bacterial cells and particulate material and the supernatant applied to a pre-wetted 500 mg C_{18} solid phase extraction column (International Sorbent Technology, UK). Trapped organic materials in the column stationery phase were eluted using 100% acetone (1 mL) for analysis by GC-MS.

Electron impact mass spectra were recorded at 70 eV using a MS 5975 Mass Selective Detector coupled to a 6890N gas chromatograph (Agilent Technologies, Santa Clara, CA) fitted with a HP-5MS fused silica capillary column (30 m x 0.25 mm i.d.; 0.25 µm film thickness) with He as carrier gas (50-55 kPa). The system was set in split mode (2:1) with a 90 min solvent delay time. Temperature programming was from 60°C at 7°/min to 220°C followed by 15°C/min to 280°C, and isothermnally for 5 min. compounds present were detected using the parameters 1.7 kV SEV, 1.1 s scan rate and identified using NISTO.5 library and authentic standards. BTEX degradation was calculated by peak area integration after calibration with authentic standards derived from benzene, toluene, ethylbenzene and xylene respectively.

3. Statistical analysis

Random block design was adopted during incubation of BTEX media and flasks were randomly selected for analysis daily with sampling carried out destructively. IBM-SPSS statistics 20 software was used to analyze all data via one way analysis of variance (ANOVA) with a 95% degree of confidence to detect significant differences between the treated groups and controls whereby the level of statistical significance was accepted at $p < 0.05$ and line diagrams constructed using Microsoft Office Excel 2010. Data are presented as the mean of at least three determinations ± standard deviation (SD).

4. Results

Preliminary screening of bacterial strains used in this study was carried out in BHM with benzene, toluene, ethylbenzene, xylene or BTEX as a carbon source. At the end of this experiment, turbidity was observed in all the media inoculated with strain ECCN 18b (*Bacillus massiliensis)* and ECCN 23b (*Proteus mirabilis*) while ECCN 19b (*Serratia nematodiphila*) and ECCN 21b (*Exiguobacterium aurantiacum)* were only able to

use benzene, toluene, ethylbenzene and BTEX as substrates for growth (Table 1). Growth inhibition was observed in benzene and toluene media inoculated with strains ECCN 20b (*Proteus penneri*) and ECCN 22b (*Mycobacterium esteraromaticum)* while at the same time, these strains grew and utilized ethylbenzene and xylene as carbon sources. The morphological characteristics and Genbank accession numbers of each strain is presented in table 1 while the composition of various bacterial consortia used in this study is presented in table 2.

Based on the results obtained for each strain inoculated into BHM that had BTEX as a substrate for growth, cells were harvested from these media, dried and the weights of dried bacterial biomass taken. Results obtained in this analysis showed the gradual but steady increase in biomass accumulation over time by each strain with ECCN 18b having the highest amount of biomass at the end of experiment (Fig. 1).

4.1. Bacterial tolerance to BTEX

For effective biodegradation, optimum concentration of BTEX that each strain can tolerate was determined. Various concentrations of BTEX were used in this regard as described in the materials and methods. Obtained results showed a gradual increase in absorbance of each inoculated medium as BTEX concentration increased (Fig. 2). At a BTEX concentration of 2000 $mg.l^{-1}$ where the highest absorbance was recorded for each medium, ECCN 20b and ECCN 21b had the lowest bacterial growth of 1.63 and 1.58 AU amongst all the strains (Fig. 2). Growth inhibition of each isolate was observed when the concentration of BTEX increased from 2000 to 3000 $mg.l^{-1}$ and this was mainly due to bacterial cell death based on the toxic nature of the substrate and on the average, each bacterial isolate required an optimum BTEX concentration of 1500 $mg.l^{-1}$ to effectively carry out biodegradation of the substrate.

4.2. Biodegradation of BTEX substrate by single bacterial strains and consortia

The degradation of BTEX in aqueous media using single bacterial strains and consortia was carried out and daily degradation was monitored for a 6 d period. Results obtained during this analysis showed the effective degradation of BTEX by both single bacterial strains and consortia. Quantitative analysis of individual components in BTEX revealed benzene degradation efficiency of ECCN 2b and ECCN 7b (bacterial consortia) to be very high when compared to other bacteria including single strains (Fig. 3A). ECCN 18b, ECCN 20b and ECCN 6b were only able to degrade benzene at a rate of 74.4 %, 78.6 % and 69.4 % respectively. The highest degradation recorded for ECCN 2b and ECC 7b were between day 0 and day 2 which is a confirmation of the preliminary results obtained during screening of bacteria strains with BTEX degradation potentials.

The degradation of toluene on the other hand was not too effective with all the bacteria except consortium ECCN 7b which degraded toluene (99 %) on the sixth day of experiment (Fig. 3B). Effective degradation of toluene by ECCN 7b was observed between day 1 and 3 and 79 % degradation was achieved within these days. Toluene breakdown was fast with consortium ECCN 2b on the first day but slowed down as experiment progressed to the fourth day and increased sharply on the fifth and sixth day to achieve a total toluene degradation of 89 % (Fig. 3B). In comparison to ECCN 7b and ECCN 2b, the single strains; ECCN 18b and ECCN 20b were not able to effectively degrade toluene and at the end the 6 d experiment, a total toluene degradation of 60 % and 68 % was achieved with isolates ECCN 18b and ECCN 20b respectively.

The same degradation pattern was observed for all bacteria with regard to ethylbenzene and xylene breakdown. Observed results at the end of the 6 d experiment showed that ethylbenzene and xylene were completely degraded within 4 days post inoculation with ECCN 7b (Fig. 3C and 3D). Although ethylbenzene degradation process was slow with treatment ECCN 2b, this consortium was able to effectively degrade xylene within 4 days of incubation achieving a total degradation of 99 % (Fig. 3D). The single isolate; ECCN 18b had the lowest ethylbenzene and xylene degradation effeciency at the end of experiment.

Daily BTEX degradation was measured during the period of experiment and observed results showed that consortia ECCN 2b and ECCN 7b were able to degrade 66.5 and 69.83 $mg.l^{-1}$ of benzene on a daily basis while the single strains; ECCN 18b and ECCN 20b had daily degradation of 35.2 and 39.3 $mg.l^{-1}.d^{-1}$ when compared to the least efficient consortium; ECCN 6b which had a daily benzene degradation of 40.33 $mg.l^{-1}.d^{-1}$ (Fig. 4). Reduction in the concentration of benzene was also observed in the control experiment (1.17 $mg.l^{-1}.d^{-1}$). Daily degradation of toluene and ethylbenzene by each inoculum was also monitored and results at the end of experiment showed that ECCN 7b was able to degrade both toluene and ethylbenzene at a rate of 66.5 and 39.16 $mg.l^{-1}.d^{-1}$ compared to the single strains; ECCN 18b and ECCN 20b which had 22.8 and 17.6 $mg.l^{-1}.d^{-1}$ for strain 18b and 25.4 and 17.9 $mg.l^{-1}.d^{-1}$ for strain 20b (Fig. 4).

5. Discussion

Published articles have stressed the toxic nature of BTEX compounds when introduced into any given environment (Attaway et al., 2002; Wang et al., 2008; Wolicka et al., 2009). With global concern being directed towards these toxic compounds and effective ways to remediate environments that are contaminated with PHC, we saw a need to use bacteria strains and consortia which were initially used in previous studies to degrade coal in carrying out this experiment (Edeki et al., 2014, unpublished Paper 2). These bacteria were isolated from coal slurry and diesel contaminated soils and according to Nikolova and Nenov (2005) and Wang et al. (2008), these bacterial isolates have been found to be the bacteria of choice with regard to PHC degradation. For a better understanding of the strains used in this study, assays were carried and their BTEX metabolic activities determined. Gram staining of each strain revealed that four out of the six strains used in this study were gram negative bacteria (Table 1) and this group of bacteria are the main group of organic pollutant degraders (MacNaughton et al., 1999).

A key deciding factor used in analysing biodegradation of PHC in any medium using bacteria, is their ability to survive in that given medium. All 15 strains sourced from the Institute for Environmental Biotechnology at Rhodes University in South Africa, were inoculated into various media made up of either benzene, toluene, ethylbenzene, xylene or BTEX and media were observed visually for turbidity. Initial observation showed that only 6 strains were able to turn each of these medium turbid (Table 1). Positive reactions were observed in media that had benzene, toluene, xylene, ethylbenzene and BTEX as the sole carbon sources when inoculated with strains ECCN 18b and ECCN 23b while strains ECCN 19b and ECCN 21b were able to utilize all the carbon sources they were supplied with except Xylene. Strains ECCN 20b and ECCN 22b were only able to show signs of turbidity in media containing xylene, ethylbenzene and BTEX. The above result confirms that of Mukherjee and Bordoloi's (2012) experiment on biodegradation of benzene, toluene, and xylene (BTX) in liquid culture and in soil by *Bacillus subtilis* and *Pseudomonas aeruginosa* strains and a formulated bacterial consortium. In their experiment, they discovered that single strains of bacteria were unable to effectively utilize individual components of BTX as substrates for growth when compared to a consortium and they concluded that the ability of all the strains to utilize BTX and show signs of turbidity in the media was due to the action of co-metabolism.The term co-metabolism has been defined by Horvath (1972) as the transformation of an organic compound by a microorganism incapable of using the substrate as a source of energy or of one of its constituent elements. Attaway and Schmidt (2002) observed same result when they inoculated

two strains of *Pseudomonas* sp. in a growth medium which comprised of benzene, toluene, ethylbenzene*, m*-xylene*, p*-xylene, *and o*-xylene. They discovered that one of the strains was able to utilise benzene, toluene and ethylbenzene as carbon sources while the other strain was only able to metabolise *m*-xylene and *p*-xylene. Neither strain was able to metabolise *o*-xylene in medium. A similar result was reported by Wang et al. (2008) when they cultured species of *Pseudomonas, Rhodococcus, Exiguobacterium* and *Bacillus* in media containing benzene, toluene and xylene. They discovered that most of the bacteria species were able to degrade *p*-xylene and ethylbenzene while difficulties in bacteria growth was observed with media that were made up of the other compounds. With regard to bacteria growth inhibition by benzene, Otenio et al. (2005) discovered that *P. putida* CCMI 852 was able to metabolise toluene and xylene in a mixture of BTX but benzene was not metabolised. When BTX was used as a carbon source, complete bacterial growth was observed and this result was a confirmation of previous observation that suggested a reaction known as co-metabolism (Attaway and Schmidt, 2002).

Tolerance levels of each bacterial strain to different concentrations of BTEX were monitored in shake flasks. At the end of experiment, it was observed that at higher concentrations of BTEX, bacterial growth was inhibited because of the toxic nature of the substrate. This result however, confirms that of Shim et al. (2005) that determined kinetics of BTEX biodegradation by a co-culture of *Pseudomonas putida* and *Pseudomonas fluorescens* under hypoxic conditions. They discovered that at higher concentrations of BTEX, metabolic activities of bacteria were inhibited. Another report by Shim et al. (2002) and Otenio et al. (2005) found the tolerance level of bacteria to BTEX to be 100 $mg.l^{-1}$. Moreover, a report by Mukherjee and Bordoloi (2012) also confirmed this result when they observed in their experiment that biodegradation was dependent on the concentration of benzene, toluene, and *m*-xylene in their culture medium.

