BACTERIAL DEGRADATION OF WASTE COAL

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ABSTRACT

As an energy source coal has one of the largest agglomerations in the world. Consequently mining of coal creates large volumes of waste in the form of low ranks coals. The complex structure of coal makes it difficult for the microorganisms to degrade and relatively few bacteria and fungi have been shown to break down coal. This study aimed to investigate bacteria not previously known to degrade coal. In this study bacteria were isolated from hydrocarbon contaminated sites and inoculated in coal medium where coal served as the only carbon source. Three strains produced a yellow – brown supernatant after 14 d of incubation at 30 °C. Bacteria generating a yellow – brown coloured supernatant were presumed to possess coal degrading capabilities and the best performing of these bacterial species was identified using 16s rDNA as *Bacillus flexus*. Scanning electron microscopy showed that there was a close association between the bacterium and substrate coal. The close association of bacteria to substrate suggested that these organisms were able to maximize solubilisation. FT-IR spectroscopic analysis demonstrated the addition of single bonded compounds COOH, OH, CN and CH that were absent prior to bacterial interaction. The increase in oxygen rich regions indicated degradation of the coal substrate. Elemental analysis showed that there was a decrease in carbon content from 47 % to 24 % during the 14 day incubation period. Reduction in coal carbon content was assumed to be due to bacterial utilization for metabolism and growth particularly as untreated coal substrate showed minimal loss of carbon. Analysis of the residual culture medium revealed that there was a linear increase in humic-like substance concentration for 8 d, coincident with increased coal biosolubilisation and colour change. Laccase activity was insignificant, and at 13 d enzyme activity was only 5×10^{-3} U/L suggesting that *B. flexus* may use a different mechanism to degrade coal. Residual culture medium remaining after bacterial action on the coal substrate appeared to possess plant growth promoting activity. This soluble biodegradation product with characteristics similar to humic acid-like substances was shown to impact growth of radish cotyledons. Expansion of isolated radish cotyledons was enhanced by 140% when incubated in coal biodegradation product.

In conclusion, this study has yielded *B. flexus* and two other unidentified bacteria, isolated from polyaromatic hydrocarbon contaminated soils, and demonstrated the ability of these microorganisms to degrade waste coal. Further studies to elucidate the mechanism of coal

breakdown by *B. flexus*, synergies with other coal degrading microorganisms, and incorporation of bacterium into Fungcoal bioprocess technology is imminent.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

ABTS	2, 2'-Azino-Bis (3-Ethylbenzothiazoline-6-Sulphonic) Acid
BLAST	Basic Local Alignment Search Tool
BP	Biodegraded Product
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleotide Triphosphate
EBRU	Institute for Environmental Biotechnology, Rhodes University
FAO	Food and Agriculture Organisation of United Nations
FT-IR	Fourier Transform Infra-Red
GE	Grahamstown Engineering
HA	Hard Coal
HA	Humic Acid
HAE	Humic Acid Extracted
HC	Hard Coal
MDD	Municipal Disposal Dump
Nm	newton meter
NCBI	National Centre for Biotechnology Information
PCR	Polymerase chain reaction
RPM	Revolutions per minute
SEM	Scan electron microscopy
SF	Strowam Farm
U/L	1 Enzyme unit per litre
WC	Waste Coal

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

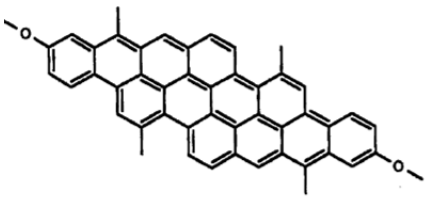
Different types of calorific value coal are obtained during mining. However, high calorific value coals are the most desired. Low calorific coal is regarded as waste and is disposed of as coal spoils which are stockpiled as dumps. These dumps do not support plant or animal life; they contain heavy metals and are a danger to aquatic life if runoff enters nearby streams/rivers. These potential harmful effects of coal dumps have resulted in the need for mining companies to rehabilitate the land used for mining. The Institute for Environmental Biotechnology, Rhodes University (EBRU) jointly worked with Anglo American Thermal Coal to develop a sustainable and efficient rehabilitation process. The process includes a grass (*Cynodon dactylon*) which was observed to grow sporadically on the coal dumps and a fungus (*Neosartorya fischeri*). The latter was isolated from the rhizosphere of this grass and was shown to have coal degrading capability. This led to the development of the Fungcoal process where plant-mycorrhizal association and the rhizospheric coal degrading fungi appear to catalyze the biodegradation of waste coal to humic-like substances (Figure 1.1). This process could be simulated in the laboratory using *Cynodon dactylon*, *N. fischeri* and weathered coal forming the basis of the Fungcoal process (Igbinigie *et al*. 2010; Mukasa-Mugerwa *et al*. 2011). To develop the system further an addition of a bacterium promises to be a valuable component of the Fungcoal process. Bacteria not only have different metabolic processes but have also been reported to have coal degrading capabilities. They play a role in biogenic methane production on anaerobic coal seams (Strapoc *et al*. 2011). Underground water in coal seams contains methanogens that closely interact with coal and in so doing produce methane. Bacterial species are implicated in the desulphurisation of coal. In some cases there is removal of structural sulphur which further breaks down the coal structure into lower molecular weight compounds (Klein *et al*. 2001). Furthermore bacteria have been documented to possess coal degradation mechanisms (Fakoussa 1981; Klein *et al*. 1999; Hofrichter and Fritsche 1997; Larboda *et al*. 1999). Therefore the addition of a bacterium will improve soil recovery in the Fungcoal rehabilitation strategy.

1.2 Economic importance of coal

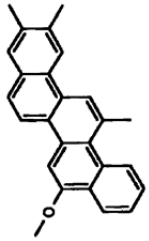
Coal is a traditional energy source for many countries and its use is projected to increase in the near future in spite of the environmental damage that this energy source causes, one of which is the emission of greenhouse gases (for example, CH_4, CO_2) into the atmosphere during burning of coal. Countries that benefit largely from the coal mining industry are the United States of America (USA), India, China and South Africa (Wang 2006; Hu 2009). South Africa is the 6th largest coal producer in the world and the second largest coal exporter (Ndaji and Thomas 1995). Coal that is not exported but used locally forms 74% of the energy usage in this country and its use is increasing (Gockay *et al*. 2001).

1.3 Structure and composition of coal

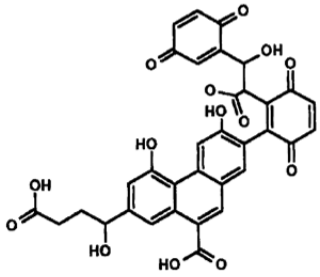
Coal is a heterogeneous organic compound bonded together by carbon-carbon linkages (Figure 1.1) (Faison 1991; Chen *et al*. 2006). Highly oxidised plant material is overtime and under high pressure in absence of water, converted to coal (Stevenson 1994; Fakoussa & Hofrichter 1999). Different coals are formed at different stages of coalification.



Anthracite



Low volatile: Bituminous



Humic acid from brown coal

Figure 1.1 Hypothetical chemical structures of different coals and humic acid.

Peat is the first product in the coalification process and is not considered a coal since it has a high moisture content and the parent plant material is in some cases still intact (Giuliani *et al*.1991; Andrews *et al*. 1994). Lignite is dark brown and is considered the lowest rank of coal. With considerably lower moisture content than peat it is used as a fuel in power stations. Leonardite on the other hand is a form of oxidised lignite and is usually classified as a coal rank which is also the case with humic acid. Subbituminous is considered the first rank of coal as it has higher carbon content, lower moisture content and higher calorific value than peat (Giuliani *et al*.1991; Andrews 1994). It is brown and normally, is neither exported nor used in chemical industries. Bituminous is the second rank of coal and is more widely used for energy and fuel (Hu 2009). The highest coal rank is anthracite which is also referred to as hard coal. It has the highest calorific value and is mostly used as fuel (Wang 2006; Hu 2009). It is black and has little to no moisture content, a high carbon content and high lustre (Hayatsu *et al*. 1979).

1.4 Bioconversion of coals

Due to the complex structure of coal only a few groups of microorganisms, the majority of which are fungi and bacteria, are able to use coal as a carbon source (Machnikowska *et al*. 2002). Degradation of coal is termed weathering which is the result of chemical and physical changes to the structure of coal (Machnikowska *et al*. 2002). Microbial activities (biodegradation) on coal, such as, depolymerisation, decolourisation, solubilisation and liquefaction were clarified in the update of the Résumé of the Bioconversion Session of the 9th International Conference on Coal Science in 1997 (Klein *et al.* 1999). Depolymerisation is defined as the catabolic reduction of higher molecular mass to smaller fractions, which could be accompanied by loss of chromophores; whereas decolourisation is the loss of chromophores without any change in the molecular size (Fakoussa & Hofrichter 1999; Klein *et al*. 1999). Liquefaction is defined as a change of physical state (solid to liquid state) and should not be confused with solubilisation, which is the dissolution of all or part of the coal molecule (Klein *et al.* 1999).

There are a number of reasons why coal is a difficult substrate for use by microorganism. Coal contains persistent compounds, for example, polyaromatic hydrocarbons (PAH) bonded together in a complex three dimensional lattice by C-C bonds that are difficult to hydrolyse (Faison 1991;

Chen *et al*. 2006). Enzyme systems in microorganisms are mostly specific thus few microorganisms are able to produce enzymes that are non-specific and can hydrolyse the heterogeneous coal structure (Mönkemann *et al*. 1997). The heterogeneous nature of coal and the hydrophobicity largely require microbial systems to degrade the coal by producing biological substances, such as enzymes, alkaline substance, and surfactants extracellularly. These are required to be in contact with the coal substrate and in an aqueous solution where these biological systems can function (Laborda *et al*. 1997; Nüske *et al*. 2002). Fakoussa (1981) and many other researchers isolated and proved the ability of a select few bacteria and fungi that are able to colonise coal and produce highly oxygenated by-products (Klein *et al*. 1999). The advantage of microbial, enzymatic, or enzyme-mimetic technology is that this process is carried out at moderate temperatures and normal pressures (Pokorný *et al*. 2005). The biocatalysts are smaller compared to conventional catalyst particles resulting in a simpler system (Quigley *et al*. 1989a; Fakoussa 1994). These are ideal conditions making the coal conversion process energy efficient and cost effective. Bioprocessing of coal is now fairly well recognized as having significant advantages over the conventional chemical methods of coal breakdown. Coal biotechnology might open new possibilities for economical production of new coal-derived products, cleaner coal, desulphurization, coal mine rehabilitation and coal methanogenesis. Coal mine rehabilitation involves high and low-grade coals with low calorific value, high ash content which pollutes the environment when burnt or abandoned (Cooke & Johnson 2002). It has been shown that the conversion of coal using microorganisms results in relatively higher oxygen content and lower molecular mass products than chemical conversion (Wadhwa & Sharma 1998). These products are usually water soluble low molecular weight compounds. A large number of fungi and bacteria capable of coal degradation/solubilisation have been identified. Some of the species are *Streptomyces viridosporous* (Strandberg & Lewis 1987), *Bacillus cereus* (Maka *et al*. 1989), *Arthrobacter sp*. (Torzilli & 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 & Catcheside 1999), *Trametes versicola* (Gotz & Fakoussa 1999),*Pseudomonas putida* (Machnikowska *et al*. 2002) and *Neosartorya fischeri* (Igbinigie *et al*. 2008).

Degradation by fungi occurs via extracellular processes to yield a darkened medium when grown in submerged culture or dark droplets of liquid on the coal's surface when grown on agar as a lawn culture (Cohen *et al*. 1990; Gupta *et al*. 1990; Fakoussa 1994; Klein *et al*. 1999). Bacteria are one of the first microorganisms that were implicated in coal degradation and were identified as belonging to *Mycobacterium* and *Norcadia* (Fakoussa 1981). Recent studies suggest that the mechanisms of coal biodegradation by microorganisms were as follows: production of ligninolytic enzymes, alkaline substances, surfactants and chelators (Fakoussa 1994; Yuan *et al*. 2006; Sekhohola *et al*. 2013). In the case of bacteria, coal biodegradation is mediated by alkaline products during bacterial growth, chelation and surface-activation, which play some role in solubilisation of low-rank coals (Willmann & Fakoussa 1997). Some of these early tests indicated that the microbial solubilisation of coal is predominantly an extracellular process. For coal degradation to occur chemical bonds that create the solid three dimensional structure must be broken and there must be a transfer of hydrogen and oxygen from media to the coal (Torzilli & Isbister 1994). Bonds include ester, ether and strong covalent bonds between aliphatic coal fractions (Szendrii *et al*. 1994). Dictated by the extent, ease, and faster rate of microbial attack and the consequential biosolubilisation, coals like lignite and leonardites are more amenable to biosolubilisation than their higher rank counterparts, namely sub-bituminous and bituminous coals (Szendrii *et al*. 1994). Biosolubilisation depends on the overall oxygen content (>30 % in lignite and leonardite), the relative number of oxygen containing linkages, difference in porosity, and water content (Machnikowska *et al*. 2002). The porosity of coal generally increases with its decreasing rank, which is directly related to biological solubilisation depending on the oxygen linkages between aromatic moieties (Hu 2006). It also makes lignite ideally suitable for microbial biosolubilisation. The importance of these oxygen linkages lies in holding the coal molecules together, leading to an increase in their solubility (Hu 2006). In view of its typical characteristics and more proneness to microbial attack, lignite has gained special attention in recent years for its biosolubilisation into a value-added product, namely humic acid.

1.5 Coal derived value added products

Some of the coal derived value added products are humic substances. Humic substances are a complex, heterogeneous mixture of organic materials arising from the decay of plant and animal

residues and represent the main carbon reservoir in the biosphere (Tripathi *et al*. 2010). Humic substances are large components of soil and are also found in marine, fresh waters, forest litter and in sediments (Adani *et al*. 2006). They can be characterized as fulvic acid, humic acid and humin (Erdogon *et al*. 2007). Fulvic acid is soluble in acid and alkali; humic acid is soluble only in alkaline conditions; humin is insoluble in both alkaline and acidic conditions (Adani *et al*. 2006; Erdogon *et al*. 2007). Humic and fulvic fractions have the same aliphatic and aromatic structures but differ in molecular weight, chemical structure and distribution of functional groups, but the structure of humin is yet to be elucidated (Tripathi *et al*. 2010; Stevenson 1994). Low rank coals appear to have the highest concentration of humic substances and are therefore a good source of humic acid (Piccolo *et al*. 1997). These, unfortunately, are locked in the coal structure and are not readily available to plants and microorganisms (Piccolo *et al*. 1997). During coal biodegradation these humic substances are released and used commercially in agriculture as an additive to fertilizer because of their benefits to plant growth (Piccolo *et al*. 1997). Humic substances increase permeability, uptake of nutrients, growth of soil microorganism, and enhance phosphate utilization and stimulate root development. Humic substances have been documented to play a crucial role in reductive and oxidative reactions, sorption, complexation, and transport of pollutants, minerals and trace elements, sustaining plant growth, soil structure and formation, and control of the biogeochemistry of organic carbon in the global ecosystem (Trevisan *et al*. 2010). Indirectly, humics also enhance root growth, have auxin-like effects, and affect the soil microflora (Trevisan *et al*. 2010). The bioactivity of coal derived humic substances has been well documented and commercial products have been formulated and applied in agricultural sectors (Trevisan *et al*. 2010). Products in the market currently are Humagra®, Bio-O-15® and Humical® and these all contain more than 50 % (by weight) humic-like substances complemented by other nutrients, such as potassium, nitrogen and phosphate, and are packed as granules and/or formula. In terms of the several bacterial species successfully employed for the biosolubilisation of lignite into humic acid, the most important and commonly employed are *Streptomyces viridisporous* T7A, *Streptomyces setonii* 75vi2, *Escherichia coli*, *Streptococci sp*., *Cornyebacterium renale*, with the most effective being *Streptomyces setonii* (Fakoussa & Hofrichter 1999; Prakash *et al*. 2010; Sekhohola *et al*. 2013).

