THE BIOTECHNOLOGY OF HARD COAL UTILIZATION AS A BIOPROCESS SUBSTRATE

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By

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ABSTRACT

The development of coal biotechnology, using hard coal as a substrate, has been impeded by its low reactivity in biological processes. As a result, the more successful application studies have focused on lignitic soft coals. However, new studies have reported using biologically or geologically oxidized hard coal as a functional substrate option for bioprocess applications on a large scale.

This study undertook a preliminary investigation into the feasibility of environmental applications of coal biotechnology using oxidized hard coal substrates in both anaerobic and aerobic processes with carbon dioxide, sulfate and oxygen as terminal electron acceptors.

A preliminary characterization of the oxidized hard coal substrates was undertaken to determine and predict their viability and behavior as electron donors and carbon sources for environmental bioprocess applications of direct interest to the coal mining industry.

Both biologically and geologically oxidized coal substrates showed loss of up to 17% and 52% carbon respectively and incorporation of oxygen ranging from 0.9 – 24%. The latter substrate showed greater loss of carbon and increased oxygenation. The biologically and geologically oxidized hard coal substrates were shown to partition readily into 23% and 32% organic humic acid, a 0.1% fulvic acid fraction and 65% and 59% inorganic and humin fractions respectively. These organic components were shown to be potentially available for biological consumption. In the unmodified hard coal substrate, partitioning was not observed and it did not perform as a functional substrate for any of the bioprocesses investigated.

Where carbon dioxide was used as a terminal electron acceptor, methane production ranging from 9 – 26 mg CH_4.g substrate^{-1} was demonstrated from both oxidized coal substrates. Geologically oxidized coal produced 30% more methane than biologically oxidized coal. Methane yields from the geologically oxidized coal in the presence and absence of a co-substrate were 5 – 13-fold higher than previous studies that used hard coal for methanogenesis. Based on these results, and that the development and optimization of the biological oxidation process is currently ongoing, further applications investigated in this study were undertaken using geologically oxidized coal.

It was shown using pyrolysis gas chromatography mass spectrometry that the methanogenic system was dependent on the presence of an effective co-substrate supporting the breakdown of the complex organic structures within the oxidized hard coal substrate. Also that the accumulation of aromatic intermediate breakdown compounds predominantly including toluene, furfural, styrene and 2-methoxy vinyl phenol appeared to become inhibitory to both methanogenic and sulfidogenic reactions. This was shown to be a more likely cause of reactor failure rather than substrate exhaustion over time. Evidence of a reductive degradation pathway of the complex organic structures within the oxidized hard coal substrates was shown through the production, accumulation and utilization of volatile fatty acids including acetic, formic, propionic, butyric and valeric acids. Comparative analysis of the volatile fatty acids produced in this system showed that geologically oxidized coal produced 20% more of the volatile fatty acids profiled and double the total concentration compared to the biologically oxidized coal.

The use of geologically oxidized hard coal as a functional substrate for biological sulfate reduction was demonstrated in the neutralization of a simulated acid mine drainage wastewater in both batch and continuous process operations. Results showed an increase in pH from pH 4.0 to ~ pH 8.0 with sulfide production rates of ~ 86 $mgL^{-1}.day^{-1}$ in the batch reactions, while the pH increased to pH 9.0 and sulfide production rates of up to 450 $mgL^{-1}.day^{-1}$ were measured in the continuous process studies using sand and coal up-flow packed bed reactors. Again, the requirement for an effective co-substrate was demonstrated with lactate shown to function as a true co-substrate in this system. However, a low cost alternative to lactate would need to emerge if the process was to function in large-scale commercial environmental treatment applications.

In this regard, the aerobic growth and production of *Neosartorya fischeri* biomass (0.64 g.biomass.g SOC^{-1}) was demonstrated using oxidized hard coal and glutamate as a co-substrate. Both can be produced from wastes generated on coal mines, with the fungal biomass generated in potentially large volumes. Preliminary demonstration of the use of the fungal biomass as a carbon and electron donor source for biological sulfate reduction was shown and thus that this could serve as an effective substrate for anaerobic environmental treatment processes.

Based on these findings, an Integrated Coal Bioprocess model was proposed using oxidized hard coal as a substrate for environmental remediation applications on coal mines. In this approach, potential applications included methane recovery from waste coal, use of waste coal in the treatment of acid mine drainage waste waters and the recovery and use of humic acids in the rehabilitation of open cast mining soils.

This study provided a first report demonstrating the use of biologically and geologically oxidized hard coals as bioprocess substrates in environmental bioremediation applications. It also provided an indication that follow-up bioengineering studies to investigate scaled-up applications of these findings would be warranted.

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LIST OF ABBREVIATIONS

%	Percentage
µg	Microgram
µL	Microlitre
µM	Micromitre
AMD	Acid mine drainage
ANOVA	Analysis of variance
BOC	Biologically oxidized coal
BOC+G	Combined biologically oxidized coal plus grass
BSR	Biological sulfate reduction
BTX	Benzene toluene xylene
CCT	Clean coal technology
CDS	Chemical data system
COC	Chemically oxidized coal
COD	Chemical oxygen demand
CSTR	Continuous stirred tank reactor
dH_2O	Distilled water
DME	Department of minerals and energy
DOE	Department of energy
EBRU	Institute for environmental biotechnology Rhodes University
EC	European community
EDTA	Ethylenediamine tetraacetic acid
EIA	Energy information administration
FA	Fulvic acid
PBR	Packed bed reactor
FID	Flame ionization detector
FSI	Free swelling index
FTIR	Fourier transform infrared spectroscopy
FTS	Fischer-Tropsch synthesis
G	Grass

GOC	Geologically oxidized coal
GOC+G	Combined geologically oxidized coal plus grass
HA	Humic acid
HC	Hard coal
HS	Humic substances
IEA	International energy agency
ICB	Integrated coal bioprocess
IFC	International finance corporation
IGCC	Integrated gasification combined cycle
IHSS	International humic substances society
kDa	Kilo Daltons
LMO	Low molecular weight organic compounds
LRC	Low rank coal
LSD	Least square difference
mA	Milliamps
mg	Milligram
mL	Mililitre
mm	Milimetre
MS	Mass spectrometer
MSD	Mass selective detector
mT	Millitorr
mV	Milivolts
NDIR	Non-dispersive infrar-red dectector
NIST	National institute of standards and technology
nm	Nanometer
PDA	Potato dextrose agar
PDB	Potato dextrose broth
ppm	Parts per million
PS	Primary sludge
Py-GCMS	Pyrolysis gas chromatography mass spectrometry
R	Carbon: sulfate ratio

RoM	Run of mine
SASOL	Suid afrikaanse steenkool en olie lidamaatskapy
SEM	Scanning electron microscopy
SHCB	Stacked heap coal bioreactor
SOC	Soluble organic carbon
SRB	Sulfate reducing bacteria
SS-NMR	Solid state nuclear magnetic resonance
ATP	Ambient temperature and pressure
TCD	Thermal conductivity detector
TIC	Total ion chromatograms
TMAH	Tetramethylammonium hydroxide
TOC	Total organic carbon
UAPB	Up-flow anaerobic packed bed
UKZN	University of Kwa-Zulu Natal
v/v	volume per volume
VFA	Volatile fatty acids
VOM	Volatile organic matter
w/v	weight per volume
WCI	World coal institute
XPS	X-ray photoelectron spectroscopy

LIST OF ANTICIPATED PUBLICATIONS

- The characterization of oxidized HC substrates for the development and design of coal biotechnological processes;
- The production of methane from oxidized HC substrates;
- Elucidation of metabolic pathways for the degradation of oxidized HC under methanogenic and sulfidogenic conditions;
- The treatment of acid mine drainage using *in-situ* oxidized hard as a carbon source and electron donor in an up flow anaerobic packed bed reactor;
- The large-scale production of fungal biomass from oxidized HC substrates for anaerobic bioprocesses.

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CHAPTER ONE

1. INTRODUCTION

1.1 Energy

The intersection in recent years of constantly rising energy demands, dwindling supply and the potentially serious environmental impacts of energy production and consumption have continued to produce a complex global crisis (World Coal Institute (WCI), 2008). The projections of the European community (EC), International energy agency (IEA), and Energy information administration (EIA) all report that energy demand is expected to increase by at least 50% worldwide in the next 20 years (Lauzon *et al.*, 2007; EIA, 2008; IEA, 2008b; Kavouridis and Koukouzas, 2008). At present, fossil fuels remain the dominant source of global primary energy supply and it is estimated that it will account for ~ 83% of overall energy demand by 2030 (IEA, 2008a). Of this, coal accounts for 63%, and will continue to be the highest consumed fossil fuel in many countries, including especially rapidly developing economies such as China and India. In South Africa, coal accounts for ~ 90% of electricity generation, with the bulk of that energy being consumed in energy-intensive mining operations and primary mineral processing industries (IEA, 2008c). South Africa has made use of its coal reserves to develop the synthetic fuels industry, which produces 30% of its petroleum needs from coal via the Fisher-Tropsch process currently in use at the Suid Afrikaanse Steenkool en Olie Lidmaatskapy (SASOL) (EIA, 2008; Van Niekerk *et al.*, 2008).

According to the Global Energy Survey (Lauzon *et al.*, 2007), the ubiquity of coal supply, improved extraction technologies and plentiful, rich and accessible reserves have led to a 'coal renaissance' with coal expected to play an increasingly dominant role in the overall global energy mix.

Because coal markets are well positioned, functioning and responsive to changes in supply and demand, an increased reliance on coal energy is anticipated. However, the major challenges confronted by the increased dependence on coal are the environmental damage and other energy related emissions, which are likely to see increased greenhouse-gas emissions and accelerated global warming. This is expected to contribute substantially to potentially irreversible climate change (IEA, 2008b; World Coal Institute (WCI), 2008). This situation requires that a balance between energy security and environmental sustainability.

The Global Energy Survey has reported that the escalating energy demand may be expected to further strengthen calls for emission controls from consumers and governments (Lauzon *et al.*, 2007). According to the survey, energy stakeholders need to enter into a period of “Sustainable Creativity” where industry executives will have to address environmental challenges while simultaneously meeting energy demand commitments. It was noted that environmental considerations of the increased energy demands for coal would influence industry economics in the short term and also impact strongly on future business decisions (Lauzon *et al.*, 2007). These concerns have emerged as the key drivers in the development of clean energy technologies in many countries (John, 2009).

It is now widely recognized that innovation and technological development are critical factors in reaching an acceptable compromise between rising energy demand and environmentally sustainable development (WCI, 2008). In recent years, Clean coal technology (CCT) development has focused on more conventionally researched clean combustion processes of coal as alternative approaches to balance energy economics with environmental requirements (Kavouridis and Koukouzas, 2008). The Global Energy Survey has noted that “coal needs to be clean to be viable and while technologies already exist, the issue is their application” (Lauzon *et al.*, 2007).

Biological options for the development of clean coal technology have been the subject of considerable research, but progress has been limited by a range of factors including low-reactivity of the coal towards microbial transformation, lack of knowledge of the metabolic pathways involved in microbial disassembly of coal, inefficient biocatalysts; ineffective bioreactor designs that are amenable to large scale continuous operation and lack of technological models for downstream processing compatible with the unique products of coal bioprocessing (Klein, 1998; Fakoussa and Hofrichter, 1999; Klein *et al.*, 1999). In progressing this initiative, it becomes important to understand the fundamentals of coal structure, its physiological and chemical properties, together with its extraction and use (Collot, 2006; Gupta, 2007; Huang and Finkelman, 2008).

1.1.1 Coal origin and formation

Coal occurs in a wide range of forms and qualities, depending on deposition, the surrounding geology and environment. It can be described as a combustible black or brownish organic rock formed in the absence of air by the accumulation, and high temperature and pressure compaction, of plant based organic polymers (Kalaitzidis *et al.*, 2006). According to Thomas (2002), the inherent constituents of any coal can be divided into 'macerals' which can be described as the organic equivalents of the mineral constituents occurring in sedimentary rocks. The inorganic portion is made up of primary and secondary minerals. The composition and ratio of the two fractions reflects the make-up of the original material and depicts the coal type (Thomas, 2002). The organic matter, or macerals, are highly heterogeneous, can be identified in all coal types, and can be divided into three main groups; vitrinite (huminite), liptinite (exinite) and intertinite (Thomas, 2002). The original classification of the maceral groups is referred to as the Stopes-Heerlen System, and is summarized in Table 1-1 (Thomas, 2002).

The bulk of South African Gondwana coals in the southern hemisphere are located in the Vryheid Formation of the Ecca Group in the Karoo Basin (Cadle *et al.*, 1993). They were deposited during the middle to late Permian era over a relatively stable continental margin and were not subjected to deep burial, intense tectonic stresses or high geothermal gradients characteristic of the northern hemisphere coals. Because of this, there is a progressive eastward increase in coal rank across the Karoo basin coal fields from sub-bituminous in the Orange Free State to anthracite and meso-anthracite in the eastern Kwa-Zulu Natal province of South Africa (Snyman and Botha, 1993; Van Niekerk *et al.*, 2008). Over 95% of South Africa's coal reserves are bituminous and only ~ 2% are anthracitic (Kershaw and Taylor, 1992; Van Niekerk *et al.*, 2008).

Coal formation and petrography is determined by the depositional environment, particularly vegetation and climate differences (Cadle *et al.*, 1993). This is the main distinguishing factor between northern hemisphere and southern hemisphere coals. The latter coals are characterized by high levels of non-reactive inertinite believed to originate from high oxidation rates and microbial degradation during peatification (Cairncross, 2001). They are also rich in mineral content, which makes them problematic for biological treatment processes. The former have minimal mineral content and low inertinite content (Snyman and Botha, 1993; Cairncross, 2001; Van Niekerk *et al.*, 2008).

Table 1-1 Group macerals recognized in hard coals adapted from Thomas (2002)**.**

Maceral group	Maceral	Morphology	Origin
Vitrinite (huminite)	Telinite	Cellular Structure	Cell walls of trunks, branches, roots and leaves
	Collinite	Structure less	Repreciptation of dissolved organic matter in a gel form
	Vitrodetrinite	Fragments of vitrinite	Very early degradation of plant and humic peat particles
	Sporinite	Fossil form	Mega and microspores
	Cutinite	Bands which may have appendages	Cuticles - outer layers of leaves, shoots and thin stems
Exinite (liptinite)	Resinite	Cell filling layers or dispersed Fossil form	Plant resins, waxes and other secretions
	Alginite	Fragments of exinite	Algae
	Liptodetrinite	Empty or mineral filled	Degradation residues
	Fusinite	cellular structure; cell structure usually well preserved Cellular structure Amorphous 'cement'	Oxidized plant material - mostly charcoal from vegetation burning
	Semifusinite		Partly oxidized plant material
	Macrinite		Oxidized gel material
Inertinite	Inertodetrinite	Small patches of fusinite, semi-fusinite or macrinite	Re-deposited inertinites
	Macrinite	Granular, rounded grains ~ 1 µm in diameter	Degradation of macerals during coalification
	Sclerotinite	Fossil form	Mainly fungal remains

The formation of coal occurs in two phases called diagenesis and catagenesis. Diagenesis involves the decomposition of organic matter into its constituent Humic acids (HA), resins, and other hydrocarbons. These compounds react with each other to generate high molecular weight compounds known as kerogens, which, depending on the source material, can be classified into type I (algal kerogen derived from algae), type II (liptinitic kerogen from plankton and some algae) and type III (humic kerogen from higher plants). These three types of compounds have distinct chemical compositions that range from high concentrations of hydrogen (H) and aliphatic hydrocarbons in type I and II and high oxygen (O) and aromatic compounds in type III kerogens (Hayatsu *et al.*, 1984; Van Krevelen, 1984; Eglinton *et al.*, 1991).

Catagenesis occurs under adverse conditions characterized by high temperatures, pressures and anoxic conditions. Complex reactions such as dehydration, decarboxylation, and hydrogen redistribution result in the formation of methane, natural gas and petroleum oils from type I and II kerogens. Type III kerogens are converted into coal (Hayatsu *et al.*, 1984; Van Krevelen, 1984; Opaprakasit, 2003).

The formation of coal from these reactions can be illustrated using the van Krevelen diagram (Figure 1-1). At the onset of the catagenesis phase during coal formation, the oxygen: carbon ratio (O:C) and hydrogen: carbon (H:C) ratio is about 0.6 and 1.4 respectively. As catagenesis progresses, O and H are removed due to increasing temperature and pressure, and so the above ratios start to decrease giving rise to the different types of coal shown along the catagenesis pathway (Figure 1-1).

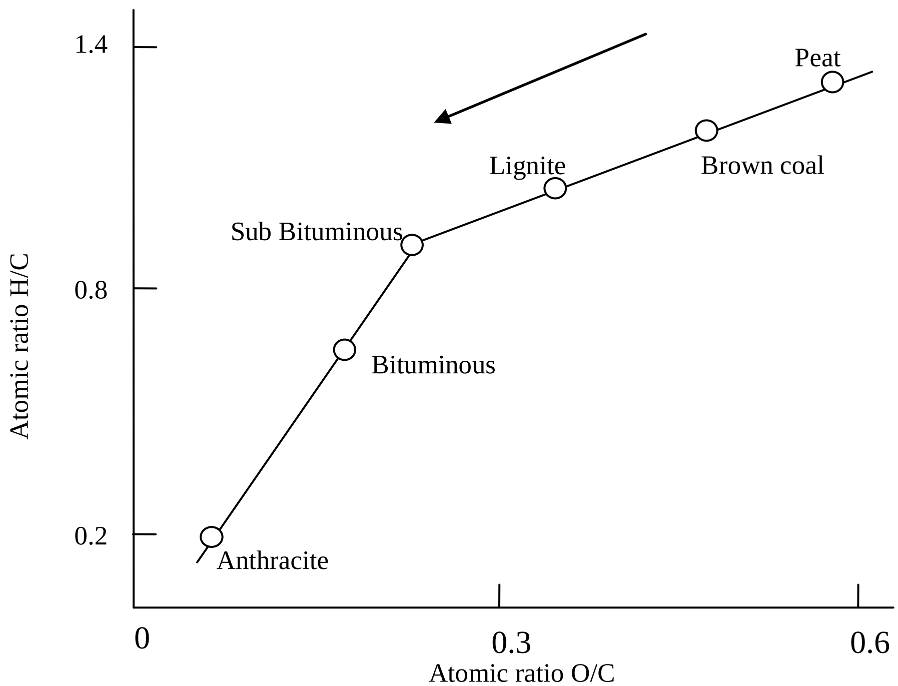


Figure 1-1 Van Krevelen diagram illustrating coal formation. Adapted from Van Krevelen (1984) and Opaprakasit (2003).

1.1.2 Classification of coal

A number of methods are used in the classification of coal based on variations in chemical composition and physical properties, and include factors such as increasing carbon content, decreasing moisture content, elemental analysis, and atomic ratio distribution (Levandowski and Kalkreuth, 2009). However, the most widely applied method categorizes coal by its carbon content or ‘rank’. Four main coal ranks are identified using this classification: lignite, sub-bituminous (high volatile coal), bituminous (low volatile hard coal), and anthracite (hard coal, HC). A summary of the coal ranks is presented below (Table 1-2).

Two other coal-related materials formed during the catagenesis stage that are not normally grouped with those four are peat and graphite. Peat occurs at the early stages of catagenesis, precedes the formation of lignite and contains about 55% C, 6% H and 35%O (Figure 1-1). Graphite is non-combustible, its carbon content is higher than 96%, and is characterized by complete removal of H and O (Opaprakasit, 2003).

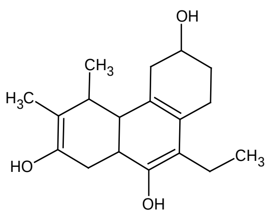
Table 1-2 Classification of coal based on carbon content, moisture and elemental analysis. Adapted from Hodek (1994) and Opaprakasit (2003) .

Coal class	Characteristics
Lignite	Formed from compaction and decomposition of peat and brown coal. It is characterized by a high moisture content coal with heat energy ranging from 8 – 10 MJ/Kg. Mainly used for electricity power generation. C – 25 to 35%; H – 6%, O – 25%
Sub-bituminous	Gradual loss of carboxyl and methoxyl groups and subsequent loss in the O:C and H:C ratios in the lignite coal results in a sooty, high moisture content and low sulfur content, which makes it attractive for use in cleaner burning applications. C – 35 to 45%; H – 5%; O – 9%
Bituminous	The later stage of catagenesis, often referred to as coalification starts at this level. This stage is characterized by redistribution of H_2 leading to the next level of coal formation. 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. C – 45 to 86%; H – 4.5% ; O – 3%
Anthracite	A shiny coal, which contains virtually no moisture content and energy content and could be 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 for heating of homes. C – 86 to 96%; H – 3.8%; O – 1.3%

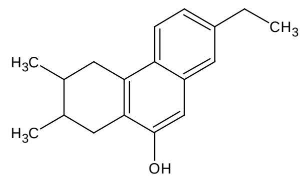
The hydrogen content of Low rank coal (LRC) remains unchanged or slightly decreases as carbon content increases. The oxygen content decreases sharply for coal with carbon contents higher than 88%. Furthermore, the nature of the oxygen functional groups changes with coal rank. During coal formation the O occurs in a variety of functional groups ranging from carboxylic acids, ethers, phenols to quinones. As the rank progresses from low to high, the number of ether and carboxylic acid groups diminishes resulting in prevalence of phenolic groups in mid-rank coal, and then finally, in high rank coal oxygen is largely present in the form of quinones and some phenols (Huffman *et al.*, 1985; Opaprakasit, 2003; Geng *et al.*, 2009).

1.1.3 Coal structure

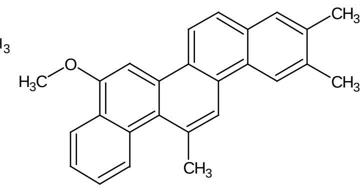
Coal is generated from a wide range of precursors under a broad range of chemical reactions, and therefore it has a predominantly heterogeneous macromolecular structure that is highly cross-linked. Its molecular structure is nevertheless still poorly understood (Fakoussa and Hofrichter, 1999; Opaprakasit, 2003; Stefanova *et al.*, 2004) . The structure is dependent on the rank and it has been demonstrated that no two coals are similar, this being influenced by geographical location as well as conditions under which diagenesis and catagenesis occur (Fakoussa and Hofrichter, 1999). Several different structural models for coal have been suggested but the variance in the coal macerals has led to researchers presenting hypothetical generic models of coal structure based on the functional groups as illustrated in Figure 1-2 (Van Krevelen, 1984; Haenel, 1992; Fakoussa and Hofrichter, 1999; Opaprakasit, 2003; Igbinigie, 2008).



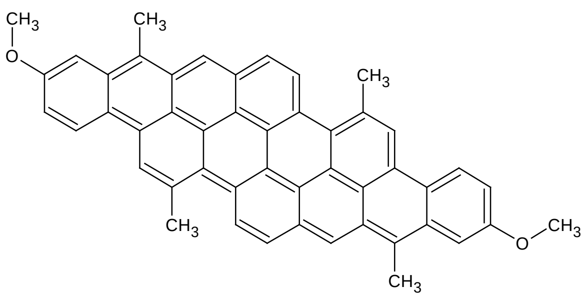
Sub bituminous coal
(High rank lignite)



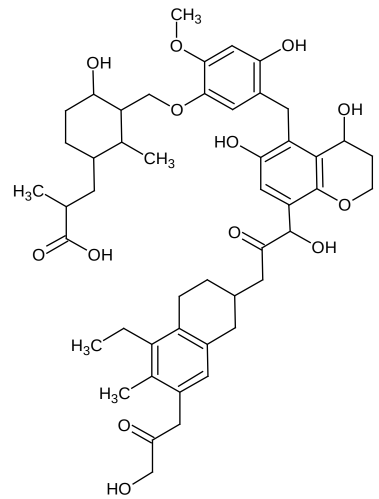
High volatile
Bituminous (hard) coal



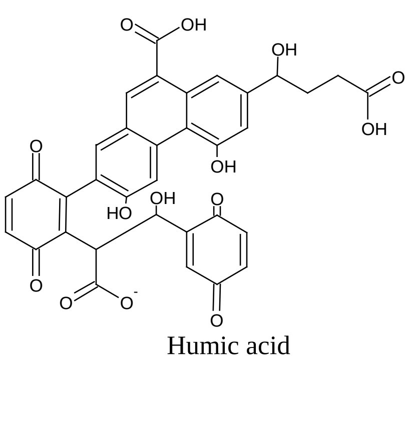
Low volatile bituminous (hard) coal



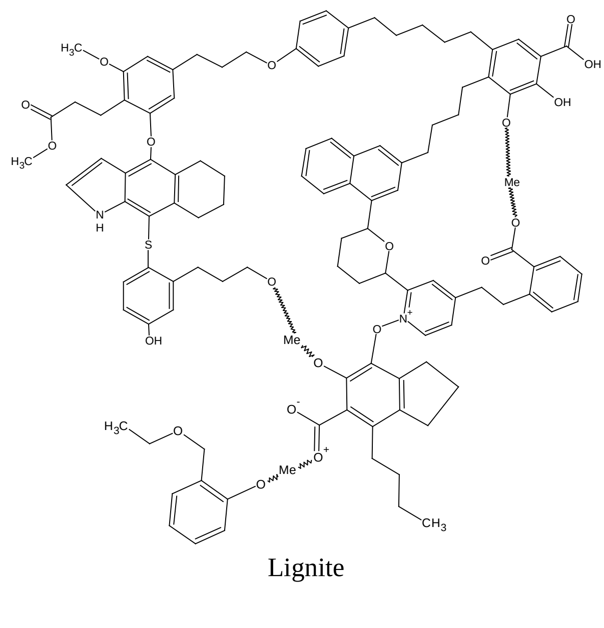
Anthracite (hard) coal



Low rank lignite



Humic acid



Lignite

Figure 1-2 Typical structure models for coals (substances) of different rank. Adapted from Fakoussa and Hofrichter (1999).

1.1.4 Coal mining and the environment

The mining of coal is dependent on the seam depth and quality of coal, which determines whether surface or underground extraction is used. Geology and environmental factors also play an important role in the extraction method to be used. Surface (strip or open cast) mining accounts for ~ 40% of global coal production. In South Africa, open cast mining is used to extract thermal and metallurgical coals located near the surface and spread over a flat terrain (Mangena and du Cann, 2007; World Coal Institute (WCI), 2008). Coal seams that were previously formed deep underground become uneconomical to extract using surface mining systems and are extracted using underground mining methods, which account for ~ 60% of the world coal production. There are five main methods used: long wall, short wall, bord and pillar, retreat mining and blast mining.