Shake flask experiment was used to measure the degradation efficiencies of various bacterial strains and consortia used in this study. Among all the consortia, only 3 showed effective BTEX metabolic activities. Chromatographic results from GC-MS analysis showed a constant and rapid reduction in BTEX peaks within 6 days. Percentage degradation of BTEX for each consortium was determined over time and compared to an un-inoculated control. There was a significant difference (IBM-SPSS statistics 20, ANOVA) between each bacteria and un-inoculated control. When a group analysis was carried out statistically, there was no significant difference between ECCN 6b, ECCN 18b and ECCN 20b. Total degradation of both ECCN 2b and ECCN 7b with respect to BTEX metabolism were significantly different from every other bacteria and a positive correlation was observed between strains ECCN 18b and ECCN 20b. At the end of experiment, it was observed that the best performing bacteria in this study were ECCN 6b and ECCN 7b. An experiment on biodegradation of benzene, toluene, and xylene (BTX) in liquid culture and in soil by *Bacillus subtilis* and *Pseudomonas aeruginosa* strains and a formulated bacterial consortium was carried out by Mukherjee and Bordoloi (2012) by comparing the metabolic activities of single bacterial isolates with a consortium in BTX media. At the end of their experiment, they however reported that a combination of two or more bacteria strains will always be the biocatalyst of choice with regard to biodegradation of petroleum hydrocarbons.

From the results obtained in this study, it was observed that bacterial consortia were the best performing biocatalysts with regard PHC degradation and this is in line with results obtained by Shim et al. (2005) when they carried out an experiment to determine the kinetics of BTEX biodegradation by a co-culture of *Pseudomonas putida* and *Pseudomonas fluorescens* under hypoxic conditions.

6. Conclusion

It is a common phenomenon that BTEX are recalcitrant group of compounds because of their high level of solubility in water. Results from this study provide evidences that the degradation of petroleum hydrocarbons can be effectively achieved by different bacterial consortia within a short space of time. Reports from previous studies showed the importance of using mixed cultures in rehabilitating contaminated sites (Babaarslan et al., 2003). Further studies involving the enzymatic actions/secretion of each bacterium during PHC degradation is needed to fully elucidate the various mechanisms involved the break-down process.

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FIGURE HEADINGS

Figure 1. Measurement of dried bacterial cell biomass ($g.l^{-1}$) obtained from BHM containing BTEX as the sole carbon source. Cells were harvested from this medium by centrifugation, dried and weights recorded. All data are the mean ± SD of three determinations and are representatives of duplicate experiments.

Figure 2. Growth and tolerance of each bacteria strain to different concentrations of BTEX in BHM. Changes in bacterial cell density were determined spectrophotometrically by measurement of absorbance at A_{600} at the end of experiment and values obtained were used to derive a measure of tolerance to BTEX. All data are the mean ± SD of three determinations and are representatives of duplicate experiments.

Figure 3. Percentage degradation of the different components in BTEX by various bacterial strains; ECCN 18b and ECCN 20b compared to established bacterial consortia; ECCN 2b, ECCN 6b and ECCN 7b. Degradation rates by bacteria was monitored daily (as described in the materials and methods) and at the end of the 6 d period of experiment, percentage degradation was calculated and all data are the mean ± SD of three determinations and are representatives of duplicate experiments.

Figure 4. Daily degradation rate of BTEX in aqueous media by various bacterial strains; ECCN 18b and ECCN 20b compared to established bacterial consortia; ECCN 2b, ECCN 6b and ECCN 7b. Average degradation rates of bacteria at the end of experiment was used to score their daily BTEX degradation rates and all data are the mean ± SD of three determinations and are representatives of duplicate experiments.

Table 1. Morphological characteristics and growth performance of bacterial strains with BTEX degrading abilities

Genbank accession numbers	Closest related Species	Gram Stain	Morphology	Benzene	Toluene	Ethyle benzene	Xylene	BTEX
KC620473	*Bacillus Massiliensis*	-ve	rod shaped	++	++	+	+	++
KC620474	*Serratia Nematodiphila*	-ve	rod shaped	+	+	+	-	++
KC620475	*Proteus Penneri*	-ve	rod shaped	-	-	+	++	+
KC620476	*Exiguobacterium aurantiacum*	+ve	rod shaped	+	++	+	-	+
KC620477	*Mycobacterium esteraromaticum*	+ve	rod shaped	-	-	++	+	+
KC620478	*Proteus mirabilis*	-ve	rod shaped	+	++	+	+	++

(+): positive turbidity, moderate growth.
(++): positive turbidity, intense growth.
(−): negative turbidity, no growth.
(+ve): positive
(-ve): negative

Table 2. GenBank accession numbers and composition of bacterial consortia

Genbank accession numbers	ECCN[A]
KC620473 + KC620475	ECCN 1b
KC620473 + KC620474	ECCN 2b
KC620473 + KC620476	ECCN 3b
KC620473 + KC620477	ECCN 4b
KC620473 + KC620474 + KC620475	ECCN 5b
KC620473 + KC620476 + KC620474	ECCN 6b
KC620473 + KC620475 + KC620476	ECCN 7b
KC620473 + KC620477 + KC620475	ECCN 8b
KC620475 + KC620476 + KC620477	ECCN 9b
KC620473	ECCN 18b
KC620474	ECCN 19b
KC620475	ECCN 20b
KC620476	ECCN 21b
KC620477	ECCN 22b
KC620478	ECCN 23b

[A]ECCN = EBRU Culture Collection Number

FIGURE 1

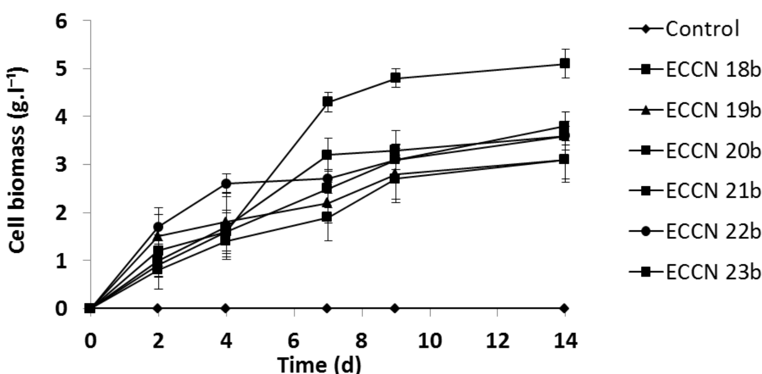


FIGURE 2

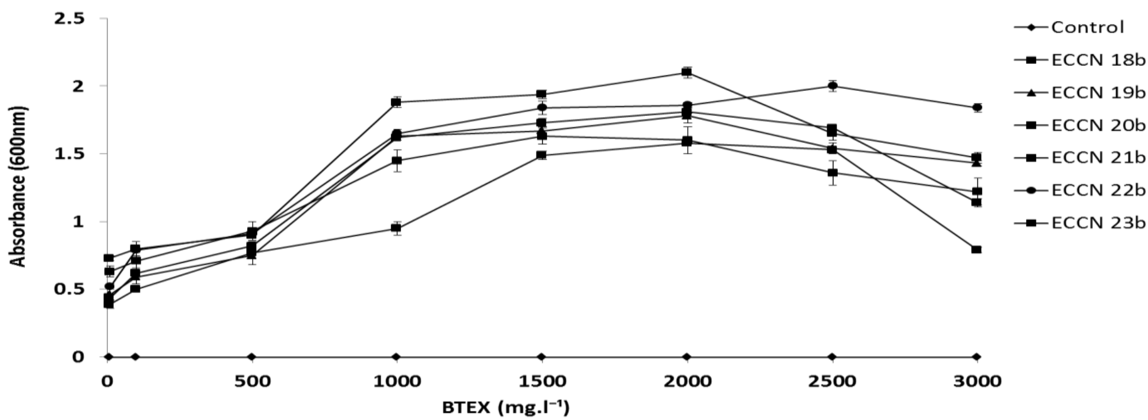


FIGURE 3

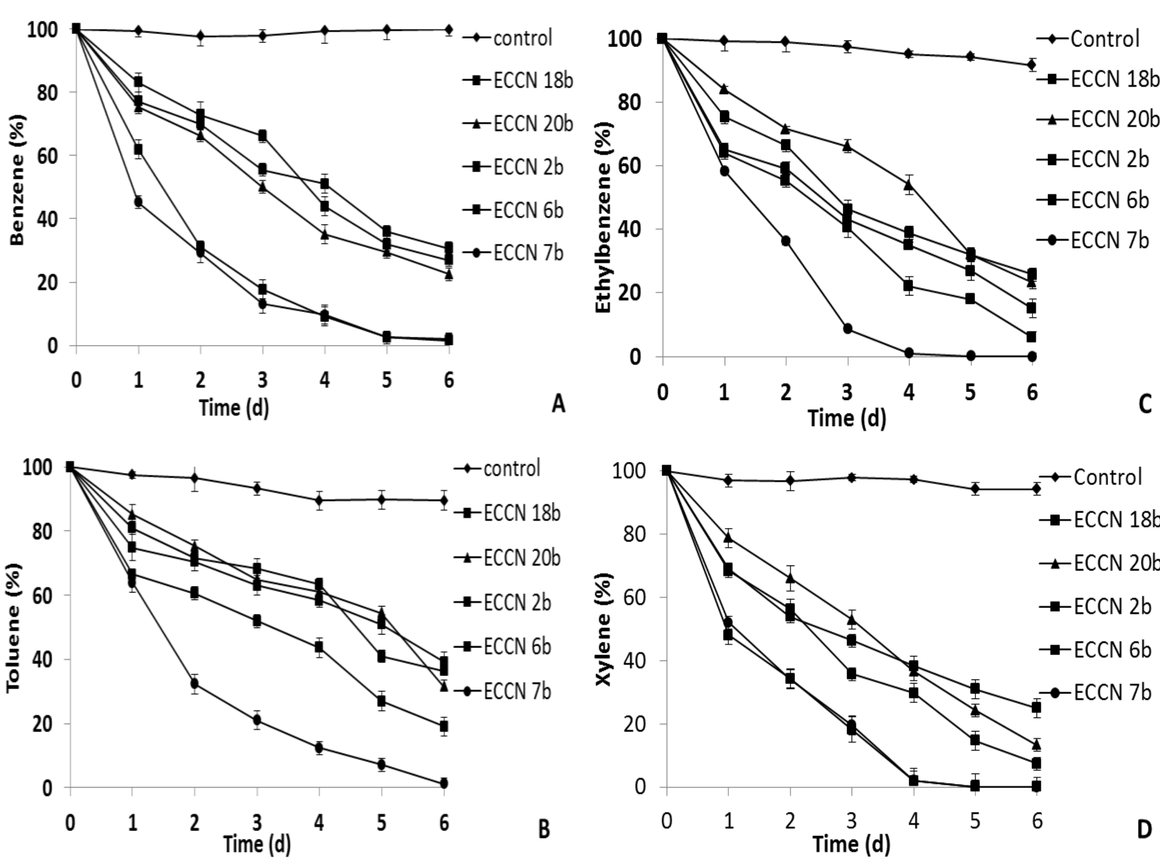
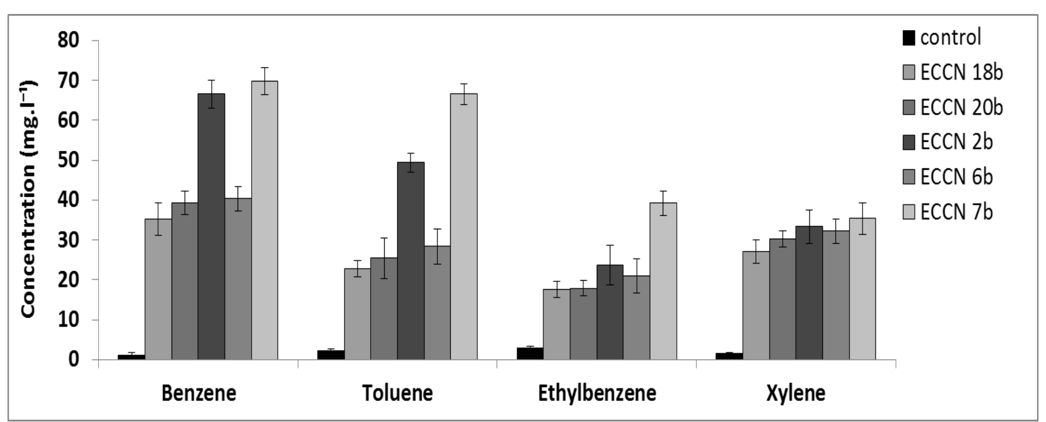


