THE RHIZOSPHERE AS A BIOPROCESS ENVIRONMENT FOR THE BIOCONVERSION OF HARD COAL

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ABSTRACT

Fundamental processes involved in the microbial degradation of coal and its derivatives have been well investigated and documented over the past two decades. However, limited progress in industrial application has been identified as bottleneck in further active development of the field. The sporadic and unanticipated growth of *Cynodon dactylon* (Bermuda grass) has been observed on the surface of some coal dumps in the Witbank coal mining area of South Africa. Preliminary investigations showed the formation of a humic soil-like material from the breakdown of hard coal in the root zone of these plants. The potential of this system to contribute to industrial scale bioprocessing of hard coal was investigated.

This study involved an investigation of the *C. dactylon*/coal rhizosphere environment and demonstrated the presence of fungal species with known coal bioconversion capability. Amongst these *Neosartorya fischeri* was identified and its activity in coal bioconversion was described for the first time. *Cynodon dactylon* plant roots were also shown to be colonized by mycorrhizal fungi including *Glomus, Paraglomus* and *Gigaspora* species.

The role of plant photosynthate translocation into the root zone, providing organic carbon supplementation of fungal coal bioconversion was investigated in deep liquid culture with the *N. fischeri* isolate used as the biocatalyst. Organic acids, sugars and complex organic carbon sources were investigated and it was shown that glutamate provided significant enhancement of bioconversion activity in this system.

The performance of *N. fischeri* in coal bioconversion was compared with *Phanaerochaete chrysosporium* and *Trametes versicolor*, both previously described fungal species in the coal bioconversion application. Fourier transform infrared spectroscopy indicated more pronounced oxidation and introduction of nitro groups in the matrix of the humic acid product of coal bioconversion in *N. fischeri* and *P. chrysosporium* than for *T. versicolor*. Macro-elemental analysis of biomass-bound humic acid obtained from the *N. fischeri* catalyzed reaction showed an increase in the oxygen and nitrogen components and coupled with a reduction in carbon and hydrogen. Pyrolysis gas chromatography mass spectroscopy further supported the proposal that the mechanism of bioconversion involves oxygen and nitrogen insertion into the coal structure.

The *C. dactylon* bituminous hard coal dump environment was simulated in a fixed-bed perfusion column bioreactor in which the contribution of organic supplement by the plant/mycorrhizal component of the system was investigated. The results enabled the proposal of a descriptive model accounting for the performance of the system in which the plant/mycorrhizal component introduces organic substances into the root zone. The non-mycorrhizal fungi utilize the organic carbon supplement in its attack on the coal

substrate, breaking it down, and releasing plant nutrients and a soil-like substrate which in turn enables the growth of *C. dactylon* in this hostile environment.

Based on these results, the Stacked Heap Coal Bioreactor concept was developed as a large-scale industrial bioprocess application based on heap-leach mineral processing technology. Field studies have confirmed that bituminous hard coal can be converted to a humic acid rich substrate in a stacked heap system inoculated with mycorrhizal and *N. fischeri* cultures and planted with *C. dactylon*.

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List of Abbreviations

%	Percentage
µL	Microlitre
µm	Micromitre
µmoL	Micromole
A	Absorbance
ABTS	2,2′-azinobis(3-ethylbenzoline-6-sulphonic acid)
ACA	Australian Coal Association
ACF	American coal foundation
Amp	Ampicillin
ANOVA	Analysis of Variance
ARC	Agricultural Research Council
BHC	Bituminous hard coal
BLAST	Basic Local Alignment Search Tool
BLC	Bio-Liquid Complex®
BM	Basal medium
BSA	Bovine serum albumin
CDS	Chemical Data System
CIAB	Coal Industry Advisory Board
CNT-Amm	Control Ammonium nitrate medium
CNT-G	Control glucose medium
CNT-Glut	control glutamate medium
CNT-R	Control rice-extract medium
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleotide triphosphate
DOE	Department of Energy
EBRU	Environmental Biotechnology Research Unit
ECCN	EBRU Culture Collection Number

EDTA	Ethylenediamine tetraacetic acid
FA	Fulvic acid
FPCB	Fixed-bed perfusion column bioreactor
FT-IR	Fourier transform infrared
GA	Glutamate agar
Geo	Global Environmental outlook
HA	Humic acid
HA-Amm	Humic acid-ammonium nitrate medium
HA-G	Humic acid-glucose medium
HA-Glut	Humic acid-glutamate medium
HA-R	Humic acid-rice extract medium
IHSS	International Humic Substances Society
IPTG	Isopropylthio-β-o-galactiside
IR	Infrared
ITS	Internal Transcribed Spacer
L	Litre
LB	Luria Bertani
LiP	Lignin peroxidase
LMO	Low molecular weight organics
LSD	Least Squared Difference
M	Molarity
mg	Milligram
min	Minute
mL	Millilitre
mm	Millimetre
mM	Millimole
MnP	Manganese peroxidase
MSC	Microbial Sequence Centre
N	Normality
NIST	National Institute of Standards and Technology

nm	Nanometer
NMR	Nuclear magnetic resonance
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PDA	Potato dextrose agar
PDB	Potato dextrose broth
Py-GCMS	Pyrolysis gas chromatography mass spectroscopy
R/P	Reserve-to-Production ratio
rDNA	Ribosomal DNA
rpm	Revolution per minute
SASOL	Suid Afrikaanse Steenkool en Olie Lidmaatskapy
SDS	Sodium dodecyl sulphate
SEC	Size Exclusion Chromatography
SEM	Scanning electron microscopy
SRC	Solvent Refined Coal
TC	Total carbon
TEM	Transmission electron microscopy
TG-FTIR	Thermogravimetric infrared spectroscopy
THF	Tetrahydrofuran
TIC	Total ion chromatogram
TMAH	Tetramethylammonium hydroxide
TMEDA	Tetramethyethelenediam
TOC	Total organic carbon
UN	United Nations
USA	United States of America
UV	Ultra violet
V	Volt
v/v	Volume per volume
w/v	Weight volume
WC	Weathered hard coal

WC-Amm	Weathered hard coal-ammonium nitrate medium
WC-G	Weathered hard coal-glucose medium
WC-Glut	Weathered hard coal-glutamate medium
WCI	World Coal Institute
WC-R	Weathered hard coal-rice extract medium
XANES	X-ray absorption near-edge structure
XAS	X-ray adsorption spectroscopy
XPS	X-ray photoelectron spectroscopy

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To my loving mother

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Research Products

Stacked-Heap Coal Bioreactor. Patent number ZA 2007/07607. South African Patent Office.

Igbinigie, E.E., Atkins, S., van Breugel, Y., van Dyke, S., Davies-Coleman, M.T. and Rose, P.D. (2007). Fungal biodegradation of hard coal by a newly reported isolate, *Neosartorya fischeri*. In prep.

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Chapter one

The bioconversion of hard coal

1. Energy

Crude oil currently supplies 37 % of world energy requirements (Department of Energy (DOE), 2007). However, Hubbert (1949 and 1956) predicted that the era of oil-based fuels would be short-lived and that the United States of America's (USA) oil reserves would peak around 1965 – 1970 and decline sharply thereafter. According to the DOE (2007), the cost per barrel of oil has experienced a gradual increase, since the mid 1990s, with an all time high approaching $100 per barrel in November, 2007. As a result, interest has shifted once again to coal (World Coal Institute (WCI), 2007). At present, coal constitutes nearly 25 % of global primary energy consumption and according to the WCI it maintains a central role in the social and economic development of the world (WCI, 2007). Coal generates 40 % of the world's electricity for lighting, cooking and refrigeration and fossil fuels all together supply over 80 % of total energy needs (Global Environmental Outlook (Geo-4), 2007).

World coal reserves are estimated at 900 billion metric tonnes with a Reserve-to-Production (R/P) ratio of around 147 years as against a world oil reserve of 164 thousand million tonnes with R/P ratio of 40.5 years (BP Statistical Review of World Energy, 2007). The recoverable coal reserve worldwide is estimated at 2500×10^9 metric tonnes and would peak around 2150, depending on how the usage graph is drawn (International Energy Outlook, 2007).

However, coal utilization is associated with serious environmental problems from its mining right through to its final utilization by consumers (Geo-4, 2007). It is a substantial source of pollution and has been identified as the largest source of

atmospheric CO_2, and thus a major contributor to global warming (BP Statistical Review of World Energy, 2007; Geo-4, 2007). It has been labeled by some as environmental enemy number one (The Economist, 2002). In the USA alone, coal power plant emissions have been implicated as the cause of tens of thousands of premature deaths annually (DOE, 2007). Contaminants arising from coal utilization include fly ash, bottom ash, boiler slag and heavy metals, which are dangerous when released into the environment. The presence of low levels of the radioactive elements (uranium and thorium) in coal could result in a cumulative radioactive waste over time, comparable to that of a nuclear power plant (Gabbard, 2007).

It has been widely argued that to minimize the deleterious effects of coal on the environment, research into more sustainable production and consumption of coal is warranted (Steel and Patrick, 2001; Coal Industry Advisory Board (CIAB), 2003; Pandey *et al.,* 2005). The necessity for a move to cleaner coal technologies was identified as a priority at the Earth Summit (2002).

1.1 Clean coal technology

According to the Australian Coal Association (ACA), the term “clean coal” is used to describe technologies that are designed to enhance the efficiency and environmental acceptability of coal extraction, preparation and utilization (ACA, 2007). Both physico-chemical and biological processes have been investigated in the development of clean coal technological processes.

Physico-chemical processes for coal conversion to synfuel (synthetic fuel), in the form of gasoline or diesel, is referred to as Coal-To-Liquid operations with technologies such as the Fischer-Tropsch process providing one of the largest current applications (Optima, 2007). The Fischer-Tropsch process, developed and in current use by Suid Afrikaanse Steenkool en Olie Lidmaatskapy (SASOL) in South Africa, is a chemically catalyzed

process in which syngas (carbon monoxide and hydrogen produced from coal gasification) are converted into liquid hydrocarbons such as petrol and diesel. Joint research by Chevron and SASOL, has indicated a significant reduction in pollution with the move from normal crude oil-based fuels to liquid-transport fuel derived from coal (Optima, 2007). Another clean coal physico-chemical process is the Bergius process, which involves a direct liquefaction of lignite by hydrogenation to synfuel, but this is currently utilized only in Germany. Other physico-chemical clean coal processes that involved direct coal liquefaction include the Solvent Refined Coal (SRC-I and SRC-II) process developed by Gulf Oil, and implemented at pilot scale in the USA, and the Karrick process, which is a low temperature carbonization process that was developed at the United States Bureau of Mines in the 1920s (US Patent number 1,958,918).

Biological processes for the production of clean coal have received particular attention but are less well developed technologies (Klasson *et al.*, 1993; Gupta and Birendra, 2000). Potential applications are numerous and end products of biological processing of coal could serve as feedstocks for a range of other biological processes, including biological wastewater treatment (Bumpus *et al.*, 1998; Steinberg, 1999), or they could be further modified by other microorganisms to produce methane, which may also be converted biologically to methanol to produce liquid fuel (Xin *et al*., 2004a and b).

The study reported here has focused on the investigation of biological approaches to coal conversion and clean coal technology, but in order to discuss the bioprocess environment, it is necessary to review the nature of coal, its structure, diagenesis and degradation functions.

1.2 Coal genesis

Coal is fossilized plant material that is chemically and physically heterogeneous (American Coal Foundation, ACF, 2001). It is an organic rock formed from plant, which

is not easily liquefied without the decomposition of its core structure. It is insoluble in common organic solvents resulting in it being chemically unreactive under mild conditions and, therefore, resistant to microbiological degradation (Fakoussa, 1981; Hodek, 1994; ACF, 2001).

Coal formation can be classified into biochemical and geochemical phases (Haenel, 1991; ACF, 2001). This process can be traced to the Devonian Period when plants with cellulose-rich stems had evolved. During the biochemical phase of coal formation known as peatification (Fakoussa, 1981), dead vegetation, usually in swampy areas, sank to the bottom of these swamps and accumulated, and with the aid of bacteria under anaerobic conditions, eventually formed a dense material known as peat.

The later geochemical phase involved the burial of the peat and the increase in pressure and temperature cause a gradual loss of water, transforming it into coal (coalification). This process occurred in stages which increased the coal rank from lignite (also known as brown coal or low rank coal) through to sub-bituminous coal, then to bituminous hard coal (BHC) and finally, to anthracite (Haenel, 1991; de la Puente *et al*., 1998). It is estimated that 0.3 million tonnes of BHC would have been produced from 0.9 million to 2.1 million tonnes of compacted plant material (ACF, 2001).

1.2.1 Coal classification

Coal is generally classified by increasing carbon content or decreasing moisture content, as well as the elemental composition and atomic ratio distribution (Table 1-1) (Hodek, 1994; ACF, 2001). Using this classification, four main coal ranks are identified; lignite, sub-bituminous (high volatile coal), bituminous (low volatile hard coal) and anthracite (hard coal). Other categories that are present during coal formation, but not generally grouped amongst the coal ranks, are peat, which precedes lignite, and graphite, which succeeds anthracite (Haenel, 1991).

The elemental composition of coal (of the same rank) can vary amongst different coal deposits, probably as a result of the variation in the composition of the original plant material and diagenetic processes (Haenel, 1991; van Krevelen, 1993; Fakoussa and Hofrichter, 1999). Nevertheless, a general classification of the coal ranks can be made based on the elemental composition (Table 1-1) (Hodek, 1994; ACF, 2001).

Table 1-1 Classification of coal based on moisture content, percentage elemental composition and atomic ratio of the component elements (Modified after Hodek, 1994 and American Coal Foundation, 2001).

Coal sample	Notes	Elemental analysis (%)					Atomic ratio				
		C	H	O	N	S	C	H	O	N	S
Lignite	A high moisture content coal with heat energy ranging from 8 – 10 MJ/Kg. Mainly used for electricity power generation.	68.60	5.00	24.50	1.56	0.31	100	87.00	27.00	1.90	0.20
Sub-bituminous	Sooty with fairly high moisture content and low sulphur content, this makes it attractive for use in cleaner burning.	82.70	5.10	9.40	1.59	0.95	100	74.00	8.50	1.70	0.40
Bituminous	Fastest growing in the coal market with heat generating value of 28 MJ/Kg and less than 3 % moisture content. Used primarily for the generation of electricity and coke for the steel industry.	89.70	4.50	2.70	1.59	1.20	100	61.00	2.30	1.50	0.50
Anthracite	A shiny coal, which has virtually no moisture content and has an energy content of up to 32 MJ/Kg. It has the lowest volatility amongst all the categories. It burns with little or no smoke, a reason for its frequent use in heating homes.	92.00	3.80	1.30	1.95	0.87	100	49.00	1.10	1.80	0.40

1.2.2 Coal structure

The structure of coal is very complex, and no two coals are the same. Coal diagenesis varies strongly with each coal deposit and depends on the nature of the source plant as well as other coal generating conditions (Haenel, 1991; van Krevelen, 1993; Fakoussa and Hofrichter, 1999). Therefore, the structural models found in texts are generally hypothetical models, which changes with the rank of coal (Fakoussa, 1981; Haenel, 1991; van Krevelen, 1993; Yağmur *et al.*, 2000). Structural models of lignite, which is regarded as being more complex than hard coal, consist of several distinct compound classes that include bitumen (mainly hydrophobic), alkaline soluble humic acid (HA) (a water soluble coal fraction), fulvic acid (FA) (soluble at any given pH) (Fakoussa and Hofrichter, 1999; Janoš, 2003) and humin, or the matrix, which is an insoluble residue regardless of pH, but soluble in organic solvents (Figure 1-1) (Moldoveanu, 1998).

In recent times, coal-derived HA has become of great interest in understanding the structure of coal as well as it having economic potential as feed stock for biological processes (Bumpus *et al.*, 1998; Steinberg, 1999) and an energy source (Xin *et al.*, 2004a and b). It has also found use as a marker in microbial coal biodegradation research (Dehorter and Blondeau, 1992; Yanagi *et al.*, 2002; Zavarzina *et al.*, 2004; Kluczek-Turpeinen *et al.*, 2005; Elbeyli *et al.*, 2006a; Yuan *et al.*, 2006). Therefore, a proper definition and understanding of HA is justified.

Humic acid, together with FA and humin, are geopolymers that are highly ubiquitous and are found naturally in soil as an integral part of humic substances (Moldoveanu, 1998). Humic substances are formed via numerous transformations of organic matter that escape complete degradation. These organic substances include cellulose, lignin, proteins, fats and low molecular weight plant components (Moldoveanu, 1998). Humic acids are also present in sewage sludge (Hernandez *et al.*, 1988) aquatic environments (Steffen *et al.*, 2002), and groundwater (Pettersson *et al.*, 1994).

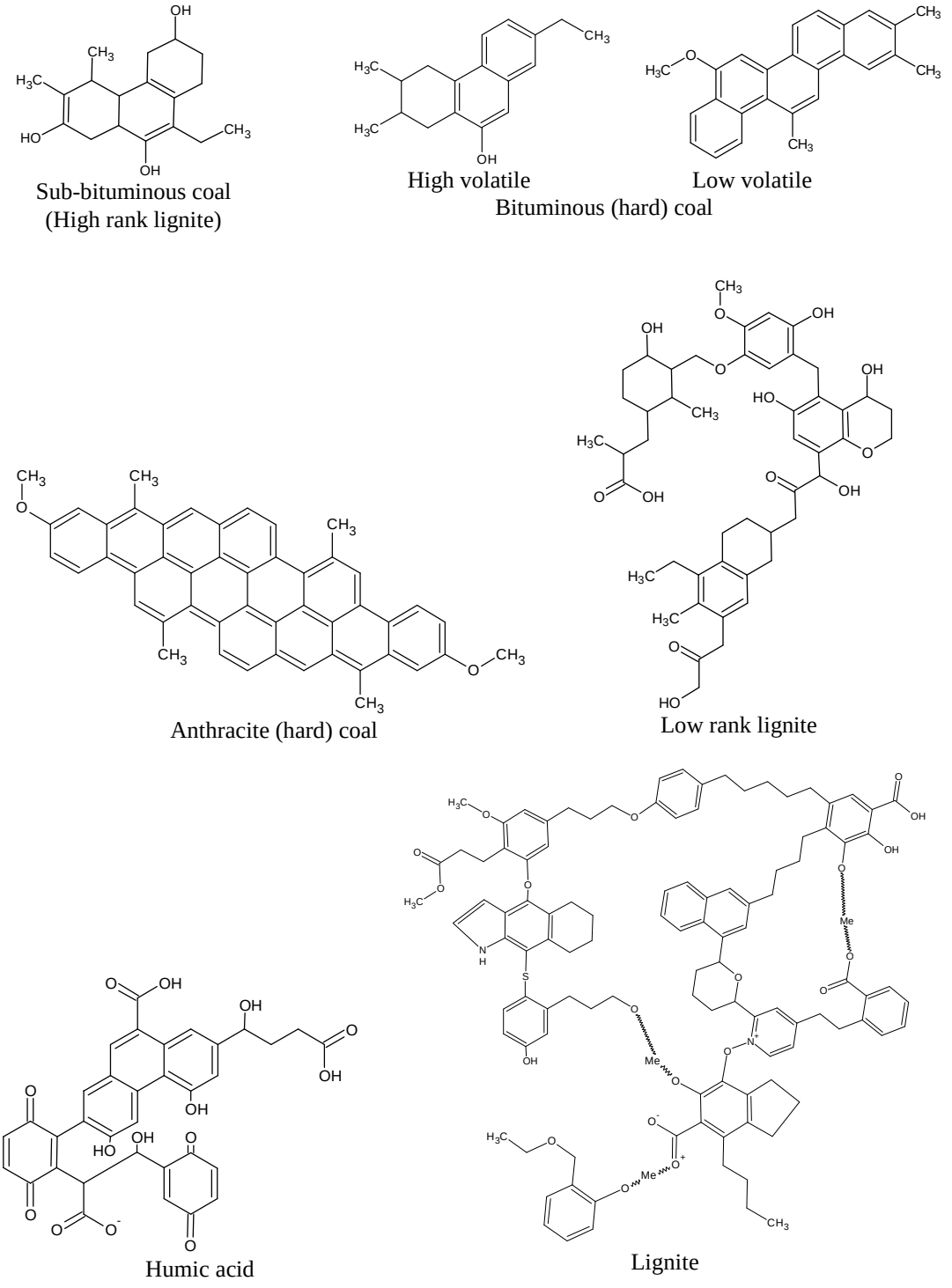


Figure 1-1 Hypothetical structural models for coals of different ranks (Fakoussa and Hofrichter, 1999).

Large amounts of HA are found in low grade coal such as lignite (Lobartini *et al.*, 1992; del Río *et al.*, 1994). They are the fraction of the humic substances that are dark brown to black in colour (Novák *et al.*, 2001), heterogeneous in composition with a wide range of molecular weight distribution and rich in structural moieties such as hydroxyl, carboxyl or carbonyl groups (Henning *et al*., 1997; Janoš, 2003). They are soluble at high pH and precipitate at low pH (Janoš, 2003). The regeneration of HA from coal, described by Piccolo *et al*. (1992) as a reversal of the diagenetic process, is facilitated in the presence of oxygen. These regenerated HAs are particularly rich in aromatic compounds and are structurally different from the HA coal precursors, but share similar solubility and reactivity properties (Piccolo *et al*., 1992). However, little information is available about the coal-derived HA (Piccolo *et al*., 1992; Henning *et al*., 1997). Fulvic acids are known for their characteristic yellow colouration and solubility at any given pH (Janoš, 2003). Fulvic acids had been characterized to be of smaller molecular weight in comparison with HA, with oxygen present as functional groups rather than as structural components as in the case of HA (Janoš, 2003).

The defining coal structure falls into one of two categories (Olivella *et al.*, 2002). One approach is to characterize coal directly by the use of techniques which allow a non-destructive investigation into the solid coal structure. The other technique applies a destructive approach aimed at defining the structure of coal by initially fragmenting the coal matrix and subsequently deriving the original coal structure through the reconstruction of the representative fragments.

Techniques used for the non-destructive investigation of the structure of coal include infrared (IR) and solid state nuclear magnetic resonance (NMR) spectrometry (del Río *et al.*, 1994; Osipowicz *et al.*,1996; Novák *et al.*, 2001; Wadhwa and Sharma, 1998), X-ray adsorption spectrometry (XAS), X-ray diffraction and transmission electron microscopy (TEM) (Haenel, 1991), X-ray photoelectron spectrometry (XPS) (Olivella *et al*., 2002; Lyubchik *et al.*, 2002) and X-ray absorption near-edge structure (XANES) (Olivella *et al*., 2002).

A non-destructive investigation of the coal macromolecule would generally define the typical structure of coal as consisting a large polymeric matrix of aromatic structures commonly called the macromolecular matrix, and a tetrahydrofuran soluble part,

referred to as the "mobile phase" of coal (Figure 1-2). The "mobile phase" usually vaporizes at 350 ^{o}C and is thought to be susceptible to microbial attack (Hodek, 1994; Ralph and Catcheside, 1994a; Solomon *et al.*, 1988). The macromolecule network consists of clusters of condensed aromatic rings that are linked to other aromatic structures by bridges thought to be mainly aliphatic in nature such as methylene and ether bridges. The C-C bond strength may be up to 218 KJ/mol and thermal requirement for cleavage may be as high as 350 – 400 ^{o}C (Shinn, 1984; Solomon *et al.*, 1988). Some bridges consist of a single bond between two aromatic clusters and are known as bi-aryl linkages. Bridges between carbon atoms can also be formed by inorganic elements, such as oxygen, sulphur, silicon, aluminum, iron, phosphorus, sodium, titanium, magnesium, potassium and calcium (Fakoussa, 1981; Solomon *et al.*, 1988). Other attachments to the aromatic clusters that do not form bridges are referred to as side chains and are thought to consist mainly of aliphatic and carbonyl functional groups. These side chains, unlike the coal core structure, are accessible to bacterial attack (Hodek, 1994; Solomon *et al.*, 1988).

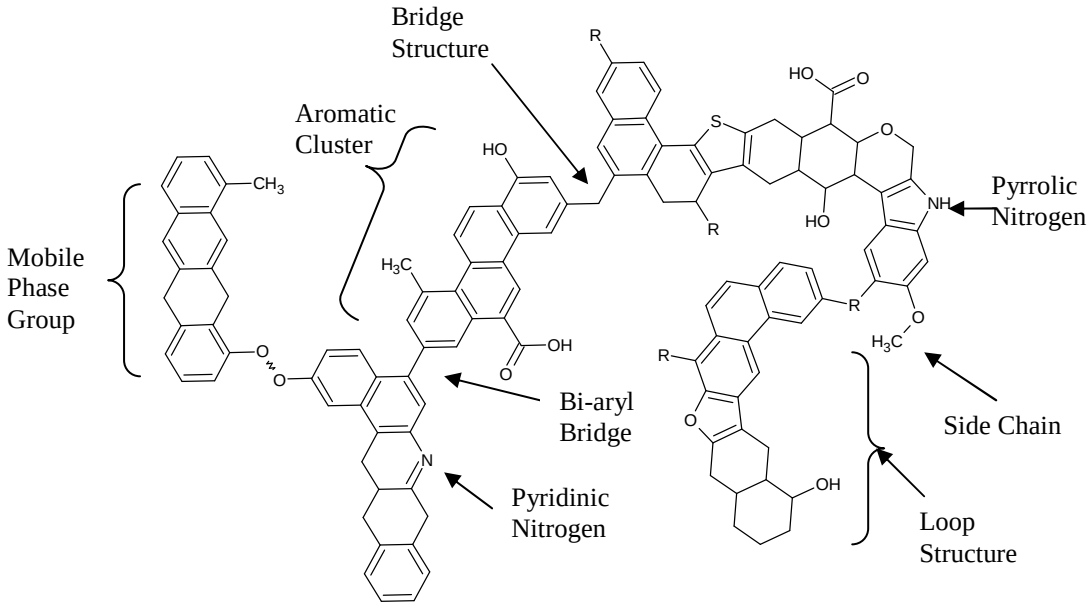


Figure 1-2 Hypothetical coal macromolecules as denoted by a non-destructive investigation. The macromolecular matrix and the mobile phase groups are pronounced and the condensed aromatic rings are linked together by aliphatic groups mainly of methylene and ether bridges (Solomon *et al.*, 1988).

Chemical degradation and pyrolysis are the two destructive approaches utilized for defining the coal structure (Olivella *et al.,* 2002). Analyses of fragmentized coal macromolecules using the pyrolysis gas chromatography mass spectrometer (Py-GCMS) usually produce various aromatic ring systems of various sizes, and aliphatic compounds of various lengths (Philp, 1982; Giuliani *et al.,* 1991; Martin *et al.,* 1994; Olivella *et al.,* 2002; Kashimura *et al.,* 2004). The chemical degradation techniques will be discussed in the subsequent section.

Destructive analysis of the coal macromolecule structure has provided evidence for the abundance of condensed aromatic ring systems numbering from two to six (Shinn, 1984; Kashimura *et al.,* 2004). Apart from the occurrence of the condensed aromatic rings, the results also indicated that solubilized coal analyzed with absorption and fluorescence spectrometry, had larger poly-aromatic ring systems than coal analyzed with pyrolysis (Kashimura *et al.* 2004). This implies that smaller poly-aromatic ring systems are lost via decarboxylation from the coal during pyrolysis, thus discrediting the use of pyrolysis as a technique for determining the coal structure. However, the introduction of tetramethylammonium hydroxide (TMAH) prior to pyrolysis prevents decarboxylation and greatly enhances the product yield, which includes methyl esters of both carboxylic acids and hydroxyl groups (Martin *et al.,* 1994; Chefetz *et al*., 2002; Olivella *et al.,* 2002). Some possible units obtained using the destructive approach is presented in Figure 1-3 (Haenel, 1991; Kashimura *et al.,* 2004).

Different methods of molecular weight determination of the coal macromolecule, such as gel permeation and size exclusion chromatography (SEC), would yield different molecular weight fragments, ranging from 300 to 800,000 D. This dismisses the relevance of a molecular weight for coal, as it is more accurate to determine the molecular weights of single fragmentized units rather than the whole coal macromolecule itself (Fakoussa, 1981; Fakoussa and Hofrichter, 1999). Kashimura *et al.* (2004) emphasized the difficulty in obtaining structural information from the entire coal macromolecule without exhaustive breakage of the linkages between the poly-condensed rings of the coal.

The fragmentation of the coal macromolecule for structural determination also indicated the presence of two vital compounds (HA and FA), which are alkaline

soluble and are responsible for the molecular weight distribution in coal (Schulten and Schintzer 1993; Stevenson, 1994; Fakoussa and Hofrichter 1999). The heterogeneity of the molecular weight of coal was suggested to be related to the inverse proportionality constant between the HA and FA in coal and it varies from coal to coal. By this factor, the properties of coal differ greatly from one another, e.g. the higher the HA in a coal sample, the higher the molecular weight, the tendency to precipitate, the ratio of carboxylic groups to the total oxygen (-COOH / Σ oxygen functions) and the lower the FA (Fakoussa and Hofrichter, 1999).

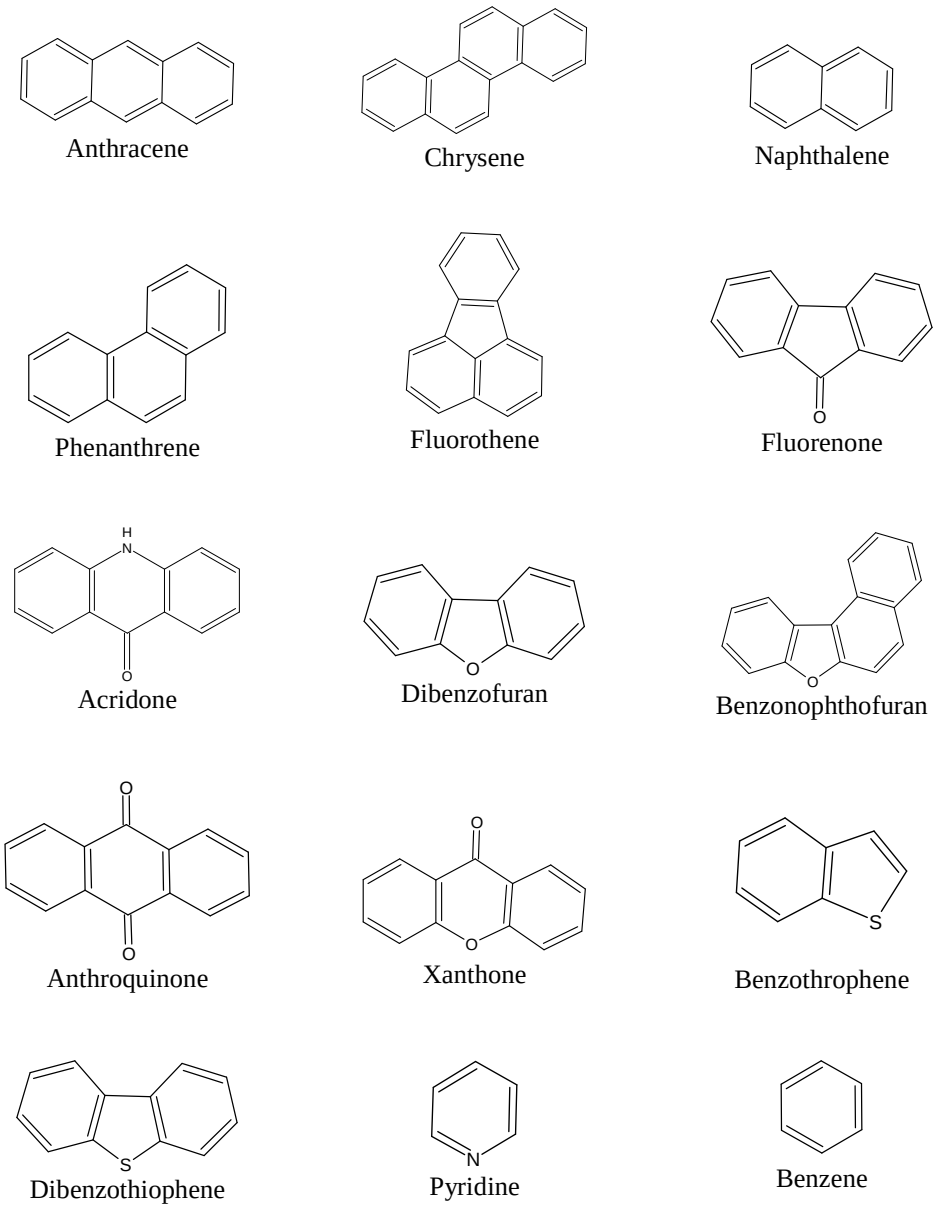


Figure 1-3 Structures of possible units of the fragmentized coal macromolecule that could be detected with destructive analysis of the coal substrate (Haenel, 1991; Kashimura *et al.*, 2004).

1.3 The biodegradation of coal

Cimadevilla *et al.* (2003) described the degradation of coal or weathering as a complex phenomenon which involves several simultaneous and interacting chemical processes, sometimes coupled with physical changes. Four categories of coal weathering are described by Cimadevilla *et al.* (2003), which include a laboratory process of low and moderate-temperature oxidation (simulated weathering), aerial oxidation of organic and mineral matter (chemical weathering), microbial oxidation of pyrite (biological weathering) and changes in the coal moisture content that may result in elevated particle size fractionation (physical weathering). In this study, the coal conversion process has been grouped into four categories, namely conversion by natural processes (weathering), conversion by thermal processes, conversion by chemical processes and conversion by microbial processes. In particular, the term conversion or bioconversion will be used to refer to any change to the coal substrate including those induced by microbial activity. This covers a wide range of terms used in the literature including solubilization, depolymerization and degradation.

1.3.1 Conversion by natural process

Coal conversion by natural processes refers to the alteration of the coal macromolecule when exposed to the atmosphere after mining (de la Puente *et al.*, 1998; Cimadevilla *et al.*, 2003). The oxidative process that occurs under this condition involves the release of a large amount of heat, which could result in spontaneous ignition of the coal material with a subsequent loss in calorific value, alteration of macromolecular properties and a modification of the coal macromolecular behaviour during the conversion processes (Ndaji and Thomas, 1995; Ibarra and Miranda, 1996; MacPhee *et al*., 2004).

It is generally understood that the natural oxidative weathering of coal usually occurs in two steps. The first step involves the utilization of oxygen to produce intermediate products while the second step involves the subsequent decay of the protracted intermediates leading to the loss of carbon as CO_2 (Equation 1). When low rank coals

such as lignite are involved, humic substances are generated as part of the intermediates (Chang and Berner, 1998).

$$\mathbf{C_{coal} + O_2 \xrightarrow{A} intermediates \xrightarrow{B} CO_2} \qquad \textbf{Equation 1}$$

The regeneration of humic substances can be achieved in the laboratory by simulating natural weathering using oxidizing agents such as HNO_3 (Quigley *et al.,* 1988; Machnikowska *et al.,* 2002; Elbeyli *et al.,* 2006a) and hydrogen peroxide (Hölker *et al.,* 1997; Hofrichter *et al.,* 1997b; Willmann and Fakoussa, 1997a), or by aerating the coal macromolecule at elevated temperature (Ndaji and Thomas, 1995). The observed regeneration of humic substances in the field via the natural oxidative processes of higher rank coal has yet to be confirmed (Chang and Berner, 1998).

The weathering of coal is characterized by the increase in oxygen functionalities and the subsequent decrease in aliphatic hydrogen content. Fourier transform infrared (FT-IR) spectrometry is widely employed to monitor and determine the extent of oxidation of the coal macromolecule (Ibarra and Miranda, 1996). The 3000 – 2700 cm^{-1} and 1800 -1500 cm^{1} regions of IR spectrum are denoted for the aliphatic and oxygen functionalities, respectively (Pavia *et al.,* 2001; Alvarez *et al.,* 2003), and their turnover is usually monitored during the coal weathering process (Ibarra and Miranda, 1996; de la Puente *et al.,* 1998; Cimadevilla *et al.,* 2003). Thermogravimetry coupled to gas analysis by infrared spectrometry (TG-FTIR) had been demonstrated to be effective in the accurate auditing of the oxygen mass balance in the three main oxygen containing gases (H_2O, CO and CO_2) during the weathering process (MacPhee *et al.,* 2004). Results from FT-IR studies of the coal weathering process suggest a slight decrease in the aliphatic hydrogen contents and an increase in carboxyl group with increasing time of storage. Significant structural changes were observed in samples obtained from areas where spontaneous heating and ignition occurred (Ibarra and Miranda, 1996). The early stages of the weathering process usually entail the formation of peroxides, which eventually decompose to generate water, at a temperature that is below the softening point of bituminous coals (MacPhee *et al.,* 2004).

1.3.2 Conversion by thermal process

As a result of the large variety of functional groups that make up the bridge structures of coal, bridges tend to have a large distribution of bond strengths (Hodek, 1994). This distribution of bond strengths is vital during pyrolysis (thermal combustion in the absence of oxygen), as the weakest bonds are broken first. Bridges that contain oxygen atoms as ethers have relatively weak bond strength and are therefore broken first (Solomon *et al.,* 1988). Coal conversion by thermal process therefore refers to any alteration to the coal structure through application of heating.

The pyrolysis behaviour of coal is known to be subject to temperature, heating rate, particle size, pressure, coal type among other factors (Solomon *et al.,* 1988). During pyrolysis of coal, devolatilization occurs. Tar and light gases (volatiles) are driven out, and a solid residue known as char, remains (Solomon *et al.,* 1988; Matsuoka *et al.,* 2005). Tar is generally defined as the volatile components that condense to a solid or liquid at room temperature (Solomon *et al.,* 1988). The morphology of the resultant char is responsible for the aerodynamic characteristics of the coal char when introduced into a reactor. Simply put, the eventual characteristic of coal char during conversion is determined by the rank of coal and petrographic composition of the parent coal, as well as the experimental conditions (Matsuoka *et al.,* 2005).

Figure 1-4 is a representation of the hypothetical pathway of coal degradation during pyrolysis (Solomon *et al*, 1990). As the coal particle temperature rises during pyrolysis, the bonds between the aromatic clusters in the coal macromolecule break, creating fragments that detach from the macromolecule (Figure 1-4). The larger fragments, referred to as metaplast, either vaporize and escape from the coal, or are reincorporated into the coal macromolecule through a process known as cross-linking. The decomposition rate and the extent of reaction of coal is a factor determined by the extent of cross-linking in the macromolecular structure. This determines the duration of chemical reaction or thermal process needed for the depolymerization of the coal macromolecule (Ndaji and Thomas, 1994). The portion of the metaplast that is vaporized usually consists of the lower molecular weight fragments; fragments consisting of light gasses of tar that can characterize the rank of coals. The side chains

on the aromatic clusters released from the coal as light gases are generally oxides (CO_2, CO, H_2O) and light hydrocarbons (C_1-C_4) (Figure 1-4). Low rank coals, such as lignite and sub-bituminous coals produce relatively high levels of light gases and very little tar. Bituminous hard coals produce significantly more tar than the low rank coals and moderate amounts of light gases. The higher rank coals (anthracite) produce relatively low levels of both light gases and tar (Solomon *et al*, 1990).

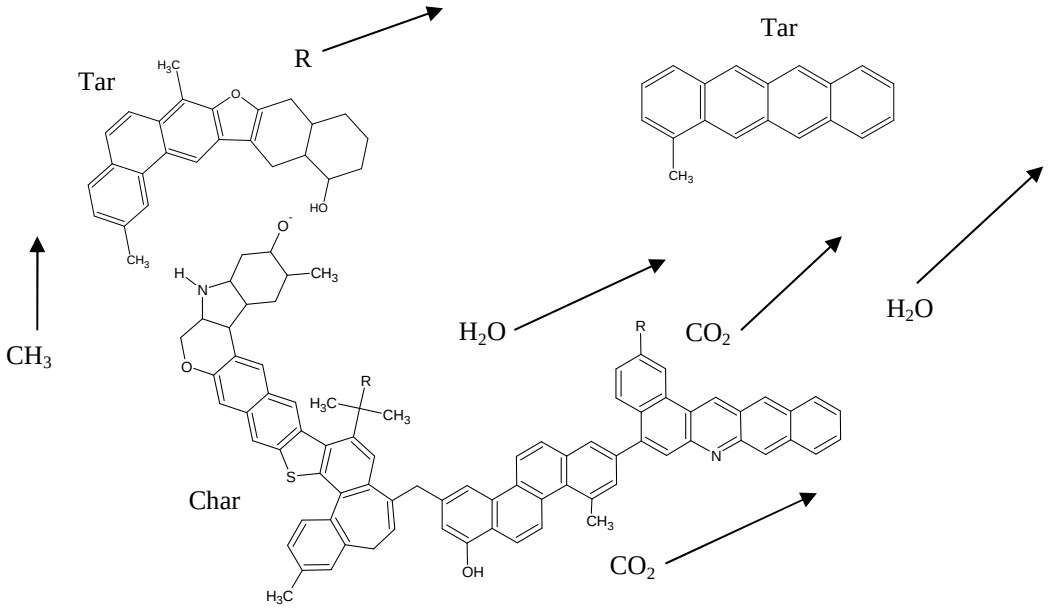


Figure 1-4 The hypothetical pathway of coal degradation during pyrolysis. A rise in temperature of the coal particle would result the cleavage of bonds between the aromatic clusters in the coal macromolecule break, creating fragments that are detached from the macromolecule (Solomon *et al*., 1990).

The general consensus in current coal degradation research is that the linkage between the aromatic clusters must be exhaustively cleaved for the structure of the coal macromolecule to be elucidated, and to make solubilization and depolymerization, as well as cleaner coal fuel production, attainable (Steel *et al.*, 2001; Kashimura *et al.*, 2004).

A major disadvantage in the industrial pyrolysis of coal is the high capital cost of the facilities and the high operational cost needed to achieve the high temperature required for bond cleavage between the aromatic clusters. Noxious gases, such as

sulphide, are released into the atmosphere during thermal combustion. The accumulation of these gases results in air pollution and acid rain (Szendrői *et al.*, 1994; Mukherjee and Borthakur, 2001). Highly reactive intermediate pyrolysis products cause uncontrollable side reactions that result in undesirable side products. These activities can be avoided by the use of ionic reactions at low temperature, which could result in derivatives that are soluble in common solvents (Hodek, 1994; Shimizu *et al.*, 1999; Steel *et al.*, 2001).

1.3.3 Conversion by chemical process

Physical methods of desulphurization and demineralization, as earlier mentioned, are limited because of environmental constrains. This is not a crucial factor in the conversion of the coal molecule by chemical process, which is becoming more acceptable as the alkali used is easily recovered though various chemical processes and the acid by simple distillation process (Stefanova *et al.*, 1999; Mukherjee and Borthakur, 2001). Though the main part of coal can not be dissolved by solvent-based methods as a result of the three-dimensional macromolecular structure of coal, partial solubility of most lignite and BHCs in benzene/ethanol and in non-specific solvents, such as chloroform, ether and acetone, are possible because of the presence of paraffinic wax and resins (Hodek, 1994). Using solvents of high polarity, such as pyridine and phenol, partial solubility of up to 20 or 40 % is achieved at temperatures below 200 ^{o}C. The solubilized products, in these cases, are said to have similar properties to the main insoluble coal macromolecule (Hodek, 1994), which undergoes a process known as “swelling” (diffusion of molecules towards the polymer structure of the coal macromolecules) (Yağmur, *et al.*, 2000).

The chemical pre-treatment of coal prior to its solubilization has been the subject of several studies. Chemical pre-treatment by reduction, alkylation, reductive alkylation, non-reductive alkylation and selective oxidative alkylation has been investigated (Bimer *et al.*, 1992). These investigations have been extensive but did not elaborate on the effect of chemical pre-treatment on the coal macromolecule (Bimer *et al.*, 1992). Acid catalyzed coal depolymerization reactions that involve the use of strong acids such as $AlCl_3$-HCl and $AlBr_3$-HBr are usually carried out at low temperatures as

a pre-treatment step prior to solubilization (Shimizu *et al.*, 1999). However, coal compounds formed with the acid are usually not easily separated into different constituents, making the recovery of the depolymerized coal products almost impossible. A Brönsted/Lewis (HF/BF_3) acid catalyst with a low boiling point was shown to be essential for coal depolymerization and the acid was easily recoverable by distillation (Shimizu *et al.,* 1999). This investigation highlighted the coal depolymerization pre-treatment step as vital for effective solubilization in selected solvents.

In another investigation of coal pre-treatment steps, coal solubilization was obtained via a stepwise process of coal activation (by alkali metal treatment), ionic oxidation, and finally, non-reductive alkylation (Stefanova *et al.,* 1999). This study proposed a chemical mechanism for an effective cleavage of C-C bonds in the coal macromolecule creating an opportunity for coal solubilization.