1.6 Coal mine rehabilitation

The problems associated with coal mining are *inter alia* the handling of the waste produced during the coal mining process and the rehabilitation of coal mines post mining. Historically, when coal mines are inactive (when production has ceased) then the collieries are boarded up and abandoned (Limpitlaw *et al*. 2005). Today it is accepted that closure requires the return of the land to viable post-mining use, such as agriculture (Cooke & Johnson 2002). Post-mining lands are characterized by water pollution from acid mine drainage, low to zero soil microbial activity, low nitrogen and phosphorus concentrations, increased levels of heavy metals and the presence of rocks that were previously buried (World Bank 2002). There are a variety of chemicals present in post-mining land soil which have different physical or chemical properties, such as, volatility, aqueous solubility, biodegradability, density and polarity (World Bank 2002; Frouz *et al*. 2008). These are the conditions that cause the land to be non-conducive for plant growth and non-productive. There are many strategies that have been developed to rehabilitate coal mine land but some of these cause further damage to the environment. When coal mining ceases in a region there are areas that are left barren and unfertile and thus not conducive for plant and animal habitation. Many mining companies use rehabilitation strategies which include extensive use of top soil imported from elsewhere. In other words top soil, which is fertile, is transplanted onto coal dumps or abandoned coal mining areas. This in turn promotes plant growth on or in the dumps and increases the microbial diversity of the area. This method improves the harsh conditions of coal dumps and in the process assists in rehabilitating coal dumps. The disadvantage of this process is that the area from which the top soil is extracted is left barren which results in the area being susceptible to soil erosion. In view of this other options which included biotechnological solutions were explored to rehabilitate coal dumps in order to derive a more sustainable, effective and environmentally friendly strategy. Microbial degradation of coal plays a role in coal mine rehabilitation when coal degrading microorganisms are included in the rehabilitation strategy. Fungcoal (Rose *et al*. 2007) is one of the few biological strategies that has been introduced as a possible solution to reduce the use of expensive chemical technologies (Igbinigie *et al*. 2010). As mentioned above, Fungcoal involves the use of fungi and grass species living in a mutualistic relationship (Igbinigie *et al*. 2010) to affect waste coal biodegradation. The mechanism postulated to be responsible is illustrated in Figure 1.2.

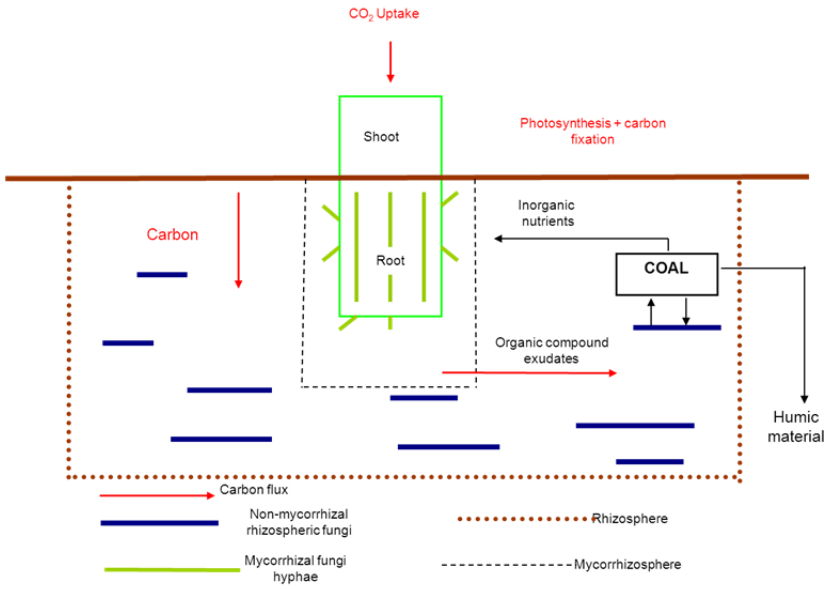


Figure 1.2 Diagram illustrating the postulated relationship between *Cynodon dactylon,* mycorrhizal fungi and non-mycorrhizal fungi in the biodegradation of waste and discard coal.

The plant provides a simple carbon source for the fungi in the form of organic acids that are part of the root exudate (Shukla *et al*. 2011). The fungi, in return, break down the coal producing a soil-like material and nutrients that the plant uses for growth and development (Igbinigie *et al*. 2010). This process promotes the recovery of soil microflora including colonization by soil bacteria.

1.7 Coal biodesulphurization

Coal desulphurization is a method used to remove sulphur bound in coal (Marinov *et al*. 2011). Sulphur is present in coal mainly as inorganic pyrite and in an organic form as sulfides, disulfides, thiols, and thiophenes (Szendrıi *et al*. 1994). Pyrite sulphur is represented as randomly distributed crystals in coal minerals; the organic sulphur is present as an integral part of the coal structure covalently bonded to the coal matrix (Saikai *et al*. 2009). During the burning of coal the sulphur in the coal reacts with oxygen and is released to the outside atmosphere as sulphur oxides (Rossi 1993). Sulphur oxides are widely known for their contribution to air pollution and acid rain (Rossi 1993). To reduce the emission of sulphur gases to the environment means that sulphur must be removed before coal combustion. The applications of coal desulphurization

techniques are a disadvantage in that they decrease the energy value of coal thereby making coal less suitable as a fuel source (Marinov *et al*. 2011).

Biodesulphurisation is a promising alternative to the current methods that have been tried and tested. Biodesulphurisation promotes the conversion of sulphur into water soluble, easy washed-out compounds (Prayuenyong 2002). Bacteria have been well documented in coal desulphurisation. *Acidothiobacillus ferrooxidans*, *Thiobacillus ferrooxidans*, *Fenobacillus ferroxidans*, *Brevibacterium sp.*, *Pseudomonas aeruginosa* OS1 and *Pseudomonas putida* are widespread bacterial cultures used in coal biodesulphurization (Myerson & Kline 1983; Tripathi *et al*. 2010). Olsson *et al.* (1993) described the desulfurization of coals with the use of *Sulfolobus acidocaldarius* DSM639, *Sulfolobus brierleyi* DMS 1651, and *Sulfolobus solfataricus* DSM1616 to be up to 90 %. These bacterial cultures possess genetic stability, reproducibility, and the ability to degrade C–S bonds in aromatic heterocyclic compounds (van Hamme *et al*. 2003). Unlike chemical methods the carbon content and the heat of combustion of coal material remained practically unchanged (Hackley *et al*. 1990). Bacterial involvement in the desulphurisation of coal strongly supports that bacteria are a good candidate for inclusion into a microbial based rehabilitation strategy such as Fungcoal.

1.8 Coal and the production of methane

The potential of bacteria using coal as a carbon source for methane production has been widely reported. Methane burns cleaner than coal and is easily transported via pipeline (Panow *et al*. 1997). The use of low rank coals, as well as waste coals, is an avenue that has been explored for coal beneficiation (Midgley *et al*. 2010). This is especially vital since the demand for natural gas is expected to increase from 6.23×10^{11} m^3 in 2004 to more than 9.34×10^{11} m^3 by 2024, according to the Energy Information Association. These microorganisms that are mostly bacteria are able to grow on low volatile bituminous coal as the sole carbon source. Methane production is mostly anaerobic and gives rise to coal bed methane (Atlas & Bartha 1998). The process of converting coal into methane is termed coal methogenesis (Madigan *et al*. 2000). In the coal beds underground water brings microbes into close proximity to the coal and in the absence of oxygen the microbes generate biogenic methane (Panow *et al*. 1997). Thermogenic methane is present in

coal beds and biologically derived methane is found in areas with low ranking coals (Atlas & Bartha 1998). Low rank coals, as mentioned above, are highly oxidized hence are able to serve as better substrate for biological methane production (Langenberg *et al*. 2001). The anaerobic degradation of coal is a more complex process involving the action of a group of bacterial strains, for example, syntrophic or acetogenic bacteria, and two types of methanogenic *Archaea* (Bumpus *et al*. 1998).

In the process of mine dump establishment waste and discard coal are highly compacted to eliminate trapped air and to prevent oxygen ingress. This is done as a preventative measure in mitigating spontaneous combustion events. As a consequence the pores between the compacted coal particles in dumps become anoxic and these conditions favour accumulation of methane. Such anoxic/anaerobic conditions have a negative impact on seed germination, which is a process that is oxygen dependent and which makes rehabilitation of such waste dumps a near impossibility unless a topsoil cladding is provided. The Fungcoal bioprocess was developed to eliminate the need to import large quantities of topsoil. Rehabilitation strategies under such adverse conditions as those of coal mine dumps require microorganisms that are able to survive these conditions. A rehabilitation strategy comprising bacteria adapted to anoxic conditions or able to survive these conditions and degrade coal could serve to supply simple carbon forms that will act to increase soil microflora to provide further carbon sources for the coal degrading fungus *N. fischeri*.

1.9 Mechanisms of coal bioconversion

Different principle descriptions are utilized for coal structural transformation and these have been distinguished by various research groups as, solubilisation and depolymerisation (Gupta *et al*. 1990; Fakoussa & Hofrichter 1999). Transformation, degradation and conversion refer to the change that occurs in the coal structure after biological attack but does not specify the kind of change that has occurred (Hofrichter *et al*. 1997b). Solubilisation by fungi refers to the production of a black liquid and in bacteria the production of water soluble by-products in a non-enzymatic reaction (Klein *et al*. 1999). The process occurs at pH 7-10 and is due to the excretion of alkaline substances, chelators, surfactants and hydrolytic enzymes (Figure 1.2) (Fakoussa &

Hofrichter 1999). Depolymerisation occurs at acidic conditions and is characterized by the production of lower molecular weight compounds due to a cleavage of major bonds (Perez *et al*. 2002). This process is an enzymatic reaction involving ligninolytic enzymes in the presence of mediators and radicals (Laborda *et al*. 1999). Mediators enhance the enzyme action increasing the substrate range of the enzyme, for example, ABTS, hydrogen peroxidase, Mn^{3+}.

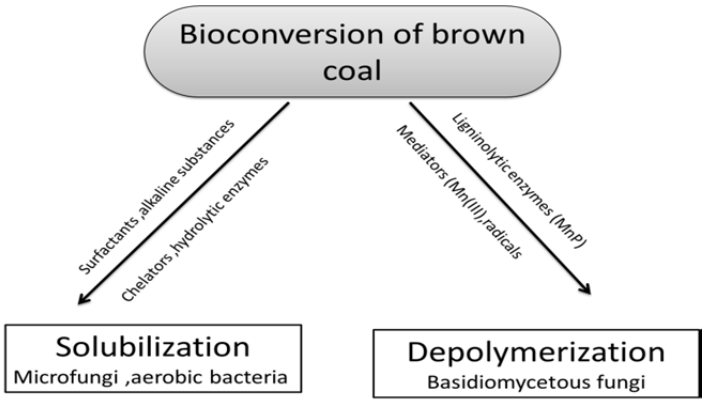


Figure 1.3 The two main structural modifications of brown coal by microorganisms.

Possible coal bioconversion mechanisms were further classified by Fakoussa and Hofrichter (1999) as ABCDE, where A=alkaline substances, B=biocatalysts (oxidative enzymes), C= chelators, D=detergents and E=Esterase.

1.9.1 Biocatalyst/enzymatic action

Biocatalytic coal degradation by enzyme action involves the production of oxidases which hydrolyze coal structural bonds thereby depolymerizing the coal macromolecule (Figure 1.3) (Fakoussa & Hofrichter 1999). The principal enzymes involved are reported to be ligninolytic (Ralph *et al*. 1996).

Manganese peroxidase, laccase and lignin peroxidase are three of the best studied enzymes produced by ligninolytic fungi, for example, *Phanerochaete chrysosporium* from the white rot fungi family (Ralph & Catcheside 1994b). Lignin peroxidases can specifically catabolise aromatic compounds that are both phenolic and non-phenolic in nature (Hayatsu *et al*. 1979; Wondrack *et al*. 1989). Laccase reduces phenolic substrate but can reduce non phenolic substances in the presence of an electron mediator normally 2,2'-azino-bis (3-

ethylbenzothiazoline-6-sulphonic) acid (ABTS) (Fahraeus & Reinhammar 1967; Quigley 1993). Laccase also occurs in prokaryotes, namely, *Azospirilium lipoferum*, *Marinomonas meditterranea*, *γ–Proteobacterium* JB and *Bacillus subtilis*. Manganese peroxidase uses manganese as a cofactor and is well distributed in ligninolytic fungi (Hofrichter & Fritsche 1997a; Nuske *et al*. 2002). Although enzymes play a role in fungal biodegradation of coal they do not appear to be involved in bacterial degradation of coal (Hofrichter & Fritsche 1996; Hofrichter & Fritsche 1997a).

The first report of enzymatic degradation of coal by a bacterium was that of *Pseudomonas capecia* (Crawford & Gupta 1991). The enzyme was shown to be extracellular, oxidative and managed to depolymerize the coal structure (Crawford & Gupta 1991). According to these authors the infra-red spectra demonstrated that the depolymerisation process was due to the action of the bacteria on the ether and ester bonds. Actinomycetes produce the enzymes, cellulase, catalase, esterase and etherase, which possibly degrade secondary metabolites which arise as a consequence of coal degradation process (Jiang *et al*. 2013). These enzymes do not form part of the ligninolytic enzyme suite but are supporting enzymes that further degrade some of the macromolecules that are produced during coal biodegradation (Jiang *et al*. 2013). *P. putida*, *Rhodococcus* RHA1 and *Rhodococcus* sp. are known to produce the ligninolytic enzyme laccase when incubated in lignin-containing media (Fang *et al*. 2012).

1.9.2 Alkaline substances, chelators and surfactants

The solubilisation of coal is a process that occurs preferentially at higher pH (pH 7-10) and can be due to microbial formation of alkaline substances and/or chelating agents and surfactants (Wilson *et al.* 1987; Fakoussa 1994). Alkaline substances and chelating agents excreted by microorganisms seem to play a crucial role during the coal solubilisation process whether or not there is dependence on extracellular enzymes (Stewart *et al*. 1990; Fakoussa & Hofrichter 1999). Alkaline substances, such as ammonium, polyamines and basic peptides, increase the pH and maintain it between 6 and 9 (Fakoussa & Hofrichter 1999). The production of alkaline substances results in the solubilisation of coal rather than depolymerisation. The action of alkaline substances largely depends on the structure of the coal, level of oxidation and the

nitrogen content (Yuan *et al*. 2006). The coal functional groups are protonated and thus made more water soluble. This process is thought to involve the transformation of NH_4^+ and amino acids to alkaline amines, or to their corresponding keto acids (Klein *et al*. 1999). Fakoussa and Truper (1983) reported that surfactants produced by the bacterium *Pseudomonao fluorescens* have a solubilizing action on coal. The surfactant lowered the surface tension to about 25.5 Nm lower than acetone and sodium dodecyl sulphate (Fakoussa & Hofrichter 1999). Bacteria, and in particular the strain *Pseudomonas stutzeri* produces a biosurfactant that was able to solubilise a portion of coal (Singh & Tripathi 2013).

Chelation involves the secretion of oxalate ions and other substances that function as metal chelators (Hodgkinson 1977; Dutton *et al*. 1993). In the presence of low rank coal oxalate sequesters polyvalent ions, such as Ca^{2+}, Fe^{3+}, Al^{3+} and Mg^{2+} causing the breaking of the links formed by metal ions (Quigley *et al*. 1989b). These metal ions link coal macromolecules and, when broken, the more polar coal macromolecules become soluble in water (Hodgkinson 1977; Gupta *et al*. 1990; Dutton *et al*. 1993). *Arthrobacter sp.* was reported to produce oxalate which caused solubilisation of coal (Fredrickson *et al*. 1990). When the coal degradation products were measured spectrophotometrically at A_{450} there was an increase in absorbance over time (Fredrickson *et al*. 1990).

1.10 Research aims and objectives

Extensive studies, as outlined above, have been published on the ability of microorganisms to degrade coal in the absence of a simpler carbon source. Furthermore the action of fungi on coal as a substrate has been highlighted and has been explored more rigorously than that of other microorganisms. In contrast, the bacterial contribution in coal breakdown has been neglected and consequently information on bacterial degradation of coal is sparse. In the Fungcoal bioprocess knowledge of the mutualistic relation between plants and fungi was exploited. Bacteria are the one candidate group that appears to have been overlooked in this mutualistic relationship which underpins Fungcoal as a rehabilitation strategy. As stated by Sekhohola *et al*. (2013) solubilisation of waste coal and lignite commences on the mutualistic relationship between plant, mycorrhizal and non-mycorrhizal fungi and it is further extended to bacteria.