FIGURE 4



Paper 5

Biodegradation of Diesel by Bacterial Isolates and Consortia in Aqueous and Solid Media

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Biodegradation of Diesel by Bacterial Isolates and Consortia in Aqueous and Solid Media

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Accepted

This paper presents a comparative study of diesel fuel biodegradation in aqueous and solid media. Bacteria used for this study were sourced from the culture collection at the Institute for Environmental Biotechnology at Rhodes University and Coastal Environmental Services, Grahamstown, South Africa. Single bacterial strains and consortia were screened in aqueous media for their diesel degradation abilities and the best performing bacteria was used to degrade diesel in soil and results obtained were compared to that of commercially available broad spectrum mixed hydrocarbon degraders. At the end of the study, it was observed that an already established bacterial consortium; ECCN 7b was able to effectively degrade both aromatic and aliphatic components of diesel in aqueous and solid media within a short period of time when compared to single bacterial isolates and commercial biocatalysts. This result was confirmed by GC-MS chromatograms obtained during analysis of residual diesel in soil. At the end of experiment, an average daily degradation rate of aromatic compounds in diesel soil that had ECCN 7b was 4.5 mg.kg^{-1}.d^{-1} while aliphatic daily degradation rate was 5.1 mg.kg^{-1}.d^{-1}.

Key words: GC-MS, Petroleum hydrocarbons, Diesel, Biodegradation, Consortia

INTRODUCTION

During exploration, production, refining, transport and storage of petroleum and petroleum products, leakage and accidental spillage are a regular occurrence (Hall et al., 2003; Pena-Castro et al., 2006; Xia et al., 2006: Sharma and Rehman, 2009; Padayachee and Lin, 2011; Kim and Lee, 2012). Among the various pollutants that enter the environment almost daily, diesel has been reported as the most significant (Li et al., 2008; Taccari et al., 2012; Zhang et al., 2013). It's significance is embedded in the fact that it is made up of a complex mixture of about 80% saturated and alicyclic hydrocarbons from C_{10} to C_{20} (primarily paraffins including the *n*-, *iso*-, and cycloparaffins), and 20% aromatic hydrocarbons (including alkylbenzenes, naphthalenes and polycyclic aromatic hydrocarbons, PAH) (Bento et al., 2005; Jonsson and Östberg, 2011; Dussán and Numpaque, 2012). According to Kaczorek et al. (2013), these compounds contribute to ecological problems due to their low levels of solubility in water. As a result of frequent oil spillages globally, the demand for efficient clean-up technologies has increased. Thus, there is concern regarding the clean-up processes in

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use at present which do not always appear to be directed towards preservation of the environment (Margesin and Schinner, 1999; Chemlal, 2012) but on development of appropriate cost effective remediation processes.

A number of techniques have been developed for clean-up of sites contaminated by petroleum hydrocarbons (PHCs) and include physical, chemical, biological, thermal, and stabilization methods and it appears that biological procedures are being accepted as the best and most cost effective (Taccari et al., 2012). Although, Yang et al. (2000) reported the use of various mechanical means such as floating booms, skimmers, sorbents and water separators in containing and recovering spilled oil in marine environments, others have suggested the use of two or more processes in cleaning up hydrocarbon spillages to enhance removal efficiency (Kim and Lee, 2012). Furthermore, the use of a multi-pronged approach avoids many of the technical restrictions apparent when used in commercial scale decontamination. The use of biological methods and particularly bioremediation in cleaning up hydrocarbon spillage is acknowledged globally as highly efficient for the treatment of sites containing high levels of biodegradable petroleum compounds (Nano et al., 2003; Fallgren and Jin, 2008). Moreover, due to complete destruction of contaminants the process is regarded as eco-friendly as it rarely produces secondary waste (Kim and Lee, 2012).

It has been widely reported that various microorganisms (i.e. bacteria and fungi), either singularly (Kaczorek et al., 2013) or as a consortium (Richard, and Vogel, 1999; Moliterni et al., 2012) can degrade/decontaminate hydrocarbons in spoiled sites (Mills et al., 1999; Olson et al., 1999; Gogoi et al., 2003; De Oteyza et al., 2004). Reports have also shown that different bacteria and fungi can be used together in a consortium to remediate polluted sites (Li et al., 2008). During the treatment process, nutrients and oxygen are often required to (bio)stimulate the resident microbial population and enhance hydrocarbon metabolism (Padayachee and Lin 2011; Taccari et al., 2012). Microorganisms have also be isolated from polluted environments, enriched, and used in a process known as bioaugmentation (Rahman et al., 2003; Bento et al., 2005; Xu and Lub, 2010).

In this study, a combination of bioaugmentation and biostimulation was adopted to investigate the ability of previously isolated coal and BTEX degrading bacteria (Edeki et al. Paper 2; Edeki et al. Paper 4) in consortia to degrade diesel. Algae biomass was used as a source of nutrient to stimulate the bioaugmentation process and the efficiency of biodegradation of diesel in soil by best performing bacterial consortium was compared to that obtained using commercially available broad spectrum mixed hydrocarbon degraders. In relation to the above, experiments were carried in aqueous media to screen for bacteria with diesel degradation potentials while results obtained from diesel soil studies were discussed in terms of developing the best performing bacterial consortium for commercial rehabilitation purposes.

MATERIALS AND METHODS

Diesel fuel was obtained locally from a fuel station (Grahamstown, South Africa) and stored in an airtight container and used as received. All other chemicals (both inorganic and organic) were of analytical grade and were purchased from Sigma-Aldrich, St Louis, MO, USA.

Bacterial Cultures, Enrichment and Nutrient amendment

Different bioaugmentation approaches were used to investigate bacterial degradation of diesel in this study. The first comprised of two commercially available broad spectrum mixed hydrocarbon degraders (C 1 and C 2 obtained from Coastal and Environmental Services, Grahamstown, South Africa). For propriety reasons, the identity of these commercial products may not be divulged. The second and third sets of bacteria were made up of single strains and consortia which were sourced from the Institute for Environmental Biotechnology, Rhodes University, Grahamstown, South Africa. These strains were isolated from coal slurry and diesel contaminated soils and various consortia were established as previously described (Edeki et al., 2014, unpublished Paper 2 and 4) and after molecular characterization, each isolate was stored in glycerol media. For the purpose of this study, all bacteria (as stated above) were resuscitated in Luria broth and washed in PBS and enriched in Bushnell Haas media (BHM) with diesel as the sole carbon source. Briefly, washed bacteria were inoculated in BHM with diesel and incubated for 5 d at 30°C after which, 1 mL of bacterial suspension was transferred to fresh enrichment medium and incubated under the same conditions for a further 5 d. This step was carried out in three cycles as described by Wyrwas et al. (2011) and cells from the last cycle were harvested by centrifugation (10 000 x g, 10 min). Each suspension was adjusted to 0.5 absorbance units (A_{600}) by dilution with phosphate buffered saline to a final concentration of 3.24-3.62 x 10^9 CFU/ml and 1 mL used as inoculum for biodegradation studies in aqueous media and 10 mL of each used for soil degradation studies.

Nutrient amendment was in the form of extracts of microalgae sourced from an integrated algae pond system (IAPS) treating municipal sewage located at the Institute for Environmental Biotechnology, Rhodes University, Grahamstown, South Africa. Algae were harvested from the high rate algae oxidation ponds, concentrated by settling and centrifugation, and processed and sterilized by autoclaving for 15 min at 121 °C. Concentrated autoclaved algal biomass was freeze dried to a constant weight, powdered, the elemental content with an empirical formula of C; 1.0, H; 1.91, N; 0.084, S; 0.003 and 0; 0.36 determined, and an amount of 2.5 $g.l^{-1}$ of algal biomass used as a soil nutrient amendment.

Determination of optimum concentration of utilizable diesel by bacterial consortia

To determine the optimum concentration of diesel for use by bacterial consortia in aqueous media, each bacterial consortium was inoculated into BHM with diesel (in varying concentrations) and incubated at 30 °C for a period of 7 d. Bacterial growth was monitored spectrophotometrically at 600 nm (Thermo Spectronic Aquamate, ThermoFisher Scientific, Waltham, MA) and the highest absorbance value used to determine the optimum diesel concentration (Fig. 2).