The wastes (solid, liquid, gaseous) generated from coal mining operations are dependent on the mining techniques, processing and end use. The production of coal results in large amounts of waste being generated annually (Table 1-3). Surface mining wastes are predominantly characterized by massive disruption of large areas of land and probable contamination of surface and ground water by Acid mine drainage (AMD). The disposal of overburden, waste rock and coal dust places substantial impacts on the environment. On the other hand, with underground mining the disturbance of land is less evident although the extent of subsidence can leave a large footprint indicative of mining activities (Cooke and Johnson, 2002; Bian *et al.*, 2008).

Table 1-3 Waste loads generated per unit of coal production based on the mining technique (tons per 1 000 tons of coal produced, adapted from Edgar (1983) and International finance corporation (IFC) (2007).

	Surface Mining		Underground Mining	
Waste characteristics	Strip	Area	Longwall	Bord and Pillar
Liquid effluents	0.24	1.2		1
Solid Waste	10	10	5	3
Dust	0.1	0.06	0.01	0.006

Coal mining exposes pyrite to oxygen and moisture, thereby initiating a series of reactions that result in the reduction of pH and accumulation of heavy metals such as Fe, Al, Mn Cd, Zn, and Cu in the surrounding water. Leaching out of these heavy metals drastically increases the

Chemical oxygen demand (COD) of the receiving water. Bacteria catalyzed reactions drive the oxidation of pyrite to form ferruginous (Fe-rich) mine water, which is deleterious to surface and ground water quality.

In light of these problems, progress has been made in the last 20 years in the development of predictive, preventative, and sustainable treatment practices to reduce the impact of liquid effluent pollution on receiving water bodies (Johnson and Hallberg, 2005; Neba, 2007). A combination of active and passive treatment technologies that have been developed for treating contaminated mine water include: chemical dosing, limestone drains, permeable reactive barriers, and more recently constructed wetlands and sulphidogenic bioreactors such as the BioSURE® Process (Younger *et al.*, 2002; Whiteley *et al.*, 2003; Johnson and Hallberg, 2005; Kalin *et al.*, 2006; Neba, 2007; Mayes *et al.*, 2008a). While these remedial technologies have been largely successful in the treatment and disposal of liquid effluents, major challenges still lie ahead in the disposal of solid and gaseous wastes.

Attempts to address the accumulating spoils and weathered coal dumps have had some degree of success although limited by the recalcitrance nature of some geological materials that cannot be readily transformed to yield soil-like materials. Methods used included cladding the surface of the dumps with viable soil from elsewhere and planting vegetation to cover the dumps in a bid prevent erosion and 'rehabilitate' the mined land. Often the vegetation grows poorly, due to lack of essential elements and the presence of heavy metals may also retard plant growth (Juwarkar and Jambhulkar, 2008). Cladding is also designed to prevent oxygen and water ingress into the discard coal spoils, by sealing the surface with clay and compacting it with heavy machinery (Smith *et al.*, 1997; Hamza and Anderson, 2005).

Downstream processing of coal results in the production of large volumes of tailings and solid waste, while storage and handling generates large amounts of coal dust (Blodau, 2006; Devasahayam, 2007). Processing of coal in beneficiation or washing plants removes non-combustible materials and pyrite to produce a cleaner coal product that has ~ 45% less ash content and a reduction of up to 25% in the inorganic sulfur levels (Mangena *et al.*, 2004). As may be expected, the processing of coal generates two large-volume waste streams; fine materials discharged as a slurry and coarse materials that are discarded onto dumps near the processing plant. Coal fines make up to 12% of the annual Run of Mine (RoM) tonnage in South

Africa, and have in the past been pumped as slurries or slimes into co-disposal discard dumps, old underground workings, or slime dams. Legislative pressure on environmental sustainability has forced mining companies to seek alternative disposal methods. One method that has been used for the agglomeration of coal fines is binder-less briquetting at high temperature and pressure, and thereby turning a waste into a useful product (Mangena *et al.*, 2004; Mangena and du Cann, 2007; Bian *et al.*, 2008).

Air pollution from mining activities is caused by fumes from spontaneous combustion and the release of toxic sulfur and nitrogenous gases. This may lead to the formation of acid rain that impacts negatively on the global climate (Bian *et al.*, 2008). Coal-generated waste will continue to accumulate and place pressure on the environment as global populations and economies expand. The need to preserve the environment through sustainable production and consumption systems have been among the main drivers for research into CCTs (DOE, 2007; Schläpfer, 2009; Wang and Nakata, 2009).

1.2 Clean Coal Technology

Clean coal technologies can be described as a range of innovative processes designed to reduce the environmental impact of coal mining, processing and consumption (World Coal Institute (WCI), 2008; Schläpfer, 2009). CCTs are specifically targeted to address different environmental problems associated with the different coal types. Moreover, they are dependent on the country's level of economic development. Highly advanced capital intensive technologies may not be suitable in developing countries where cheaper readily available alternatives can have a larger and more beneficial environmental impact (World Coal Institute (WCI), 2008). CCTs have developed in three strategic areas; (i) beneficiation of coal for waste reduction; (ii) increasing the energy value of products by removing impurities and (iii) production of cleaner fuels and value-added products. Both physico-chemical and biological approaches have been developed.

Within the physico-chemical systems a number of CCTs have been developed in a bid to reduce the environmental impact of coal and promote the non-fuel use of coal. Coal gasification is one of the easiest and most resourceful ways of extracting energy from coal (DOE, 2007). Several processes for conversion of coal to gas have been developed to commercial application (Zoeller, 2004; Collot, 2006; Christou *et al.*, 2008). SASOL produces synthesis gas from coal using a Lurgi gasifier, which is then converted to paraffinic liquid fuels and chemical feed stocks by

Fischer-Tropsch synthesis (FTS) over iron based catalysts (Schobert and Song, 2002). Another important CCT is the highly efficient Integrated gasification combined cycle (IGCC), which combines coal gasification with electricity generation resulting in near zero emission and is easily adapted to all coal types at low capital input (Wang and Nakata, 2009).

A second strategy involves direct conversion of coals to liquids or tars through carbonization, pyrolysis, liquefaction and extraction processes for downstream conversion into higher value products such as phenol, naphthalene, phenanthrene, pyrene, Benzene, toluene, xylene (BTX) and their derivatives (Schobert and Song, 2002). While this process is feasible, the major obstacle is that the liquefied products contain hundreds of components which are time consuming and expensive to separate. A third approach involves direct conversion of coal to high-value products by selectively cleaving bonds to remove the structural fragments of interest. In this strategy certain aromatic-aliphatic C-C bonds that are stronger than those bonds which would be readily cleaved in non-catalytic reactions are selectively cleaved by specific inorganic catalysts, and. Therefore, by tailoring the reaction conditions (reagents and catalysts) there is potential to target site-specific cleavage of the required compounds (Schobert and Song, 2002).

While inorganic catalytic processes are applicable in coal conversion and production of value-added products, biological processes offer some advantages over the physico-chemical processes, such as higher specificity, higher yields, lower energy requirements and better resistance to poisoning. In addition, the irreversible nature of biological reactions allows complete conversion and tends to circumvent thermodynamic equilibrium dynamics (Klasson *et al.*, 1992; Klasson *et al.*, 1993; Silva-Stenico *et al.*, 2007; Oboirien *et al.*, 2008). The study reported here has focused on the investigation of biological approaches to CCTs and the bioprocess implications of the products derived from the associated processes. Therefore, it is necessary to review the fundamentals underpinning coal biotechnology.

1.3 Coal Biotechnology

Third wave biotechnology has ushered in, and extended the application of microbe catalyzed processes, and although it is still in its infancy, computational reaction pathways and experimental data have developed processes that involve the sustainable use of natural resources ranging from the production of specialized chemicals, foodstuffs and drugs to waste treatment (Mullin, 2003; Mirasol, 2004). Among these, coal bioprocessing or coal biotechnology has attracted considerable interest, with its potential for use in CCT (Olson and Brinckman, 1986; Couch, 1988). Coal bioprocessing has focused on two broad areas: beneficiation of coal to remove impurities and coal transformation which involves microbial solubilization and depolymerization, decolorization, gasification and pretreatment (Olson and Brinckman, 1986). The former processes involve removal of sulfur, nitrogen and reduction of ash content using mild microbial processes. Microbial coal conversion is not well-defined, although the overall goal of these conversions is the production of value-added products such as cleaner fuel and specialized chemicals. However, the functionality of these two areas is dependent on a solid understanding of the coal structure and its reactivity (Catcheside and Ralph, 1999).

Coal biotransformation activities (solubilization, depolymerization, decolorization and liquefaction) were clarified at the Bioconversion Session of the 9th International Conference on Coal Science in 1997 (Klein *et al.*, 1999). According to Klein *et al.* (1999) these activities describe totally different phenomena and should therefore be clearly distinguished. Liquefaction was defined as the mere conversion of coal into another physical state (solid to liquid) without process implications, while solubilization was defined as the dissolution of all or part of the coal molecule by alkali or organic solvents. Depolymerization was defined as the catabolic reduction of higher molecular weight compounds into smaller components, which could be coupled to the loss of chromophore, whereas, decolorization is the loss of chromophore without any change in relative molecular weight. In this review, the terms biotransformation or bioconversion and conversion will be used to refer to any coal modifications brought about by microbial activity (solubilization, depolymerization and degradation) and chemical treatment respectively.

1.3.1 Microbial conversion of coal

Studies into the biological action of fungi and bacteria on coal started as early as 1920s but became constrained by the limitations of coal structure and physiology (Olson and Brinckman, 1986). Since then small but significant strides have been made in the last 3 decades (Table 1-4) since renewed efforts to characterize and optimize coal conversion followed the first reported (Fakoussa, 1981) use of coal as the sole carbon and energy source in a microbial study (Table 1-4). Fakoussa (1981) demonstrated the ability of certain bacterial strains to partially utilize the organic fraction of HC as the sole source of carbon and energy, with observations of brown colored supernatants (Fakoussa and Hofrichter, 1999). This was closely followed by Cohen and Gabriele (1982), who reported the complete dissolution of a highly oxidized lignite (leonardite) into a black liquid by wood decaying fungi *Trametes versicolor* and *Poria monticola*. This black liquid was not observed in the controls that had no fungi or coal.

This instigated intensified studies by various research groups with the aim of establishing a better understanding of the mechanisms underpinning the biological transformation of coal coupled to production of value-added products (Fakoussa and Hofrichter, 1999). In recent years, most of the studies have focused on the ability of different microbial cultures to modify and/or degrade LRC (Faison and Lewis, 1990; Catcheside and Ralph, 1999; Hőlker *et al.*, 2002). The geological conditions that occur during LRC formation result in the development of heterogeneous polyaromatic and polycyclic complexes linked together by ether linkages and methoxy groups, which are more amenable to microbial action than higher-ranking HCs (Narayan and Ho, 1988; Gokcay *et al.*, 2001). It is therefore not surprising that lignin degrading micro-organisms and enzymes were investigated first in microbial coal conversion studies. As a result filamentous fungi have been found to be capable of transforming coal due to their ability to secrete extracellular non-specific enzyme systems. Some filamentous bacteria, including members of the actinomycetes and occasionally eubacteria, have been implicated in coal bioconversion (Catcheside and Ralph, 1999). At the same time, little or no success has been made in the degradation of higher rank coals, although modification of HC has generally only been achieved after pre-treatment (Laborda *et al.*, 1999).

Table 1-4 Advances in coal bioconversion. Adapted from Horichter and Fakoussa, (2002).

Year	Step of Progress	Reference
1927	Ability of micro-organisms to grow on LRC and modify its physico-chemical properties	(Fischer and Fuchs, 1927a; Fischer and Fuchs, 1927b)
1981	Effects on hard coals by *Pseudomonas* strains, simultaneous biotenside-excretion	(Fakoussa, 1981)
1982	Solubilization of lignite to droplets on agar plates by fungal action	(Cohen and Gabriele, 1982)
1986f	Acceleration of solubilization by pre-treatment of coal	(Scott, 1986; Grethlein, 1990) and others
1987f	First solubilization mechanism elucidated: production of alkaline substances (fungi + bacteria)	(Quigley *et al.*, 1988; Quigley *et al.*, 1989; Quigley *et al.*, 1991)
1988f	Second mechanism elucidated: production of cheaters (fungi)	(Quigley *et al.*, 1988; Quigley *et al.*, 1989; Cohen *et al.*, 1990; Quigley *et al.*, 1991)
1989	First product on market: Solubilized lignite as fertilizer	(Arctech Inc., 2007)
1991f	Evidence that chelators alone are not responsible for all effects	(Fakoussa, 1994)
1994	Decolorization and reduction of molecular weight of soluble lignite-derived humic acids proves catalytic enzymatic attack	(Ralph and Catcheside, 1994 a; Hofrichter and Fritsche, 1997b a)
1994	Analysis of low-molecular mass products from biosolubilized coal	(Toth-Allen *et al.*, 1994)
1991	Improved analysis by 13C-solid state-NMR, MW-determination, ultra-filtration, etc.	(Polman and Quigley, 1991; Ralph and Catcheside, 1996b; Henning *et al.*, 1997; Hofrichter and Fritsche, 1997b; Willmann and Fakoussa, 1997a)
1997	In vitro systems shown to degrade humic acids and attack matrix and coal particles	(Hofrichter and Fritsche, 1997a b) Hofrichter and Fritsche (1997b)
1997	First fine chemical produced successfully from heterogeneous humic acid mixtures to polyhydroxyalkanoates (PHA, ``Bioplastic") by pure cultures	(Steinbüchel and Fűchtenbusch, 1997; Fűchtenbusch and Steinbűchel, 1999)
1999	Involvement of laccase in depolymerization of coal implied by conversion of coal humic acid to fulvic acids *in vivo* by *T. versicolor* (basidiomycetous fungi)	(Fakoussa and Frost, 1999)
2001	Microbial solubilization lignites. Preliminary gasification tests with solubilized coal yielding 21% energy recovery from methane	(Gokcay *et al.*, 2001)
2002	9.3% solubilization of lignite by solid state fermentation with *T. atrovide* in a new trickle bed reactor	(Hőlker *et al.*, 2002)

Year	Step of Progress	Reference
2006	Mechanisms of coal solubilization in *P. decumbens* P6 combination of production of alkaline materials, peroxidase and esterase. First report on involvement of biosurfactant in coal solubilization by fungi	(Yuan *et al.*, 2006)
2007	Degradation of LRC by *T. atrovide* (ES 11)	(Silva-Stenico *et al.*, 2007)
2007	Phytoremediation of coal mine spoil dump through integrated biotechnological approach	(Juwarkar and Jambhulkar, 2008)
2008	The effect of the particulate phase on coal biosolubilization mediated by *T. atrovide* in a slurry bioreactor	(Oboirien *et al.*, 2008)
2008	Fungal biodegradation of hard coal by a newly reported isolate, *Neosartorya fischeri.*	(Igbinigie *et al.*, 2008)

f; following years

1.3.2 Coal solubilization

The ability to solubilize coal has been predominantly associated with filamentous fungi although some filamentous bacteria, members of the actinomycetes and occasionally eubacteria, have been reported (Fakoussa and Hofrichter, 1999; Oncu *et al.*, 2007). The mechanism of microbial solubilization is yet to be fully understood. Researchers have postulated that several compounds are produced through microbial action such as oxidative and hydrolytic enzymes, alkaline substances and chelators (Cohen and Gabriele, 1982; Fakoussa, 1988; Quigley *et al.*, 1991; Laborda *et al.*, 1999). It is important to note that microbial solubilization of coal is dependent on the level of coal oxidation, nitrogen content and, in some cases, presence of a co-substrate such as glutamate or gluconate (Holker *et al.*, 1995; Willmann and Fakoussa, 1997b). Coals with higher levels of oxidation such as the Victorian brown coals, like leonardite were found to be more susceptible to biosolubilization by a wide range of fungal species (Catcheside and Mallett, 1991). In their studies Catheside and Mallet (1991) found that the most oxidized coals were located near the surface of deposits at Loy Yang, achieved solubilization rates of up to 70% while the run of mine coals at Morwell were hardly affected by fungi. Only after they were oxidized by treatment with nitric acid did solubilization (~ 90%) occur (Catcheside and Mallett, 1991; Catcheside and Ralph, 1999). According to Catheside and Ralph (1999), two mechanisms have been established for coal solubilization. The first mechanism proposed by Quigley and co-workers (1989), suggested that the generation of alkaline metabolic products by a diversified range of fungi growing on an enriched medium, led to ionization of the acidic groups on LRC, thereby releasing water-soluble humic components. The second mechanism was presented by Cohen and Gabriele (1982) who reported that the coal solubilizing ability of *Corelus versicolor* was due to the production of chelating agents such as oxalates, that sequestered polyvalent metal ions such as Fe^{3+}, Mg^{2+}, and Ca^{2+} which are inherent in the coal and form part of the ionic linkages in water soluble humates. The removal of the metal ions is thought to have a dual function in increasing solubility of the coal humates; by disruption of the ionic linkages that form the structural linkages and increasing the number of free acidic groups (Cohen *et al.*, 1990; Ralph and Catcheside, 1996b; Ralph and Catcheside, 1996b; Ralph *et al.*, 1996; Catcheside and Ralph, 1999).

In both mechanisms, fungal coal solubilization will only occur in the presence of an enriched nutrient medium containing high N concentrations in the form of glutamate or ammonia (Holker *et al.*, 1995; Hofrichter *et al.*, 1997a). While the presence of an additional C source is important for the growth of fungi, it was shown to cause inhibition of coal solubilization in studies by Hőlker *et al.* (1995; 1997). On the other hand, Blondeau (1995) reported enhanced decolorization of humic acids by 15 strains of *Streptomyces* grown in the presence of an enrichment medium with glucose. In spite of this, LRC can be solubilized by selected fungi and bacteria when grown on a mineral medium but, the solubilization of HC will still require the addition of an enriched growth medium. This results in preferred solubilization of lignites over HC as it becomes uneconomical to use expensive enrichment medium containing high concentrations of N and C (Polman *et al.*, 1991; Ralph and Catcheside, 1996a; Catcheside and Ralph, 1999; Igbinigie, 2008).

Coal solubilization can be explained by a descriptive model, in which the solubilization process is effected by Deuteromycetes and during which guttation droplets are formed by the fungus growing in the proximity of the coal particle (Figure 1-3). Solubilization reactions occur in the guttation droplet mediated by alkaline substances present in the fungal growth medium. The reaction takes place because of production of alkaline agents, NH_4^+ or chelating agents such as dicarboxylic acids (Klein *et al.*, 1999; Hőlker *et al.*, 2002). The alkaline substances function to solubilize coal by deprotonation of acidic groups to enhance water solubility, whereas chelating agents serve to remove metals from the structure that serve to aggregate molecules together as a complex.

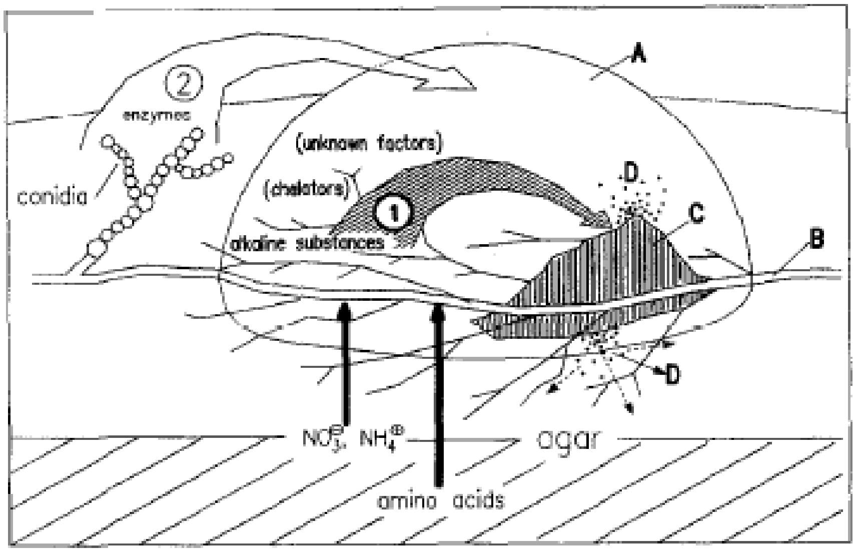


Figure 1-3 Proposed mechanism for the solubilization of low rank coal by Deuteromycetous fungi (moulds). 1 – Formation of guttation droplets. 2 – Secondary non-lignolytic, radical generating oxidases and peroxidases, which are preferentially formed during melanin synthesis, can be secreted into the droplets of solubilized coal and lead to re-polymerization reactions (additionally enhanced by the presence of certain metal ions such as Fe^{3+}, Mn^{2+}, Cu^{2+}). A – Guttation droplet, B – fungal hyphae, C – coal particle, D – solubilized coal in the form a black liquid. Adapted from Hofrichter *et al.* (1997b) .

1.3.3 Coal depolymerization

According to Hofrichter *et al.* (1999), the coal depolymerization reaction is mediated by the activity of manganese peroxidase acting in conjunction with a number of supporting factors (Figure 1-4). The enzyme non- specifically oxidizes a wide range of aromatic and aliphatic compounds via chelated Mn^{3+} (a strong oxidant which carries single electron and H^+ abstractions). The radical groups formed from the one-step electron oxidation process undergo various non-enzymatic reactions leading to cleavage of the covalent bonds and fission of aromatic rings. The presence of unsaturated fatty acids and thiols enhances the oxidative effect of Mn^{3+}, which is transformed into aggressive radicals that can oxidize structures not normally oxidized by the manganese peroxidase/Mn^{3+} complex. Manganese peroxidase attack results in the depolymerization of high molecular weight fractions i.e. humic acids and coal matrix to lower molecular mass fulvic acids. Other lignin-modifying enzymes such as laccase and lignin peroxidase may act synergistically with Mn^{3+}. These enzymes are only expressed in conditions with low nitrogen and pH in the range pH – 6 (Catcheside and Ralph, 1999).

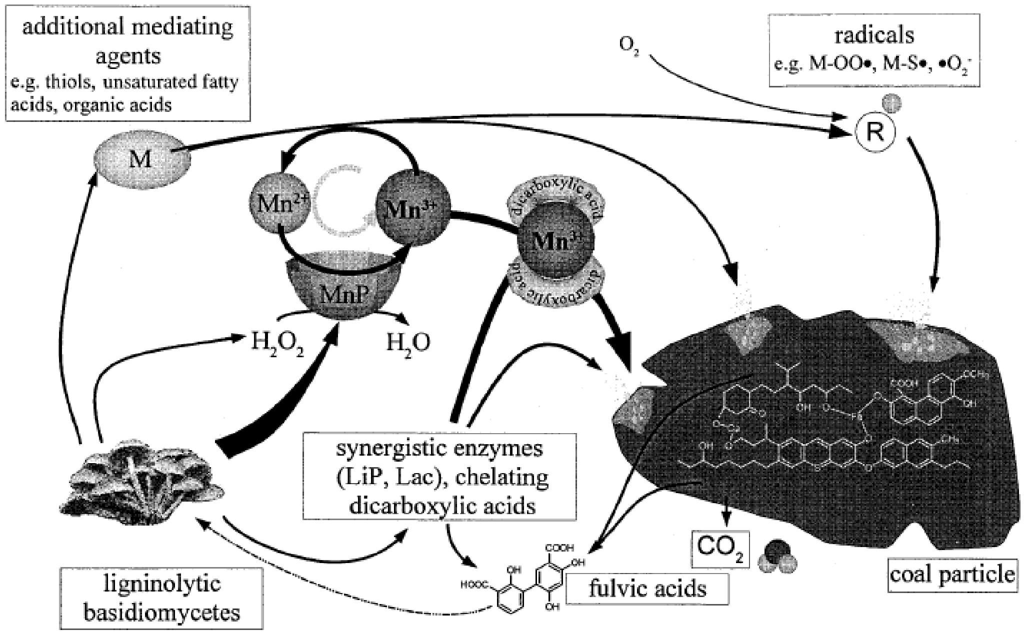


Figure 1-4 Proposed scheme for the degradation of low-rank coal (lignite) by ligninolytic basidiomycetes; MnP-manganese peroxidase, LiP-lignin. Adapted from Hofrichter *et al.* (1999).

The aerobic solubilization and depolymerization by white rot fungi results in the production of water-soluble humates, which can be regarded as a cleaner form of coal when compared to the original coal. These breakdown products can be used as carbon sources for micro-organisms including synthesis of specialized chemicals such as polyesters of hydroxyalkanoic acids, which were derived from the growth of bacteria on solubilized LRC (Fϋchtenbusch and Steinbϋchel, 1999). This study also investigated the use of solubilized LRC humates as carbon sources for energy generation and mine waste treatment using anaerobic pathways.

1.3.4 Production of value-added compounds

Several researchers have reported the appearance of ‘black viscous liquid substances as breakdown products of microbial coal degradation and/ or modification (Cohen and Gabriele, 1982; Hӧlker *et al.*, 1997; Catcheside and Ralph, 1999). To a large extent, this has influenced coal bioconversion research with the aim of recovering intermediate products such as humic acids, volatile fatty acids and low molecular weight specialized compounds such as methanol (Catcheside and Mallett, 1991; Toth-Allen *et al.*, 1994; Fakoussa and Hofrichter, 1999; Ortega and Fernández, 2007; Silva-Stenico *et al.*, 2007). Of particular interest, is the generation of Humic substances (HS) from coal, because they constitute a major component of the coal macro-structure and secondly they play an important role in agriculture. Humic substances can best be described as refractory dark colored heterogeneous, polyelectrolytic compounds of high molecular weight (McDonald *et al.*, 2004). Research into non-fuel usage of coal for the production of low molecular weight compounds has been receiving greater attention in recent years (Avena *et al.*, 1999; Janoš and Tokarová, 2002; Skybová *et al.*, 2007; Giannouli *et al.*, 2009). Traditionally, LRC have been preferred over high rank coals for the production of HS as they account for ~ 70% (wt) of the organic content in LRC (Ibarra and Miranda, 1996; Chassapis and Roulia, 2008; Giannouli *et al.*, 2009). According to Peuravuori *et al*. (2006), lignites contain the highest geologically oxidized HA among the coal ranks. This has since led to the development of several processes to synthetically oxidize other coals such as sub-bituminous and bituminous with the aim of increasing the content of oxidized functional groups, thereby making them amenable to microbial transformation (Peuravuori *et al.*, 2006; Skybová *et al.*, 2007). Aerobic studies by Dong *et al.* (2006) on Chinese lignite using a *Penicillium* strain demonstrated increased concentration of HA and water-soluble HS in the lignite extract after microbial transformation. Similar studies by Yuan *et al* (2006) also showed that the microbial transformation of coal generated HA from LRC. According to a report by Ziegler and van Heek (1998), at the 9th International Conference on Coal Science there was a general consensus that work into coal characterization and general fundamental science may have reached a plateau, although there was need to interrogate future and unexpected constraints through collaborative efforts with national governments (Ziegler and Van Heek, 1998). Research over the last 2 decades has in effect, provided platforms for better understanding of coal bioconversion (Table 1-4), but with little or no progress in the bioprocessing application field over the same period (Klein *et al.*, 1999).