This study therefore aimed to isolate bacterial strains from environmental samples obtained from sites contaminated with PAH-type pollutants. Bacteria were screened for coal degrading capability using waste coal as the substrate and as the sole carbon source. Subsequently bacteria were inoculated in waste coal media and the coal degradation products tentatively identified using a combination of physicochemical and biological assays. The results are discussed with a view to utilizing this coal degrading bacterium to supplement the bioprocess technology known as Fungcoal.

CHAPTER 2
SCREENING FOR AND ISOLATION OF COAL DEGRADING BACTERIA

2.1 Introduction

Microorganisms have been widely used in the degradation and remediation of recalcitrant substances such as hydrocarbons, oil spillages and heavy metals from the environment (Prenafeta-Bold 2004). Many of these microorganisms are bacteria that have been isolated from extreme environments such as swamps, forest litter, coal dumps and hydrocarbon contaminated sites (Moolick *et al.* 1989). Bacteria in extreme conditions develop the ability to utilize readily available contaminants from these sites as a carbon source. This enables bacteria to survive in the absence of simpler carbon sources such as glucose, sucrose and organic acids (Klein *et al.* 2001). In addition to bacteria, other microorganisms, such as white rot fungi, have been used to degrade coal macromolecules into lower molecular weight compounds (Torzilli & Isbister 1994).

Coal is very resistant to microbial attack because of its complex structure and therefore only a few species of bacteria have been shown to degrade coal (Moolick *et al.* 1989; Sekhohola *et al*. 2013). It is uncommon to find bacteria that can degrade both aliphatic and aromatic compounds (Salam *et al*. 2011). This is probably due to the different metabolic pathways employed by the bacteria for the two classes of hydrocarbon (Salam *et al*. 2011). However, some reports have shown that there are bacterial strains that have the ability to degrade both classes of hydrocarbons simultaneously which are present in coal (Salam *et al*. 2011). Highly oxidized coal contains greater amounts of oxygen in the form of carboxyl and hydroxyl groups (Levine *et al.* 1982) and it is believed that the presence of these terminal oxygen groups facilitate microbial attack (Tripathi *et al.* 2010).

Research on the bacterial colonization of coal dates back to 1908 when a strain was reported to have the ability to degrade lignite (Klein *et al.* 2001). Research in this area of coal degradation using other microbes such as fungi, moulds and yeasts has been recorded (Klein *et al.* 2001, Sekhohola *et al*. 2013). Some of the strains that successfully colonize and degrade coal are *Bacillus* spp., *Piptoporus betulinus*, and *Pseudomonas aureofaciens* (Torzilli & Isbister 1994;

Machnikowska *et al*. 2002; Yuan *et al*. 2006). These bacteria degrade highly oxidized coals such as lignite and leonardite. Degradation of the coal occurs to a limited extent but these strains have been reported to be able to modify the chemical and physical structure of the coal (Machnikowska *et al.* 2002).

Microbial degradation of coal has been considered as an effective and economical way to transform coal into lower molecular weight compounds (Prayuenyong 2002). Low rank coals, such as lignite and waste coal from mining activity, are discarded in large quantities (Fuchtenbusch & Steinbuchel 1999). Some of the major impurities in coal are heavy metals, clay, and sulphur containing minerals like pyrites (Reich-Walber *et al*. 1997). On the other hand unremediated mining areas filled with oxidized waste coal are considered sterile (Igbinigie *et al*. 2010). New conversion technologies are thus required to convert low rank coals into value added products which can be used by the industry. Microbial separation of sulphur from pyrites (desulphurization) has been extensively studied in the past years (Skvarla *et al*. 1998). *Thiobacillus ferroxidans* has been used successfully in coal desulphurization through the process of biosurface modification (Reich-Walber *et al*. 1997; Skvarla *et al*. 1998). Other bacterial species, such as *Mycobacterium,* have been implicated to successfully flocculate fine coals removing ash forming minerals (Skvarla *et al*. 1998). Biological processes are required because they operate under low temperatures and pressures (Cartmell & Lancaster 1983). The systems are small and more efficient compared to chemical and physical conversion technologies (Cartmell & Lancaster 1983; Reich-Walber *et al*. 1997; Skvarla *et al*. 1998). Chemical techniques employed in remediation cause problems, such as runoff to nearby streams and disturbance of aquatic ecosystems (Igbinigie *et al*. 2010). Biological methods promise to be a more environmentally friendly alternative. In coal mine rehabilitation practices bacteria and other microorganism are able to interact with the coal substrate forming a soil like material (Igbinigie *et al*. 2010). As the below ground chemistry changes the substrate is able to support a more diverse community of microorganisms (Claassens *et al*. 2005) and higher plants (Musaka–Mugerwa *et al*. 2011; Sekhohola *et al*. 2013). In view of the potential for mutualism between coal degrading microorganisms, plant growth promoting bacteria, arbuscular mycorrhizal fungi and higher plants, there is reason to suggest that together these organisms are able to support remediation of the disturbed land and restoration.

Early work at the Institute for Environmental Biotechnology, Rhodes University (EBRU) isolated a fungus that was able to degrade waste and weathered coal (Igbinigie *et al.* 2010; Mukasa-Mugerwa *et al*. 2011; Sekhohola *et al*. 2013). These studies resulted in the emergence of a bioprocess technology termed Fungcoal (Rose *et al*. 2007). A limiting factor in Fungcoal technology is not only the recalcitrance of the coal substrate but the time taken by the biocatalysts to carry out the coal degradation process. To speed up and improve the efficiency of the coal breakdown process it seemed pertinent to bio-prospect for bacteria which are known to promote plant growth and contribute to mutualism between higher and lower organisms. Ideal candidates might be bacteria capable of degrading polyaromatic hydrocarbons (PAH) as these organisms may also be able to utilize the coal. The isolates thus obtained were maintained in culture and investigated for their ability to degrade and/or biosolubilise waste and weathered coal. The most efficient bacterial strains were used for further studies.

2.2 Materials and Methods

2.2.1 Bioprospecting, strain isolation and culture maintenance

Samples were collected from diesel contaminated soils and from the rhizosphere of grasses growing on contaminated soils on the premises of Grahamstown Engineering (GE), Grahamstown, South Africa. Wet soil and liquid samples were also taken from contaminated streams near the Municipal Disposal Dump (MDD), Grahamstown, South Africa. Other sample sites included old tractor engines at Strowam farm (SF).

Soil samples were added to sterile saline water which was then serially diluted, and aliquots inoculated on nutrient, malt extract, potatoes dextrose, and MacConkey agars prepared according to the manufactures' instruction (Appendix 1). This was to ensure that bacterium colonies from poly aromatic hydrocarbon contaminated sites would grow successfully to allow for screening using a coal based media. Malt extract and potato dextrose agar are media specifically suitable for fungal growth and these were used to distinguish between bacterial and fungal species.

Nutrient and MacConkey agar were used for bacterial growth. MaConkey agar is selective for gram positive and gram negative bacteria.

The cultures were incubated at 30 °C overnight in a constant environment chamber and discrete colonies were aseptically picked using a sterile tooth pick and inoculated immediately into 100 mL nitrogen-rich coal media (Igbinigie *et al.* 2010) for adaptation. The flasks were placed on a rotary shaker at 150 rpm at 30 °C in a constant environment room for 14 days (d) to select for coal degrading bacteria. Nitrogen-rich coal medium, with no additional carbon source, consisted of KH_2PO_4, 12.7 g; $NaNO_3$, 3 g; K_2HPO_4, 2 g; $MgSO_4.7H_2O$, 0.5 g; KCl, 0.5 g and 0.5 g of waste coal. Only strains that were able to survive and/or grow in this nitrogen-rich coal supplemented medium were retained for further analysis (see below). Unless otherwise stated all isolated strains were, in addition, maintained in Luria broth and subsequently stored as glycerol stocks (Appendix 1).

2.2.2 Screening and selecting for coal degrading capability

Waste coal obtained from Witbank, South Africa was powdered using a HP-M 100 Pulverizer (HERZOG Maschinenfabrik GmbH Co., Osnabrück, Germany) to yield particles of approximately 0.5 mm in diameter for addition to flasks containing nitrogen-enriched minimal salts medium as described above. For coal degrading capability visual observations were made daily to determine qualitative changes in the culture medium including presence of precipitates, increased browning-yellowing (i.e. colour change), and extent of mycellial proliferation. Based on the examination of 120 isolates, Strain GE37 and SF41 were selected together with one less efficient strain, GE18, and these were transferred and maintained in Luria broth.

2.2.3 Morphology

Scanning electron microscopy (SEM) was used to determine bacterial morphology. Sample preparation for SEM was according to the method by Cross (2001). A drop of coal media was fixed in a 2.5 % glutaraldehyde solution prepared from a 25 % glutaric dialdehyde stock reagent by diluting it with phosphate buffer (0.1 M, pH 7) (Appendix 2) after 12 hours (h).

Glutaraldehyde solution was decanted and samples were washed twice with phosphate buffer (0.1 M, pH 7) (Appendix 2) for 10 minutes (min). Fixed samples were then dehydrated using a graded ethanol series of 30, 50, 70, 80, 90 and 100 % for 15 min for each followed by two changes of 100 % ethanol for 15 min each. Samples were then placed in a Polaron critical point dryer (Watford, England) and dried for 2 h. Dried samples were mounted on 12 mm diameter aluminium posts with 12 mm carbon conducting adhesive tabs. Samples were then transferred to a sputtering device (Balzers Union) and gold coated for 160 s at 80 mT pressure at 45 mA. Carbon paint was applied from the surface of the gold coating to the aluminium post. This was done to ensure conductive paths from electrons on the surface reach ground state. Samples were examined with a VEGA LMU (VEGA © Tescan) scanning electron microscope.

2.2.4 DNA extraction

Method was conducted as described by Head *et al*. (1998). Samples were extracted from Luria broth by centrifugation (17950 × g for 5 min) using an Eppendorf 5415D desktop centrifuge. The supernatant was discarded and the nucleic acid extracted from the pellet after re-suspension in 500 µL of TE buffer (Appendix 3) to which was added 6 µL of 50 mg/mL of lysozyme stock solution. The tubes were then incubated for 3 h at 37 °C with periodic vortexing.

The samples were heated at 100 ºC for one minute and then five freeze-thaw cycles were performed by transferring samples between liquid nitrogen and an 80 °C water bath. After the last freeze-thaw cycle, the samples were allowed to cool to room temperature and then 50 µL of 10 % Sodium Dodecyl Sulphate (SDS) (Appendix 3) and 2.5 µL of 50 µg/mL Proteinase K stock solution were added; the contents were mixed and then incubated at 37 °C overnight prior to the addition of 100 µL of 10 % CTAB (Appendix 3) and 200 µL of 5 M NaCl (Appendix 3) followed by incubation at 55 ºC for 1 h. Aliquots (500 µL) were then transferred into sterile Eppendorf centrifuge tubes; an equal amount of buffer saturated phenol, pH 8 (Sigma-Aldrich St Louis, MO) was added. Samples were vortexed for 30 seconds and then centrifuged for two minutes at 17950 g; the upper aqueous layer was then transferred to a fresh tube and an equal volume of phenol: chloroform: isoamyl alcohol (24:24:1,

v/v/v) added. The samples were then mixed and centrifuged as above. The upper aqueous layer was then transferred to a fresh Eppendorf tube and an equal amount of chloroform: isoamyl alcohol (24:1, v/v) was added; the samples were vortexed and centrifuged as previously described: this step was repeated until the upper aqueous layer was clear. The final clear upper aqueous layer was placed in a sterile Eppendorf tube and 2.5 volumes of ice- cold 96 % rectified ethanol was added. The samples were then placed at 20 °C for 12 h. Samples were then centrifuged at 17950 × g for 20 min and the supernatant was discarded. Pellets were allowed to air dry and then re-suspended in triple distilled water, the volume depending on the size of the pellet, but usually 500 µL. DNA samples were placed at 4 °C for short-term storage and for long-term storage they were placed at 20 °C.

2.2.5 Agarose gel electrophoresis

The results of the DNA extractions and PCR product were viewed by using agarose gel electrophoresis. First a 1 % agarose gel was prepared, which consisted of 0.5 g of molecular grade agarose and 50 mL × TE Buffer (Appendix 3) and was heated until the agarose was completely dissolved and then cooled to approximately 50°C. 50 µL of ethidium bromide stock solution (Appendix 3) was added and then the agarose gel was poured into a gel mould and a comb was placed into the gel. The gel was allowed to set and then it was placed in a gel tank containing 1 × TBE buffer (Appendix 3). Approximately 5 µL of DNA and 5 µL of DNA loading buffer were added in each well. A λ*Pst I* molecular weight marker was added in the first well to determine the size of the band on the gel by drawing a standard curve (Appendix 3). The gel was developed for 1 h at 100V and then viewed using a UV light box.

2.2.6 Polymerase chain reaction

Each 25 µL PCR reaction contained: 2 µL of 10 µM GM5F primer stock solution (Muyzer *et al.* 1993; Santegoeds *et al*.1998) (Appendix 3), 2 µL of 10 µM 907R primer stock solution (Muyzer *et al.* 1993; Santegoeds *et al*. 1998) (Appendix 3), 2 µL of 10 µM dNTP stock solution (Appendix 3), 1 µL BSA, 3 µL DMSO, 6 µL triple distilled H_2O (Sigma) and 1 µL DNA. PCR

reactions were placed in and the GeneAmp PCR system 9700. As shown in Table 2.1 a touchdown thermocycle programme was used.

Table 2.1 Thermocycler cycles used for PCR amplification.

Initial Denaturation	95 °C	2 min	1 cycle
Denaturation	94 °C	30 s	4 cycles
Annealing	68 °C	45 s	
Extension	72 °C	2 min	
Denaturation	94 °C	30 s	4 cycles
Annealing	66 °C	45 s	
Extension	72 °C	2 min	
Denaturation	94 °C	30 s	4 cycles
Annealing	64 °C	45 s	
Extension	72 °C	2 min	
Denaturation	94 °C	30 s	4 cycles
Annealing	62 °C	45 s	
Extension	72 °C	2 min	

2.2.7 DNA Sequencing

The 16S rDNA gene was amplified by PCR and the PCR product purified and subsequently sequenced using an ABI 3130XL sequencer (Inqaba Biotech). These chromatograms were converted into text format using Gene Tools and then put into the NCBI BLAST database (Appendix 4). BLAST or Basic Local Alignment Search Tool is a set of similarity search programs designed to explore all of the available sequence databases (Altschul *et al.* 1990) (Appendix 4).

2.3 Results

2.3.1 Isolation of bacteria with coal degrading capabilities

Bacterial strains were isolated in the bio-prospecting exercise and each of these was propagated in liquid culture media to which was added waste coal without an alternative carbon source. The ability of microorganisms to degrade the coal substrate was determined qualitatively by observing precipitation and colour change (Figure 2.1). As shown, the introduction of strains into a medium containing coal resulted in the formation of a yellow to brown supernatant. In the flask inoculated with strain GE 37 waste coal settled out of solution when compared to the uninoculated control (Figure 2.1). Furthermore, flasks containing strain GE 37 demonstrated a distinct colour change from between brown to yellow. Strain SF 41 showed similar results (data not shown).