Coal activation by alkali treatment using potassium as an electron transfer agent was achieved in the presence of tetrahydrofuran (THF) and naphthalene. This was followed step-wise by the oxidation of the activated coal with dry oxygen, acidification to pH 4 using n-BuOH, and finally, distillation by the use of water vapour. The non-reductive alkylation was accomplished by introducing 2 mL freshly distilled THF to 1 g of coal and adding 10 mL Tetramethyethelenediam (TMEDA) under argon atmosphere while stirring the mixture in liquid nitrogen. This was followed by the drop-wise addition of 10 mL of n-butyl lithium (n-BuLi) solution in heptane. Stirring continued at room temperature for 3 hrs and an excess of n-Butyl iodide (n-BuI) (three fold of potassium), which was distilled over copper wire, was introduced. Finally, the product was acidified using 3 % HCl and steam distilled to recover the treated coal.

Solubility of chemically treated coal in trichloromethane ($CHCl_3$) increased up to 84 %, depending on the activation procedure adopted. Spectral analysis of the solubilized coal by FT-IR spectrometry and –H and ^{13}C CP/MAS NMR revealed that the solubilization of the coal was effected by C-alkylation, and consequently, the weakening of strong C-C bond interactions present in coal. Here the solubilization of

the coal was effected by the weakening of strong C-C bond interactions present in coal (Stefanova *et al.,* 1999).

Other forms of chemical pre-treatment of coal such as metallic potassium (K-THF-isopropanol) could increase coal susceptibility to solubilization in methanol-NaOH, while reductive methylation with potassium tetrahydrofuran methyl iodide (K-THF-CH_3I), decarboxylation, reduction with lithium aluminium hydride ($LiAlH_4$) and O-methylation has been investigated and were found to greatly reduce coal reactivity (Bimer *et al.,* 1992). Also, the use of aqueous sodium hydroxide followed by hydrochloric acid pre-treatment has been investigated as a pre-treatment step for the desulphurization and demineralization of coal (Mukherjee and Borthakur, 2001). Results indicate a 43 – 50 % removal of total inorganic sulphur and around 10 % of organic sulphur from the coal sample that was of Indian origin. Desulphurization and demineralization were found to increase with the increase in alkali concentration.

The degree of modification, mainly by insertion and oxidation, depends on various factors, such as the nature of chemical agents, treatment time and temperature, as well as the nature of the coal used (Lyubchik *et al*., 2002; Alvarez *et al*., 2003). HNO_3 oxidative pre-treatment of coal has been shown to desulphurize the coal macromolecule (Alvarez *et al*., 1996 and 2003), and to incorporate carbonyl/carboxyl functionalities (Alvarez *et al*., 1996 and 2003; Mae *et al*., 2005). In addition, the introduction of aromatic nitrogen was observed after HNO_3 pre-treatment of coal (Machnikowska *et al*., 2002; Alvarez *et al*., 2003; Elbeyli *et al.,* 2006a and b), and an increased aliphatic hydrogen content under more energetic conditions (Alvarez *et al*., 2003). Interestingly, HNO_3 treatment does not seem to have any influence on the methylene and methyl groups present in the macromolecule (Alvarez *et al*., 2003).

The oxidative pre-treatment of the coal samples using HNO_3 greatly elevated the coal solubilization potential for microbial interaction (Achi, 1993, 1994a and b; Fakoussa, 1994; Machnikowska *et al.,* 2002; Başaran *et al.*, 2003). For example, Machnikowska *et al.* (2002) found 90 and 40 % enhanced solubilization of lignite and sub-bituminous coal, respectively, after HNO_3 treatment.

1.3.4 Conversion by microbial process (bioconversion)

Although the biological reactivity of coal has been known for some time, more recent investigations of the microbial action on coal date from 1979 when the structural similarities between wood lignin and coal were reported by Hayatsu *et al*. (1979). Fakoussa (1981) provided the first direct evidence that filamentous fungi, yeast and bacteria utilize coal as the sole carbon source. This was followed shortly by evidence that fungi can liquefy coal (Cohen and Gabriele, 1982). Over the years several investigations resulted in the descriptive postulation of fungal activity on coal and coal related macromolecules. Most of these investigations focused on fungal activity on low rank coal such as lignite (Scott *et al.,* 1992; Ralph and Catcheside, 1993; Hölker *et al.,* 1995; Hölker and Höfer, 2002; Yuan *et al.,* 2006). This was mainly due to the accessibility of the coal macromolecule to microorganisms, because of the lesser compactness of the aromatic ring system as well as the higher moisture content of low rank coal when compared with higher rank coal or hard coal (Hodek, 1994). Oxidative pre-treatment of the low rank coal greatly enhanced the microbial interaction resulting in the solubilization of the coal macromolecule (Quigley *et al.,* 1988 and 1989; Achi, 1994a; Fakoussa, 1994; Hofrichter *et al.,* 1997b; Laborda *et al*., 1997; Machnikowska *et al.,* 2002). Only a few investigations have focused on microbial solubilization of untreated hard coal (Fakoussa, 1988; Osipowicz *et al.,* 1994; Monistrol and Laborda, 1994; Hofrichter *et al.,* 1997a). Both bacterial consortia and pure bacterial isolates have been implicated in coal bioconversion (Fakoussa, 1988; Andrews *et al.,* 1994; Torzilli and Isbister, 1994; Hölker *et al.,* 1997).

Findings on the microbial degradation of coal are, however, not conclusive and in some cases were found to be contradictory and very limiting (Torzilli and Isbister, 1994). Willmann and Fakoussa (1997a) maintained that extracellular enzymes (peroxidases and/or laccases) were a key factor responsible for coal solubilization, but that the enzymes were induced by some mediators. They found that the excretion of these enzymes starts shortly before the solubilization of the coal and the enzymes are rendered inactive during the course of solubilization. These enzymes could be responsible for modifying the structure of coal which eventually results in a decrease in the octanol-water partition coefficient of coal (an indication of increased

solubility). The application of purified peroxidases and enriched laccases, as well as commercial samples of these enzymes, was not able to effect *in vitro* coal solubilization. These results led to the conclusion that other enzymes or factors help to promote coal solubilization. The oxidation state of coal was, however, found to be a major factor that determines the extent of its solubilization using fungi (Willmann and Fakoussa, 1997a).

At present there are no standardized protocols for the screening of microbial species with coal bioconversion potential. Research groups have generated and customized screening protocols that meets their various requirements (Klein *et al*., 1999). In most investigations that involved the microbial degradation of coal, the secretion of extracellular oxidative enzymes had been targeted and incorporated in the screening protocol to determine organisms with coal depolymerization potential. The presence of extracellular oxidative enzyme-secreting organism can be confirmed by the addition of the substrate 2,2′-azinobis(3-ethylbenzoline-6-sulphonic acid) (ABTS) to the growing agar plates (Hofrichter and Fritsche, 1996 and 1997a; Hofrichter *et al.*, 1997b; Willmann and Fakoussa, 1997a). A colour transformation of the agar plate that contained the ABTS, compared to a control plate without ABTS, is usually interpreted as laccase activity, an indication of the fungal ability to depolymerize coal and its derivatives. However, organisms that lack the ability to degrade ABTS have been implicated and documented in coal depolymerization studies (Odom *et al.*, 1991; Hofrichter *et al.*, 1997a). The presence of extracellular manganese peroxidases (MnP) activity can be screened in a non-agitated submerged culture system in the presence of hydrogen peroxide, by the oxidation of Mn^{+2} to Mn^{+3} (Lundell and Hataka, 1994; Hofrichter and Fritsche, 1997a and b; Willmann and Fakoussa, 1997a). Lignin peroxidase (LiP) activity can be detected by the oxidation of veratryl alcohol (3,4-dimethyoxylbenzyl alcohol) to veratraldehyde (Dehorter and Blondeau, 1993; Lundell and Hataka, 1994; Ralph and Catcheside, 1994a; Hofrichter and Fritsche, 1997a). Besides the screening methods for extracellular oxidative enzymes, other screening methods for the identification of potential coal degraders target the release of water-soluble products from coal particles, or the liquefaction of the coal particle when in contact with mature fungal cultures, which is described as gutation (Hofrichter *et al.*, 1997b; Hölker *et al*., 1997; Yuan *et al.*, 2006). Additional method include clearance zones around colonies on agar containing alkali-solubilized coal (Hofrichter and

Fritsche, 1996 and 1997b) and the clearance of a nutrient broth medium containing alkali-soluble coal when inoculated with microorganisms (Willmann and Fakoussa, 1997b). It is evident that the identification of a larger array of organisms is much needed for the advancement of coal bioconversion technology and is currently based on a limited understanding of the microbial coal bioconversion mechanism, and of the final microbial coal bioconversion products (Klein *et al*., 1999).

Microbial activities on coal such as depolymerization, decolorization, solubilization 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). Depolymerization was defined as the catabolic reduction of higher molecular mass to smaller fractions, which could be accompanied by loss of chromophore; whereas, decolourization is the loss of chromophore without any change in the molecular size. Liquefaction was defined as a change of physical state (solid to liquid state) and should not be confused with solubilization, which is the dissolution of all or part of the coal molecule.

Various investigations have been conducted in a bid to elucidate the microbial coal degradation mechanism. Findings on the microbial enzymatic coal degradation indicated extracellular oxidative enzymes, such as laccases (which includes phenoloxidases) (Eggert *et al*., 1996; Fakoussa and Frost, 1999; Laborda *et al*., 1999; Zavarzina *et al*., 2004; Kluczek-Turpeinen *et al*, 2005), peroxidases and oxidases (Hofrichter and Fritsche, 1996 and 1997a and b; Willmann and Fakoussa, 1997a and b) capable of ligninolytic activities, were utilized by fungi in the degradation of coal and its derivatives. Hydrolytic enzymes such as esterases were also found in association with coal degradation (Fakoussa and Hofrichter, 1999; Laborda *et al*., 1999; Hölker *et al.*, 2002). Other microbial mechanisms utilized for the breakdown of coal and its constituents include the production of alkaline substances (Strandberg and Lewis, 1987; Quigley *et al.*, 1988 and 1989; Stefanova *et al.*, 1999), biological extrusion of metal ion chelators (Quigley *et al.*, 1989; Cohen *et al.*, 1990; Fredrickson *et al*., 1990; Fakoussa, 1994; Hölker *et al*., 1999) and biotensides (Strandberg and Lewis, 1988).

The process of coal biodegradation (solubilization and depolymerization), that had been misconstrued by most researchers to be a simultaneous process, was clarified and redefined to be a two-stage immobilization process (Hofrichter *et al.*, 1997b). Conditions warranting solubilization were preferential under non-enzymatic, alkaline (pH 6 – 9) and at high nitrogen concentrations, whereas, depolymerization of coal humic substances was achieved through the action of ligninolytic enzymes in acidic (pH 3 – 6) medium at low nitrogen concentrations. Biological systems need not be alkaline to support coal solubilization and coal solubilization is increased when microbial growth is supported by aromatic compounds structurally related to lignin (Faison and Lewis, 1990). Also, the solubilization of coal could only be associated with depolymerization of coal HAs under ligninolytic conditions (Pyne *et al.*, 1988; Wondrack *et al.*, 1989; Hofrichter *et al.*, 1997b), since HA are more easily utilized as substrates by microorganisms than the coal matrix (Willmann and Fakoussa, 1997b).

1.3.4.1 Solubilization

A single defined mechanism for microbial coal degradation has been elusive and the common microbial strategy exhibited for coal biosolubilization is yet to be exhaustively revealed (Hölker *et al.*, 1995 and 1997). The production of alkaline substances and the extrusion of chelators are mechanisms that were known to be utilized by microorganisms for coal solubilization. Certain organisms use extracellular oxidative enzymes in coal solubilization when conditions are favourable. When different coal solubilizing organisms were combined in a mixed culture, a synergistic effect on coal biosolubilization was observed, and the predictability of the mechanism of coal degradation with a microbial mixed culture becomes impossible (Pyne *et al.*, 1988; Hofrichter *et al.*, 1997b; Hölker *et al.*, 1999; Klein *et al.*, 1999; Hölker *et al.*, 2002). The utilization of pure cultures could provide a suitable level of predictability coupled with efficient manipulation of regulatory mechanisms. An example of a regulatory mechanism suitable for manipulative means is the pigment bikaverin excreted by *Fusarium oxysporum*, indicating a physiological condition that is inhibitory to coal solubilization (Hölker *et al.*, 1995 and 1997). However, a mixed culture of organisms that include fungi and bacteria are more likely to be more efficient at coal solubilization (Wainwright, 1992).

Microbial solubilization of coal is dependant on the level of the coal oxidation (Willmann and Fakoussa, 1997a), nitrogen content, which determines the pH (Hölker *et al.*, 1999), and certain nutrient sources such as glutamate and gluconate, essential for organisms as a carbon and nitrogen source (Hölker *et al*., 1995, 1997 and 1999; Klein *et al*., 1999; Hölker *et al*., 2002).While fungal growth on glucose and other sugars do promote cell growth, it has been demonstrated to inhibit coal solubilization (Hölker *et al*., 1995 and 1997). In contrast, Dari *et al.* (1995) demonstrated the ability of 15 *Streptomyces* strains to decolorize HA in the presence of glucose on a mineral medium. Low rank coal is easily solubilized by certain bacteria and fungi when grown on mineral media, but solubilization of higher rank coal is heavily dependent on the microbial growth on a complete growth medium (Laborda *et al*., 1997).

Under optimized conditions, the complete solubilization of coal material could take about a week and result in end products that are usually liquid. This can vary from clear to black in colour, contain complex mixtures of primarily polar organic solutes with molecular weights ranging from moderate to high, and have a considerable degree of aromaticity (Wainwright, 1992).

A descriptive account of solubilization of the coal particle has been provided by Hofrichter *et al*. (1997b) (Figure 1-5). The organism would generally secrete gutation droplets in close proximity to the coal particle. Within these droplets, coal solubilization occurs by means of alkaline substances derived from nitrogen containing compounds in the medium. This process, which may be facilitated by the presence of chelators and/or hydrolytic enzymes, is thought to involve the transformation of NH_4^+ and amino acids to alkaline amines, or to their corresponding keto acids and NH_4^+. Re-polymerization reactions (a reversal of depolymerization) may also occur as a result of melanin synthesis, which gives rise to the non-ligninolytic radical generating oxidases and peroxidases that are released into the gutation droplets.

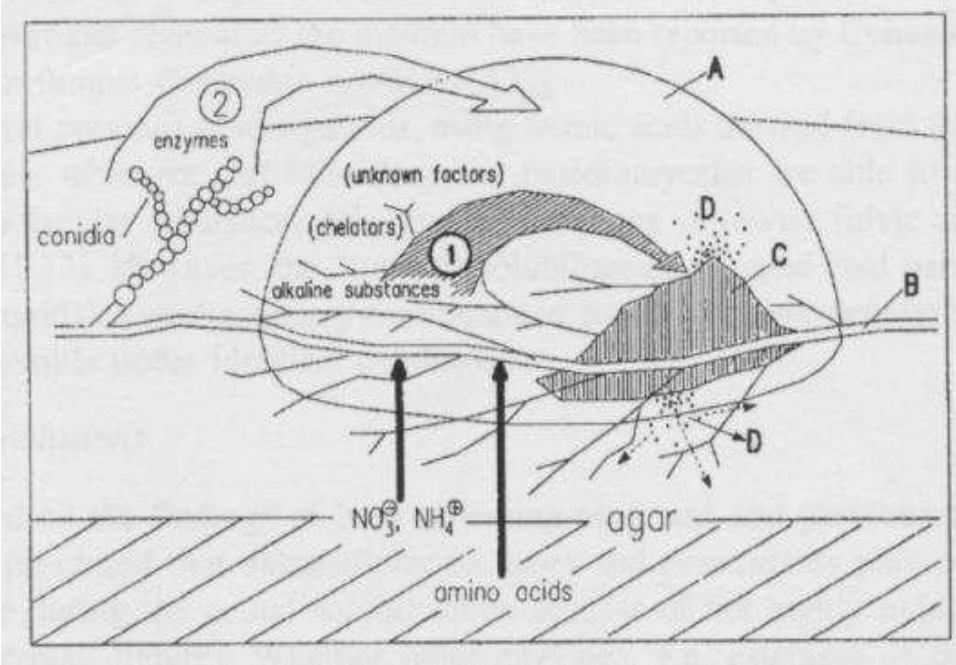


Figure 1-5 Proposed mechanism for the solubilization of low-rank coal by deuteromycetous fungi (moulds). (1) Gutation formation on coal particle (2) Secondary non-lignolytic, radical generating oxidases and peroxidases, which are preferentially formed during the conidiogenesis (melanin synthesis), can be secreted into the droplets of solubilized coal and lead to re-polymerization reactions (additionally enhanced by certain metal ions, e.g. Fe^{2+}, Mn^{2+}, Cu^{2+}). A – gutation droplet, B – fungal hypha, C – coal particle, D – solubilized coal as black liquid (Hofrichter *et al.*, 1997b).

1.3.4.2 Depolymerization

For microorganisms to successfully utilize coal as a carbon and electron source, the complex macromolecular structure of the coal would have to be fragmentized. Microorganisms are able to achieve coal depolymerization by means of non-specific oxidases in conjunction with a number of supporting factors (Hofrichter *et al*., 1999). Basidiomycetes utilize the MnP enzyme complex in the presence of Mn^{3+} chelators (Figure 1-6) to depolymerize low grade coal (Hofrichter *et al*., 1999). The enzyme is a non-specific oxidase and, in the presence of the chelators Mn^{3+}, enables the organism to oxidize a range of high-molecular-mass aromatic and aliphatic compounds when activated by H_2O_2 generated by the fungus (Hofrichter *et al*., 1999). The oxidative strength of the chelators is thought to be increased by the introduction of thiols or unsaturated fatty acids (mediating agents) (Hofrichter *et al*., 1999). An attack on higher molecular weight coal-derived HA would result in the depolymerization and generation of FA of lower molecular weight (Hofrichter *et al*., 1999; Klein *et al*., 1999). Oxidative enzymes such as laccase and LiP have also been shown to catalyze

the depolymerization of HA both *in vivo* and *in vitro* (Hofrichter and Fritsche, 1997b; Klein *et al.,* 1999).

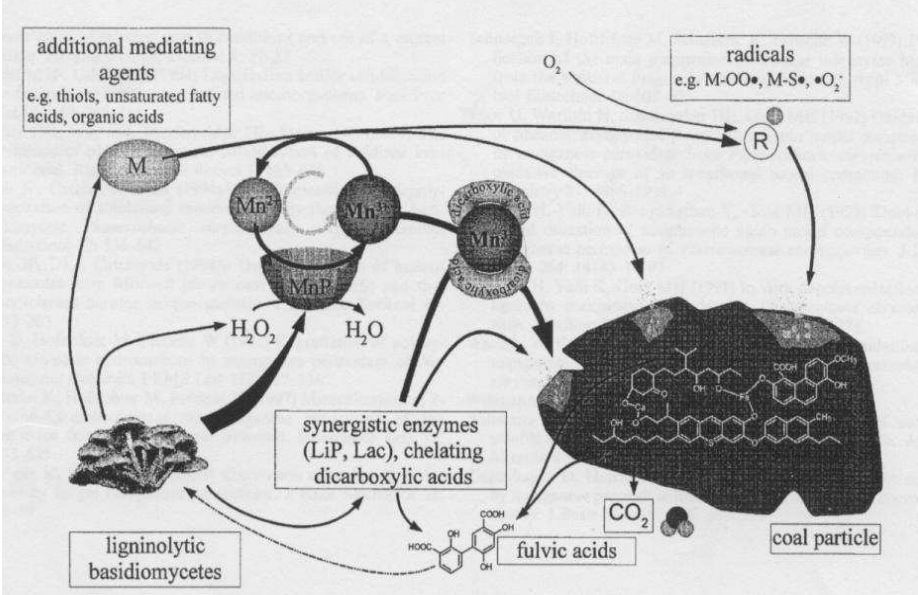


Figure 1-6 Proposed scheme for the degradation of low-rank coal (lignite) by lignolytic basidiomycetes; MnP – manganese peroxidase; LiP – lignin peroxidase; Lac – laccase; M – mediator; R – radical (Hofrichter *et al*., 1999.).

1.4 Industrial application of coal bioconversion technology

The industrial application of coal bioconversion has advanced little since the first implications of fungal bioconversion of coal were noted by Fakoussa (1981). Progress has mainly been made in fundamental research and with an emphasis on laboratory scale processes (Table 1-2). Several basic research and screening methodologies designed to understand and explain the coal bioconversion mechanism have been developed by various research groups to identify microorganisms with the ability to convert coal to valuable by-products or gaseous fuels (Bublitz *et al.,* 1994; Monistrol and Laborda, 1994; Hofrichter and Fritsche, 1996; Osipowicz *et al.,* 1996; Hofrichter *et al.,* 1997a; Klein, *et al.,* 1999; Arctech, 2004; Xin *et al*., 2004a and b). Major drawbacks exist which mainly pertain to the economic viability, the slow conversion rates in the production of biogenic methane, the choice of most active and stable enzymes, the possible inhibition by products, and scale-up of the processes (Fakoussa and Hofrichter, 1999; Klein, *et al.,* 1999). Klein (1999) concluded that future progress

in the field would be dependent on advances made in the area of process development.

Table 1-2 Advances in coal microbiology (Modified from Fakoussa and Hofrichter, 1999).

Year	Step of progress	Reference
1981	Effects on hard coals by *Pseudomonas* strains, simultaneous biotensides-excretion	Fakoussa (1981)
1982	Solubilization of lignite to droplets on agar plates by fungal action	Cohen and Gabriele (1982)
1986f	Acceleration of solubilization by pretreatment of coal	Scott *et al*. (1986), Grethlein (1990).
1987f	First solubilization mechanism elucidated: production of alkaline substances (fungi + bacteria)	Quigley *et al*. (1988, 1989).
1988f	Second mechanism elucidated: production of chelators (fungi)	Cohen *et al*. (1990).
1989	First product on market: Solubilized lignite as fertilizer	Arctech (2004).
1991f	Evidence that chelators alone are not responsible for all effects	Fakoussa and Willmann (1991), Fakoussa (1994).
1994f	Decolorization and reduction of molecular weight of soluble lignite-derived humic acids proves catalytic = enzymatic attack	Ralph and Catcheside (1994b), Hofrichter and Fritsche (1997a).
1997	*In vitro* systems shown to degrade humic acids and attack matrix and coal particles	Hofrichter and Fritsche (1997b).
1999	First fine chemical produced successfully from heterogeneous humic acid mixtures to polyhydroxyalkanoates (Bioplastic) by pure cultures!	Füchtenbusch and Steinbüchel (1999).
Future	Turnover-rates, choice of most active and stable enzyme, possible inhibition by products and scale-up are remaining problems to be solved	

f; Following years

1.4.1 Electricity generation: The Rheinbraun's concept

The biotechnology of lignite conversion for use in electricity generation was investigated in some detail by an international group of researchers sponsored by Rheinbraun, Germany's largest lignite producer (Reich-Walber *et al*., 1997). While substantial progress was made in understanding the mechanisms of coal

biosolubilization, process application remained theoretical. In the proposed process, the biosolubilization of 27 million tonnes of annual run-of-mine lignite would be undertaken in a 150 000 m^3 bioreactor which would be fed at the rate of 4000 t/hr. The feed was to be milled and screened to an average size of 10 mm before contact with the fungal biocatalysts. Water, sulphur and ash would be removed from the solubilized product before it was piped to a steam power plant, 22 km away. A cost-benefit exercise was conducted for the entire process and Reich-Walber *et al*. (1997) reported that approximately 1 % of energy loss would be due to biochemical insertion of oxygen into the coal matrix

Although a number of reactor configurations had been investigated in this study, a granular bed dual-phase system incorporating both trickling bed and fluidized bed operations was selected (Reich-Walber *et al*., 1997). In the first phase, the granular bed would be operated as a trickling filter and the milled lignite fed semi-continuously from above. Bioconversion of the lignite would take place in this phase of the operation. The bed is then fluidized with water and air fed from the bottom, allowing the bed to expand and discharge the solubilized product. The growing agglomeration of the granular bed in the first phase, a drawback of fixed bed systems, is overcome by breaking up clogging particles and thus ensuring a homogenous distribution of nutrients. This provides the particle residence time required for solubilization (Reich-Walber *et al*., 1997).

Despite the study showing a number of unfavorable factors, primarily the organism's carbon requirement which consumes an estimated 25 % of coal input, the provisional costing showed a cost reduction for power generation when compared to the best available coal power plant technology. The cost benefits arise primarily in the use of smaller boilers, simplified fuel handling, an almost ash-free fuel and the virtual elimination of sulphur emissions. In spite of the perceived benefits, the Rheinbraun system has yet to be implemented.

1.4.2 Coal bioconversion bioreactor designs

Microbial processes are used in various remediation operations and are gradually gaining acceptance in large scale industrial processes (Acevedo, 2000). Heap-leach and open and enclosed bioreactors are process environments that are commonly used in the large-scale industrial application of microorganisms (Scott, 1990; Bredwell *et al.*, 1999; Shumkov, 1999; Acevedo, 2000; Hölker and Höfer, 2002). Heap-leach operations are simple to operate and are usually adopted for large scale processing of minerals in the extraction of copper, gold and uranium. However, the large volume of materials processed makes it difficult to ensure effective monitoring and operational controls. Bioreactor operations are generally used for the processing of smaller volumes of materials and in wastewater treatment operations. These allow for close monitoring and manipulation of operational parameters, resulting in more stringent control of process performance and product yield (Acevedo, 2000).

Various bioreactor designs for use in coal process technology have been investigated and reported in the literature (Table 1-3). Particular consideration has been given to column reactor systems such as the fluidized and fixed bed bioreactors for coal biosolubilization processes (Figure 1-7) (Scott, 1990; Wainwright, 1992). These reactors were designed to retain bioreagents and reportedly delivered high productivities while allowing effective operational control at ambient temperatures and pressures (Scott, 1990). In the fluidized-bed bioreactor (Figure 1-7i), small particles of coal are suspended in an up-flowing aqueous stream containing the inoculated fungus and appropriate nutrients required for vegetative growth. Air is supplied at the bottom of the bioreactor and the solids are removed, leaving the liquid product for further modification. In the fixed-bed (or packed-bed) bioreactor system for coal solubilization (Figure 1-7ii), the fungus is grown on a stationary bed of coal at the top of a column exposed to humid air and a nutrient solution is introduced at the top of the column. The liquid product generated is eluted at the bottom of the column.

Of particular interest is the solubilization of coal carried out in a non-sterile columnar solid substrate fermentation bioreactor inoculated with *Trichoderma atroviride* (CDS 349) (Table 1-3) (Hölker and Höfer, 2002). Over a 40 day period, 1.5 kg of lignite

packed in a 25 L columnar bioreactor was solubilized by the fungus. After a six day lag phase, solubilization commenced and a product containing 70 % HA and 30 % FA in a 4 g/L liquid effluent/day was produced. Steady state coal solubilization was maintained for a further period of 30 days, after which the solubilization decreased until day 40 as indicated by the decrease in absorbance at 450nm. Upon shut down, 41g dry weight of residual lignite and 14.8 L residual solution remained in the bioreactor. The lignite was not pre-treated prior to solubilization and because the system was non-sterile, bacterial contamination of bioreactor content was observed after 20 days and subsequently became well established in the bioreactor. Consequently, *T. atroviride* became overwhelmed and could not be detected after 40 days using light microscopy (Hölker and Höfer, 2002).

Table 1-3 Bioreactor types for a sustainable coal process technology.

Bioreactor type	Aim/method	Author
Coal slurry reactor	Pre-combustion microbial desulphurization of coal by means of a 60 L coal slurry reactor inoculated with a mixed culture of *Thiobacillus ferrooxidans* and *Thiobacillus thiooxidans*	Pandey *et al.*, 2005
Stirred tank bioreactor	Production of manganese peroxidases (active in oxidizing humic substance) in a 300 L stirred tank bioreactor using a mixed culture of *Nematoloma (Hypoloma) frowardii* strain b19 and *Clitocybula dusenii* strain b11	Nüske *et al.*, 2002
Solid substrate fermenter	Solubilization of coal in a semi-continuous fed-batch culture of *T. atroviride*	Hölker and Höfer, 2002
Flood rain reactor	Coal depyritization via a combined physical and microbial processes, which involved a periodic fluidization with a mixed bacterial culture and draining of the coal bed	Andrews *et al.*, 1994
Fluidized-bed bioreactor	Solubilization of coal by means of a modified hydrogenase enzyme extracted from *Proteus vulgaris*. The modification of the enzyme used 2,4-dinitrofluoro-benzene which attaches dinitrophenyl groups to the enzyme, there by increasing its hydrophobicity and solubility	Scott *et al.*, 1992

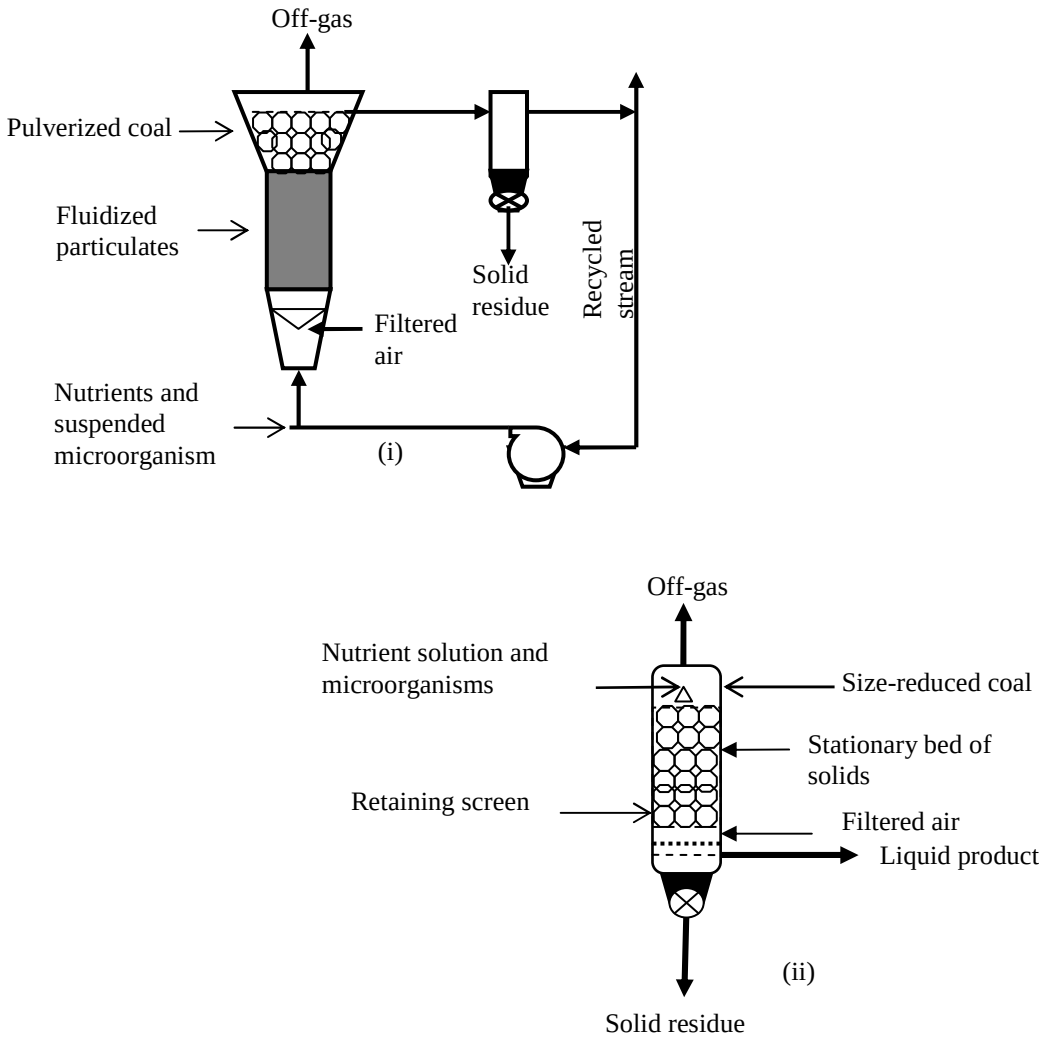


Figure 1-7 (i) Fluidized bed with continuous liquid phase and (ii) Fixed-bed contactor with continuous gas phase.

1.4.3 Biomethanation of coal

Methane is a major component of natural gas (Golombok *et al.,* 2004), it is one of the most abundant carbon feed stocks available (Periana *et al.,* 2005) and has been identified as the most desired and environmentally benign source of fossil fuel energy available (Luca Technologies, 2005). A number of groups have investigated and reported success in the bioconversion of lignite to methane (Isbister and Barik, 1991). The anaerobic degradation of Texan lignite to methane using a termite derived microbial mixed culture has been reported by Isbister and Barik (1991). However, research on German and Australian lignite yielded no substantial degradation, and biosolubilized extracts from lignite were found to strongly inhibit methane production

from sewage sludge (Klein *et al*., 1999). The toxicity of lignite is thought to be a limiting factor in lignite methanogenesis and low loading rates, as well as the addition of an organic nutrient supplement, have been shown to be required (Klein *et al*., 1999). The enhanced oxidation state of the lignite is considered to be an important requirement for bioconversion (Quigley *et al.*, 1988 and 1989; Achi, 1994a; Fakoussa, 1994; Hofrichter *et al.*, 1997b; Laborda *et al*., 1997; Machnikowska *et al.*, 2002; Başaran *et al.*, 2003). A two-step process design has thus been proposed that would incorporate an aerobic oxidative cleavage step of the lignite macromolecule followed by an anaerobic fermentation/methanogenesis step for the production of methane. Most of the biogasification work has focused on lignite with little success being reported for hard coals (Arctech, 2004). Klein (1999) has identified that much fundamental research and developmental work is required to see large scale progress in this area.

Arctech Inc. USA, a spin-off company of the Environmental Science and Technology division of the Atlantic Research Corporation, had been in the field of coal-derived HA research and beneficiation since 1992 (Arctech, 2004). MicGas^{TM}, a patented bioconversion process technology, developed by Arctech Inc, is a widely publicized coal technology (Figure 1-8). The lignite bioconversion technology is based on the application of natural microorganisms that have been adapted to anaerobically convert lignite into clean fuels, coupled with the reduction of greenhouse carbon dioxide gas emission. Unlike conventional coal gasifiers, the solid residue generated from MicGas^{TM} anaerobic treatments are rich in organic humic matter (Arctech, 2004). The residual coal left in the bioreactor is collected and subjected to further biochemical processes, extracting HA, which is used in environmental remediation and fertilizer application in agriculture (Arctech, 2004).

The organisms utilized in the MicGas^{TM} biotechnology were generally isolated from the gut of wood and humus eating termites, and adapted for coal bioconversion in the presence of appropriate nutrients. The bioconversion process is accomplished in a three-step process. In the first step, a hydrolytic and fermentation process, the microbes are conditioned in the presence of the appropriate nutrients to convert the coal substrate into volatile organic acids, primarily acetate and CO_2. In the second step, a methanogenic process, the gas and liquid effluents generated from the first step

are converted by the microbes to methane through hydrogenating the acetate and CO_2. The methane gas is collected and the residual coal which remains unconverted is sent to the third step, where it is converted, via a biochemical process, to products that are utilized in agriculture and environmental remediation (Figure 1-8).

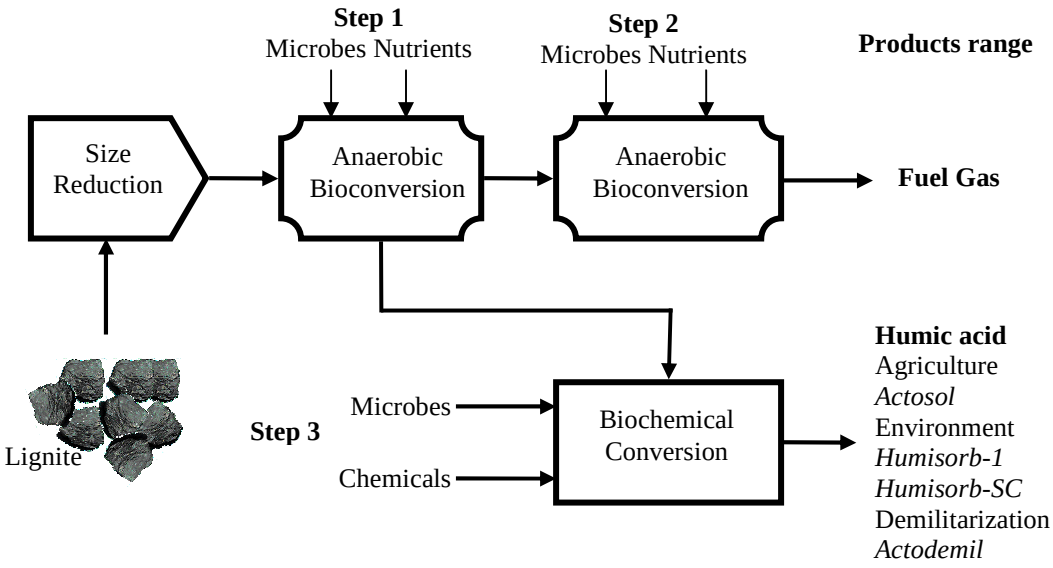


Figure 1-8 Schematic illustration of MicGas™ coal bioconversion technology designed to produce clean energy from coal coupled with the reduction of greenhouse carbon dioxide gas emission (Arctech, 2004).

In 2004, Luca Technologies reported the discovery of real-time natural gas production in Wyoming's Powder River Basin located in the USA (Haas and Kureczka, 2004). These naturally occurring methane production zones, referred to as geobioreactors, are subject to the presence of certain indigenous anaerobic organisms that are capable of degrading the large hydrocarbon molecules present in oil, oil shales and coal, to methane (Haas and Kureczka, 2004 and 2005). Prior to this discovery, it had been generally accepted that much of the natural gas present in coal fields was generated millions of years ago when the organic deposits were less mature and closer to the surface. However, recent research findings conducted by Luca Technologies suggest the presence of active methanogens in the anaerobic core samples from the Powder River Basin, that are capable of converting coal to methane in present conditions (Luca Technologies, 2004). The biogenic methane generation from coal by these *in situ* methanogens was shown to be elevated by the introduction of specific nutrients and other amendments, and suppressed either by sterilization or introduction of

oxygen. The introduction of radio-labeled CO_2 to the anaerobic core of the Powder River Basin was demonstrated to be effectively converted to radio-labeled methane. This indicated a real-time production of methane from coal *in situ* by the methanogens present there (Luca Technologies, 2004).

The occurrence of biogenic methane was initially believed to be ubiquitous in marine and freshwater sediments at depths of several kilometers, in tertiary basins that had high sedimentation rates (Martini *et al*., 1996). Martini *et al.* (1996) first reported the occurrence of significant volumes of bacterially generated methane gas in organic-rich shales at depths of less than 600 meters in the Upper Devonian Antrim shale, along the northern margin of the Michigan basin (USA). In a similar report to the real-time natural gas production in Wyoming's Powder River Basin, Luca Technologies has also reported the confirmation of *in situ* real-time methane generation in Utah's Monument Butte Oil Field (Haas and Kureczka, 2005). Molecular studies performed on the microbial population responsible for the *in situ* methane gas generation revealed the presence of *Clostridia* and *Thermotoga* spp. Both organisms are established anaerobic thermophiles with *Clostridia* spp. known to have diverse metabolic pathways and *Thermotoga* spp. known to oxidize hydrocarbons to alcohols, carbon dioxide and organic acids such as acetic acid (Orphan *et al.*, 2000).

14.4 Humic acid production

The extraction of HA from low grade coal prompted large scale HA production for agricultural purpose (Lobartini *et al*., 1992). The bulk of the scientific publications on the agricultural effectiveness of coal-derived HA have been made mainly in local publications in the Far East with, apparently, very promising prospects. The most extensive and well documented work on coal-derived HA as a soil amendment, which included a cost benefit estimation with viable economic possibilities, was conducted and documented in India (Mazumdar, 1982). In this investigation, coal-derived HA was obtained via a two-step process of HNO_3 oxidation and ammonification (NH_4OH) of the coal material, and resulted in the formulation of the product "nitrohumic" (Mazumdar, 1982). The agricultural soil amendment efficacy of the coal-derived HA was then demonstrated in both pot and field trials. The effects could

be optimized when a 10 – 20 % mixture with traditional fertilizer was applied (Mazumdar, 1982).

Lobartini *et al.* (1992) investigated and characterized the commercial HA that were obtained from lignite and non-lignite deposits, and their effects on plant (*Zea mays*) growth were studied in a greenhouse. The results demonstrated that commercial humates (lignite and non-lignite) were similar in C and H content but with higher N and carbohydrate content than in the lignite-derived HA. The growth effect of commercial HA (lignite and non-lignite) on *Zea mays* were comparable with soil derived HA.

AGRON Co. (Marble Hall, South Africa), a domestic coal-derived HA production company, and a supplier of HA fertilizer to local farmers in its locality in Mpumalanga, South Africa, attested to the successful increase in crop production as a result of the introduction of coal-derived HA to fertilizer. Bio Ag technologies (1999) made use of HA as one of the most important components of one of its fertilizer products Bio-Liquid Complex® (BLC). Bio Ag Technologies attributed the success of BLC in agriculture to the molecular structure of HA.

Coal-derived HA had been widely publicized for the purpose of soil amendment; however, most claims on the agricultural effectiveness of coal-derived HA as soil amendments are found mainly in magazines and on internet web pages, but not in scientific journals and publications (Fulvica Bioscience's Health Alert; Lobartini *et al.*, 1992; Kline and Wilson, 1994). Negative publicity on the deleterious nature of coal-derived HA on agricultural produce, increased toxicity in plants and distorted and retarded shoots are also a concern (Humate.net). A fraction of the components of coal-derived HA may have the ability to stimulate plant growth; however, accumulation in soil may immobilize soil phosphorus under certain conditions creating a negative effect on plant performance (Anglo Coal South Africa, pers. comm.). It was also suggested that coal-derived HA as soil amendments are based on sound principles and that the potentials for their beneficiation does exist. The economics and time involved to increase organic matter through commercial products, rather than through the more traditional organic-matter-building programs, should be seriously considered (Anglo Coal South Africa, pers. comm.). Furthermore,

implications that the HA is a precursor for carcinogens are a major cause for concern (Oliver and Visser, 1980).

Except for the few citations on coal-derived HA for soil amendments mentioned above, it would appear that critical scientific researching and publications on coal-derived HA as soil amendments for agricultural purposes ended in the early 1990s. This indicates a discontinued scientific research funding possibly due to the negative publicity in some sectors (Kline and Wilson, 1994). Kline and Wilson (1994) argued that the continuous utilization of coal-derived HA for soil amendment by farmers is an indication of its effectiveness and warrants continued scientific investigation and documentation.

1.5 The phytobioconversion of hard coal

Given the implications of coal bioconversion in both the energy and environmental remediation sectors, the Environmental Biotechnology Research Unit (EBRU) at Rhodes University, South Africa, undertook an investigation of the fundamental and applied processes associated with coal bioconversion.

During a broad based sampling of hard coal waste dumps for coal degrading organisms, the sporadic growth of *Cynodon dactylon* (Bermuda grass) was observed to occur on these dumps (Figure 1-9). This unanticipated observation raised a number of questions concerning the biological processes involved in enabling the grass to establish itself in this harsh environment. It was evident from initial observations that the root zone of *C. dactylon* was well populated with both mycorrhiza and possibly free living rhizospheric fungi (Rose, pers. comm.).

The need to understand what was happening in this system indicated both academic interest and possible commercial application. The presence of a fungal-dominated system in the sub-layer had the potential to generate considerable knowledge in the field of hard coal biosolubilization process development in which little progress has been reported (Klein, 1999).

Figure 1-9 *Cynodon dactylon* growing on the bituminous hard coal surface of an unrehabilitated coal dump site.

Subsequent investigation showed that the *C. dactylon* root zone did contain a heavy mycorrhizal infection with the breakdown of the surrounding coal to a humic soil-like material which had an earthy smell, possible a geosmin odour, which was not present in adjacent coal sub-layers. Characterization of the humic material in the root zone demonstrated a 20 – 40 % HA content (Appendix 1-A).

It appeared in this system that microorganisms were apparently associated with an oxidative weathering and breakdown of hard coal structure. Given the growth of the grass plant (in this case *C. dactylon*) at the same location, it was proposed that an exchange of nutrients between the plants and the rhizospheric organisms could be occurring. The requirement for an organic carbon source for fungal-coal biosolubilization has been reported (Hölker *et al*., 1995, 1997, 1999 and 2002; Willmann and Fakoussa, 1997a; Hofrichter *et al*., 1997b; Klein *et al*., 1999) and it appeared that the conditions for the solubilization and depolymerization of the coal substrate thus possibly existed in the *C. dactylon* root zone.