Biodegradation of diesel in aqueous and solid media

For biodegradation of diesel in aqueous media, 1 mL of enriched bacterial culture (commercial bacteria, single strains and consortia) was inoculated into 100 mL mineral salts medium (MSM, K_2HPO_4, 1.71 g.l^{-1}; KH_2PO_4, 1.32 g.l^{-1}; $NHCl_4$, 1.26 g.l^{-1}; $MgCl_2$ $6H_2O$, 0.011 g.l^{-1}; $CaCl_2$, 0.02 g.l^{-1} containing 4 mL trace mineral solution (containing in g.l^{-1}, $MgSO_4.7H_20$, 30.0; $MnSO_4$.H20, 5.0; NaCl, 10.0; $FeSO_4.H_20$, 5.0; $CoCl_2$, 1.0; $CaCl_2$, 1.0; $ZnSO_4.7H_20$, 1.0; $CuSO_4.5H_20$, 0.1; $AlK(SO_4).12H_20$, 0.8; H_3BO_3, 0.1; $NaMoO_4.2H_20$, 0.9)) with pH adjusted to 7.0 and diesel (1.0 % v/v). Flasks were completely sealed to prevent loss of diesel through volatilization and incubated in a controlled environment at 30 °C on an orbital shaker (110 rpm) for 5 d and destructively sampled at the intervals specified in the Results.

For biodegradation of diesel in soil, pots containing 1 kg of soil were spiked with 100 g diesel and covered with aluminum foil for 10 d. The concentration of diesel (TPH) in each pot was determined gravimetrically by comparing the weight with that of un-spiked soils. Loss of diesel in each pot was compensated for by re-spiking with the amount of diesel that had been lost during the 10 d period. This process was repeated (3x) to obtain a diesel saturated soil environment. Sterile algae powder (20 mL) was added to diesel containing soil as a nutrient amendment followed by 10 mL of bacterial suspension. The commercial products, C1 and C2 were used as specified by the manufacturer. Diesel soil inoculated with bacterial consortium (without nutrient amendments) was used as control.

Pots were arranged in a complete randomized design in a polycarbonate-covered tunnel (Ulma Agricola, Spain) and moisture content maintained at 50% as recommended by Dibble and Bartha (1979). Aeration of soils in all pots was by churning at weekly intervals and the experiment was allowed to proceed for 42 d.

Extraction and analysis of residual diesel fuel

For analysis of residual diesel in aqueous media, 10 ml of MSM from each incubate was filtered using a 0.22 μm glass fibre filter and the filtrate applied to a pre-wetted 500 mg C_{18} solid phase extraction column (International Sorbent Technology, UK), eluted using 100% acetone, and analyzed by combined GC-MS. The rate of degradation of diesel in

soil was monitored by extracting residual diesel from soil. Briefly, 1 g of diesel soil was weighed into tubes and 5 ml 100% acetone was added to each tube with constant agitation for 2 h. Soil was sedimented under gravity for 1 h and a 1 ml aliquot of the clear acetone fraction was collected and analyzed by combined GC-MS.
Electron impact mass spectra were recorded at 70 eV using a MS 5975 Mass Selective Detector coupled to a 6890N gas chromatograph (Agilent Technologies, Santa Clara, CA) fitted with a HP-5MS fused silica capillary column (30 m x 0.25 mm i.d.; 0.25 µm film thickness) with He as carrier gas (50-55 kPa). The system was set in split mode (2:1) with a 90 min solvent delay time. Temperature programming was from 60°C at 7°/min to 220°C followed by 15°C/min to 280°C, and isothermnally for 5 min. compounds present were detected using the parameters 1.7 kV SEV, 1.1 s scan rate and identified using NISTO.5 library and authentic standards. Separation of diesel into aromatic and aliphatic fractions was determined by analysis of the total ion chromatograms (TIC) and the rate of diesel degradation calculated by peak area integration after calibration with the authentic standards benzene, pentadecane toluene and heptadecane.

Statistical analysis

IBM-SPSS statistics 20 software was used to analyze all data via one way analysis of variance (ANOVA) with a 95% degree of confidence to detect significant differences between the treated groups and controls whereby the level of statistical significance was accepted at $p < 0.05$ and line diagrams constructed using Sigma Plot version 11.2 (SPSS Inc., Chicago, IL). Data are presented as the mean of at least three determinations ± standard deviation (SD).

Results

Characterization of bacterial strains

16S rDNA sequence analyses showed that bacterial strains isolated from diesel contaminated soils belonged to four genera; *Bacillus, Proteus, Exiguobacterium* and *Enterobacter* (Fig. 1) Among these genera, ECCN 18b belonged to *Bacillus* which effectively turned benzene, toluene, ethylbenzene, xylene and BTEX media turbid with an increase in cell biomass (Edeki and Cowan, 2014, unpublished Paper 4), Isolate ECCN 18b was close to *Bacillus massiliensis* (KC620473, 99 %), ECCN 20b was close to *Proteus penneri* (KC620475, 98 %) which also increased in biomass and turned ethylbenzene, xylene and BTEX media turbid. ECCN 21b was closely related to *Exiguobacterium aurantiacum* (KC620476 99 %) and turned benzene, toluene, ethylbenzene and BTEX media turbid while ECCN 25b was closely related to

Enterobacter asburiae (KC700329, 99 %) and was only able to turn benzene medim turbid. Bacterial strains were also isolated from coal mine dumps and used in combination with the above bacteria to establish different consortia. These isolate belonged to five genera; *Serratia, Mycobacterium, Proteus, Citrobacter* and *Bacillus* (Fig. 1). Among these genera, ECCN 19b was closely related to *Serratia nematodiphila* (KC620474, 98 %) and turned all media turbid except xylene, ECCN 22b was closely related to *Mycobacterium esteraromaticum* (KC620477, 99 %) and was able to turn ethylbenzene, xylene and BTEX media turbid, ECCN 23b was closely related to *Proteus mirabilis* (KC620478, 99 %) and turned all media turbid, ECCN 25b was closely related to *Citrobacter freundi* (KC700328, 99 %) and ECCN 26b, closely related to *Bacillus odysseyi* (KC700330, 99 %) respectively.
All results of bacteria characterization are summarized in Table 1 (Paper 4; Edeki and Cowan, 2014).

Biodegradation of diesel fuel in aqueous and solid media

Biodegradation of diesel in aqueous media over a 5 d period showed a progressive decline with regard to pentadecane, hexadecane and toluene for the commercial products C1 and C2, individual bacteria isolates and the various consortia (Fig. 3). The rate of degradation for all isolates and consortia was similar to that observed for the commercial products and aside from ECCN 7b; none were capable of completely metabolizing the diesel substrate. Statistical analysis confirmed that there was no significant difference between the inoculated control and the commercial biocatalysts; C1 and C2 with regard to pentadecane degradation. A total pentadecane degradation rate of 64.7 % was recorded for strain ECCN 21b as compared to 39.7 % for strain ECCN 18b which was the least performing single isolate (Fig. 3B). Consortium ECCN 7b; showed 100 % degradation of pentadecane (Fig. 3C).

Based on the screening results obtained for experiments involving diesel degradation in aqueous media, the best performing bacterial consortium; ECCN 7b was used to set up diesel soil experiment in comparison with commercial biocatalysts; C 1 and C 2. The ability of these bacteria to degrade diesel in soil was analysed by GC-MS. Results obtained from this experiment showed a complete degradation of pentadecane by both C1 and ECCN 7b while C2 was unable to completely degrade this compound (Fig. 5). Effective degradation of hexadecane (100%) by ECCN 7b was achieved within the 42 d period of experiment and when compared to each commercial biocatalysts (C1 and C2), these bacteria were only able to degrade hexadecane at a rate of 72 and 60% respectively (Fig. 5). Toluene on the other hand was effectively degraded by ECCN 7b reaching a 98% degradation rate at the end of experiment. C1 and C2 were only able to achieve 77% and 50% toluene degradation rates.

Daily degradation rates of aliphatic and aromatic compounds in diesel was calculated and results obtained at the end of 42 d period of experiment, showed C1 to have a benzene daily degradation rate of 3.59 ± 0.01 $mg.kg^{-1}.d^{-1}$ while that of C2 was 3.60 ± 0.05 $mg.kg^{-1}.d^{-1}$ (Table 2). The bacterial consortium; ECCN 7b had the highest daily degradation rate for each compound in diesel and these rates are presented in Table 2.

Discussion

Bacterial strains and consortia used in this study were screened for their BTEX degradation potentials (Edeki and Cowan, 2014, unpublished Paper 4) and enriched in diesel for further use. This enrichment technique has been the method of choice for isolating bacteria expressing specific phenotypes and has been successfully used to enrich bacteria capable of degrading hydrocarbons (Golovlev, 2001). These bacterial strains and consortia were used in this study to carry out biodegradation of diesel both in aqueous and solid media and results obtained from the experiment involving aqueous media, showed that the single bacterial strains were not a promising set of biocatalysts with regard to the biodegradation of diesel. Various studies have also been carried out on the degradation of diesel and published results have shown the inability of single bacterial strains to effectively carry out this process (Márquez-Rocha et al., 2001; Ghazali et al., 2004; Li et al., 2008; Sharma and Rehman, 2009; Moliterni et al., 2012).

In addition to bacterial isolates and consortia used in this study, commercial bacteria with PHC degradation potentials were also used and results obtained in aqueous media showed an effective degradation of both aromatic and aliphatic compounds contained in diesel. However, when the above results obtained for single strains and commercial bacteria were compared to already establish bacterial consortia which were also used in this study; it was observed that the bacterial consortia were more efficient diesel degraders than single strains and commercial bacteria. The above result confirmed that already published by Sathishkumar et al. (2006) and Hawle-Ambrosch et al. (2007).

The number of isolates used to establish bacterial consortium for biodegradation purposes is a major criterion which must be considered as well. For instance, Moliterni et al. (2012) observed 90% breakdown of diesel when using a bacterial consortium comprised of three isolates. Ghazali et al. (2004) used consortia of three and six isolates and showed that the consortium of six was more efficient petroleum hydrocarbon degrader. In agreement with these observations, data from the present study demonstrated that by increasing the number of isolates in a consortium the rate of diesel breakdown was enhanced. As shown, consortia of two isolates were not different from single isolates in terms of diesel biodegradation measured using the organic fingerprint of pentadecane, hexadecane and toluene. By comparison, a consortium of three isolates (i.e. ECCN 7b) completely removed pentadecane and hexadecane from the media within 5 d and reduced toluene levels by almost 95%. There was a significant

difference (IBM-SPSS statistics 20, ANOVA) between un-inoculated control and ECCN 3b, ECCN 5b, ECCN 6b, ECCN 7b and ECCN 9b with regard to hexadecane and toluene degradation and the following order of efficacy was established for metabolism of pentadecane and hexadecane; ECCN 7b>ECCN 6b >ECCN 5b=ECCN 9b>ECCN 3b. With benzene degradation, there was no significant difference between un-inoculated control and the above consortia.

Degradation of diesel in soil was also carried out in this study and different bacteria were used in this regard. Nutrient amendment in the form of algae was used to enhance the degradation potentials of bacteria used in this experiment and obtained results showed the effectiveness of these amendments in inoculated diesel soils. The effects of nutrients on the degradation of hydrocarbons by bacteria have been emphasized by different authors in various publications (Chaineau et al., 2005; Wrenn et al., 2006: Östberg et al., 2007). In this study, the addition of nutrients enhanced the degradation efficiency of ECCN 7b when compared to inoculated control without nutrients. The above observation was highlighted by Edeki and Cowan (2014, unpublished Paper 1) when they stated that for effective degradation of PHC to take place, optimum conditions such as nutrient amendments must be met.