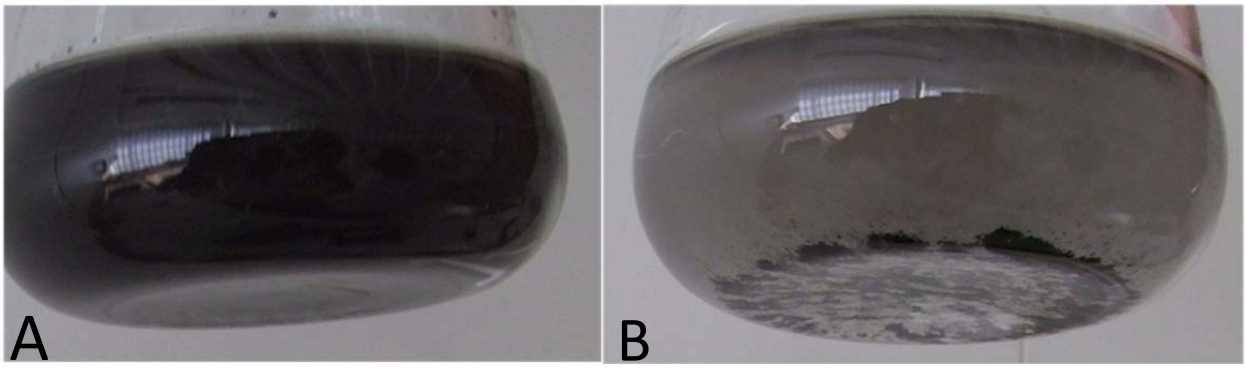


Figure 2.1 Bacterially induced colour change of coal containing minimal medium. GE 37 was introduced in minimal salts medium containing 0.1 g of waste coal and incubated for 14 d at 30 °C in continuous light on a rotary shaker (A) uninoculated control (B) GE 37.

Isolated bacterial strains were inoculated into coal media to determine their coal biodegradation potential. In the flasks inoculated with strain GE 37, the coal formed a precipitate with a brown-yellow supernatant; in the control no precipitate was formed nor was there any visible colour change. Typically a microorganism induced precipitation of coal in liquid medium coupled with colour change indicates an association between coal particles and microorganisms. Furthermore

the colour change suggested that a change in the chemical and physical structure of the coal substrate had occurred.

2.3.2 Characterization of bacteria with coal degrading capability

Isolated bacterial strains were characterized by observing their morphological appearance. Table 2.2 shows these characteristics of strains growing on nutrient and MacConkey agars.

Table 2.2 Morphological characteristics of strain GE 37 and SF 41.

Characteristics	Strain GE 37	Strain SF 41
Colour	Yellow	White
Colony shape	Circular	Circular
Diameter	4 mm	2 mm
Margin	Entire	Entire
Elevation	Raised	Raised
Gram stain	Positive	Positive
Shape	Rod	Rod
Motility	+	+

Colonies were differentiated on the basis of morphological appearance. Dilution plate count technique resulted in 120 isolated colony forming units in all the agars and only two strains were further developed. Developed isolated strains from Strowam farm (SF 41) and Grahamstown Engineering (GE 37) indicated the potential for coal degradation with different colony morphologies. Both biocatalysts were confirmed to be bacteria, circular, with an entire margin, elevation raised, rod shaped and flagellated. Gram staining revealed that GE 37 to be gram positive with yellow colonies and strain SF 41 to be gram positive with white colonies. In each case colony diameter was 4 mm for GE 37 and 2 mm for SF 41.

2.3.3 Bacteria -coal particle interaction in liquid media

Many studies in coal biodegradation have indicated a close association between substrate and bacteria and typically this association involves the encapsulation of coal particles by the microorganisms' concerned (Midgley *et al*. 2010). Thus it was of interest to determine the nature

of the interaction between the strains and waste coal provided as the sole carbon source. This was particularly important considering the precipitate that was formed (see Figure 2.1).

Scanning electron microscopy revealed the interactions shown in Figure 2.2. Clearly visible is bacterial colonization of coal.

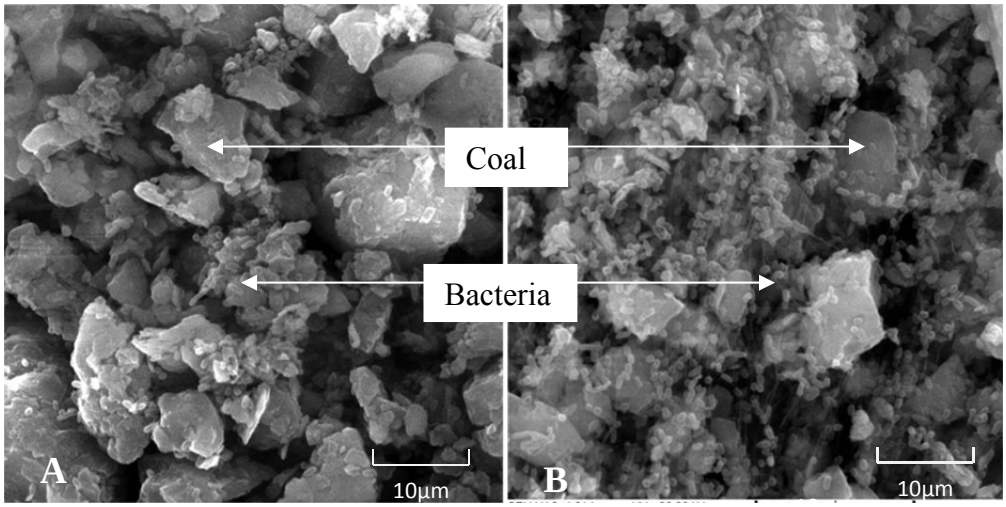


Figure 2.2 Scanning electron micrographs of strain GE 37 (A) and SF 41 (B) illustrating the interaction between bacteria and waste coal particles.

As can be seen in Figure 2.2A cells of SF 41 show an association indicating colonisation of coal substrate. This was however less prolific than for strain GE 37 (Figure 2.2B) which shows intense colonisation of coal indicating a close interaction between species and coal particle.

2.3.4 Identification of strain GE 37 and SF 41

For identification of the unknown strains DNA was extracted from SF 41 and GE 37 and further amplified to acquire the identity of the two unknown strains. Computer analysis using BLAST identified both strains as belonging to the genus *Bacillus* with the highest sequence similarity with *Bacillus flexus* and *Bacillus megaterium* with 99 % identification. The phylogenetic tree of strain SF 41 and GE 37 was constructed based on the gene sequences which are presented in Appendix 4.

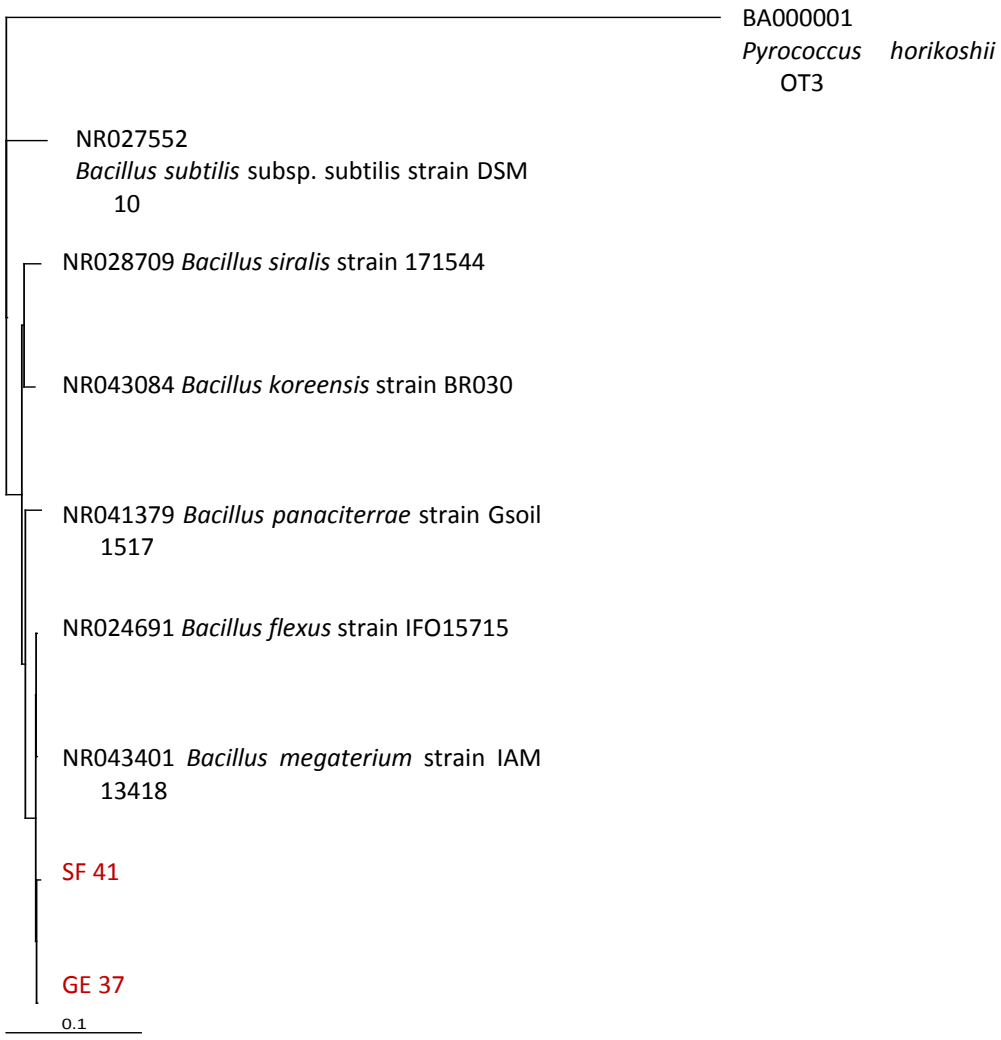


Figure 2.3 Phylogenetic tree based on the 16S DNA of strain SF 41 and GE 37.

The phylogenetic tree, which was rooted with the 16S DNA sequence of *Pyrococcus horikoshii* 0T3 and generated with ClustalW and TreeView, shows that the two strains are highly similar to *Bacillus flexus* and *Bacillus megaterium*. Based on Gram staining and the above PCR analysis coupled with microscopic study it was decided that any differences between SF 41 and GE 37 were so slight as to be insignificant. As a consequence and for the purposes of this research all further studies were carried out using *B. flexus*.

2.4 Discussion and Conclusion

Hydrocarbon degrading bacteria are distributed in diverse ecosystems. Using bacteria that inhabit hydrocarbon contaminated sites for biodegradation has been widely accepted alluding to the successes by other authors (Salam *et al*. 2011). The bacteria found in these niches have distinct characteristics; one of which is inducible enzymes and another that allows them to survive is nucleotide single or multiple mutations (Reich-Walber *et al*. 1997). These rare abilities are caused by long exposure to a pollutant and as a result they acquire appropriate degradative genes (Salam *et al*. 2011). These features allow the bacteria to degrade the compounds found in hydrocarbon contaminated areas (Salam *et al*. 2011).

From the environmental samples collected in this study a total of 120 bacteria were isolated and of these only three showed potential to degrade coal. Two of these were chosen for further studies based on results in initial screening trials. The two isolated species were identified by PCR analysis as *Bacillus flexus* and *Bacillus megaterium* and exhibited the ability to degrade coal evidenced by the production of brown-yellow precipitate when inoculated in coal media.

Bacillus species have been documented to be able to degrade recalcitrant material (Al-Sarida *et al* 2000). *Bacillus ceresus*, *Bacillus pumilus* and *Bacillus subtilis* are among a few that have been reported to have the ability to degrade coal (Sekhohola *et al*. 2013). *B. flexus* has been reported to be able to inhabit highly saline conditions (Alamri 2012). They have the ability to degrade recalcitrant compounds and are found in sea water as well as in stationary water bodies, such as lakes (Sivraprakasm *et al*. 2008). This bacterium is mainly found in aquatic environments which represent the location from which samples for this study were sourced. Sivraprakasm *et al*. (2008) demonstrated the ability of *B. flexus* in degrading tannery wastewater which contains high concentrations of phenol and halogenated organics. *B. flexus* was also documented to possess genes that encode for an enzyme with the ability to hydrolyze mycrocystin-RR (Alamri 2012). These are toxic cyclic structures found in lakes. The characteristics of the bacteria demonstrate their potential for coal degradation although *B. flexus* has until now not been documented to have coal degrading capability. *Bacillus megaterium* is closely related to *B. flexus* and upon analysis

by PCR was similarly matched. *B. megaterium* is found mainly in the soil and is said to have plant growth regulation capabilities (Zou *et al*. 2010).

Bacterial cells are sensitive to nutritional requirements therefore in extreme conditions the surface and the nature of the cell wall change in order to adapt to the new environment. Gram negative bacteria have been documented to produce membrane vesicles (Beveridge 1999). These membrane vesicles have the ability to produce/carry extracellular substances in extreme conditions (Beveridge 1999) and, in a coal environment have a potential to assist in the release of extracellular enzymes, alkaline substances and chelating agents. All of these are reported strategies that bacteria and fungi use in coal degradation (Fakoussa 1988). All efforts to demonstrate the production of and/or presence of membrane vesicles by cultures of *B. flexus* and *B. megatarium* challenged with waste coal as substrate proved unsuccessful (data not shown).

The change in colour of coal media to brown-yellow solution might indicate the presence of humic-like substances. The production of humic-like substances has been used in many studies as an indicator of the biodegradation of coal (Kai-yi *et al*. 2009). Scanning electron microscope images demonstrated a close association of the bacteria to coal. Raichur and Vijayalakshmi (2003) explain the phenomenon of coal bacteria adhesion as dependent on the surface charge of the bacteria in relation to the surface charge of the coal particle. Both coal and bacteria facilitate the bacteria adhering to the coal particle (electrostatic attraction) (Raichur & Vijayialakshmi 2003; Boks *et al*. 2008). *B. flexus* was publicized to be able to adhere to metals such as manganese in manganese oxidation producing a biofilm (Anandkumar *et al*. 2011). These bio-surfactants increase cells surface hydrophobicity thus bacteria are likely to prefer hydrophobic surfaces, for example, coal surfaces, metal, etc. (Anandkumar *et al*. 2011). Bacteria together with other microorganisms are known to degrade coal by producing enzymes, chelating agents, and alkaline substances (Torzilli & Isbister 1994). Close association with the coal particle allows the extracellular substances produced by bacteria to be in close proximity to targeted substrate making the process of degradation more efficient.

In this study bacteria were successfully isolated from hydrocarbon contaminated sites. The two bacteria isolated were identified as *Bacillus flexus* and *Bacillus megatarium* with a 99 %

similarity. *B. flexus* and *B. megatarium* are known to overlap with gene distribution and therefore show a close relationship (Berrada *et al*. 2011). Both strains demonstrated an ability to degrade waste coal into a brown-yellow like soluble product. Furthermore, residual waste coal settled at the bottom of the flask and SEM images showed a close association of the bacteria with this residual coal. The results strongly suggest that both strains have the potential to be incorporated into bioprocess technologies aimed at coal degradation for land rehabilitation.

CHAPTER 3

DEGRADATION OF WASTE COAL BY *BACILLUS FLEXUS*

3.1 Introduction

One of the current challenges in the world is how to use traditional fossil fuels, especially low-rank coal such as lignite or brown coal as clean fuel (Polman *et al.* 1991). Since the first report of the microbial transformation of coal into liquid clean coal and lower molecular weight compounds, efforts have focused on identifying other microorganisms capable of transforming this largely recalcitrant substrate. In addition to characterisation of the by-products and exposition of the underlying biochemical mechanism of coal degradation by white rot fungi, mostly from the *Basidiomycetes* class, effort is required to explore bacteria and the role played by these organisms in biodegradation of coal (Kai-yi *et al.* 2009; Tao *et al.* 2010). To date very little research has been conducted to elucidate the role that bacteria play in coal biodegradation (Gupta & Birenda 2000) particularly as these organisms are able to flourish under the harshest of conditions, for example, extreme pH and temperature (Abd-Elsalam & El-Hanafy 2009). According to these authors bacteria are also biochemically adaptable and have the ability to acquire traits that permit them to persist under stressful conditions.