It was thus proposed that bioconversion of coal observed in the *C. dactylon*/coal system, in itself a novel observation, may also produce a novel approach to a longstanding and difficult problem associated with the development of coal bioconversion into an industrializable process. It was proposed that the investigation and development of this system and the ability to manipulate the process of coal bioconversion on a large industrial scale may provide the elements of a platform technology that would enable the beneficiation of waste coal in a range of possible applications. Based on these insights the hypothesis for the current research study was developed.

1.6 Research hypothesis

The colonization of the open coal dump surface by grass species such as *C. dactylon* is associated with microbial activity and coal bioconversion within the rhizosphere of the plant root/coal system.

The system provides a functional model for both understanding the microbially-driven bioconversion of bituminous and anthracitic hard coals in the natural environment and this also has potential applications in the development of a functional bioprocess environment for the treatment of large coal volume throughput.

1.6.1 Research objectives

In order to understand the various conditions promoting the growth of *C. dactylon* on BHC dumps, and then to address potential process applications, the following research objectives were identified:

- To undertake the screening of the plant/coal dump environment to identify and characterize microorganisms in the rhizosphere of this system, which are active in coal bioconversion;

- To develop and evaluate experimental systems in which rhizospheric microbial coal degradation activity can be investigated under controlled laboratory conditions;
- To interrogate these experimental systems as well as the natural environment, to characterize the biological processes active within the *C. dactylon*/coal rhizospheric system and to derive an explanatory model accounting for the activities within this system;
- To evaluate the potential application of this system in advancing process development of coal biotechnology and specifically with the use of bituminous and anthracitic hard coals as feedstock.

Chapter two

Screening of the *Cynodon dactylon*/coal rhizosphere for microorganism active in coal bioconversion

2. Introduction

Microorganisms that have been implicated in the coal bioconversion process have mainly been sourced from sites such as coal outcrops, coal fines, coal waste dumps, oil wells, settling ponds, and fresh and marine water sediments (Gupta and Birendra, 2000). Fungal isolates that have been implicated in coal bioconversion include *Phanerochaete chrysosporium* (Achi, 1993; Ralph and Catcheside, 1994a; Bumpus *et al*., 1998; Başaran *et al*., 2003; Elbeyli *et al*., 2006a), *Trametes (Polyporus) versicolor* (Cohen and Gabriele, 1982; Pyne *et al*., 1988; Cohen *et al*., 1990; Bumpus *et al*., 1998; Fakoussa and Frost, 1999; Götz and Fakoussa, 1999; Başaran *et al*., 2003), *T. atroviride* (Hölker *et al*., 1997, 1999 and 2002; Hölker and Höfer 2002; Pokorný *et al*., 2005), *Fusarium oxysporum* (Hölker *et al*., 1995, 1997 and 1999) and *Penicillium* spp. (Achi, 1994a and b; Laborda *et al*., 1999; Pokorný *et al*., 2005; Yuan *et al*., 2006). *Aspergillus* spp. have also been demonstrated to be active in coal solubilization (Monistrol and Laborda, 1994; Torzilli and Isbister, 1994; Laborda *et al*., 1999) as well as in coal desulphurization (Acharya *et al*., 2005). In some cases bacterial cultures have been demonstrated to be active in coal solubilization (Fakoussa, 1988; Andrews *et al*., 1994; Torzilli and Isbister, 1994; Hölker *et al*., 1997)**.**

While protocols utilized for the screening and identification of microorganisms that are active in coal degradation have not been standardized, Klein *et al*. (1999) noted that various research groups have established customized screening protocols that are based on the aims and objectives of their particular research program. In general, the design of the various customized screening protocols have included the identification of the extracellular oxidative enzymes (Eggert *et al*., 1996; Hofrichter and Fritsche, 1996 and 1997a and b; Willmann and Fakoussa, 1997a and b; Fakoussa and Frost, 1999; Fakoussa

and Hofrichter, 1999; Laborda *et al*., 1999; Hölker *et al.*, 2002; Zavarzina *et al*., 2004; Kluczek-Turpeinen *et al.*, 2005), the secretion of alkaline substances (Strandberg and Lewis, 1987; Quigley *et al*., 1988 and 1989; Hofrichter *et al*., 1997b; Stefanova *et al*., 1999) and the biological extrusion of metal ion chelators and biotensides (Strandberg and Lewis, 1988; Quigley *et al*., 1989; Cohen *et al*., 1990; Fredrickson *et al*., 1990; Fakoussa, 1994; Hölker *et al*., 1999). Aspects of these studies were followed in the development of a standardized protocol for the screening of microorganisms from the South African coal dump environment reported in this study.

NOTE: The role of the mycorrhiza fungal components of the *C. dactylon*/coal rhizosphere system was investigated in a separate study undertaken concurrently in this laboratory.

2.1 Objectives

The objectives of the screening and strain selection study were to:

- Develop and use a standardized protocol for the rapid screening of a large number of samples and the identification of a potentially broad array of microbial species showing activity in coal bioconversion;
- Conduct a survey of different South African geographical areas and environmental conditions, that could harbour microorganisms with the potential to break down coal and its derivatives;
- Characterize the most active microbial species isolated.

2.2 Materials and methods

2.2.1 Sample collection

Samples were collected from the field in various coal associated environments in South Africa, and the geographical locations of these screened areas are shown in Table 2-1. Sample collection at these sites was narrowed down to areas which showed apparent

microbial activity. Of particular interest was the sporadic invasion of *C. dactylon* that was observed to grow directly on the surface of unrehabilitated BHC dumps (Figure 1-9). Root material was sampled where coal breakdown to a humic soil-like material was observed and where a geosmin odour possibly indicated the presence of fungal activity. Collected samples were first stored in a 25 mL sterile sample bottle, sealed and kept in a cooler box containing ice for a day before being transported back to the laboratory where they were stored at 4 ^{o}C and screened within two weeks.

Table 2-1 Geographical location of environmental samples collected in the screening study.

Sampling sites	**Site description and geographical location**
Coal plume (Carolina)	Closed underground mine site in Mpumalanga. (Latitude: 26^{o} 07'S;Longitude: 30^{o} 12'E)
Excelsior Mine	Closed coal mine Witbank area, Mpumalanga. (Latitude: 29^{o} 90'S;Longitude: 27^{o} 15'E)
Greenside Colliery	Operational coal mine, Witbank area, Mpumalanga. (Latitude: 26^{o} 48'S;Longitude: 28^{o} 77'E)
Good Hope Colliery	Witbank area, Mpumalanga.
Klein Kopje Colliery	Operational coal mine, Witbank area, Mpumalanga (Latitude: 26^{o} 48'S;Longitude: 28^{o} 77'E)
Kromdraai Mine	Operational coal mine, Witbank area, Mpumalanga. (Latitude: 25^{o} 98'S;Longitude: 27^{o} 77'E)
Navigation Colliery	Mpumalanga (Witbank area). (Latitude :25^{o} 55'S;Longitude :29^{o} 8'E)
Old coal mine (Leeunek)	Closed coal mine site located in the Vryheid area, KwaZulu Natal. (Latitude:28^{o} 33'S;Longitude:30^{o} 57'E)
Walker's Mine (Leeunek)	Closed coal mine site located in Vryheid area, KwaZulu Natal. (Latitude :28^{o} 33'S;Longitude :30^{o} 57'E)

Two fungal isolates that had been demonstrated to exhibit coal bioconversion potential were obtained from the South African Agricultural Research Council (ARC) to serve as comparative reference control cultures. These isolates included *Trametes versicolor* (South African: PPRI 4835) (Cohen *et al*., 1990; Bumpus *et al*., 1998; Götz and Fakoussa, 1999) and *Phanerochaete chrysosporium* (South African: PPRI 5328) (Ralph and Catcheside, 1994a; Mönkemann *et al*., 1997; Başaran *et al*., 2003).

2.2.2 Screening program

2.2.2.1 Step 1: The 2 % Duff-agar selective medium

All reagents used in this study, unless otherwise stated, were sourced from Merck, South Africa. A 2 % Duff-agar medium was developed for use in the first screening step to simulate the coal environment that is deprived of other available carbon sources. Duff is fine dry coal produced in coal-washing plants with an upper size limit ~ 0.95 mm. The 2 % Duff-agar was prepared as 20 g of Duff added to 15 g agar-agar in 1 L of milliQ H_2O (Synergy^{TM}, Millipore). The 2 % Duff-agar was autoclaved at 120 ^{o}C for 15 minute and poured into 90 mm Petri dishes. Plates were inoculated and then sealed with parafilm, inverted and incubated at 28 ^{o}C. In the first round of selection, fungal isolates were selected on the basis of any growth on the 2 % Duff-agar medium. Plates with visible colonies were picked and transferred to freshly prepared 2 % Duff-agar plates after 4 weeks of incubation. The second selection was made on the basis of the demonstration of a rapid luxuriant growth on 2 % Duff-agar. Controls were set up using water agar plates to which the coal Duff had not been added to exclude false positives due to the ability of some organisms to grow on agar alone.

Cultures selected for their vigorous growth on 2 % Duff-agar were then transferred for maintenance on 2.5 % potato dextrose agar (PDA) plates and assigned an EBRU Culture Collection Number (ECCN). For temporary storage, inoculated PDA slants were prepared in triplicate and refrigerated at 4 ^{o}C. For permanent storage, five agar plugs of 5 mm, obtained from actively growing zones of the PDA plates, were collected and submerged in 50 % glycerol (Analar, BDH) in sterilized 1.5 mL microfuge tubes (Eppendorf, Merck South Africa) and stored at - 80 ^{o}C. All permanently stored cultures were re-inoculated once every six months to test for regeneration. The competence of the reference control cultures were tested for growth on 2 % Duff-agar before their inclusion in the culture collection. Ludwig's insect spray was used for mite control.

2.2.2.2 Step 2: Extracellular enzymatic activity

In the second screening step, the extracellular oxidative enzyme activity of the cultures selected in step one was assayed following the method described by Hofrichter *et al*. (1997b). However, a complete growth medium was used in this study compared to a nutrient medium suitable for deuteromycetes and basidiomycetes that was reported by Hofrichter *et al*. (1997b). The substrate ABTS (0.02 %) was filter sterilized (MAGNA 0.22 µm nylon filters) and introduced to previously sterilized (autoclaved at 120 ^{o}C for 15 min) 2.5 % PDA held at 40-45 ^{o}C. The ABTS/PDA preparation was poured into 90 mm Petri dishes and allowed to solidify. Prepared plates were inoculated with a 5 mm plug inoculum of pure culture, sealed with parafilm, inverted and incubated at 28 ^{o}C. Colour transformation from the characteristic yellow PDA colour to indigo-green or purple, which is indicative of the oxidative degradation of ABTS, was monitored. Control plates were prepared in which ABTS was omitted.

2.2.2.3 Step 3: Gutation test

In the third screening step, gutation droplet formation on the coal particle was monitored as described by Monistrol and Laborda (1994). The coal pre-treatment method was as described by Machnikowska *et al*. (2002). Bituminous hard coal samples (Klein Kopje Colliery, Witbank, South Africa), were utilized for the gutation studies. These were first autoclaved at 120 ^{o}C for 15 minute and then pre-treated with 4.69 N HNO_3 (1:50 w/v) in a 200 mL Erlenmeyer flask for 48 hours at room temperature on a rotary shaker at 150 rpm (Labcon 3100u). The pre-treated coal was then washed repeatedly with sterilized water until a clear rinsate of pH > 5 was obtained, and dried in an oven at 40 ^{o}C. Several particles of pre-treated coal (~ 5 mm^3) were placed on the fungal mat growing on PDA plates. Gutation droplet formation, as well as the response of the culture to the coal substrate, was recorded. Cultures were then ranked based on gutation formation, from no reaction, moderate gutation formation, to complete or heavy gutation formation, within three to seven days contact time. Untreated coal particles were similarly deposited on the fungal mat as a control in each study.

2.2.2.4 Step 4: Submerged growth in liquid culture

In the fourth screening step, the activity of the selected cultures was examined in nitrogen-rich and nitrogen-deficient conditions, in a method following Hofrichter *et al*. (1997b). Here glucose was omitted and a commercial grade HA (AGRON Co. Marble Hall, South Africa) was introduced to create a carbon-stressed environment deficient in easily accessible and degradable carbon. The nitrogen-rich broth medium consisted of sodium glutamate (0.2 %), $NaNO_3$ (0.3 %), K_2HPO_4 (0.2 %), $MgSO_4.7H_2O$ (0.05 %), KCl (0.05 %) and HA (0.1 %) and pH adjusted to pH 7.0. For the nitrogen-deficient medium, sodium glutamate was excluded from the formula but all other conditions remained the same.

The inoculum was prepared by washing actively growing fungal mats on PDA plates with sterilized water. This was repeated until a concentrated suspension of spores and mycelia was obtained. A 2 mL concentrate was used to inoculate 100 mL medium in a 250 mL Erlenmeyer flask. Samples were incubated at 28 ^{o}C for five days on a rotary shaker at 150 rpm. The formation of fungal biomass resulting from the spore inoculation was monitored visually. The pH of the medium was measured and HA ($A_{450\ nm}$) and FA ($A_{280\ nm}$) in the resultant culture supernatant was analyzed spectrophotometrically (ThermoSpectronic Aquamate v4.60), as described by Fakoussa and Frost (1999). Uninoculated flasks containing medium served as controls.

Analysis of humic and fulvic acid

The flask samples were first filtered using a 36 µm mesh. Humic acid in the filtrate was precipitated by reducing the pH (< 1) using a minimal volume of concentrated HCl (32 %). The filtrate was allowed to stand for 1 hour before it was centrifuged (Eppendorf 5415D desktop centrifuge) at 3220 × g for 90 mins at 10 ^{o}C. The supernatant was decanted and spectrophotometrically analyzed at 280 nm for FA (Fakoussa and Frost, 1999). The pellet, containing HA, was resuspended in 0.1 M NaOH and made up to 10

mL following which it was centrifuged at 3220 × g for 90 mins at 10 ^{o}C to remove any suspended material before the supernatant was spectrophotometrically analyzed at 450 nm for HA (Fakoussa and Frost, 1999).

2.2.2.5 Step 5: Comparison with reference control cultures

2.5 % PDA and glutamate agar (GA) were prepared as in Hofrichter *et al*. (1997b). Glutamate agar was made up in milliQ water using $NaNO_3$ (0.6 %), K_2HPO_4 (0.31 %), $MgSO_4.7H_2O$ (0.05 %), KCl (0.05 %), L-glutamate (0.2 %), agar-agar (1.5 %). The pH was adjusted to 5.7. The media were prepared in Schott bottles and autoclaved at 120 ^{o}C for 15 minute after which the molten agar was poured into 90 mm Petri dishes and allowed to solidify. Prepared PDA and GA plates were inoculated with a 5 mm plug of inoculum punched out from a Petri dish culture which included the selected culture organisms, *Chrysosporium* (PPRI 5328, ARC, South Africa) and *T. versicolor* (PPRI 4835, ARC, South Africa). Culture plates were sealed with parafilm, inverted and incubated at 28 ^{o}C for 5 days before use.

In the pre-treatment of the BHC, 6 N, 4 N, 2 N, 1 N and 0.1 N HNO_3 solutions, prepared from the stock concentrate of 65 % HNO_3 (Appendix 2-A), were used to determine the least concentration of HNO_3 pre-treatment required for gutation formation. The pre-treatment method was as described in Section 2.2.2.3 and it followed that of Machnikowska *et al*. (2002). The BHC samples (finely ground and particulate) were pre-treated and washed until pH > 5. To each of the PDA and GA culture plates, 1 g of ground (< 1 mm) and a single particle of about 5 g pre-treated BHC were placed on the fully grown fungal mat. About 5 g of commercial grade HA (AGRON Co.) was also deposited on each culture mat. Washed and sterilized untreated ground and particle BHC served as the control. The cultures were observed for gutation droplet formation and the response of the culture to the different coal substrates was monitored over time as described by Monistrol and Laborda (1994).

2.3 Results and discussions

The screening and characterization of the environmental samples continued throughout the study and cultures were isolated, characterized and added to the culture collection.

2.3.1 2 % Duff-agar (Selective media) screening

From over 2000 environmental samples collected in the field, 109 isolates showed successful growth on the 2 % Duff-agar (Figure 2-1), and were entered into the culture collection for follow-up evaluation.

Hobbie *et al*. (2004) have reported the ability of saprophytic fungi to obtain some of their carbon requirement from agar. However, none of the 109 isolates that were entered in the culture collection from this study showed any growth on water agar control plates. Thus, isolates were assumed to access nutrients from the coal Duff only. This observation strengthened the validity of the Duff-agar screening methodology. The growth on Duff-agar medium provided an initial indication that the selected cultures were able to utilize the coal substrate as a carbon source. Notable amongst the 109 cultures, ECCN 84, sourced from the Good Hope Colliery (Witbank, South Africa), demonstrated a vigorous and luxuriant growth that covered the 90 mm Petri dish within two week of inoculation.

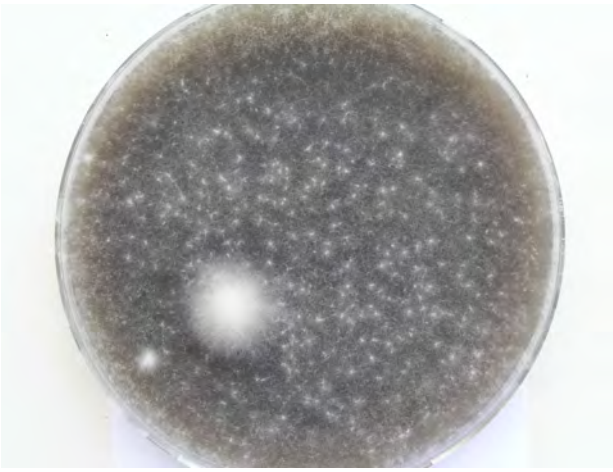


Figure 2-1 Growth of fungal culture on a 2 % Duff-agar at 28 ^{o}C.

2.3.2 Extracellular enzyme activity

Extracellular enzyme activity was monitored by observing the colour change on the inoculated plate, which indicated the oxidative degradation of the substrate ABTS (Figure 2-2). 39 of the 109 cultures recovered in the first screening step showed activity (Table 2-2). Of the 39 cultures that showed ABTS activity, 11 demonstrated a delayed reaction and showed pigment production only after storage at 4 ^{o}C (Table 2-2). Possibly, a delayed secretion of the oxidative enzymes was triggered by the stress of the cold storage conditions. The remaining 28 cultures showed immediate reaction within 24 hours of inoculation at 28 ^{o}C (Table 2-2). Of the cultures with ABTS activity, 38 were fungal cultures and one was a fungal/bacterial co-culture that was isolated from the Greenside Colliery. A number of bacteria have been reported to be active in coal bioconversion, including *Pseudomonas* spp (Osipowicz *et al*., 1994; Torzilli and Isbister, 1994; Laor *et al*., 1999, Machnikowska *et al*., 2002), *Bacillus* and *Arthrobacter* spp. (Torzilli and Isbister, 1994) as well as a fungal/bacterial co-culture (Boonchan *et al*., 2000) had been demonstrated to actively degrade coal and its derivatives.

Figure 2-2 A pure fungal culture plugged in the centre of a potato dextrose agar plate containing the substrate 2,2′-azinobis(3-ethylbenzoline-6-sulphonic acid), showing a purple pigmentation diffusing from the centre. This is an indication of the presence of extracellular oxidative enzymes acting on the substrate 2,2′-azinobis(3-ethylbenzoline-6-sulphonic acid).

Table 2-2 A tabulation of isolates that were capable of oxidizing 2,2′-azinobis(3-ethylbenzoline-6-sulphonic acid) and the location where the isolates originated from.

Source environment	Activity on ABTS		Number of cultures
	1^o	2^o	
Coal based environment			
Coal Plume (Carolina)	4	2	6
Excelsior coal mine	13	-	13
Greenside coal mine	4	-	4
Good Hope Colliery	-	1	1
Klein kopje Colliery	2	4	6
Kromdraai	3	1	4
Navigation Colliery	-	1	1
Old Anglo coal mine field (Leeunek)	1	1	2
Walker's Mine (Leeunek)	1	1	2
TOTAL	28	11	39

1^o, immediate reaction; 2^o, delayed reaction after storing the plates at 4 ^oC

Despite the luxuriant growth on Duff-agar demonstrated by the ECCN 84 isolate, the culture exhibited no activity against the substrate ABTS, thus indicating the absence of extracellular oxidative enzymes known to be utilized in the depolymerization of coal and its derivatives (Hofrichter and Fritsche, 1996 and 1997a; Hofrichter *et al.*, 1997b; Willmann and Fakoussa, 1997a). Indeed, organisms such as *Neurospora crassa* (Odom *et al*., 1991) and *Coprinus sclerotigenis* (Hofrichter *et al*., 1997a) were inactive towards ABTS, but otherwise were promising in coal depolymerization activity.

2.3.3 Gutation droplet formation

Further screening of organisms which emerged from the second step, was based on their ability to form gutation droplets and to some extent degrade HNO_3 treated bituminous hard coal (BHC). Five cultures emerged from this selection step as the best performing organisms and their preliminary identification was based on morphology in microscopic examination (Table 2-3).

Table 2-3 Five isolates selected on their gutation and growth performance on HNO_3 treated and untreated bituminous hard coal material, and their preliminary identification based on morphology.

ECCN	Morphological identification	Activity on bituminous hard coal	
		Treated	**Untreated**
84	*Aspergillus* sp.	Heavy gutation droplets formed in 3 days on the treated coal samples with mycelia engulfment after 40 days.	Mycelia engulfment within 30 days.
88	*Trichoderma* sp.	Moderate gutation on treated coal sample after 7 days. Mycelia engulfment after 21 days.	Mycelia encroachment on the untreated coal.
98	*Aspergillus* sp.	Moderate gutation formed on treated coal in 7 days.	Observed wetness of untreated coal after 14 days.
105	*Alternaria* sp.	Heavy gutation droplets formed in 7 days on the treated coal samples. Change in areas around treated coal and mycelia engulfment of the treated coal after 14 days.	Mycelia engulfment after 7 days.
115	*Penicillium* sp.	Moderate gutation formed on treated coal in 14 days.	Mycelia engulfment of the untreated coal after 14 days.

ECCN; EBRU Culture Collection Number.

All the organisms presented in Table 2-3, have been reported in the literature to be active in the bioconversion of coal and its derivatives, and in most cases, were reported to be indigenous to the coal environment (Hölker and Höfer, 2002; Acharya *et al*., 2005**;** Elhottová *et al.*, 2006; Řezácová *et al.*, 2006; Yuan *et al*., 2006). These observations suggest that the rhizosphere of *C. dactylon* growing in the coal dump environment harbours microorganisms that are actively involved in coal bioconversion processes and that the presence of these organisms may be associated in some way with the ability of the grass to grow in the hostile coal dump environment.

In general, the selection was based on visual observation of gutation formation. These varied from observing no reaction, moderate gutation droplet formation, to complete or heavy gutation droplets formation covering the entire treated coal samples within three to seven days contact time (Figure 2-3).

Complete mycelia engulfment of the coal particle was also observed and considered. Invariably, performance on HNO_3 treated coal was more pronounced and faster than on

untreated coal. Fungal ability to secrete gutation droplets on coal material as a preliminary step to solubilize and/or liquefy the coal material has been widely reported (Monistrol and Laborda, 1994; Hofrichter *et al*., 1997b; Hölker *et al*., 1997; Başaran *et al*., 2003; Yuan *et al*., 2006). The mechanisms involved in the solubilization process have been described as the secretion of gutation droplets containing alkaline substances, and which may be facilitated by the presence of chelators and/or hydrolytic enzymes (Hofrichter *et al*., 1997b).

Figure 2-3 Gutation droplet formation on 4.69 N HNO_3 treated bituminous hard coal (right) after 3 days contact with a fully grown culture of ECCN 84. No change yet was observed in the untreated bituminous hard coal (left).

2.3.4 Submerged growth in liquid culture

Certain cultures were found to grow as fungal pellets in liquid shake cultures and adsorb humic acid from the medium. Clarification of the nitrogen-rich culture medium after five day incubation was taken to indicate culture activity (Figure 2-4). An increased pH measurement in the nitrogen-rich flask suggested the secretion of alkaline substances that has previously been noted as a requirement for the effective solubilization of coal and its derivatives (Hofrichter *et al*., 1997b; Hölker *et al*., 1999). In active cultures, the pH of the medium rose to ~ pH 8 by day 5, and when left for an extended incubation time (21

days), the spherical pellets of biomass (mean diameter ~ 8 mm) collapsed and degenerated, releasing HA material back into the medium.

Figure 2-4 Glutamate medium containing humic acid when inoculated with ECCN 84 (right) as compared to the uninoculated control (left), after five days incubation time at 28 ^{o}C.

Under nitrogen-deficient conditions, the submerged culture supernatant showed no change in the pH or in the HA or FA concentration, when compared to the nitrogen-enriched medium after the five days of incubation. The stable pH condition provided an indication that nitrogen is required by the microorganism for the secretion of alkaline substances needed for the solubilization of coal and its derivatives. In addition, a supplementary organic carbon source is probably required for effective microbial degradation of coal and its derivatives. Glutamate and gluconate have been demonstrated to be effective supplementary organic carbon and a nitrogen sources for microbial solubilization of coal and its derivatives (Hölker *et al*., 1995, 1997 and 1999; Klein *et al*., 1999; Hölker *et al*., 2002).

All inoculated nitrogen-rich flasks showed a decrease of HA ranging from 20 – 98 % and an increase of FA ranging from 60 - 85 % (Figure 2-5). The change in HA and FA absorbance obtained from the 10 most active inocula is reported in Figure 2-5.

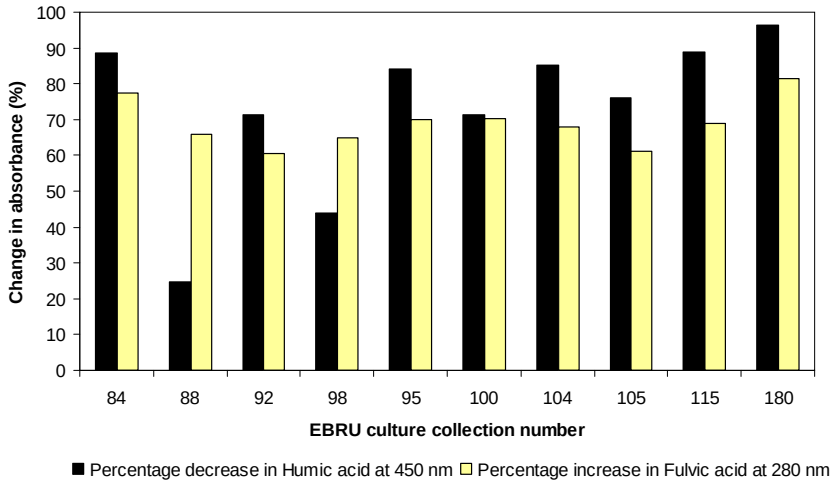


Figure 2-5 Percentage increase in fulvic acid ($A_{280\ nm}$) and decrease in humic acid ($A_{450\ nm}$) levels after 5 days incubation in 100 mL glutamate medium containing humic acid as used for the selection of culture isolates.

The changes in HA levels could be the result of simple biosorption and desorption of the HA, as previously demonstrated by Zhou (1992), but depolymerization of HA to form FA or decolorization of HA may also be involved (Klein *et al*., 1999). The decrease in absorbance at 450 nm coupled with an increase in absorbance at 280 nm is indicative of HA depolymerization (Hofrichter *et al*., 1999; Klein *et al*., 1999). However, without data to demonstrate the relative changes in molecular sizes fractions, the interpretation of the decrease in absorbance at 450 nm should be limited to an indication of adsorption and/or decolorization of the HA.

The ECCN 84 isolate was among the organisms that showed an elevated pH in submerged liquid culture, indicating the ability of the organism to secrete alkaline substances which might be used for the solubilization of coal and its derivatives. This was coupled with the effective clarification of the nitrogen-rich HA medium (Figure 2-4) coupled with the significant increase in the FA levels (77 %) and a corresponding decrease in the HA (87 %) of the culture supernatant (Figure 2-5). The increase in the absorbance at 280 nm of the ECCN 84 culture supernatant cannot be solely attributed to the increase in FA, because subsequent investigation (Chapter four) has indicated the possible spectral interference caused by an ergochrome biosynthesized by the organism.

The light microscopic observation of the surface of the ECCN 84 pellets showed adsorbed material, presumed to be a humic acid precipitate (Figure 2-6).

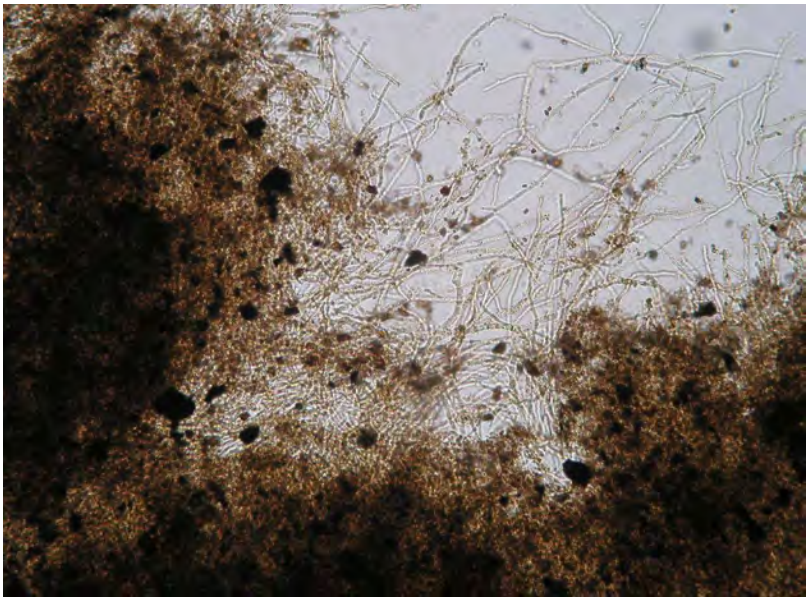


Figure 2-6 Light microscopic view of the surface of the fungal pellets of isolate ECCN 84 grown in liquid culture and showing the adsorption of an apparent humic acid precipitate (X40).

Among the 109 selected strains in the culture collection, ECCN 84 was found to be the most promising isolate and was used exclusively in the final screening step and the studies which followed.

2.3.5 Comparison with reference control cultures

The formation of gutation on the different pre-treated BHC samples by isolate ECCN 84 was compared to the two reference cultures grown on PDA and GA plates. In general, gutation was observed to be formed by ECCN 84 on all the ground HNO_3 pre-treated BHC samples within three days of contact with the fungal mat on PDA, and which was eventually engulfed by the mycelia (Figure 2-7i). The HNO_3 pre-treated BHC particle samples showed traces of gutation within three days, with mycelium encroachment observed on the BHC particles (Figure 2-7ii). The HNO_3 pre-treated and untreated BHC

samples (ground and particle) were observed to be fully engulfed by mycelia growth after an average of 45 days of contact with the fungal mat (Figure 2-7). Besides the gutation formation on all the treated coal samples, the ability of ECCN 84 to engulf both the pre-treated and untreated BHC samples is an indication of an active interaction existing between the isolate and the coal material.

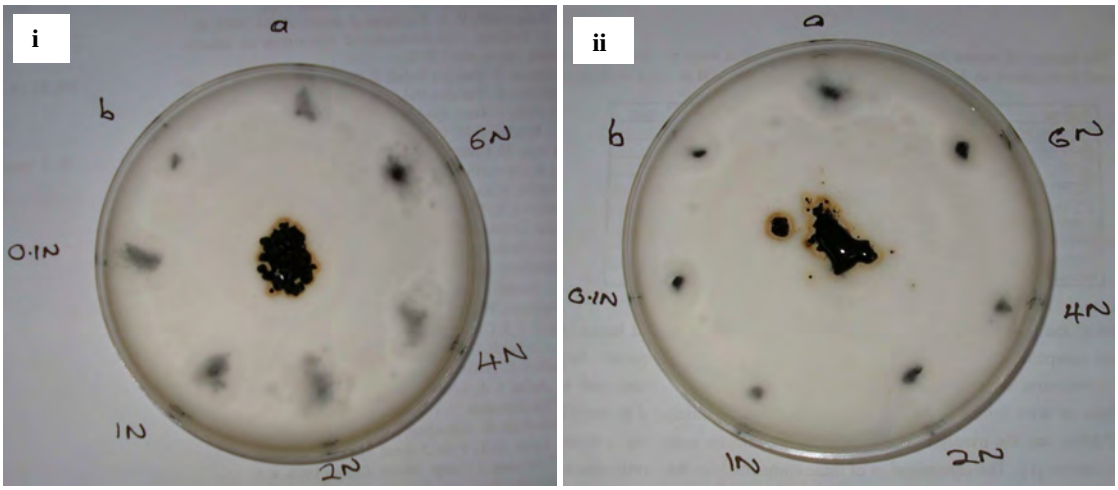


Figure 2-7 ECCN 84 fungal mat on Potato dextrose agar plates with deposited (i) ground and (ii) particle 6 N, 4 N, 2 N, 1 N, and 0.1 N HNO_3 pre-treated bituminous hard coal samples and humic acid that was placed at the centre of the fungal mat after four days of contact. Positions a and b represent untreated ground and particle samples respectively. The engulfment of the ground pre-treated samples (i) was faster than that of the pre-treated particle samples (ii).

The *P. chrysosporium* fungal mat cultured on PDA was observed to form gutation on the ground HNO_3 pre-treated BHC samples within three days of contact, and the mycelia completely engulfed the 0.1 N and 1 N HNO_3 pre-treated coal particle after eight days. The mycelia were observed to have encroached on the 2 N and 4 N HNO_3 pre-treated BHC ground samples within eight days of contact. In the 6 N HNO_3 pre-treated BHC ground coal sample, gutation was observed within three days, and 14 days later it was engulfed completely by the fungal mycelia. Only the 6 N HNO_3 pre-treated particle of BHC coal demonstrated the presence of gutation within three days, while on the other HNO_3 pre-treated samples (particle), no gutation was observed, but encroachment of fungal mycelia was evident after four days of contact.

In all cases of contact with the fungal mat of *P. chrysosporium*, the HA was observed to be completely liquefied within six hours, after which diffusion of the HA into the agar occurred and an eventual mycelial engulfment of the pastey HA residue became evident after 30 days (Figure 2-8).

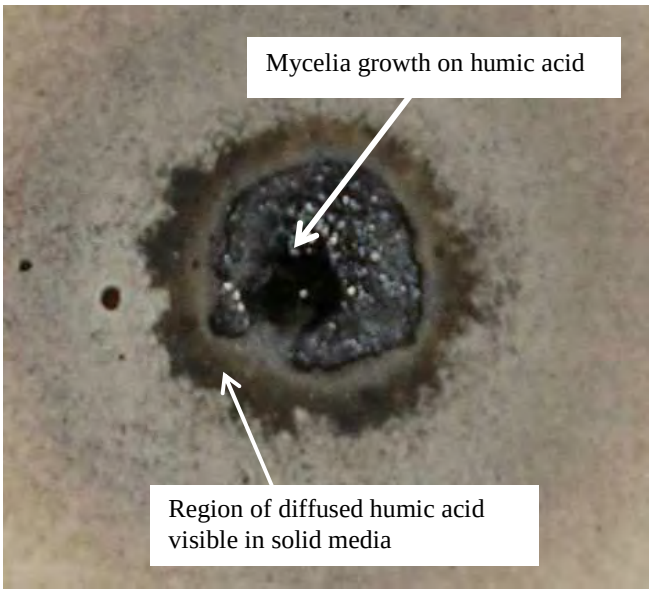


Figure 2-8 Fungal mat of *Phanerochaete chrysosporium* on potato dextrose agar with evidence of humic acid diffusion into the medium and mycelia growth on the pastey residue of humic acid after a 30 day contact time.

No gutation formed on the ground and HNO_3 pre-treated and untreated BHC samples that were contacted with the fungal mat of *T. versicolor*. None of the particle samples (pre-treated and untreated) showed any evidence of gutation and there was no visible mycelia presence on any of the coal samples (pre-treated and untreated). Within three days of contact with HA, liquefaction of the HA was comparable with that observed in the case of *P. chrysosporium*. However, the diffusion of the liquefied HA into the PDA was not as intense as that which was demonstrated on the *P. chrysosporium* (Figure 2-8). The results for 4 N pre-treated samples HNO_3 on PDA and GA plate study are summarized in Table 2-4.

Table 2-4 The activity of ECCN 84, *Trametes versicolor* and *Phanerochaete chrysosporium* grown on potato dextrose agar and glutamate agar comparing pre-treated and untreated ground coal (1 g of < 1 mm) and single coal particle (~ 5 g).

PDA plates	**Growth**	**4 N HNO_3 pre-treated bituminous hard coal**		**untreated bituminous hard coal**		**Humic acid**
		Ground coal	Particle	Ground coal	Particle	
ECCN 84	* * *	Gutation formed with mycelia engulfment within three days	Heavy gutation formed in three days and mycelia engulfed after 45 days	Mycelia engulfment within three days	Mycelia engulfment within 30 days	Gutation formed in three days and becomes pastey after 30 days. Very little diffusion into the media.
T. versicolor	* * *	No effect	No effect	No effect	No effect	Gutation within three days with slight diffusion into the media
P. chrysosporium	* * *	Gutation formed within three days with mycelia engulfment after eight days	Moderate gutation formed within three days	No effect	No effect	Gutation formed in six hours and heavy diffusion into the media. Gutation becomes pastey after 30 days
GA plates						
ECCN 84	* * *	Mild gutation after five days	No effect	No effect	No effect	Gutation formed after seven days
T. versicolor	*	No effect	No effect	No effect	No effect	Heavy gutation formed within six hours and diffusion into the media
P. chrysosporium	* *	No effect	No effect	No effect	No effect	Gutation formed within six hours and diffusion into the media with mycelia engulfment after five days

PDA, Potato dextrose agar; GA, glutamate agar.

A summary of results of the GA plate studies showed no evidence of gutation in any of the pre-treated coal samples, except for the samples that were contacted with ECCN 84. Gutation formation for ECCN 84 was, however, very minimal when compared with the culture grown on PDA plates. No mycelial growth on the coal (pre-treated and untreated) occurred in any of the GA plates. Gutation formation on HA and liquefaction was more rapid for all cultures on GA plates than it was on PDA plates.

The results of PDA and the GA plate study of the 4 N HNO_3 pre-treatment (Machnikowska *et al.,* 2002) are also summarized in Table 2-4. On PDA plates, gutation of HA granules was effected by all three strains, but for BHC, only ECCN 84 and *P. chrysosporium* formed gutation droplets. On the selective GA, all three strains formed gutation on HA, but only ECCN 84 produced gutation on HNO_3 pre-treated BHC (Table 2-4). The gutation formation on HA by ECCN 84 was slow compared to the gutation formation by *P. chrysosporium* and *T. versicolor* grown on GA. On the other hand, ECCN 84 was the only strain to form gutation with BHC on GA. This finding contrasts with observations by Laborda *et al.* (1997) who reported that low rank coal materials were more easily solubilized by fungi than hard coal material when grown on mineral media, and that solubilization of higher rank coal was dependent on a complete medium. These results indicate a selective capacity of ECCN 84 to initiate bioconversion of hard coal (possibly by a solubilization reaction), with a comparably lower capacity to dismantle the HA molecule (possibly by a depolymerization reaction) (Klein *et al.,* 1999). This may relate to the site-specific adaptations that *P. chrysosporium* and *T. versicolor* have for degrading lignin in rotting wood compared to ECCN 84, which was isolated from coal dumps.

In summary, the ECCN 84 and *P. chrysosporium* cultures exhibited good growth in the presence of the pre-treated coal samples with a better performance on the HNO_3 pre-treated ground samples (Table 2-4). Interestingly, the ECCN 84 culture was observed to engulf both the pre-treated and untreated ground and particle BHC samples indicating a strong affinity for coal substrate regardless of pre-treatment. *T. versicolor* performed the

worst in comparison with the ECCN 84 and *P. chrysosporium* cultures, in terms of gutation formation ability and growth on the coal samples.

Generally, results obtained from the screening study indicated the superior performance of ECCN 84 in all tests, except for the ABTS assay. This underscores the importance that screening tests should not be interpreted in isolation, but rather as part of an integral screening process. As only probably a fraction of microorganisms that are capable of coal bioconversion have been studied, it should be remembered that an understanding of the potentials of these organisms, as well as the products of their activities, remains limited (Klein *et al.*, 1999). A screening process has to be robust, simple and reliable to enable more effective screening of high numbers of isolates. The screening program results reported here indicate ECCN 84 is active in coal bioconversion with activity equal to, and in some cases superior to, that previously described for the two reference cultures.

2.4 Conclusions

- A standardized five-step screening protocol for the identification of microorganisms capable of coal bioconversion was developed.
- The 2 % Duff-agar screening system enabled the rapid identification of 109 rhizospheric organisms with coal bioconversion potentials.
- Fungal genera with previously described coal bioconversion capabilities were isolated from the *C. dactylon*/coal rhizosphere system. This indicated that bioconversion of coal may be a component process involved in the rhizosphere microenvironment.
- The ECCN 84 isolate emerged from the screening study as the best all round performer of all the environmental isolates, and with coal bioconversion activity comparable to the *P. chrysosporium* (PPRI 5328) and *T. versicolor* (PPRI 4835) reference cultures.
- The ECCN 84 isolate showed the ability to engulf untreated BHC, unlike the reference cultures. This suggests a strong affinity of ECCN 84 isolate to coal even without its pre-oxidation.

Given these results, the ECCN 84 isolate was selected to be used exclusively in the subsequent studies reported here.

Chapter three

Characterization of the fungal population in the *Cynodon dactylon*/coal rhizosphere

3. Introduction

The conventional microbiological approach followed in the screening study of the *C. dactylon*/coal rhizosphere, reported in the previous chapter, and had indicated the participation of a number of fungi with coal bioconversion activity. The ECCN 84 isolate emerged from the screening study as the most bioactive organism for all criteria tested. However, it is generally known that only a limited number of organisms can be isolated from the natural environment using conventional enriched media techniques, where the microbial community exists in a complex population structure. This has led to the development of total genomic Deoxyribonucleic acid (DNA) analysis using molecular techniques (Wagner *et al.*, 1994; Amann *et al.*, 1995).

In a previous screening study, the ECCN 84 strain had been isolated together with several other strains which also demonstrated good coal bioconversion activity. However, it would be necessary to know whether the coal bioconversion activity was an artifact of the screening study, before proceeding to use the ECCN 84 isolate as a model organism for subsequent interrogation of the *C. dactylon*/coal rhizosphere system. It would also be important to compare the types of organisms which emerged in the screening study with those identified using a more representative molecular characterization methodology.

3.1 Objectives

The objective of the study reported here was to investigate the rhizosphere population using total genomic DNA analysis and to determine the possible presence and identity of ECCN 84 as a component of the rhizosphere consortium.

3.2 Material and methods

3.2.1 Sample collection and site description

Navigation Colliery (Witbank, South Africa) was identified as a suitable site for sampling the *C. dactylon*/coal rhizosphere and sourcing material for total genome extraction analysis due to long-established and undisturbed *C. dactylon* growth. A vertical profile of the root system was exposed and ~ 40 g of the rhizospheric coal material was collected in 50 mL sterile sample bottles. This was transported on ice back to the laboratory and stored at 4 ^{o}C prior to being examined within 2 weeks of collection. A stock culture of the ECCN 84 isolate was sourced from the EBRU culture collection for use in the subsequent investigation.

3.2.2 DNA extraction

The total DNA extraction followed the method of Sambrook *et al.* (1989) and Bond *et al.* (2000), with some adaptations. Identical steps were followed for both rhizospheric material and ECCN 84 DNA extraction. 1 g of mycelia growth obtained from the ECCN 84 culture mat and 1 g of the rhizospheric sample material were utilized. Each sample was suspended in 5 mL Phosphate Buffered Saline (PBS) at pH 1.2, vortexed and a 500 µL aliquot of each was transferred to sterile 1.5 mL microfuge tubes (Eppendorf, Merck). Both samples were centrifuged (Eppendorf 5415D desktop centrifuge) at 17949 × g for 2 minute at 4 ^{o}C and the supernatants decanted, while the pellets were resuspended in 250 µL 50 % glycerol and 250 µL Buffer A (200 mM Tris/HCl, 50 mM Ethylenediamine tetraacetic acid (EDTA), 200 mM NaCl, 2 mM sodium citrate and 10 mM $CaCl_2$, pH 8) (Bond *et al.*, 2000). Centrifugation was repeated as described above and the supernatant decanted and discarded, while the pellets were resuspended in 250 µL of Buffer A. Both samples were lysed further by adding 15 µL of a 50 µg/mL lysosyme enzyme stock and incubating while shaking in a Labcon water bath at 37 ^{o}C for three hours. 15 µL of 50

µg/mL Proteinase K solution was then added to the samples which were further incubated at 50 ^{o}C for one hour. For the cellular fracture, 500 µL of 10 % sodium dodecyl sulphate (SDS) was added to the samples followed by five freeze-thaw cycles, which entailed 1 minute freeze in liquid nitrogen and 1 minute thaw in 100 ^{o}C water.