1.3.5 Bioprocess development constraints and solutions?

Fakoussa and Hofrichter (1999) and Klein *et al*. (1999) have noted that the primary impediment to coal bioprocessing is the coal itself. Ziegler and Van Heek (1998) stated that “the application of biotechnology for coal conversion can only be expected in the far future’’ but acknowledged the large-scale conversion of lignite for electricity generation concept by Reich-Walber *et al.* (1997). In addition Klein *et al.* (1999) reported that only a few biological systems for coal processing have been applied economically and reiterated the same constraints highlighted by Fakoussa (1988) summarized in Table 1-5. Coal biodesulfurization is an example of a technically feasible coal bioprocess with inorganic sulfur removal rates of up to 90% being reported

(Kilbane, 1989; Gonsalvesh *et al.*, 2008). However, the removal of organically bound sulfur has not been convincingly demonstrated, and furthermore the economics of the technology are not yet favorable, due to the elevated energy demands of the reactor systems (Klein *et al.*, 1999).

Table 1-5 Problems associated with the process upscale of the coal bioconversion operation. Adapted from Ziegler and van Heek, (1998) and Fakoussa and Hofrichter, (1999)

Impediment	
Coal property	
• Heterogeneous complex structure of coal	• Most enzymes systems are highly substrate specific.
• Contains recalcitrant compounds such as polycyclic aromatic hydrocarbons	• Lack of adapted microbial cultures, enzyme systems or transport shuttles
• Hydrophobicity	• Most biochemical reactions require aqueous conditions
• Particle size or surface area	• High molecular mass requires extracellular mechanism
• Porosity	• Poor adsorption of cells and /or enzymes on coal surface
• Stoichiometric ratio o High C:N o Low O:C	• Does not support microbial growth, co-substrate required to adjust ratio
Analytical constraints	
• Complex coal chemistry	• Lack of suitable analytical methods • Heterogenous mixture with several unknown compounds • Insufficient quantities of sample material for accurate analysis of reactions
Technical process constraints	
• Scale-up	• minimal amounts of products generated

The successful implementation of coal bioprocess application hinges on technical (substrate, reaction dynamics and equilibrium) and economic feasibilities (Klein *et al.*, 1999). Fundamental constraints required to demonstrate economic viability include:

- Highly efficient biocatalysts capable of generating higher reaction rates and yields;
- The establishment of an adaptive reaction environment;
- Effective bioreactor configurations that can withstand continuous operation and enhanced mass transfer in an environment compatible with coal bioprocessing;
- Technological models for downstream processing that are compatible with the unique products derived from coal bioprocessing (Klein *et al.*, 1999).

Low stoichiometric ratios of (C, H, O and N) in coal, more so in HC than the LRC, and complexity of the aromatic substrate to be degraded are the two major obstacles that have been reported as constraints in the development of coal biotechnology. Several researchers have tried to address this problem by introducing enriched medium and co-substrates, which, despite having conflicting results in terms of enhancing or inhibiting coal conversion, has not proved economically feasible for large-scale operation (Blondeau, 1995; Holker *et al.*, 1995).

The current status of coal bioprocessing is such that several micro-organisms (fungi and bacteria) are capable of solubilizing LRC and HC depending on coal structure and oxygenation state. Coal scientists have a basic understanding of the mechanisms of bioconversion of coal, which are complex and consist of a synergistic interaction between enzymes and chelators that facilitate cleavage of bonds within the coal structure. Finally, there are some preliminary reports on the nature of the compounds produced from coal bioconversion (Ralph and Catcheside, 1997; Klein *et al.*, 1999; vanHeek, 2000; Oboirien *et al.*, 2008).

Given low reaction rates and volume through-put in biologically based processes, the use of physico-chemical pre-treatment methods on the coal substrate to enhance the availability of reactive functional groups has been a strategy that has been pursued to improve bioprocess feasibility (Jain *et al.*, 1991; Wadhwa and Sharma, 1998; Catcheside and Ralph, 1999; Klein *et al.*, 1999). To date, attempts to improve the reactivity of coal prior to microbial conversion have focused mainly on pre-treatments using oxidizing chemicals such as HNO_3 in lab-scale studies (Achi, 1994; Fakoussa, 1994; Alvarez *et al.*, 2003; Başaran *et al.*, 2003). Work by Machnikowska *et al.* (2002) reported enhanced biosolubilization rates of 90% and 40% in lignites and sub-bituminous coals, respectively, after pre-treatment with HNO_3. Unfortunately, these have not been proved feasible to scale-up to industrially competitive processes. To the best knowledge of this author, large-scale biological pre-oxidation of coal has not been previously reported as an alternative pretreatment strategy to enhance subsequent coal bioconversion reactions.

In previous studies at the Institute for environmental biotechnology Rhodes university, (EBRU), Igbinigie (2008) and Mukasa-Mugerwa (2008) proposed and developed a novel systematic bio-weathering process aimed at oxidizing and/or degrading HC into a substrate they termed Fungcoal that can then be used in a series of downstream coal bioprocess applications. In this approach, a low-cost bulk-volume biologically catalyzed oxidation of the coal substrate was targeted, in which the O:C ratio was increasing thereby enhancing reactivity for biotechnological processing. In further development of this concept it is important to understand the details of the processes leading to coal weathering.

1.4 Coal Weathering

The process of coal weathering can be defined as the progressive degradation of coal involving complex, simultaneous, interlinked chemical processes accompanied by meteorological factors (Cimadevilla *et al.*, 2005; Wagner, 2007). The weathering of coal affects its chemical and structural properties and consequently has an impact on its technological applications such as combustion, coking, hydrogenation and pyrolysis (Ibarra and Miranda, 1996; Waanders *et al.*, 2003; MacPhee *et al.*, 2004). In addition, coal weathering affects the commercial value of coal in several ways:

- Bioreactivity – in terms of combustion properties, many authors have concluded weathered coal becomes more reactive than fresh coals (Pisupati and Scaroni, 1993; Lo and Cardott, 1995; Waanders *et al.*, 2003; Wagner, 2007), while others have indicated that weathering can enhance reactivity through the formation of oxygenated functional groups (Iglesias *et al.*, 1998). It is important to note that coals with similar properties can combust differently due to petrographic variations, degree and type of oxidation, and the combustion environment (Waanders *et al.*, 2003; Wagner, 2008).
- Increase in caking properties or loss of swelling properties – which affects the classification of mined coal. For example a fresh coal can be commercially classified as a metallurgical coal, but after the weathering process, the same coal may be graded as a thermal grade coal (Wagner, 2007).
- Weathering increases friability of the coal thereby facilitating the production of fines. In addition, high moisture content after weathering makes it difficult to handle, process and store the coal.
- According to MacPhee *et al.* (2004), weathering diminishes the rheological properties of coal.

Exposure of coal to environmental conditions will initiate the weathering process. The oxidation of coal occurs before mining when the coal is still buried beneath the surface, during mining and post mining activities. The impact of this process in the alteration of the structural and chemical properties has been the impetus of most research in coal weathering and oxidation (MacPhee *et al.*, 2004).

Weathering of coal can be classified into four main categories: natural weathering due to uncontrolled environmental factors; chemical weathering which involves aerial oxidation of organic and mineral matter; simulated or artificial weathering under carefully controlled low and moderate temperature oxidation reactions; and biological weathering due to the interaction of plants and microbiological oxidation of organic and inorganic components of the coal (Seoane and Leirós, 2001; Casal *et al.*, 2003; Cimadevilla *et al.*, 2005; Igbinigie *et al.*, 2008; Mukasa-Mugerwa, 2008; Ruiz *et al.*, 2009).

Several methods have been used to monitor weathering and oxidation of coals ranging from highly complex and advanced techniques such as X-ray photoelectron spectroscopy to rudimentary elemental analysis (Wagner, 2007; Geng *et al.*, 2009). The sensitivity levels of these techniques are dependent on the degree of oxidation in the coal. The Gieseler fluidity analysis (GFA, Gieseler Plastometer) used in the characterization of coal by Lin *et al.* (1983) and later by Huffman *et al.* (1985) was proposed to be the most sensitive technique for detecting preliminary oxidation in coal (Wagner et al 2007). The dilometric analysis and Mossbauer spectroscopy were similarly sensitive to monitoring early oxidation while the Free swelling index (FSI) was the least sensitive (Huffman *et al.*, 1985; Wagner, 2007).

Widely used techniques, such as Fourier transform infrared spectroscopy (FTIR), flotation analysis, and coke abrasion, were found to be insensitive to early oxidation of coal but highly responsive in the tracking of oxygen functionalities and aliphatic alterations during the later stages of coal weathering. More detailed techniques used to investigate the reactions of functional groups in weathered and oxidized coals include Solid-state nuclear magnetic resonance (SS-NMR) and X-ray photoelectron spectroscopy (XPS) (Geng *et al.*, 2009). XPS studies by Kelemen and Freund (1990) on bituminous coal exposed to low temperature oxidation showed that carbonyl groups were formed during the preliminary stages of oxidation, while the carboxyl groups were only formed at higher temperature in the later stages of oxidation. In their studies on coal oxidation kinetics, the authors indicated that the oxidation of LRC resulted in increased levels of carboxyl groups and a decrease in the hydroxyl groups, while the levels of carboxyl, carbonyl and other reactive groups increase upon oxidation of higher rank coals, including bituminous coal (Kelemen and Freund, 1989; Kelemen and Freund, 1990; Kelemen and Kwiatek, 1995; Geng *et al.*, 2009).

1.4.1 Natural weathering

Coal is unstable when exposed to atmospheric moisture and oxygen (Wagner, 2007). The natural weathering of coal occurs *in-situ* whilst it is still buried beneath the surface and the induced or secondary weathering usually follows extraction, preparation, processing and storage (Cimadevilla *et al.*, 2005; Wagner, 2008). According to Ibarra and Miranda (1996), the coals undergo exothermic oxidative reactions that emit large amounts of energy, which could trigger spontaneous combustion. This often results in decline of the value of the coal for technological applications.

The chemistry of natural weathering and oxidation is poorly understood mainly because of the high number of uncontrolled environmental variables that include temperature, moisture, pile porosity, oxygen ingress, photochemical reactions, and local self-ignition processes. Nevertheless, it is believed that oxidative weathering of coal occurs in two steps (equation 1): (i) the initial phase involves ingress and assimilation of oxygen by the coal to form reactive products, and (ii) the decay of the prolonged intermediates accompanied by formation and release of CO_2 (Chang and Berner, 1999).

$$C_{coal} + O_2 \xrightarrow{(i)} \text{Intermediates} \xrightarrow{(ii)} CO_2 \quad (1)$$

The nature of the intermediate products formed during natural coal weathering is dependent on the coal rank. Oxidative weathering of low rank lignites yields HS. Weathered lignites, often referred to as leonardite, have 85% alkaline extractable organic matter (Chang and Berner, 1999). To date, there have been no field observations regarding extensive HS formation via oxidative weathering of higher rank coals such as bituminous coal. On the other hand, chemical oxidation of higher rank coals under laboratory-controlled conditions has been known to yield HS. It therefore becomes important to ascertain the possibility of producing HS from high rank coals via oxidative weathering considering the relative abundance of this sedimentary organic matter exposed to the earth's surficial environment (Gokcay *et al.*, 2001; Chang *et al.*, 2003; Demirbas, 2007; Giannouli *et al.*, 2009).

1.4.2 Simulated weathering

Most studies on coal weathering have attempted to simulate the natural weathering process under controlled conditions in order to gain a fundamental understanding on the mechanisms involved during coal weathering and oxidation. Knowledge acquired to date using simulated reactions points to the existence of a link between natural weathering and artificial weathering processes, although, the processes have distinct characteristics in their mechanism (Casal *et al.*, 2003). It becomes imperative to highlight the level of complexity in simulating a process that has many unknown variables.

Casal and co-workers (2003) reported that experimental conditions play a crucial role in the course of oxidation reactions. For example, in simulated weathering, reaction rates are accelerated under well-controlled conditions whereas under natural conditions the degree of environmental variability and meteorological fluctuations determines the rate of the reactions (Casal *et al.*, 2003). Moreover, artificial conditions have been shown to be more severe than natural conditions, favoring reactions that have a higher activation energy. The weathering rate is dependent on temperature, with reactions occurring rapidly at $80^{o}C$, moderately fast at $50^{o}C$ and very slowly at $25^{o}C$ (Wu *et al.*, 1988; Wang *et al.*, 2003; Wagner, 2008). Most reported coal weathering simulation studies have been conducted at temperatures greater than $100^{o}C$ to increase oxidation rates, however, the mechanism and chemical nature of coal behave differently at temperatures above and below $80^{o}C$ (Wu *et al.*, 1988; Wang *et al.*, 2003).

In spite of these arguments, comparative FTIR studies on natural and artificial oxidation have provided fundamental qualitative and semi-quantitative analysis of the two mechanisms. The loss of swelling properties in coal treated to simulated oxidation reactions involve the formation of carbonyl groups with varying functionalities such as ketones and carboxylic acids, and decrease in aliphatic C-H bonds (Liotta *et al.*, 1983; Casal *et al.*, 2003). However, some authors have reported an absence of carbonyl group formation during simulated oxidation; instead, they attribute loss of swelling properties to formation of ether cross-links and the decrease in methylene groups (Casal *et al.*, 2003).

1.4.3 Chemical weathering

The treatment of coal for improved processing and beneficiation have been the main drivers of research into the chemical weathering of coal. The removal of organic and inorganic sulfur from coal prior to downstream use (combustion, syngas synthesis) has been a main target in coal beneficiation. Previous studies that have focused on chemical desulfurization of coal have not been effective (Tripathy *et al.*, 1998). Problems cited include high chemical input, intensive energy requirements, and disintegration of the coal into slurries (Tripathy *et al.*, 1998). Severe chemical treatment during clean up processes, has been reported to cause reduction in the combustible volatile content of the coal (Chang and Berner, 1999; Geng *et al.*, 2009; Levandowski and Kalkreuth, 2009). Tripathy *et al*. (1998) reported that the use of alkaline preconditioning of pyrite and arsenopyritic refractory sulfidic minerals was very effective for subsequent bioleaching with bacteria. Combining chemical and biological processes for coal beneficiation seems to offer efficient removal of the impurities without adversely compromising the value of the coal.

Coal pre-treatment for further transformation, can be either physico-chemical or biological. This involves introduction of important modifications to the chemical and structural composition of the coal. Widely used chemical pre-treatment techniques such as reduction with metallic potassium (K-THF-isopropanol), reductive methylation (K-THF-CH_3I), oxidation, decarboxylation, reduction with $LiAlH_4$, *O*-methylation and methanol-NaOH solubilization have been used to provide a fundamental understanding of the chemistry and relationship between coal structure and reactivity (Boudou *et al.*, 1995; Tripathy *et al.*, 1998; Ruiz *et al.*, 2006). Chemical weathering paves the way for biogenic processing by availing reactive functional groups for microbial attack.

1.4.4 Biological weathering

As mentioned earlier, the complex and heterogeneous organic matter of coal is rather resistant to microbial degradation. However, many of the inorganic and organic components in themselves intrinsic to the coal macro-structure are amenable to biotransformation thereby presenting opportunities for microbial bioconversion of coal for clean energy production or bioconversion. In this regard, investigations into biological weathering of coal have been focused on the removal and/or modification of the inorganic components of coal such as pyrite and other sulfide minerals (Klein, 1998; Chang and Berner, 1999; Benner *et al.*, 2000; Malik *et al.*, 2001; Van Dyk *et al.*, 2009).

Biodesulfurization involving atmospheric O_2 and bacteria is one of the most important weathering processes that results in the oxidation of sulfide minerals. High pyrite removal efficiencies and low waste generation have been reported from the microbial desulfurization studies (Olson and Brinckman, 1986; Klein *et al.*, 1999). Two mechanisms have been proposed for biological oxidation of pyrite from LRC.

The first mechanism is a direct approach, in which the bacteria, typically *Thiobacillus ferroxidans,* attach to the sulfur crystal surface and oxidation occurs in the thin film located in the interspaces between the bacterial outer wall and sulfide surface (McIlwain and Dugan, 1990; Klein *et al.*, 1999; Johnson and Hallberg, 2005).

The second mechanism is referred to as the indirect oxidation, the pyrite is oxidized by exposure to atmospheric air and water to form acid and ferrous ion. The bacteria oxidize the ferrous ion at low pH as a source of metabolic energy (equation 2).

$4FeS_2 + 15\ O_2 + 2H_2O \rightarrow 2Fe_2\ (SO_4)_3 + 2H_2SO_4$ (2)

The bacteria are thought to accelerate the process of pyrite dissolution, (which is normally the rate-limiting step) by catalyzing aerobic oxidation of the ferrous ion in solution to its ferric state. The ferric ion in solution then oxidizes pyrite to the ferrous state resulting in generation of acidity by release of protons (Benner *et al.,* 2000; Blodau, 2006).

Although fundamental data on the biodesulfurization of intrinsic inorganic components of coal have been elucidated for coal beneficiation processes, there has been little to no progress in the biological weathering of the organic coal matrix. Over the past years, the increasing awareness of the environmental impact of coal derived energy has instigated research at EBRU into exploring the mechanisms underpinning biological weathering of organic fraction of the coal.

1.4.5 A case study of biological enhanced weathering

Ongoing research at EBRU, has led to the development of biotechnologically enhanced processes for the beneficiation of waste coal, and rehabilitation and restoration of affected mining environments. Site visits to the Anglo mine dumps in Mpumalanga, South Africa, led to the observations of grass (*Cynodon dactylon*) growing on HC dumps (Figure 1-5). Further investigations into the rhizosphere of the system revealed apparent break down of the HC into soil-like humic material and extensive root establishment in the top 120 cm of the coal dump

(Figure 1-6). This led to a two pronged investigation into the biotransformation of coal, which involved the mycorrhizal component (Mukasa-Mugerwa, 2008) and the non-mycorrhizal rhizosphere component (Igbinigie, 2008 a), with an overall objective of extending these observations into bioprocess development.

Mukasa-Mugerwa (2008) simulated the *C. dactylon*-coal dump system in pot and column trials and demonstrated the role of arbuscular mycorrhizal fungi in the biodegradation of coal. He confirmed the colonization of the *C. dactylon* root system with fungal mycorrhizal species: *Glomus clarum*, *Paraglomus occultum*, *Gigaspora gigantea* and *Glomus mosseae* in his simulation of the simulation of the *C. dactylon*-coal dump system in pot and column trials.

Molecular characterization of the non-mycorrhizal rhizosphere confirmed the presence of potential coal-degrading fungal species also reported by Igbinigie (2008). The addition of both mycorrhizal and non-mycorrhizal microbial cultures in the simulated studies was shown to enhance the process of coal biotransformation over a 44-week period.

Figure 1-5 *Cynodon dactylon* growing on hard coal dump at Navigation Colliery (Mpumalanga, South Africa).

Figure 1-6 Cross section of a hard coal discard dump that has been transformed into a soil like material through the interaction of *Cynodon dactylon* and associated fungi at Navigation Colliery dump site (Mpumalanga, South Africa). The field of view is 45 – 50 cm

In the colonization of the *C. dactylon* roots, Mukasa-Mugerwa (2008) also demonstrated that the extraradicular mycorrhizal occurrence was reduced while the intra-radicular colonization was increased when grown on coal inoculated with *Neosartorya fischeri.* The reduction in mycorrhizal activity appeared to suggest the establishment of a mutualistic process between the non-mycorrhizal fungi and the rhizosphere fungi in the biotransformation of coal (Mukasa-Mugerwa, 2008). Chemical analysis of the degraded HC further confirmed the existence of a mutualistic system of mycorrhizal and non-mycorrhizal rhizospheric fungi. An increase in extractable HA was observed in the dual fungal system and was accompanied by a decrease in the Low molecular weight organic compounds (LMOs), which was absent without the non-mycorrhizal fungi. The study also noted that the HA was bound to the non-mycorrhizal fungi in the dual system but freely available in the single system. These results were comparable with the results from submerged liquid culture studies by Igbinigie (2008), who went on to show the bioconversion of HC into a soil-like humic material (Fungcoal Product), comparable to the observations on the coal dump. Confirmatory Scanning electron microscopy (SEM) studies showed the fungal weathering of the coal by hyphal penetration and fracturing along the penetration zones.

With a standardized 5-step screening protocol (Igbinigie, 2008), 109 non-mycorrhizal micro-organisms were shown to exhibit some coal degrading abilities from 2000 samples collected at various sites on Anglo Coal mines in Mpumalanga, South Africa. Igbinigie (2008) was the first to report the occurrence of the fungus *N. fischeri* as a good performer in coal degradation comparable to *Phanaerochaete chrysosporium* and *Trametes versicolor* (Silva-Stenico *et al.*, 2007; Gonsalvesh *et al.*, 2008). The mechanism of the biological weathering of HC was demonstrated through FTIR and Pyrolysis gas chromatography mass spectroscopy (Py-GCMS) analysis which showed that biotransformation of coal involved oxidation and nitration of the coal (Igbinigie, 2008).

The role of *C. dactylon* in coal weathering and modification was demonstrated by replacing the plant with a synthetic feed composed of LMOs in perfusion fixed-bed column bioreactor studies packed with HC. Results obtained showed the appearance and disappearance of pyrolysates, which suggested the release, and consumption of coal breakdown products in the micro-environment. However, the extent of coal breakdown was not comparable with the column studies by Mukasa-Mugerwa (2008) suggesting a lack of particular exudates that were supplied by the plant but were absent in the synthetic feed.

The results obtained for both the mycorrhizal and non-mycorrhizal components of the *C. dactylon*/coal system provided the basis of a descriptive model accounting for the mechanisms involved in the bioconversion of coal in this system (Figure 1-7). According to Igbinigie (2008) and Mukasa-Mugerwa (2008), the entire process is facilitated and driven by the translocation of photosynthetic organic carbon into the rhizosphere by *C. dactylon*. The translocation is mediated by the associated mycorrhizal fungi, with possible regulation of the exudate release into the rhizosphere playing active or passive roles in the weathering process. It is assumed that the exudate is either in the form of LMOs such as glutamic acid and oxalates, or is converted into intermediates by the microorganisms associated in the rhizosphere, and simultaneously metabolized as a co-substrate for the biotransformation of the coal (Renella *et al.*, 2006; Kuzyakov *et al.*, 2007; Paterson *et al.*, 2007). The degradation of the coal results in the release of essential minerals (such as phosphate) and nutrients for assimilation by the plant. The Fungcoal product generated would then improve the fertility of the 'soil' through enhanced nutrient and water holding capacity (Wan and Liu, 2006; Ortega and Fernández, 2007).

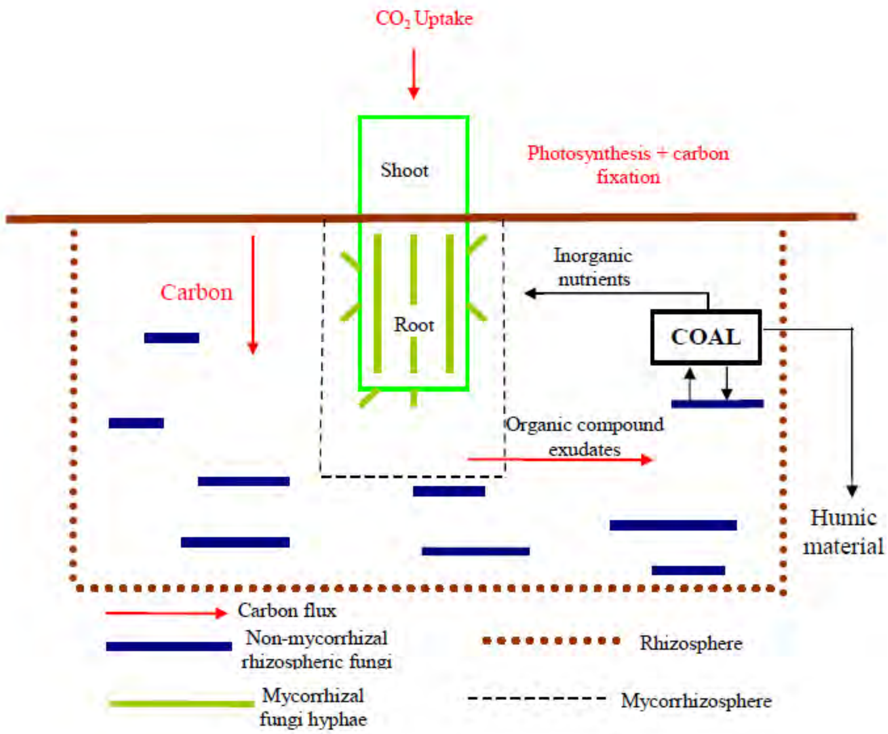


Figure 1-7 Schematic diagram illustrating the possible association between *Cynodon dactylon*, mycorrhizal fungi and rhizospheric non-mycorrhizal fungi that leads to the bioconversion of coal *in situ*. Igbinigie (2008) and Mukasa-Mugerwa (2008) have proposed that photosynthetically produced carbon is translocated into the rhizosphere via the root mycorrhizal system. This provides a co-substrate for coal degradation where non-mycorrhizal rhizosphere microorganisms are responsible for coal breakdown and release of inorganic nutrients utilized in turn by *C. dactylon* to support plant growth. Hard coal becomes oxidized during this process.

1.4.5.1 The stacked heap coal bioreactor

The descriptive model outlined above led to the proposal of a large-scale bioreactor system in the bioconversion of HC known as the Stacked heap coal bioreactor (SHCB) (Rose *et al.*, 2008). The bioprocess involves stacking bituminous HC, and inoculating the surface layers with the mycorrhizal and non-mycorrhizal fungal species (Figure 1-8). The *C. dactylon* is then planted and irrigated to ensure prolific growth. After a period of time, the upper 150 cm layer becomes oxidized and is harvested for downstream applications among which, use as a fertilizer, soil rehabilitation, methane production and AMD remediation have been suggested. Laboratory and field studies were undertaken at EBRU and Klein Kopje Colliery discard coal dump (Mpumalanga, South Africa). It was shown that a conversion of 30 – 40% bituminous coal into HA occurred over 40 weeks (Rose *et al.*, 2007; Mukasa-Mugerwa, 2008).