Coal degradation mechanisms that microorganisms utilize to successfully degrade coal comprise enzymatic actions and non-enzymatic actions. Enzymatic actions include ligninolytic enzymes, phenol oxidase and the supporting enzymes, esterase and etherase for example (Acten *et al.* 2011). Since lignin is a constituent of coal (Sekhohola *et al*. 2013), ligninolytic enzymes are expected to play a major role in coal degradation (Willmann & Fakoussa 1997; Monkemann *et al*. 1997). These enzymes are able to degrade lignin as well as compounds that are structurally similar to lignin (Scott *et al*. 1986; Willmann & Fakoussa 1997). They are produced by specialised organisms referred to as ligninolytic fungi and ligninolytic bacteria (Scott *et al*. 1986; Willmann & Fakoussa 1997; Steffen *et al*. 2002). Bacteria involved in lignin degradation are good candidates for coal degradation. Amongst others the following have been documented to possess ligninolytic properties: *Azotobacter, Bacillus megatarium* and *Serratia marcescens*

(Abd-Elsalam & El-Hanafy 2009). Principal ligninolytic enzymes are lignin peroxidase, manganese-dependent peroxidase and laccase (Scott *et al*. 1986; Willmann & Fakoussa 1997; Steffen *et al*. 2002; Singh & Tripathi 2013).

In addition to enzymes, non-enzymatic actions include the production of alkaline metabolites, chelators, surfactants and siderophore-like substances (Torzilli & Isbister 1994). In most bacteria alkalinity and chelation play more of an essential role than the production of enzymes (Yuan *et al*. 2006). Both these mechanisms are known to be utilized by bacteria and other microorganisms specifically in coal biosolubilisation as opposed to biodepolymerisation (Strandberg & Lewis 1987). Alkaline metabolites are released by bacteria into the media and pH is increased. The alkaline media cause protonation of coal side chains making coal more soluble (Quigley *et al*. 1989 a, 1989b). Alkaline compounds, such as ammonia and amines, are released by bacteria in the presence of coal. A study by Maka *et al*. (1989) identified three bacterial species in a mixed culture that produced a supernatant with high pH after a period of incubation with lignite, namely *Bacillus cereus, Bacillus pumilus,* and *Bacillus subtilis*. Chelators are in many cases low molecular weight organic acids, ammonium oxalate, for example. They sequester cations, for example, Ca^{2+}, Mg^{2+} by removing these from the coal structure thereby breaking bonds and linkages making coal more soluble (Cohen *et al*. 1990). Biosurfactants are excreted by bacteria, yeast, and fungi extracellularly; these include compounds such as rhamnolipids, lipopeptides and sophorolipids (Hofrichter & Fakoussa 2001; Yuan *et al*. 2006; Sekhohola *et al*. 2013). They reduce surface tension making the coal more wettable and more water soluble (Fakoussa & Hofrichter 1999; Yuan *et al*. 2006; Sekhohola *et al*. 2013). Siderophore-like substances have been recently discovered in coal degradation with extensive research yet to be done (Torzilli & Isbister 1994). Most bacterial systems use more than one mode of degradation. One mode can however be used.

A reduction in the carbon content of coal particles is one indication that coal biodegradation has occurred. The by-products of degradation can be highly oxygenated aromatic or aliphatic low molecular weight compounds, low solubility in organic solvents but highly soluble in water (Davison *et al*. 1990: Machnikowska *et al*. 2002). The products of degradation are typically heterogeneous, depend on coal rank, and the location in which it was mined (Strąpoc *et al*.

2011). Degradation products also depend on the biocatalyst that is being utilized since each species uses a different bioconversion strategy (Gupta & Birenda 2000).

The aim of the studies described in this chapter was to determine and characterise the mode used by *B. flexus* to transform waste coal. Evaluation of the coal degrading capability of *B. flexus* was undertaken in this research (experiment) in terms of the aim of the study. In order to do this evaluation, cultures of *B. flexus* were grown with waste coal as the sole carbon source and any change in the coal structure was determined by a combination of elemental and FT - IR analysis.

3.2 Materials and Methods

3.2.1 Culture and maintenance of *B. flexus*

Bacteria were cultivated on Luria broth (Appendix 1) overnight. The cells were harvested by centrifugation (Eppendorf 5415D desktop centrifuge) at 1610 × g for 30 min. The harvested cells were washed with 10 mL phosphate buffered saline (Appendix 1) and then collected by centrifugation at 1610 × g for 30 min for three cycles. The cells were re-suspended in 5 mL of phosphate buffered saline (Appendix 1) and a 1 mL aliquot was used for each inoculation.

Growth curves were generated by inoculating bacteria in Luria broth and incubating overnight on a rotary shaker at 30 °C at 150 rpm. A typical example of bacterial growth is illustrated in Figure 3.1.

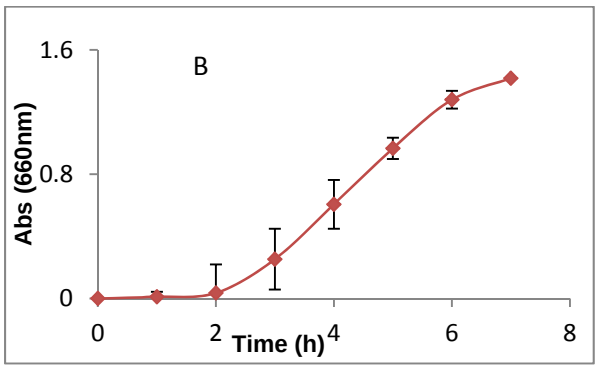


Figure 3.1 Growth of *B. flexus* in liquid culture. *B. flexus* cells were inoculated in 100 mL Luria broth and incubated for 7 h at 30 °C.

During the 2 h (hour) lag phase no discernible change in cell growth was observed. Exponential growth commenced between 2 and 4 h after culture initiation and was linear for approximately 4 h (Figure 3.1). Thereafter, growth slowed as cells appeared to enter a stationary phase. Increased growth rate means that the strain will produce essential protein in higher quantities due to the increase in cell density.

3.2.2 Preparation of coal and coal-containing media

Waste coal obtained from a coal dump in Witbank, South Africa was sterilized to exclude any bacterial activity by freeze thawing with liquid nitrogen for three cycles. Minimal media were sterilized by autoclaving at 121 °C for 15 min. Colonies were aseptically picked using a sterile tooth pick and inoculated into a nitrogen rich coal containing media (KH_2PO_4, 12.7 g; $NaNO_3$, 3 g; K_2HPO_4, 2 g; $MgSO_4.7H_2O$, 0.5 g; KCl, 0.5 g and 0.5 g of waste coal) prepared as described by Igbinigie *et al.* (2010). Controls were composed of nitrogen rich coal media excluding bacteria. Inoculums were incubated on a rotary shaker at 150 rpm in a constant environment (30 °C) for 14 days.

3.2.3 Characterization of the waste coal substrate and waste coal product

A pulverizer HP-M 100, HERZOG (Maschinenfabrik, GmbH Co., Osnabrück, Germany) was used to powder both waste and discard hard coal obtained from a coal mine in Witbank, South Africa to particle sizes of 0.5 mm which were stored at room temperature in a dark area. Elemental analysis was carried out using a viro micro cube V1.6.2 instrument. The elemental analysis was carried out in triplicate for S, N, and H composition for waste coal and discard hard coal. After 14 d of incubation of *B. flexus* in coal containing media, 14 mL of coal degraded liquid was centrifuged (Eppendorf 5415D desktop centrifuge) at 1610 × g for 30 min. The pellet was frozen in liquid nitrogen and placed in a freeze dryer for 24 h. The freeze dried pellet was then subjected to elemental analysis.

Coal samples (waste coal and discard hard coal) were dried in an oven at 50 °C to remove excess moisture. One (1) g was weighed into a pre-weighed crucible and placed in a muffle furnace

(Carbolite, Labotech) at 600 °C overnight. Ash content was calculated using the following equation.

$$\textbf{\% Dry ash} = \textbf{(final mass/initial mass)} \times \textbf{100}$$

A Perkin Elmer FT-IR Spectrometer (Spectrum 2000) was used to generate spectra of waste coal, discard hard coal, and coal derived humic acids and commercial humic acid (Sigma). Coal-derived humic acid was extracted using the alkaline extraction method.

3.2.3.1 Humic acid extraction

Coal samples (0.25 g) were added in flasks containing 100 mL of 0.1M NaOH and left to rotate at 150 rpm overnight. Minimal volume of concentrated HCl (32 %) was added in the solution until the pH was less than 1. The solution was allowed to stand for 1 h before it was centrifuged (Eppendorf 5415D desktop centrifuge) at 3220 × g for 90 min at 4 °C. The supernatant was discarded and the pellet was freeze dried at -70 °C over night in a freeze dryer (VirTis benchtop SLC).

3.2.4 Laccase assay

Laccase was assayed by a method modified from that described by Li *et al*. (2008). The reaction mixture consisted of 1.5 mL sodium acetate buffer (1 mM, pH 5.0), 1.5 mL 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid (ABTS) (Sigma) (0.5 mM) and 1.5 mL culture medium and the oxidation of ABTS monitored spectrophotometrically (ThermoSpectronic Aquamate v4.60) at 420 nm using a molar extinction coefficient of ε= 36 000 M^{-1} cm^{-1} at 30 °C. ABTS was prepared fresh for each assay by dissolving in distilled water and filter sterilization to eliminate un-dissolved particulates. The experiment was performed in triplicates and controls were without substrate. Enzyme activity was determined by the following equations:

$$\Delta A/\min = \frac{A(\text{final}) - A(\text{initial})}{\text{Time (final)} - \text{Time(initial)}} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots 1$$

$$\text{Laccase } \left(\frac{U}{L}\right) \frac{\frac{\Delta A}{min} \text{ x Total volume x 1000}}{\text{Coefficient x Path lenght x Sample volume}} \quad \text{.....2}$$

3.3 Results

3.3.1 Elemental analysis and ash content of coal substrate

Waste coal and hard coal were analyzed before contact with the microorganism. This was done to determine the compounds that make up the two coals. Coals are heterogeneous in nature therefore it was necessary that substrate analysis be carried out to assist in monitoring coal biodegradation more accurately. Elemental analysis provided the elemental percentages found in the coal substrate (Table 3.1). The percentages of S, C, N and hydrogen are shown in Table 3.1. Ash content represents the inorganic portion of the coal substrate and is presented in Table 3.1.

Table 3.1.Characterisation of waste and discard hard coal from Witbank, South Africa.

Coal	C	H	N	S	Ash (%)
Waste	47	2	1	ND*	7.2
Discard hard	67	4	2	0.5	33%

**ND-not detected*

As shown in Table 3.1 and as might be expected the amount of carbon in waste coal is lower than in discard hard coal. As expected sulphur, hydrogen and nitrogen are also lower in waste coal than in discard hard coal. The percentage difference in carbon between waste coal and discard hard coal is 20 %. For nitrogen and hydrogen the percentage difference is 2% and 1%, respectively. Discard hard coal therefore had higher amounts of the basic elements. Sulphur levels in waste coal were below detection limits. This indicated that the distribution of sulphur in the waste coal was very low. Ash content in waste coal was higher than in hard coal.

3.3.2 Fourier transform infra red spectroscopy of coal substrate

Fourier transform infrared (FT-IR) was used to determine the functional groups present in both waste and discard hard coal as seen in Figure 3.1 before bacterial attack. The peak intensities

reflect the changes in the functional groups of substrate post bacterial attack. Bacterial degradation of coal is reported to produce changes such as increase in oxygenated functional groups, for example, carboxylic, ethers and hydroxyl groups in the waste coal substrate.

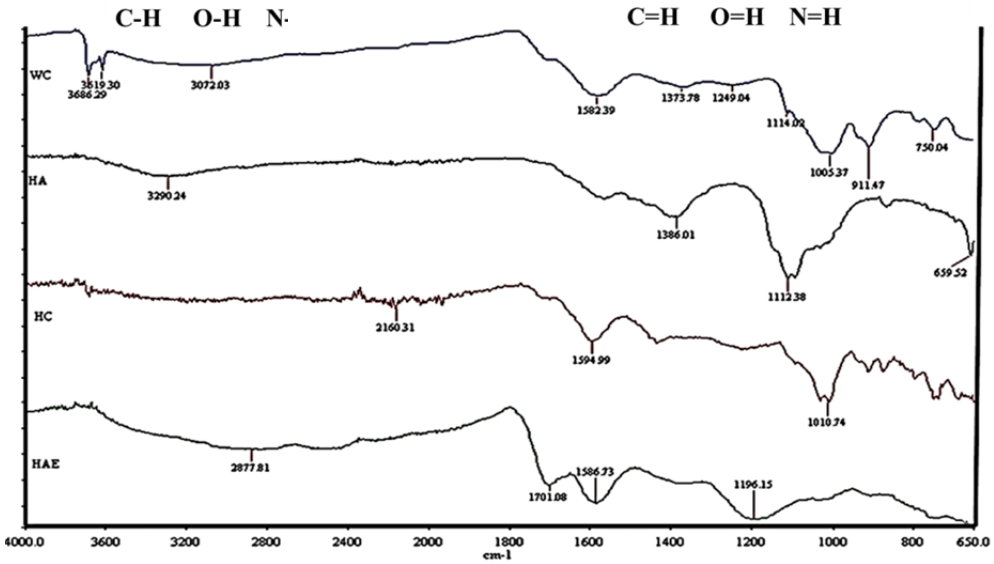


Figure 3.2 Fourier transform infrared spectra of commercial humic acid (HA), alkaline extracted humic acid (HAE), waste coal (WC) and discard hard coal (HC) without prior exposure to microbial activity.

FT-IR for waste coal, coal derived humic acid, discard hard coal and commercial humic acid is shown in Figure 3.2. The spectra for discard hard coal differed from that of waste coal. Bands from 3600 cm^{-1} to 2000 cm^{-1} showed the presence of carbonyl, hydroxyl, carboxyl and amide groups. These groups were absent in discard hard coal but present in waste coal (WC) confirming that the latter is more degraded. Commercial humic acid and coal derived humic acid presented similar spectra in that they share similar functional groups and their intensity differed. This result indicates the possibility that WC could serve as a humic-like substrate particle when exposed to coal degrading bacteria.

3.3.3 Elemental analysis of coal degraded product

B. flexus was inoculated into coal media and incubated for 14 d. A yellow–brown supernatant was formed and the residual pellet was extracted and analysed for elemental composition which is presented in Table 3.2. Functional group identities are shown in Figure 3.4.

Table 3.2 Elemental and chemical composition of original waste coal and biodegraded waste coal 14 d after inoculation with *B. flexus.*

Coal type	**Elements %**			
	C	H	N	S
WC	47	2	1	*ND*
WC-product	24.6	2.1	1.2	0.3

The results in Table 3.2 indicate a substantial reduction in carbon content of WC substrate which is most likely the result of bacterial action. There was little or no change in N, H and S content. Carbon content of the waste coal decreased from 47 % to 24.59 % after 14 d of bacterial attack. This means that *B. flexus* has the ability to extract carbon from a complex substrate such as waste coal in the absence of an alternative carbon source. In addition the supernatant contained material with a yellow to brown appearance indicating a colour change and that much of the degradation product was present in a soluble form. Thus coal had been biosolubilized in liquid media by B. *flexus*. Such soluble material derived from a WC substrate is usually indicative of humic-like substance formation. Measurement of humic- like substances is shown in Figure 3.3.

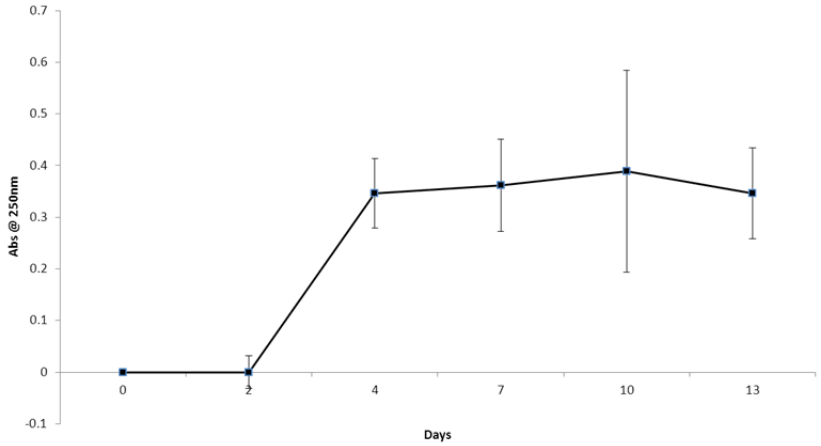


Figure 3.3 The production of humic like substances by *B. flexus* in a coal media (K_2HPO_4, KH_2PO_4, KCl, $NaNO_3$, $MgSO_4$, 0.5 g waste coal) the background was subtracted where a control was used with no bacteria present. Absorbance was determined at A_{250nm}.