500 µL aliquots were removed from both samples and added to 1.5 mL sterile microfuge tubes. Cell lysates were extracted with an equal volume of phenol, vortexed and centrifuged at 17949 × g for 2 min. The upper aqueous layer was collected and extracted in an equal volume of phenol:chloroform:isoamyl alcohol (24:24:1) solvent mixture, vortexed, and centrifuged at 17949 × g for 2 min. This procedure was repeated until the pink colour was removed from the aqueous layer. The upper aqueous layer was again removed and the nucleic acids precipitated by mixing with 50 µL of 3 M sodium acetate in 1.5 mL sterile microfuge tubes filled with cold 96 % rectified ethanol. The samples were then refrigerated over night at -20 ^{o}C.

The two DNA samples were concentrated by centrifugation at 17949 × g for 25 minute at 4 ^{o}C and the pellet resuspended in 50 µL distilled water. 30 µL aliquot of each DNA sample was stored at 4ºC for immediate use and short-term storage, while the remainder was stored at -20 ^{o}C. The DNA was electrophoresed on a 1 % agarose gel in TAE buffer (BIO-RAD, Cat. # 161-0073). 100 µL of 0.5 g/mL ethidium bromide in milliQ water was added to the agarose upon cooling and prior to gel casting. A λ *Pst*1 molecular weight marker was utilized to establish the molecular weight of the product. Preparation was by digesting 200 µL λDNA (0.25 µL/mL) with 24 µL of 10 × Buffer H and 10 µL of *Pst*1 enzyme for three hours at 37 °C. 550 µL of 10 mM TE buffer (pH 8.0) and 150 µL of 6 × loading buffer (0.25 % Bromophenol blue, 0.25 % xylene cyanol and 30 % glycerol) was added. Extracted DNA was electrophoresed (BIO-RAD POWER PAC 3000) at 120 V for 45 minute and visualized with a UV transilluminator (UVP BioDoc-It^{TM} System) fitted with a digital camera.

3.2.3 Polymerase Chain Reaction amplification of fungal Internal Transcribed Spacer region

Polymerase Chain Reaction **(**PCR) amplification of the extracted genomic DNA was carried out using a Taq DNA Polymerase kit (Promega). The concentrations used were 2 µL of 5 mM ITS1-F Primer, 2 µL ITS4 Primer (5 mM), 1 µL deoxyribonucleotide triphosphate (dNTPs) (10 mM), 1 µL Bovine serum albumin (BSA) (50 mg/mL), 2 µL 25 % DMSO, 2.5 µL magnesium-free buffer, 1.5 µL $MgCl_{2,}$ 0.2 µL *Taq* DNA polymerase and 10.8 µL sterile distilled water in a 25 µL PCR reaction.

Amplification was performed on a GeneAmp 9700 PCR Thermocycler using a touch-down PCR procedure. The touch-down program utilized for the PCR amplification used an initial denaturation step at 94 °C for 2.5 minute, which was followed by 40 cycles of 15 seconds denaturation at 94 °C, 30 seconds annealing at 53 °C and 90 seconds elongation at 72 °C. A final elongation step at 72 °C for 10 minute completed the reaction. A 1 % agarose gel containing ethidium bromide was prepared. The PCR products were electrophoresed (BIO-RAD POWER PAC 3000; 120 V for 45 min) and visualized on a UV transilluminator (UVP BioDoc-It™ System) fitted with a digital camera.

3.2.4 Ligation of Polymerase Chain Reaction products

The individual PCR products that were derived from the genomic extraction of the rhizospheric material and the ECCN 84 isolate was cloned into a pGEM®-T Easy Vector system (Promega, USA). The method was as described by the manufacturer (Table 3-1). The ligation samples were incubated over night at 4 ^{o}C.

Table 3-1 Ligation Reactions for pGEM-T Easy Vector.

	Standard Reaction	**Positive Control**	**Background Control**
2 x Rapid Ligation Buffer	1 µL	1 µL	1 µL
pGEM-T Easy Vector	0.2 µL	0.2 µL	0.2 µL
PCR product	0.6 µL	-	-
Control Insert	-	0.6 µL	-
T4 DNA Ligase 3	0.2 µL	0.2 µL	0.2 µL

3.2.5 Transformation and selection of competent cells with clones

The transformation into high efficiency *E. coli* JM 109 competent cells followed the method of Sambrook *et al*. (1989). After the overnight incubation period, 2 µL of the ligation reaction was added to 50 µL thawed competent *E. coli* cells in a 1.5 mL sterile microfuge tube and were left on ice for 20 min. To allow for plasmid uptake, the cells were heat-shocked at 42 ºC for 45 seconds and then cooled on ice for 5 min. 900 µL of SOC medium was added to the transformed cells and the tubes were incubated at 37 ^{o}C with shaking at 150 rpm for 1 hour to allow the cells to recover. 100 µL of the transformant were plated and screened on Luria Bertani (LB) agar (30 g LB agar/1 L milliQ) plates containing 100 µg/mL ampicillin (Amp). Prior to plating, the LB/Amp plates were spread with IPTG (1M Isopropylthio-β-o-galactiside) and X-Gal (20 mg of 5-Bromo-4-chloro-3-indolyl-β-o-galactoside in 1 mL dimethylformamide). The plates were incubated overnight at 37 °C and transformants with an insert in the β–glycosidase gene appeared white on the X-Gal plates as opposed to blue colonies, which had a plasmid but no insert in the β–glycosidase gene.

3.2.6 Plasmid preparation from transformed cells

Five white transformed bacterial colonies were selected from each of the two DNA sources using a sterile blood lancet to lift sample material from the LB/Amp plates. Each colony was inoculated into sterile 5 mL LB in a test tube and incubated overnight at 37 ^{o}C while agitating at 150 rpm. The plasmid was extracted using the plasmid extraction kit

QIAprep® Spin Miniprep Kit (Qiagen Cat. # 27104). The extraction procedure was as described by the manufacturer. 1 mL of each culture was placed into a sterile 1.5 mL microfuge tubes and pelleted by centrifugation at 17949 × g for 5 minute at 4 ^{o}C, after which the supernatant was decanted and discarded, while the pellet was resuspended in 250 µL buffer P1 (Tris-EDTA buffer). 250 µL of buffer P2 (lysis buffer) was added and complete mixing was achieved by inverting the tube six times. 350 µL of buffer N3 (Neutralization buffer) was then added and further mixed by gently inverting the tube six times. The mixed samples were centrifuged at 17949 × g for 10 minute at 4 ^{o}C and the resulting supernatant was introduced onto a QIAprep spin column and centrifugation repeated for 1 min. The flow-through was discarded and 0.5 mL buffer PB was added onto the column and centrifuged at 17949 × g for 1 minute at 4 ^{o}C. The flow-through was discarded again. The column was then washed by introducing of 0.75 mL buffer PE, centrifuging at 17949 × g for 1 minute at 4 ^{o}C and discarding the flow-through. Centrifugation was repeated to remove residual wash buffer.

DNA elution was achieved by placing the QIAprep column into a sterile 1.5 mL microfuge tube and adding 50 µL of sterile distilled water at the centre of the column. The column was left to stand for 2 minute before it was centrifuged at 17949 × g for 1 minute at 4 ^{o}C and the eluted plasmid DNA was placed in 4 ^{o}C for short term and in -20 ^{o}C for long term storage.

3.2.7 Cycle sequencing of cloned polymerase chain reaction products

The individual PCR products ligated into pGEM-T were cycle sequenced using the BigDye V3.1 Terminator Cycle Sequencing kit (Applied Biosystems). A 20 µL thermocycling reaction sample was prepared in a 100 µL PCR tubes. The reaction constituent was made up of 4 µL BigDye, 4 µL 5 × Buffer, 2 µL 10 µM SP6 primer stock, 6 µL plasmid DNA and 4 µL distilled water. The cycle sequencing reactions were carried out using a GeneAmp® PCR system 9700 Thermocycler and the cycle sequencing routine is shown in Table 3-2.

Table 3-2 Cycle Sequencing on the 9700 PCR System.

Step	Action	Cycle
1	Initial denaturation step at 96 ^{o}C for 1 sec	
2	Denaturation at 96 ^{o}C for 10 sec	25
3	Annealing at 50 ^{o}C for 5 sec	25
4	Elongation at 60 ^{o}C for 4 min	25

The purification of the extension products were achieved by alcohol precipitation. 6 µL of EDTA (125 mM) and 70 µL of 100 % rectified ethanol was added to each sequencing reaction and incubated at room temperature for 15 minute after which the PCR tubes were centrifuged (17949 × g for 30 minute at 4 ^{o}C). The supernatant was carefully decanted and discarded without disturbing the pellet and 100 µL 70 % rectified ethanol was added to the PCR tubes and samples centrifuged at 17949 × g for 15 minute at 4 ^{o}C. The supernatant was then carefully decanted and discarded.

The PCR tubes were then placed in an open GeneAmp 9700 PCR Thermocycler with their lids open and the pellets dried at 95 °C for 5 minute in the dark after which they were stored at 4 °C.

3.2.8 Automated DNA sequencing

The alcohol precipitated DNA samples were resuspended in 10 µL deionised formamide, vortexed, and briefly centrifuged at 17949 × g. The samples were then heated to 95 ^{o}C for 2 minute to denature the DNA and then placed on ice until utilization. The extension products were sequenced using an ABI PRISM® 3100 Genetic Analyzer and the method was as described in the User's Manual (P/N 903565).

The sequence generated from the ABI PRISM® 3100 Genetic Analyzer was read using the BioEdit v7.07 Sequence Alignment Editor. Sequences were copied into text format using Chromas and the identity of the organism was derived using the Basic Local Alignment Search Tool (BLAST) available online from the National Centre for

Biotechnology Information (http://www.ncbi.nlm.nih.gov/). The BLAST is designed to explore regions of local similarity between sequences (Altschul *et al.*, 1990).

The phylogenetic tree was rooted with the 16S sequence of *Pyrococcus horikoshii* 0T3. For the phylogenetic tree calculations, sequences were aligned using the CLUSTAL X multiple sequence alignment program and phylogenetic trees were generated with the aid of Treeview. Graphics were finalized with the aid of CanvasX from the ACD systems.

3.2.9 Morphological characterization of cultures

Morphological characterization of fungal isolates followed the method described by Onions *et al*. (1981). Culture morphology was recorded as described in Table 3-3. Tape mounts of the cultures were prepared as follows for microscopic examination under an Olympus BX50 light microscope. 1 mm^2 of sample from a pure culture plate was carefully picked and placed in a drop of ethanol on a glass slide. The sample was teased out carefully with a pair of sterile blood lancets and one or two drops of lactophenol blue was added before the cover-glass was placed. A clear lacquer (nail varnish) was used to fix the cover-glass for storage.

Table 3-3 Culture morphology adapted from Onions *et al*. (1981).

Criteria	Description
Growth rate	Diameter of the fungal mat resulting from a 5 mm plug inoculum (mm/day).
Pattern of colony colour	Whether uniform, in zones or patchy or evanescent.
Pattern of colour change as viewed from the underside of the plate	Whether uniform, in zones or patchy.
Colour change in the medium	Whether confined to area of fungal growth or diffused from area of fungal growth.
Texture of fungi surface	Loose or compact, plain, wrinkled, velvety, matted, floccose, hairy or leathery.

3.3 Results and discussions

In the field, extensive *C. dactylon* root penetration of > 2 m deep into the coal layers was observed. The coal material within the *C. dactylon* rhizosphere appeared to be broken down into smaller size fractions (~ 0.5 – 5 mm) compared to coal in the adjacent areas not colonized by root growth. A brownish humic soil-like material was observed to have been produced in the root zone. Mycorrhizal rhizoid formation was associated with the plant root zone and appeared as large fungal rhizoid mats covering the plant roots and incorporating soil and coal particles.

The DNA was successfully extracted from both the ECCN 84 isolate and the *C. dactylon* rhizospheric coal samples. The PCR reaction methodology was first optimized in order to effectively amplify the extracted DNA. Polymerase Chain Reaction results showed that the 560 bp section of DNA selected for by the specific primers was successfully amplified (Figure 3-1).

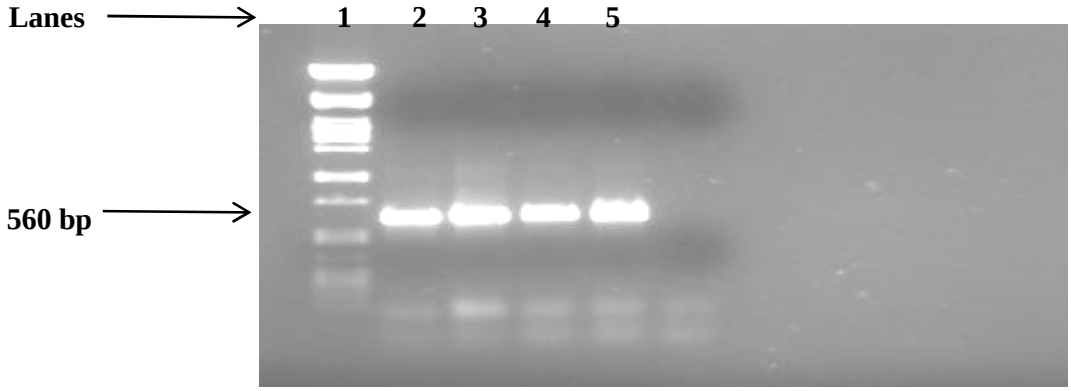


Figure 3-1 Agarose gel showing the result of the Polymerase Chain Reaction for the ECCN 84 isolate and the rhizospheric coal samples of *Cynodon dactylon*. Lane 1, λ *Pst*1 molecular weight marker showing the 560 bp; lanes 2 and 3, the PCR products obtained from genomic DNA that was extracted from rhizospheric coal samples of *Cynodon dactylon* and 4 and 5, the PCR products obtained from genomic DNA that was extracted from the ECCN 84 culture isolate.

The PCR products were cloned and sequenced and the results, illustrated in the form of a phylogenetic dendrogram, are represented in Figures 3-2 and 3-3 for the total genomic extract of rhizospheric microorganisms and the ECCN 84 culture, respectively.

Figure 3-2 The phylogenetic dendrogram of total genomic extract sourced from the rhizosphere of *Cynodon dactylon* grown on the bituminous hard coal dump at Navigation Colliery. Clones are highlighted in red. The phylogenetic tree was rooted with the 16S DNA sequence of *Pyrococcus horikoshii* 0T3.

The genomic extract of the *C. dactylon*/coal rhizospheric samples showed an array of organisms that are likely to be involved in some form of coal interaction (Figure 3-2). A number of the genera identified in the phylogenetic dendrogram (Figure 3-2) had previously been reported to be involved in the coal bioconversion process. These organisms were also isolated and selected during the screening study, thus further strengthening the validity of the screening program that was implemented. These organisms include *Penicillium* spp. (Achi, 1994a and b; Laborda *et al*., 1999; Pokorný *et al.,* 2005; Yuan *et al*., 2006), *Trichoderma* spp. (Hölker *et al*., 1997, 1999 and 2002; Hölker and Höfer 2002; Pokorný *et al.,* 2005) and *Aspergillus* spp. (Monistrol and Laborda, 1994; Torzilli and Isbister, 1994; Acharya *et al*., 2005; Elhottová *et al.,* 2006) (Figure 3-2). Other genera present in the phylogenetic dendrogram that have been linked to some form of bioconversion of coal and its derivatives are discussed here. Řezácová *et al.* (2006) reported the implication of *Exophiala* in the degradation of HA and FA. *Cladosporium* has been isolated from a pristine lignite environment in Southern Slovakia (Pokorný *et al.,* 2005). *Rhodotorula* has been reported in close association with mycorrhiza in the fermentation of mucilage (Fracchia *et al*., 2003; Aksu and Eren, 2005; Boby *et al.,* 2006) and implicated in the degradation of pyrene (a polycyclic aromatic hydrocarbon) (Romero *et al.,* 2002), and *Neosartorya,* isolated from petroleum polluted soil has been shown to be capable of degrading HC (Chaillan *et al.,* 2004). Other root-promoting soil fungal genera present in the phylogenetic dendrogram that had not been implicated in the bioconversion of coal and its derivatives, included *Piriformospora, Nematoctonus, Phialophora*, *Scolecobasidium* and *Hohenbuehelia.* This is the first report implicating these organisms as being involved in some form of coal bioconversion activity.

The Internal Transcribed Spacer (ITS) regions of fungal ribosomal DNA (rDNA) is composed of highly variable sequences which are specific to fungal species, and hence their importance in distinguishing fungal species by PCR analysis (Martin and Rygiewicz, 2005). Sequence analysis of the intergenic spacer region of the rDNA of ECCN 84 demonstrated a 98 % match with *Neosartorya fischeri* (Figure 3-3), a species which groups phylogenetically with *Aspergillus fumigatus* (Girardin *et al*., 1995; Varga *et*

al., 2000). Interestingly, clones 3D, 9C and 10B, shown on the genomic dendrogram (Figure 3-2), are close relatives of *N. fischeri*. *N. fischeri* has been isolated repeatedly, by this group, from the *C. dactylon/*coal rhizosphere samples sourced from the various coal related locations in South Africa (Table 2-1). This indicates that the organism is likely to be involved and play some role in coal bioconversion activity (Chapter two) and also may contribute in some way to the growth of *C. dactylon* in the harsh environment of waste coal dumps. This is the first report of the occurrence of this organism in this environment and its implication in coal bioconversion.

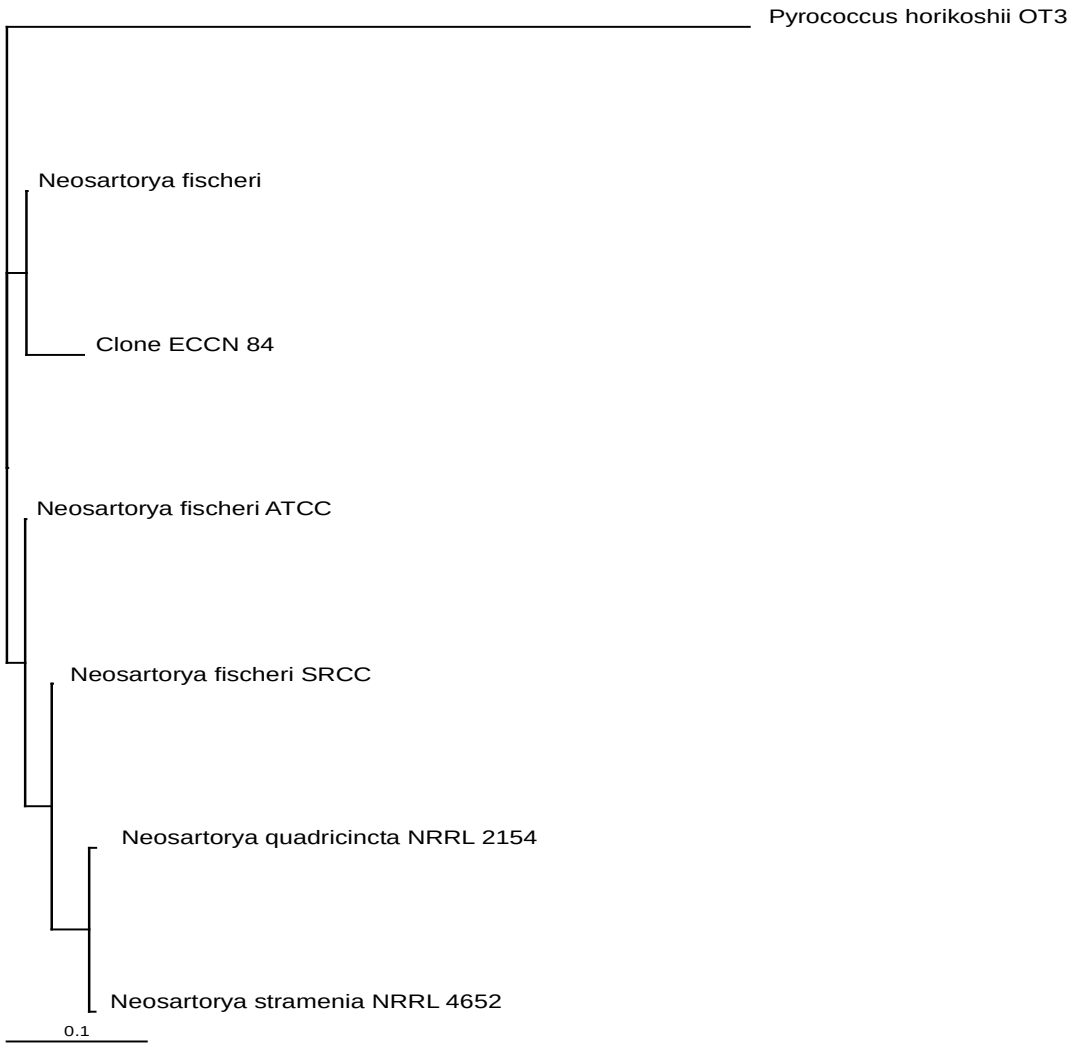


Figure 3-3 The phylogenetic dendrogram of the ECCN 84 isolate shown to be closely related to *Neosartorya fischeri.* The isolate was original sourced from the Good Hope Colliery, Witbank, South Africa and maintained on potato dextrose agar. The phylogenetic tree was rooted with the 16S DNA sequence of *Pyrococcus horikoshii* 0T3.

Previous characterization had shown the organism (*N. fischeri*) to be closely related to *A. fumigatus* (Raper and Fennell, 1965; Girardin *et al.*, 1995; Geiser *et al*., 1998, Wang *et al*., 2000; Varga *et al*., 2000; Balajee *et al.*, 2005; Wortman *et al.*, 2006). Members of the ascomycetous genus *Neosartorya* have anamorphs similar to *A. fumigatus* (Summerbell *et al.*, 1992) and were first placed in the *A. fumigatus* group by Raper and Fennell (1965). Phylogenetic analysis of *N. fischeri* groups the fungus with *A. fumigatus* (Girardin *et al.*, 1995; Geiser *et al*., 1998; Varga *et al*., 2000; Wang *et al*., 2000; Wortman *et al.*, 2006) and they have been shown to be a close teleomorphic relative with a remarkably low genetic diversity (Balajee *et al.*, 2005; Rydholm *et al*., 2006; Wortman *et al.*, 2006; Jones, 2007). The phylogenetic dendrogram (Figure 3-2) also shows the close relationship between *N. fischeri* and *A. fumigatus*. *N. pseudofischeri* has been cited as another teleomorphic relative of *N. fischeri* and they could only be differentiated by electron microscope analysis of the ascospore structure (Balajee *et al.*, 2005). *A. fumigatus* is a widely distributed mould which has been isolated from natural and residential environments and which is thought to be the *Aspergillus* species most pathogenic to humans (Wang *et al.*, 2000). The close relationship of *N. fischeri* to *A. fumigatus* is therefore of concern as a potential health risk where future studies are anticipated. The assembled genome sequence of *N. fischeri* at the time of writing was incomplete, and progress to completion can be monitored at the Microbial Sequence Centre (MSC) (http://msc.tigr.org/aspergillus/neosartorya_fischeri_nrrl_181/index.shtml).

In addition to morphological similarities between *N. fischeri* and *A. fumigatus*, which include uniseriate, greenish tinted, phialidic conidial heads with pyriform vesicles and columnar conidial masses (Balajee *et al.*, 2005), they also have similar thermo-tolerance characteristics (Piecková *et al*., 1993 and 1994; Piecková and Jesenská, 1997; Salomão *et al.*, 2007). *In vitro*, *N. fischeri* can grow at a temperature up to 51 ^{o}C, which may account for its survival in the mesophilic to thermophilic environment of coal dumps. It differs from *A. fumigatus* by forming large numbers of pale coloured ascomata bearing equatorially furrowed ascospores with prominent crests arising along the margins of the furrow (Summerbell *et al*., 1992).

The mycelium of the *N. fischeri* isolate, when grown on PDA plates, appears very hairy and compacted with a uniform white tint (Figure 3-4i). Light microscopy (Figure 3-4ii) indicated that the isolate was closely related to *Aspergillus* (Onions *et al.,* 1981). The underside of the culture showed a light brown coloration and the growth rate of a 5 mm plug was determined to be 11.1 mm/day (radially) at 28 ^{o}C.

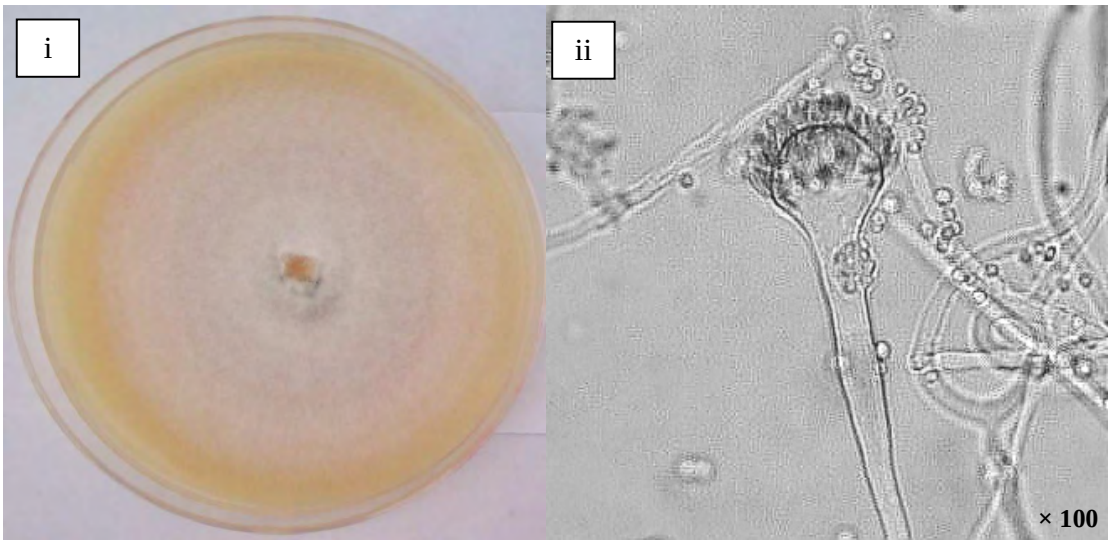


Figure 3-4 *Neosartorya fischeri* (ECCN 84) (i) maintained on potato dextrose agar plate and (ii) micrograph showing its phialidic and conidial structures. The isolate was sourced from the Good Hope Colliery, South Africa.

3.3.1 *Neosartorya fischeri* and safety considerations

N. fischeri (Wehmer, Malloch and Cain, 1972), sometimes referred to as *Aspergillus fischeri* (Senthilkumar *et al.,* 2005), has been ubiquitously isolated from damp environments, such as soil, decaying vegetation and organic debris where it produces large numbers of ascospores, which are highly heat resistant (Ugwuanyi and Obeta, 1991; Jesenská *et al.,* 1992 and 1993; Piecková *et al.,* 1993 and 1994; Wortman *et al.,* 2006). *N. fischeri* has been studied in some detail in the food industry and medical applications and the results provide useful indicators in the health implications of its potential application in coal bioconversion technology.

3.3.1.1 Neosartorya fischeri in the food industry

N. fischeri is widely reported as a food-related fungus (Wortman *et al.,* 2006). Literature indicates that ascospores of *N. fischeri* in soil can survive heating for 175 minute at 70 ^{o}C (Piecková *et al*., 1993 and 1994; Piecková and Jesenská, 1997). It is this ability of the thermostabile ascospores to survive heating that has generated research interest in this fungus. Complete destruction of *N. fischeri* during commercial heat processing is not always possible and the organism had been implicated in the spoilage of fruit products (Ugwuanyi and Obeta, 1991; Piecková *et al*., 1993 and 1994; Piecková and Jesenská, 1997; Rajashekhara *et al.*, 2000; Slongo and de Aragão, 2006; Salomão *et al.,* 2007). A number of mycotoxins that are of concern to the food industry have been isolated from *N. fischeri* and characterized. Amongst them are fischerin, a biologically active component that causes peritonitis in mice (Fujimoto *et al*., 1993) and α-sarcin-like ribotoxin (type II ribotoxins) that exhibit ribotoxic activity by cleaving ribosomes and generating α-fragment (Lin *et al*., 1995).

3.3.1.2 Neosartorya fischeri in medicine

A. fumigatus is capable of causing a number of diseases in humans, ranging from allergic bronchopulmonary aspergillosis and aspergilloma to invasive aspergillosis and systemic infection due to hematogenous dissemination. Infection in the immunocompromised host is often fatal (Wang *et al.,* 2000).

However, a literature search showed limited documented reports of infection by *N. fischeri* in the past 25 years. In the small number of cases where *N. fischeri* was shown to be associated in disease conditions, most of these were opportunistic infections in immunocompromised individuals (Summerbell *et al*., 1992; Lonial *et al*., 1997; Gori *et al.,* 1998). Only one case in a non-immunocompromised individual has been reported to be associated with *N. fischeri* (Coriglione *et al*., 1990). Due to its abundance in the environment it may be possible that the presence of the fungus could be due to contamination of the sample rather than isolation from the infective site. Furthermore,

identification of the causative agent had relied on morphological identification rather than molecular analysis, which is not easy with such closely related organisms and a misdiagnosis with *A. fumigatus,* remains a possibility (Summerbell *et al*., 1992; Gori *et al.,* 1998).

Although this one case is of concern, the patient was highly susceptible to infection, he had undergone a number of previous heart surgeries, already had pre-existing lesions, and the operation requires the grafting of a foreign body into the patient. The fungus was identified by plating out and by morphological identification, and although the accuracy and aseptic techniques of the laboratory staff was not questioned, it always remains a possibility that contamination and misidentification could have taken place. Molecular identification could have been performed for a more accurate determination but this technique was unavailable at that time.

N. fischeri has the potential to be an opportunistic pathogen, but if due care is taken when working with the organism it should not present a problem. Many fungal strains have the potential to be opportunistic pathogens with the right circumstances and according to literature reports, *N. fischeri* had demonstrated no highly pathogenic traits, and its reported incidences of causing disease are rare. It is, however, recommended that standard laboratory care should be exercised during handling of the organism. In addition, cases where immunocompromised conditions, such as pulmonary infections, are suspected such persons should avoid all areas where work is performed using *N. fischeri*.

3.3.2 Genus *Aspergillus* and *Neosartorya fischeri* in coal bioconversion

3.3.2.1 Genus Aspergillus

Limited literature is available on the activity of the genus *Aspergillus* in relation to coal bioconversion. All the reported cases of *Aspergillus* strains capable of coal bioconversion were observed to have been sourced from a coal environment, which suggests a possible

coal induction effect that results in the adaptation of the organism to this environment. Most recently, *Aspergillus* was isolated from a viable pristine microbial community flourishing in the Miocene lacustrine clay of a Cypris formation, which was excavated from 200 m below the surface as spoils during open-cast mining of brown coal in Czech Republic (Elhottová *et al.*, 2006). Organisms isolated from such environments could have originated from microbial communities that were deposited millions of years ago, and results suggest that the high fatty-acids content in the non-viable microbial biomass may have been easily available as a carbon source that helped sustain the pioneer microbial communities.

Acharya *et al*. (2005) had demonstrated the biodesulphurization of coal from the North Eastern coalfield of Assam (India), by the use of *Aspergillus*-like fungi. 78 % of the total sulphur content was shown to be eliminated by the *Aspergillus* sp. within 10 days when coal particle size of less than 74 µm was utilized. The *Aspergillus*-like fungus was indigenous to the coal environment suggesting its adaptation to growth in coal. However, in that study the species of the culture was not stated. Also, Monistrol and Laborda (1994) isolated an *Aspergillus* sp., indigenous to the coal environment, which had some liquefaction and/or solubilization effect on Spanish coals. The isolate was only partially characterized and identified as S10-H.

In a comparative study, the solubilization of leonardite by an *Aspergillus* sp. appeared to be similar to that of a bacterial consortium, but contrasted with the solubilization products obtained from *T. versicolor* and *P. chrysosporium* (Torzilli and Isbister, 1994). The *Aspergillus* isolate, used in the study, was partially characterized and identified as CP4 and was indigenous to coal environments. Subjecting the microbial solubilized products to gel permeation yielded a molecular mass profile that was unique to each organism, indicating substantial product diversity (Torzilli and Isbister, 1994).

3.3.2.2 Neosartorya fischeri

Studies have demonstrated the ability of *N. fischeri* to secrete a wide range of compounds under both acidic and alkaline conditions. These include acid proteinase, which is secreted under acidic conditions (Wu and Hang, 1998; Wu and Hang, 2000), and xylanase, an alkali-stable enzyme, secreted under alkaline conditions (Senthilkumar *et al*., 2005). The xylanase had been described as a novel enzyme with potential as a bleaching agent in the pulp and paper industry (Anuradha *et al*., 2007; Sharma *et al.*, 2007).

Senthilkumar *et al*. (2005) outlined a solid-state fermentation process for the production of the alkaline stable xylanase. That study may provide important details for the design of a fermentation system optimizing the enzyme's activity that might be important in the breakdown of coal and its derivatives. Results emanating from the present study have demonstrated the potential of *N. fischeri* in the coal bioconversion process. Although, still in its early stages, further investigation is required to confirm the mechanisms involved in the *N. fischeri* coal bioconversion activity and also to clarify the role of this organism in the *C. dactylon*/coal rhizosphere. Apart from advancing an understanding of coal solubilization reactions occurring in the natural environment, further development of the system may assist in the development of sustainable industrial biotechnology applications and coal mine rehabilitation.

A recent publication has implicated *N. fischeri* as effective for the biovolatilization of arsenic (Čerňanský *et al*., 2007). Results from the study demonstrated that nearly all the biosorbed arsenic was transformed into volatile derivatives. Previously, a screening of aerobic hydrocarbon-degrading microorganisms from petroleum-polluted soil had revealed the presence of *N. fischeri* which was shown to be capable of degrading saturated hydrocarbons (Chaillan *et al*., 2004). These reports highlight the potential of *N. fischeri* for the bioremediation of contaminated sites.

3.4 Conclusions

- The total genomic DNA analysis result shows comparisons to the isolate screening study with the identification of similar genera present in each case.
- Fungal species related to those identified in the total genome DNA analysis have been described in the literature as organisms active in coal bioconversion. Their presence in the *C. dactylon*/coal rhizosphere suggests their activity in coal bioconversion in this system.
- The ECCN 84 culture was shown to be closely related to *N. fischeri* (98%) and had been isolated repeatedly in environmental samples sourced during the screening of the *C. dactylon*/coal rhizosphere. This is the first report implicating *N. fischeri* in coal bioconversion activity.
- Health considerations with coal bioconversion applications of *N. fischeri* (ECCN 84) were noted due to its close relationship to *A. fumigatus*. However, the few cases of infection reported for *N. fischeri* were apparently opportunistic in nature. In most cases, precise characterization using molecular techniques were not utilized, leaving room for incorrect identification and confusion with the closely related but pathogenic *A. fumigatus.*
- *N. fischeri* has previously been implicated in the biovolatilization of arsenic and in the degradation of saturated hydrocarbon. In addition, in the present study, the isolate has shown coal bioconversion activity and was thus considered a useful model organism for further interrogation of coal bioconversion functions occurring in the *C. dactylon*/coal rhizosphere.

Chapter four

Organic carbon supplementation in the *Cynodon dactylon*/coal system

4. Introduction

The hypothesis on which this study was based proposed that the microbial community present in the rhizosphere of the *C. dactylon*/coal system may act in some way to facilitate the growth of *C. dactylon* observed in the coal dump surface. The screening study of organisms in the rhizosphere indicated an array of fungi present in the system, including *N. fischeri*, and also other species that have been previously implicated in coal bioconversion activity (Achi, 1994a and b; Monistrol and Laborda, 1994; Torzilli and Isbister, 1994; Hölker *et al*., 1997, 1999 and 2002; Laborda *et al*., 1999; Hölker and Höfer 2002; Acharya *et al*., 2005; Pokorný *et al.*, 2005; Elhottová *et al.*, 2006; Yuan *et al*., 2006). These findings alone provide a reasonable indication that active fungal-driven coal bioconversion activity may be occurring in the rhizosphere of the *C. dactylon*/coal system and in some way contributing to the ability of *C. dactylon* to grow in this environment.

In a separate investigation undertaken concurrently with this study Mukasa-Mugerwa (2007) has shown that the *C. dactylon* growth in the coal is associated with a prolific infection of the root system by *Glomus*, *Paraglomus* and *Gigaspora* mycorrhizal species. These observations provided additional support for the fungal coal bioconversion idea and the suggestion that photosynthetically produced nutrients, supplied into the system by the plant, may provide organic supplements such as glutamate (Griffiths *et al.*, 1999; Baudoin *et al.*, 2003; Falchini *et al*., 2003), which has been shown to be required for hard coal bioconversion (Hölker *et al*., 1995, 1997 and 1999; Klein *et al*., 1999; Hölker *et al*., 2002). Organic supplementation may have a major impact on the non-mycorrhizal microbial community structure and activity in the root zone (Curl and Truelove, 1986;

Hodge *et al.,* 1998; Griffiths *et al.,* 1999; Kuzyakov *et al.*, 2001; Nardi *et al.,* 2002; Baudoin *et al.,* 2003; Falchini *et al.*, 2003).

This functional microenvironment has been shown to include the so-called “rock eating” fungi which are active in the breakdown of minerals to produce clays and soils (Jongmans *et al.,* 1997; van Breemen *et al.,* 2000a and b; Landeweert *et al.,* 2001). These observations implicate a possible complex interaction of physico-chemical and biological processes occurring in the *C. dactylon* rhizosphere and resulting in the bioconversion of the coal substrate.

In order to test these assumptions under controlled conditions, it was considered important to attempt to simulate the *C. dactylon*/coal rhizosphere environment as a model system in laboratory-based investigations. The need for a reductive approach was identified so that the non-mycorrhizal/rhizospheric fungal components of the system could be isolated and the role of organic carbon supplementation possibly provided by the plant/mycorrhizal component of the system could be separately investigated. The development of a column bioreactor to replicate a vertical profile through the coal dump surface is described in Chapter six.

To undertake this development, it would be necessary to first understand the proposed organic carbon supplementation process that may be involved in the system in order to be able to optimize the reaction. Thereafter, mechanistic studies would be required to further inform the design and operation of the coal environment simulation exercise. These investigations are reported in Chapters four and five.

Previous laboratory studies on hard coal bioconversion had used coal which had been pre-oxidized with HNO_3 (Achi, 1993, 1994a and b; Fakoussa, 1994; Machnikowska *et al.,* 2002; Başaran *et al.*, 2003). It was considered important in the simulation exercise to consider replacement of this in part or completely with hard coal which had been oxidized by natural processes (de la Puente *et al.,* 1998; Cimadevilla *et al.* 2003), and thus the use of weathered hard coal (WC) was investigated. In addition, the coal substrate in the waste coal dump environment is thought to include a free humic acids component

in addition to un-oxidized raw hard coal and oxidized hard coal. This could play a role in enabling the establishment of the non-mycorrhizal rhizospheric component of the population as an initial stage in the coal dump colonization process.

4.1 Objectives

The following research objectives were identified:

- To investigate the bioconversion of humic acid and WC by *N. fischeri*;
- To determine if WC would provide an effective oxidized coal substrate to replace HNO_3 treated hard coal in the waste coal dump simulation environment;
- To evaluate a suitable organic carbon source for use as a supplement in the bioconversion of HA and WC by *N. fischeri.*

4.2 Materials and methods

Commercial grade HA (AGRON Co. Marble Hall, South Africa), WC (sourced from Kromdraai Colliery, South Africa), glutamate and glucose were used in medium formulation. Cresta Rice^{TM} was used for the rice extract formulation and sourced from a local supermarket.

4.2.1 Growth media preparation and characterization

A basal medium (BM) in 1 L demineralized H_2O was formulated to include K_2HPO_4 (0.31 %), KH_2PO_4 (1.27 %), $NaNO_3$ (0.3 %), $MgSO_4.7H_2O$ (0.05 %), KCl (0.05 %), and the pH was adjusted to 5.7. Glutamate (0.2 %), glucose (0.257 %) or rice extract (868 mg/L total organic carbon (TOC)) was used as the organic carbon source included to establish optimal fungal growth and ensure the effective degradation of 0.1 % HA and 0.25 % WC in a submerged liquid culture system. Either spores or pre-grown biomass of *N. fischeri* were used as the inoculum. Controls were setup that excluded the organic carbon supplement, but included HA or WC as the sole carbon source. 0.2 % ammonium nitrate was added as the nitrogen source (Table 4-1). Control media were set up as

described in Table 4-1 but were not inoculated, and the medium formulation that excluded the addition of HA and WC served as the biomass control.

Table 4-1 Tabulation of the medium formulation used in the submerged liquid culture growth studies.

Controls	Glucose	Glutamate	Rice-extract
BM + NH_4NO_3 + HA	BM + glucose + HA	BM + Glutamate + HA	BM + rice-extract + HA
BM + NH_4NO_3 + WC	BM + glucose + WC	BM + Glutamate + WC	BM + rice-extract + WC

BM, Basal medium; HA, Humic acid; WC, Weathered hard coal.

For a complete solubilization of the HA, it was first added to a volume of dimineralized water and stirred. The settled particulate fraction was discarded before the remaining components were then added and the final volume made up to 1L. 100 mL of completely mixed media in 250 mL Erlenmeyer flasks were autoclaved at 120 ^{o}C for 15 min.

4.2.1.1 Total organic carbon

An Apollo 900 combustion TOC analyzer (Teledyne Tekmar) equipped with a boat sampler (Model 183) and calibrated with potassium hydrogen phthalate ($C_8H_5KO_4$) was utilized for TOC analysis. The TOC was measured by initially removing the inorganic carbon fraction by sparging a 10 mL (1 %) solution of sample in distilled water acidified with 40 µL of 0.1 M phosphoric acid for 5 min. This was followed by combusting a 40 µL aliquot of the acidified sample in the boat sampler.

4.2.1.2 Rice extracts

A rice extract was prepared as a stock concentrate by adding 500 mL tap water to 100 g rice and heating in a water bath at 100 ^{o}C for 30 min. Before use, the stock concentrate was diluted with tap water to produce a working standard solution with a TOC concentration of 868 mg/L. This was determined by TOC measurement to be an equivalent TOC concentration to a 0.257 % glucose and 0.2 % glutamate solution.

4.2.1.3 Weathered hard coal, humic and fulvic acid.

The WC sample was crushed with a pestle and mortar, dried in an oven at 60 ^{o}C and stored in a desiccator before use. Percentage composition of humin, HA and FA was determined by acid precipitation of the alkaline extracts of the WC (Appendix 1-A). Standard curves for HA and FA were derived. A Suwannee River HA standard (2S101H) (Appendix 4-A) and Waskish Peat FA standard (IR107F) (Appendix 4-B), both sourced from the International Humic Substances Society (IHSS), were used for the derivation of HA and FA standard curves respectively. Humic acid was measured spectrophometrically by absorbance at 450 nm (ThermoSpectronic Aquamate v4.60) and quantified against the standard curve. Fulvic acid was measured by absorbance at 280 nm and also using Folin-Ciocalteau phenol reagent (Box, 1983) with absorbance at 260 nm and at 750 nm.

4.2.2 Growth studies

4.2.2.1 Inoculum preparation

The spores of *N. fischeri* were harvested by flushing an actively growing fungal mat on PDA in a Petri dish, with sterile water. The spore suspension was then transferred by sterile pipette into a sterile Schott bottle containing 100 mL distilled water and then vortexed. This was repeated until a high-concentrate spore suspension was attained. A spore suspension of ~1.64 x 10^{9} spores/mL was used and the spore count was measured using a haemocytometer (Neubauer, Merck).

100 mL potato dextrose broth (PDB) was used as the growth medium for biomass inoculum preparation. The PDB was inoculated with a 2 mL spore suspension (~1.64 x 10^{9} spores/mL) of *N. fischeri* and placed on a rotary shaker (150 rpm, 28 ^{o}C) and harvested when the fungal growth was in the logarithmic phase. This was reached after two days and was determined gravimetrically. The biomass was harvested by filtration (sterile 36 μm nylon mesh) and washed several times with sterile milliQ water until the rinsate became clear. This served as the pre-grown inoculum. Inoculation was performed aseptically with a flamed forceps to transfer the washed pellets of *N. fischeri* into the

prepared medium. The dry weight of the inoculum was determined gravimetrically by drying an equivalent inoculum sample for 24 hours at 60 ^{o}C.