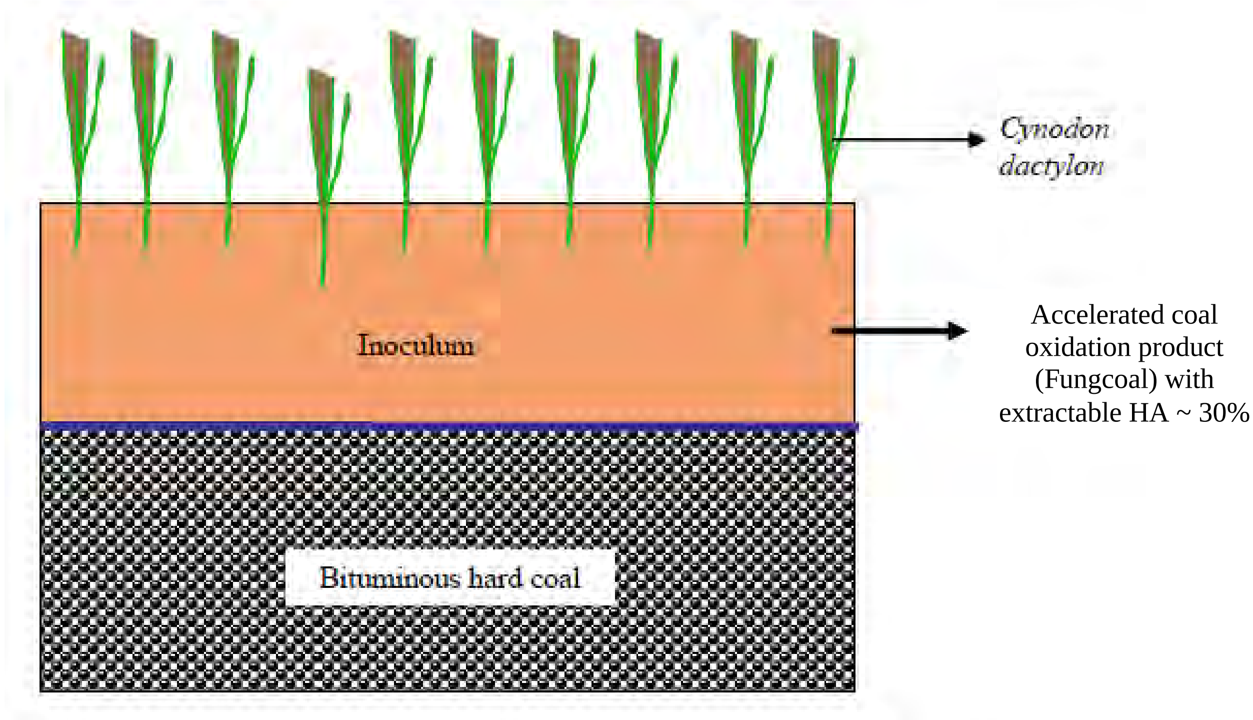


Figure 1-8 Schematic diagram illustrating the Stacked-Heap Coal Bioreactor for the large-scale biotransformation of bituminous hard coal. In this system hard coal is stacked into a heap, inoculated with a mixed rhizosphere population including appropriate mycorrhizal population and then planted with *C. dactylon* and irrigated. Adapted from Igbinigie (2008).

It was shown that the bituminous HC in the SHCB had been partially oxidized and extractable humic acid content elevated from near zero to 30% (w/w).

1.5 Research Objectives

As noted, the increased biological reactivity of coal has been shown to be directly dependent on its oxidation state (Machnikowska *et al.*, 2002). For these reasons, most successful applications in coal bioprocessing have focused on lignites (Catcheside and Mallett, 1991; Ralph *et al.*, 1996; Gokcay *et al.*, 2001; Başaran *et al.*, 2003). A few studies have investigated chemically oxidized HC (Fakoussa, 1988; Hofrichter *et al.*, 1997b). However, to the best of this author's knowledge and in the case of HCs, no studies have reported bioprocess applications using Geologically oxidized coal (GOC) or Biologically oxidized coal (BOC). The weathering processes act to increase the reactivity of the coal by increasing the O:C ratio. Although, this weathering of coal has been demonstrated at lab-scale using chemical oxidants such as HNO_3 (Alvarez *et al.*, 2003;

Du *et al.*, 2008), Igbinigie (2008) and Mukasa-Mugerwa (2008) have shown in principle that accelerated oxidation can be achieved biologically on a large scale using the SHCB system. While work on the further development of the SHCB is ongoing, no investigation has been undertaken, to date, to investigate the application of the accelerated coal oxidation product (Fungcoal) as a substrate in downstream bioprocess development. This study is the first to report an investigation of bioprocess applications of the biologically oxidized coal product. The primary objective of this study was thus to undertake a preliminary investigation of the feasibility of both GOC and BOC use in a number of bioprocess applications that included both anaerobic and aerobic process environments. The following detailed objectives were identified:

- To characterize both GOC and BOC in order to determine and predict the behavior of these substrates under various biotechnological processes;
- To explore recovery of the energy fraction from the oxidized HC substrates;
- To develop and evaluate application of the oxidized HC product and the extractable HA fraction in prioritized coal mining environmental remediation processes including AMD treatment and rehabilitation of compacted mined land;
- To investigate cost effective option for co-substrates required to support biodegradation of the HC substrate.

1.5.1 Research Hypothesis

Oxidized HC, including BOC and GOC, can be used as effective substrates for the development of viable bioprocess applications.

CHAPTER TWO

CHARACTERIZATION OF OXIDIZED HARD COAL SUBSTRATES

2. INTRODUCTION

The geochemical transformations of organic carbon in terrestrial ecosystems is an important starting point for the formation of brown coal and lignites, from which coalification arises (Francioso *et al.*, 2003). HS represent the major organic components of coal and are the biologically refractory degradation compounds of coal transformation (Skybová *et al.*, 2007). The exposure of coal to natural weathering and other oxidative conditions affects the physical and chemical properties of HS and results in an increase in oxygen functionalities concurrent with a decrease in aliphatic and aromatic hydrocarbon content (Ibarra and Miranda, 1996; Peuravuori *et al.*, 2006; Wagner, 2007). The bulk of South African coal reserves are prone to weathering because they are deposited near the surface (World Coal Institute (WCI), 2008), which results in a large amount of coal being dumped as discard. In 2001, the Department of minerals and energy (DME) reported 1,121 Mt of dumped discard and slurry coal (DME, 2001; Wagner, 2007; Van Dyk *et al.*, 2009). South African discard coals are characterized by high ash content (10 – 70%), low calorific value (8.0 – 26.0 $MJkg^{-1}$), low volatile organic matter (8 – 30%) and high sulfur content (0.8 – 8%) (Grobbelaar *et al.*, 1995; DME, 2001; Wagner, 2007).

HS can be partitioned into three fractions based on their solubility in alkali and acid, namely HA, Fulvic acids (FA) and humin (Brigante *et al.*, 2007; Bratskaya *et al.*, 2008). The HA fraction is soluble in alkaline solutions and insoluble in acidic solutions (pH< 2). They are characterized by high molecular weight compounds ranging from 50 – 500 kDa (McDonald *et al.*, 2004). FAs are soluble in aqueous medium in all pH ranges and are characterized by moderate molecular weight compounds ranging from 1 – 5 kDa. Humin is the humic fraction that is not soluble at any pH.

On this basis, many studies have characterized and used HS for interpreting both physiological and biochemical pathways of coalification (Francioso *et al.*, 2003). Furthermore, the mechanism of coal weathering, also referred to as decoalification, has been investigated using alkali-soluble HA as marker compounds. Therefore, the partitioning studies of HS in LRC such as lignites have been mainly driven by a need to understand the geochemical transformation of coal to optimize its commercial utilization (Wagner, 2008; Levandowski and Kalkreuth, 2009). On the other hand, the petrography of coal has not been extensively investigated for bioprocess application mainly due to its complexity and heterogeneity (Fakoussa and Hofrichter, 1999; Hofrichter, 2002). One of the main limitations of coal bioprocessing on an industrial scale has been the inconsistency in coal from different geographic locations, which has prohibited application of innovative processes associated with CCT. Little work has been reported on the characterization of oxidized HC and as far as this author could establish, no reports are available for geologically oxidized HC substrates in bioprocess applications. It was thus necessary to characterize the oxidized HC substrate that was to be used in the various investigations undertaken in this study.

2.1 Objectives

- To determine the concentration of the soluble organic matter in BOC and GOC and potentially available for biodegradation;
- To investigate fractionation of carbon between the aqueous and solid phases of GOC;
- To determine the temporal solubility fractions of both coal substrates;
- To determine the concentration of the insoluble and unavailable carbon in both coal substrates;
- To simulate and predict the amount of SOC, that may serves as a carbon source for biological activity.

2.2 Materials and Methods

2.2.1 Coal sample preparation

All coal samples used in this study were collected from Navigation and Kroomdraai collieries that are located in the same geological area (Mpumalanga, South Africa).

2.2.1.1 Hard coal

The HC used in this experiment was collected from Navigation colliery mine (Mpumalanga, South Africa). The HC was crushed into small fragments using a pestle and mortar, and then sieved through a 250 µm sieve to remove the larger coal fractions, followed by drying in an oven at 50°C for 24 h. the coal was then stored under nitrogen, in a dry place for subsequent use.

2.2.1.2 Biologically oxidized hard coal

The BOC used in this experiment was collected from Navigation Colliery mine, (Mpumalanga, South Africa) where it had been observed that the grass *Cynodon dactylon* was actively growing on the surface of the waste coal mine dumps (Figure 2-1). Further investigations led to the elucidation of soil-like material forming under the roots of the grass and breakdown of the coal in the surrounding environment (Figure 1-7). The BOC is formed as a result of a biologically mediated plant-rhizosphere interaction on coal mine dumps and the resultant soil-like humic material contains a wide range of compounds including humic acids and LMOs that could serve as precursors for metabolic activity (Igbinigie *et al.*, 2008). The BOC was sieved through a 250 µm sieve to remove plants roots and debris, followed by drying in an oven at 50°C for 24 h. the coal was then stored under nitrogen, in a dry place for subsequent use.

Figure 2-1 Formation of biologically oxidized coal from interaction of plants and microbial rhizosphere at Navigation Colliery, Witbank, South Africa. The samples were collected from a 45 – 50 cm cross section of a hard coal discard dump.

2.2.1.3 Geologically oxidized hard coal

The GOC used in this section, and all subsequent studies, was sourced from Kromdraai Colliery, Mpumalanga, South Africa. The coal used was selected by picking the darker humic substance fractions (Figure 2-2) from the lighter clay rich fractions generated from the physical weathering process of coal (Devasahayam, 2007; Wagner, 2008). The GOC was sieved through a 250 µm sieve to remove debris and dried in an oven at 50^{o}C for 24 h. The coal was then stored under nitrogen, in a dry place for subsequent use.

Figure 2-2 Coal face at Kromdraai Colliery Mine showing an oxidized hard coal seam. The darker layers of the seam representing the geologically oxidized coal were sampled for this study.

2.2.2 Macro-elemental analysis of the oxidized hard coal substrate

Two mg samples of both coal substrates in duplicate were prepared for elemental analysis as described in section 2.2.1 and elemental analysis undertaken at the School of Pure and Applied Chemistry, University of KwaZulu-Natal (UKZN), South Africa. A LECO CHNS-932 was used for determination of carbon and sulfur. Oxygen analysis was performed using a VTF-900 furnace attachment. Instrument calibration was carried out using blank samples and Ethylenediamine tetraacetic acid (EDTA), with helium as a carrier gas.

2.2.3 Humic acid characterization

The alkaline extraction of HA has been reported from a wide range of complex macromolecular compounds that include soil, peat, sedimentary rocks, and LRC (Neyroud and Schnitzer, 1975; Avena and Wilkinson, 2002; Adani *et al.*, 2006). The nature of these compounds has resulted in the development of various extraction methods. It was therefore necessary to develop a standard method suited for the alkaline extraction of oxidized HC to be used in this study. Based on the elemental analysis results (section 2.3.1), GOC was the most oxidized of the coal and therefore, it was assumed that this coal would contain sufficient amounts of humic substances for alkaline fractionation studies, in comparison to HC and GOC.

Three extraction methods outlined below were investigated and compared using Analysis of variance (ANOVA). Standard curves were prepared using International humic substances society (IHSS) Leonardite HA standard 1S104H-5 (Appendix 1-A) and HA extracted from Kromdraai GOC in a dilution series and compared (Appendix 1-B).

2.2.3.1 Method 1

The weight percentage composition of the humic component (HA and FA), and humin fraction in GOC was determined by alkaline extraction of the HS in triplicate as show below.

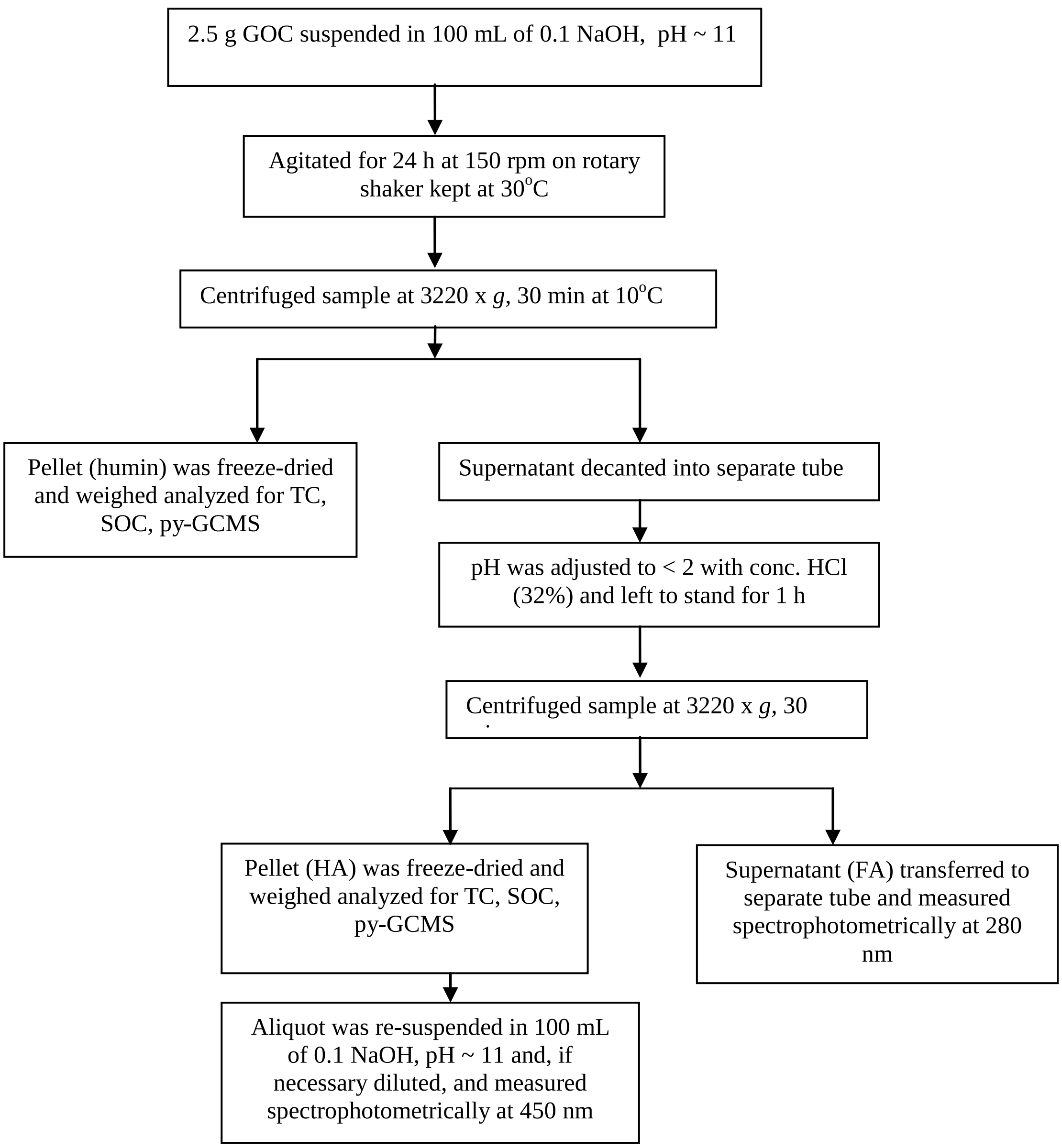


Figure 2-3 Flow diagram of the experimental protocol used in the characterization of biologically oxidized hard coal and geologically oxidized hard coal.

2.2.3.2 Method 2

The GOC sample (2.5g) was repeatedly extracted with (3 x 35 mL) of 0.1 M NaOH and agitated for 24 h. The three aliquots were combined into one sample, which was processed as described in the flow diagram (Figure 2-3) used in method 1.

2.2.3.3 Method 3

GOC (2.5 g) was successively washed (3 times) with 40 mL of Distilled water (dH_2O) to remove non-humic water-soluble substances that interfere with the extraction process. The washed sample was then extracted (3 times) with 35 mL of 0.1 M NaOH and analyzed as described in section 2.2.3.1.

Fractionation of the HC and BOC was then conducted using the most effective and efficient alkaline extraction method. After determination of the organic matter that could be potentially available for biological utilization, detailed investigations into its predicted behavior in a bioprocess system was continued using GOC. The results obtained would be applied to BOC.

2.2.4 Solubility of oxidized hard coal in water

The water solubility studies at neutral pH were undertaken to determine the fraction of unbound organic matter in the GOC. Different concentrations of the oxidized HC (0.2 – 10%, w/v) were suspended in 100 mL of dH_2O. The different coal concentrations in duplicate were agitated for 1 h and 24 h on a rotary shaker (120 rpm, Labcon, 3100u) at 30^oC. The soluble fraction was separated from the pellet by centrifugation at 3220 × g for 15 min at 10^oC (Eppendorf 5810R centrifuge). The supernatant containing the soluble BOC and GOC were analyzed for humic acid and total carbon. The remaining pellet from the respective coal samples were dried (50^oC, 24 h), weighed and analyzed for humic acid and total carbon.

2.2.5 Simulated extraction of oxidized hard coal at different pH values

Fractionation studies were carried out a using 1% (w/v) coal amount because it demonstrated the best operational qualities in comparison to the other concentrations. The lower concentrations yielded products insufficient for analysis while the higher concentrations inhibited efficient harvesting of partition products due to their viscosity. The fractionation experiment was performed by suspending GOC in the respective solutions (Table 2-1) and mixing for 1 h, 24 h, 96 h and 334 h.

The choice of buffer in this experiment was critical. Initial trials were undertaken with Tris-HCl buffer, but it was found to be highly unstable and introduced carbon, which contributed to the overall carbon mass balance. A potassium phosphate buffer (~0.1M, Merck, South Africa) was prepared at different pH ranges (Table 2-1) based on the optimum working range of aerobic and anaerobic micro-organisms (Brigante *et al.*, 2007; Liu *et al.*, 2008). The flasks were agitated and treated in the same manner as described in section 2.2.4.

Table 2-1 Experimental set up of flasks used in the partitioning studies

Flask	**1**	**2**	**3**	**4**	**5**
dH_2O (mL)	100	100	100	100	100
GOC-1h (% w/v)	1.0	1.0	1.0	1.0	1.0
GOC-24h(% w/v)	1.0	1.0	1.0	1.0	1.0
GOC-334h(% w/v)	1.0	1.0	1.0	1.0	1.0
NaOH (1M,pH)	-	7.5			
Potassium Phosphate Buffer	-	-	7.5	8.0	8.4

2.2.6 Analysis

2.2.6.1 pH analysis

The pH of the experimental flasks was measured using a pH 330 meter (model WTW 82362, Germany).

2.2.6.2 Determination of volatile organic matter

Pre-dried coal samples were weighed into crucibles and the Volatile organic matter (VOM) burnt off in a blast furnace set at 900^oC for 6 h. The crucibles were cooled in a desiccator, weighed and the amount of VOM was calculated.

2.2.6.3 Soluble organic carbon analysis-liquid extract

The Soluble organic carbon (SOC) was determined by measuring the Total organic carbon (TOC) of the liquid extract using the Apollo 9000 TOC analyzer. Samples with concentrations higher than 400 ppm were diluted with Milli-pore dH_2O before analysis. The samples were analyzed immediately after sampling. TOC analysis involved acidification of the sample with phosphoric acid to remove all the inorganic carbon using air (Oxygen – 19-22%, Nitrogen – 78-81%, Afrox, South Africa). This was followed by conversion of carbon in the sample into CO_2 in the combustion furnace at 680^oC. The derived CO_2 was swept by air (Afrox, South Africa) through a Non-dispersive infrared (NDIR) detector, which generates a non-linear signal, which is proportional to the instantaneous concentration of CO_2 in the carrier gas. That signal was then linearized and integrated over the sample analysis time. The resulting area was compared to stored calibration data and a sample concentration in parts per million (ppm) was calculated. A stock solution of phthalic acid was used to calibrate the TOC analyzer.

2.3 Results and Discussion

2.3.1 Elemental analysis

The elemental characterization undertaken in this study provided information on the extent of coal oxidation through biological and geological processes in comparison to the untreated HC. Figure 2-4 showed a decrease in the carbon content and an increase in the oxygen content between the untreated HC and the weathered coals. Compared to HC from the same mine, BOC showed a 25% decrease in carbon content and a 76% decrease in carbon content was measured for the GOC. Oxygen content between the HC and BOC increased 7%, with a 3-fold increase measured for the GOC to 37% (Figure 2-4A). Sulfur content for BOC was 10% higher than HC but GOC was 95% lower than HC (Figure 2-4B). Nitrogen and hydrogen were not analyzed in this study since the composition of these two elements had been found to be constant at ~ 1.4% and ~ 4% respectively in previous studies (Igbinigie, 2008; Mukasa-Mugerwa, 2008).

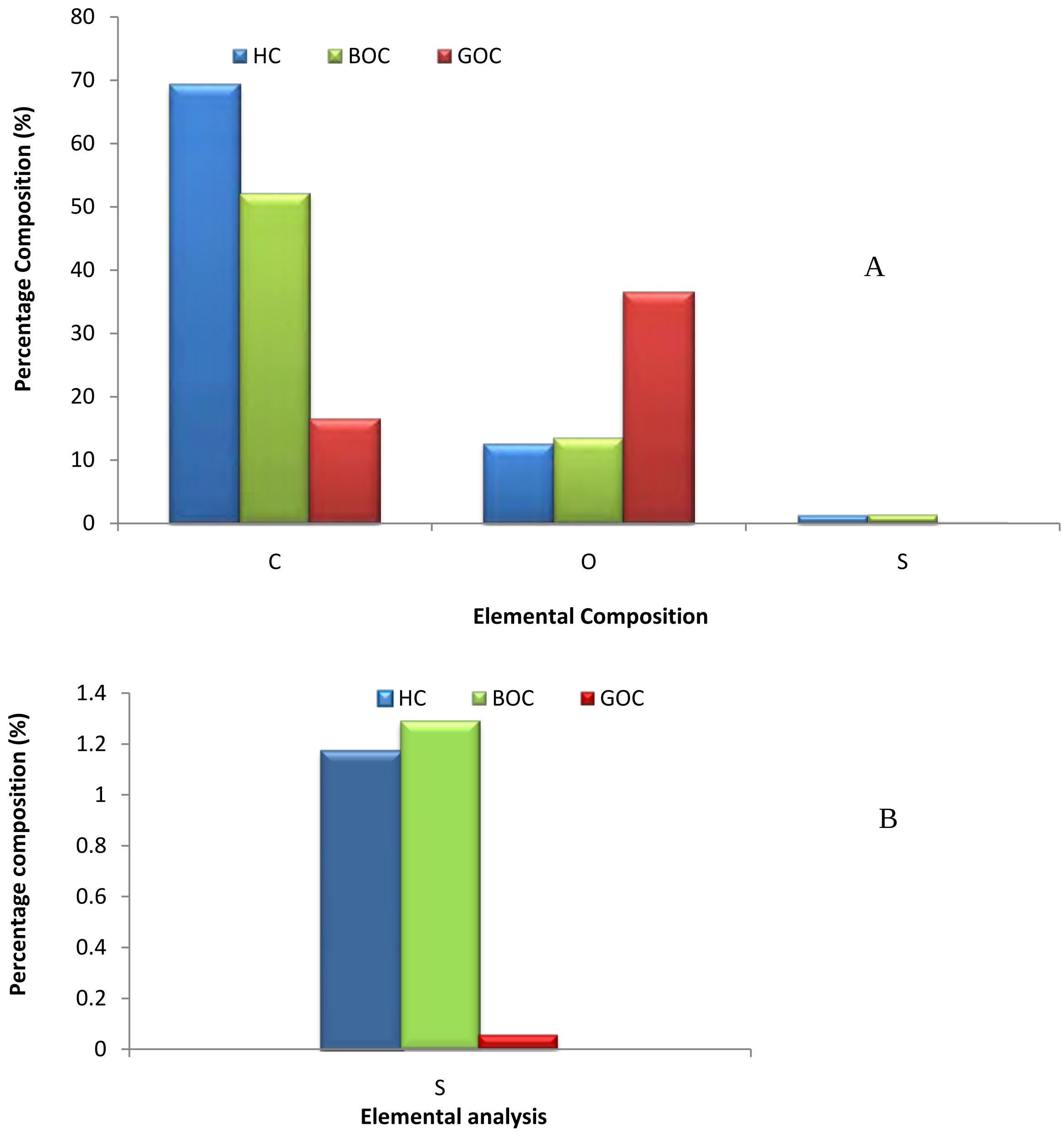


Figure 2-4 A – Macro-elemental analysis of coal samples collected from Kromdraai Colliery, South Africa. C= carbon; O = oxygen; S = sulfur. B – enlarged layout graph of the sulfur elemental composition.

The carbon content of HC measured in this study falls in the range 45 – 86% and is comparable with elemental analysis data previously reported by other researchers (Hodek, 1994; Opaprakasit, 2003). However, the oxygen content is ~ 3 times higher than that reported for typical bituminous coal (<3%). This could have occurred when the coal was extracted and exposed to oxygen and moisture, or the oxidative processes could have been initiated while the coal was still in the seam or outcropping (Seoane and Leirós, 2001; Cimadevilla *et al.*, 2005). Oxidation during storage on the mine dump cannot be ruled out, since this contributes to the loss of commercial value of coals globally (Chang and Berner, 1999; Casal *et al.*, 2003; Wang *et al.*, 2003; Wagner, 2008). The weathering of HC results in the loss of carbon and an increase in oxygen and moisture content (Pisupati and Scaroni, 1993; Opaprakasit, 2003).

In the same way, the reduction in the carbon content of the BOC (~ 52%) compared to the HC, may be attributed to the biological weathering process occurring in the SHCB. During weathering, HC undergoes a decoalification process that is driven by ingress of water and air with varying temperature conditions resulting in modification of the physical structure of the coal to produce highly oxidized lignite-like derivatives (Pisupati and Scaroni, 1993; Chang and Berner, 1999; Cimadevilla *et al.*, 2005) which is evident in the elemental analysis observed in this study. Previously, micro-organisms have been implicated in the oxidative weathering of coal, although most studies have focused on their role in pyrite oxidation (Olson and Brinckman, 1986; Fakoussa and Hofrichter, 1999; Klein *et al.*, 1999; Malik *et al.*, 2001; Demirbas, 2007).