As shown in Figure 3.3 humic-like substance production by *B. flexus* commenced 2 d after culture initiation, reached a maximum at 4 d and then remained constant for the remainder of the experiment. Humic acid-like substance accumulation was constant for approximately 8 d.

Thereafter, humic acid-like substance production slows and/or stops presumably due to utilization or metabolism of this newly formed material.

3.3.4 Fourier transform infrared spectroscopy of coal degraded product

The residual pellet or insoluble fraction remaining after bacterial action was precipitated by centrifugation washed in fresh media, freeze dried and analysed by FT – IR. The spectrum is shown in Figure 3.4

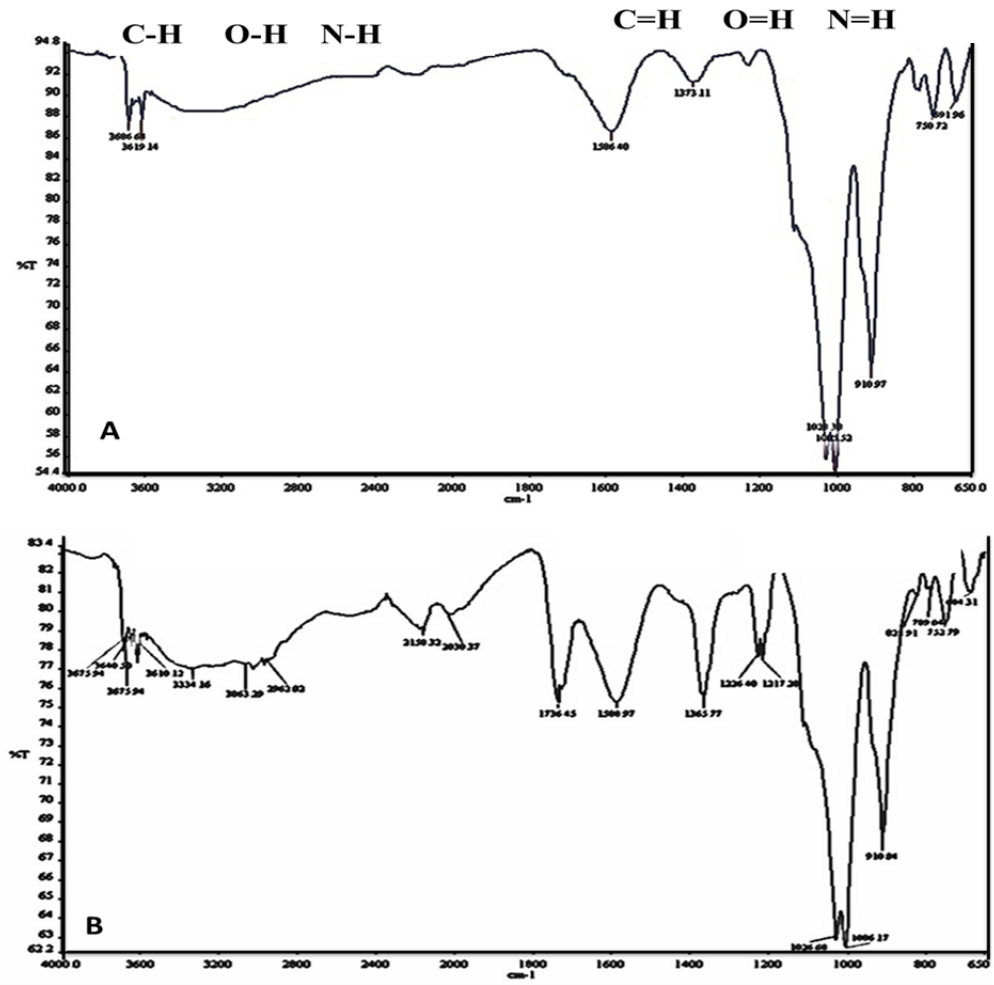


Figure 3.4 Fourier transform infrared spectra of biodegraded coal extract. Waste coal substrate prior to bacteria inoculation (A) Product after 14 d of exposure to *B. flexus* (B).

Analysis revealed that the residual insoluble substrate exhibited a typically highly oxidized coal with an increase in the number of bands and peaks associated with oxygenation, in the 1734 cm^{-1} region which represent double bonded compounds such as C=C,C=O and C=N (Figure 3.4B). Whereas bands in the region of 3600 cm^{-1} to 2500 cm^{-1} were increased and represent the presence of single bonded hydrogen groups such as OH, COOH, C-H and C-N. Aliphatic stretches were stronger in the degraded waste coal compared to humic acid and discard hard coal (Figure. 3.1). There were also stronger peaks compared to the control in the COOH and C=C region (Figure 3.4A). This confirms increased oxygenation of waste coal post bacterial degradation.

3.3.5 Laccase activity (LAC, E.C. 1.10.3.2)

Laccase is one of the principal ligninolytic enzymes implicated in coal biodegradation. The presence of laccase was investigated by collecting supernatant from cultures incubated with and without waste coal substrate for 7, 10, and 13 d after inoculation with *B. flexus*. Since laccase is produced extracellularly by ligninolytic bacteria it is expected that the enzyme be present in the media. The levels of laccase were determined spectrophotometrically at A_{420} (Table 3.3). Plate assays were also conducted according to the method described by Pointing (1999) (data not shown) where the presence of laccase is indicated by a change in color from opaque to green. When *B. flexus* was incubated in agar containing ABTS no color change was observed.

Table 3.3 Laccase activity in culture of *B. flexus* in coal containing media at days 7, 10, 13. Absorbance was taken at $A_{420nm.}$

Time	Enzyme activity (U/L)
Day 0	0
Day 7	0
Day 10	25×10^{-4}
Day 13	5×10^{-3}

As shown in Table 3.3 when the culture medium was incubated with ABTS there was no apparent indication of enzyme activity on day 0 and day 7. However, samples taken from day 10 and 13 cultures demonstrated a slight increase suggesting increased enzyme activity.

3.4 Discussion and Conclusion

Waste coal has more oxygen content in the periphery of the coal compound when compared to discard hard coal (Ibarra & Miranda 1996; Alvarez *et al.* 2003). Oxygen functional groups in low rank coals, for example, weathered coal, lignite, and waste coal, are in the form carboxylic groups, ethers and phenols (Tao *et al*. 2010; Moolick *et al*. 1989). Discard hard coal displayed the presence of C=C in the band region of 1600 cm^{-1} and there were also peaks in the aromatic region 1000 cm^{-1} to 650 cm^{-1}. This confirmed that the discard hard coal is more aromatic than waste coal. High rank coals typically have reduced oxygen containing compounds and in the instances where they contain oxygen it is normally in the form of quinones (Tao *et al*. 2010). As the rank increases there is a reduction in the oxygen containing functional groups. It is recognized that low rank coals and oxidized coals are more susceptible to degradation by the wood-rot fungus *Coriolus versicolor* and a range of other fungal species (Moolick *et al*.1989). These findings suggest that the vulnerability of coals to bio liquefaction/solubilisation decreases with an increase of rank. In this study discard hard coal ranks higher than waste coal and thus is expected to have higher aromaticity. The presence of aromatic compounds and the absence of oxygen rich regions in the discard hard coal suggest that is it less oxidized than waste coal and is a less preferred substrate for bacteria.

The current results for ash content analysis suggest that waste coal has less concentration of inorganic compounds than discard hard coal. The distribution of inorganic components assists in the coal - bacteria association (Boks *et al*. 2008) and coals with a low ash content are a conducive environment for bacterial attachment (Singh & Tripathi 2013). Close contact with coal suggests that the bacteria can easy access the carbon and make an efficient coal degradation system.

Sediments, soils, sedimentary rocks and interstitial waters contain more than 30 genera and 100 species of various bacteria, fungi and yeast which can utilise hydrocarbons as a sole source of energy in their metabolism (Hunt 1996). Abd-Elsalam and El-Hanafy (2009) reported three isolated bacteria from soil compost that have an ability to degrade lignin which is a coal precursor and constituent, namely *Azactobacter, B. megatarium* and *S. macescens*.

Microorganisms may either attack the carbonaceous coal matrix or the dispersed inorganic materials within the structure of coal (Basaran *et al*. 2003). The reduction in carbon presented by the elemental analysis could be attributed to the bacteria utilizing the coal as a carbon source. Carbon reduction also increases the carbon hydrogen ration which was used by Dong *et al*. (2006) to determine the aromaticity of the coal. Higher hydrogen carbon ratio depicts low aromaticity and high hydrogen carbon ratio depicts higher aromaticity.

Ultraviolet-visible (UV) spectroscopy analysis showed that the main components of bio-liquefied lignite (black liquid) were phenol derivatives, ketones and aldehydes (Yin *et al.* 2011). In addition to increase in coal product, *n*-Alkanes present in coal structure are oxidized to ketones, acids and organic acids (Yin *et al.* 2011). FT - IR shows an increase in aliphatic groups and a decrease in aromatic groups. There is an increase in carboxyl carbons and hydroxyl groups compared to under-graded coal. Increase in oxygen content also suggests an alteration in the initial coal material (Kai-yi *et al*. 2009). It has been established that treatment of low-ranked coals with aerobic coal solubilizing microorganisms results in the production of high molecular weight, polar, heterogeneous material with a relatively high oxygen content (Davison *et al*. 1990). Aliphatic stretches were stronger in biodegraded samples. This means that the presence of *Bacillus flexus* has chemically changed the structure of coal.

The presence of humic substances as products of coal degradation suggests that there is a breakdown of coal from high molecular weight compounds to lower molecular weight compounds. The absence of strong laccase activity in the culture media suggests that *B. flexus* uses an alternative strategy for degradation. Steffen *et al*. (2002) reported laccase activity to be 15 U/L on day 14 when *Collybia dryophila* was incubated in humic acid rich media. After 42 d laccase reached a maximum 40 U/L. Research by Singh *et al.* (2011) showed laccase activity to be 8U/L on day 14 of incubation in lignin media. These values are however 10 000 more active than laccase activity during bacterial degradation by *B. flexus*. To date there is no known evidence of extracellular laccase production in *B. flexus.* Bacteria have been reported to prefer the production of alkaline substances, chelators and surfactants for coal degradation (Yuan *et al*. 2006).

In conclusion, this chapter has shown that *B. flexus* possesses coal degrading capabilities by evidently degrading waste coal. In addition much of the residue remains soluble in the media which was transformed from a black to a brown-yellow like colour. Spectrometric analysis indicated that some of the material could be humic-like substance. Humic acids are known to support plant growth and are an integral component of the Fungcoal process which has been developed to facilitate rehabilitation of land post-mining.

CHAPTER 4

PLANT GROWTH REGULATION POTENTIAL OF COAL BIODEGRADATION PRODUCT

4.1 Introduction

Humic substances are found in composts, soil and inter-locked in coals (Piccolo *et al*. 1992; Prakash *et al*. 2010). These humic substances may be in forms of fulvic and humic acids and humins (Fong *et al*. 2006; Szczepanek & Wilczewski 2011). Fulvic acids are soluble in water under all pH conditions whereas humic acids are soluble in alkaline conditions and humin is the insoluble portion (Davies & Ghabbhour 2001; Farouk *et al*. 2011). Fulvic acids are lower in molecular weight than humic acids. The structure of humin is not yet elucidated (Davies & Ghabbour, 2001; Fong *et al*. 2006; Farouk *et al*. 2011). The colour of humic acids is black to brown; brown has been recorded as the colour for fulvic acids and humin (Fong *et al*. 2006). Fulvic and humic acids differ in molecular weights, carboxyl phenolic and hydroxyl groups as well as the level of polymerisation (Piccolo *et al*. 1993). Humic acids are mostly used for their documented benefits in plant growth (Davies & Ghabbour 2001).

Coal, especially low grade coal, is one of the richest sources of humic substances (Fong *et al*. 2006). Humic substances can be released as a by-product of the coal degradation process (Piccolo *et al*. 1993). Coal derived humic substances have also been reported in increasing concentration in weathered coal (Holker *et al*. 1997). Methods for extracting humic substances vary but chemical extractions have been predominantly used (Chen *et al*. 2006). However, recent studies have proved that biological extraction is also possible (Basaran *et al*. 2003; Fong *et al*. 2006; Prakash *et al*. 2010). The quality of produced humic substances greatly depends on the coal source (Gonzalez-Perez 2004; Erdogan *et al*. 2007). Humic substances play important roles in agriculture, especially in plant growth regulation. These substances influence the physical, chemical and biological properties of soil. This indirectly contributes in plant growth (Davies & Ghabbour 2001). Plant responses to influences of humic substances depend on their chemical composition and molecular weight (Farouk *et al*. 2011). Humic substances, with high ash

content, little humic acid content and less oxygen functional groups, are not ideal for agricultural purposes (Gonzalez-Perez 2004).

This study investigated the bioavailability of coal biodegraded products formed by the action of *Bacillus flexus* using the radish (*Raphanus sativus*) cotyledon bioassay which exploits cotyledon expansion growth as a measure of plant growth regulation or activity.

4.2 Materials and methods

4.2.1 Preparation and screening of coal degradation by-product

B. flexus was incubated in waste coal media (see Chapter 2) for 14 days after which the coal media was centrifuged at 17950 × g for 30 min. The pellet was discarded and the supernatant termed biodegraded product (BP) further diluted using deionised water. One litre of deionised water was placed into two volumetric flasks and 10 mL/L (v/v) and 1 mL/L (v/v) of BP was added respectively. The two preparations were subsequently used to determine optimal BP concentration for use in the radish cotyledon seed bioassay.

4.2.2 Radish cotyledon expansion bioassay

Method was performed as described in Hong *et al*. (2009). Radish seeds (*Raphanus sativus* L. cv. Cherry Belle) were surface sterilized by gentle shaking in 50 mL of 0.5 % sodium hyperchlorite for 10 min. After extensive washing with deionised water, 0.75 g seed was added to sterile Petri dishes containing Whatman 2 filter paper and 8 mL of deionised water. The Petri dishes were then covered in foil and incubated at 25 °C for 48 h (hours).

After 48 h Hoagland's solution (4 mL) (Appendix 1) was prepared and placed in an empty Petri dish containing Whatman 2 filter paper. Hoagland's solution was used as a nutrient base in all the treatments. Furthermore, four treatments were prepared from which three were controls: deionized water (D water) (control), 10 mM potassium hydroxide (KOH) (negative control), 300 mg/L humic acid (HA) (positive control) and, BP. Two days after germination the smaller of the

two cotyledons was excised from germinated seeds, pre-weighed and placed into prepared Petri dishes containing 4 mL of Hoagland's solution and treatments. Cotyledons in Hoagland's solution and treatments were incubated at 25°C and final weight was determined after 72 h. Effect of the treatments was determined by calculation of percentage difference according to the equation below:

$$\frac{\textbf{final weight} - \textbf{initial weight}}{\textbf{intial weight}} \times \textbf{100} = \%\,\textbf{difference}$$

4.2.4 Statistical analysis

Data from all treatments were subject to the Shapiro test to test for normal distribution. Means of all the treatments were compared using Welch's t-test at $p < 0.005$. Statistical analysis was conducted using the R software package (Faraway 2005).

4.3 Results

Several lines of evidence indicate that products of coal biosolubilisation serve to support plant growth. In the present study *B. flexus* was shown to solubilise waste coal and the degradation products accumulated in the culture medium were tentatively identified as humics (Chapter 3). To determine the effect of these on plant growth, the effect of the coal BP was compared to that of commercial humic acid (Fluka) using the radish cotyledon bioassay. Figure 4.1 illustrates the effect of different concentrations of BP on radish cotyledon growth. Clearly, at a concentration of 10 mL/L both de-etiolation, namely, chlorophyll synthesis and chloroplast differentiation, and growth of the cotyledons was inhibited suggesting that at this concentration BP negatively impacted plant growth. At 1 mL/L however, de-etiolation and expansion growth proceeded and as a consequence this concentration was used for all further experiments.