4.2.2.2 Experimental protocol

A total of 224 Erlenmeyer flasks, which included 128 experimental flasks and 96 control flasks, were set up as a single study. The flasks were placed on rotary shakers Labcon (3100u) at 150 rpm and incubated at 28 ^{o}C in a controlled temperature room for 14 days. Reactions were then terminated at two day intervals from 0 to 14 days and the flasks were destructively sampled. Termination was by filtration (36 μm nylon mesh), and the filtrate and biomass were collected and analyzed for biomass, humin, HA and FA. A stepwise representation of the experimental protocol utilized is shown as a flow chart in Figure 4-1.

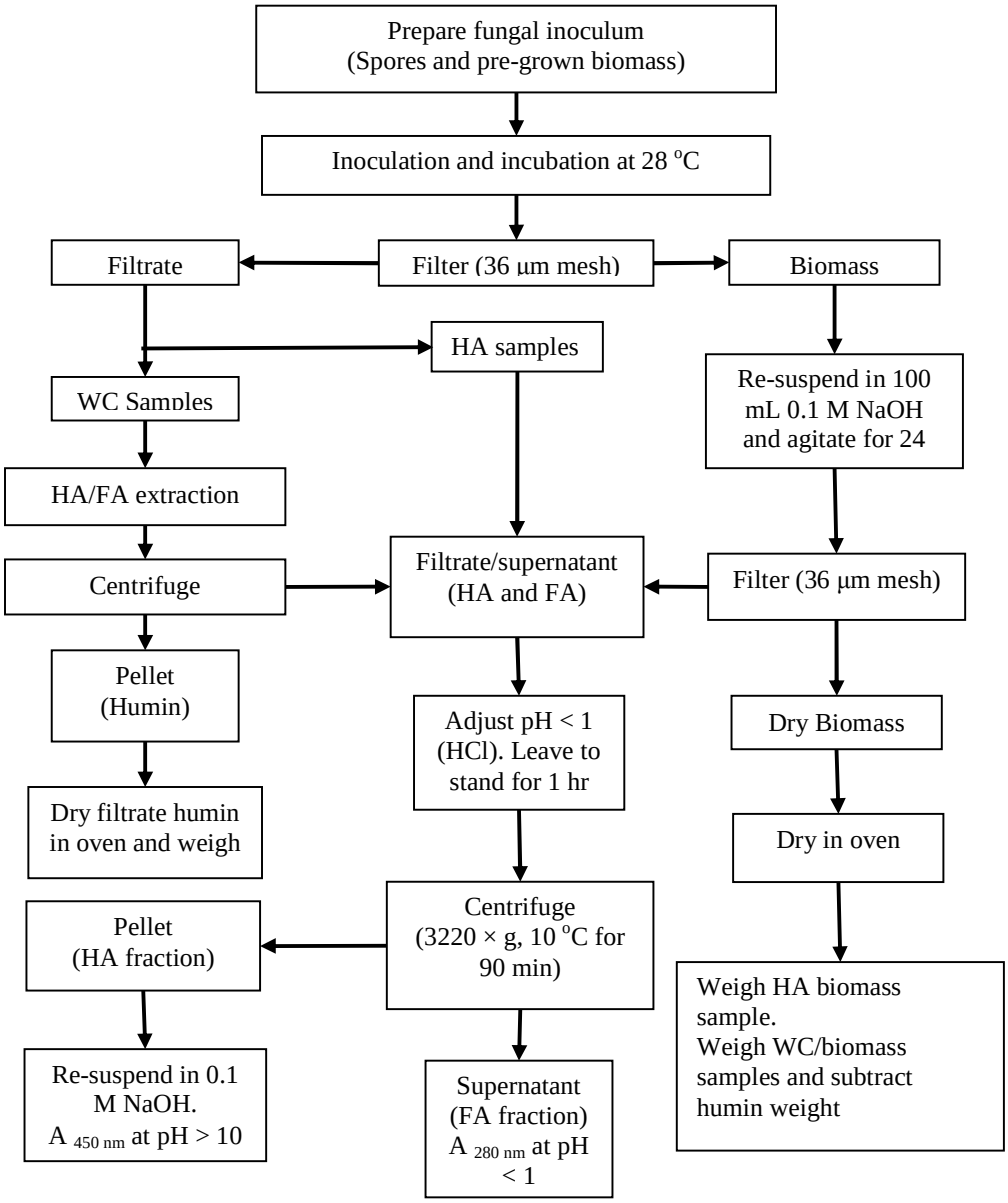


Figure 4-1 Flow diagram of the experimental protocol utilized for the determination of biomass, humic acid and fulvic acid in the *Neosartorya fischeri* growth study.

4.2.2.3 Analysis of biomass, humic acid, fulvic acid and humin

For analysis of medium containing the HA substrate, the extraction and analysis of HA and FA was as described in Section 2.2.2.4. In addition, the HA (biomass-bound HA) and FA (biomass-bound FA) fractions absorbed onto the biomass sample were extracted by resuspending the biomass in 100 mL 0.1 M NaOH for extraction and analysis. The collected biomass was dried (60 ^{o}C for 24 hours) and stored in a desiccator to cool before it was weighed.

For analysis of medium containing the WC substrate, the pH of the filtrate, after removing the biomass, was first adjusted to > 11 with a minimal volume of 10 M NaOH and placed on a rotary shaker at 150 rpm for 24 hours. The filtrate sample was then centrifuged (3220 × g for 90 mins at 10 ^{o}C) and the supernatant, which contained the soluble HA and FA fractions was decanted and a 10 mL aliquot collected. The pellet (filtrate humin) was dried (60 ^{o}C for 24 hrs) in the oven and left to cool in a desiccator before it was weighed. The HA and FA in the 10 mL aliquot sample were separated and analyzed as described.

The biomass plus the adsorbed humin fraction (biomass-bound humin) of the WC was dried (60 ^{o}C for 24 hours) and stored in a desiccator to cool before it was weighed. An average humin content of 0.25 % WC medium was measured to be 0.1 g/100 mL of medium as described in Appendix 4-C. The biomass-bound humin was then derived by subtracting the weight of the filtrate humin from the average humin weight that was determined to be contained in 0.25 % WC medium. The weight of the fungal biomass contained in the WC-biomass was subsequently calculated by subtracting the average weight of the biomass-bound humin from that of the WC-biomass (Appendix 4-C).

The concentration of FA, which had been referred to as a degradation product of HA (Hofrichter and Fritsche, 1997b; Hofrichter *et al*., 1999; Klein *et al*., 1999), could not readily be determined in all cases, and was found to be observed due to the secretion of the ergochrome, neosartorin, biosynthesized by the *N. fischeri* (Proksa, *et al.*, 1998). The

absorbance maxima of neosartorin are observed at 279 nm (Proksa, *et al.*, 1998) and interfere with that of FA at 280 nm. Also the use of Folin-Ciocalteau phenol reagent (Box, 1983) with absorbance at 750 nm to differentiate FA from neosartorin did not yield a positive differential result (Appendix 4-B). The secretion of neosartorin was evident in all the control systems, thus preventing quantitative analysis of the FA.

4.2.2.4 Statistical analysis

STATISTICA, version 8.0 software (StatSoft, Inc. 2007) was utilized for statistical analysis. The Fisher Post-hoc Least Squared Difference (LSD) test or the planned comparison test of breakdown of one-way Analysis of Variance (ANOVA) with a 95 % degree of confidence was adopted in which the level of statistical significant difference was accepted as $p < 0.05$. Standard deviations were calculated for the stationary phase and growth phase results where a large sample size was available. This was not done for the logarithmic growth phase results due to the short period and the low sample number available.

4.3 Results and discussions

Visual observation of the flasks in the study showed the growth of *N. fischeri* as fungal pellets in the liquid medium and the rapid adsorption of HA with the clearing of the dark coloured medium as shown in Figure 2-4. Changes in the substrate were analyzed and are reported below. However, given the internal consistency of each experiment, a degree of comparability is available. The biomass productivities for the studies comparing the various organic carbon supplements added to the HA and WC media are shown in Table 4-2. The use of spore and biomass inocula was also compared. Table 4-3 shows the rate calculations of filtrate HA removal and adsorption to biomass, coupled with the percentage increase of the biomass-bound HA fraction. Given the high variability of extractable HA inherent in the WC samples, it was found that the total HA introduced in the various experiments also varied.

Table 4-2 Measurement of biomass production and growth rates obtained for the various media investigated in the *Neosartorya fischeri* growth study at logarithmic and stationary phase.

Media recipe	Logarithmic phase			Stationary phase			
	Maximum biomass (mg/100 mL)	Time (d)	Growth rate (mg biomass/day)	Average biomass (mg/100 mL)	Standard deviation	Time (d)	Growth rate (mg biomass/day)
Glutamate media							
Spore inoculum							
[1]CNT-Glut	65	4	16.3	66	10	10	6.6
[1]HA-Glut	84	4	21.0	71	8	10	7.1
[1]WC-Glut	84	2	42.0	66	13	12	5.5
Biomass inoculum							
[2]CNT-Glut	228	10	22.8	195	29	4	48.7
[2]HA-Glut	110	2	55.0	106	14	12	8.8
[2]WC-Glut	187	2	93.5	160	31	12	13.3
Glucose media							
Spore inoculum							
[1]CNT-G	153	6	25.5	143	7	8	17.9
[1]HA-G	124	4	31.0	110	11	10	11.0
[1]WC-G	186	14	13.3	na	na	na	na
Biomass inoculum							
[2]CNT-G	124	2	62.0	125	5	12	10.4
[2]HA-G	115	4	28.8	119	4	10	11.9
[2]WC-G	96	4	24.0	85	28	10	8.5
Rice extract media							
Spore inoculum							
[1]CNT-R	181	2	90.5	147	15	12	12.3
[1]HA-R	92	2	46.0	73	12	12	6.1
[1]WC-R	81	4	20.3	60	15	8	7.5
Biomass inoculum							
[2]CNT-R	211	2	105.5	189	30	12	15.8
[2]HA-R	170	4	42.5	113	38	10	11.3
[2]WC-R	151	6	25.2	116	40	8	14.6
Ammonium nitrate media							
Biomass inoculum							
[2]CNT-Amm	112	4	28.0	76	26	10	7.3
[2]HA-Amm	34	2	17.0	24	5	12	2.0
[2]WC-Amm	22	6	3.7	20	21	8	2.5

Biomass figures exclude inoculum. na, not ascertained; d, day; [1] spore inoculum; [2] biomass inoculum; CNT-Glut, control glutamate medium; HA-Glut, humic acid-glutamate medium; WC-Glut, weathered hard coal-glutamate medium; CNT-G, control glucose medium; HA-G, humic acid-glucose medium; WC-G, weathered hard coal-glucose medium; CNT-R, control rice-extract medium; HA-R, Humic acid-rice extract medium; WC-R, weathered hard coal-rice extract medium; CNT-Amm, control ammonium nitrate medium; HA-Amm, humic acid-ammonium nitrate medium; WC-Amm, weathered hard coal-ammonium nitrate medium.

Table 4-3 Measurement of rates of humic acid removal from the filtrate and adsorption to biomass during the logarithmic phase culture for the various media investigated in the *Neosartorya fischeri* growth study.

		Filtrate humic acid removal					Biomass adsorption				
Media recipe	**Humic acid (Start) (mg/100 mL)**	**HA (mg/100 mL)**	**Time (d)**	**HA/d**	**biomass/d**	**Removal rates (mg/mg biomass/day)**	**HA (mg/100 mL)**	**Time (d)**	**HA/d**	**Adsorption rates (mg/mg biomass/day)**	**% HA after 2d**
Glutamate media											
Spore inoculum											
[1]HA-Glut	127.3	98.8	2	49.4	21.0	2.4	122.6	2	61.3	2.9	
[1]WC-Glut	80.0	76.7	2	38.4	42.0	0.9	118	2	59.0	1.4	32.2
Biomass inoculum											
[2]HA-Glut	127.7	120.5	2	60.3	55.0	1.1	71.9	2	35.9	0.7	
[2]WC-Glut	26.7	25.6	2	12.8	93.5	0.1	59.3	2	29.7	0.3	55
Glucose media											
Spore inoculum											
[1]HA-G	116.0	112.6	6	18.8	31.0	0.6	74.5	6	12.4	0.4	
[1]WC-G	61.0	59.3	2	29.7	13.3	1.8	48.2	2	24.1	2.2	7.1
Biomass inoculum											
[2]HA-G	123.2	105.2	4	26.3	28.8	0.9	62.5	4	15.6	0.5	
[2]WC-G	39.0	38.6	2	19.3	24.0	0.8	66.6	2	33.3	1.4	41.4
Rice extract media											
Spore inoculum											
[1]HA-R	124.5	62.6	2	31.3	46.0	0.7	49.3	2	24.7	0.5	
[1]WC-R	57.8	57.3	2	28.6	20.3	1.4	89.7	2	44.9	2.2	35.6
Biomass inoculum											
[2]HA-R	134.1	129.9	2	65.0	42.5	1.5	65.8	2	32.9	0.8	
[2]WC-R	14.3	12.6	2	6.3	25.2	0.2	41.8	2	20.9	0.6	65.8
Ammonium nitrate media											
Biomass inoculum											
[2]HA-Amm	126.5	79.3	2	39.7	17.0	2.3	47.2	2	23.6	1.4	
[2]WC-Amm	38.0	37.8	2	18.9	3.7	5.2	57.4	2	28.7	7.8	33.8

HA, humic acid; d, day; na, not ascertained; [1], spore inoculum; [2], biomass inoculum; %, indicate the increment in the biomass-bound humic acid after two days; HA-Glut, humic acid-glutamate medium; WC-Glut, weathered hard coal-glutamate medium; HA-G, humic acid-glucose medium; WC-G, weathered hard coal-glucose medium; HA-R, Humic acid-rice extract medium; WC-R, weathered hard coal-rice extract medium; HA-Amm, humic acid-ammonium nitrate medium; WC-Amm, weathered hard coal-ammonium nitrate

4.3.2 Glutamate growth study

The use of glutamate as a supplementary carbon source was investigated for both HA and WC substrates inoculated with *N. fischeri* spores and biomass.

4.3.2.1 Spore inoculum

A rapid removal of HA (Figure 4-2) from the medium was observed in the humic acid-glutamate (HA-Glut) growth study and indicated an adsorption by the fungal biomass which was partly reversible with alkaline reaction. The filtrate HA declined from 1273 mg/L to 285 mg/L within two days of inoculation with spores of *N. fischeri*. During this initial reaction period, a rate HA removal from the filtrate of 2.4 mg/mg biomass/day was measured. This compares with a rate of adsorption onto the biomass of 2.9 mg/mg biomass/day, with the comparability between the two measurements suggesting a linkage between the two observations. After day two, the reduction in biomass-bound HA, without a concomitant rise in the filtrate HA levels, suggested that some breakdown of the HA substrate may have occurred during this period. After day eight, some desorption of the HA was observed, which was associated with a decline in biomass-bound HA and a concomitant rise in filtrate HA (Figure 4-2). A break-up of the fungal pellets in the flasks at this time indicated the cultures had entered the decline phase. This observation was not as clear cut in the subsequent studies.

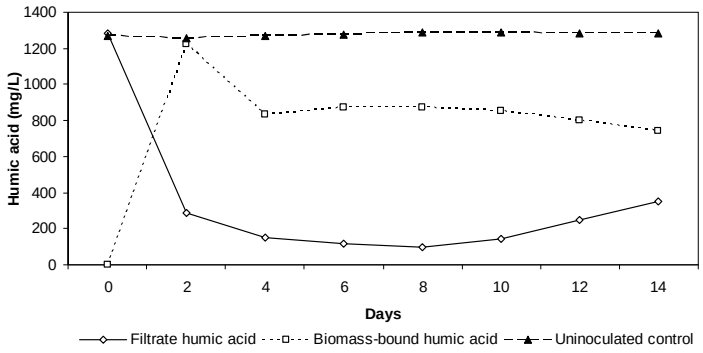


Figure 4-2 The 14 day profile of the filtrate extractable humic acid and biomass-bound humic acid in the humic acid-glutamate medium inoculated with *Neosartorya fischeri* spores.

The results for the WC-Glut medium study with spore inoculum are reported in Figure 4-3. Here HA was observed to be released from the WC and its subsequent removal from the medium occurred within two days, with a decline of the filtrate HA from 819 mg/L to 33 mg/L. A rate of HA adsorption within the first two days of 1.4 mg/mg biomass/day was measured. This is greater than the 0.9 mg/mg biomass/day removal rate of the filtrate HA. A 32.2 % increase in biomass-bound HA was measured on day two for the WC-Glut medium which was not seen in the HA-Glut medium. This increase in biomass-bound HA suggests the bioconversion of WC in the medium with the release of additional HA (over and above that present from the outset) due to fungal activity (Figure 4-3). A decline in HA was then observed between day two and four, which may indicate a breakdown of HA. However, with the fluctuating levels of biomass-bound HA, the results were not as clear cut for the WC study. This response may be due to ongoing breakdown and simultaneous utilization of the WC resulting in release of HA over the course of the experiment. This observation of additional HA release in the WC-medium was seen in all of the other WC-media studies.

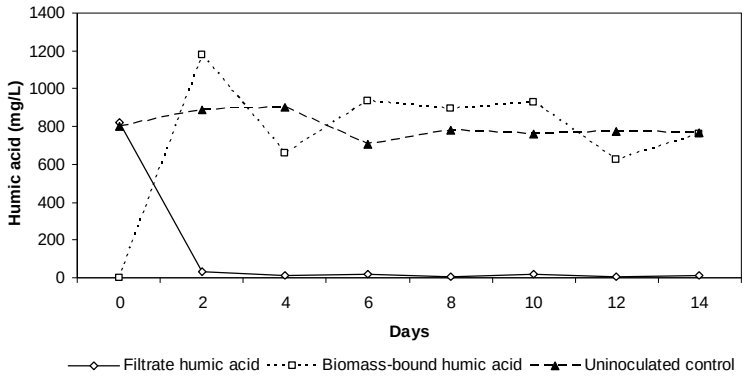


Figure 4-3 The 14 day profile of the filtrate extractable humic acid and biomass-bound humic acid in the weathered hard coal-glutamate medium inoculated with *Neosartorya fischeri* spores.

In both studies the HA and WC media performed better than glutamate on its own during logarithmic growth phase (Table 4-2). This indicated that the HA and WC are likely to have provided *N. fischeri* with certain growth requirements absent in the glutamate medium. While the logarithmic growth rate of *N. fischeri* in WC-Glut medium was twice

that of HA-Glut growth medium, the biomass productivity by the end of the stationary phase was broadly comparable with no significant difference observed ($p > 0.05$, Appendix 4-D). This result suggests that the humin fraction in the coal (WC – HA) does not exercise an inhibitory effect on *N. fischeri* growth.

4.3.2.2 Biomass inoculum

The HA-Glut and WC-Glut studies were repeated to compare biomass inoculation with spore inoculation of the experimental system (Figures 4-4 and 4-5). For the HA-Glut growth medium, the filtrate HA removal rate of 1.1 mg/mg biomass/day was greater than the adsorption rate of 0.7 mg/mg biomass/day, suggesting possible utilization of HA by *N. fischeri* activity. However, for the WC-Glut a 0.1 mg/mg biomass/day HA removal rate was lower than the adsorption rate of 0.3 mg/mg biomass/day, suggesting breakdown of WC to release the additional HA (Table 4-3). Here again, no biomass-bound HA peak occurred at day two in the HA-Glut medium (Figure 4-4), while a 55 % increase in biomass-bound HA was measured after day two in the biomass inoculated WC-Glut medium (Figure 4-5).

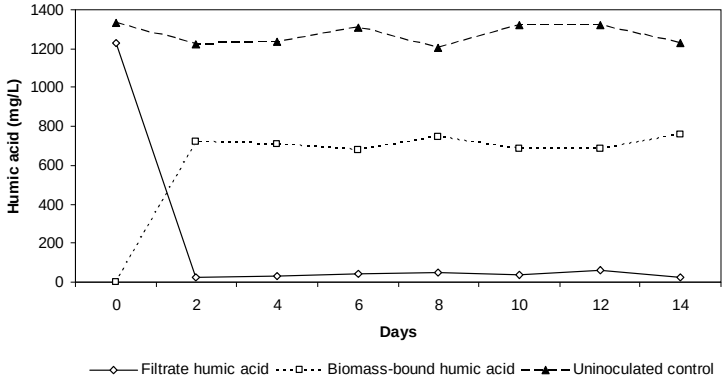


Figure 4-4 The 14 day profile of the filtrate extractable humic acid and biomass-bound humic acid in the humic acid-glutamate medium inoculated with *Neosartorya fischeri* biomass.

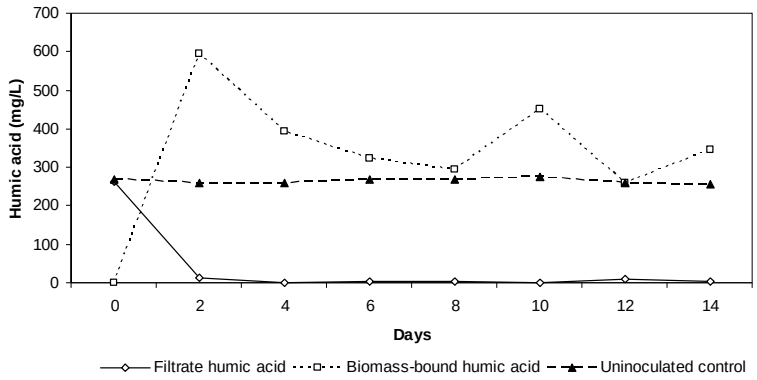


Figure 4-5 The 14 day profile of the filtrate extractable humic acid and biomass-bound humic acid in the weathered hard coal-glutamate medium inoculated with *Neosartorya fischeri* biomass.

As observed in the spore inoculum, the HA and WC media performed better than the glutamate control during the logarithmic growth phase (Table 4-2). Also, the biomass production of 187 mg/100 mL was almost double that of HA-Glut medium 110 mg/100 mL (Table 4-2). In addition, biomass productivity by the end of the stationary growth phase was also significantly higher ($p < 0.05$, Appendix 4-D). The results for the biomass inoculation study showed significantly better growth in the WC-Glut medium than for the spore inoculum ($p < 0.05$, Appendix 4-D).

4.3.3 Glucose growth study

4.3.3.1 Spore inoculum

The clarification of the humic acid-glucose (HA-G) growth medium was observed to be slower than the WC-glucose (WC-G) medium and occurred within six days of inoculation with *N. fischeri* spores. The filtrate HA declined from 1160 mg/L to 34 mg/L within this period (Figure 4-6). The rate of filtrate HA removal of 0.6 mg/mg biomass/day was measured and compares broadly with the HA adsorption rate of 0.4 mg/mg biomass/day, but suggests some breakdown may have occurred. In contrast to previous observations, there was no decline of the biomass-bound HA from day six onwards (Figure 4-6).

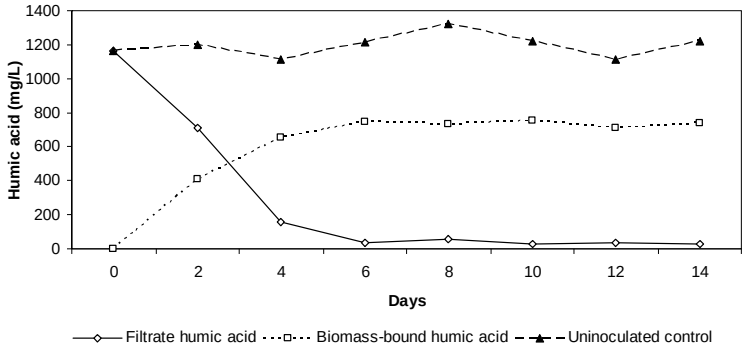


Figure 4-6 The 14 day profile of the filtrate extractable humic acid and biomass-bound humic acid in the humic acid-glucose medium inoculated with *Neosartorya fischeri* spores.

A more rapid clearance of the medium occurred within two days when HA was replaced with WC (Figure 4-7). The filtrate HA declined from 610 mg/L to 17 mg/L within two days of inoculation and the growth of biomass continued until day 14 at a rate of 13.3 mg/day (Table 4-2). The filtrate HA removal rate of 1.8 mg/mg biomass/day was slower than the adsorption rate of 2.2 mg/mg biomass/day (Table 4-3). The peak in the biomass-bound HA over and above the HA levels present in the uninoculated control which had been observed after two days in the WC-Glut medium study, occurred only after eight days in the WC-G medium and with an increase of 29 % (Figure 4-7). After that HA declined with no concomitant rise in the filtrate HA, possibly indicating breakdown by *N. fischeri* at the end of the 14 day experimental period. No significant difference was observed for biomass productivity in the HA-G, WC-G and the glucose media ($p > 0.05$, Appendix 4-D).

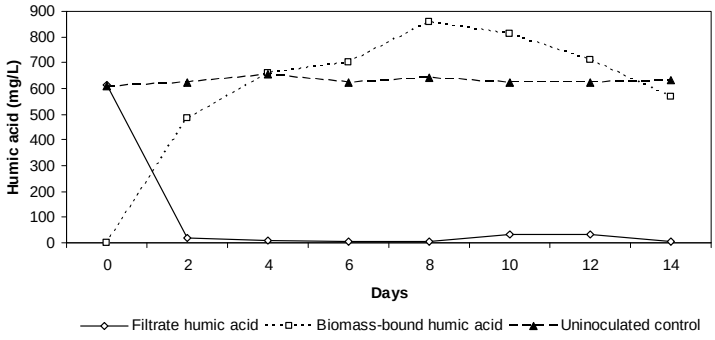


Figure 4-7 The 14 day profile of the filtrate extractable humic acid and biomass-bound humic acid in the weathered hard coal-glucose medium inoculated with *Neosartorya fischeri* spores.

4.3.3.2 Biomass inoculum

The evaluations of the performance of the biomass inoculum in the HA-G and WC-G media are shown in Figures 4-8 and 4-9, respectively. For the HA-G medium, the filtrate HA declined within the first four days and the removal rate of 0.9 mg/mg biomass/day was greater than the adsorption rate of 0.5 mg/mg biomass/day measured, suggesting that some breakdown of HA had occurred (Table 4-3). However, in the WC-G growth medium, the HA removal rate of 0.8 mg/mg biomass/day was lower than the adsorption rate of 1.4 mg/mg biomass/day measured within this period (Table 4-3). An increase in biomass-bound HA of 41.4 % was measured after two days, suggesting release of HA from the WC substrate by *N. fischeri* activity (Table 4-3).

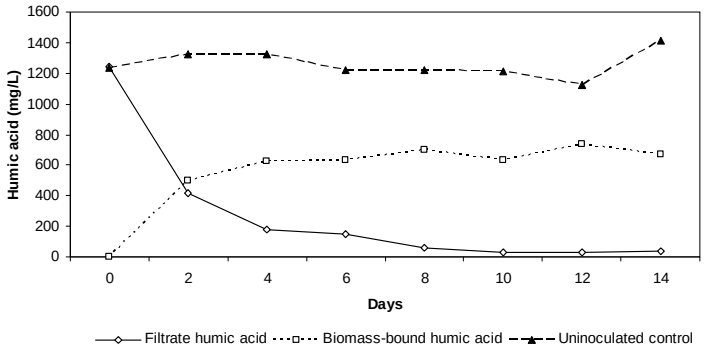


Figure 4-8 The 14 day profile of the filtrate extractable humic acid and biomass-bound humic acid in the humic acid-glucose medium inoculated with *Neosartorya fischeri* biomass.

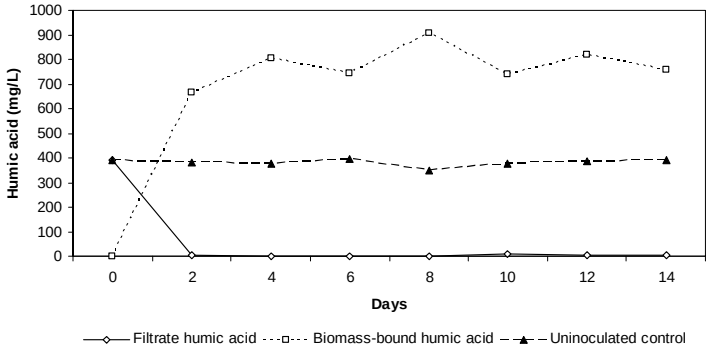


Figure 4-9 The 14 day profile of the filtrate extractable humic acid and biomass-bound humic acid in the weathered hard coal-glucose medium inoculated with *Neosartorya fischeri* biomass.

Both the glucose HA and WC media inoculated with *N. fischeri* biomass performed better than the spore inoculum during the logarithmic growth phase (Table 4-2). However, by the end of the stationary growth phase no significant difference was observed ($p > 0.05$) between the spore and biomass inocula (Appendix 4-D).

4.3.4 Rice extract growth studies

4.3.4.1 Spore inoculum

In the HA-rice extract (HA-R) growth study, the clarification of filtrate HA did not occur with *N. fischeri* spore inoculum (Figure 4-10). The filtrate HA only declined from 1245 mg/L to 619 mg/L within two days at a removal rate of 0.7 mg/mg biomass/day, which was broadly comparable to the adsorption rate of 0.5 mg/mg biomass/day measured within this period (Table 4-3). No biomass-bound HA peak was observed in this medium (Figure 4-10).

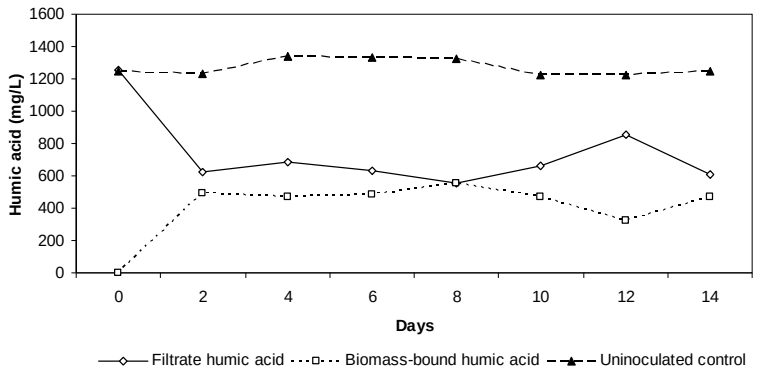


Figure 4-10 The 14 day profile of the filtrate extractable humic acid and biomass-bound humic acid in the humic acid-rice extract medium inoculated with *Neosartorya fischeri* spores.

The results for the WC-rice extract (WC-R) represented in Figure 4-11 showed filtrate HA reduction from 578 mg/L to 5 mg/L within two days. The HA filtrate removal rate for this period was 1.4 mg/mg biomass/day and was lower than the adsorption rate of 2.2 mg/mg biomass/day (Table 4-3). The peak in HA exceeding the control of 35.6 % was measured within two days after which it declined on day four, which is comparable to the

WC-Glut and WC-G media and suggests HA release from WC by *N. fischeri* activity (Figure 4-11).

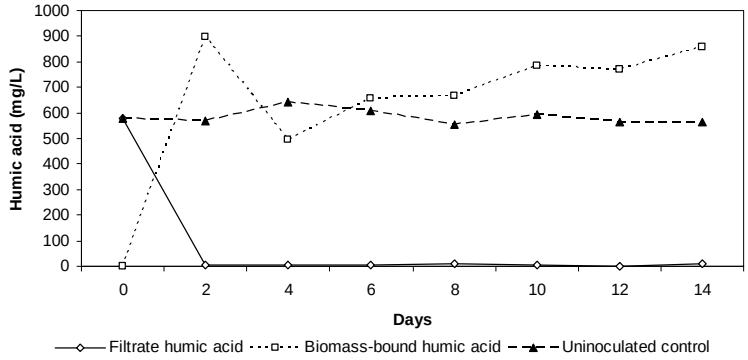


Figure 4-11 The 14 day profile of the filtrate extractable humic acid and biomass-bound humic acid in the weathered hard coal-rice extract medium inoculated with *Neosartorya fischeri* spores.

In both studies, the biomass productivity in the rice extract growth medium was significantly greater than the HA and WC media during the logarithmic and stationary growth phases ($p < 0.05$, Appendix 4-D).

4.3.4.2 Biomass inoculum

In the study to compare biomass with spore inocula, the filtrate HA in the HA-R growth medium declined from 1341 mg/L to 42 mg/L during the first two days. The removal rate of 1.5 mg/mg biomass/day that was greater than the adsorption rate of 0.8 mg/mg biomass/day measured during this initial period suggesting HA breakdown by *N. fischeri* activity (Table 4-3). No biomass-bound HA peak was observed after day two (Figure 4-12).

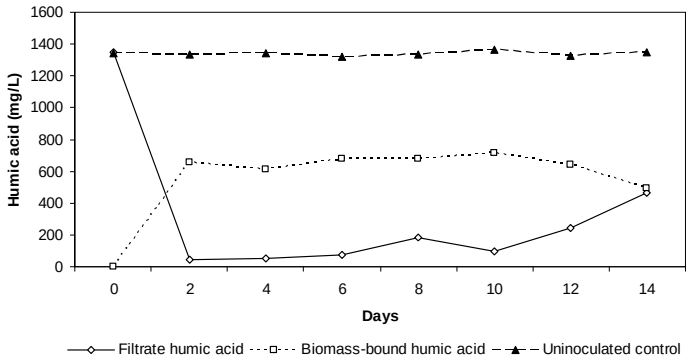


Figure 4-12 The 14 days profile of the filtrate extractable humic acid and biomass-bound humic acid in the humic acid-rice extract medium inoculated with *Neosartorya fischeri* biomass.

In the presence of WC, the filtrate HA declined from 143 mg/L to 17 mg/L two days after inoculating with *N. fischeri* biomass, with the concomitant rise in the biomass-bound HA producing a 65.8 % increase in HA compared to the uninoculated control (Figure 4-13). However, the filtrate HA removal rate of 0.2 mg/mg biomass/day was lower than the adsorption rate of 0.6 mg/mg biomass/day within this period (Table 4-3).

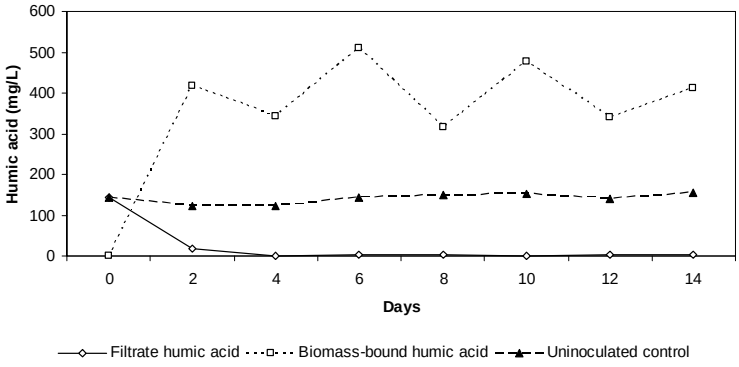


Figure 4-13 The 14 days profile of the filtrate extractable humic acid and biomass-bound humic acid in the weathered hard coal-rice extract medium inoculated with *Neosartorya fischeri* biomass.

The rice extract medium inoculated with biomass performed better than the spore inoculum in this category during the logarithmic phase (Table 4-2) and the biomass performance of the biomass inoculum was also significantly higher at the end of the stationary phase ($p < 0.05$, Appendix 4-D).

4.3.5 Ammonium nitrate growth study

A control study was set up to compare *N. fischeri* growth in the absence of an added organic carbon supplement. Ammonium nitrate was provided as an additional N source, since it has been identified as a bioconversion requirement (Hofrichter *et al*., 1997b). For each medium, the *N. fischeri* spore inoculum failed to grow and produce biomass, while the biomass inoculum did produce the least growth amongst the different media (Table 4-2) and in most cases significantly lower than where organic carbon supplements were added ($p < 0.05$, Appendix 4-D).

The results for the biomass inoculated HA-Amm and WC-Amm growth studies are shown in Figures 4-14 and 4-15, respectively, with an adsorption and removal pattern comparable to previous biomass inoculum studies. For the HA-Amm growth medium, a decline in the filtrate HA from 1265 mg/L to 472 mg/L occurred within two days (Figure 4-14). The removal rate of 2.3 mg/mg biomass/day was higher than the adsorption rate of 1.4 mg/mg biomass/day (Table 4-3) suggesting HA breakdown by *N. fischeri* activity. No biomass-bound HA peak appeared above the control background and the HA adsorption was low (Figure 4-14) compared to the WC-Amm study (Figure 4-15).

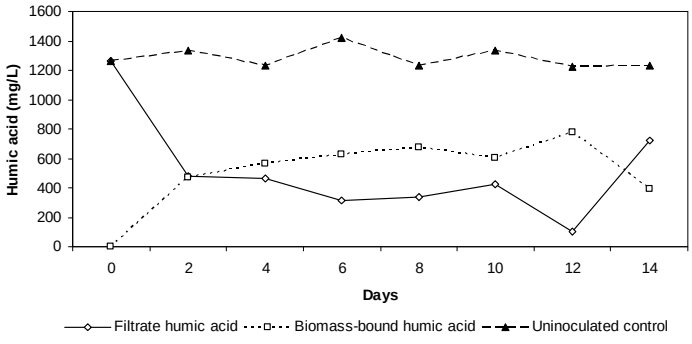


Figure 4-14 The 14 day profile of filtrate extractable humic acid and biomass-bound humic acid in humic acid-ammonium nitrate medium inoculated with *Neosartorya fischeri* biomass.

In the presence of WC a decline of the filtrate HA occurred at the rate of 5.2 mg/mg biomass/day, with an adsorption rate of 7.8 mg/mg biomass/day (Table 4-3). Although total biomass productivity was lower, this was a substantially higher rate than for any of

the organic carbon supplemented studies and suggests HA was not only released from WC as previously observed, but also probably used as a nutrient source. A gradual and continuous increase in the biomass-bound HA was observed after the 33.8 % increase at day two (Figure 4-15).

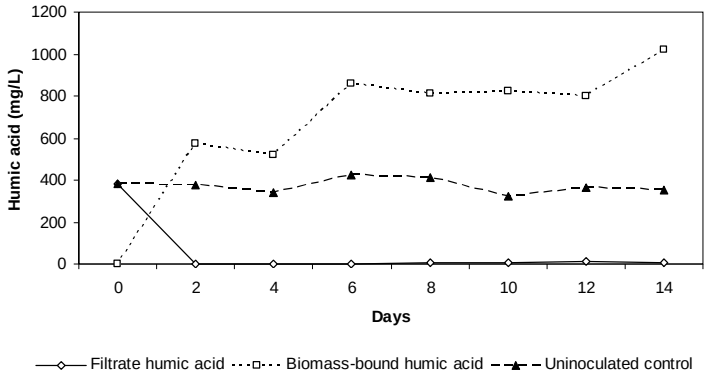


Figure 4-15 The 14 day profile of the filtrate extractable humic acid and biomass-bound humic acid in weathered coal-ammonium nitrate medium inoculated with *Neosartorya fischeri* biomass.

In summary, glutamate, when used as an organic carbon supplement gave the best result. Biomass production of 187 mg/100 mL was achieved in the presence of WC when inoculated with *N. fischeri* biomass. The biomass produced in the WC-Glut growth study was significantly greater ($p < 0.05$, Appendix 4-D) than the biomass produced in the other carbon supplemented growth media (Table 4-2). The next best productivity was 170 mg/100 mL which was measured in HA-R inoculated with spores. In terms of HA release from WC, WC-R medium performed the best with a 65.8 % increase measured at day two after inoculation with *N. fischeri* biomass, followed by WC-Glut medium which recorded a 55 % increase in HA after inoculation with *N. fischeri* biomass (Table 4-3).

In all the cases involving glucose, cultures showed a reduced effect on the alkaline extractable HA. This was particularly evident in the HA-G medium inoculated with spores (Table 4-2 and Figure 4-6). Hölker *et al.* (1995) had previously reported that the fungal-coal interaction was inhibited in the presence of glucose. The release and possible breakdown of HA and its use as a nutrient source by *N. fischeri* was observed in all the WC studies. The growth of *N. fischeri* in the WC-Amm study, where no organic carbon

source was available, supported this conclusion, although reduced biomass productivity was observed under these conditions.

4.4 Conclusions

The investigation of the *C. dactylon*/coal rhizosphere system using *N. fischeri* as a single model inoculum provided an indication of the processes that may occur within this microenvironment:

- Biomass production was significantly higher in the WC-Glut medium using biomass inoculum than in all the other media.
- The presence of humin in the WC studies produces no significant inhibitory effects on growth.
- A direct correlation does not exist between the filtrate HA removal and HA adsorption rate onto biomass in the media.
- In the presence of WC, the filtrate HA removal rate was observed to be lower than the adsorption rate and this suggests the bioconversion of the WC with the subsequent release and possible consumption of HA.

These conclusions present the first insights into the nutritional importance of organic carbon supplemented as plant exudates in the *C. dactylon*/coal system. Furthermore, this lays the foundations for a more detailed interrogation of WC-Glut medium in defining the coal bioconversion mechanism of *N. fischeri*. In addition to providing insights into the activity occurring in the *C. dactylon*/coal rhizosphere, results reported here enabled the formulation of a growth medium that could be used in further studies of the system and led to the setting up of a laboratory scale simulation of the *C. dactylon*/coal rhizosphere microenvironment.

Chapter five

The bioconversion of hard coal by *Neosartorya fischeri*

5. Introduction

The microbial population screening program demonstrated the presence of *N. fischeri* in the *C. dactylon*/coal microenvironment. Among the performance indicators investigated, *N. fischeri* was found to have coal bioconversion activity comparable to that of *P. chrysosporium* and *T. versicolor,* which have been previously described in this role (Dehorter and Blondeau, 1992; Torzilli and Isbister, 1994; Mönkemann *et al.,* 1997; Bumpus *et al.,* 1998; Başaran *et al.,* 2003). Investigations reported in Chapters two and four had indicated the ability of *N. fischeri* to convert both HNO_3-oxidized and naturally weathered oxidized BHC. In liquid culture, its growth was shown to be associated with the release of HA from the coal matrix and that in addition, other components present in the coal may be utilized as nutrients. Also, the mechanism resulting in the increase in the alkaline extractable HA may be understood as a reversal of the diagenetic process of coal formation (Piccolo *et al*., 1992; Chang and Berner, 1998). This process may be compared to the chemical regeneration of HA by the use of HNO_3 (Quigley *et al.*, 1988; Machnikowska *et al.,* 2002; Elbeyli *et al.,* 2006a), hydrogen peroxide (Hölker *et al.*, 1997; Hofrichter *et al.*, 1997b; Willmann and Fakoussa, 1997a) and the aeration of the coal macromolecule at elevated temperatures (Ndaji and Thomas, 1995).

The study reported here was undertaken to investigate the mechanisms involved in the *N. fischeri* coal bioconversion activity in a submerged liquid culture system, in order to inform a descriptive model accounting for the activities observed in the coal dump environment. The results reported in the previous chapter had indicated the WC-Glut medium inoculated with pre-grown biomass would provide a functional experimental system in which to follow changes in HA and its release from the WC substrate by *N. fischeri* activity. Here again the performance was compared with the known strains, *P. chrysosporium* and *T. versicolor*.

5.1 Objectives

The objectives of the study reported in this chapter include:

- To investigate mechanisms utilized by *N. fischeri* in coal bioconversion activity by monitoring the modification of the HA substrate in submerged liquid culture;
- To undertake a comparative study of mechanisms utilized in coal bioconversion by *N. fischeri* and the previously described species, *P. chrysosporium* and *T. versicolor*.

5.2 Materials and methods

5.2.1 Medium formulation

The WC-Glut medium was prepared in 1 L demineralized H_2O and included WC (0.25 %), glutamate (0.2 %), K_2HPO_4 (0.31 %), KH_2PO_4 (1.27 %), $NaNO_3$ (0.3 %), $MgSO_4.7H_2O$ (0.05 %), KCl (0.05 %). The pH of the medium was adjusted to 5.7. A total of 20 Erlenmeyer flasks, which included 5 experimental flasks for each set and 5 control flasks, were set up as a single study.

5.2.2 Preparation of inoculum

Cultures used in the study included *N. fischeri* (ECCN 84), *P. chrysosporium* (PPRI 5328), *T. versicolor* (PPRI 4835) and a mixed culture of all three organisms. The preparation of *N. fischeri* biomass inoculum was as previously described in Section 4.2.2.1. For the mixed culture, 300 µL of each spore suspension was added to 100 mL PDB from which the pre-grown mixed culture inoculum was harvested and used as inoculum.