Figure 2-5 shows an increase in the ratio of oxygen: carbon, between the untreated HC and the oxidized BOC and GOC respectively. A 1.4-fold increase in the O:C ratio was measured between the HC (0.18) and the BOC (0.26), while an ~ 12 fold increase in the ratio was measured in the GOC (2.2).

The O:C ratio presented in Figure 2-5 suggests the oxygenation of the coal substrates as result of the weathering processes. These results provide further evidence to support the decoalification process, discussed earlier. The O:C ratio of the HC falls in the range described by van Krevelen (1984), who reported a ratio of 0.2 for bituminous coal. The O:C ratio for BOC may be correlated to the lignite (0.27) in the van Krevelen diagram (Figure 1-1), which indicates the presence of ~ 20% oxygen (Van Krevelen, 1984; Opaprakasit, 2003). However, the O:C ratio measured in the GOC substrate was substantially higher than the lowest ranked coals such as peat (0.6). (Van Krevelen, 1984; Opaprakasit, 2003). While the oxygen content reported by Van Krevelen (1984) and Opapraksit (2003) is comparable to the results obtained in this study (Figure 2-5), the loss in carbon due to geological weathering was much higher than previously reported.

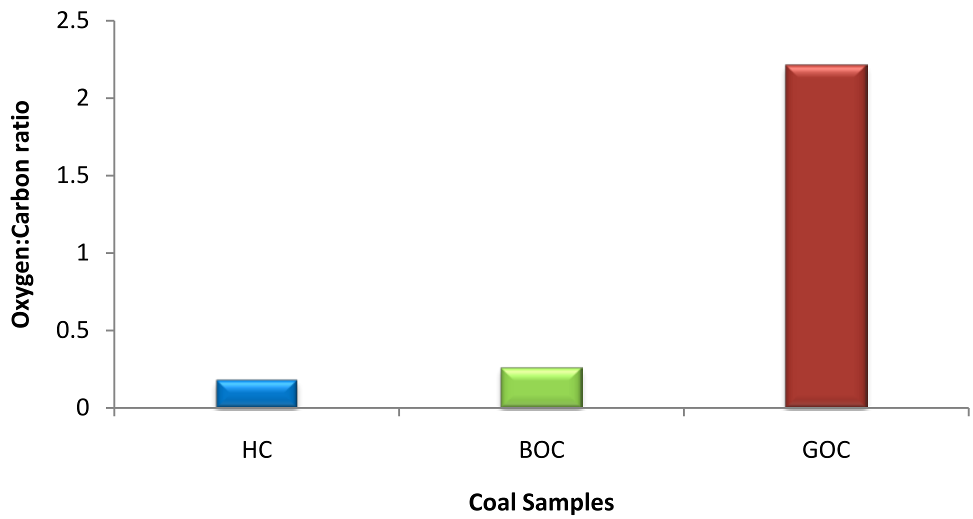


Figure 2-5 Elemental analysis of the oxygen: carbon ratios of coal samples collected from Kromdraai Colliery, South Africa, hard coal, which had been previously been subjected to biological oxidation (BOC) and geological oxidation (GOC).

2.3.2 Standardization of alkaline extraction method

Extraction of HS from the GOC was compared using three different methods. A higher humin concentration (60.1% w/v) was observed in method 1 (single extraction) and a lower concentration of ~ 58% (w/v) was observed in extraction methods 2 (multiple extraction) and 3 (pre-wash+ multiple extraction) (Figure 2-6). Concentrations of FA (~0.1% w/v) in all three extraction methods were minimal (Appendix 1-C). A 32% - 34% humic acid extraction was comparable for all the methods. Spectrophotometric measurement of the extracted HA from all three methods was found to be 35 ± 0.5% (Figure 2-6) which was higher than the dry HA weight equivalent in all three methods (Appendix 1-C).

Comparison of the three extraction methods showed no significant differences ($p > 0.05$) in the humin content of the GOC (Figure 2-6). Similarly there was no significant ($p > 0.05$) improvement in the extraction efficiency of HA between the three methods, although method 3 showed higher HA recovery than method 1 and 2. It has been reported that in general multiple extractions ensure maximum recovery of the soluble HA through the continuous displacement of divalent and polyvalent ions by hydrogen (Stevenson, 1994). An average recovery weight of 94% was observed in all three methods, the remaining 6% could be attributed to the displacement and removal of inherent inorganic minerals such as pyrite (Kilbane, 1989; Seoane and Leirós, 2001). Spectrophotometric analysis of the HA fraction at 450 nm presented higher concentrations above the dry weight analysis, probably due to the interference of suspended particles in the sample (Adani *et al.*, 2006). An average recovery of 91% was observed in all 3 methods using the spectrophotometric analysis.

Based on these results the single extraction method was used in subsequent work since there was no significant (T-test p > 0.05) difference between single and multiple extraction. Further determination of extractable HA was conducted using the weight analysis since it provided a better unit recovery than the spectrophotometric analysis.

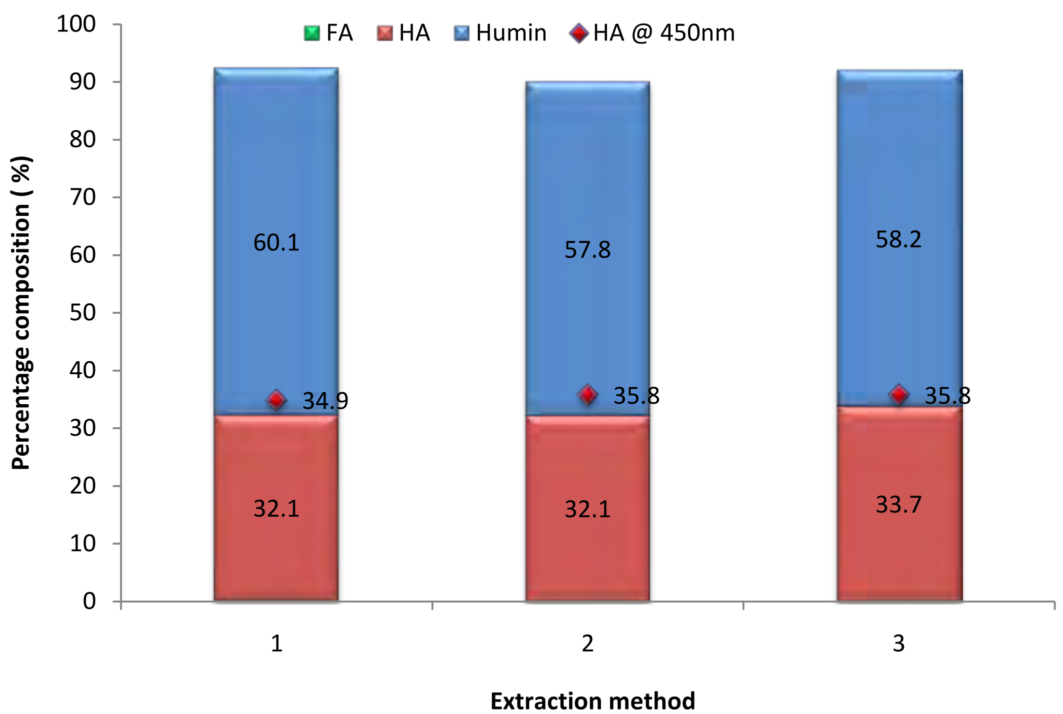


Figure 2-6 Comparison of the dry weight analysis of the alkaline extraction methods used in optimizing the partitioning of geologically oxidized coal. Humic acid @ 450 nm represents the spectrophotometric measurement of the extracted humic acid. Method 1 is the single extraction, method 2 is a multiple extraction; method 3 is a pre-washing of the coal sample followed by a multiple extraction.

2.3.3 Alkaline fractionation of coal substrates

Figure 2-7 shows the fractionation of HA, FA and humin in the HC, BOC and GOC. HA could not be extracted from the HC. A 23% (w/w) HA was recovered from BOC, which increased to 32% in the GOC. Humin concentration was relative to all 3 coal samples and fluctuated according to the HA content in an inversely proportional relationship.

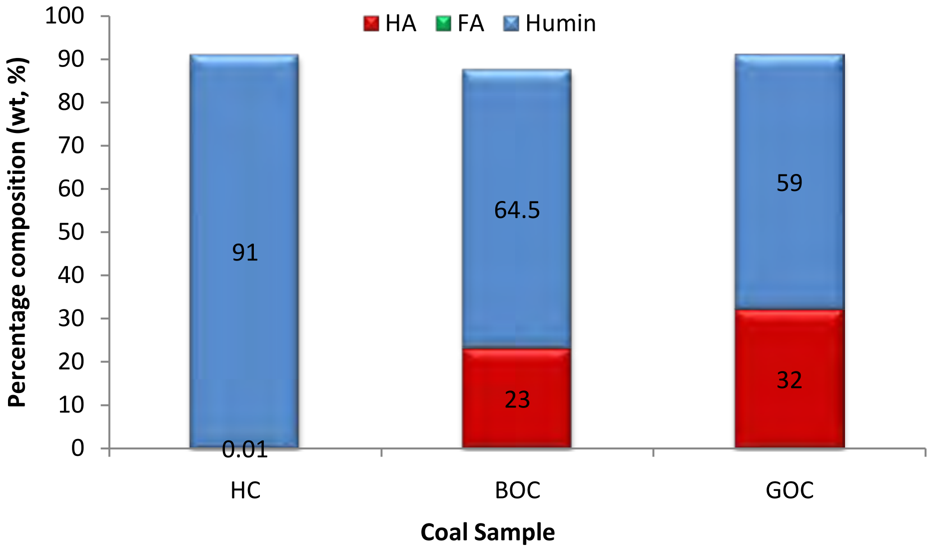


Figure 2-7 Comparison of the fractionation of coal substrates using dry weight analysis of the single alkaline extraction method. HC = hard coal; BOC = biologically oxidized coal; GOC = geologically oxidized coal.

The results presented above are comparable with the elemental analysis results section 2.3.1 and suggest that an increase in oxidation results in an increase in the humic acid content, and may be linked to the presence of reactive functional groups previously reported in other studies (Petersen *et al.*, 2008). Where the HC has not been oxidized, the carbon is bound in highly condensed structures characterized by a high C:H ratio (Piccolo *et al.*, 1992; Martin *et al.*, 1998).

2.3.4 Volatile organic matter determination

The coal samples were ashed to determine the fraction of organic matter in the coal samples that could be available to microbial utilization. Figure 2-8 shows a decrease in the VOM from the HC to the oxidized HCs, and as may be expected the ash content relative to each coal sample increased from the HC to the oxidized HCs.

According to Martinez-Garcia *et al.* (2007), the VOM of coal is made up of hydrocarbons containing only carbon and hydrogen, as well as substituted hydrocarbons composed partly of oxygen, nitrogen, sulfur, halogens and other atoms. The results presented above indicate that the HC contains up to 80% (w/w) relative VOM, which was reduced to 73% due to biological weathering (BOC). In the GOC, the VOM (45%) was lower, compared to the HC and BOC. It should be noted that although the oxidation and weathering are responsible for the loss of organic matter, it is unlikely that all the measured organic matter would be available for biodegradation, since the VOM value was derived at high temperature. However the results do provide an indication of the amount of hydrocarbon that may be oxidized either geologically or biologically.

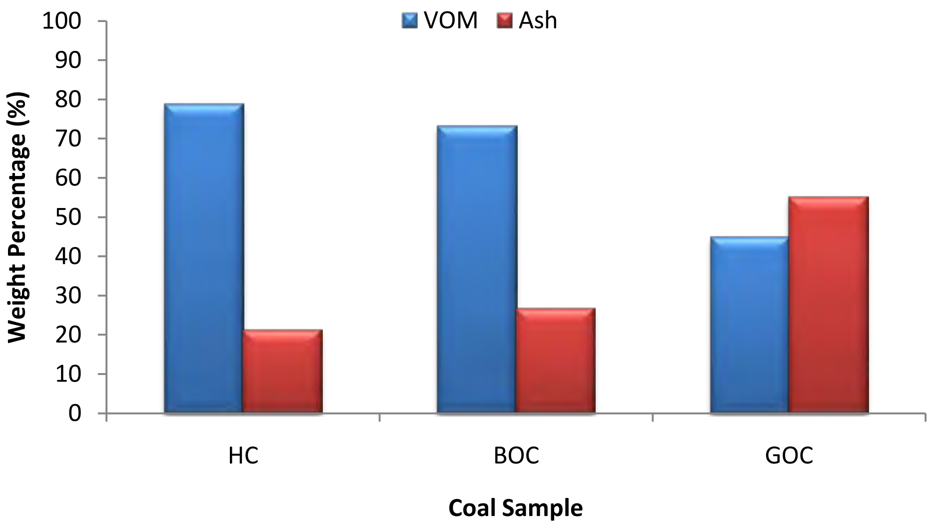


Figure 2-8 Volatile organic matter and ash content determination of hard coal (HC), biologically oxidized coal (BOC) and geologically oxidized coal (GOC).

The relative increase in ash content as the HC becomes oxidized (Figure 2-8) is related to the loss in carbon due to the oxidation processes. The ash content, comprising the inorganic mineral components was comparable to the reported range (10 – 70%) characteristic of South African GOCs (DME, 2001; Wagner, 2007). According to Van Dyk *et al.* (2009), the ash content gives an indication of the amount of inorganic and alkali insoluble material in coal, which would be crucial for the bioprocess reactor design using coal as a substrate.

2.3.5 Soluble organic carbon in oxidized hard coal as a function of coal concentration and time

The solubility of GOC in water was investigated to determine the amount of organic carbon available in an aqueous reaction environment. The initial pH of dH_2O (pH 7.2 ± 0.5) was reduced to pH 4.7 ± 0.5 after the addition of the coal, which may have decreased the solubility of coal in an aqueous medium. Figure 2-9 showed that agitating the GOC for 1 h did not yield significantly more solubilized product than agitating for 24 h. Similarly an increase of the GOC in suspension from 0.2 – 10% (w/v) did not increase the amount of SOC in solution.

The SOC concentration was found to be independent of agitation time and GOC concentration under neutral pH conditions. It has been previously observed that reduced solubility of HA in aqueous solutions was due to hydrogen bonding, cation bridging and hydrophobic interactions to form insoluble aggregates at low pH, high ionic strength and increased HA concentration (Avena *et al.*, 1999; Brigante *et al.*, 2007).

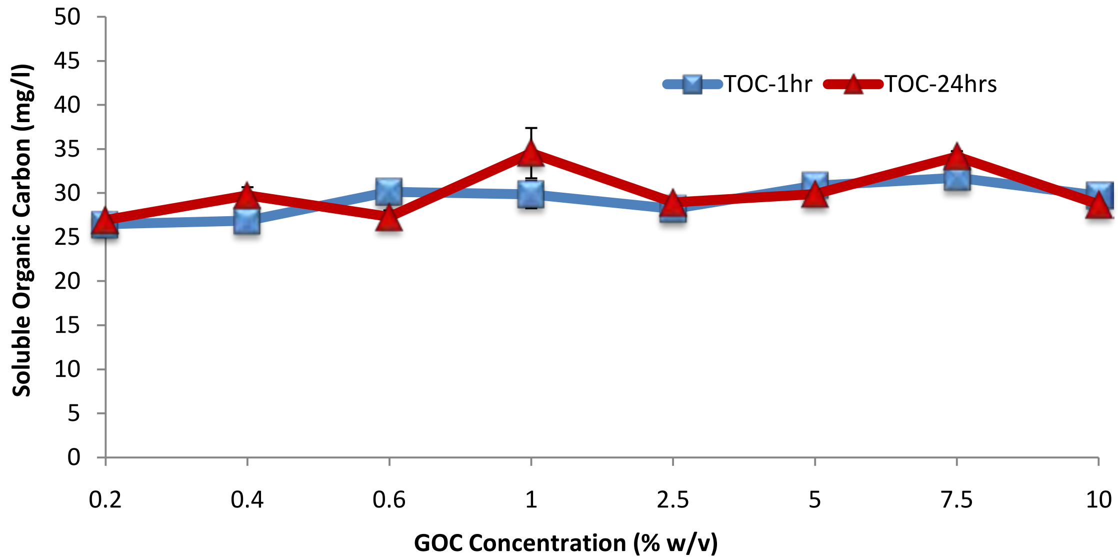


Figure 2-9 Soluble organic carbon analysis of geologically oxidized coal suspended in distilled water and agitated for 1 h and 24 h in a controlled environment laboratory maintained at 30°C, pH ~ 4.7.

2.3.6 Extractable humic acid in oxidized hard coal as a function of pH and time

GOC was shown to be the most oxidized substrate, and contained the highest HA content above BOC and HC. GOC was therefore chosen as an ideal substrate for detailed investigations of the availability of the soluble organic matter for microbial degradation. The extraction of HA in a bioprocess reactor system was simulated to predict the behavior of the oxidized HC substrate. The experimental pH ranges were derived from, and representative, of the optimum working pH of micro-organisms associated with aerobic and anaerobic coal bioconversion processes (Crawford *et al.*, 1990; Liu *et al.*, 2008). Previous alkaline extraction under optimum conditions with 0.1 M NaOH (pH >11) had shown that the maximum extractable HA from the GOC was ~ 35% (w/v) (Figure 2-6).

In Figure 2-10, it was shown that the extraction and solubility of HA was primarily dependent on pH and time. Separate flasks with low pH solutions below 6.5 (acidic range), that were agitated for 1 h showed no extraction of HA into solution. When the pH was increased above 6.5 over the same period, the highest HA extraction of 14.3% (w/v), at pH 8.0 was observed after 1 h of agitation. When compared to flasks agitated for 24 h, similarly, there was also no HA extraction in the acidic range, but the extractable HA increased from 6.5% at pH 6.5 to 67.2% (w/v), at pH 8.0. It was interesting to note that extended agitation for 334 h at a lower pH of 6.5 resulted in a 20.5% (w/v) extraction, while up to 80% of the extractable HA became soluble at pH 8.0.

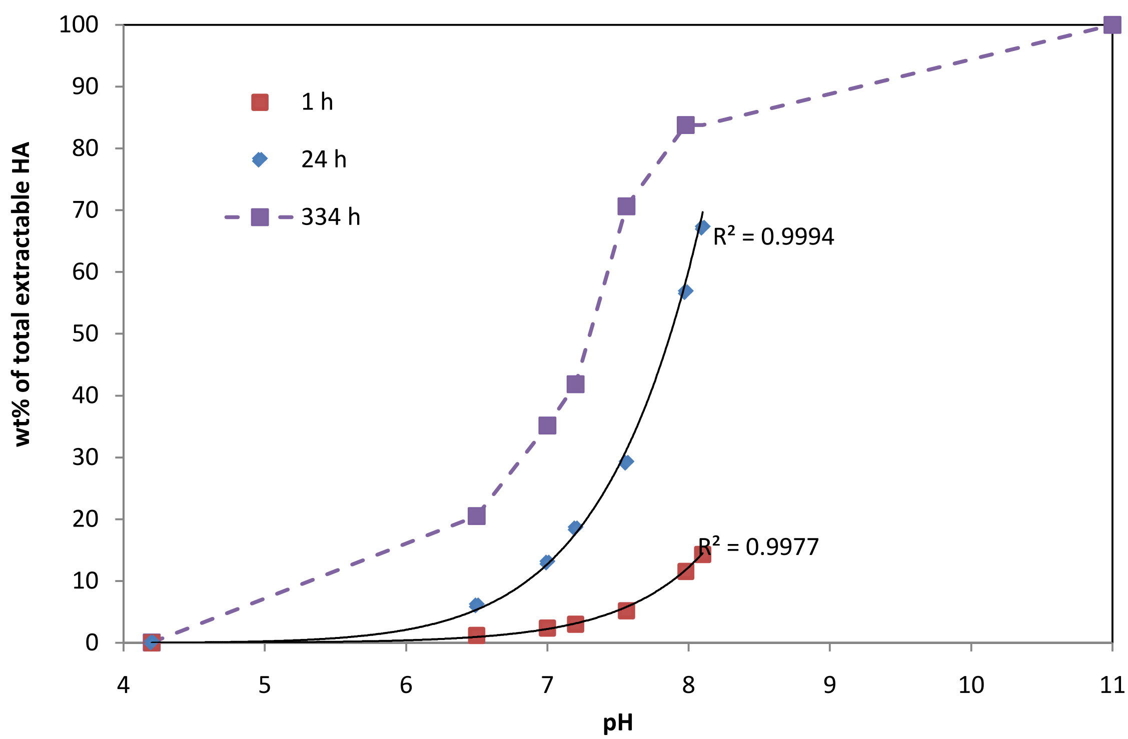


Figure 2-10 Humic acid extraction as a function of pH and time using phosphate buffer (~ 0.1 M). The R^2 values for the 1 h and the 24 h experiments are lines of best fit derived from exponential and power curves respectively. These values can be used to accurately predict the amount of extractable humic acid at a given pH value.

A sigmoidal extraction curve was observed over extended agitation times of 334 h (Figure 2-10), in which there was low extraction efficiency below pH 6.5 and above 8.0, but there was an exponential increase in extraction efficiency in between these values with increasing pH (Figure 2-10). The bulk of the extractable HA (~80%) was dissolved into solution during the exponential phase. Previous researchers have reported extraction periods ranging from 12 h to 7 days (168 h), although 24 h is generally considered optimal (Goh, 1970; Goh and Reid, 1975; Kakezawa *et al.*, 1992; Kasim *et al.*, 2007). Chemical extraction of HA is normally performed under highly alkaline conditions (pH > 11) (You *et al.*, 2006). At such pH, the mechanism of HA extraction and solubility is assumed to be strongly linked to the deprotonation of the carboxylic and phenolic functional groups of the HA macro-molecule which results in the cleavage of some of the hydrogen bonds that maintain the structural integrity of HA (Brigante *et al.*, 2007; Cooke *et al.*, 2007). The reaction of the aqueous hydroxyl ions with acidic functional groups in the GOC initiates the HA dissolution process, and involves the chemisorption of alkali-metal ions onto the coal structure. The separate HA fractions in the coal structure thus became or solubilized, depending on the steric orientation of the functional groups within the coal (Camier and Siemon, 1978; McDonald *et al.*, 2004).

Based on these results, the optimal pH and time for efficient recovery of extractable HA from oxidized HC under biological conditions would be ~ pH 8 over a 24 h period, as prolonged incubation at that pH would only result in a 3% increase in the extractable HA being released into solution. Both aerobic and anaerobic microbial systems have been reported to generate alkaline pH. In aerobic studies on *Fusarium oxysporum,* Holker *et al*. (1999) reported that the fungi increased the pH to 9 in 7 days. In anaerobic systems, the generation of alkalinity and increase in pH has been demonstrated by several researchers (Johnson and Hallberg, 2005; Krohn, 2007; Costa *et al.*, 2008; Prasad and Henry, 2009). In light of these reports, the feasibility of developing commercial bioprocesses for the extraction of HA from oxidized HC substrates would depend on extending the incubation period, to allow adaptation and generation of the required pH.

2.3.7 Effect of buffer on alkaline extraction

The solubility of GOC in phosphate buffer and NaOH solution was investigated and compared over time to determine the role of buffered and alkaline solutions in solubilizing HA and was therefore represented as SOC (Figure 2-11). The maximum extractable HA under alkaline conditions (Appendix 1-A) was ~ 2600 mgl^{-1}. Flasks that were agitated for 1 h with GOC in dH_2O extracted 1.2% of the maximum extractable HA which remained unchanged after 24 h at 1.2%. Flasks containing 0.1 M NaOH at pH 7.5 extracted 1.6% HA into solution, which increased to 5.5% after 24 h. Phosphate buffer at the same molarity and pH extracted 16% of the extractable HA into solution after 1 h, which subsequently increased to 60% HA after 24 h.

When the pH was increased to pH 8.0 at the same molarity, there was no increase in the extraction efficiency after 1h of agitation, although there was a slight increase in extractable HA after 24 h to 64%.

The remaining pellets at the various pH ranges were then totally extracted using the standard 0.1M NaOH (pH > 12) to solubilize the remaining HA. It was observed that most of the HA had remained in the pellet in the dH_2O-GOC solution where 98% and 97% of HA was dissolved into solution after 1 h and 24 h respectively. Similar results were observed for the pellets remaining after extraction with NaOH solution where 93% and 91% were dissolved into solution (Figure 2-11). In the phosphate buffered pellets (pH 7.5) there was a 37% increase in extraction efficiency over time with 77% and 40% HA dissolved into solution after 1 h and 24 h respectively. At pH 8.0, in phosphate buffer, there was a 36% increase in soluble HA from 73% to 37% after 1 h and 24 h respectively.

A 96% average recovery of the total extractable HA was observed in the different extraction solutions after complete extraction of the pellets.

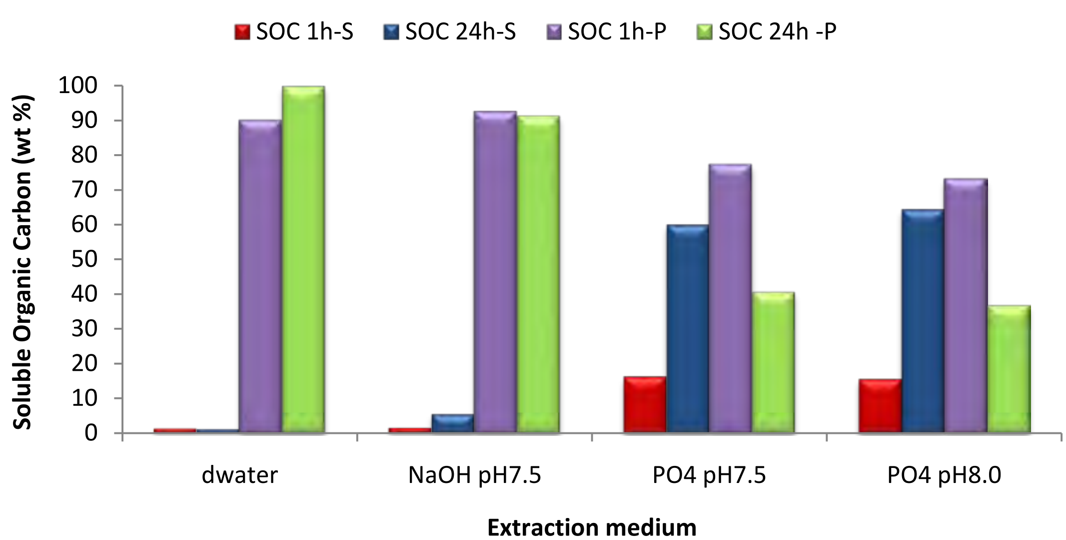


Figure 2-11 Distribution of the organic carbon fraction of humic acids from 1% oxidized hard coal in different extraction solutions agitated for 1 h and 24 h. S = supernatant; P = pellet. The humic substances were measured as soluble organic carbon.