Conclusion

The presence of bacteria in any given contaminated environment enables the biotransformation and elimination of contaminants from such environment. Results presented in this study suggested that obtained bacteria sourced from Environmental Biotechnology Research Unit at Rhodes University in South Africa, have the ability to degrade diesel both in aqueous and solid media within a very short space of time. Compared to other remediation strategies, biological treatment of contaminated sites is simple, cost effective, efficient, and environmentally friendly. This can be explained from the fact that the raw materials such as algal extract and bacteria used in this study to enhance biodegradation of diesel was sourced from the natural environment which is an indication of an efficient biotechnological process. Different authors in their publications have stressed the importance of isolating microorganisms that have degradation abilities from contaminated environments (Ghazali et al., 2004; Mancera-Lopez et al., 2008: Luo et al., 2012). Although these organisms play vital roles in mineralizing contaminants, the overall success of bioremediation is centered on optimizing various physical, chemical, and biological conditions in the contaminated environments.

With the optimization of relevant parameters in this experiment, consortium ECCN 7b could be said to be the best treatment of choice with regard to bioremediation of PHC contaminated sites. It is believed that these results provides a database for further evaluations of the various mechanisms involved in PHC degradation and an understanding of the optimum treatment applications with regard to oil spilled sites.

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FIGURE HEADINGS

Figure 1. Phylogenetic tree based on 16S rDNA sequences of both coal and PHC degrading bacteria. Database sequence accession numbers are as follows: *Bacillus* sp.; KC620473 (ECCN 18B), *Seratia* sp.; KC620474 (ECCN 19b), *Proteus* sp.; KC620475 (ECCN 20b), *Exiguobacterium* sp.; KC620476 (ECCN 21b), *Mycobacterium* sp.; KC620477 (ECCN 22b), *Proteus* sp.; KC620478 (ECCN 23b), *Citrobacter* sp.; KC700328 (ECCN 24b), *Enterobacter* sp.; KC700329 (ECCN 25b) and *Bacillus* sp.; KC700330 (ECCN 26b).

Figure 2. Determination of optimum concentration of diesel for effective metabolism by each established bacterial consortium. Different concentrations (g/v) of diesel were added to MSM and various bacterial consortia were inoculated in the media. These media were incubated at 30 ºC for 7 d after which, absorbance (600nm) readings were taken to determine bacterial activities in the media. All data are the mean ± SD of three determinations and are representatives of duplicate experiments.

Figure 3. Time course of biodegradation of selected compounds in diesel using different bacterial in aqueous medium. 1A; commercial biocatalysts used as positive control, 1B; single bacterial isolates and 1C; different bacterial consortia derived by mixing two or three bacterial isolates together. Aliquots were collected from diesel media on a daily basis and residual diesel was extracted by solid phase extraction method using C_{18} columns. Trapped extracts were eluted from the columns into GC vials using acetone and analyzed with the GC-MS. Peak areas were converted to various concentrations of compounds in diesel using already plotted standard curves for each compound. All data are the mean ± SD of three determinations and are representatives of duplicate experiments.

Figure 4. A comparison of degradation rates of both aromatic and aliphatic compounds in diesel over time in aqueous media. Representative organisms are the best performing organisms; ECCN 21b, single bacterial isolate; C 1, commercially available biocatalysts (positive control); ECCN 7b, bacterial consortium and Control (negative control). Aliquots were collected from diesel media daily and residual diesel was extracted by solid phase extraction method using C_{18} columns. Trapped extracts were eluted from the columns into GC vials using acetone and analyzed with the GC-MS. Peak areas were converted to various concentrations of compounds in diesel using already plotted standard curves for each compound. All data are the mean ± SD of three determinations and are representatives of duplicate experiments.

Figure 5. Time course of biodegradation of selected compounds in diesel in spiked soils using different bacterial treatments. C 1 and C2; commercial biocatalysts (positive control) and ECCN 7b; best performing bacteria. GC-MS was used to analyze residual diesel in soil by collecting aliquots obtained from clear acetone fraction filtered using a 0.22 µm glass filter. Peak areas were converted to various concentrations of compounds in diesel using already plotted standard curves for each compound. All data are the mean ± SD of three determinations and are representatives of duplicate experiments.

Table 1. GenBank accession numbers and composition of bacterial consortia

Genbank accession numbers	ECCN[A]
KC620473 + KC620475	ECCN 1b
KC620473 + KC620474	ECCN 2b
KC620473 + KC620476	ECCN 3b
KC620473 + KC620477	ECCN 4b
KC620473 + KC620474 + KC620475	ECCN 5b
KC620473 + KC620476 + KC620474	ECCN 6b
KC620473 + KC620475 + KC620476	ECCN 7b
KC620473 + KC620477 + KC620475	ECCN 8b
KC620475 + KC620476 + KC620477	ECCN 9b
KC620473	ECCN 18b
KC620474	ECCN 19b
KC620475	ECCN 20b
KC620476	ECCN 21b
KC620477	ECCN 22b
KC620478	ECCN 23b
KC700328	ECCN 24b
KC700329	ECCN 25b
KC700330	ECCN 26b

[A]ECCN = EBRU Culture Collection Number

Table 2. Daily degradation rates of various compounds in diesel contaminated soil by best performing bacteria; ECCN 7b and the commercial products C-1 and C-2.

Compound	Control	C -1	C -2	ECCN 7b
	mg/kg/d			
Benzene	0.23±0.01	3.59±0.01	3.60±0.05	4.78±0.04
Toluene	0.29±0.01	3.22±0.03	2.50±0.02	4.45±0.04
Xylene	0.19±0.03	4.02±0.03	2.62±0.03	4.45±0.02
Pentadecane	0.35±0.02	4.88±0.05	2.41±0.04	4.76±0.05
Hexadecane	0.25±0.02	3.44±0.02	3.02±0.03	4.67±0.04
Heptadecane	0.23±0.01	5.17±0.01	3.41±0.03	5.71±0.02
Octadecane	0.23±0.02	3.94±0.03	3.18±0.04	3.88±0.04
Nonadecane	0.35±0.01	4.75±0.02	4.22±0.05	5.51±0.04
Tetradecane	0.27±0.01	2.15±0.01	1.96±0.02	2.54±0.01