5.2.3 Sampling method

Fakoussa and Hofrichter (1999) reported that only the HA attached to fungal hyphae is degraded in agitated, submerged liquid culture systems. Therefore, the biomass-bound HA fraction obtained from the four systems was investigated for coal bioconversion using FT-IR, macro-elemental analysis and Py-GCMS. The reactions were terminated by filtration (36 μm nylon mesh) and destructively sampled at day 0, 2, 4, 9 and 14 as described in Section 4.2.2.3. The total HA (alkaline extractable filtrate HA plus alkaline extractable biomass-bound HA) and total carbon (TC) (TC of filtrate plus TC of alkaline extractable biomass-bound HA) of the system was determined.

5.2.4 Analysis

5.2.4.1 Glutamic acid

The glutamic acid analysis followed that described by Hwang and Ederer (1975). A 0.35 % ninhydrin solution in ethanol (96 %) was prepared and 1 mL was added to a 5 mL culture supernatant in a test tube and covered with parafilm. This was heated and stirred in a water bath at 90 ^{o}C for 6 min. Samples were cooled to room temperature under running water after which they were analyzed spectrophotometrically (ThermoSpectronic Aquamate v4.60). Distilled water prepared in the same manner served as the blank. Absorbance was read at 570 nm and glutamate quantified from a standard curve ($y = 0.004x$, where $R^2 = 0.9831$) (Appendix 5-A).

5.2.4.2 Total Carbon

The TC was monitored as an indicator for microbial utilization of carbon within the system. The Apollo 9000 combustion TOC analyzer (Teledyne Tekmar) equipped with a boat sampler (Model 183) was utilized for the TC analysis. The TC measure was obtained by a direct combustion of 40 μL of 1 % solution of the sample in distilled water. Carbon in the sample is first converted to CO_2 in the combustion furnace at 680 ºC. The derived CO_2 is swept by a carrier gas through a non-dispersive

infrared (NDIR) detector which generates a non-linear signal that is proportional to the instantaneous concentration of CO_2 in the carrier gas. That signal is then linearized and integrated over the sample analysis time. The resulting area is then compared to stored calibration data and a sample concentration in parts per million (ppm) is calculated.

5.2.4.3 Fourier transform infrared spectroscopy

Fourier transform infrared spectrometry (FT-IR) was used to monitor modification of the HA after microbial interaction. The extracted biomass-bound HA utilized was freeze dried (VirTis benchtop SLC) and preserved in a desiccator with activated silica gel before use, in order to prevent moisture interference in the FT-IR spectra generated (Yang and Simms, 1995; Cimadevilla *et al*., 2003; Elbeyli *et al*., 2006b). Humic acid extracted from uninoculated media served as the control.

The KBr method used in the FT-IR analysis followed that of Başaran *et al.* (2003). A mid IR (4000 – 400cm^{-1}) scan was used in the analysis. Pure KBr was dried in a muffle furnace (Carbolite Labotec®) at 600 ^{o}C for 12 hrs and preserved in a desiccator with activated silica gel and allowed to cool before it was utilized. A 1 % HA concentrate in KBr was prepared and ground with a clean pestle and mortar and then transferred into a suitable die (nuts and bolts) where pressure (± 30 ton/in^2) was applied to form an adequately consistent disc. Prepared discs were stored in a desiccator and then transferred to a Perkin Elmer FT-IR Spectrometer (Spectrum 2000) where spectra were generated and processed.

5.2.4.4 Macro-elemental analysis

Macro-elemental analysis was undertaken for the biomass-bound HA fraction. Samples were prepared by alkaline extraction followed by lyophilization (VirTis benchtop SLC) and were analyzed at the School of Pure and Applied Chemistry, University of KwaZulu-Natal (South Africa). The LECO CHNS-932 was utilized for the analysis of carbon, hydrogen and nitrogen, while a VTF-900 furnace attachment was used for oxygen analysis. The instrument was calibrated with blank samples and

EDTA, and helium served as the carrier gas. In each case a 2 mg sample was analyzed.

5.2.4.5 Pyrolysis-gas chromatography mass spectroscopy

Thermochemolysis in the presence of a methylating agent (TMAH) was used to characterize the pyrolysates of the biomass-bound HA extracts obtained from the *N. fischeri*.

Methods used for the derivatization and pyrolysis of the freeze dried biomass-bound HA extract follow Martin *et al.* (1994) and Lehtonen *et al.* (2000). A 25 % aqueous solution of tetramethylammonium hydroxide at a 2:1 (w/v) ratio was added to ~ 2 mg of the extracted HA in a quartz tube supported at both ends with fused silica wool (Restek, Cat. # 24324). 1 µL of poly(*tert*-butylstyrene) (0.05 mg/mL in hexane) was introduced to serve as an internal standard. Samples were then dried at 100 ^{o}C for 10 min. The sample was pyrolyzed at 700 ^{o}C for 15 s in a Chemical Data System (CDS) 5000 series Pyroprobe with a CDS 1500 valve interface (CDS Analytica, Inc) that was kept at 250 ^{o}C and purged continuously with helium gas at 1.5 mL/min (Appendix 5-B). The 6890N gas chromatograph system (Agilent Technologies) was programmed to ramp from 40 ^{o}C to 200 °C at 6 °C/min and then at 10 °C/min to 300 °C, where it was held for 4 minute. The system was set at split mode 2:1 with a 3 minute solvent delay time. The capillary column (Agilent 19091s-433) specification was as followed; 30 m length and 250 µm internal diameter with a film thickness of 0.25 µm. Four sample replicates were used and detected with the MS 5975 inert Mass Selective Detector using the parameters 70eV, 1.7kV SEV, 1.1 s scan rate. The identification of the microbial bioconversion products was based on the National Institute of Standards and Technology (NIST) library search and some of the selected spectra were confirmed using standards. The results were evaluated qualitatively and semi-quantitatively as described by Fabbri *et al.* (2002).

5.2.4.6 Statistical analysis

STATISTICA, version 8.0 software (StatSoft, Inc. 2007) was utilized for statistical analysis. The Post-hoc LSD (ANOVA) with a 95 % degree of confidence was adopted whereby the level of statistical significance was accepted at $p < 0.05$.

5.3 Results and discussions

5.3.1 Bioconversion of weathered hard coal

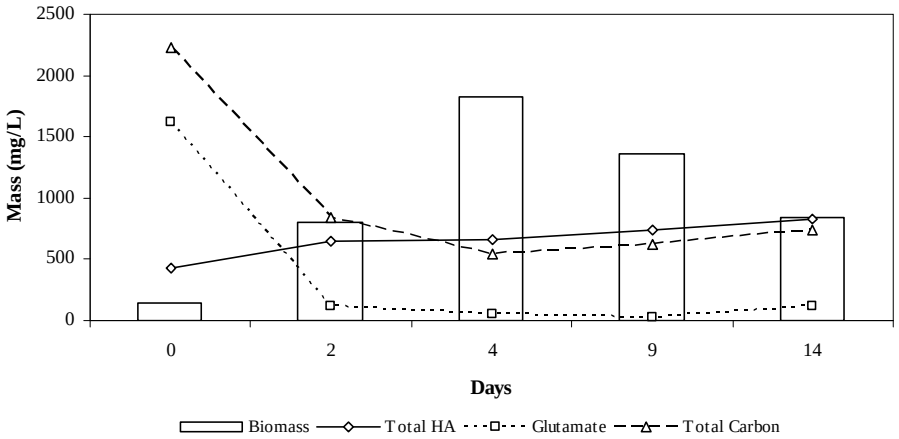
The performances of the *N. fischeri*, *P. chrysosporium*, *T. versicolor* and the mixed culture in the bioconversion of WC, using glutamate as an organic carbon supplement were investigated and compared. The study showed comparable broad trends, suggesting similar processes maybe involved in the coal bioconversion activity (Figure 5-1i – iv). Within each system, the TC and glutamate were reduced indicating that WC breakdown had occurred, while the increase in total HA indicated the release of HA from the WC breakdown process. The measurement of TC provided an indication of composite change in WC breakdown and HA and glutamate net removal from the flask system

In comparison with the other cultures, *N. fischeri* demonstrated the highest increase in biomass coupled with the rapid utilization of glutamate within two days of incubation at a removal rate of 2.3 mg/mg biomass/day (Figure 5-1i). A 62 % reduction of the TC from 2230 to 839 mg/L at the rate of 2.1 mg/mg biomass/day was also observed within this period. Also, a 35 % increase in the total alkaline extractable HA was observed by the second day at a production rate of 0.4 mg/mg biomass/day (Figure 5-1i). The total alkaline extractable HA within the *N. fischeri* system gradually increased by 49 % from day 0 to day 14 (Figure 5-1i). Laborda *et al.* (1999) had previously demonstrated a 45 % increase in the alkaline extractable HA of a lignite sample after microbial interaction on a solid medium using S10H fungal strain (*Aspergillus* sp.). In this study, they found that the average molecular weight of the HA in the reaction medium appeared to be lower than that of the original HA and suggested that either a partial digestion of HA by the microorganisms or a selective

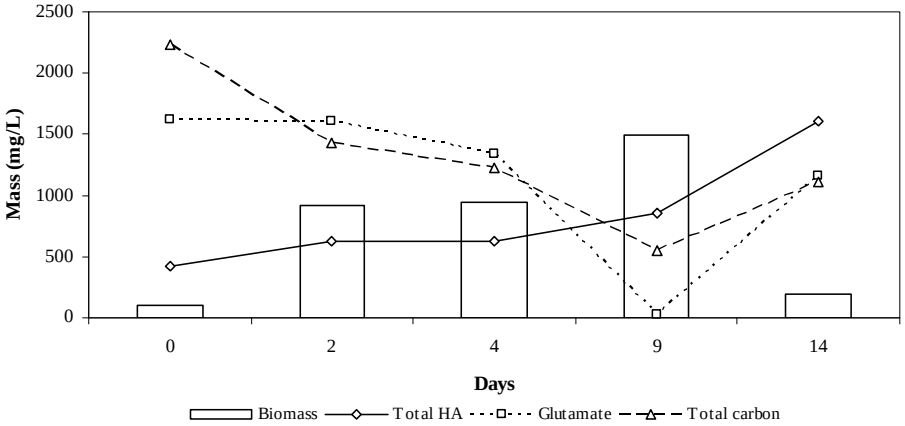
release of lower-molecular-weight HA during lignite liquefaction had occurred (Laborda *et al.,* 1999). This suggests some similarities in the bioconversion properties of lignite and weathered hard coal. In the present study, the total TC was observed to gradually decrease to a value lower than the total alkaline extractable HA (Figure 5-1i–iv). This is suggestive of the bioutilization of carbon present in the HA and is accounted for by the reduced TC in the systems.

In comparison with *N. fischeri*, the *P. chrysosporium* and *T. versicolor* systems in the present study showed a higher alkaline extractable HA production, although at a slower rate. In the *P. chrysosporium* system, the total alkaline extractable HA increased by 32 % within two days, at the rate 0.2 mg/mg biomass/day, and gradually increased by 74 % on day 14 when compared with the starting concentration (Figure 5-1ii). However, the utilization rate of glutamate within the first two days in the *P. chrysosporium* system was 0.01 mg/mg biomass day, and that of TC was 1 mg/mg biomass/day. The slow glutamate utilization rate persisted until day four after which it reduced rapidly from 1345 to 30 mg/L on day nine, with the biomass rising to 1494 mg/L. This was coupled to an increase in the total extractable HA of 47 % (Figure 5-1ii).

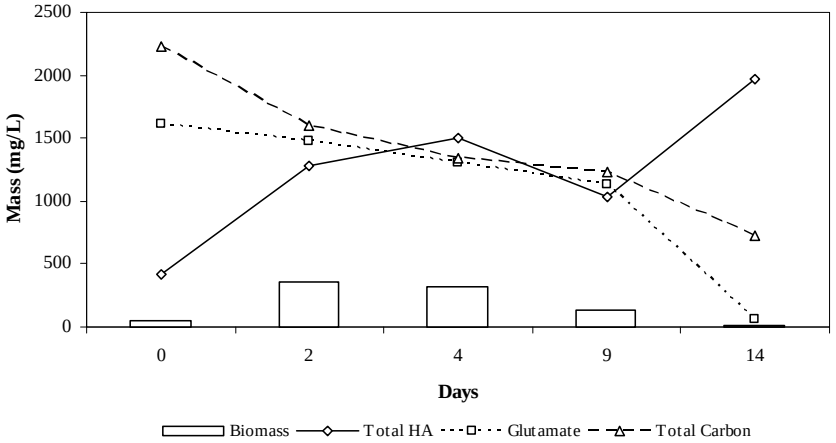
In the *T. versicolor* system, total extractable HA increased by 67 % at the rate of 2.8 mg/mg biomass/day by the second day and by 78 % by day 14 (Figure 5-1iii). The lowest biomass formed over the experimental period was seen in this system and the glutamate also showed slow utilization at the rate of 0.5 mg/mg biomass/day. The TC was reduced at the rate of 2.1 mg/mg biomass/day within the first two days (Figure 5-1iii). The slow utilization rate of glutamic acid and the low biomass production by *T. versicolor* in comparison with *N. fischeri* and *P. chrysosporium* (Figure 5-1iii) is contrasted by a rate of HA release which was 85 % faster than *N. fischeri* and 94 % faster than *P. chrysosporium* over the first two days. This suggests different mechanisms may be involved in the strategy of coal bioconversion by this organism (Cohen *et al.,* 1990).



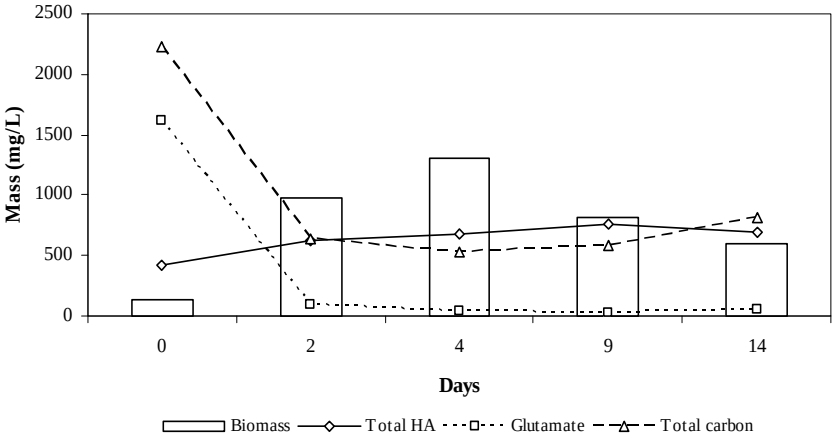
(i) *Neosartorya fischeri*



(ii) *Phanerochaete chrysosporium*



(iii) *Trametes versicolor*



(iv) Mixed culture

Figure 5-1 Bioconversion of weathered hard coal showing changes in the total humic acid (alkaline extractable filtrate HA plus alkaline extractable biomass-bound HA), Total carbon (TC of filtrate plus TC of alkaline extractable biomass-bound humic acid), glutamate and biomass production as demonstrated by (i) *Neosartorya fischeri*, (ii) *Phanerochaete chrysosporium*, (iii) *Trametes versicolor* and (iv) a mixed culture of all three cultures grown in a 100 mg/L weathered hard coal-glutamate media.

Finally, the results of the mixed culture system were comparable to the performance of the *N. fischeri* inoculum alone, suggesting the dominance of the *N. fischeri* bioconversion processes in competition with *P. chrysosporium* and *T. versicolor* (Figure 5-1iv). The slower release of HA, faster TC consumption and higher biomass production in the *N. fischeri* and *P. chrysosporium* systems compared to *T. versicolor* suggests possible consumption of HA by these organisms and that similar mechanisms of WC bioconversion may be involved. FT-IR and Py-GCMS analysis were undertaken to shed further light on these observations.

5.3.2 Fourier transformed infrared spectrometry

Structural changes with alteration of functional groups in the biomass-bound HA fraction compared to the HA control were observed for all cultures (Figures 5-2 to 5-5). The FT-IR spectra for the biomass-bound HA in the *N. fischeri* (Figure 5-2) and *P. chrysosporium* (Figure 5-3) studies again showed similarities with the changes in functional groups maximized by day nine. Overall, the mixed culture demonstrated maximum modification of functional groups by the second day of contact (Figure 5-5).

5.3.2.1 O-H band

In the HA control, the hydrogen bonded O-H peak at the 3400 cm^{-1} to 3300 cm^{-1} region (Pavia *et al*., 2001), while present, was not as intense as HA extracted from the various fungal inoculated cultures. For *N. fischeri*, the band gradually increased until day nine with a simultaneous disappearance of the aliphatic band (Figure 5-2). This suggested a gradual and complete oxidation of the aliphatic groups present in the substrate. The hydrogen-bonded O-H band also appeared to have reached a maximum by day nine for the *P. chrysosporium* HA sample (Figure 5-3). With *T. versicolor*, modification of the original HA occurred by day two and then persisted unchanged throughout the remainder of the experimental period (Figure 5-4). In this case, the intensity of the hydrogen-bonded O-H band appeared to be less pronounced compared to the other fungal cultures. It increased slightly on the second day and extended to merge with the 3000 - 2700 cm^{-1} region attributed to the aliphatic (C-H) stretching

frequencies (de la Puente *et al*., 1998) (Figure 5-4). The HA sample obtained from the mixed culture system (Figure 5-5) showed a broad and intensive O-H band (3000 - 2700 cm $^{-1}$) that appeared by the second day and reached its highest intensity by day nine, which was comparable to the *N. fischeri* and *P. chrysosporium* systems. Laborda *et al.* (1999) had reported an increase in the bound and unbound O-H groups associated with the biological oxidation of HA by *Doratomyces* sp.

5.3.2.2 C-H band

The sharp aliphatic C-H stretching peaks that appeared at 2916 cm^{-1} (asymmetrical) and 2843 cm^{-1} (symmetrical) (Pavia *et al.*, 2001) in the control HA sample was revealed to have gradually decreased in the *P. chrysosporium* samples and had weakened by day nine (Figure 5-3iii). In the *N. fischeri* (Figure 5-2) and the mixed culture (Figure 5-5) samples, the aliphatic band appeared to have weakened by the second day. This aliphatic band weakness was consistent with observations by Ralph and Catcheside (1996) from analysis of solubilized brown coal using wood-rot fungi. The aliphatic band disappeared by the second and ninth day in the *N. fischeri* and *P. chrysosporium* samples, respectively (Figure 5-2ii and 5-3iii). This indicates the oxidation of aliphatic groups, which result in the broad and intense O-H stretching vibration, or a valence vibration of N-H bonds, such as amines (Willmann and Fakoussa, 1997a).

In all cases, a disappearance of the aliphatic group was observed compared to the HA control. The 3000 – 2700 cm^{-1} aliphatic region (de la Puente *et al.*, 1998) had been reported to be unaffected by HNO_3 oxidation (Ndaji and Thomas, 1995; de la Puente *et al.*, 1998; Alvarez *et al.*, 2003; Elbeyli *et al.*, 2006b), but has shown here to be affected by microbial interaction. Similar results of the microbial oxidation of the aliphatic groups present in HA has been reported by Bien and Sandler (1983).

5.3.2.3 C=O and –COO bands

Absorbances in the 1600 cm^{-1} region consistent with alkene (C=C) stretching frequencies (Laborda *et al*., 1999; Lyubchik *et al*., 2002) was observed in the *N.*

fischeri and *P. chrysosporium* reacted samples. The band at 1630 cm^{-1} denoting C=O and –COO groups (Yuan *et al.,* 2006) appeared to intensify over time for *N. fischeri* (Figure 5-2), *P. chrysosporium* (Figure 5-3) and the mixed culture (Figure 5-5) samples. At the same time, in the HA control and *T. versicolor* samples (Figure 5-4), the band appeared to be less conjugated and absorbed at a higher frequency (1605 cm^{-1} and 1585 cm^{-1} respectively). In comparison, the absorption at 1630 cm^{-1} indicated the presence of C=O and –COO groups (Yuan *et al.,* 2006) for the *N. fischeri* (Figure 5-2iii) and *P. chrysosporium* samples (Figure 5-3iii). The increased appearance of carbonyl functionalities and hydroxyl moieties was consistent with oxidative weathering of the coal macromolecule as previously reported (Ibarra and Miranda, 1996; de la Puente *et al.,* 1998; Cimadevilla *et al.,* 2003; MacPhee *et al.,* 2004). Also, the microbial oxidation of the coal macromolecule suggested in this present work is consistent with previous reports (Fakoussa, 1988; Bublitz *et al.,* 1994; Ralph and Catcheside, 1996; Yuan *et al*., 2006).

5.3.2.4 –CH_2– and –CH_3 band

The prominent peaks at 1431 cm^{-1} and 1447 cm^{-1} that were evident in the spectra for the control HA and *T. versicolor* samples respectively, suggest the characteristic bending absorption peaks for un-oxidized methylene (–CH_2–) and methyl (–CH_3) groups that occur in the 1475 – 1365 cm^{-1} range (Pavia et al., 2001). In the *N. fischeri* samples, the un-oxidized –CH_2– and –CH_3 groups appear to persist until day nine (Figure 5-2). In the *P. chrysosporium* samples, the absorbance appears to decrease over time (Figure 5-3), while the band was absent in all the mixed culture samples (Figure 5-5).

5.3.2.5 N=O bands

The absorption at 1350 cm^{-1} which is attributed to symmetric stretch of aliphatic nitro compounds (N=O) in the 1390 – 1300 cm^{-1} region (Pavia *et al.,* 2001) appeared by day nine for the *N. fischeri* (Figure 5-2iii) and *P. chrysosporium* (Figure 5-3iii) samples and by day two in the mixed culture (Figure 5-5ii) samples. This band was absent in the control and *T. versicolor* samples.

HNO_3 treatment of coal has been shown to result in the incorporation of nitro groups into the coal macromolecules (Patti *et al*., 1992; Alvarez *et al.*, 2003; Elbeyli *et al.*, 2006a and b). However, in this study, naturally oxidized WC was utilized instead of HNO_3 oxidized coal. The presence of the absorption band at 1350 cm^{-1} therefore suggests a possible nitration of the coal substrate by *N. fischeri* and *P. chrysosporium* (Figure 5-2iii and 5-3iii).

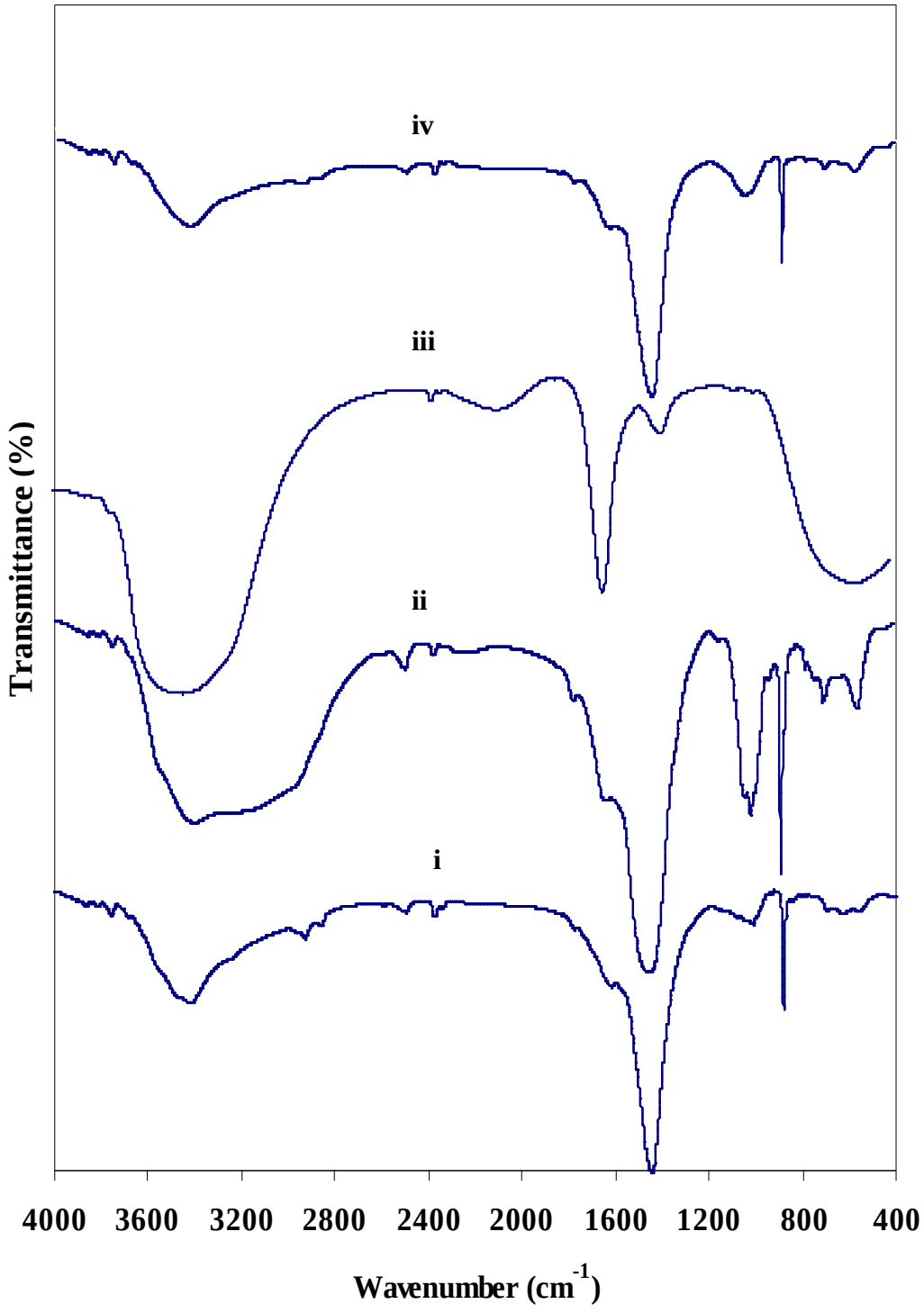


Figure 5-2 Fourier transform infrared spectra of (i) humic acid extracted from weathered hard coal-glutamate media before inoculation with pre-grown biomass of *Neosartorya fischeri* and biomass-bound HA extract of *Neosartorya fischeri* after (ii) 2 days; (iii) 9 days and (iv) 14 days of reaction time.

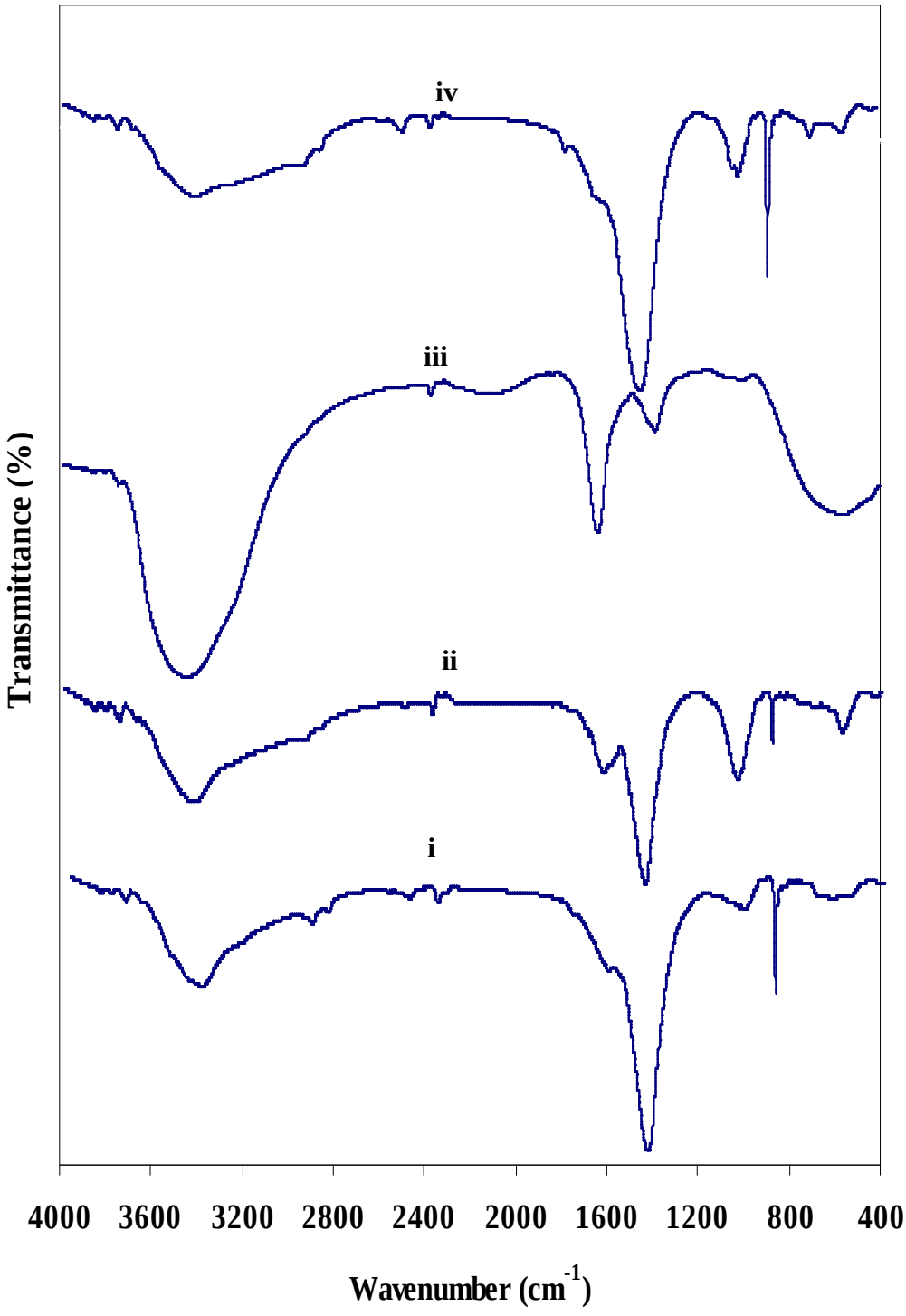


Figure 5-3 Fourier transform infrared spectra of (i) humic acid extracted from weathered hard coal-glutamate media before inoculation with pre-grown biomass of *Phanerochaete chrysosporium* and biomass-bound HA extract of *Phanerochaete chrysosporium* after (ii) 2 days; (iii) 9 days and (iv) 14 days of reaction time.

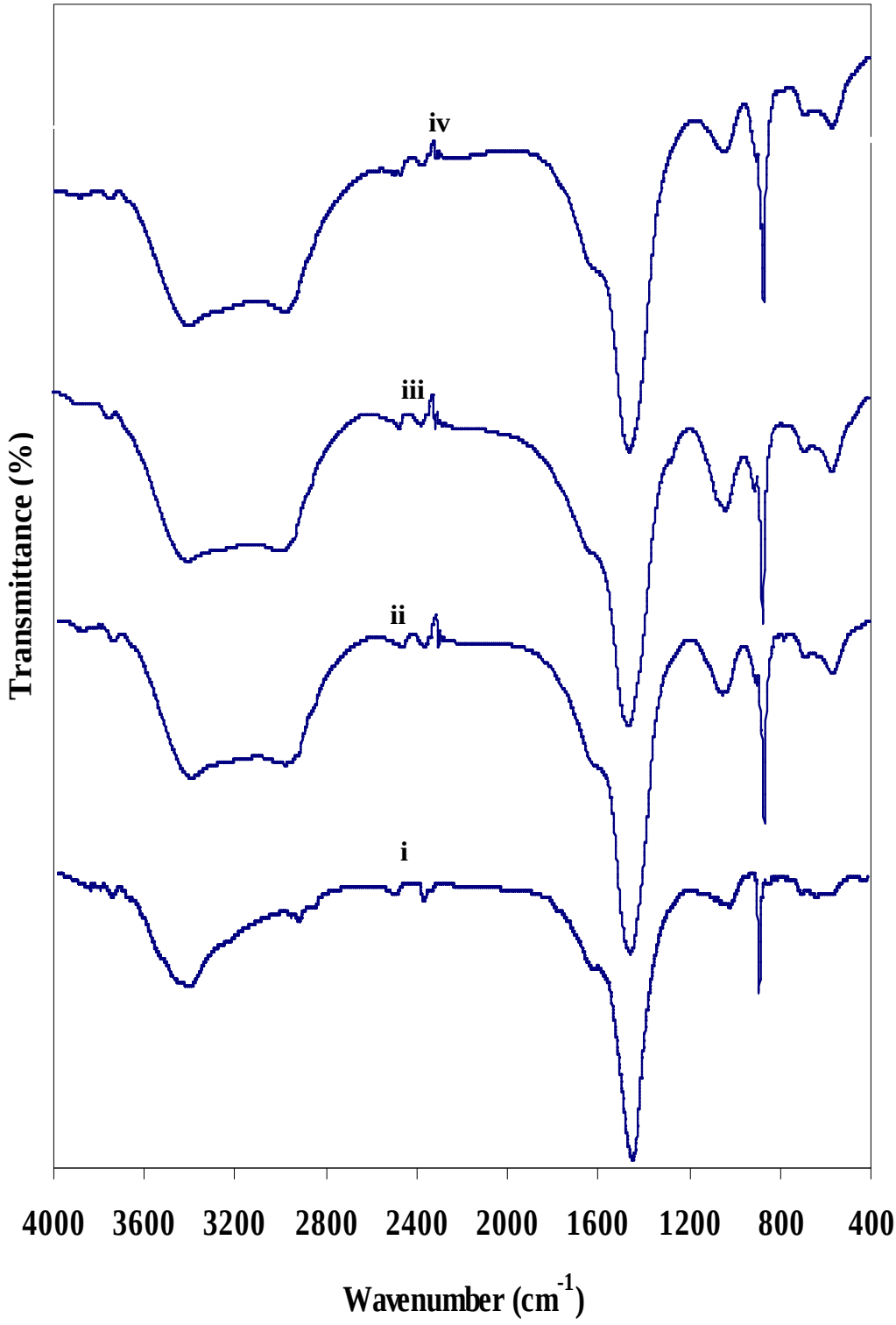


Figure 5-4 Fourier transform infrared spectra of (i) humic acid extracted from weathered hard coal-glutamate media before inoculation with pre-grown biomass of *Trametes versicolor* and biomass-bound HA extract of *Trametes versicolor* after (ii) 2 days; (iii) 9 days and (iv) 14 days of reaction time.

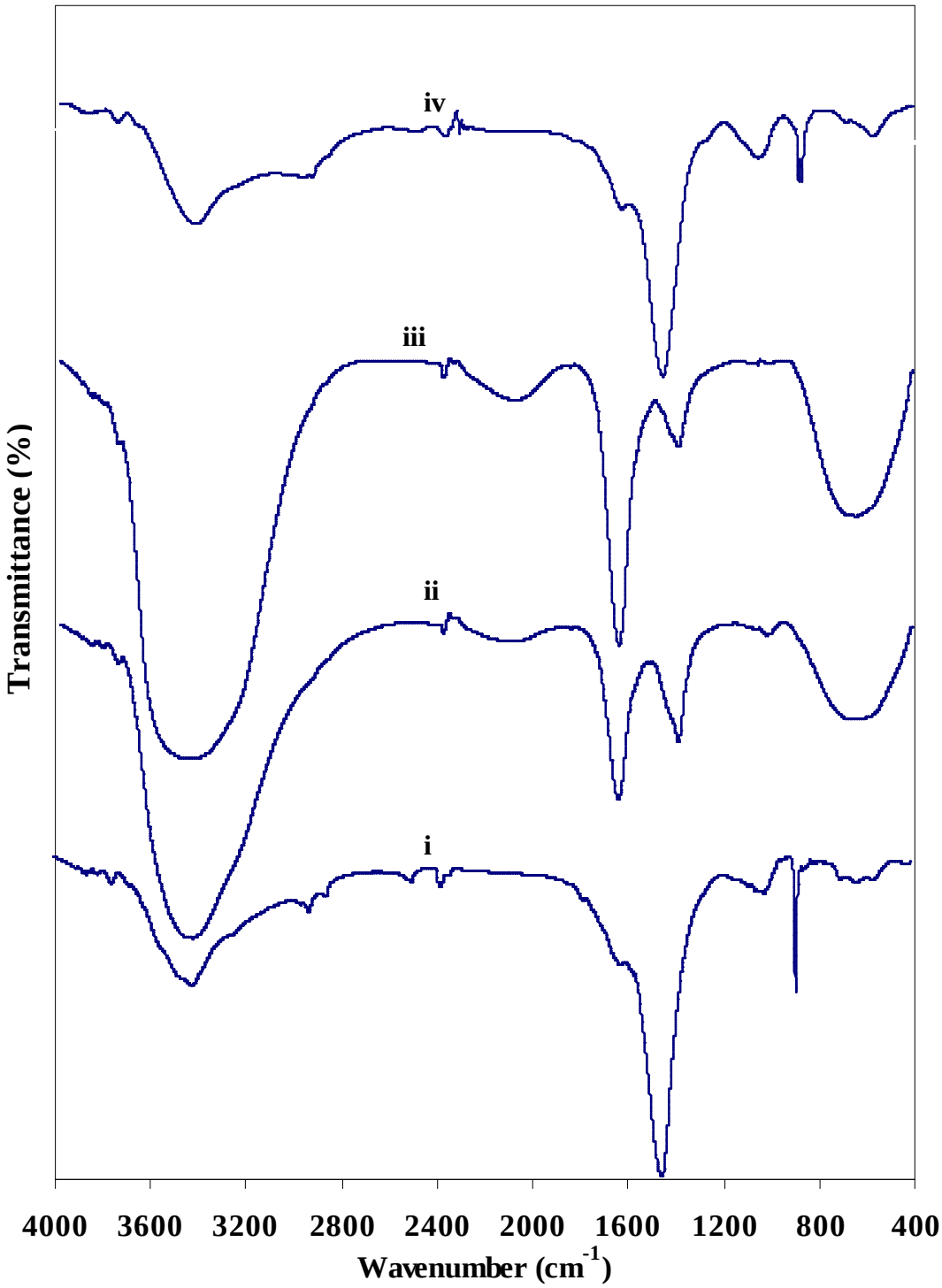


Figure 5-5 Fourier transform infrared spectra of (i) control humic acid extract from weathered hard coal-glutamate media before inoculation with pre-grown biomass of the mixed culture compared with biomass-bound HA extract of the mixed culture after (ii) 2 days; (iii) 9 days and (iv) 14 days of reaction time.

5.3.3 Macro-elemental analysis

Elemental analysis of *N. fischeri* biomass-bound HA (day 14) are presented in Table 5-1 and the results showed a 20.52 % and 1.02 % reduction in the carbon and hydrogen content coupled with a 14.3 % and 0.92 % increase in the oxygen and nitrogen, respectively, after 14 days incubation. Machnikowska *et al.* (2002) has previously shown the increase in nitrogen and oxygen content coupled with a reduction in carbon in the biomass-bound HA after microbial interaction with low rank coals.

Table 5-1 Macro-elemental analysis of control humic acid and of biomass-bound humic acid after 14 days in deep liquid culture with *Neosartorya fischeri*.

Sample	**% C**	**% H**	**% N**	**% O**
Commercial grade humic acid (AGRON Co.)	45.97	4.18	1.05	34.06
14 day humic acid extract (Experiment)	25.45	3.16	1.97	48.36
14 day humic acid extract (Control)	36.68	4.57	1.07	39.24

5.3.4 Pyrolysis gas chromatography mass spectrometry

Given the focus of the study of the BHC dump system on the role of *N. fischeri* isolate, the reaction products generated by this organism in the bioconversion experiment were investigated further. Products generated in the bioconversion of WC were analyzed by Py-GCMS chromatography (Figure 5-6). Pyrolysis products showing change over the course of the reaction are summarised in Table 5-2. The semi-quantification of the selected pyrolysates of *N. fischeri* biomass-bound HA using poly(tert-butylstyrene) as internal standard showed change over the course of the reaction time (Figure 5-7). The methyl esters of C_{16} and C_{18} saturated and unsaturated fatty acids (FAMe) were likely to have been derived from the fungal biomass itself and the possibility that peaks 1 and 6 were contaminants could not be excluded (Figure 5-6).

Semi-quantitative results of the analysis of selected total ion chromatograms (TIC) are presented in Figure 5-7 with significant increases shown for the concentration of all

selected compounds over the initial 4 day reaction time, except for indole and N-methylphthalimide (ANOVA post hoc LSD test, $p < 0.05$). The Box and Whisker plots are reported in Figure 5-8 and their respective Post-hoc test is presented in Appendix 5-C. Consumption of breakdown products may occur over time and by day 14 pyrolysis products, such as benzoic acid (methyl ester), had been completely removed (Figure 5-6ii and iii).

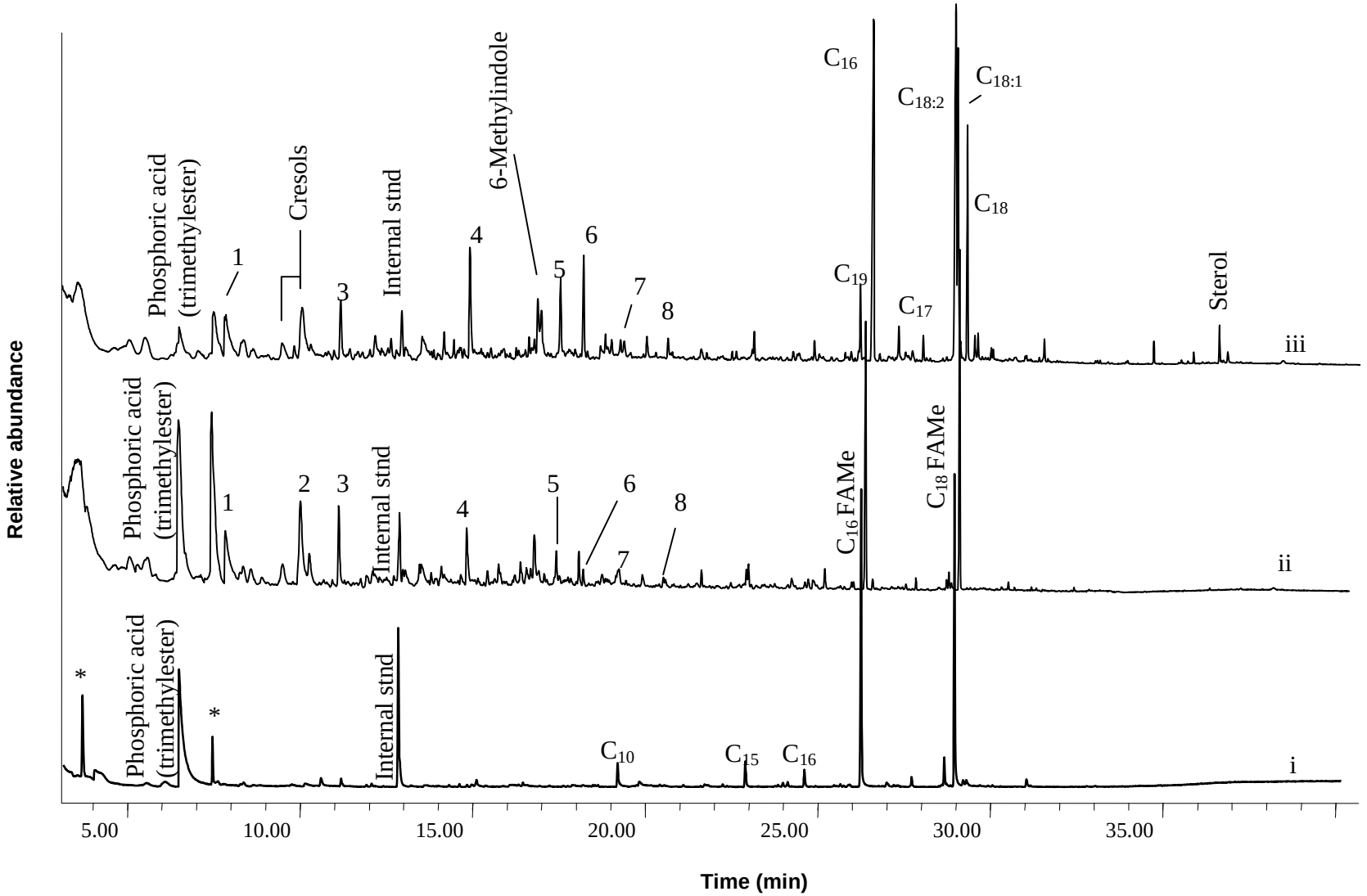
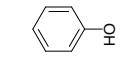
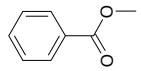
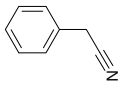
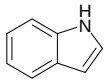
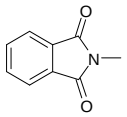
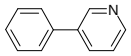
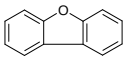
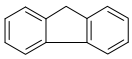


Figure 5-6 The total ion chromatogram of humic acid (i) from control weathered hard coal and humic acid in solution after 4 (ii) and 14 (iii) days reaction of weathered hard coal with *Neosartorya fischeri*. See Table 5-2 for the labeling of the peaks and compound structures. (*) Suspected contaminants.