Hoagland's solution was used both as a nutrient supplement and a baseline in all the treatments. Deionised water, potassium hydroxide and humic acid were controls. Humic acid functioned as a positive control because of the well documented effect of humic acid on plant growth promotion.

Figure 4.3 depicts the already expected positive growth of radish cotyledons in humic acid. Alkaline substances are usually produced by bacteria during coal degradation (Yuan *et al.* 2006; Sekhohola *et al*. 2013) and were used in this experiment to monitor the effect of alkaline conditions on radish cotyledon growth. Alkaline conditions have been documented to inhibit the growth of the seedlings (Lin *et al*. 2012), as such, the alkaline solution also served as a negative control. KOH demonstrated poor expansion of radish growth as expected and when compared to other treatment conditions.

4.3.1 Growth of radish cotyledons in coal degradation by product

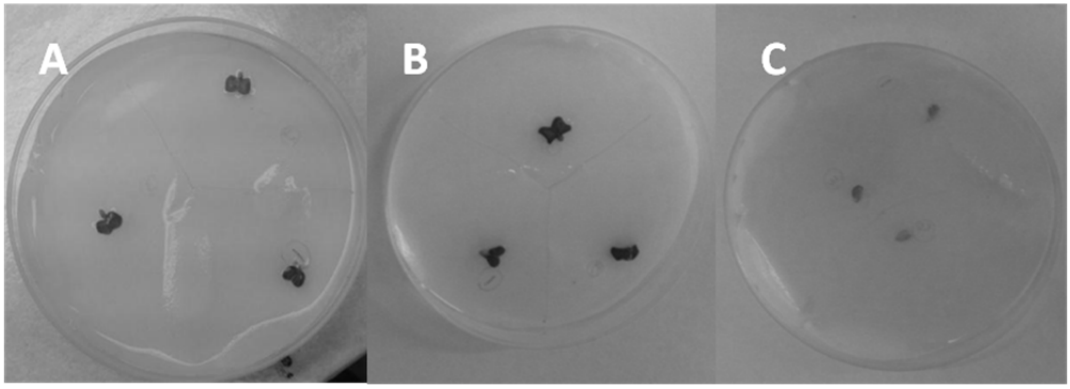


Figure 4.1 Three day old cotyledons treated with the soluble coal biodegradation products at (A) 0 mL/L (B) 1 mL/L (v/v) and (C) 10 mL/L (v/v) concentration**.**

A visual observation of the effect of different concentrations of humic substances was carried out to determine optimal humic acid concentration for radish cotyledon expansion growth. Growth in 10 mL/L BP was stunted with no visible increase in cotyledon width and height. Radish cotyledons have pale yellowish-white coloration and no visible expansion growth was observed. Cotyledons in the 1 mL/L solution of BP showed a positive response to BP treatment. The radish cotyledon in B showed expansion growth and a visible increase in cotyledon width and height. The cotyledons show a green colouring over time, namely de-etiolation has occurred. In light of the observed results 1mL/L concentration was used in the bioassay for further tests.

4.3.2 Radish cotyledon expansion bioassay

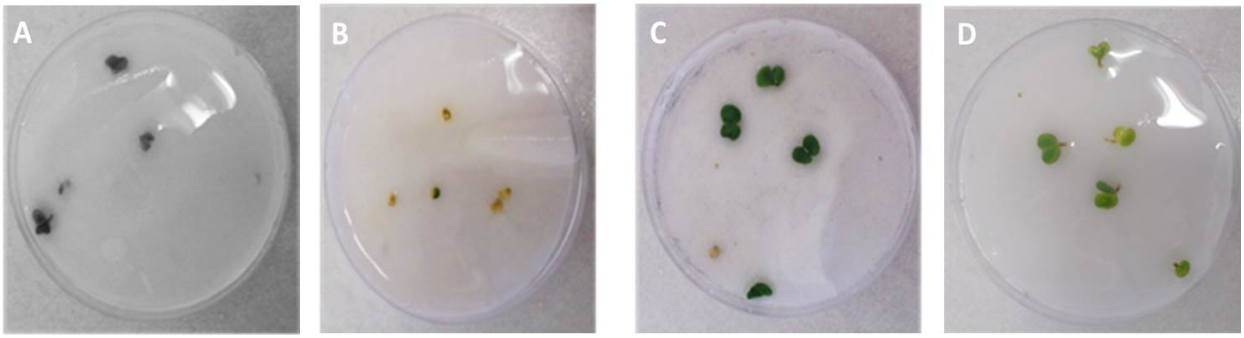


Figure 4.2. Radish cotyledon expansion after 72 h in (A) deionised water (Dwater), (B) 0.1 M Potassium hydroxide (KOH), (C) commercial humic acid (300 mg/L) (HA) and (D) soluble coal biodegradation product (BP).

As shown in Figure 4.2 radish cotyledons exhibited different responses to KOH, BP, HA and D water. Figure 4.2B shows that in KOH only 20% of the radish cotyledons manage to survive. Alkaline conditions had a negative impact on the development of cotyledon expansion. The cotyledon had a pale yellowish colouration with no visible increase in cotyledon width and height. In commercial HA 80 % of cotyledons survived and showed positive growth responses (Figure 4.2C). The cotyledons had a green colouring with a visible increase in width and height. There was 100 % survival of cotyledons in the BP treatment (Figure 4.2D). The cotyledons had a green colouring with a visible increase in width and height. The cotyledons response improved in BP compared to all the other treatments with 100 % survival and KOH had the worse response at 20% survival. De-etiolation occurred in all the treatments where there was greening of the cotyledon except in the KOH treatment where etiolation occurred. Figure 4.3 further shows the changes in fresh weight of cotyledons after 72 d of incubation.

4.3.3 Effect of coal biodegradation product on the weight of radish cotyledons

As shown in Figure 4.3 there is an increase in fresh weight across all the treatments, namely KOH, HA, BP and Dwater at 22 %, 115 %, 140 % and 93 % , respectively. A t-test comparing the treatments to the Dwater was performed. HA showed no significant difference against Dwater the p =0.88 which was above level of significance therefore cotyledon growth in Dwater and HA is the same KOH and BP showed a significant difference against Dwater with p =0.0078 and 0.01 respectively.

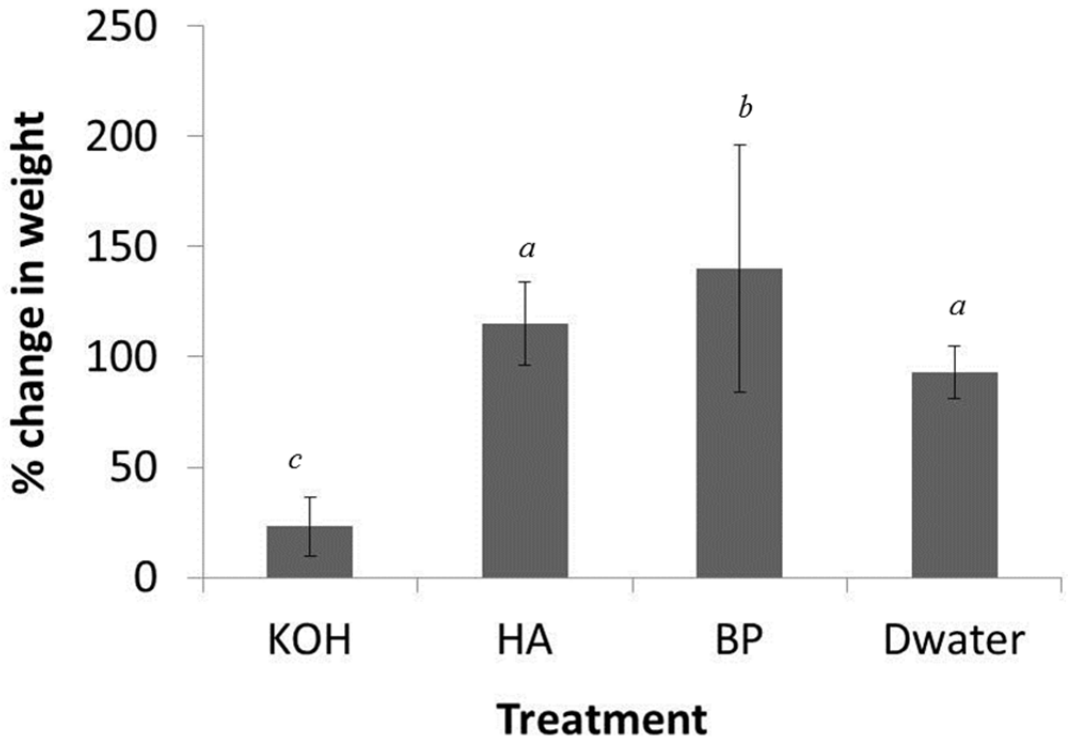


Figure 4.3 Effect of alkaline and acid substances and soluble coal biodegradation product on expansion growth of excised radish cotyledons. *a*- no significant difference, *b*- significantly different and *c*- significantly different.

4.4 Discussion and Conclusion

Lignite and waste from the coal industry are promising sources for biologically active compounds: humic acids and fulvic acid, for example (Piccolo *et al*. 1992). Humic substances as a plant growth regulator compounds are well documented (Atiyeh *et al*. 2002) and commercial products as a result are widely available. Humic substances contain up to 65% organic matter and have long been recognized as having a positive influence on plant growth regulation (Cacco & Dell'Agnola 1984; Canellas *et al*. 2002; Paksoy *et al*. 2010). Biodegradation of coal has largely been marked by the production of humic substances as degradation products (Goa *et al*. 2012). Radish bioassay was designed to test the bioavailability of coal derived humic substances produced during coal degradation by *B. flexus* where the changes in radish cotyledon was observed over 72 h. Radish cotyledons were allowed to grow in media with coal derived humic substances, potassium hydroxides, commercial humic acid and distilled water. Figure 4.1 shows that 1 mL/L had a positive effect on radish cotyledon expansion growth demonstrated by cotyledon greening and a visible increase in width and height. Higher concentrations had a

negative impact on the growth of cotyledons. Atiyeh *et al*. (2002) achieved similar results where they observed increasing concentrations of humic acid plant growth decreased significantly. Similarly Figure 4.1 shows that when 10 mL/L of coal biodegradation product was utilized in radish cotyledons no visible increase in greening, width and height was observed. Therefore, humic acid concentration affects the plant growth significantly.

Radish cotyledon in BP demonstrated the highest fresh weight increase over time with 140 % weight change. This is due to the ability of humic substances in penetrating the plasmalemma of the plant cells thus regulating processes that influence plant growth (Pinton *et al*. 1999). Carboxylic and hydroxyl groups of humic substances are believed to facilitate this process (Piccolo *et al*. 1992; Piccolo *et al*. 1997; Trevisan *et al*. 2010). Therefore, more oxygenated humic substances are able to penetrate the cell plasmalemma. In a study conducted by Piccolo (2002) an increase in cotyledon fresh weight indicated that fractions of coal derived humic substances with a low molecular weight and a high content of acidic functional groups generally were responsible for promoting cell elongation compared to the control without humic substances. They also observed that fractions with a high molecular weight did not behave differently from the control.

Addition of humic acid in the growth of radish seeds was reported to act as a chelator by Farouka *et al*. (2011). In their study radish seeds were exposed to high concentrations of cadmium and those that grew in the presence of humic acid showed an improved growth compared to the control where no humic substances were added (Farouka *et al*. 2011). Humic acids can therefore play an important role in plant establishment in heavy metal contaminated sites. Land with high concentrations of heavy metals is a typical environmental condition found in coal dumps. The presence of humic acid therefore serves as a protective measure to the plants from heavy metal uptake which results in under development of plants.

This study indicated that the addition of coal derived humic substances improved the growth of radish cotyledons when compared to the control. Alkaline conditions did not have a positive effect on the growth when compared to the control.

CHAPTER 5

GENERAL DISCUSSION AND CONCLUSIONS

5.1 General discussion

Microorganisms, specifically bacteria, have been documented to survive in harsh conditions (Prenafeta-Bold *et al*. 2004). They possess the necessary mechanisms that enable them to grow in toxic, disturbed environments using the contaminant/pollutant as a substrate for cell metabolism (Khan *et al*. 2013). Contaminated land or extreme conditions are boundless sources of bacteria that have inimitable genes that can breakdown recalcitrant material (El-Deeb 2009). Bacteria that have the ability to degrade oil and thus potentially act to remediate the area, have been isolated in oil spills (El-Deeb 2009; Das & Chandran 2011). In heavy metal contaminated sites bacteria have also been used to reduce the toxic levels to normal levels for animal, plants and humans (Suhadolc *et al*. 2004).

In this study, environmental samples obtained from diesel contaminated sites were collected and screened for bacteria that were able to withstand harsh conditions for growth in a waste coal substrate. Bacterial screening resulted in 120 isolates of which only three were confirmed to show coal degradation potential. Only one was further investigated for use as a biocatalyst for coal degradation. This isolate produced a yellow to brown supernatant after 14 days of incubation when waste coal was supplied as the only carbon source. Molecular identification was performed using 16S rDNA which was amplified, sequenced and the BLAST results showed the strain to have high sequence similarity with that of *Bacillus flexus* with a 99 % percentage similarity. This indicated that the isolated strain was phylogenetically related to *B. flexus*. Bacterial characterisation studies showed that the isolated bacterium was rod shaped and gram positive. Kanna *et al*. (2009) viewed similar description of *B. flexus* as a rod shaped and gram positive bacterium when identification studies were conducted on a bacterium isolated from mangrove soils. *B. flexus* is reported to be an alkaliphile that is largely found in saline and alkaline environments (Sivaprakasm *et al*. 2008; Tambekar and Dhundale 2012). It produces active enzyme under alkaline conditions, two or more units above neutrality (Sanchez-Gonzalez

et al. 2011; Tambekar & Dhundale 2012). Mulla *et al*. (2011) reported a novel 2-dioxygenative pathway that was discovered to be utilized by *B. flexus* when exposed to nitroaromatic rich media. A 2, 3-dioxygenase enzyme enabled *B. flexus* to degrade nitroaromatics at higher concentrations when compared to other gram positive and gram negative bacteria. *B. flexus* has also been reported to possess hydrolysing enzymes such as cell bound phenolic acid esterase and cellulases that have the ability to hydrolyse complex carbon compounds (Sanchez-Gonzalez *et al*. 2011; Trivedi *et al*. 2011; Perez *et al*. 2002). Esterases are one of the few hydrolytic enzymes that are believed to play a role in coal degradation (Gupta *et al*. 1988; Fakoussa 1981; Sugoro *et al*. 2012). The involvement of cellulase has not previously been reported but can be assumed since much of the coal parent material comes from plants which are high in cellulose (Fakoussa & Hofrichter 1999). It can be suggested that cellulase could be one of the enzymes, among others, that is viewed as supporting hydrolytic enzyme activities. These enzymes aid and play a secondary role in the process of coal degradation. On the other hand *B. flexus* has been reported to be able to bioremediate waste water from maize processing factories, a substrate rich in complex carbohydrate (Sanchez-Gonzalez *et al*. 2011). Maize waste water contains plant based recalcitrant material high in complex carbon compounds. The presence of *B. flexus* in extreme environments makes it a good candidate for coal degradation. There have not been any reports on this strain possessing any coal degrading abilities. However, certain *Bacillus* species have been reported to be able to degrade coal as pure strains and in a mixed culture (Jiang *et al*. 2013). A closely related bacterium to *B. flexus* is *B. megatarium* which has many overlapping sequences within its genomes (Berrada *et al*. 2011). *B. megatarium* has lignin degrading capabilities which function by decolourisation and/or solubilisation (Morii *et al*. 1995). It is also known to be a plant growth promoting rhizobacteria (Zou *et al*. 2011). These bacteria are able to stimulate plant growth in association with root systems. *B. megatarium* has the ability to remarkably promote plant growth of *Aradiposis* by producing a plant growth promoter compound 2-pentylfuran (Zou *et al*. 2010). Jiang *et al.* (2013) isolated a bacillus strain that was identified as *Bacillus cereus* ATCC 14579. Maka *et al.* (1989) also recorded the production of alkaline solubilizing substance by *Bacillus cereus, Bacillus plumis* and *Bacillus subtilus* when inoculated in media with coal.