The minimal amounts of HA detected in the dH_2O-GOC flasks were representative of loosely bound or freely soluble HS. This was further confirmed by the lack of increase in HA after prolonged agitation as the remaining HA was bound in the GOC matrix is not easily released into solution. The extraction using a NaOH (pH – 7.5) solution was not comparable to a buffer solution of the same pH. A likely explanation based on experimental observations could be that the oxidizing potential of the NaOH was quenched by the oxidized carboxylic functional groups in the GOC whereas the buffer maintained its oxidizing potential to some extent (Alvarez-Puebla and Garrido, 2005; Peuravuori *et al.*, 2006; Giannouli *et al.*, 2009). The mechanism of the phosphate buffer can be compared to the buffering effect of soil, in which its buffering capacity relation to the changes in its natural pH is directly related to the protonation or deprotonation of organic materials and minerals in the soil (Weaver *et al.*, 2004; Vázquez *et al.*, 2009). From these results it became apparent that the length of agitation at a particular pH was important. Moreover, the extraction medium, was also critical towards achieving efficient recovery of the soluble fraction of the coal. Phosphate buffer was found to be suitable for this study, since one of the aims was to simulate the condition under which the GOC would be exposed in transformations mediated by micro-organisms (Cooke *et al.*, 2007; Soetaert *et al.*, 2007). Again, a 24 h agitation period at pH 8.0 (which is physiologically possible in bioreactor system of interest) would be appropriate for this process.

In summary, the heterogeneous nature of the oxidized HC substrates necessitated the characterization of the substrates for subsequent use in microbial conversion. While it may serve as a substrate for microbial degradation, it is important to note that only a fraction of the coal is amenable to biological attack. The rate at which this becomes available for utilization was therefore shown to occur as closely related functions of time and pH.

2.4 Conclusions

The following deductions can be drawn from this study:

- The oxidation and weathering process causes loss of carbon and increase in oxygen in the coal substrates, and increases the O:C ratio;
- BOC and GOC were partitioned into HA, FA, and humin but HC could not be partitioned. The alkali soluble HA and FA constituted hydrophilic organic matter while the humin constituted the hydrophobic organic and insoluble inorganic fraction;
- A fraction of the organic matter may be available for biological consumption;

- pH and time play a fundamental role in the extraction of organic C from oxidized HC.

These conclusions provided insights into the actual amount of extractable organic C from GOC, and provided a basis for developing bioprocesses targeted at utilizing the available organic carbon in this substrate. Furthermore, the results reported here lays the foundations for the formulation of a growth medium that could be used for bioprocess development by separating out the inorganic and organic fractions.

CHAPTER THREE

BIOGASIFICATION OF OXIDIZED HARD COAL SUBSTRATES 1: GAS PRODUCTION

3. INTRODUCTION

Coal biogasification is a complex microbial process involving the metabolic activity of an integrated consortium of anaerobic microorganisms adapted, to some degree, to the biodegradation of complex hydrocarbons (Gupta, 2007; Ulrich and Bower, 2008; Lozano *et al.*, 2009).

Most laboratory studies have focused on the biogasification of LRC (Luca Technologies, 2004; Green *et al.*, 2008). These contain readily leachable LMO that may be susceptible to microbial degradation (Green *et al.*, 2008; Harris *et al.*, 2008). On the other hand, higher rank coals such as bituminous and anthracitic coals contain highly condensed aromatic structures, are low in soluble fractions and are not as amenable to biogasification. However, a number of reports have shown that these coals may support some level of methanogenic growth with relatively low yields (Johnson *et al.*, 1994; Volkwein *et al.*, 1994; Budwill, 2003).

Jones *et al.* (2008) have identified process factors which may enhance the commercial feasibility of coal biogasification including: (i) increasing the bioavailability of carbon in the coal, (ii) development of well adapted bacterial communities that can degrade the carbon substrates in coal (iii) establishing process conditions that will promote microbial growth in these substrates such as nutrient supply, the presence of suitable co-metabolites and the elimination of inhibitory factors.

Mukasa-Mugerwa (2008) and Igbinigie (2008) have shown that the pre-oxidation of HC substrates may substantially enhance its biological reactivity and in this regard that both geological and biological oxidation processes may be effective. While the SHCB (Rose *et al.*, 2008) has been developed to enhance the biological oxidation process, no studies to date have investigated the use of the oxidized HC product for biogasification and methane production.

The development of specific anaerobic microbial consortia for the biotreatment of different organic substrates has been comprehensively reviewed by (Mata-Alvarez *et al.*, 2000; Rockne and Strand, 2001; Maukonen *et al.*, 2003; Akram and Stuckey, 2008; Green *et al.*, 2008; Lee *et al.*, 2009). The development of microbial cultures for biodegradation of low rank coals has been studied using microorganisms from a range of environments such as abandoned coal mines, underground coal seams and surrounding rocks, aquatic sediments, petrochemical processing plants effluents, insects, cow dung, and domestic and industrial effluents (Panow *et al.*, 1997; Pérez *et al.*, 1997; Gupta and Birendra, 2000; Chang *et al.*, 2003; Green *et al.*, 2008; Jones *et al.*, 2008). Aspects of these approaches were followed in the development and adaptation of a consortium of anaerobic methanogenic bacteria used for the biogasification of oxidized HC substrates undertaken in this study.

3.1 Objectives

The objectives of this study were to:

- Develop a methane producing anaerobic microbial consortium capable of coal biodegradation. This would be composed of microorganisms sourced from environments in which aromatic, phenolic and lignocellulose compounds dominate as carbon and electron donors;
- Evaluate the potential application of the adapted consortium of bacteria in the production of methane from the oxidized HC products including BOC and GOC;
- Investigate the requirement for a co-metabolite in the production of methane from oxidized HC substrates.

3.2 Materials and Methods

3.2.1 Development of methanogenic cultures

3.2.1.1 Sample collection

Environmental samples for the development of a robust anaerobic bacterial consortium adapted to the degradation of complex aromatic hydrocarbon compounds were collected from a range of sources in South Africa (Table 3-1).

Table 3-1 Sites from which samples collected for the development of the methanogenic consortia used in the coal biogasification study.

Sampling site	Culture sample
Grahamstown[1] (Makana Municipality treatment works)	Anaerobic digester treating domestic wastewater
East London[1] (Potsdam and Buffalo City Municipality treatment works)	Anaerobic digesters treating domestic and textile dye-house industrial effluent
Port Elizabeth[1] (Fish Water Treatment Works)	Anaerobic digester treating industrial and domestic wastewater
Port Elizabeth[1] (Ibhayi Brewery)	Anaerobic digester treating brewery effluent
Stellenbosch University[2]	Anaerobic digester treating wine distillery effluent
Grahamstown Abattoir[1]	Anaerobic ruminant gut contents
Termite mounds Graham College, Sports grounds[1]	Dissection of hind gut of termites
Grahamstown[1], EBRU culture collection	Lignocellulose degrading anaerobic culture

[1] – Eastern Cape Province, South Africa
[2] - Western Cape Province, South Africa

Anaerobic digesters treating domestic and industrial effluents contain established consortia that may be adapted to the degradation and biogasification of complex hydrocarbons (Liu *et al.*, 2008; Ward *et al.*, 2008; Ruiz *et al.*, 2009). The digester at Potsdam received textile dye-house effluent and was selected for the presence of microorganisms adapted to the degradation of recalcitrant aromatic compounds, such as azo dyes and anthraquinones, used in the manufacture of dyes (Cervantes *et al.*, 2001; Kasai *et al.*, 2007). The digesters at Port Elizabeth and Stellenbosch treated brewery and winery effluent and, in addition to a range of microbial types, may contain *Clostridium* adapted to the anaerobic degradation of aromatic compounds (Fang *et al.*, 2004; Akram and Stuckey, 2008; Dolfing *et al.*, 2008). Ruminant gut populations and those from the hindgut of termites in particular are adapted to wood and lignocellulose degradation (Pareek *et al.*, 2000; Adams and Boopathy, 2005; Wolin and Miller, 2006; Green *et al.*, 2008). Fresh ruminant pouch wastes sourced from Grahamstown Abattoir, and the hind gut of *Psammotermes* termites were dissected and added to the cell generators.

3.2.1.2 Coal preparation

HC, GOC and BOC used in the various studies were prepared as previously described (section 2.2.1).

3.2.1.3 Growth medium and stock cell generator

A modified basal medium for the cultivation of anaerobic micro-organisms, adapted from Jackson-Moss (1990), was mixed in tap water and formulated to include: lactate (0.2% w/v, as a once-off addition to initiate the logarithmic growth phase of the mixed consortium) (Kolmert *et al.*, 1997; Riffat *et al.*, 1999; Li *et al.*, 2006; Costa *et al.*, 2008), GOC (0.2%), BOC (0.2%) and grass 0.05% as a possible co-substrate. The grass (*Cynodon Dactylon)* was used as a co-substrate because it plays an integral role in the Stacked Heap Coal Bioreactor section 1.4.5.1, and is thus harvested as part of the BOC. The grass was ground into a powder, mixed in 0.5 L dH_2O and boiled for 20 min to assist cell-wall rupture. It was then cooled and added to the cell generator. In addition, the medium contained: yeast extract (0.2%, w/v), KNO_3 (0.2%), $CaCl_2$ (0.02%), $FeCl_3$ (0.02%), $MnSO_4.4H_2O$ (0.002%), $ZnSO_4.7H_2O$ (0.001%), $Na_2MoO_4.2H_2O$ (0.0004%), $Na_2B_4O_7.10H_2O$ (0.0002%). The medium was sterilized at 121^oC for 15 min before inoculation.

An inoculum (0.5 L) from each of the collected samples was added together with 6.8 L of growth medium in duplicate 10 L cell generators. The reactors were sealed with a rubber bung, connected to a gasometer (Figure 3-1), and purged with N_2:CO_2 (80:20%) (Afrox, South Africa) for 15 min and agitated on rotary shaker (100 rpm, Labcon, 3100u), in a constant environmental laboratory maintained at 30^oC. Reactor samples were drawn to determine the volume of gas produced and its composition (CH_4:CO_2 ratio) (Figure 3-2). The cell generators were operated for 100 days and replenished with fresh medium (coal, grass and nutrients) every 14 days or when gas volume production and methane composition showed signs of decline (Ruiz *et al.*, 2009). Culture development continued until a methane:carbon dioxide ratio of around 60:40% was obtained.

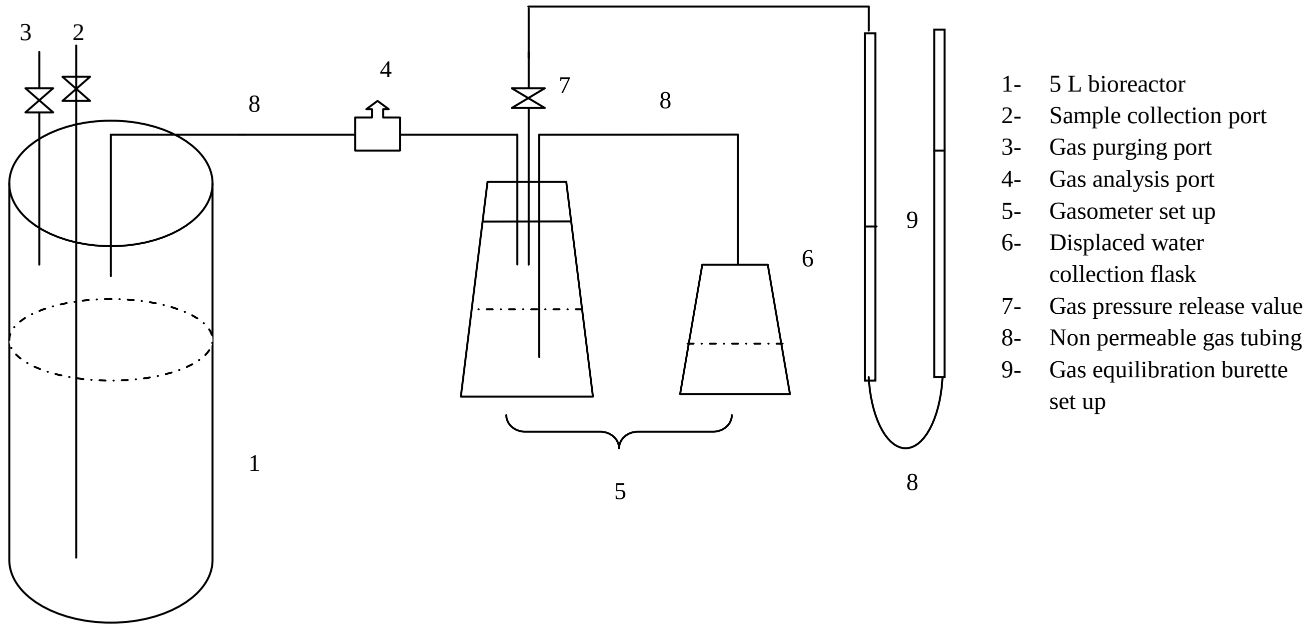


Figure 3-1 Schematic diagram of the experimental set up used in the studies on the biogasification of coal biologically oxidized coal and geologically oxidized coal with and without grass co-metabolite. The figure shows gas from the reactor passing to a gasometer with a pressure equalization column. At ambient temperature and pressure, water displaced from the gasometer vessel was taken as an indicator of total gas production.

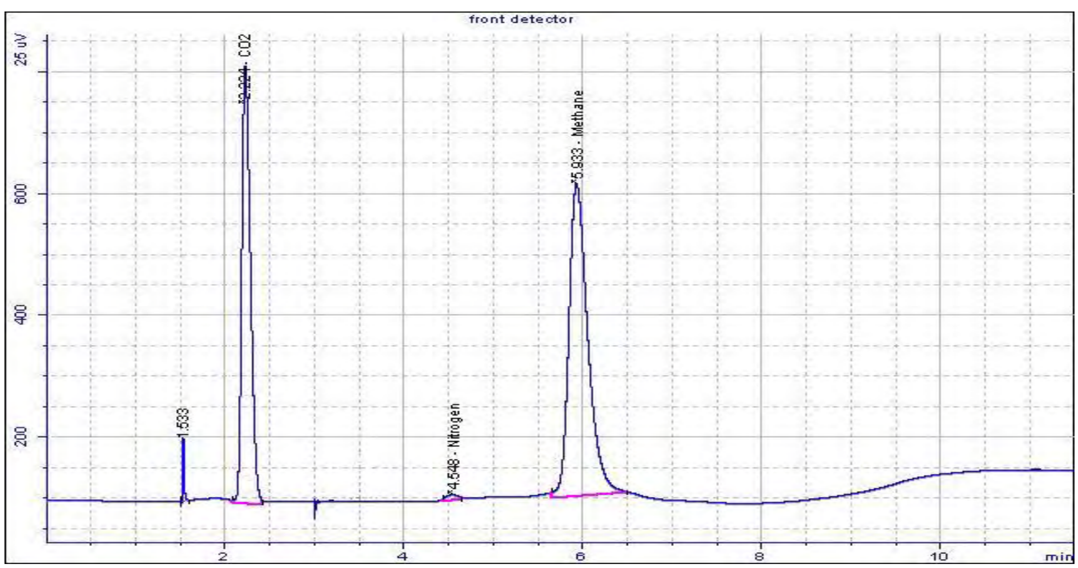


Figure 3-2 Gas chromatogram of gaseous samples collected from the stock cell generator containing oxidized hard coal and an enriched medium, which was run for 100 days.

3.2.2 Experimental outline

A series of 5 L anaerobic batch shaker flask reactors containing various loadings of BOC, GOC, HC (Mpumalanga, South Africa) were set up (Table 3-2). Grass (*Cynodon dactylon*) was added to the basal medium as described in section 3.2.1.3, but without lactate and yeast extract. The growth medium was adjusted to pH 7.5 using 1M NaOH or 1M HCl before inoculation. The experimental layout is outlined in Table 3-2.

Table 3-2 Experimental configuration of the reactor set up for the anaerobic biogasification of coal including control reactors (2,3,4,6,7,8) that contained the respective substrates alone.

	Reactors								
Input	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
GOC	√		√	√					
BOC					√	√			
HC									√
Grass	√	√		√	√		√	√	√
EBRU Inoculum	√	√	√		√	√	√	√	√

All experimental reactors were inoculated with an active methane-producing (10% v/v) culture from the stock cell generator as outlined in section 3.2.1. Drawing of the inoculum was undertaken during optimal methane production in the stock cell generator. This was achieved by adding new feed to the cell generators 7 days prior to commencement of the experiment.

The first sample was drawn at day 0 immediately after inoculation and then the reactors were sealed with rubber bungs, connected to a gasometer and the headspace purged with N_2 gas for 15 min (Figure 3-1). The reactors were agitated continuously on a rotary shaker (120 rpm, Labcon, 3100u) and incubated in a controlled environment laboratory at 30^{o}C.

Control reactors that included inoculated and un-inoculated media and containing the respective substrates alone were also set up and run concurrently with the experimental reactors. Reactor performance and methanogenic activity was monitored by daily and weekly measurements of gas volume production, gas composition, Volatile fatty acid (VFA) analysis and pyrolysis products by Py-GCMS. The experiment was repeated and both studies produced comparable results. Only one set of results (gas volume and composition) is reported here and was run for 49 days after which gas production ceased.

3.2.3 Analytical methods

3.2.3.1 Gas production

Gas production was measured using a gas equilibration burette and gasometer system as illustrated in Figure 3-1 and Figure 3-3. The pressure generated from gas production was equilibrated to Ambient temperature and pressure (ATP) at 30^{o}C using a gas equilibration burette filled with acidified dH_2O (0.1M HCl, pH <2). The volume of displaced water was measured from the burette. In the gasometer (Figure 3-3), the gas produced in the reactor displaced the acidified dH_2O to give a gas volume equivalent. The two volumes were combined to give an indication of the total gas produced in the system at ambient temperature and pressure.

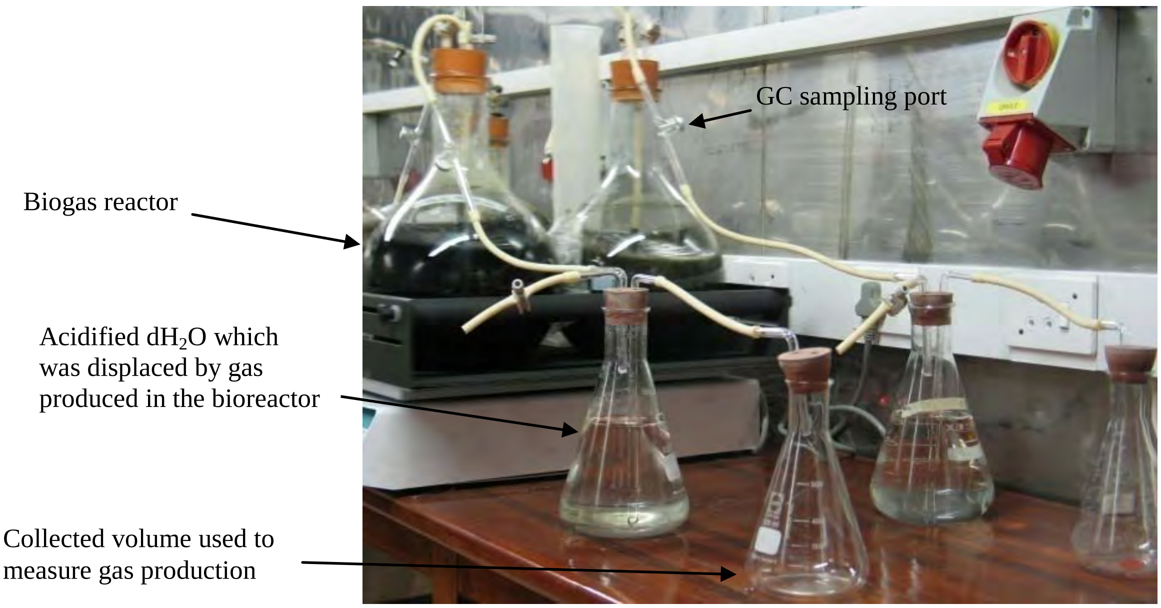


Figure 3-3 Photograph of the experimental set up used in the studies on the biogasification of geologically and biologically oxidized coal substrates showing shaker flask batch reactors and gasometers, and collection vessels for displaced water.

3.2.3.2 Gas analysis

Gas samples were withdrawn weekly and analyzed by gas chromatography to identify the gases produced within the system both qualitatively and quantitatively. A gas sample of 150 µL was extracted from the reactor using a gas-tight syringe (Agilent, South Africa) and injected into an Agilent 6820 gas chromatograph equipped with a 30 m x 0.32 mm x 0.5 micron 19091N-213 HP-Innowax column (Agilent, South Africa). The oven temperature profile was set at 50^{o}C for 4 min and then ramped to 160^{o}C at 20^{o}C.min^{-1}. The total run time was 11.5 min. A split-less injection mode was used. A Thermal conductivity detector (TCD) set at 300^oC was used and Helium was used as a carrier gas with a flow rate of 0.9 $mL.min^{-1}$. An Alpha gas standard (Afrox, South Africa) containing 20% H_2S; 20% CO_2; 20% N_2 and 40% CH_4, was used to identify and quantify the gases using a standard curve relating gas mass to relative area under the curve (Appendix 2-A).

3.2.3.3 Methane yield

The gas chromatography results were used to calculate the ratio of methane per volume of gas produced. The moles of methane gas were calculated using the Ideal Gas Law (Avsec *et al.*, 2002):

$pV = nRT$

where: p – pressure of the gas at ATP; V – volume of the gas produced; n – moles of gas; R – Ideal gas constant; T – temperature.

The mass of methane was determined using the formula:

Moles of CH_4 = mass $_{(methane)}$/ $M_{r\ (methane)}$

The $CH_{4\ yield}$ was determined using the formula: mass $_{(methane)}$/ mass $_{(Total\ substrate\ removed)}$

3.2.3.4 Statistical analysis

STATISTICA, version 8.0 software (StatSoft, Inc. 2008) was utilized for statistical analysis. The Fisher Post-hoc Least squared difference (LSD) test with a 95% degree ($p < 0.05$) of confidence was used to compare the impact of a co-substrate in coal breakdown. Box and Whisker plots were adopted for comparison of gas production of methane in the coal substrates used in this study.

3.3 Results and Discussion

3.3.1 Development of methanogenic cultures

The composition of biogas collected from the cell generators changed over time (Figure 3-4). The addition of lactate provided a carbon source which, it is assumed, was used first and gave rise to methane gas production observed between days 0 – 21. Within this period CH_4:CO_2 ratio peaked at 62:38% (day 7) and then declined as the readily available lactate became exhausted. The remaining available carbon sources in the system were grass, BOC and GOC. An adaptation period followed shortly between days 30 and 70 where production of CH_4 and CO_2 was minimal (Figure 3-4). As the culture became adapted to the less readily available additional grass and coal carbon sources, the ratio of methane production increased over the next 30 days and only declined again after 100 days of reactor operation. This was presumed to be due to the exhaustion of available electron donors. The methane content in the cell generator increased over time with the highest CH_4:CO_2 ratio of 77:23% being observed after 75 days (Figure 3-4). The addition of grass as a co-substrate was used to simulate BOC production in the SHCB.

These results are comparable to reports of similar biogasification experiments using non-coal substrates, where CH_4 content ranged between 36 – 65% (Nopharatana *et al.*, 2007; Rasi *et al.*, 2007). The highest ratio of 77:23% CH_4:CO_2 observed in this study is somewhat unusual and thereby indicated the adaptation of the starter culture to partially hydrolyze complex macro – molecules present in the coal and grass substrates. The adaptation of a culture to the metabolism of complex substrates requires time and may result in extended lag phases in these systems (Banks and Wang, 1999; Borja *et al.*, 2005) as was observed during this study where it took up to 60 days for maximal methane production to be observed.

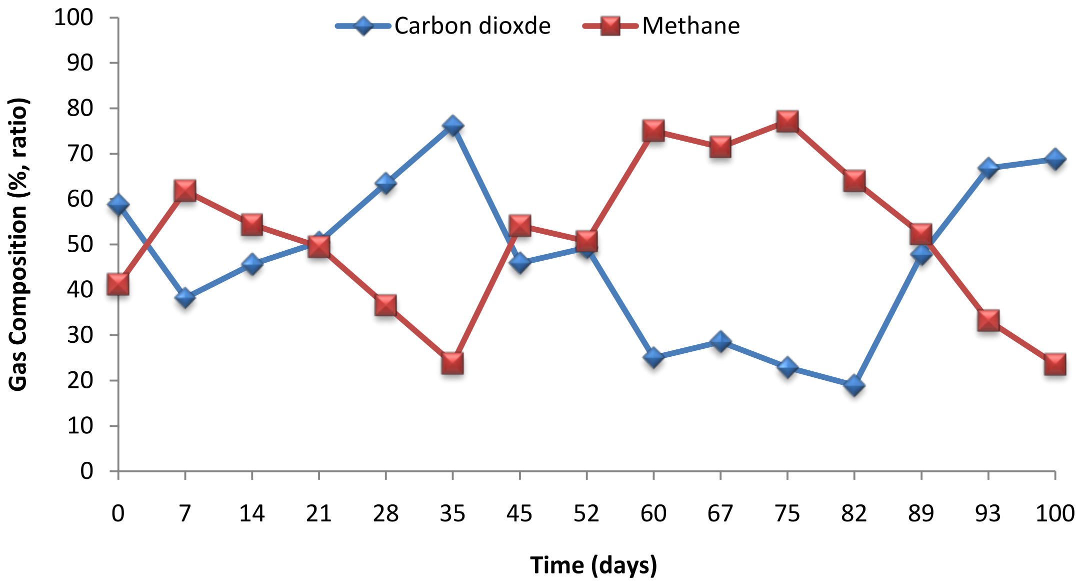


Figure 3-4 Production of methane and carbon dioxide gases in a cell generator containing grass, biologically and geologically oxidized coal with an enriched medium, seeded with the inocula sourced from different environmental and industrial sites over a 100 day period.

The adapted stock culture was then used as the inoculum for all the subsequent anaerobic studies reported below. The successful demonstration of the development of a methanogenic bacterial consortium and its probable ability to produce CH_4 from the oxidized HC and grass substrates, then led to detailed studies on the utilization of these substrates by the methanogenic consortium.