Table 5-2 Compound structures of the pyrolysis products from humic acid extracts derived from the reaction of weathered hard coal with *Neosartorya fischeri*. Peak numbers refer to the labeled total ion chromatogram in Figure 5-6.

Peak no.	Compound name	Compound structure
1	Phenol	
2	Benzoic acid, methyl ester	
3	Benzylnitrile	
4	Indole	
5	N-Methylphthalimide	
6	3-Phenylpiridine	
7	Dibenzofuran	
8	Fluorene	

The appearance of an increasing phenol and fluorene concentration over the course of the reaction period may be indicative of the breakdown of aromatic structures in the complex WC substrate in the presence of *N. fischeri*. Of particular note is the appearance of aromatic nitrogen-containing pyrolysis products including benzylnitrile (peak 3), indole (peak 4), N-methylphthalimide (peak 5), and 3-phenylpiridine (peak 6) which were not observed to be present in the WC substrate (Figure 5-6). The possible insertion of nitrogen groups into the aromatic structure correlates with the observations of the changes in the FT-IR absorption band at 1350 cm^{-1} which indicates the appearance of a symmetric stretch of aliphatic nitro compounds (N=O) within the system. This observation may provide an indication of a mechanism by which depolymerization of the condensed macromolecule progresses in the bioconversion reaction catalyzed by *N. fischeri*.

While of considerable interest to the current study, the above would need to be the subject of a further detailed confirmatory investigation. Bearing in mind that these compounds are derivatized pyrolysates of more complex molecules in the medium and

so, although observation of their presence provided an indicative value, a more detailed investigation is needed to confirm their actual presence.

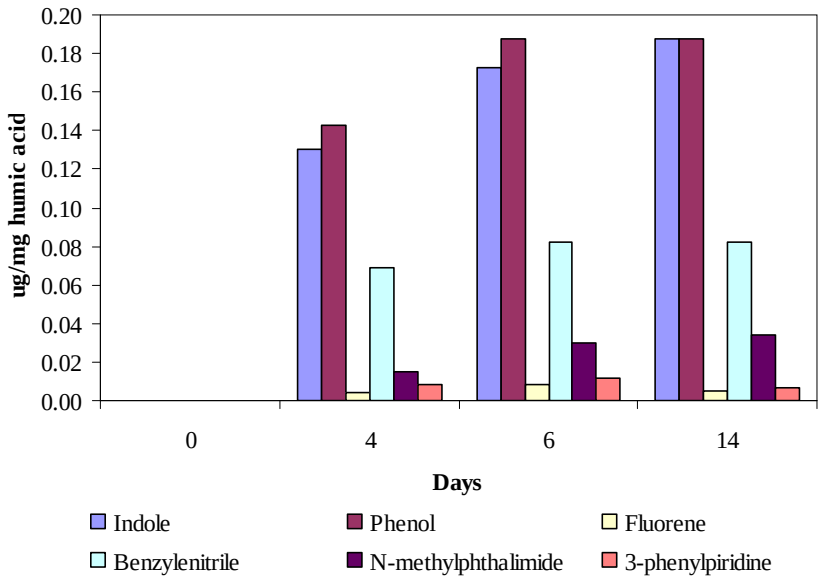


Figure 5-7 Semi-quantitative results of pyrolysis products in µg/mg humic acid.

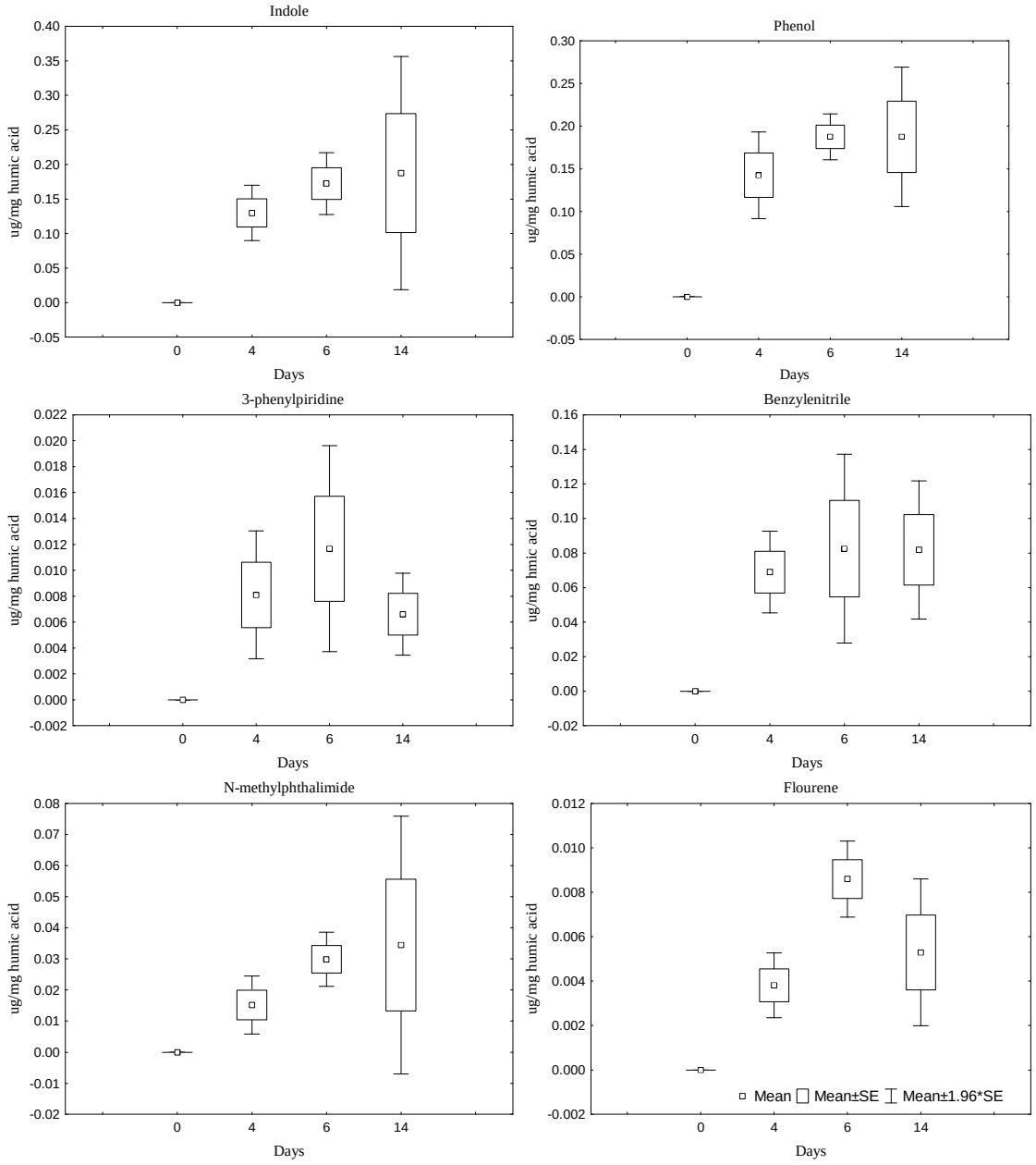


Figure 5-8 Box and Whisker plot showing the semi-quantitation for indole, phenol, 3-phenylpiridine, benzylnitrile, N-methtylphthalimide and fluorine in the breakdown of humic acid in the *Neosartorya fischeri* study.

5.4 Conclusions

- Comparable reaction profiles for bioconversion of WC by *N. fischeri* and *P. chrysosporium* suggested similar mechanisms may be involved, while differences compared to *T. versicolor* suggested a different strategy is used by this organism.
- Biomass production linked TC and glutamate removal from the reaction medium indicates that a breakdown and consumption of these substrates by *N. fischeri* and *P. chrysosporium* may occur, suggesting an extracellular reaction component.
- Low biomass production and high conversion of WC to HA indicate the action of primarily extracellular depolymerization events in *T. versicolor*.
- The FT-IR result showed a much more pronounced oxidation of the biomass-bound HA substrate occurring in the *N. fischeri, P. chrysosporium* and mixed culture systems. Macro-elemental analysis also suggested a reduction occurred in carbon and hydrogen which is coupled with an increase in the oxygen and nitrogen contents in the *N. fischeri* biomass bound-HA.
- The FT-IR results suggest that in addition to oxidation of the coal surface, the bioconversion reaction may also progress through the insertion of nitrogen groups into the condensed aromatic structure of the coal substrate. This is further strengthened by the results obtained from the Py-GCMS analysis of the *N. fischeri* system, indicating the presence of nitrogenous compounds.
- The PY-GCMS results for *N. fischeri* also indicate that a breakdown of the aromatic structure of the WC/HA substrate occurs over time.
- The mechanism of N insertion may be a structural depolymerization approach.
- Mixture of the three cultures showed comparability to *N. fischeri* in the bioconversion of WC to HA, indicating the probable dominance *N. fischeri* in the combined inoculum system. This observation supports its use as a single model organism for the further investigation of changes occurring in the BHC dump environment.

Chapter six

Simulation of the *Cynodon dactylon*/coal rhizosphere

6. Introduction

The performance and mechanism of action of *N. fischeri* in coal bioconversion was investigated in submerged liquid culture flask studies and compared against two species, *P. chrysosporium* and *T. versicolor*, which have been previously described as being active in coal biosolubilization (Dehorter and Blondeau, 1992; Torzilli and Isbister, 1994; Mönkemann *et al*., 1997; Bumpus *et al*., 1998; Başaran *et al*., 2003). Mukasa-Mugerwa *et al.* (2007) has recently reported the presence of mycorrhizal fungi in the roots of the *C. dactylon*/coal system. It was proposed that the coal bioconversion observed in the *C. dactylon* root zone may involve a mutual dependence on organic carbon extrusion by the plant-mycorrhizal system enabling the breakdown of coal by the non-mycorrhizal fungal population, thereby releasing essential nutrients for plant growth.

It is known that plants translocate large amounts of their photosynthetically fixed carbon into the soil as insoluble and soluble organic carbon compounds (Griffiths *et al.,* 1999; Kuzyakov *et al*., 2001; Nardi *et al.,* 2002; Baudoin *et al.,* 2003; Falchini *et al*., 2003; Kuzyakov and Jones, 2006). These exudates are usually complex and include a wide range of low molecular weight organic compounds which are used to selectively define the microbial population found in the rhizosphere (Curl and Truelove, 1986; Hodge *et al.,* 1998; Griffiths *et al.,* 1999; Baudoin *et al.,* 2003; Falchini *et al*., 2003; Landi *et al*, 2006). However, the dynamics of the *C. dactylon*/coal relationship are not yet clearly understood.

The submerged liquid culture flask studies provided a reductive approach to the investigation of the processes occurring in this complex microenvironment. In order to

understand the events occurring in the root zone, it was considered important to attempt to simulate the *C. dactylon*/coal association observed at the coal dump in an experiment representative of the natural environment, while carrying out the investigation under controlled laboratory conditions. A fixed-bed perfusion column bioreactor (FPCB) was developed in which coal was packed to represent a cross-section of the top layer of the dump. The perfusate was supplemented with organic carbon nutrient to simulate the contribution of *C. dactylon* photosynthate to the system. A mixed inoculum was used to inoculate the FPCB and was sourced from the root zone of *C. dactylon* growing in the coal dump environment. It included *N. fischeri* among other fungal forms.

6.1 Objectives

The objectives of the study reported here were to:

- Develop a fixed-bed column reactor to simulate the *C. dactylon*/coal rhizosphere microenvironment and to enable its investigation under controlled laboratory conditions;
- Investigate the bioconversion of hard coal using a mixed microbial species inoculum;
- To investigate the role of supplementation with organic carbon and WC and to compare this with results obtained for the submerged culture liquid flask studies previously reported.

6.2 Materials and methods

6.2.1 Fixed-bed perfusion column bioreactor

The FPCB was set up to simulate the vertical profile of the *C. dactylon*/coal rhizosphere environment under controlled laboratory conditions.

A schematic representation of the FPCB is shown in Figure 6-1. In the bioreactor column (120 × 750 mm) 3 layers of 30 µm mesh were placed at the bottom of the bioreactor column as a filter. Glass beads (10 mm diameter) were placed to support the mesh and to serve as an interface that would prevent the bioreactor bed from clogging the pores of the mesh and blocking the outlet. The bioreactor was aerated from the bottom of the column with humidified air. The bioreactor column discharged into a stirred tank and the medium recycled back to the bioreactor by peristaltic pump at a 5 mL/min flow rate. Sterilized air filters were connected in series before and after the water trap. Control bioreactors were prepared as above but fed as described below.

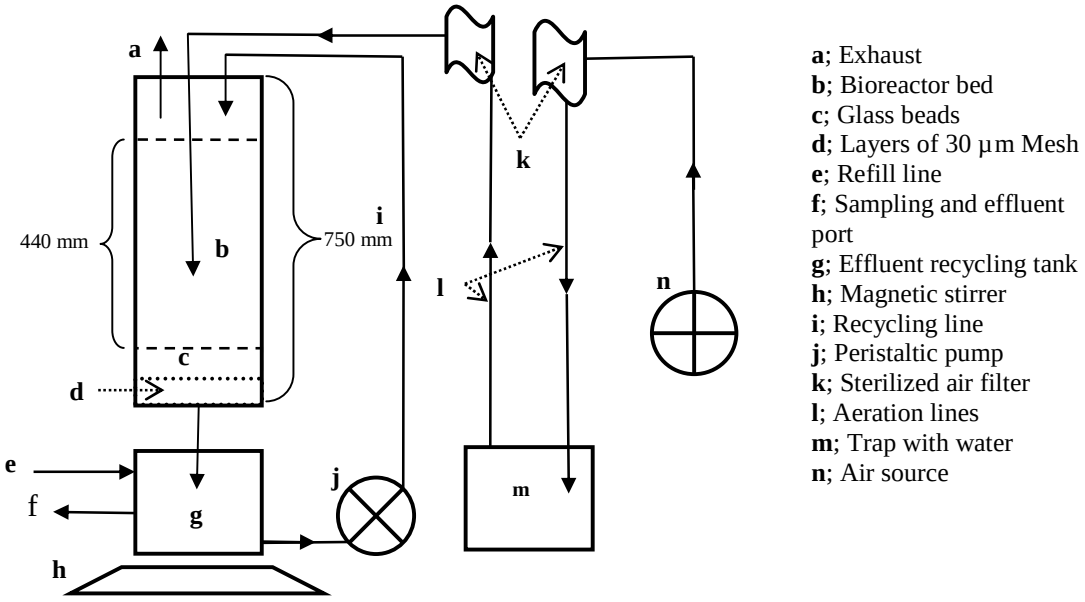


Figure 6-1 Schematic diagram of the fixed-bed perfusion column bioreactor designed to simulate the *Cynodon dactylon*/coal rhizosphere in coal dump environment.

6.2.1.1 Bioreactor bed preparation

Freshly ground BHC obtained from Klein Kopje Colliery was used as the bioreactor bed packing. The BHC sample was crushed into small fragments and sieved through a sieve stack to achieve a size fraction within the range of < 2 but > 1 mm. After sizing, it was washed repeatedly with milliQ water until a clear rinsate was obtained. It was then autoclaved at 120 ^{o}C for 15 min, before it was packed in the bioreactor.

6.2.1.2 Inoculum preparation

Although a biomass inoculum had been shown to give the best results in the flask studies, it was decided to use spore inoculum in the simulated system, in order to accurately reflect natural conditions. A mixed culture inoculum was prepared as a suspension of *C. dactylon* root zone material sourced from Navigation Colliery.

6.2.1.3 Perfusion medium make up

The perfusion medium was formulated as shown in Table 6-1. With the exception of an inorganic nitrogen source, the medium did not contain other minerals. The objective here was to induce the microbial population that established in the system to derive the essential minerals and trace elements required for growth from the breakdown of the coal substrate itself. However, a nitrogen source was added to ensure that this was not limiting in the system, given the indication of its role in a possibly essential first step in coal bioconversion reported in Chapter four.

Table 6-1 Media formulation for perfusion bioreactors 1 – 4, designed to simulate the *Cynodon dactylon*/coal rhizosphere under controlled laboratory conditions.

Input/Feed	**FPCB-1**	**FPCB-2**	**FPCB-3**	**FPCB-4**
WC	X	O	O	O
LMOs*	X	X	O	O
Potassium nitrate	X	X	X	X
dH_2O	X	X	X	X
Inoculum	X	X	X	O
Bituminous hard coal	X	X	X	X

(FPCB) Fixed-bed perfusion column bioreactor, (WC) weathered hard coal, (X) present, (O) absent, (*) for detailed make up of low molecular weight organics (LMO) refer to Table 6-2.

The organic carbon supplementation of the synthetic feed (Table 6-2) was designed to simulate components and concentration of exudate known to be released into the rhizosphere and included amino acids, sugars and other organic acids (Griffiths *et al*.,

1999; Falchini *et al*., 2003; Kuzyakov *et al*., 2007)**.** In this study glutamate, glucose and oxalic acids were used.

Table 6-2 The low molecular weight organics carbon supplement added to the perfusion medium.

	Per Litre (%)	Mass (g/L)
Glutamate	0.2	2
Glucose	0.05	0.5
Oxalic acid	0.2	2

The FPCB-1 contained all reactants, including WC as an oxidized coal source, which was shown to play a role in initiating the *C. dactylon*/coal interaction. In FPCB-2, the WC was excluded, and thus the low molecular weight organic (LMO) provided the sole nutrient supplement. The FPCB-3 provided the control for WC and LMO addition, and FPCB-4 as a control for the use of inoculum.

6.2.1.4 Operation

The set up of the FPCB is shown in Figures 6-2 and 6-3 in a constant environment laboratory held at a temperature of 28 ^{o}C. The columns were inoculated with the mixed culture inoculum in 1 L of the relevant feed medium and allowed to stand for 24 hours to enable adhesion to the coal surface. Perfusion with feed then commenced at a rate of 5 mL/min. Bioreactor effluent was collected weekly for analysis and the bioreactor bed packing was sampled at the conclusion of the experiment and investigated using scanning electron microscopy (SEM).

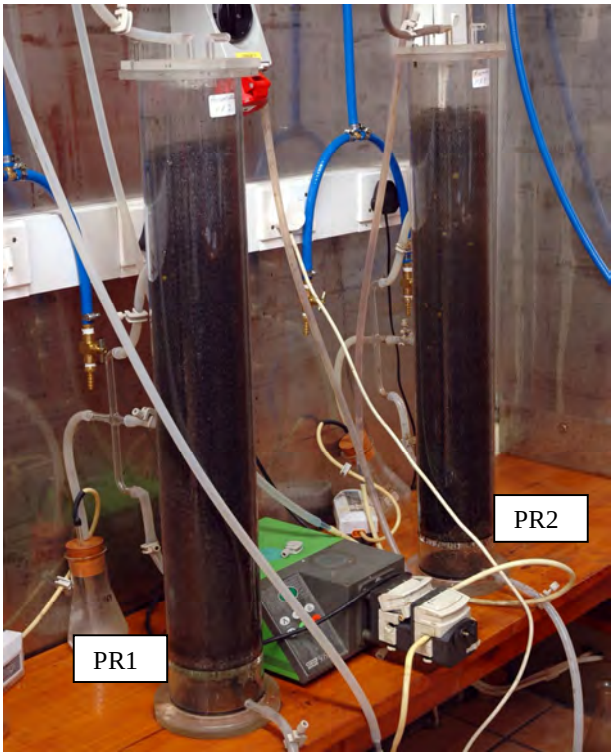


Figure 6-2 Experimental set up for the fixed-bed perfusion column bioreactor that was packed with bituminous hard coal and perfused with a feed described in Table 6-1.

Figure 6-3 Experimental set up of the fixed-bed perfusion column bioreactors in a constant environmental laboratory held at 28 ^{o}C.

6.2.2 Analysis

6.2.2.1 Scanning Electron Microscopy

Preparation of samples for SEM was performed according to the method of Cross (2001). The collected samples were fixed overnight in a 2.5 % glutaraldehyde solution prepared from a 25 % glutaric dialdehyde stock reagent by diluting with phosphate buffer (0.1 M, pH 7.0 phosphate buffer). Glutaraldehyde solution was decanted and the samples washed twice with phosphate buffer (0.1 M, pH 7.0, 10 min). The samples were then dehydrated using an ethanol series (30 %, 50 %, 70 %, 80 %, 90 %, 100 %, with two changes of 100 % ethanol; 15 minute each step). Samples were then placed in a Polaron critical point dryer (Watford England) and dried for 2 hours. The dried samples were mounted on 12 mm diameter aluminium posts with 12 mm carbon conducting adhesive tabs and transferred to a sputtering device (Balzers Union) and gold coated for 160 sec at 80 mT pressure at an applied current of 45 mA. Carbon paint was applied from the surface of the gold coating to the aluminium post to ensure a conductive path from electrons on the surface to reach ground state. The samples were examined with a VEGA LMU (VEGA © Tescan) scanning electron microscope.

6.2.2.2 Total carbon analysis

The TC analysis was conducted as previously described in Section 5.2.4.2.

6.2.2.3 Pyrolysis-Gas Chromatography Mass Spectroscopy

Bioreactor-effluent (100 mL) was collected weekly from each bioreactor, freeze dried and stored in a desiccator before it was analyzed. The method used for the Py-GCMS followed Martin *et al.* (1994) and Lehtonen *et al.* (2000) and was applied as previously described in Section 5.2.4.5.

6.3 Results and discussions

6.3.1 Scanning electron microscopy

Well-developed biofilm formation could be observed visually on the hard coal when the LMO solution was introduced into the FPCB-1 and FPCB-2 to simulate exudation of photosynthate by *C. dactylon* (Figure 6-4). In the absence of the LMO (FPCB-3), the formation of a luxuriant fungal biofilm was not observed. Scanning electron microscopy of the coal surface, however, did reveal fungal colonization of the coal particles in both the presence and absence of LMO (Figure 6-5). However, this was reduced in the absence of LMO. Similar observations were made by Laborda *et al.* (1997) who described the fungal biofilm formation on the coal surface as an "extracellular polymer-like structure" that was strongly involved in the fungal attachment to the coal particle as well as a tool for the attack on the coal material and for nutrient uptake. This result further suggests that LMOs, as a phyto-organic carbon supplement, stimulates the attachment and growth of fungi present in the coal environment, thereby enhancing coal bioconversion. It has been demonstrated that plant exudates impact on the microbial community structure in bulk soil (Curl and Truelove, 1986; Hodge *et al.*, 1998; Baudoin *et al.*, 2003), which in turn may release nutrients which enhances plant growth in a mutualistic manner.

Figure 6-4 Biofilm formation on the surface of bituminous hard coal in the fixed-bed perfusion column bioreactor that was perfused with a low molecular weight organics carbon supplement.

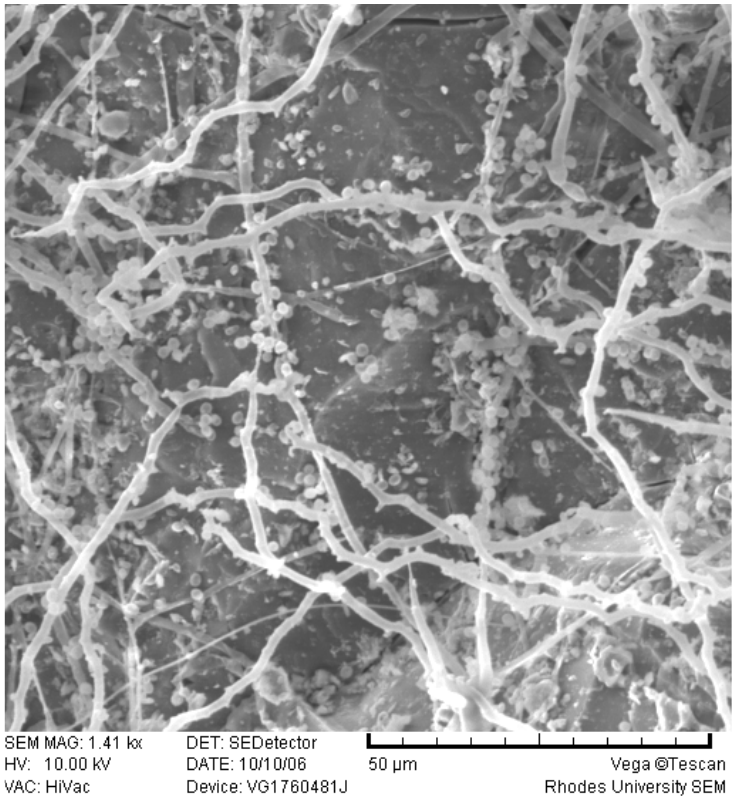


Figure 6-5 Scanning electron micrograph showing heavy colonization of the surface of bituminous hard coal particles in the fixed-bed perfusion column bioreactor.

6.3.2 Total carbon of leachates

The change in the TC in the perfusion medium in FPCB-1 and FPCB-2 is shown in Figure 6-6. The control bioreactors FPCB-3 and FPCB-4 are shown in Figure 6-7 where little change occurs. During the first week, the TC for FPCB-1 (containing WC and LMO) was reduced by 60 % at the rate of 189 mg/day. During this initial period the TC reduction that occurred in FPCB-1 was greater than that of FPCB-2 indicating an enhancement of LMO supplemented activity in the presence of WC. Previous studies indicate that this may be due to the initial release of humic acids from the WC substrate. This may be related to the rise in TC from week one to week four with a 31 % TC reduction at the rate of 76 mg/day (FPCB-1). Although occurring at a lower rate of 76 mg/day in week one, TC in FPCB-2 continued to decline until week two and then increased at a rate of 183 mg/day with a 96 % TC reduction by week two. The rise in TC in FPCB-2, without WC, indicates the possible release of carbon from the BHC substrate

during the bioconversion process. By week six the TC in both bioreactors had been utilized.

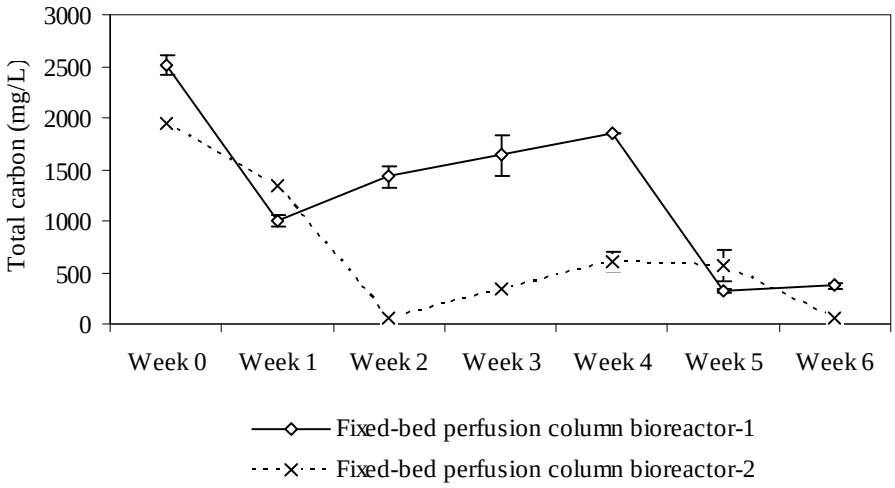


Figure 6-6 The change in the total carbon in the fixed-bed perfusion column bioreactors FPCB-1 and FPCB-2, in which both were supplemented with the low molecular weight organic compound mix and weathered hard coal, was excluded in FPCB-2.

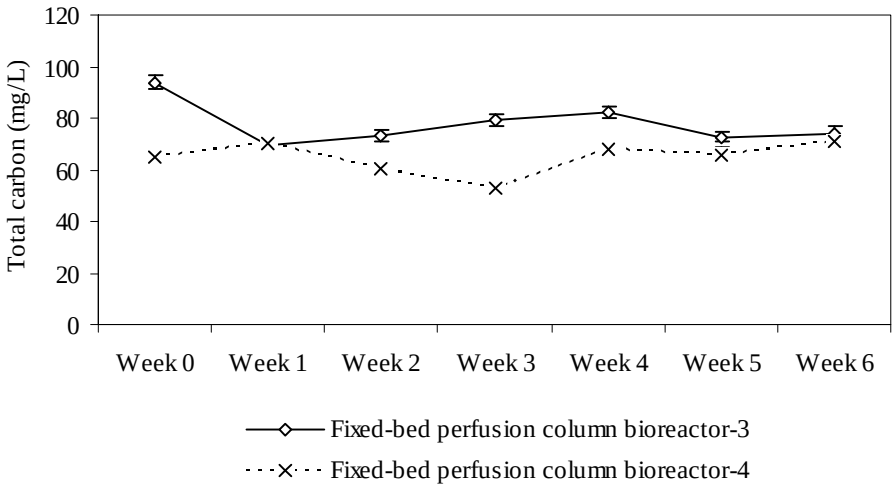


Figure 6-7 The change in the total carbon in the fixed-bed perfusion column bioreactors FPCB-3 and FPCB-4 that provided controls for weathered hard coal and low molecular weight organics, and the inoculum, respectively.

The profile of the TC results for FPCB-1 and FPCB-2 are comparable to the bioconversion of WC observed in submerged liquid culture inoculated with *N. fischeri*.

With the exclusion of both LMO and WC, no appreciable reaction was observed, either with inoculum or without.

6.3.3 Pyrolysis gas chromatography mass spectrometry

The Py-GCMS traces for FPCB-1 and FPCB-2 are presented in Figures 6-8 and 6-9, respectively, compound names of labeled TIC are shown in Table 6-3. Both reactors showed the presence of nitrated and oxidized aromatic compounds. Nitrated compounds which were not present at week 0 appeared earlier in the presence of WC in FPCB-1, while in FPCB-2, where WC was excluded the nitrated compounds appeared, but more slowly. In each bioreactor there was removal of nitrated compounds, possibly utilized by the organism within the system.

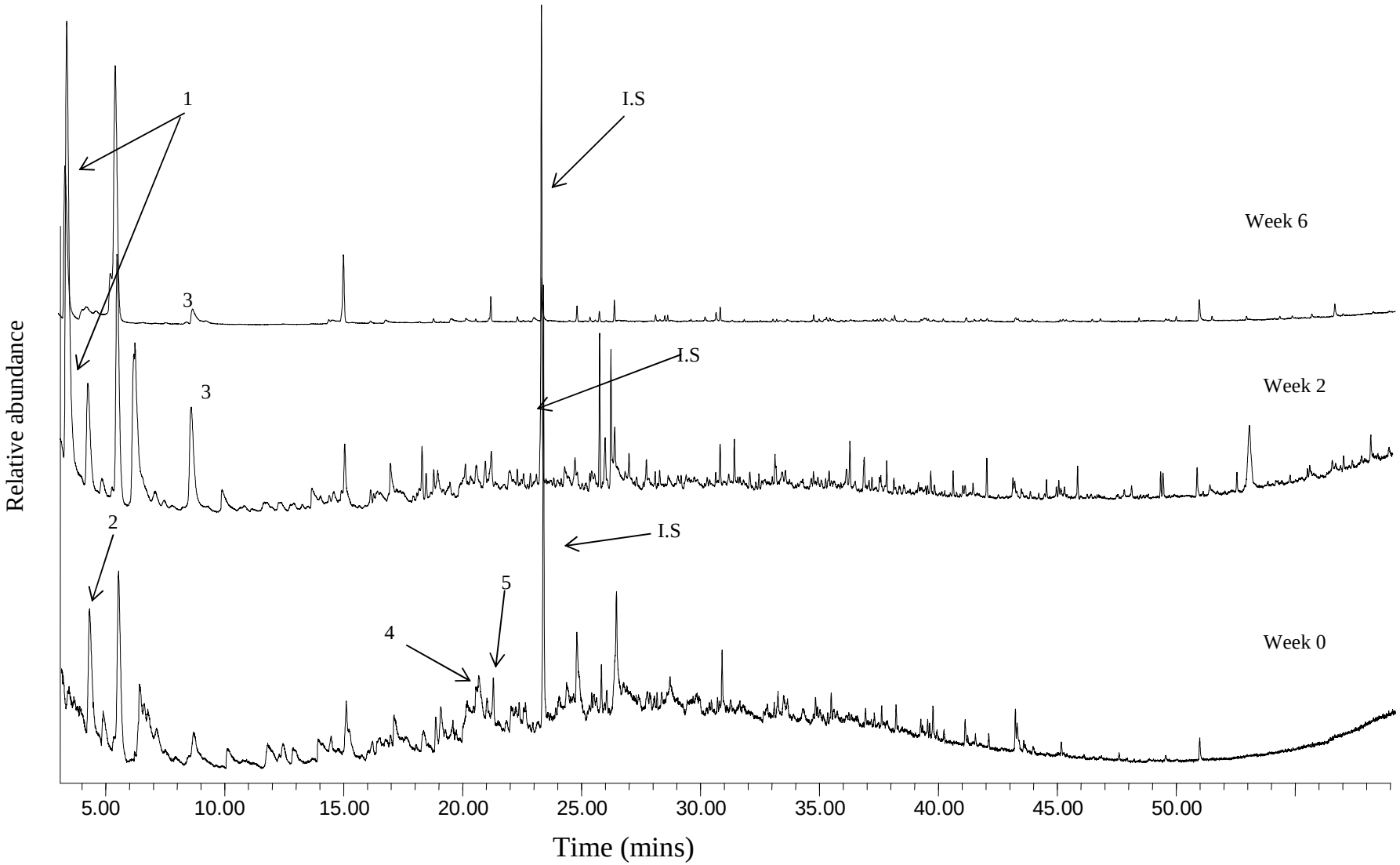


Figure 6-8 A stacked total ion chromatogram of the effluent from fixed-bed perfusion column bioreactor (FPCB-1) for weeks 0, 2 and 6. I.S, Internal standard (poly(*tert*-butylstyrene)). For numbers refer to Table 6-3.

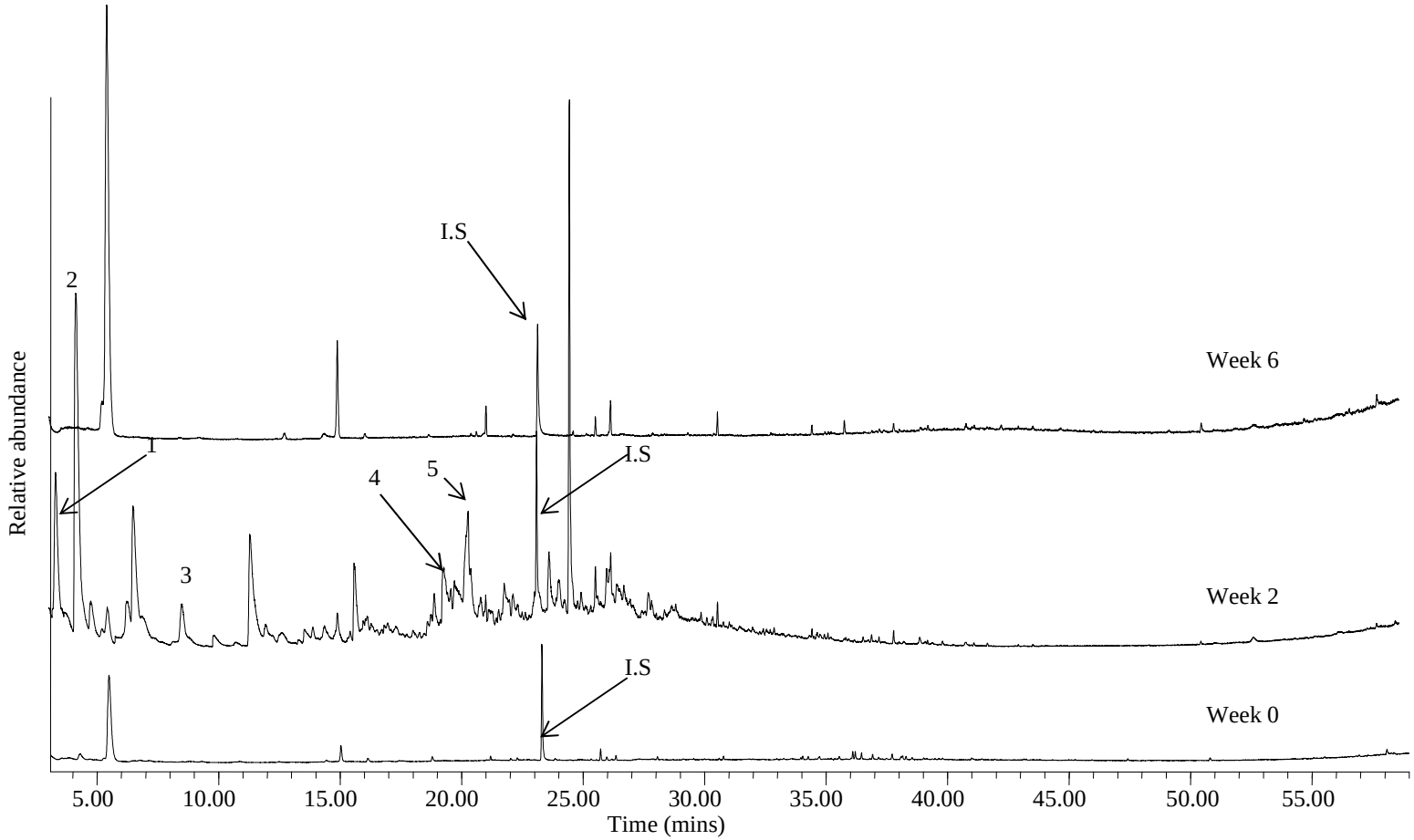


Figure 6-9 A stacked total ion chromatogram of the effluent from fixed-bed perfusion column bioreactor (FPCB-2) for week 0, 2 and 6. . I.S, Internal standard (poly(*tert*-butylstyrene)). For numbers refer to Table 6-3.

Table 6-3 Compounds in pyrolysates generic to the fixed-bed perfusion column bioreactors FPCB-1 and FPCB-2. Peak numbers refer to the labeled total ion chromatogram in Figures 6-8 and 6-9.

Peak numbers	Retention times (mins)	Compounds
1	3.3	Butenoic acid
2	4.2	Pyrrole
3	8.7	Styrene
4	19.8	Pyrrolidinone
5	22.7	Amino-methyl pyrazole
Internal standard	23.7	poly(*tert*-butylstyrene)

The changes in detectable pyrolysates shown in the stacked TICs in Figures 6-8 and 6-9 were analyzed semi-quantitatively as shown in Figures 6-10 to 6-15. In FPCB-1, the majority of the detectable pyrolysates that were present in the effluent of the bioreactor at week 0 were observed to disappear over time. These include the nitrated compounds such as amino-methyl pyrazole, pyrrole and pyrrolidinone (Figure 6-10), which were broken down and possibly utilized by the fungal culture present in the reactor. Pyrolysates such as butenoic acid and styrene which were not detectable at the start of the operation, appeared by week 2, disappeared and then reappeared over time (Figure 6-11), possibly indicating the action of an inducible enzyme degradation pathway. In each case these compounds appear in week 2 and in smaller concentrations in FPCB-2 where WC is excluded.

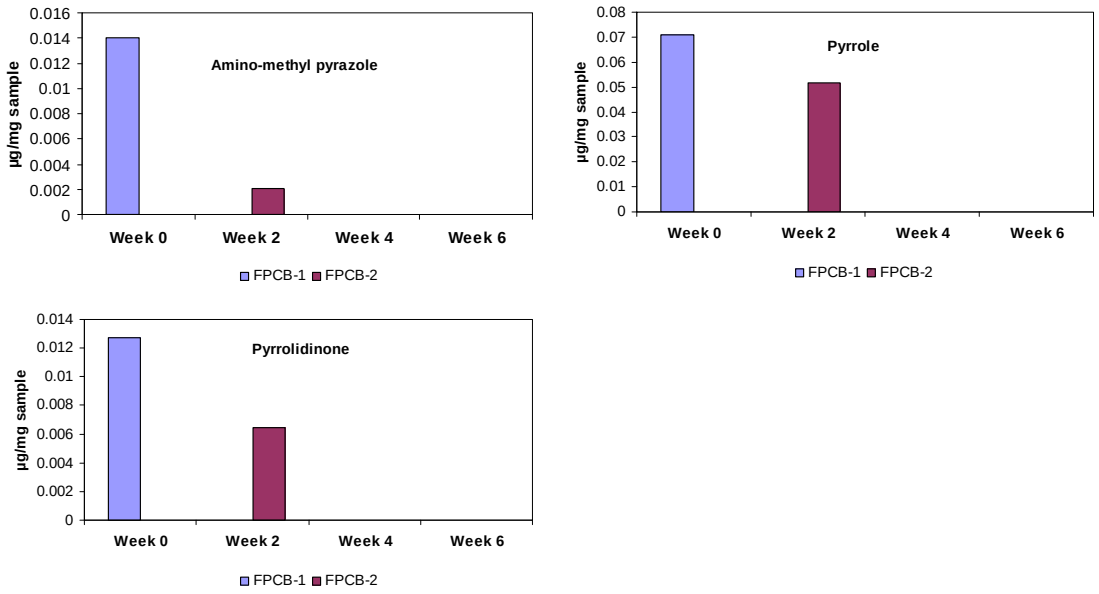


Figure 6-10 Semi-quantitative analysis of pyrolysates that were common to the fixed-bed perfusion column bioreactors FPCB-1 and FPCB-2, showing a trend of appearance and then removal from the system.

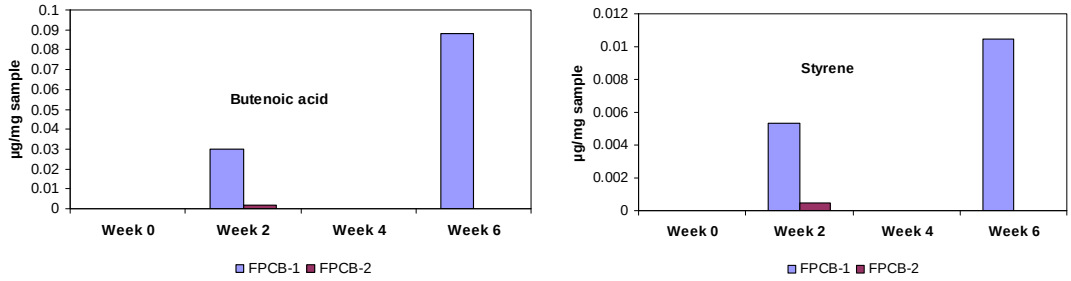


Figure 6-11 Semi-quantitative analysis of butenoic acid and styrene detected in both fixed-bed perfusion column bioreactors FPCB-1 and FPCB-2.

In addition to the above, a number of compounds were specific to either FPCB-1 (Figures 6-12 to 6-14) or FPCB-2 (Figure 6-15). Figure 6-12 shows semi-quantification analysis of benzeneamine, diazine, dimethyl pyranone, furfural, glucopyranose, hexadecanoic, methyl aminophenol and propanenitrile, which were observed in FPCB-1 in week one, but disappeared by the second week. This may indicate their utilization within the system.

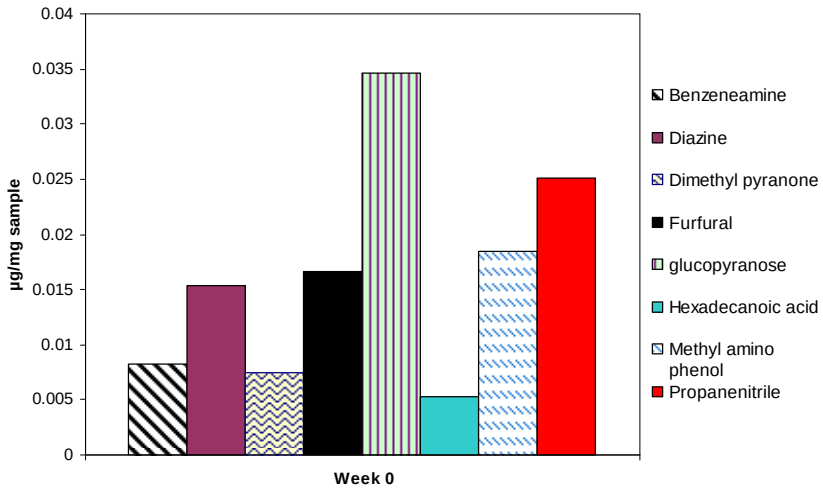


Figure 6-12 Semi-quantitative analysis of pyrolysates that appeared in week 0 in the fixed-bed perfusion column bioreactor-1 and then disappeared.

4-methyl phenol, methyldecene, naphthalene and toluene were observed in the second week only, indicating that their precursors were generated by the second week but were subsequently, possibly utilized (Figure 6-13) (Said-Pullicino and Gigliotti, 2007).