FIGURE 1

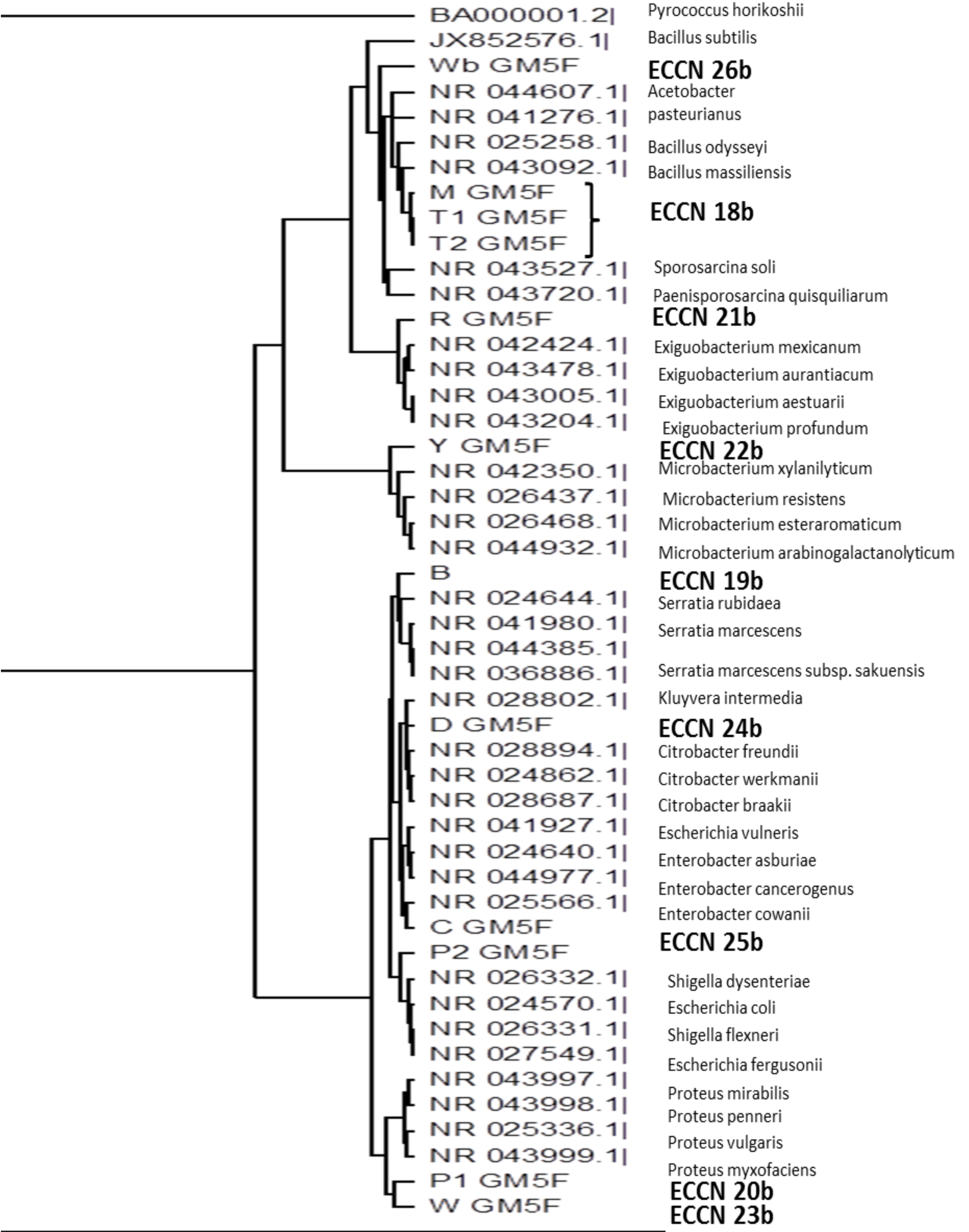


FIGURE 2

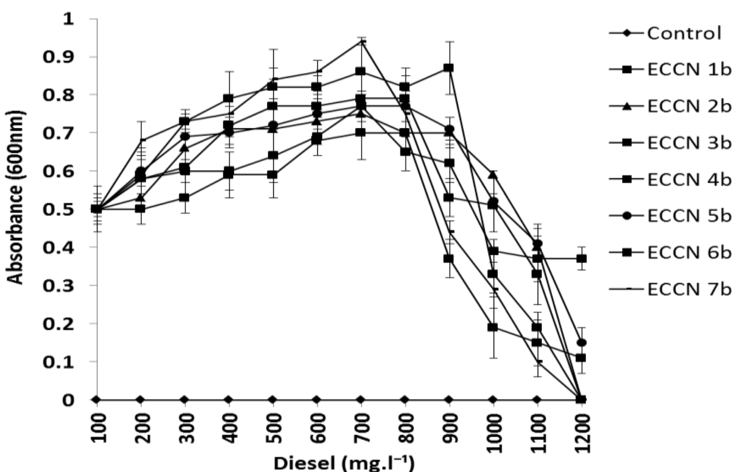


FIGURE 3

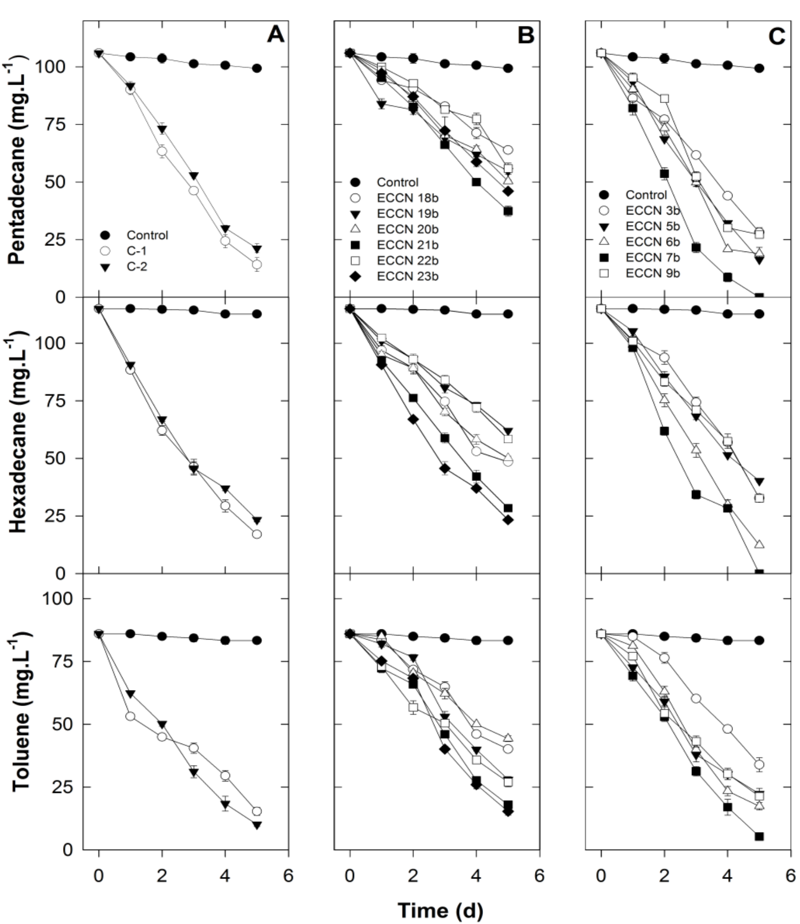
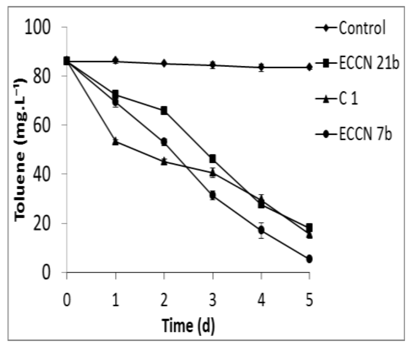
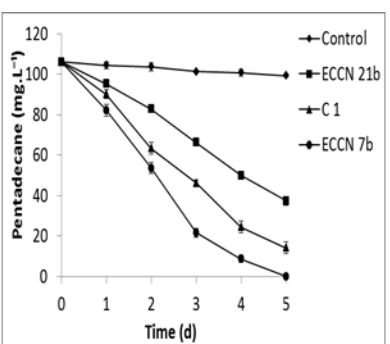


FIGURE 4





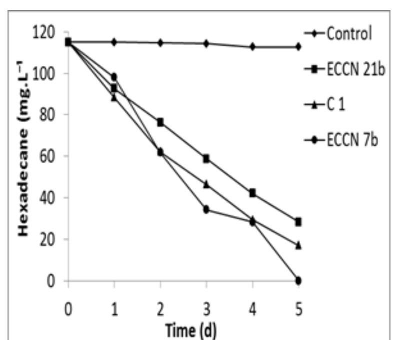
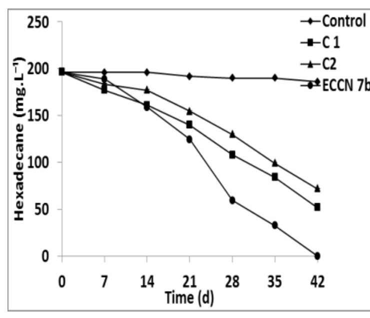
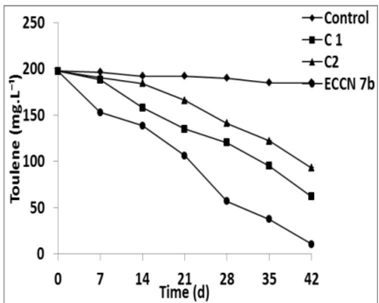


FIGURE 5





Supplimentary information

1. Growth media preparations

Luria Broth (LB)

5g Tryptone

2.5g Yeast Extract

2.5g NaCl

Make up to 1L with dH2O. Sterilize with autoclaving at 121 C for 15min.

Nutrient agar

Make up to 1L with dH2O. Sterilize with autoclaving at 121 C for 15min.

Bushnell Hass medium (BHM)

Content	Grams/Litre
Magnesium Sulfate	0.2
Calcium Chloride	0.02
Monopotassium Phosphate	1.0
Dipotassium Phosphate	1.0
Ammonium Nitrate	1.0
Ferric Chloride	0.05

Final pH 7.0 +/- 0.2 at 25°C

Directions:

Suspend 3.27 g of Bushnell Haas Broth in 1000 mL of distilled water. Boil to dissolve the medium completely. Sterilize by autoclaving at 121°C for 15 minutes before use.

Mineral Salts Medium (MSM)

Content	Grams/Litre
K_2HPO_4	1.71
KH_2PO_4	1.32
$NHCl_4$	1.26
$MgCl_2.6H_2O$	0.01
$CaCl_2$	0.02
Ferric Chloride	0.05

Add 4 ml trace element solution and 20 g agar (if solid media is required). Mix well and adjust pH to 7.0 ± 0.2 and autoclave at 121°C for 15 minutes before use.

Trace element solution (TES) 100 x composition

Content	mg/Litre
$MnSO_4.H20$	5.0
NaCl	10.0
$FeSO_4.H_20$	5.0
$CoCl_2$	1.0
$CaCl_2$	1.0
$ZnSO_4.7H_20$	1.0
$CuSO_4.5H_20$	0.1
$AlK(SO_4).12H_20$	0.8
H_3BO_3	0.1
$NaMoO_4.2H_20$	0.9

2. DNA extractions and agarose gel electrophoresis

Tris-EDTA Buffer (0.01M Tris; 0.05M EDTA)

Content	Grams/500 mL
Tris (hydroxymethyl) aminomethane	0.61
EDTA	0.093

Adjust pH to 8 then sterilize by autoclaving at 121º C for 15min.

10 % SDS

Content	Grams/100 mL
SDS	10

Warm to 65° C to allow SDS dissolve.

10 % CTAB

Content	Grams/100 mL
Cetyltrimethylammonium bromide	10

Warm to 65° C to allow CTAB dissolve.

5 M NaCl

Content	Grams/L
NaCl	292

Sterilize by autoclaving at 121 ºC for 15 min.

Chloroform: Isoamyl alcohol (24:1) (100 ml)

96 mL chloroform

4 mL isoamyl alcohol 78

10 x TBE buffer (1l)

Content	Grams/L
Tris base	107.8
Boric Acid	55
di-sodium EDTA	7.44

Adjust pH to 8.3 with boric acid and autoclave at 121 ºC for 15 min. Dilute 1 in 10 for 1 x TBE.

Ethidium bromide (500 mg/mL)

Dissolve 0.5 g of ethidium bromide powder in 1mL of sterile distilled and store in a dark bottle at room temperature.

DNA loading buffer (6 x)

0.25 % Bromophenol blue

0.25 % Xylene cyanol

30 % Glycerol

***Pst1* Molecular Weight Marker**

Digest 200 µL µDNA (0.25 µg/uL) with 24 µL of 10 x buffer H and 10 µL of *Pst1* enzyme for 3 hours at 37 ºC. Add 550 µL TE buffer (pH 8) and 150 µL 6 x loading buffer. Dispense 100 µL aliquote in eppendorfs and store at -20 °C.

Primers

907R 5'- primer 907R (5'- CCCCGTCAATTCCTTTGAGTTT-3')
GM5F 5'-CCTACGGGAGGCAGCAG-3'
Both primers were manufactured by Integrated DNA technology, Iowa, USA.

10 µM dNTP`S

30 µL of each nucleotide (dATP, dTTP, dCTP & dGTP) was added into an eppendorf. 180 µL of sterile distilled water was also added, bringing the total volume to 300 µL. 60 µL aliquots were dispensed in eppendorf tubes and store at –20 ºC.

Band sizes of *lPst1* Molecular Weight Marker:

- 14057bp
- 5077bp
- 4749bp
- 4507bp
- 2838bp
- 2459bp
- 2443bp
- 2140bp
- 1986bp
- 1700bp
- 1159bp
- 1093bp
- 805bp
- 514bp
- 468bp
- 448bp
- 339bp
- 264bp
- 247bp
- 216bp
- 211bp
- 200bp
- 164bp

3. Humic substances in waste coal before, during and after inoculation with microorganisms were extracted using the following steps;