3.3.2 Biogas production from the biologically oxidized coal and grass substrate

3.3.2.1 Total gas production

The production of gas from the combined biologically oxidized coal plus grass (BOC+G) substrates using the developed methanogenic consortium was compared to the controls of BOC and grass alone in separate reactors (Figure 3-5). In general, gas production in all reactors followed a cyclic pattern at the beginning of the study but declined over time as the study proceeded (Figure 3-5). A total gas production of 1203 mL was observed in the reactor containing BOC+G over a 49 day period, while 621 mL and 658 mL was observed in the BOC and grass controls respectively (Table 3-3). The daily average gas production rate over the duration of the experiment was 24.6 $mL.d^{-1}$ in the combined BOC+G reactor, while the BOC and grass controls produced 12.7 and 13.4 $mL.d^{-1}$ respectively (Table 3-3). This represents 51% and 54% of the combined substrates and suggested that the grass may function as a co-metabolite by increasing the gas yield from BOC as opposed to providing the total substrate for methane production observed in the combined substrate reactor.

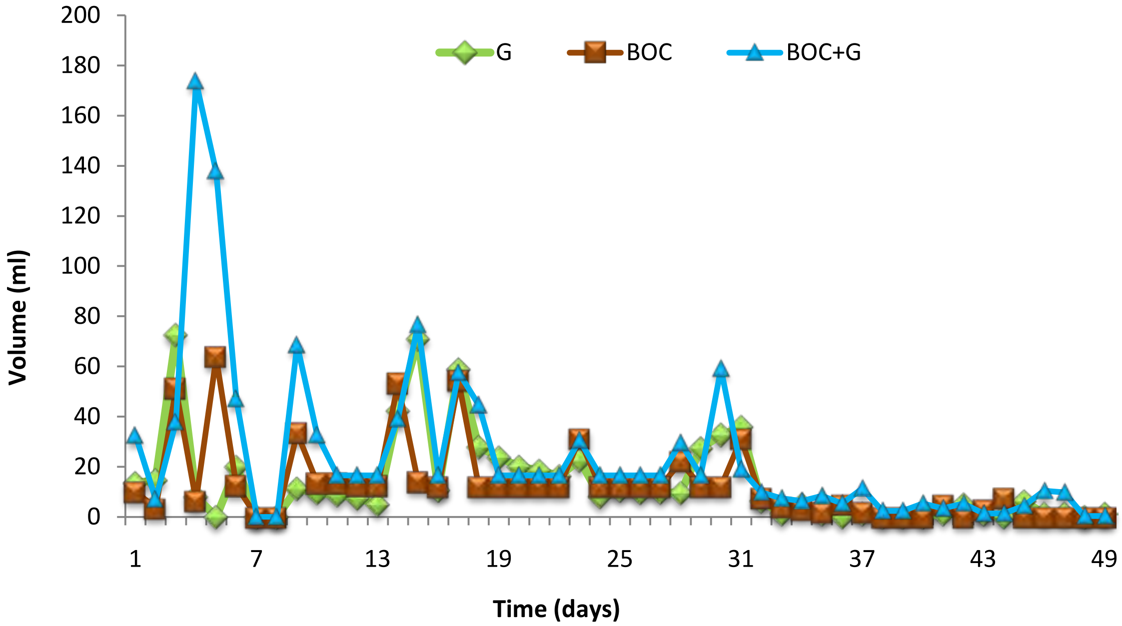


Figure 3-5 Total daily gas production in the reactors containing biologically oxidized coal and grass, and biologically oxidized coal and grass controls separately.

A highest daily gas production rate of 173 $mL.d^{-1}$ was recorded after 4 days in the combined BOC+G substrate reactor (Table 3-3). The controls containing BOC and grass separately achieved maximal daily gas production rates of 63 $mL.d^{-1}$ and 72 $mL.d^{-1}$ of gas after 5 and 3 days respectively (Figure 3-5). It is important to note that the gas production peaked first in the grass reactor, before the BOC and the combined BOC+G reactor indicating that the grass was an easier substrate for gas production.

Between 32 – 49 days, the daily gas production rates in all three batch reactors declined to ~ 5 $mL.d^{-1}$ in the combined substrate reactor, ~ 1.8 $mL.d^{-1}$ and 1.5 $mL.d^{-1}$ in the BOC and grass reactors respectively. Very likely, this was due to the depletion of available carbon. Spiking of the reactors with lactate resulted in resumption of gas production (data not shown) which further indicated that depletion of accessible carbon occurred during the experiment and was responsible for the declining gas yields observed over time.

Table 3-3 Gas production rates of the biogas reactor with biologically oxidized coal plus grass combined substrate and the respective controls, biologically oxidized coal and grass alone.

		Gas Production	
Reactors	Total Gas (mL)	Highest Daily rate ($mL.d^{-1}$)	Total Average gas ($mL.d^{-1}$)
BOC + G	1203	173	24.55
BOC	658	63	12.67
G	621	72	13.43

3.3.2.2 Gas composition

Gas distribution in the combined BOC+G reactor showed an increase in the CH_4:CO_2 ratio (22:78%) after 14 days (Figure 3-6). Thereafter, the methane production, fluctuated for the duration of the study, and achieved a maximum CH_4:CO_2 ratio 27:73% after 35 days (Figure 3-6). In the BOC control reactor (Figure 3-7), the CH_4:CO_2 ratio peaked at 37:63% after 21 days, and declined to < 10:90% until the end of the experiment. The CO_2 content of the biogas remained high through the study in both the combined substrate and the BOC control reactor, indicating effective activity of fermentative bacteria (Wolin and Miller, 2006; Chen *et al.*, 2008; Dolfing *et al.*, 2008). In the grass control reactor (Figure 3-8), the CH_4:CO_2 ratio increased to 34:66% after 14 days and then peaked at 37:63% after 21 days, where it then declined gradually for the remainder of the experiment.

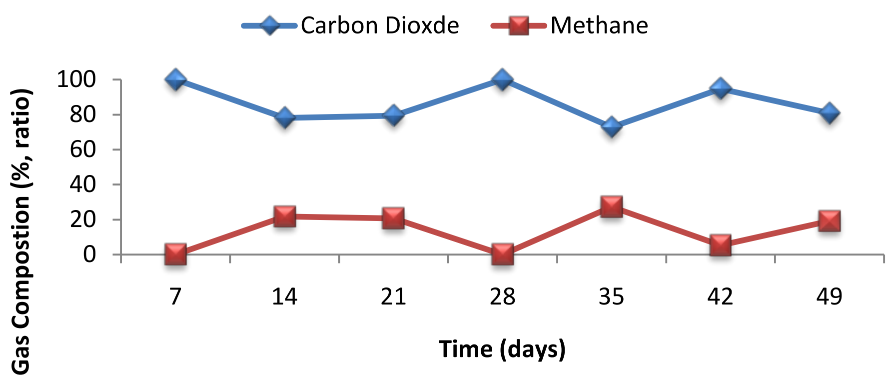


Figure 3-6 Relative amounts of the changing methane: carbon dioxide ratio over time in the headspace of the combined biologically oxidized coal plus grass reactor.

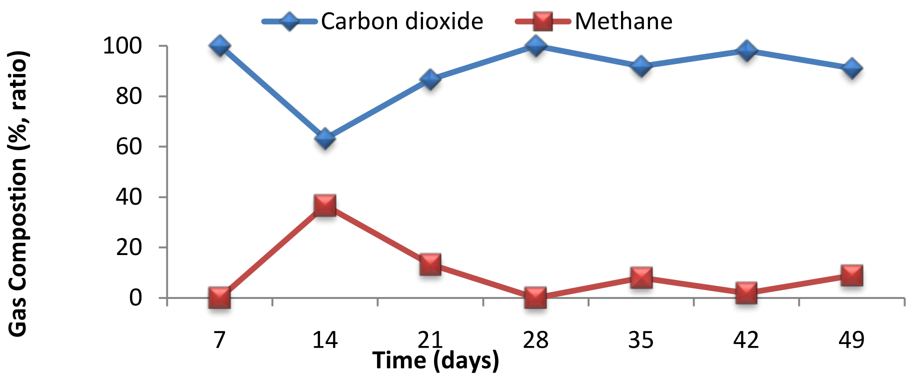


Figure 3-7 Relative amounts of the changing methane: carbon dioxide ratio over time in the headspace of the biologically oxidized coal control reactor.

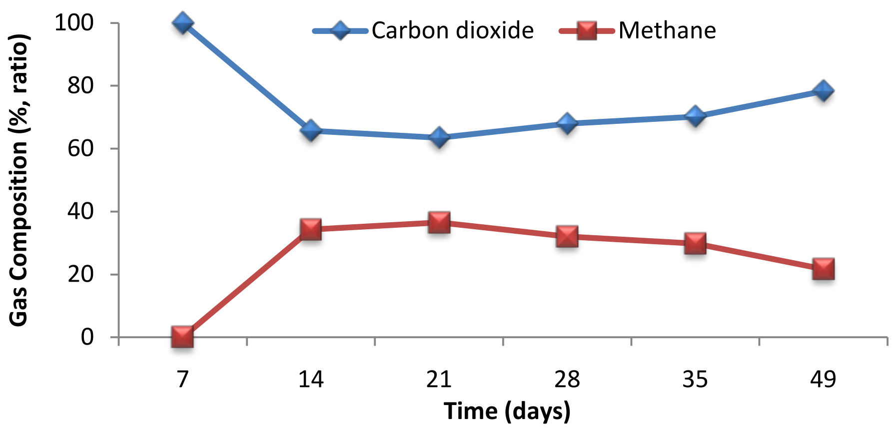


Figure 3-8 Relative amounts of the changing methane: carbon dioxide ratio over time in the headspace of the grass control reactor.

Statistical analysis of the total gas production volumes using Box and Whisker plots of the combined BOC+G and the controls showed a significant difference in gas production between the combined substrate reactor and the grass control alone (Figure 3-9). No significant difference was observed between the combined substrate BOC+G and the BOC control alone. Although there was an apparent difference in gas production between the control reactors BOC and G, the level of significance could not be ascertained by the Box and Whisker plot (Figure 3-9). The Fisher LSD test however, indicated that there was a significant difference between the combined BOC+G and grass control (Fisher LSD; $p < 0.05$) but there was no significant difference between the BOC+G and the BOC control and the control reactors BOC and G alone ($p > 0.05$, Appendix 2-B). This indicates that the presence of the grass together with coal is crucial for the production of more gas by providing the required substrates for the anaerobic process to proceed thereby emphasizing the need for a co-substrate system in the bioconversion of coal.

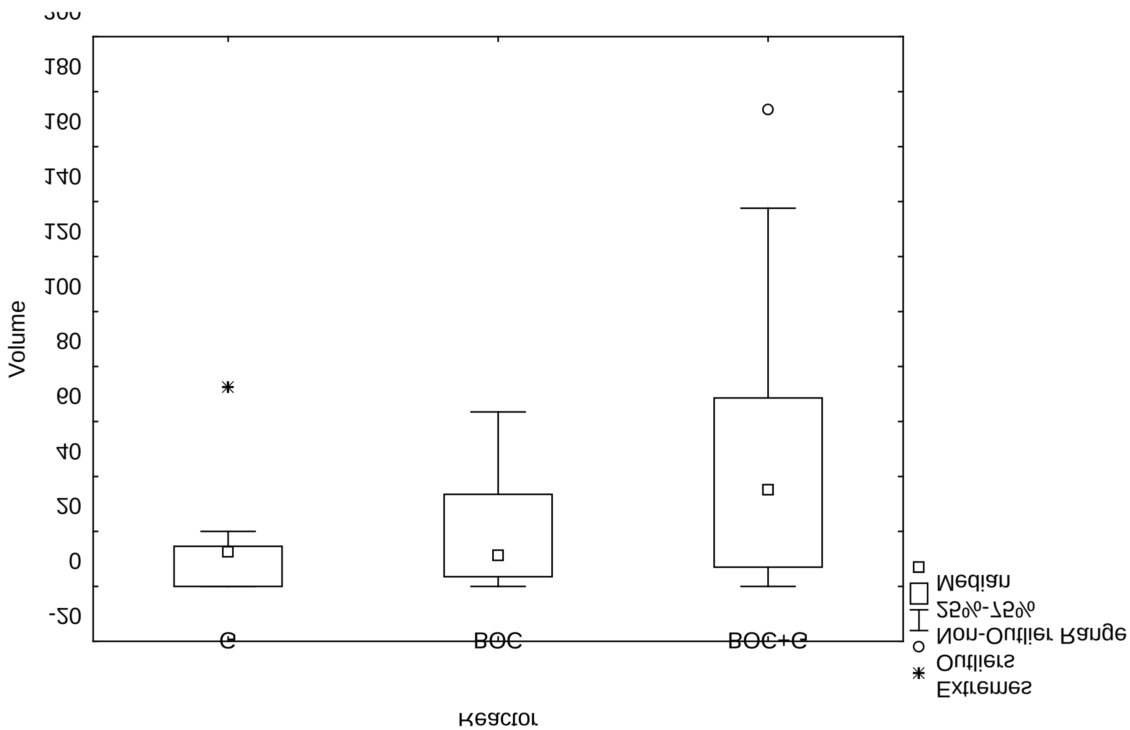


Figure 3-9 Box and Whisker plot showing the total gas production from biologically oxidized coal (BOC) with and without grass (G) and the grass control.

3.3.2.3 Methane yields

The yield of methane produced from the combined BOC+G and the respective controls alone is presented in Figure 3-10. In the combined substrate reactor, the methane yield increases ~ 2 mg CH_4.g substrate^{-1} after 14 days and decreased to 1.8 mg.CH_4.g substrate^{-1} after 28 days. Thereafter the methane yield measured followed the cyclic pattern observed in Figure 3-5. The reactor containing BOC increased methane yield to 1.9 mg CH_4.g substrate^{-1} after 7 days and was followed by a 64% decrease in yield after 21 days (Figure 3-10). No methane yield was recorded after 28 days and 42 days, although, 0.41 mg CH_4.g substrate^{-1} and 0.45 mg CH_4.g substrate^{-1} was recorded after 35 days and 49 days respectively suggesting cycling pattern of methane generation (Figure 3-10).

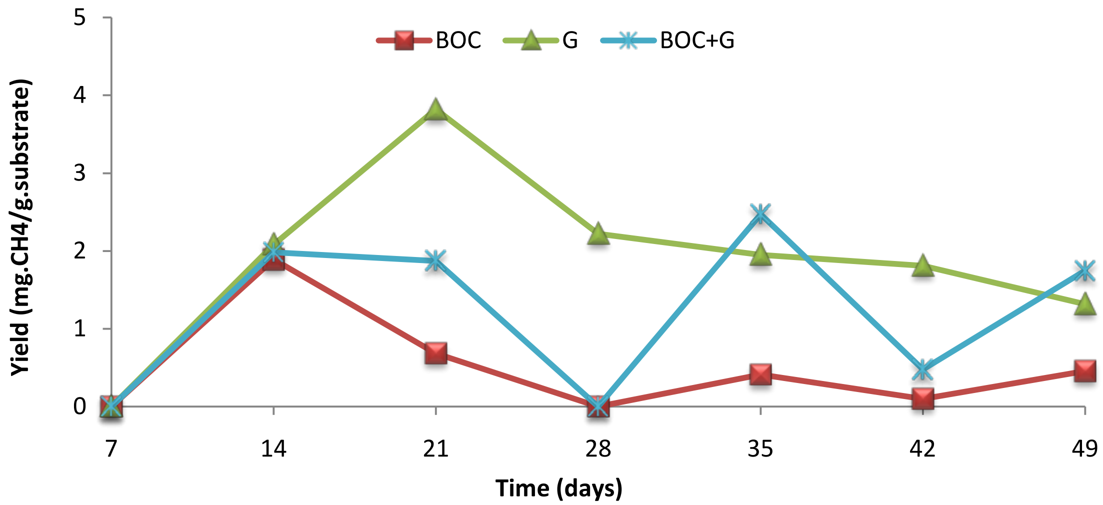


Figure 3-10 Methane yield from the degradation of combined biologically oxidized coal plus grass including the biologically oxidized coal and grass control reactors alone.

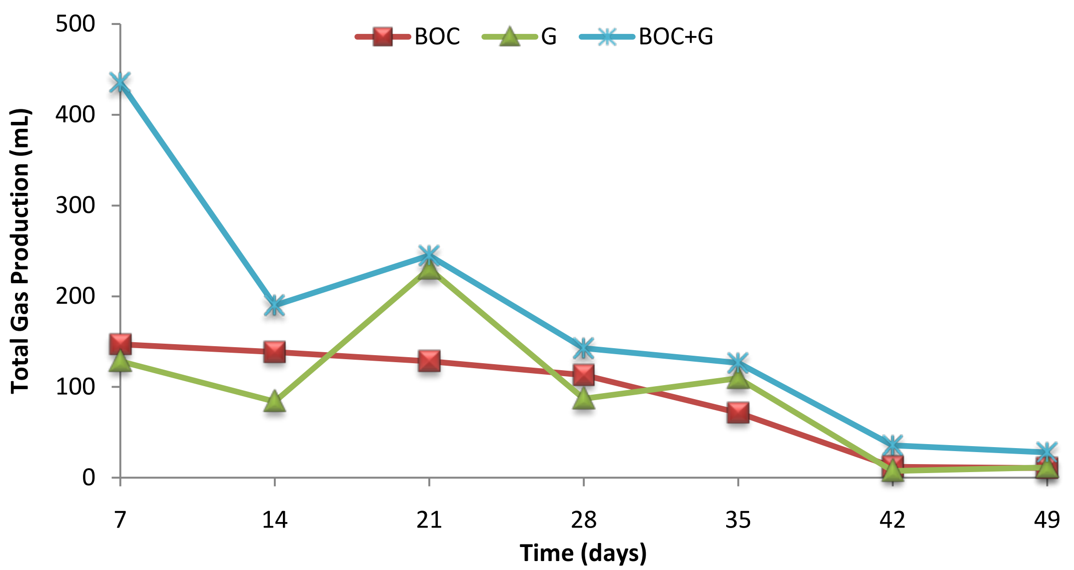


Figure 3-11 Weekly total gas production in the reactors containing combined biologically oxidized coal plus grass including the biologically oxidized coal and grass control reactors alone.

The overall methane yield for the combined BOC+G reactor was 9 mg CH_4.g substrate^{-1} and 4 mg CH_4.g substrate^{-1} for the BOC control. Interestingly, the grass control generated a higher methane yield (13 mg CH_4.g substrate^{-1}) than both the combined BOC+G, and BOC control reactor. A statistical analysis of the methane yields of these substrates using the Fisher LSD test showed that there was no significant difference ($p > 0.05$) between the combined substrate BOC+G and the BOC alone (Appendix 2-C). Although the methane yield from the grass control reactor was 44% higher than the combined BOC+G, no significant difference was found between the two reactors. The methane yield from the grass control reactor was significantly higher than BOC control reactor ($p < 0.05$).

The grass alone may have contained more readily available carbon substrates for methane generation than BOC, and a possible reason that methane yield was less in the combined BOC+G could be that the grass substrates were used up for anabolic processes by the faster growing bacteria in the methanogenic consortium. It has been previously reported that the fast growing bacteria use up the readily available carbon sources (producing CO_2 and could have accounted for the increased total gas production) (Figure 3-5), leaving insufficient carbon substrates for the slower growing methanogens (Pérez *et al.*, 1997; Reeve *et al.*, 1997; Chang *et al.*, 2003; Akram and Stuckey, 2008; Buendía *et al.*, 2009). It has also been reported that bacteria seem to utilize carbon principally to build the initial biopolymer matrix (i.e. anabolism) thereby reducing the methane yield (Pérez *et al.*, 1997; Michaud *et al.*, 2005), which may have occurred in this experiment with the BOC.

3.3.2.4 Comparison of total gas volume vs methane yield

A comparison of the total gas production (Figure 3-11) and the methane yield (Figure 3-10) showed that quantitatively, the combined BOC+G produced significantly more gas than the grass control reactor, but qualitatively yielded less methane than the grass control reactor. It is known that the early stages of anaerobic digestion may be characterized by production of LMOs and CO_2 during which the hydrolysis and fermentation processes dominate (Griffin *et al.*, 1998; Riffat *et al.*, 1999; Borja *et al.*, 2005; Siegert and Banks, 2005; Cooney *et al.*, 2007). As the LMOs become available in the form of VFA among others, methanogenesis is initiated, which may be occurring in this study. Perhaps the exhaustion of the readily available VFAs or the appearance of inhibiting factors in the combined BOC+G reactor prevented continued methane production (Siegert and Banks, 2005; Moosa and Harrison, 2006; Chen *et al.*, 2008), although this suggestion would require further clarification. However, in the grass control there seemed to be continued methane production for the duration of the study. Possible reasons for this are that the grass contained more readily available carbon substrates for methanogenesis and lacked inhibiting compounds, when compared to the coal substrate. Inhibiting compounds such as phenols and aromatic macromolecular structures could be produced from BOC biodegradation (Chang *et al.*, 2006; Jin *et al.*, 2007; Chen *et al.*, 2008).

However, methane production was lower than in the combined reactor. Furthermore, this study supports the proposal that grass functions as a co-metabolite in gas production from coal.

3.3.3 Gas production from geologically oxidized coal and grass co-substrate

3.3.3.1 Total gas production

Studies on the production of methane gas using biologically oxidized coal with grass provided a preliminary indication that the presence of a co-substrate (grass) enhanced total gas production from the coal substrate. This result provided the basis for a comparison of the use of geologically oxidized coal in a similar approach.

Figure 3-12 shows total gas production over 49 days of the study using GOC both alone and in the presence of grass. The general trend of gas production followed a cyclic pattern observed previously in the BOC study (Figure 3-5) but continued throughout the experiment in the reactor with combined geologically oxidized coal plus grass (GOC+G), although a gradual decline in gas production was also observed as the study proceeded. The grass control was the same as that used in the BOC study. The results are repeated here for ease of comparison.

In the combined GOC+G reactor, total gas production of 1710 mL was observed over 49 days while 817 mL and 658 mL was observed in the GOC and grass control reactors over the same period. A daily average gas production rate of 35 $mL.d^{-1}$ was measured in the combined GOC+G (Table 3-4), while the GOC and grass controls alone recorded 48% and 38% of the combined GOC and grass substrates. Between 32 – 49 days, the daily gas production rate declined by 41% and 45% to 20.5 and 9.1 $mL.d^{-1}$ in the combined GOC+G and the GOC control respectively. In the grass control reactor, the rate decreased by 89% to ~ 1.5 $mL.d^{-1}$. This may suggest exhaustion of readily available substrates in the reactors. The combined GOC+G and the GOC control reactors both recorded the highest daily gas production rates of 112 and 58 $mL.d^{-1}$ after 5 days of the study, respectively (Table 3-4). In the grass control reactor, a highest daily gas production rate of 72 $mL.d^{-1}$ was recorded after 3 days. Again, this indicates that the grass is easier to degrade in comparison to the GOC substrate for gas production. Furthermore, this suggests that 51% of the gas produced here was derived from the oxidized HC substrate and the remaining 49% was derived from grass. The grass may serve the purpose of enhancing gas production from coal in the combined substrate reactor.

Table 3-4 Gas production rates of the biogas reactor with geologically oxidized coal with grass and the respective controls, geologically oxidized coal and grass.

		Gas Production	
Reactors	Total Gas (mL)	Highest Daily rate ($mL.d^{-1}$)	Total Average gas ($mL.d^{-1}$)
GOC + G	1710	112	35
GOC	817	58	17
G	621	72	13

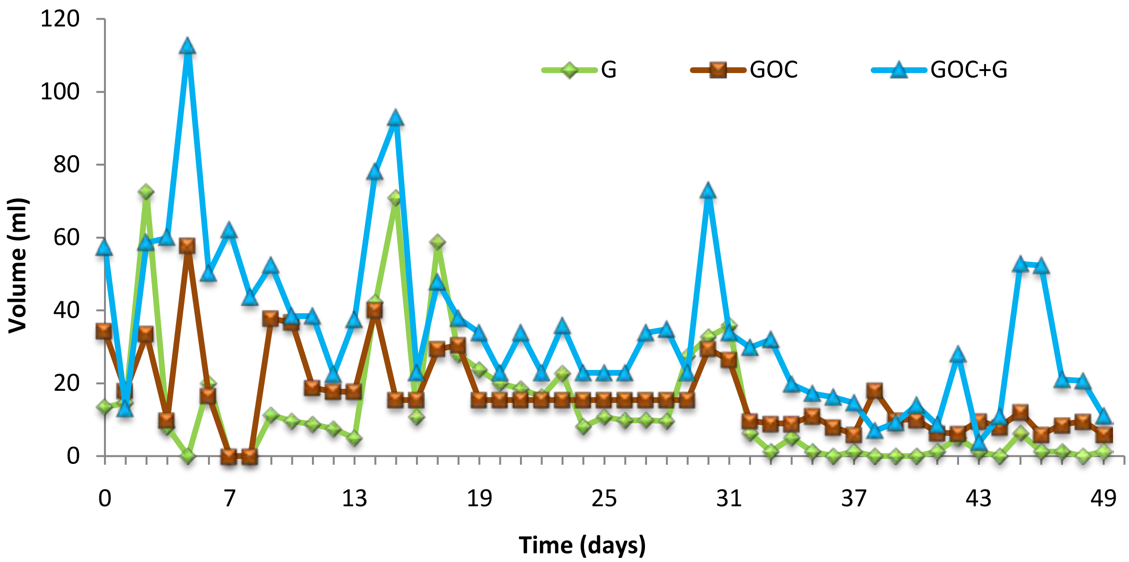


Figure 3-12 Total daily gas production in the batch reactors containing combined geologically oxidized coal plus grass substrates, including controls for geologically oxidized coal and grass separately.

3.3.3.2 Gas composition

Figure 3-13 shows the distribution of CH_4 and CO_2 in the headspace of the combined reactor with GOC+G substrate. Weekly analysis of the CH_4:CO_2 ratio showed a gradual increase in the CH_4 content and a consequent decrease in CO_2 over time. The CH_4:CO_2 ratio peaked at 59:41% after 42 days. Towards the end of the experiment, the CH_4:CO_2 ratio had decreased to 38:62%. As noted previously, CH_4:CO_2 ratios ranging from 50 – 70% indicate optimal function of the methanogenic component of the anaerobic consortium (Safinowski *et al.*, 2006; Matteson and Jenkins, 2007; Liu *et al.*, 2008). The results observed in this study fell well within that range.