When hard coal was used as the sole carbon source in the presence of bacteria the production of a yellow-brown supernatant was typically observed and taken to indicate utilization of carbon

present in coal (Fakoussa 1981). The yellow-brown colour was attributed to the presence of humic-like substances (Hofrichter & Fritsche 1996). The presence of humic-like substances has been reported as one of the major indicators of the ability of bacteria to degrade coal. Screening studies described in Chapter 2 demonstrated the production of a yellow-brown supernatant that resembles what is typically reported as being produced in the process of coal degradation. Isolated bacteria from environmental samples that showed similar results were therefore assumed to possess coal degrading capabilities. The presence of humic substances is also an indication of oxygenation of the coal particles (Quigley *et al*. 1988). Oxygenated coal particles are known to be more biodegradable than hard coal which has a more condensed structure compared to oxygenated coal (Ralph & Catcheside 1997; Machnikowska *et al*. 2002). The availability of oxygenated coal therefore encourages further biosolubilisation which appears to have been the case in this study (Hayatsu *et al*. 1979; Fakoussa & Hofrichter 1999).

Bacteria were incubated in coal media for 14 days and waste coal concentration in the media was 1 %. These growth conditions were similar to those that were used by Jiang *et al.* (2013) where lignite concentration and incubation period was discovered to play a major role in the degree of biosolubilisation. In their study they discovered that there was a substantial growth of *Bacillus sp*. observed within 12 days in low lignite concentrations (Jiang *et al*. 2013). Bacterial strain *Pseudomonas putida* was reported to degrade lignite in 14 days (Machnikowska *et al*. 2002.). Maka *et al*. (1989) reported that a mixed bacterial culture (including *Bacillus cereus*, *Bacillus pumilus* and *Bacillus subtilis*) could solubilize lignite when incubated for two weeks. Similar trends were observed in a study where coal biosolubilisation was facilitated by the action of *Trichoderma sp*. which is one of the most effective biosolubilisation fungal species (Holker *et al*. 1997). In other cases pre-treated coal was used and this greatly influenced biosolubilisation. In all other mentioned cases no pre-treatment was performed. Any changes that are observed on the coal structure are therefore due to the action of the bacterial/fungal strains.

The production of alkaline substances and chelating agents has been largely discovered to be responsible for bacterial coal degradation (Strandberg & Lewis 1987; Acten *et al.* 2011). This however does not rule out the presence of enzyme involvement even though they have been reported to play a minor role in bacterial degradation of coal. In the presence of ABTS solid

media *B. flexus* did not demonstrate production of extracellular laccase. The presence of laccase can be confirmed by a change in colour of the media from opaque to green. This is due to the release of radicals in the media that emit a green colour. Furthermore, when spectrophotometric tests were done where coal media supernatant was incubated with ABTS there was no change in colour and thus no increase in enzyme activity.

Humic substances make up a large organic component of soil (Trevisan *et al*. 2010). They contribute to the soil fertility and prevent the soil from degradation (Trevisan *et al*. 2010). Humic substances from soil and low rank coals are normally extracted by using a strong alkali which in most cases is either sodium hydroxide or potassium hydroxide (Veeken *et al*. 2000). Fakoussa (1981) reported the ability of microorganisms, namely bacteria such as *Rhodococuss*, *Bacillus* and *Pseudomonas*, to extract humic substances from low rank coals. Maka *et al*. (1989) reported that a mixed bacterial culture (including *Bacillus cereus*, *Bacillus pumilus* and *Bacillus subtilis*) could solubilize lignite when incubated for two weeks. Machnikowska *et al*. (2002) reported that *Pseudomonas putida* could solubilize 11 % of a Polish lignite sample's K and 25 % of the sample's S in two weeks and that the pH gradually increased as the process progressed. Humic substances are widely known to have an auxin like effect on seedling germination and plant growth development in higher plants (Trevisan *et al*. 2010; Rzepka-Plevnes *et al*. 2011). In addition it is well known that expansion of radish cotyledons is largely influenced by cell expansion hormones, namely, auxin and gibberellin (Thomas *et al*. 1981). The presence of humic substances increases the hormone effect on the cotyledons and thus assists in cotyledon expansion. It has been documented that plant growth can be stimulated by low concentrations of humic substances (Prakash *et al*. 2010). These humic substances are said to influence the availability to nutrients that would otherwise not be readily available to the plant (Farouk *et al*. 2011). It increases the transport system by affecting the production of proteins involved in the transportation of ions (Vaughan & MacDonald 1971). The increase in nutrient uptake by plants in the presence of humic substances therefore promotes growth of healthier individuals compared to those that are not exposed to humic substances. In a study by Piccolo *et al*. (1993) it was shown that increases in germination percentage and germination rate were typical when tomato and lettuce seeds were treated with coal derived humic substances. After 72 hours of growth in humic substances maize roots were able to develop lateral roots in the primary root region. Those

that grew in a nutrient solution had no lateral root development (Piccolo *et al*. 1993). Growth promotion potential of coal derived humic substances can therefore be an advantage in the rehabilitation of coal mine dumps by inducing plant growth and increasing plant cover of the dumps by serving as a biostimulant.

5.1 Research conclusions

- Hydrocarbon contaminated sites and extreme environments possess unique bacteria that are well adapted to expand in the absence of a simpler carbon source.
- These bacteria can be used as biocatalysts in degrading and utilizing recalcitrant material.
- *B. flexus* was isolated from a diesel contaminated site.
- *B. flexus* was shown to have coal degrading capabilities by producing yellow – brown supernatant post incubation in coal media in the absence of a simpler carbon source.
- Byproducts of the bacterial degradation were oxygenated and less aromatic in structure when analyzed using FT-IR.
- Elemental analysis showed that there was a reduction in weight percentage of carbon after bacterial degradation.
- There was an increase in humic-like substances in the supernatant over time.
- Biologically derived humic-like substances significantly improved the growth of radish cotyledons
- Degradation products were therefore bioavailable.
- *B. flexus* is a good bacterial candidate to be included in the Fungcoal process.

5.2 Applications

Fungcoal process was establish in 2007(ZA 2007/07607) and involves the application of *N. fischeri*, a rhizospheric coal degrading fungi, and *C. dactylon*, a perennial grass that was observed to grow sporadically on coal mine dumps. The process has been applied commercially to coal mine dumps in Witbank to reduce the use of top soil which has proven to be expensive to transport and which causes more damage in the areas in which top soil is excavated. This makes

rehabilitation a challenge to mining companies. Processes like Fungcoal are therefore ideal to mitigate some of the challenges posed by current rehabilitation strategies.

Bacteria form an important part of the rhizosphere in plants because they contribute to the symbiotic relationships present in the rhizosphere. Rhizospheric bacteria produce plant growth stimulating compounds. Increased soil/substrate fertility in the rhizosphere also improves nutrient availability to the plant and improves the growth of the plant. The addition of a soil bacterium isolated from diesel contaminated soils might prove to be a valuable contribution to the already existing Fungcoal process. *B. flexus* should increase microflora in the rhizosphere contributing to the symbiotic relationships in the rhizosphere. In agricultural practises plant growth promoting bacteria are incorporated in seed mixes to improve survival. *B. flexus* can also be included in the seed coating to create a better contact and an efficient symbiotic relationship with the plant.

The production of humic acid by *B. flexus* and its potential use in land rehabilitation strategies can also address the current problem reported in the recently published *The Soil Atlas of Africa* (FAO, 2013). After a five year research project African soils were found to be gradually degrading due to erosion and nutrient depletion. This poses a threat to the agricultural sector and subsequently affects the livelihood of most African households and farmers who depend mostly on soil for produce and sustenance. The rehabilitation of land previously used for mining not only increases available arable land but provides a substrate for use as a soil amendment to restore soil organic carbon content in regions severely affected by erosion and nutrient depletion. In other words, the use of biologically based soil conditioning products and/or strategies.

5.3 Future study

Intensive work has been implemented to expand the Fungcoal process and one of the recommendations is to advance the inoculum for an effective degradative formula. The improved Fungcoal which includes bacteria will be applied for a better rehabilitation results in the dumps. Further research investigating the following can be attempted with *B. flexus*:

- Laboratory scale co-habitation studies could be done to investigate the ability of *N. fischeri* and *B. flexus* in the degradation of the coal substrate.
- Further studies could be conducted to establish the efficiency of *B. flexus* in degrading hard coal a more recalcitrant coal substrate.
- A genome analysis of *B. flexus* could be undertaken to confirm the genes that code for coal degrading agents.
- An investigation and analysis of coal degradation proteins and related degradative agents in *B. flexus* could be done.
- Coal degrading mechanism of *B. flexus* could be determined.

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APPENDIX 1

Growth media preparations

Luria Broth (LB)
5g Tryptone

2.5g Yeast Extract

2.5g NaCl

Make up to 1L with dH_2O. Sterilize with autoclaving at 12℉C for 15min.

Nutrient agar

Make up to 1L with dH_2O. Sterilize with autoclaving at 12℉C for 15min

Malt extract agar

Make up to 1L with dH_2O. Sterilize with autoclaving at 12℉C for 15min.

Potatoes dextrose agar

Make up to 1L with dH_2O. Sterilize with autoclaving at 12℉C for 15min.

MacConkey agars

Make up to 1L with dH_2O. Sterilize with autoclaving at 12℉C for 15min.

80 % Glycerol stocks
8 mL dH_2O
10 mL glycerol

Phosphate buffer saline (pH 7.4)

Compound	g/L
NaCl	8.01
KCl	0.20
$Na_2HPO_4.2H_20$	1.78
KH_2PO_4	0.27

Hoaglands solutions

Hoagland's solutions was prepared by making up 1M solutions of macronutrients as follows: (1) $NH_4H_2PO_4$ (80 g/L), (2) KNO_3(101 g/L) ,(3) $Ca(NO3)_2$ (136 g/L),(4) $MgSO_4$ (393 g/L). Micronutrient were also prepared in different concentrations as follows: H_3BO_3 (2.86 g/L), $MnCl_2$.$4H_2O$ (1.81 g/L), $ZnSO_4$.$7H_2$ O (0.22 g/L), $CuSO_4$. 5H2O (0.08 g/L), H_2MoO_4. H_2O (0.02 g/L) (5). Iron stock (6) was prepared by adding 26.1 g EDTA and dissolved in 286 ml distilled water with approximately 19 g KOH. Then 24.9g $FeSO_4.7H_2O$ was dissolved in 500 ml water and was slowly added to the potassium EDTA solution and left on the magnetic stirrer until pH reaches 7. Macronutrient were combined in the following amounts (1) 1 mL, (2) 6 mL (3) 4 mL, (4) 2 mL, an aliquot of 1 mL was taken from solution (5) and solution (6). The salt solutions were combined and made up to a total of 1 L of nutrient solution.

APPENDIX 2

Electron Microscopy

Phosphate buffer

Solution A 35.8 g/L $Na_2HPO_4.2H_2O$

Solution B 13.6 g/L KH_2PO_4

To achieve a molarity of 0.1 M and a pH 7.3 these solutions are mixed in the ratio 4 parts.

Solution A: 1 part Solution B.

Buffered glutaraldehyde

10 mL 25 % ultrastructure grade glutaraldehyde

90 mL Phosphate buffer

This solution is stored in a dark bottle at 4° C.

1 % Buffered Osmium Tetroxide

Solution 1:2.55 % $Na_2HPO_4.2H_2O$

Solution 2:2.52 % NaOH

Solution 3:5.4 % glucose

Solution 4:41.5 mL Solution 1

8.5 mL Solution 2

Fixative consists of 45 mL Solution 4, 5 mL Solution 3 and 0.5 g OsO_4. Dissolve the osmium tetroxide crystals in Solution 4 by warming in the fume hood. Cool and decant into a clean flask, add Solution 3 and pH to 7.3 with 0.1 M HCl. Store in a dark bottle at 4 ° C. (Cross 2001).

APPENDIX 3

DNA extractions and agarose gel electrophoresis.

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

0.61 g Tris (hydroxymethyl) aminomethane

0.093 g EDTA

Add 400 mL of dddH2O and pH to 8, then make up to 500 mL volume with dddH2O.

Sterilize by autoclaving.

10 % SDS

10 g SDS

100 mL dddH2O

Add 10 g of SDS to 100 mL of $dddH_20$ Warm to 65° C to allow SDS to dissolve.

10 % CTAB

10 g Cetyltrimethylammonium bromide

100 mL $dddH_2O$

Add 10 g of CTAB to 100 mL of $dddH_2O$ .Warm to 65° C to allow CTAB to dissolve.

5 M NaCl

292 g NaCl

1 L $dddH_2O$

Autoclave to sterilize.

Chloroform: Isoamyl alcohol (24:1, v/v) (100 ml)

96 mL chloroform

4 mL isoamyl alcohol

10 × TBE buffer (1 L)

107.8 g Tris base

55 g Boric Acid

7.44 g di-sodium EDTA

Make up to 800 mL with ddd H_2O and pH to 8.3 with boric acid. Make up to 1 L and autoclave. Dilute 1 in 10 for 1 × TBE.

Ethidium bromide (500 mg/mL)

Dissolve 0.5 g of ethidium bromide powder in 1mL of dddH2O, 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 × buffer H and 10 µL of *Pst1* enzyme for 3 hours at 37º C. Add 550 µL TE buffer (pH 8) and 150 µL 6 × loading buffer. Aliquot 100 µL into Eppendorfs and store at -20° C.

Primers

907R 5'-cgc ccg ccg cgc ccc gcg gtc ccg ccg ccc ccg ccc gcc gtc aat tcc ttt gag ttt-'3

GM5F 5'-cct acg gga ggc agc ag-'3

Both primers were manufactured by Integrated DNA technology, Iowa, USA.

10 µM dNTP`S

30 µL of each nucleotide (dATP, dTTP, dCTP & dGTP) is added into an eppendorf. 180 µL of dddH2O is added, bringing the total volume to 300 µL. Aliquot into eppendorfs (60 µL volumes) and store at –20º C.

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

APPENDIX 4

DNA sequencing

SF 41

GGTMGGAGTCTGACGGAGCACGCCGCGTGAGTGATGAAGGCTTTCGGGTCGTAA
AACTCTGTTGTTAGGGAAGAACAAGTACAAGAGTAACTGCTTGTACCTTGACGGT
ACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAG
GTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGTTTCTTA
AGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGGA
ACTTGAGTGCAGAAGAGAAAAGCGGAATTCCACGTGTAGCGGTGAAATGCGTAG
AGATGTGGAGGAACACCAGTGGCGAAGGCGGCTTTTTGGTCTGTAACTGACGCT
GAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCC
GTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTGCTGCAGCTAA
CGCATTAAGCACTCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAA
TTGACGGCGGGCGGGGCSGGGGR

GE37

CGGGGMSAAGYSTGACGGAGCRCGCCGCGTGAGTGATGAAGGCTTTCGGGTCGT
AAAACTCTGTTGTTMGGGAAGAACAAGTACAAGAGTAACTGCTTGTACCTTGAC
GGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACG
TAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGTTT
CTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTG
GGGAACTTGAGTGCAGAAGAGAAAAGCGGAATTCCACGTGTAGCGGTGAAATGC
GTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGGCTTTTTGGTCTGTAACTGA
CGCTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCA
CGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTGCTGCAG
CTAACGCATTAAGCACTCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAA
GGAATTGACGGCGGGCGGGGSSGG

BLAST

SF 41

Sequences producing significant alignments:						
Accession	Description	Max score	Total score	Query coverage	E value	Max ident
NR_024691.1	Bacillus flexus strain IFO15715 16S ribosomal RNA, partial sequence	843	843	98%	0.0	99%
NR_043401.1	Bacillus megaterium strain IAM 13418 16S ribosomal RNA, partial sequence	843	843	98%	0.0	99%

GE37

Sequences producing significant alignments:						
Accession	Description	Max score	Total score	Query coverage	E value	Max ident
NR_024691.1	Bacillus flexus strain IFO15715 16S ribosomal RNA, partial sequence	736	736	100%	0.0	99%
NR_043401.1	Bacillus megaterium strain IAM 13418 16S ribosomal RNA, partial sequence	736	736	100%	0.0	99%