· Suspend 1 g WC in 0.1 M NaOH and make up to 100 mL

· Agitate for 24 hours at 150 rpm

· Centrifuge at 3220 × g for 90 mins at 10 ℃

· Collect supernatant and freeze dry sediment

· Drop pH of supernatant to > 1 using minimal volume of 32 % HCl

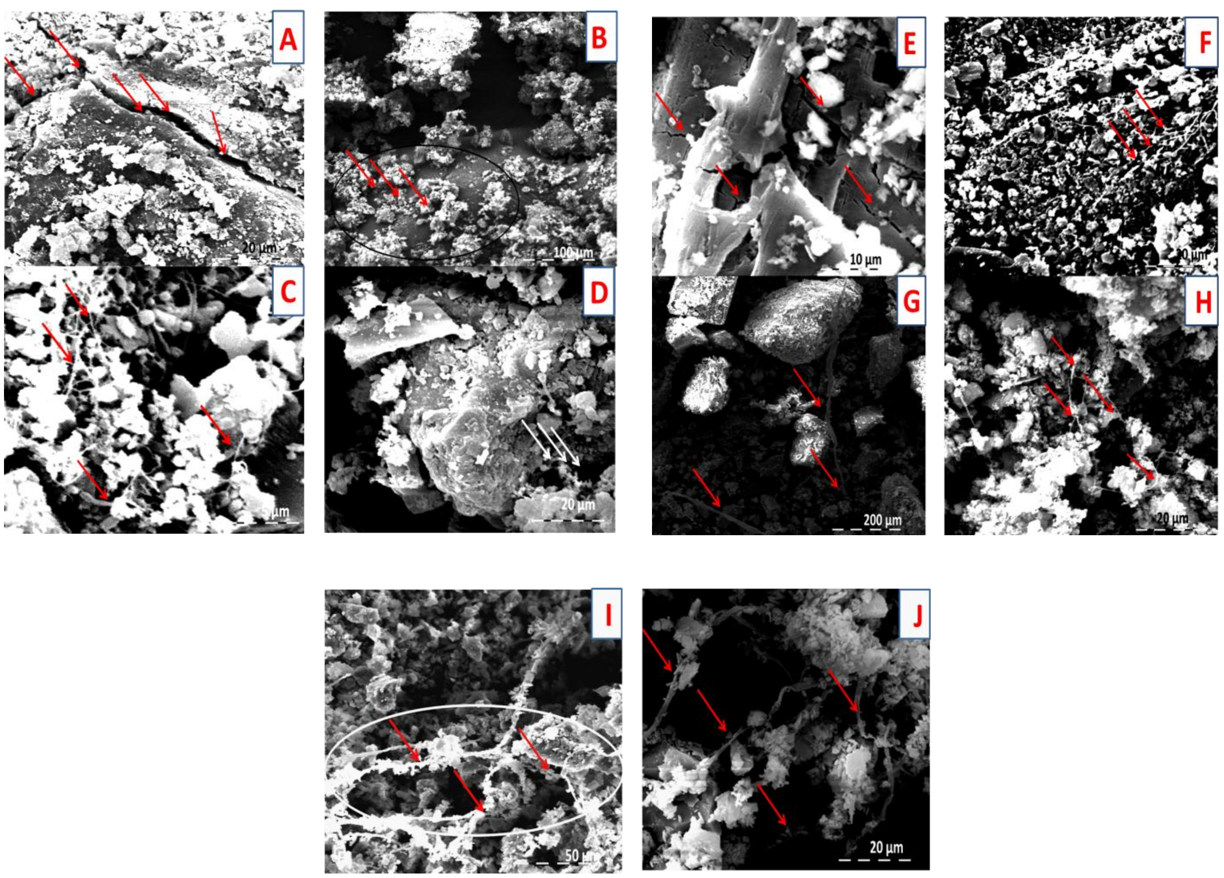
· Stand for 1 hour and repeat centrifugation

· Decant supernatant (FA fraction) and measure absorbance 380 nm

· Re-suspend pellet (HA fraction) in 100 mL 0.1 M NaOH and measure absorbance at 450 nm.

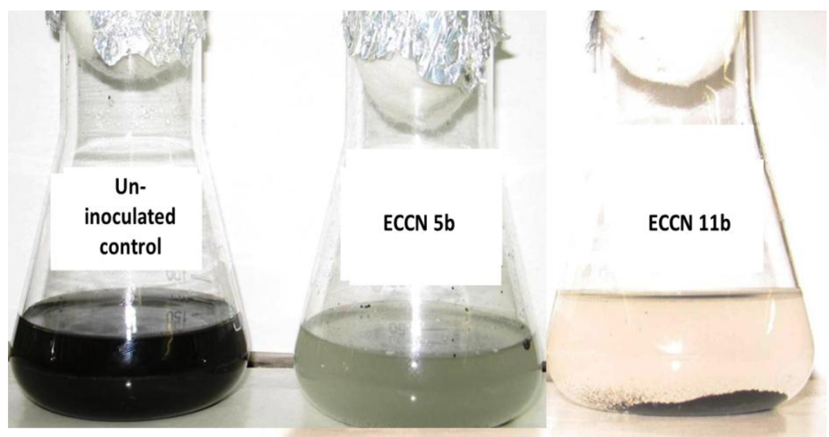
· Freeze dry both fractions and and analyze using FT-IR

4. Scanning electron microscopy

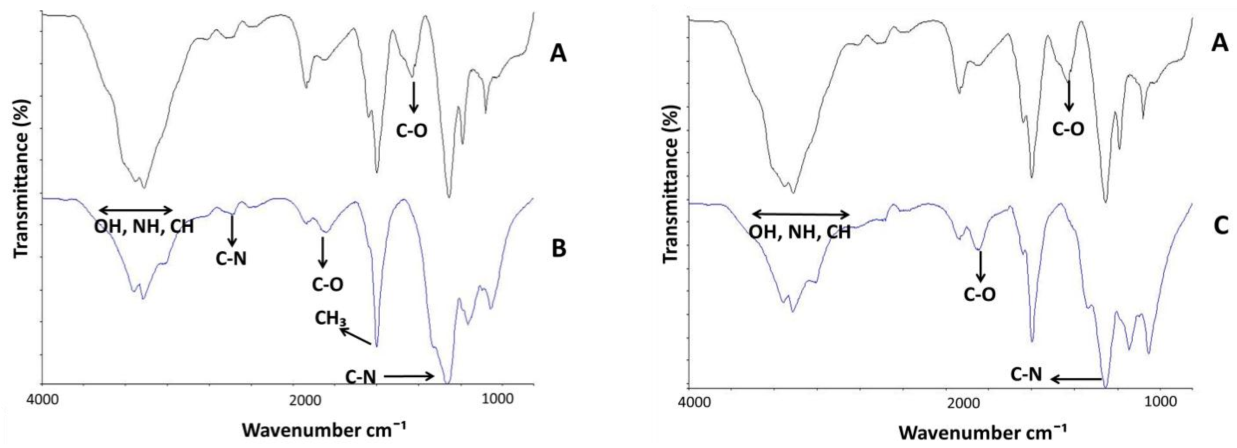


Scanning electron micrographs showing disintegration and colonization of coal particles by bacteria from the various consortia prepared as described in materials and methods of chapter 3. Arrows indicate attachment to and disintegration/colonization of the coal surface by bacteria. A & F) ECCN 1b; B & G) ECCN 2b; C & H) ECCN 5b; D & I) ECCN 6b; and, E &J) ECCN 11b

5a. Waste coal media with different treatments and FT-IR spectra of coal substrates in aqueous media

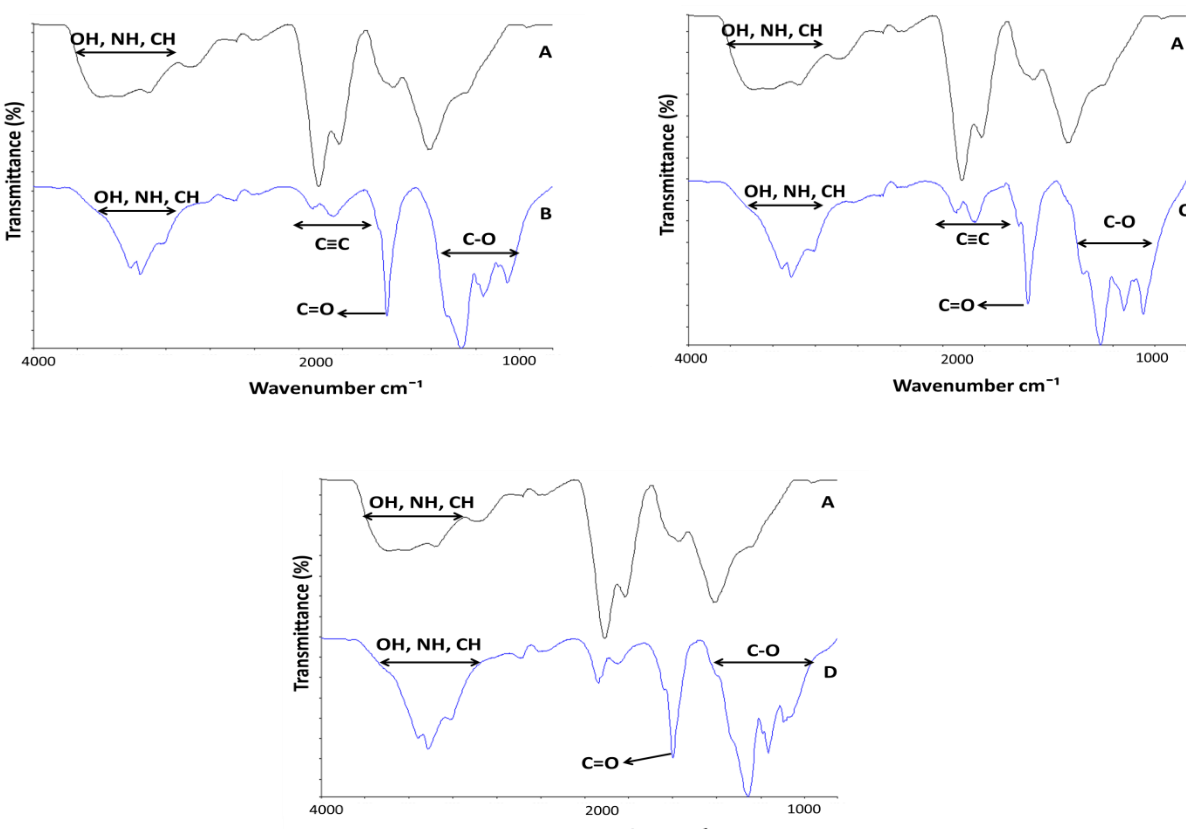


Waste coal media treated with different bacterial consortia over a period of 12 d.