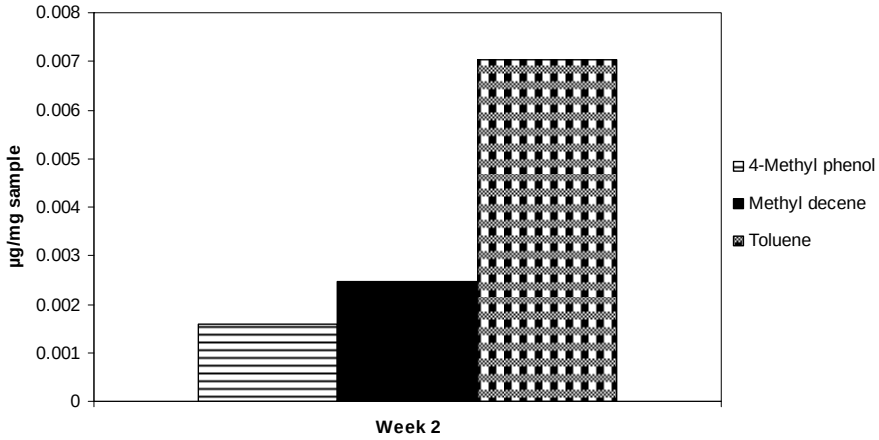


Figure 6-13 Semi-quantitative analysis of pyrolysates that appeared in the fixed-bed perfusion column bioreactor-1 by week 2 and then disappeared.

Benzoic acid and quinolin-9-one were detected as pyrolysis products only in week six compared to the controls, indicating the later production of their precursors by the organisms in the mixed inoculum (Figure 6-14).

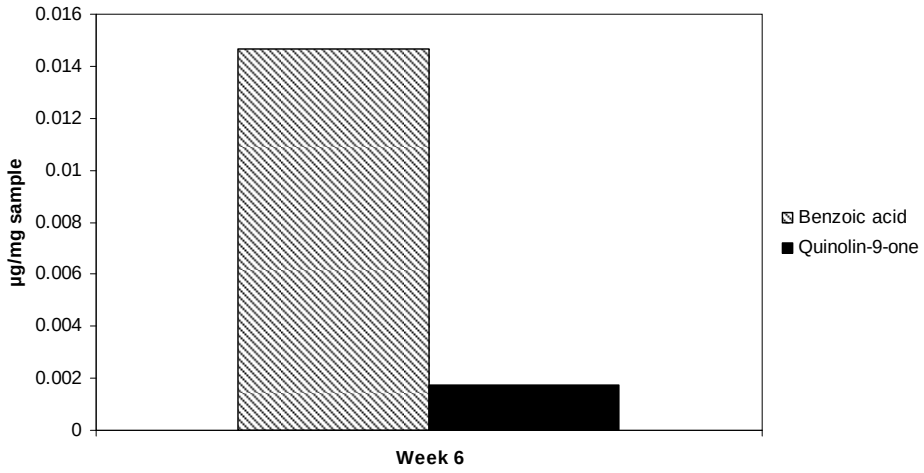


Figure 6-14 Semi-quantitative analysis of pyrolysates that appeared in fixed-bed perfusion column bioreactor-1 by week 6.

It is interesting to note that while a number of nitrated aromatic compounds were observed to be common for both FPCB-1 and FPCB-2 (Figure 6-11), the further appearance of ethyl pyrrole, methyl pyrrole and methyl pyrrolidinone was observed in FPCB-2 (Figure 6-15), where the more challenging environment of LMO without WC supplement was encountered. No build up of unutilized compounds was seen in FPCB-2.

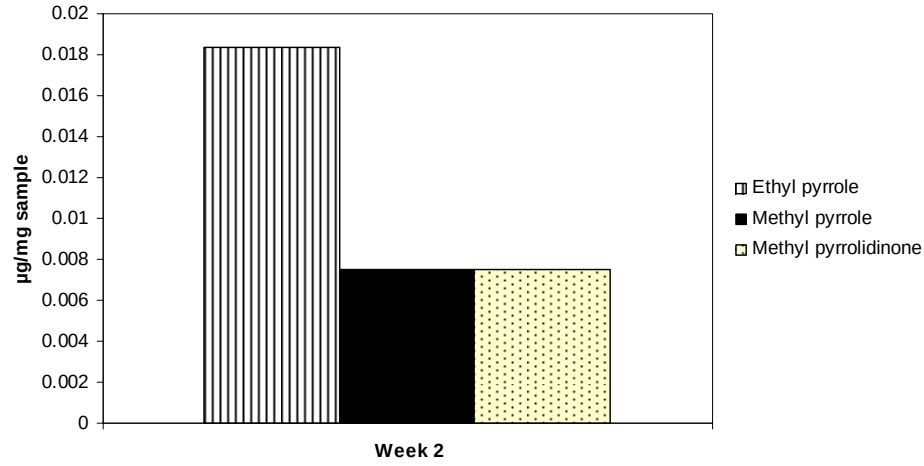


Figure 6-15 Semi-quantification of pyrolysates that were observed only after two weeks of operating fixed-bed perfusion column bioreactor-2.

Although more detailed confirmatory studies are required, the initial results indicate that the release of nitrated aromatic compounds is due to microbial activity. No activity was observed in the uninoculated control. Machnikowska *et al*. (2002) has shown an increase in the nitrogen content of biomass-bound HA in comparison to the unattended residue during microbial interaction. The insertion of nitro groups into the aromatic macromolecular structure of coal may in fact be a step in the initiation of microbial attack, thereby producing nitrated aromatic compounds. Said-Pullicino and Gigliotti (2007) have shown that aromatic compounds are readily degraded and used as an organic carbon source by micro-organisms under aerobic conditions. It may thus be proposed that a similar mechanism may be seen in action in this system and that the compounds observed are coal breakdown products generated by microbial action in the BHC bed. Further support for this conclusion is provided by the submerged liquid culture flask studies where the single *N. fischeri* inoculum also showed the production of nitrated aromatic compounds at the start of the coal bioconversion process. While it is evident that both humic acid, present in WC and LMOs in both FPCB-1 and FPCB-2 would provide a functional organic source for certain components of the microbial population, the production of compounds from the coal suggest their use by these organisms in the provision of certain essential nutrient requirements not present in the feed perfusate.

6.4 Conclusions

Clearly the results reported here provide a provisional indication of the processes occurring in the system and substantial further work is required to validate the assumptions made. However, the results do provide a first order approach to the problem of understanding the *C. dactylon*/coal system and enable initial steps in developing an explanatory model accounting for the mechanisms involved in coal bioconversion. The results generated from the FPCB studies, using mixed culture inoculum, have indicated that:

- The activity of the single isolate *N. fischeri* in the coal bioconversion process in the submerged liquid culture system is comparable to the activity of the mixed

inoculum in the perfusion system where WC and LMO are supplemented to simulate the plant/mycorrhizal component of the *C. dactylon*/coal dump system.

- While WC performs better, the presence of LMOs is required to facilitate coal bioconversion in the bioreactors.
- No activity is seen where LMO and microorganism are excluded.
- The trends in the appearance and breakdown of pyrolysates seen in the simulation studies suggest a nitration of aromatic compounds is involved in initiating coal bioconversion activity.
- The pattern of appearance and disappearance of pyrolysates suggests product released from coal bioconversion is utilized for microbial growth in the system.

Chapter seven

Conclusions

7. The bioconversion of coal in the *Cynodon dactylon*/coal rhizosphere

While the bioconversion of the coal substrate by certain fungi has been the subject of thorough investigation especially since the early 1980s (Fakoussa, 1981; Cohen and Gabriele, 1982; Scott *et al*., 1986; Quigley *et al*., 1988 and 1989; Cohen *et al*., 1990; Grethlein, 1990; Fakoussa and Willmann, 1991; Fakoussa, 1994; Ralph and Catcheside, 1994b; Hofrichter and Fritsche, 1997a and b), the large-scale industrial application of this system has made little progress (Fakoussa and Hofrichter, 1999; Klein *et al.*, 1999). The development of a cost effective reaction environment has been identified as one of the major constrains (Klein *et al.*, 1999).

The sporadic and unanticipated growth of *C. dactylon* has been reported to occur on the surface of hard coal dumps in the Witbank coal mining area of South Africa (Figure 1-9). Preliminary investigation of the *C. dactylon* root zone showed the breakdown of the coal in this area into a humic soil-like material that was absent in the adjacent coal sub-layer. A vertical profile of the root system showed an extensive penetration of > 2 m depth into the coal layer. Preliminary studies showed mycorrhizal colonization of the *C. dactylon* root system and the presence of extensive non-mycorrhizal fungi growth in the rhizosphere. A two-pronged investigation of the *C. dactylon*/coal system was undertaken in this laboratory. The investigation of the non-mycorrhizal fungal population has been reported here, parallel with this study, the role of the mycorrhizal fungi population has been reported by Mukasa-Mugerwa (2007).

This study investigated the processes involved in coal breakdown occurring specifically in the *C. dactylon* rhizosphere (as opposed to mycorrhizosphere), and was based on the hypothesis that its growth in the hostile hard coal dump environment may be associated

with the activity of coal degrading fungi. The range of mechanisms already described in the literature for these organisms including, possibly, biosolubilization (Strandberg and Lewis, 1987; Quigley *et al.,* 1988 and 1989; Hofrichter *et al*., 1997b; Stefanova *et al.,* 1999), liquefaction (Hofrichter *et al.,* 1997b; Hölker *et al*., 1997; Yuan *et al.,* 2006) and depolymerization (Hofrichter and Fritsche, 1996 and 1997a; Hofrichter *et al.,* 1997b; Willmann and Fakoussa, 1997a) amongst others, have been collectively termed bioconversion activity here.

7.1 Research Conclusions

A number of conclusions may be drawn from this study that the construction of a descriptive model of coal bioconversion that may occur in the *C. dactylon*/coal rhizosphere:

- A standardized five-step protocol was developed and utilized to screen over 2000 coal-based environmental samples with coal bioconversion capability;
- Amongst the screened and selected cultures were genera with previously described coal bioconversion capabilities;
- Of the 109 cultures that were selected, the ECCN 84 isolate, later identified as *N. fischeri*, was ranked the best overall performer with coal bioconversion activity comparable to well described isolates in coal bioconversion such as *P. chrysosporium* (PPRI 5328) and *T. versicolor* (PPRI 4835);
- The ability of *N. fischeri* to engulf BHC particles regardless of pre-oxidation suggested a strong affinity of the isolate to coal. This is the first report that implicates *N. fischeri* in coal bioconversion;
- The use of total genomic analysis to characterize the rhizosphere of undisturbed *C. dactylon* growing on coal dump showed an array of genera that included cultures selected in the screening study as well as those with previously described coal bioconversion capabilities. The presence of these organisms in the

rhizosphere therefore suggests coal bioconversion activity does occur in the *C. dactylon*/coal rhizosphere;

- *N. fischeri* was shown to be among the organisms identified in the total genomic analysis of the *C. dactylon*/coal rhizosphere and repeated analyses also confirmed the presence of *N. fischeri*;
- The development of the simulation studies utilized a reductive approach to investigate the organic carbon supplementation provided by the plant/mycorrhizal component in the rhizosphere;
- In the deep liquid culture flask studies used to interrogate the modification of HA during coal bioconversion, the activity of *N. fischeri* was observed to be comparable to that of *P. chrysosporium* and *T. versicolor*. *N. fischeri* was subsequently selected as a single model organism for use in the *C. dactylon*/coal rhizosphere simulation studies;
- Using the selected WC-Glut medium in the deep liquid culture flask studies, an increase in HA content after fungal interaction was demonstrated, which suggests a bioconversion of the WC substrate to release HA;
- The FT-IR results showed that the coal bioconversion process involves oxidation and nitration, and this observation was strengthened by Py-GCMS and macro-elemental analysis results;
- Finally, in the absence of LMOs, the nitrated and oxidized compounds were not found in the pyrolysis product, which indicates that the coal bioconversion process is dependent to some degree on the LMOs introduced into the system by plant photosynthesis.

7.2 Descriptive model

Mukasa-Mugerwa (2007) demonstrated the colonization of the *C. dactylon* root system by mycorrhizal fungi that included *Glomus, Paraglomus* and *Gigaspora* species. He simulated the *C. dactylon*/coal dump system in pot and column studies and the bioconversion of the coal to a humic soil-like substrate was demonstrated over a 20 – 40

week period, using an inoculum including *N. fischeri* as the sole representative of the non-mycorrhizal rhizospheric fungi. Atkins and Horan (pers. comm.) have also demonstrated this phenomenon in field studies undertaken on the Klein Kopje Colliery waste coal dump, Witbank, South Africa. In addition, Mukasa-Mugerwa (2007) showed that the extraradicular mycorrhizal presence was reduced and the intraradicular colonization of the *C. dactylon* roots was increased when grown on coal with *N. fischeri* inoculum compared to soil controls. This reduction of mycorrhizal activity in the rhizosphere seemed to indicate an increased role for the coal bioconverting fungi in establishing a mutualistic process, enabling the combined system to function. He also showed that humic acid released from WC in the system remained bound where *N. fischeri* was present, but was substantially reduced and also freely available in the perfusate of columns where non-mycorrhizal fungal growth was absent. This correlates with the observation made in this report of strong humic acid adsorption by *N. fischeri* hyphae.

The mechanism of action of *N. fischeri* as a representative of the non-mycorrhizal rhizospheric fungal population in the *C. dactylon*/coal system, together with the insight into the role of the mycorrhizal population, provided at least a provisional foundation on which to develop an integrated descriptive model accounting for the bioconversion of coal to the humic soil-like substrate that has been observed.

Based on the results obtained for both the mycorrhizal and non-mycorrhizal components of the *C. dactylon*/coal system, a descriptive model was developed to elucidate the mechanisms by which the coal weathering in the system could be explained and is shown in Figure 7-1. The results obtained from the various studies suggest that the entire process is facilitated and driven primarily by the translocation of photosynthetic organic carbon into the rhizosphere, and the key component of the system in this regard is *C. dactylon*. This process is mediated by the associated mycorrhizal fungi with their possible control of exudate release into the rhizosphere playing either a direct or an indirect role in the oxidative weathering of the coal. The translocated photosynthate is thought to be converted to organic acids, amongst other compounds, and subsequently released by

these fungi. The organic carbon exudates could then be utilized by the non-mycorrhizal fungi such as *N. fischeri* as an organic carbon source to facilitate the bioconversion of the coal substrate. In return, nutrients released from the coal substrates such as possibly phosphate are made available for uptake by the plant. This interactive process would lead to the generation of the humic soil-like material derived from the BHC substrate.

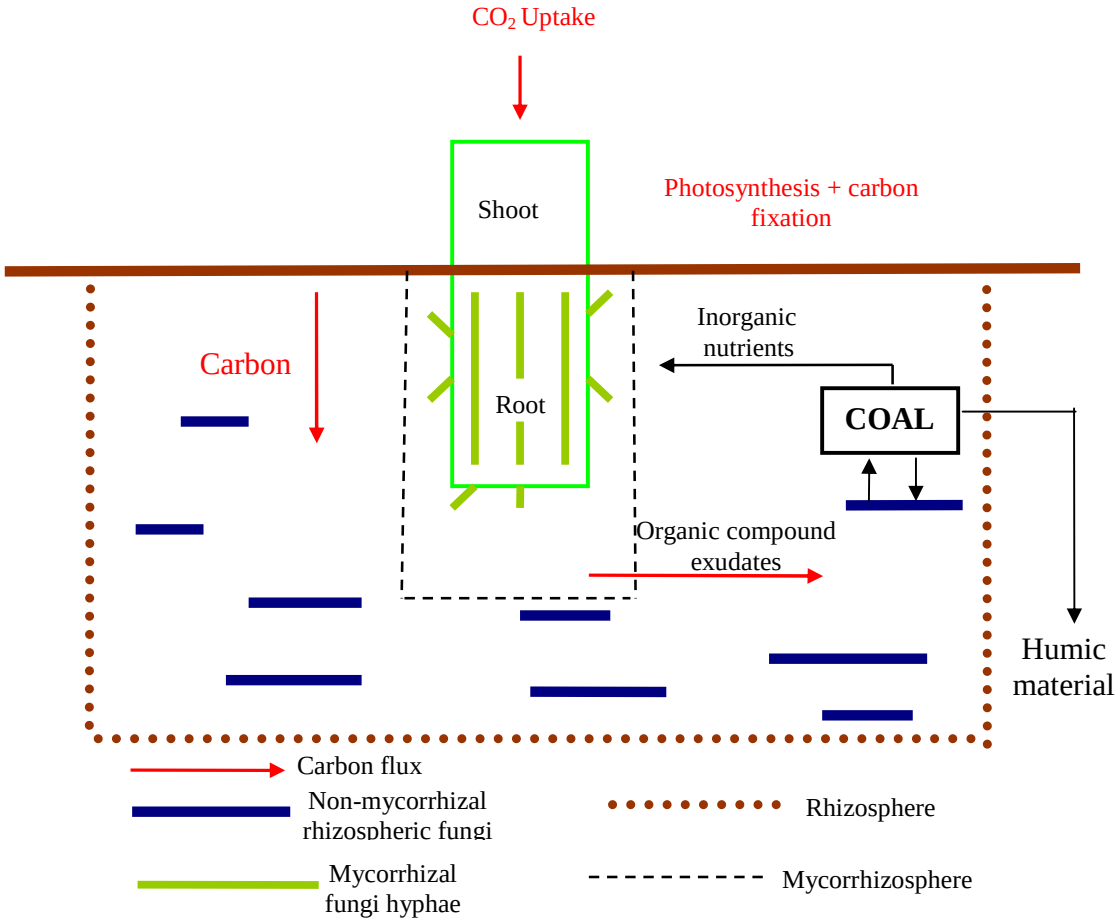


Figure 7-1 Schematic diagram illustrating the possible association between *Cynodon dactylon*, mycorrhizal fungi and rhizospheric non-mycorrhizal fungi that leads to the bioconversion of coal *in situ*.

7.3 Application

The experimental results described above have led to the development of the "Stacked-Heap Coal Bioreactor" (Patent ZA 2007/07607) in which a heap-leach process

environment is used for the large-scale bioconversion of the BHC substrate (Figure 7-2). In this system (Figure 7-2) the waste BHC is stacked, the surface layers inoculated with the appropriate fungal cultures (including mycorrhizal and non-mycorrhizal rhizospheric forms including *N. fischeri*) and then planted with *C. dactylon*. The system is then irrigated to ensure prolific growth of *C. dactylon* and after a period of time the upper 1.5 – 20 m transformed layer is harvested and passed to further downstream applications. The underlying un-harvested layer is then planted and the process repeated. Concurrent with these investigations, field trials have been implemented at the Klein Kopje Colliery discard coal dump (Witbank South Africa) and the bioconversion of BHC was replicated in the presence of mycorrhizal fungi and *N. fischeri* inocula (Horan, pers. comm.).

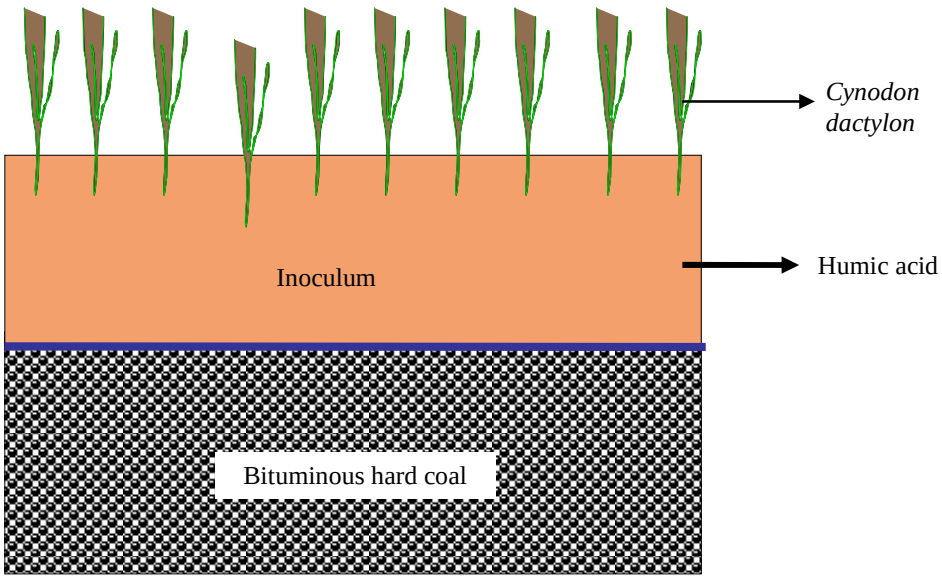


Figure 7-2 Schematic diagram illustrating the Stacked-Heap Coal Bioreactor for the large scale bioconversion of bituminous hard coal.

Under these conditions it has been shown that BHC can be converted to 30 - 40 % HA within 40 weeks and that the HA rich product may be recovered and used in a variety of application ways including:

- Its use as a fertilizer in the agricultural sector. Horan (pers. comm.) has showed enhanced growth of plants and the decompaction of soils in the rehabilitation of open-cast mining areas.

- Its use in the *in situ* breakdown of coal to establish a self-cladding layer on discard coal dumps as an alternative to the large volumes of topsoil that are currently used to rehabilitate these dumps. In the self-cladding operation, the coal surface is converted to the humic soil-like substrate and in addition to stabilizing the dump surface it also controls water and oxygen ingress into the system. Preliminary studies have indicated that the successful application of the process and cost saving over topsoil translocation would reduce rehabilitation costs by ~ 8 fold (Horan, pers. comm.).

- Its use in the production of methane from the humic product in the presence of adapted anaerobic bacterial consortium (Mutambanengwe, pers. comm.).

7.4 Future work

The studies described here adopted a reductive approach to the description of what is clearly a complex system. The advances in commercial application of coal bioconversion proposed here are preliminary and substantial further work is required to investigate and develop the potential of the Stacked Heap Coal Bioreactor system for large-scale practical applications of coal bioconversion as an industrial process. This would include its piloting under a wider range of environmental conditions and using different types of coal and grass. A substantial effort should be made to further select functional strains and improve the inoculum. In addition it would be necessary to improve the understanding of processes occurring in the *C. dactylon*/coal microenvironment. These investigations would include among others:

- The use of a single isolate to represent the complex population needs to be confirmed and further studies inclusive of bacteria, protozoa and archaea possibly present in the system needs to be undertaken;
- Boundaries between the functions of the mycorrhizal fungi and the non-mycorrhizal fungi in the *C. dactylon* root zone need to be described;
- In a complex environment of mixed populations different mechanisms of coal bioconversion needs to be understood;
- Radio-labeled studies describing C-flux through the system need to be undertaken.

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Appendices

Appendix 1-A

Percentage composition of HA, FA and humin fraction in a 2.5 g sample of weathered hard coal was determined in triplicate as follow;

- Suspend 2.5 g WC in 0.1 M NaOH and make up to 100 mL
- Agitate for 24 hours at 150 rpm
- Centrifuge at 3220 × g for 90 mins at 10 ^{o}C
- Collect supernatant and freeze dry sediment
- Drop pH of supernatant to > 1 using minimal volume of 32 % HCl
- Stand for 1 hour and repeat centrifugation
- Decant supernatant (FA fraction) and measure absorbance 280 nm
- Re-suspend pellet (HA fraction) in 100 mL 0.1 M NaOH and measure absorbance at 450 nm.
- Freeze dry both fractions and weigh

Results obtained showed ~ 59 % humin, ~ 40 % HA and ~ 1 % FA as constituent of the WC (Kromdraai) sample that was used for the entire work.

Appendix 2-A

Preparation of Normal HNO_3 from 65 % HNO_3 stock concentration

The equilibrium dissociation constant for HNO_3 is represented by;

$$HNO_3 \rightarrow H^- + NO_3^-$$

It follows that:

$$1\ M\ HNO_3 = 1\ N\ HNO_3$$

Calculation:

Relative molecular mass of HNO_3

H (1) + N (14.007) + O_3 (15.999 × 3) = 63.004 g/L

63.004 g/L HNO_3 = 1 M HNO_3

63.004 g/L HNO_3 = 6.3004 % HNO_3

Molarity of 65 % HNO_3

65 / 6.3004 = 10.317 M = 10.317 N

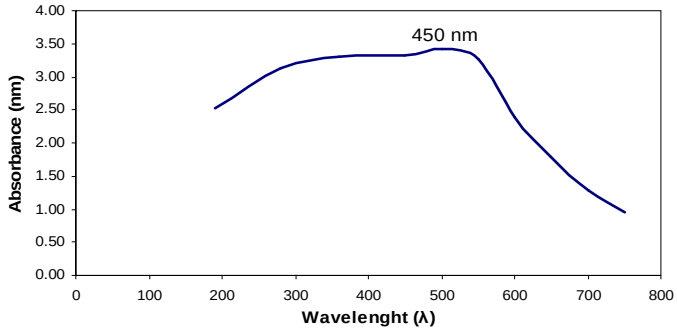
Volume of stock required for preparation of X N HNO_3

= Required Normality × final volume / Stock Normality

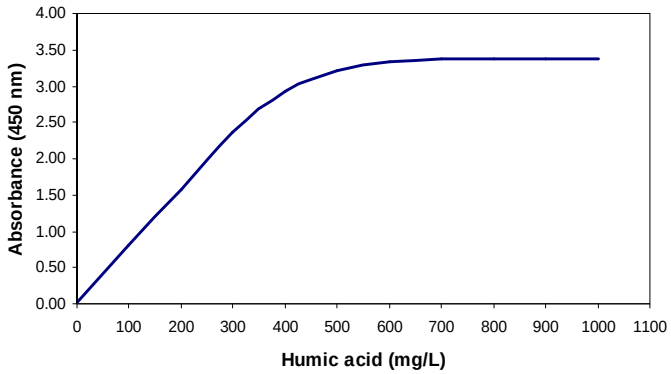
Normality	**Final Volume**	**Stock Normality**	**Volume of stock**
6	100	10.32	58.16
4	100	10.32	38.77
2	100	10.32	19.39
1	100	10.32	9.69
0.1	100	10.32	0.97

Appendix 4-A

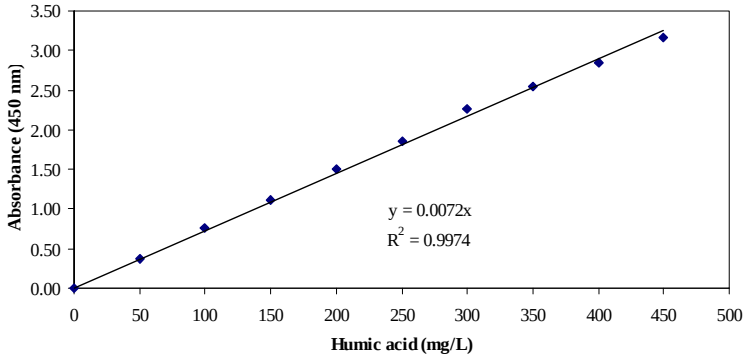
UV maxima, saturation point and standard curve plot for Suwannee River HA standard (IHSS, 2S101H)



UV analysis of 500 mg/L Suwannee River humic acid standard showed a maximum peak at 450 nm with a 3.2 nm absorbance reading



The determination of the saturation point of humic acid at 450 nm showed a 450 mg/L set limit. Further increase in concentrations demonstrated no relative increase in absorbance.



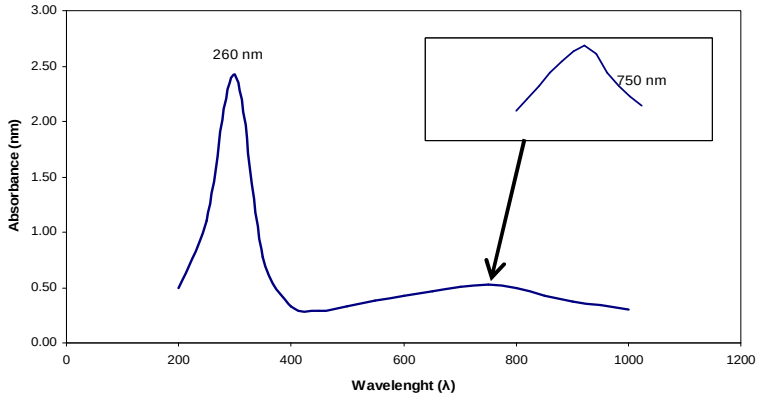
Suwannee River humic acid standard curve plot. Absorbance values were converted to mass by extrapolation made from the plot.

Appendix 4-B

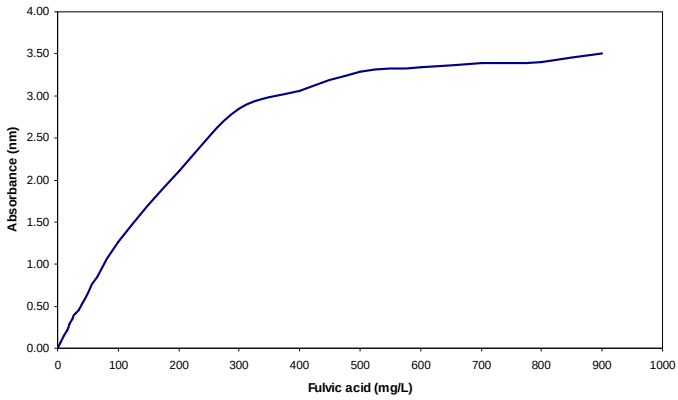
UV maxima, saturation point and standard curve plot for Waskish Peat FA as determined by Folin-Ciocalteau phenol reaction with the FA.

Reaction preparation;

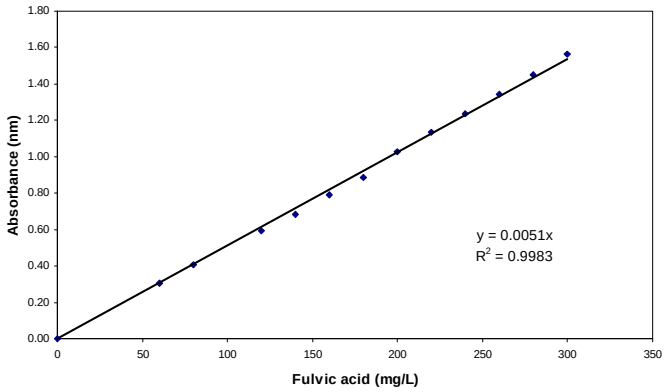
- To 10 mL of samples, add 1.5 mL of 200 g/L sodium carbonate and 0.5 mL of Folin-Ciocalteau phenol reagent
- Incubate at 20 ^{o}C for 60 min
- Measure absorbance at 750 nm in a 4 cm cell immediately after incubation as a decrease in sensitivity occurs over time if left to stand
- Distilled water served as blank



UV scan of 40 mg/L Waskish Peat Fulvic acid using Folin-Ciocalteau phenol reagent showed peaks at 260 nm and at 750 nm maxima. Inserted is a close-up on the 750 nm region which demonstrated a sharp peak proposed by Box, 1983.



The determination of saturation point of Waskish Peat Fulvic acid at 750 nm showed a 300 mg/L set limit after which further increase in fulvic acid concentration demonstrated no relative increase in absorbance.



Waskish Peat Fulvic acid standard curve plot at 750 nm. Absorbance values were converted to mass by extrapolations made on the standard plot.

Appendix 4-C

For sample sets containing HA, a direct use of the biomass weight measured after drying in the oven was adopted. The Tables represent the method utilized to deduce the biomass formed in WC-Glut media using spore inoculum, WC-rice extract media using pre-grown biomass inoculum and biomass formed in HA-rice extract media using spore inoculum. Similar method was utilized for the media recipe containing WC.

Biomass formed in WC-glutamate media using spore inoculum.

Days	Humin + Biomass (mg)	Total Humin (mg)	Filtrate Humin (mg)	Biomass Humin (mg)	Biomass (mg/100ml)
0	0	100	100	0	0
2	154	100	30	70	84
4	154	100	30	70	84
6	131	100	30	70	61
8	112	100	20	80	32
10	136	100	20	80	56
12	124	100	30	70	54
14	130	100	30	70	60

Biomass formed in WC-rice-extract media using pre-grown biomass inoculum.

Days	Humin + Biomass (mg)	Total Humin (mg)	Filtrate Humin (mg)	Biomass Humin (mg)	Biomass (mg/100ml)
0	14*	100	100.00	0.00	14
2	208	100	3.66	96.33	111.67
4	224	100	6.33	93.67	130.33
6	263	100	2.33	97.67	165.33
8	246	100	4.33	95.67	150.33
10	152	100	5.33	94.67	57.33
12	192	100	4.00	96.00	96.00
14	224	100	4.00	96.00	128.00

*Weight of pre-grown biomass inoculum

Biomass formed in HA-rice-extract media using spore inoculum

Days	Biomass (g/100ml)	Biomass (mg/100ml)
0	0	0
2	0.092	92
4	0.085	85
6	0.078	78
8	0.067	67
10	0.065	65
12	0.058	58
14	0.071	71

Appendix 4-D

The breakdown of One-way Analysis of Variance showing the Post-hoc LSD test results for the biomass formed in the various media recipes.

LSD test marked differences significant at p < 0.05 are highlighted in red

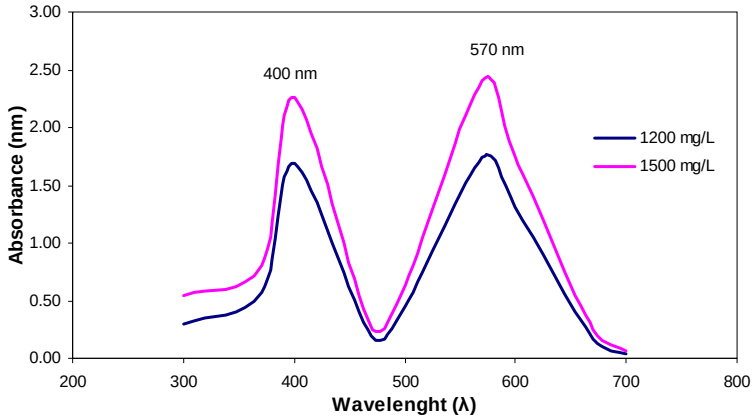
	{1}	{2}	{3}	{4}	{5}	{6}	{7}	{8}	{9}	{10}	{11}	{12}	{13}	{14}	{15}	{16}	{17}	{18}	{19}	{20}	{21}
[1]CNT-Glut {1}		0.6992	0.9349	0.0140	0.0973	0.1466	0.0000	0.5864	0.6476	0.0000	0.0174	0.0000	0.0022	0.0079	0.1246	0.0000	0.0179	0.0184	0.4317	0.4681	0.0944
[1]HA-Glut {2}	0.6992		0.7605	0.0375	0.2021	0.2853	0.0002	0.8745	0.3994	0.0000	0.0454	0.0000	0.0071	0.0226	0.2490	0.0000	0.0466	0.0478	0.6888	0.2669	0.0401
[1]WC-Glut {3}	0.9349	0.7605		0.0174	0.1147	0.1704	0.0000	0.6437	0.5901	0.0000	0.0215	0.0000	0.0028	0.0100	0.1457	0.0000	0.0221	0.0228	0.4809	0.4197	0.0796
[1]CNT-G {4}	0.0140	0.0375	0.0174		0.4147	0.3061	0.0920	0.0541	0.0037	0.0251	0.9349	0.0488	0.5295	0.8378	0.3478	0.0025	0.9262	0.9176	0.0916	0.0016	0.0000
[1]HA-G {5}	0.0973	0.2021	0.1147	0.4147		0.8347	0.0130	0.2632	0.0351	0.0024	0.4628	0.0057	0.1497	0.3080	0.9015	0.0001	0.4695	0.4762	0.3803	0.0178	0.0010
[1]WC-G {6}	0.1466	0.2853	0.1704	0.3061	0.8347		0.0072	0.3621	0.0571	0.0012	0.3461	0.0030	0.0996	0.2199	0.9323	0.0000	0.3517	0.3574	0.5032	0.0303	0.0020
[1]CNT-R {7}	0.0000	0.0002	0.0000	0.0920	0.0130	0.0072		0.0003	0.0000	0.5715	0.0775	0.7716	0.2884	0.1382	0.0092	0.1698	0.0757	0.0740	0.0008	0.0000	0.0000
[1]HA-R {8}	0.5864	0.8745	0.6437	0.0541	0.2632	0.3621	0.0003		0.3174	0.0000	0.0650	0.0001	0.0111	0.0335	0.3193	0.0000	0.0665	0.0681	0.8082	0.2052	0.0273
[1]WC-R {9}	0.6476	0.3994	0.5901	0.0037	0.0351	0.0571	0.0000	0.3174		0.0000	0.0048	0.0000	0.0004	0.0019	0.0470	0.0000	0.0049	0.0051	0.2146	0.7880	0.2224
[2]CNT-Glut {10}	0.0000	0.0000	0.0000	0.0251	0.0024	0.0012	0.5715	0.0000	0.0000		0.0203	0.7827	0.1047	0.0413	0.0016	0.4179	0.0198	0.0192	0.0001	0.0000	0.0000
[2]HA-Glut {11}	0.0174	0.0454	0.0215	0.9349	0.4628	0.3461	0.0775	0.0650	0.0048	0.0203		0.0403	0.4775	0.7746	0.3912	0.0019	0.9913	0.9826	0.1082	0.0020	0.0000
[2]WC-Glut {12}	0.0000	0.0000	0.0000	0.0488	0.0057	0.0030	0.7716	0.0001	0.0000	0.7827	0.0403		0.1771	0.0769	0.0039	0.2781	0.0393	0.0383	0.0003	0.0000	0.0000
[2]CNT-G {13}	0.0022	0.0071	0.0028	0.5295	0.1497	0.0996	0.2884	0.0111	0.0004	0.1047	0.4775	0.1771		0.6712	0.1180	0.0156	0.4708	0.4642	0.0212	0.0001	0.0000
[2]HA-G {14}	0.0079	0.0226	0.0100	0.8378	0.3080	0.2199	0.1382	0.0335	0.0019	0.0413	0.7746	0.0769	0.6712		0.2533	0.0047	0.7663	0.7580	0.0590	0.0008	0.0000
[2]WC-G {15}	0.1246	0.2490	0.1457	0.3478	0.9015	0.9323	0.0092	0.3193	0.0470	0.0016	0.3912	0.0039	0.1180	0.2533		0.0000	0.3972	0.4033	0.4508	0.0245	0.0015
[2]CNT-R {16}	0.0000	0.0000	0.0000	0.0025	0.0001	0.0000	0.1698	0.0000	0.0000	0.4179	0.0019	0.2781	0.0156	0.0047	0.0000		0.0018	0.0018	0.0000	0.0000	0.0000
[2]HA-R {17}	0.0179	0.0466	0.0221	0.9262	0.4695	0.3517	0.0757	0.0665	0.0049	0.0198	0.9913	0.0393	0.4708	0.7663	0.3972	0.0018		0.9913	0.1106	0.0021	0.0000
[2]WC-R {18}	0.0184	0.0478	0.0228	0.9176	0.4762	0.3574	0.0740	0.0681	0.0051	0.0192	0.9826	0.0383	0.4642	0.7580	0.4033	0.0018	0.9913		0.1130	0.0022	0.0000
[2]CNT-Amm {19}	0.4317	0.6888	0.4809	0.0916	0.3803	0.5032	0.0008	0.8082	0.2146	0.0001	0.1082	0.0003	0.0212	0.0590	0.4508	0.0000	0.1106	0.1130		0.1317	0.0145
[2]HA-Amm {20}	0.4681	0.2669	0.4197	0.0016	0.0178	0.0303	0.0000	0.2052	0.7880	0.0000	0.0020	0.0000	0.0001	0.0008	0.0245	0.0000	0.0021	0.0022	0.1317		0.3406
[2]WC-Amm {21}	0.0944	0.0401	0.0796	0.000	0.0010	0.0020	0.0000	0.0273	0.2224	0.0000	0.0000	0.0000	0.0000	0.0000	0.0015	0.0000	0.0000	0.0000	0.0145	0.3406	

[1], Spore inoculum; [2], pre-grown biomass inoculum; CNT-Glut, control glutamate media; HA-Glut, Humic acid-glutamate media; WC-Glut, Weathered hard coal-glutamate media; CNT-G, Control glucose media; HA-G, Humic acid-glucose media; WC-G, Weathered hard coal-glucose media; CNT-R, Control rice-extract media; HA-R, Humic acid-rice extract media; WC-R, Weathered hard coal-rice extract media; CNT-Amm, Control Ammonium nitrate media; HA-Amm, Humic acid-ammonium nitrate media; WC-Amm, Weathered hard coal-ammonium nitrate media

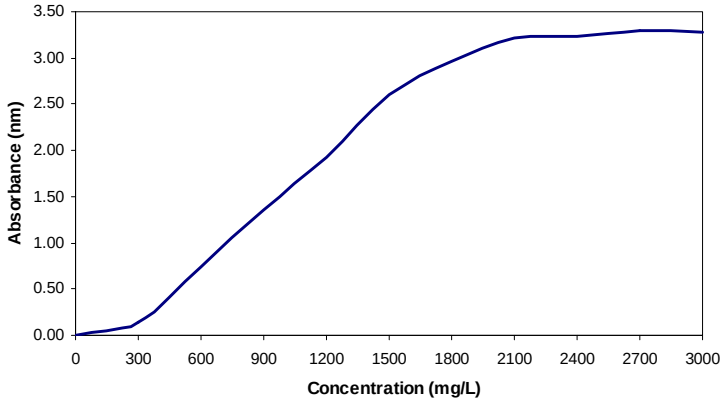
Appendix 5-A

0.35 % Ninhydrin reagent preparation for glutamic acid determination (Colorimetric method)

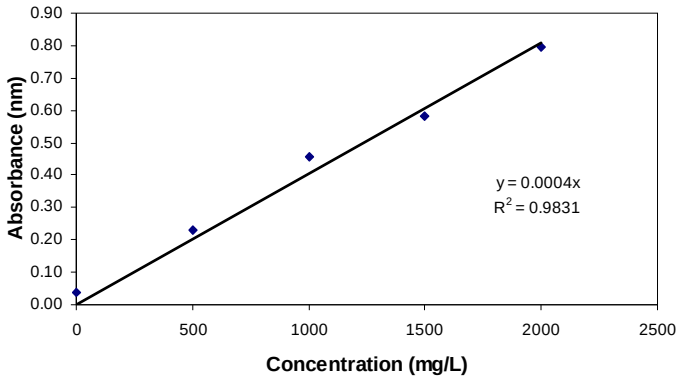
Ninhydrin reagent was prepared by dissolving 0.35 g of Ninhydrin in 100 mL of ethanol and stirred at room temperature until completely dissolved.



The UV scan of 1200 mg/L and 1500 mg/L of L-glutamic acid showed maxima at 400 nm and at 570 nm. However, the optima maxima at 570 nm (Hwang and Ederer, 1975) was selected and utilized throughout for glutamate analysis.



The determination of saturation point of L-glutamic acid at 570 nm showed a 2100 mg/L set limit after which further increase in L-glutamic acid concentration demonstrated no relative increase in absorbance.



L-glutamic acid standard curve obtained at 570 nm. Absorbance values were converted to mass by extrapolation made on the standard curve plot.

Appendix 5-B

The CDS 5000 Pyroprobe parameters

		Temperature (^{o}C)	Time
Pyroprobe	Initial	50	1.00 Seconds
	Ramp	15	per mSeconds
	Final	700	15 Seconds
	Clean	1200	5 Seconds
	Dry	80	60 Seconds
Interface	Rest	250	Enabled
	Initial	250	0.00 minute
	Ramp	0	per minute
	Final	250	0.00 minute

Appendix 5-C

Analysis of variance Post-hoc test of the variables with marked differences (highlighted in red) that are significant at $p < 0.05$ (ANOVA; LSD test).

	Indole				**Phenol**			
Days	**0**	**4**	**6**	**14**	**0**	**4**	**6**	**14**
0		0.067171	0.020397	0.013263		0.001926	0.000223	0.000223
4	0.067171		0.523014	0.390899	0.001926		0.235984	0.235984
6	0.020397	0.523014		0.820292	0.000223	0.235984		1.000000
14	0.013263	0.390899	0.820292		0.000223	0.235984	1.000000	
	3-phenylpiridine				**Benzylenitrile**			
0		0.042113	0.006660	0.088169		0.020453	0.007757	0.008175
4	0.042113		0.337395	0.683142	0.020453		0.610909	0.630148
6	0.006660	0.337395		0.181770	0.007757	0.610909		0.977907
14	0.088169	0.683142	0.181770		0.008175	0.630148	0.977907	
	N-methylphthalimide				**Flourene**			
0		0.351099	0.080443	0.048169		0.021559	0.000066	0.003217
4	0.351099		0.366282	0.242469	0.021559		0.006124	0.324223
6	0.080443	0.366282		0.776387	0.000066	0.006124		0.040871
14	0.048169	0.242469	0.776387		0.003217	0.324223	0.040871	