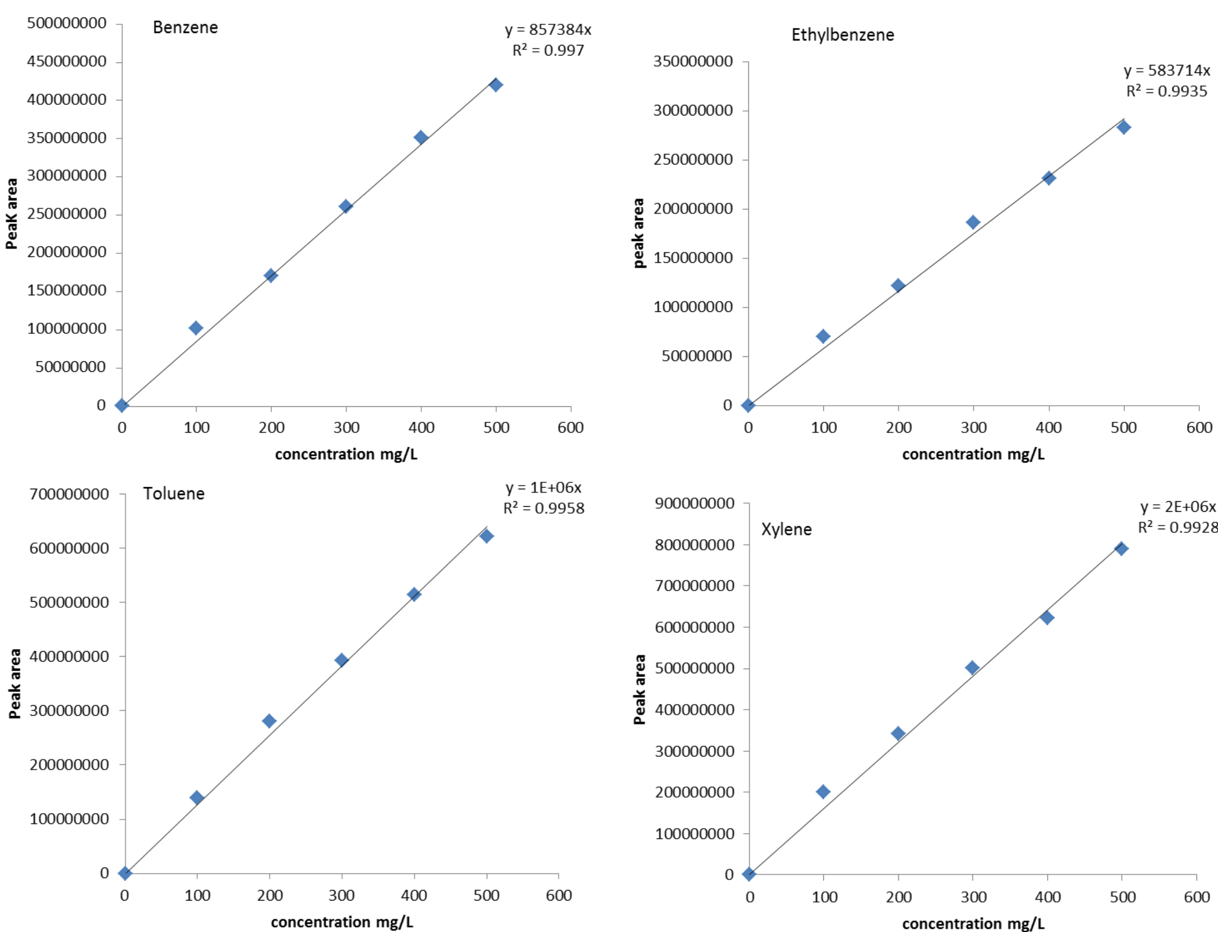
FTIR spectra of humic acid-like products produced by bacterial degradation of waste coal in aqueous media. Mineral salts medium containing waste coal as substrate was inoculated with different bacterial consortia and incubated at 30°C for 21 d on a rotary shaker. Humic and fulvic acid-like substances were extracted, dried and analyzed by FT-IR. (A) Commercial HA, (B) humic acid-like substances produced from waste coal by ECCN 1b and (C) humic acid-like substances produced from waste coal by ECCN 5b.

5b. **FT-IR spectra of coal substrates in aqueous media**



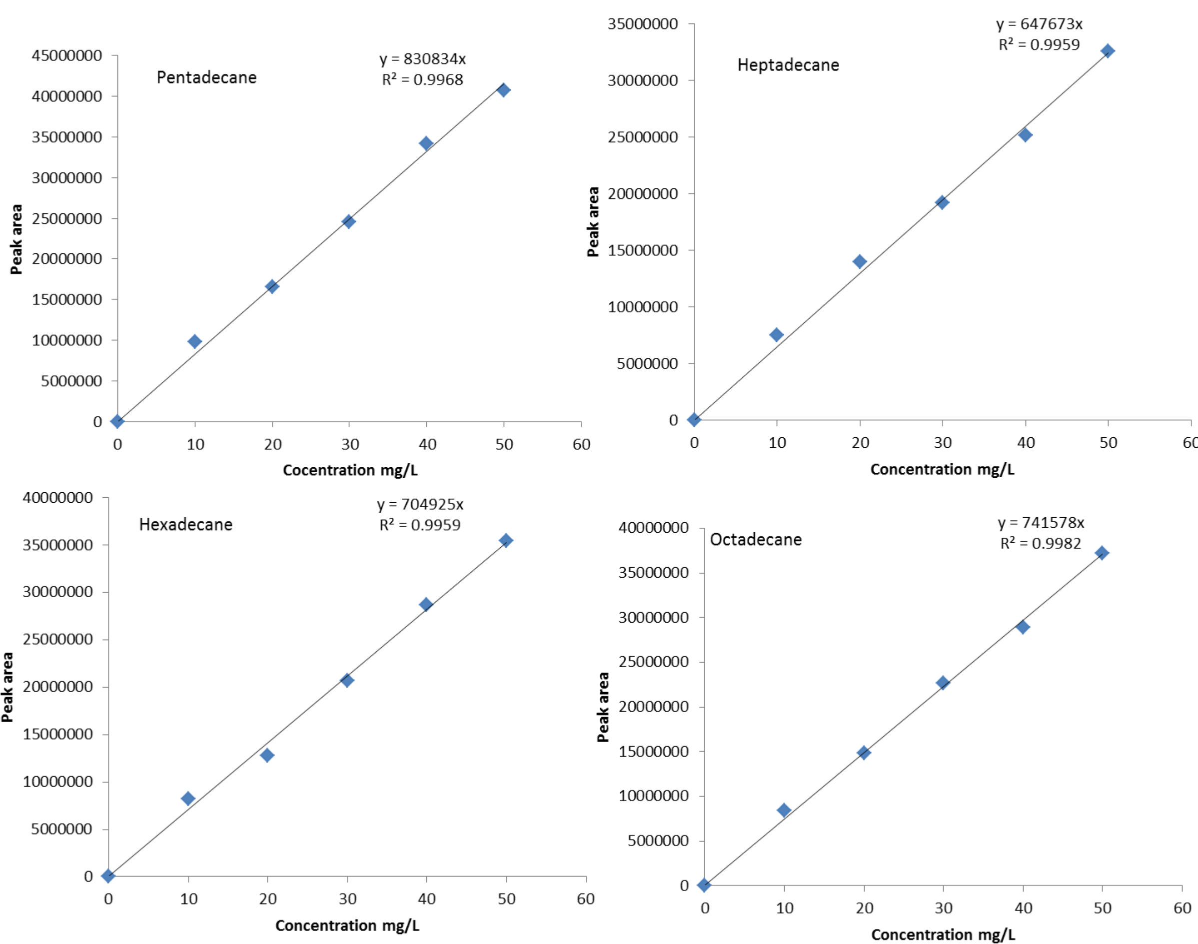
FTIR spectra of fulvic acid-like products produced by bacterial degradation of waste coal in aqueous media. Mineral salts medium containing waste coal as substrate was inoculated with different bacterial cosortia and incubated at 30°C for 21 d on a rotary shaker. Humic and fulvic acid-like substances were extracted, dried and analyzed by FT-IR. (A)) fulvic acids extracted from commercial humic acid, (B) fulvic acid-like substances extracted from the humic acid-like substances produced from waste coal substrate by ECCN 1b, (C) fulvic acid-like substances extracted from the humic acid-like substances produced from waste coal substrate by ECCN 5b and (D) fulvic acid-like substances extracted from the humic acid-like substances produced from waste coal substrate by ECCN 11b,

6a. Standard curves for various aromatic compounds



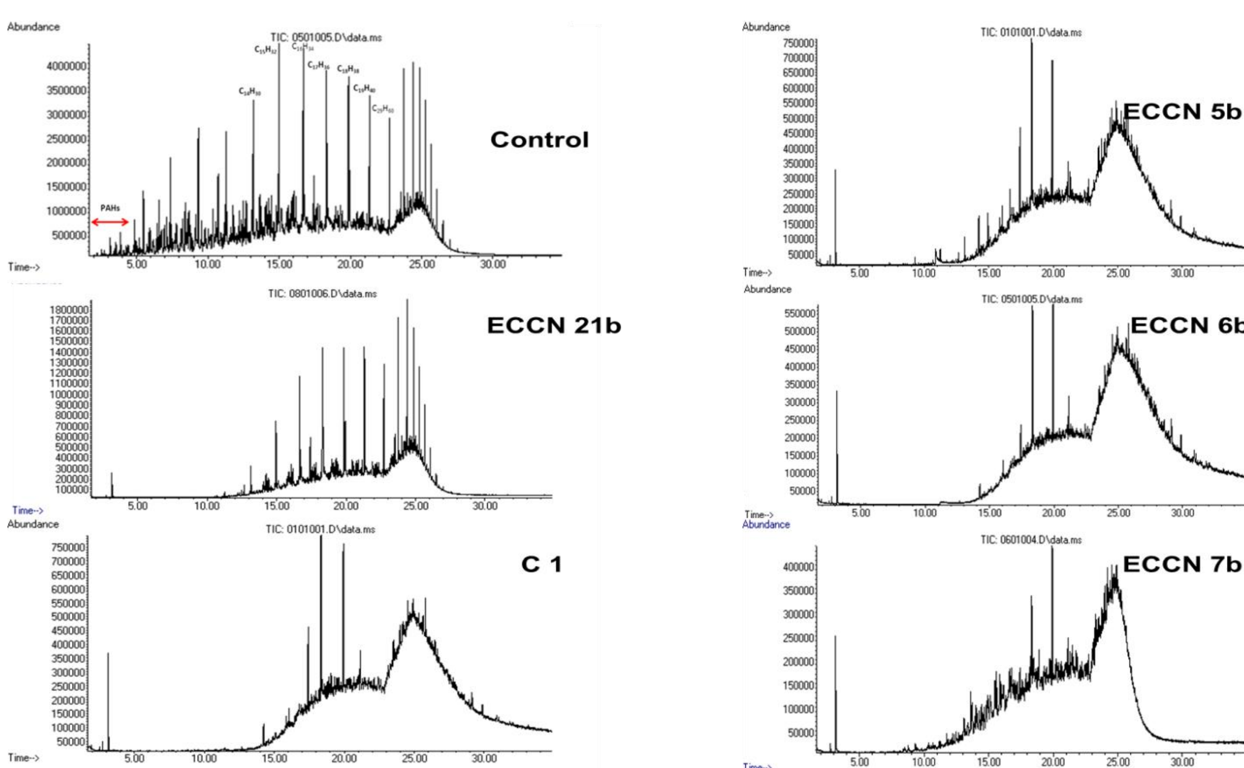
Standard curves of various compounds representing aromatic hydrocarbons in diesel. Absorbance values were converted to mass by extrapolation made from the plot.

6b. Standard curves for various aliphatic compounds



Standard curves of various compounds representing aliphatic hydrocarbons in diesel. Absorbance values were converted to mass by extrapolation made from the plot.

7. Residual diesel in soil after a period of 42 d experiment



GC-MS chromatograms of different bacteria used in the biodegradation of diesel in soils over a period of 42 d. Residual diesel in soil was extracted as described in materials and method of manuscript 5 and analyzed with the GC-MS after 42 days. ECCN 21b; best performing single bacterial isolate, C 1; best performing commercial biocatalysts, ECCN 5b, 6b and 7b; formulated bacterial consortia.