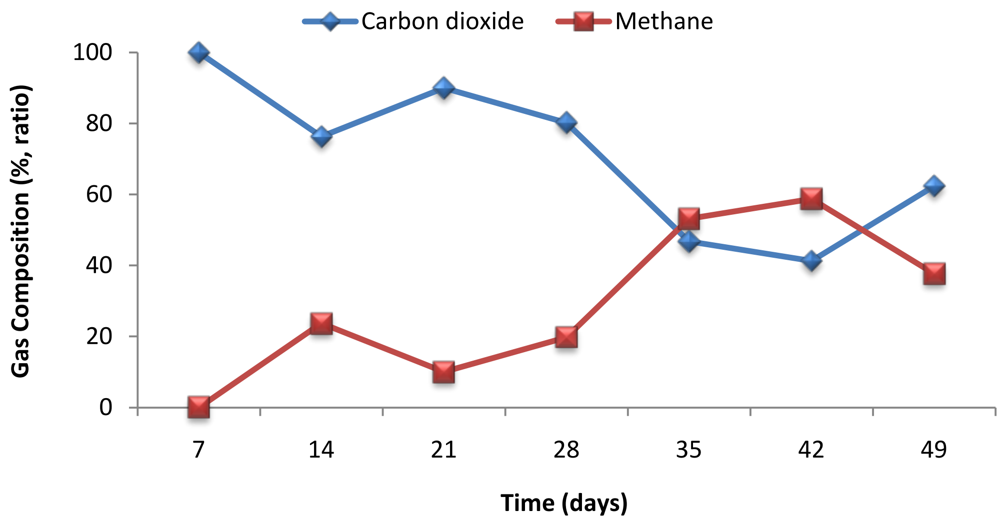


Figure 3-13 Relative amounts of changing methane: carbon dioxide ratio over time in the headspace of the combined reactor with geologically oxidized coal plus grass.

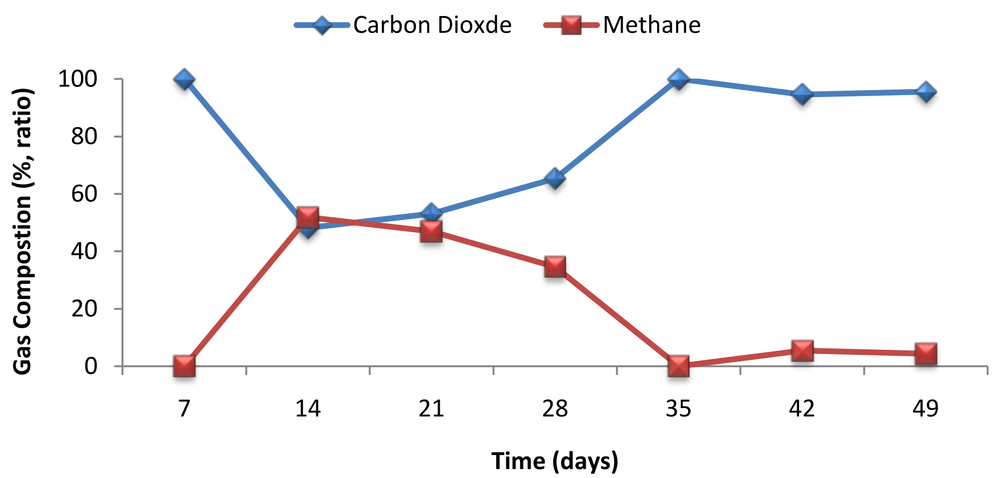


Figure 3-14 Relative amounts of the changing methane: carbon dioxide ratio over time in the headspace of the geologically oxidized coal control reactor.

Where GOC was used as the sole substrate, the CH_4:CO_2 ratio peaked at 51:49% after 14 days of the experiment and then declined to levels below detection by the end of the study (Figure 3-14). The CO_2 content decreased initially to 49% at day 14 and then gradually increased again until the end of the experiment. This may indicate active fermentation without methanogenesis (Lee *et al.*, 2009).

In the GOC control reactor, where the coal substrate was the sole carbon source, production of methane was derived from the soluble fraction of the coal, and ceased once the substrate became exhausted. This was also confirmed by the overall decrease in the volume of gas produced over time (Figure 3-12). As mentioned earlier in section 2.3.5, the fraction of GOC soluble in water is minimal (~ 35 $mg.L^{-1}$), and would have been removed from solution during the early stages of the experiment. Therefore, the microorganisms probably had to degrade the high molecular weight compounds of the coal in order to achieve the gas production observed. Figure 3-14 shows an increase in the CO_2 content as the CH_4 decreases, which may suggest the action of fermentative and hydrolyzing bacteria on the macromolecular structures of the coal substrate. This action can be correlated to the cyclical consumption pattern of gas production (Figure 3-12) and possibly indicates intermittent production of readily available substrates that eventually led to achieving an optimal CH_4:CO_2 ratio of 59:41% in the combined substrate reactor.

Towards the end of the experiment, the declining rate of gas production suggests that the hydrolytic bacteria in the methanogenic consortium were unable to degrade the remaining complex coal substrate or probably became inhibited by the presence of precursor compounds in the coal. It has been reported that bacterial activity and subsequent production of gas (methane in particular), can be inhibited by physical-chemical conditions that arise in the medium as metabolism proceeds (VanDenHeuvel and Beeftink, 1988; Siegert and Banks, 2005), and this could have occurred during this study.

The Box and Whisker plot for gas production showed significant differences between the combined GOC+G and its controls (Figure 3-15). This was also confirmed by the more sensitive Fisher LSD test in which the combination of GOC and grass showed increased production of gas, which was highly significant when compared to separate GOC and grass reactors ($p < 0.001$, Appendix 2-D). This reinforced the proposal that a co-substrate leads to improved gas production compared to a single substrate on its own (Boddy *et al.*, 2007). In this study, the grass could have provided the co-substrate enabling progressive degradation of the oxidized HC substrate and increased gas production.

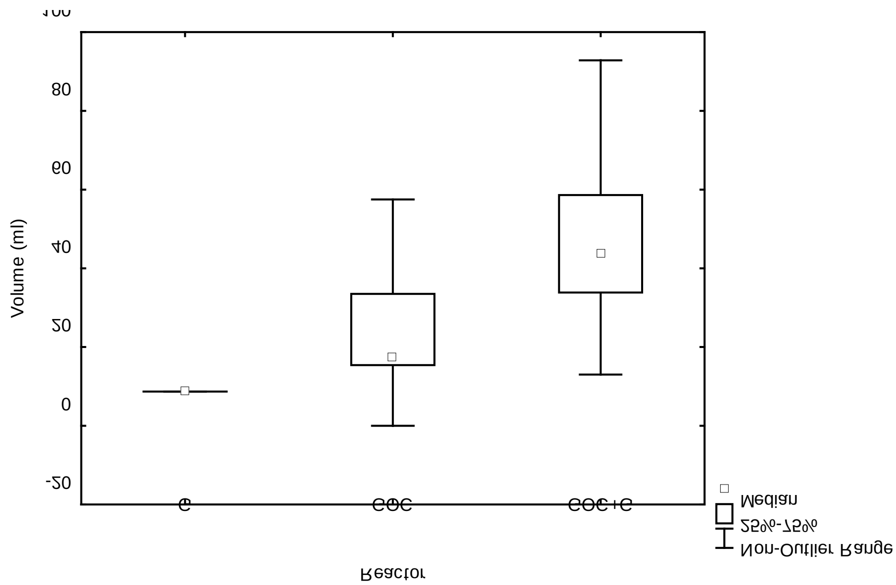


Figure 3-15 Box and Whisker plot showing the total gas production from geologically oxidized coal (GOC) with and without grass (G) and the grass control.

3.3.3.3 Methane yields

Figure 3-16 shows the weekly methane yield from the combined substrate GOC+G and the controls GOC and grass alone. In the combined substrate reactor, the methane yield increased over time and followed a cyclic increase and decrease pattern that was also observed in the total gas production results (Figure 3-12). A 2.5-fold increase in the methane yield from 3.1 mg CH_4.g substrate^{-1} to 7.6 mg CH_4.g substrate^{-1} was observed between 14 and 42 days, before decreasing by 36% to 4.8 mg CH_4.g substrate^{-1} at the end of the experiment (Figure 3-16). The methane yield in the GOC control peaked at 3.5 mg CH_4.g substrate^{-1} after 14 days, and then declined until no methane was detected at 35 days. Thereafter, 0.4 mg CH_4. g.substrate^{-1} and 0.3 mg CH_4. g.substrate^{-1} were measured after 42 and 49 days respectively. In the grass control reactor, methane yield peaked at 3.8 mg CH_4.g substrate^{-1} after 21 days, before gradually declined by 65% to 1.3 mg CH_4.g substrate^{-1} by the end of the experiment (Figure 3-16).

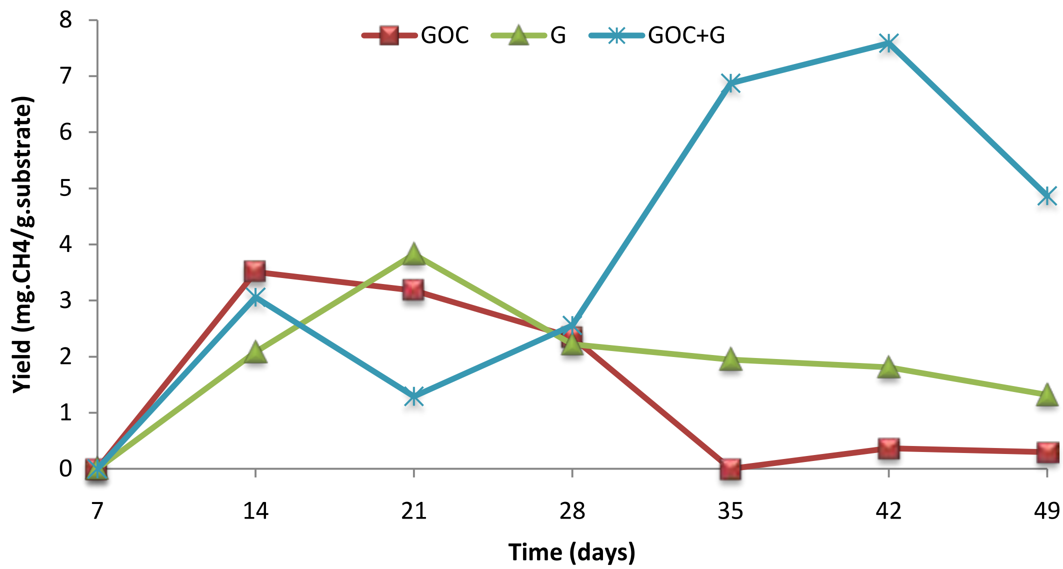


Figure 3-16 Methane yield from the biogas reactor with combined geologically oxidized coal plus grass including the geologically oxidized coal and grass control reactors alone.

The overall methane yield in the combined GOC+G reactor was 26 mg CH_4.g substrate^{-1}, while the controls for GOC and grass reactors yielded 10 and 13 mg CH_4.g substrate^{-1}, respectively. Statistical analysis of the methane yield showed a significant difference between the combined substrate reactor and the GOC control alone (Fisher, LSD $p < 0.05$) suggesting that the combined degradation of both GOC and grass substrates enhanced methane yield. However, there was no significant difference between the combined substrate reactor and the grass control alone. There was also no significant difference in methane yield between the GOC and grass controls alone (Appendix 2-E).

3.3.3.4 Comparison of total gas volume vs methane yield

A comparison of the total gas production (Figure 3-17) and the methane yield (Figure 3-16) showed that, quantitatively, the combined GOC+G produced significantly more gas than the grass control reactor, but qualitatively yielded less methane than the grass control reactor. It is known that the early stages of anaerobic digestion may be characterized by production of LMOs and CO_2 during which the hydrolysis and fermentation processes dominate (Griffin *et al.*, 1998; Riffat *et al.*, 1999; Borja *et al.*, 2005; Siegert and Banks, 2005; Cooney *et al.*, 2007). As the LMOs become available in the form of VFA among others, methanogenesis is initiated, which may be occurring in this study. It is likely that the exhaustion of the readily available VFAs or the appearance of inhibiting factors in the combined GOC+G reactor prevented continued methane production (Siegert and Banks, 2005; Moosa and Harrison, 2006; Chen *et al.*, 2008), although this suggestion would require further clarification. However, in the grass control there seemed to be continued methane production for the duration of the study. This suggest that the grass contained more readily available substrates for methanogenesis and lacked inhibiting compounds, when compared to the coal substrate. However, methane production was lower than in the combined reactor. Furthermore, it supports the proposal that grass functions as a co-metabolite in gas production from coal.

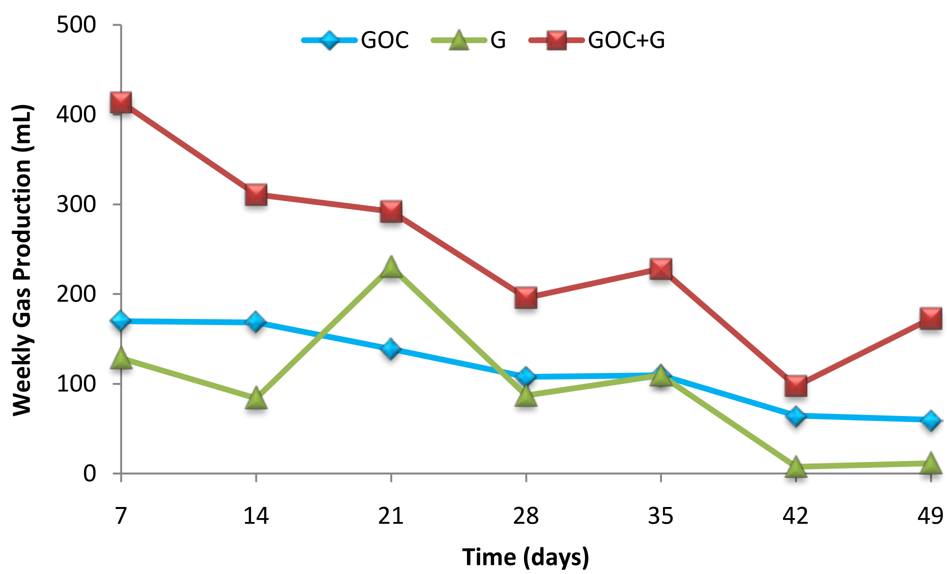


Figure 3-17 Weekly total gas production in the reactors containing combined geologically oxidized coal plus grass including the geologically oxidized coal and grass control reactors alone.

3.3.4 Comparison of biologically and geologically oxidized coal biogas reactors

A statistical comparison using the Fischer LSD test of both combinations of grass with BOC and grass with GOC, showed no significant difference ($p > 0.05$) between the BOC and GOC, although there was on average 20% more gas production in the GOC than in the BOC biogas reactors. Moreover, qualitatively, more methane was evolved in the GOC (58%) than in the BOC (28%). This suggests that more substrates readily available for methane production are present in the GOC than in the BOC substrate. This may have resulted from reduced precursor organic matter such as acetate and organic acids being available in the BOC due to consumption during the oxidation phase of the coal by rhizosphere community in the SHCB (Devasahayam, 2007;

Askaer *et al.*, 2008; Igbinigie, 2008; Wagner, 2008). Alternatively, the biological oxidation process was incomplete and would have benefited from a longer reaction period in the SHCB.
On the other hand, the biological oxidation of HC generated a substrate that was considerably more functional and amenable to methane gas production in comparison to HC, which demonstrated an inability to function as a substrate for biological processes in this study.

Previous studies by Green *et al.* (2008) on the development and optimization of a methanogenic consortium for the degradation of sub-bituminous coal (sourced from Wyodak, USA) supplemented with yeast extract as a co-substrate, reported methane yields of 0. 56 mg CH_4.g coal^{-1}, 0.13 mg CH_4.g coal^{-1} and 2, 2 mg CH_4.g coal^{-1} at a temperature of 22^{o}C, 30^{o}C and 38^{o}C respectively. Since the current study was performed at 30^{o}C, methane yields from the combined GOC+G (26 mg CH_4.g substrate^{-1}) and GOC alone (10 mg CH_4.g substrate^{-1}) are comparatively higher than those reported by Green *et al* (2008), even after they had raised the temperature of their system to 38 ^{o}C. Furthermore, an increase in the coal surface area improved their daily yields by 214%, to 0.1 mg CH_4. g substrate^{-1}. day^{-1}, while the daily yield for the GOC studies was 0.5 mg CH_4.g substrate^{-1}. day^{-1}.

In other studies on Texas Lignite involving the MicGAS process, Walia and Srivastava, (1996) reported a 7-fold increase in methane yields to 230 mL.g coal^{-1} (recalculated to 152 mg CH_4.g substrate^{-1}) after adapting their methanogenic cultures to utilize the Texas lignite as the sole source of carbon. These yields are ~ 6 fold higher than those obtained in this study, and may be due to the rank of coal. LRC such as lignite are more amenable to microbial conversions at near ambient temperatures, and would therefore provide a comparatively economical advantage over oxidized HC (Ulrich and Bower 2008). These findings point to the reactivity of the coal for methanogenesis being dependent on the presence of increased oxygen content as confirmed in section 2.3.1, and demonstrated by the results presented in this chapter. Increased oxidation of the coal substrates improves the methane yield only up to a point, however, greater performance seems to be achieved by a combination of factors including; physiological parameters such as temperature and coal surface area, and adapting microbial cultures to the specific coal (Walia and Srivastava, 1994; Ulrich and Bower, 2008).

3.4 Conclusions

The following conclusions may be drawn from these results:

- Methane gas can be produced from oxidized HC substrates using anaerobic cultures developed from environmental and industrial sources;
- It was demonstrated that both BOC and GOC could function as substrates for methane generation, and that yields for oxidized HCs are significantly better than for un-oxidized HCs;
- The recovery of methane from biologically and geologically oxidized HC substrates is a first report, since previous studies on methane production have focused on the lignites while those that have looked at HCs yield minimal amounts;
- No significant difference was demonstrated in total volume of gas production between the GOC and BOC;
- Yield of methane from GOC was substantially higher than BOC;
- It was shown that methane production from the coal substrates was enhanced in the presence of grass, which could have functioned as a co-metabolite in the biodegradation of the coal substrate.

To further investigate this proposal, VFA and Py-GCMS profiles of the reactor products were investigated in the following chapter.

CHAPTER FOUR

BIOGASIFICATION OF OXIDIZED HARD COAL SUBSTRATES 2: INTERMEDIATE METABOLITES

4. INTRODUCTION

The demonstration of methane gas production from oxidized HC raised the possibility of developing the system for energy recovery from the beneficiation of waste coal. Whether these findings would provide a basis for feasible bioprocess development would depend on a more detailed understanding of the factors limiting methane gas production in the system, which terminated after 50 days in a batch unit operation. The concerns in this regard related to observations that substantial quantities of both coal and grass co-substrate remained apparently available in the reactors when gas production declined. Questions that required attention included whether decline in gas production could be related to factors such as substrate exhaustion or end product inhibition of the biocatalyst.

In order to address these questions, investigations of the intermediate catabolic pathways in the methanogenic systems were undertaken. The anaerobic fermentation of organic matter comprises four broadly defined steps, namely hydrolysis, acidogenesis, acetogenesis and methanogenesis, and involves the catalytic action of hydrolytic, acidogenic, acetogenic and methanogenic bacteria (Fang *et al.*, 2004; Buendía *et al.*, 2009; Wang *et al.*, 2009). However, the initial hydrolysis of complex organic molecules in anaerobic systems has been frequently identified as the rate limiting step (Mata-Alvarez 2000). Both BOC and GOC are complex substrates where hydrolytic breakdown of the macromolecular structure would determine the success the subsequent steps.

VFAs are important intermediate products in methane production, and the particular forms and their concentrations affect the efficiency of the methanogenic process (Ulrich and Bower, 2008; Wang *et al.*, 2009). VFA analysis has been reported, in combination with other assays, as an important indicator of anaerobic aromatic hydrocarbon biodegradation in contaminated aquifers (Elshahed *et al.*, 2001; Ulrich and Bower, 2008).

The anaerobic degradation of the oxidized HC and grass substrates in the biogas reactors reported in the previous chapter was monitored by following the changes in the profiles of the hydrolysis and VFA products generated within the system.

The grass co-substrate had been included in the reaction process to simulate the conditions under which BOC was produced in the SHCB. As a result, it had been shown to contribute towards gas production from oxidized HC breakdown. It was therefore considered important to establish whether the grass functioned as a true co-substrate in this system enabling breakdown of the complex coal substrate or whether gas production was simply the sum of the products of the individual substrates.

4.1 Objectives

The objectives of this study were to:

- Determine the principal metabolic products of the breakdown of both oxidized HC and grass substrates;
- Investigate the fermentation products of the acidogenesis and acetogenesis steps in the anaerobic digestion pathway;
- Determine whether grass functioned as a true co-substrate in oxidized HC breakdown;
- Attempt to determine possible causes of the termination of methane production in the system.

4.2 Materials and Methods

4.2.1 Pyrolysis gas chromatography mass spectrometry

Py-GCMS of the coal and grass substrates was conducted in order to establish the transformation of the macromolecular structures under the influence of the anaerobic microbial consortium. This would provide a qualitative analysis of the reaction products, their formation and depletion over time, and to provide a diagnostic tool for prediction of methane pathways. Aliquot samples were withdrawn from the biogas reactors weekly, and freeze dried for 24 h. The dried samples were weighed into a quartz tube (~ 1 mg) and sealed with quartz glass.

The samples were then derivatized in order to preserve the functional groups of the coal fractions during pyrolysis for detection by mass spectroscopy. Tetramethylammonium hydroxide (TMAH) was used as the methylating agent (Martin *et al.*, 1994; Lehtonen *et al.*, 2000). A 25% aqueous solution of tetramethylammonium hydroxide at a 1:1 (w/v) ratio was added to ~ 1 mg of the sample in a quartz tube supported at both ends with quartz glass. One µL of poly (*tert*-butyl styrene) (0.05 $mg.mL^{-1}$ in hexane) was introduced to serve as an internal standard for semi-quantitative determination of the pyrolysates. Samples were then dried at 100°C for 10 min. The sample was pyrolyzed at 610°C for 10 s in a Chemical data system (CDS) 5000 series Pyroprobe with a CDS 1500 valve interface (CDS Analytical, Inc) that was set at 250°C and purged continuously with helium gas at 1.5 $mL.min^{-1}$. A split injection mode (1:300) with a 3 min solvent delay time was used. The oven temperature was set at 40°C and ramped up to 300°C at a rate of $6^{o}C.min^{-1}$. A capillary column (30 m x 250 µm x 0.25 µm Agilent 19091s-433; South Africa) was used. The pyrolysates were detected with the Mass spectrometer (MS) 5975 inert Mass selective detector (MSD) using the parameters 70 eV, 1.7 kV SEV, 1.1 s scan rate. The chromatographic peaks were identified using the National institute of standards and technology (NIST) library licensed to Agilent, South Africa. Further confirmation of peaks was carried out using commercial standards.

4.2.2 Volatile fatty acid analysis

VFAs were analyzed using 2 mL samples drawn from the reactors every second day. The sample was acidified with 6 N HCl (3 drops) to pH < 1.0 (Jackson-Moss, 1990) and then centrifuged at 10000 rpm for 15 min (JA -20 rotor, Beckman, USA, 25 min, 4ºC). The supernatant (0.5 µL) was injected into a Hewlett Packard 6820 gas chromatograph equipped with a Flame ionization detector (FID), and a HP-Innowax 19091N-213, 30 m x 0.32 mm, 0.5 µm column. The oven temperature was set at 150°C and the detector at 180°C. A VFA standard mix, (Supelco, South Africa) was used for calibration and the identification and quantification of VFAs produced in the reactors (Appendix 3-A).

4.2.3 pH analysis

The pH of the reactors was measured using a pH 330 meter (model WTW 82362, Germany), and throughout the experiments remained in the range of 6.5 – 7.5 which is in the optimal range for anaerobic digestion (Liu *et al.*, 2008) the actual measurements have not been reported here.

4.3 Results and Discussion

4.3.1 Pyrolysis gas chromatography mass spectroscopy of biologically oxidized coal substrates

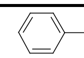
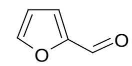
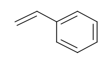
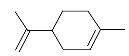
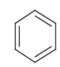
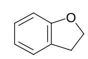
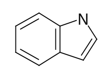
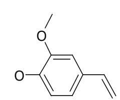
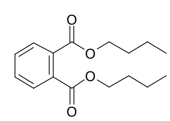
The reaction products generated from the anaerobic degradation of the combined substrates BOC+G, including the respective controls BOC and G alone, were analyzed by Py-GCMS chromatography (Figure 4-1 to Figure 4-3) and all shown at the same attenuation. Major pyrolysate products are listed in order of elution time in Table 4-1. In general, the pyrolysate products showed a trend of production, accumulation and consumption throughout the reaction process suggesting the breakdown of the oxidized HC substrate. Figure 4-1 shows the Total ion chromatograms (TIC) of the combined substrate reactor over time where toluene, limonene, furfural and benzene were apparent at the start of the study. After 21 days, a range of aromatic and long chain precursor compounds were observed but were not detected in the reactor at the end of study after 49 days (Figure 4-1).

In the BOC control reactor, oxidized precursor compounds such as furfural appeared throughout the experiment and followed a degradation pattern suggesting that it was being removed from solution by the microbial system (Figure 4-2). It was not detected after 49 days in both coal reactors possibly due to depletion of the precursor compound.

Benzene and phenolic precursor compounds including toluene and styrene were detected in the BOC control reactor at day 0 (Figure 4-2). Over time (21 days), the benzene peak decreased while, the phenol and styrene peaks increased in intensity. Carboxylated benzene derivatives and aliphatic long chain fatty acid compounds were also detected over this period. At the end of the experiment, benzene was no longer detected in the system, whilst the phenolic compound peak had increased in intensity. More aliphatic long chain compounds (C14 – 15) were also observed, while C16 was not detected after 49 days. An interesting observation was made with the appearance of limonene after day 49, which had not been detected during the earlier stages of the experiment (Figure 4-2).

In the grass control reactor, aromatic compounds such as toluene, cyclopentenone and phenolic derivatives were detected at the start of the experiment (Figure 4-3). After 21 days, an increased range of benzene and phenolic precursor compounds, that included benzyl nitrile, benzoic acid, imidazole and methoxy vinyl phenol, were observed in the reactor indicating active breakdown of the substrate by the methanogenic consortium. At the end of the experiment, methoxy vinyl phenol was still detected, while the benzene precursor such as benzyl nitrile and benzoic acid were not detected possibly due to consumption (Figure 4-3).

Table 4-1 Peak identification and compound structures of the pyrolysis products derived from the reaction of the oxidized hard coal substrates with and without grass co-substrate, with the adapted methanogenic culture. Peak numbers refer to the labeled total ion chromatogram in Figure 4-1 to Figure 4-5. Peak no. 9 may be a contaminant.

Peak No.	Compound	MW	Structure
1	Toluene	92	
2	Furfural	96	
3	Styrene	104	
4	Limonene	136	
5	Benzene	78	
6	Benzofuran	120	
7	Indole	117	
8	2-Methoxy-4-vinyl phenol	150	
9	Benzene dicarboxylic acid	278	

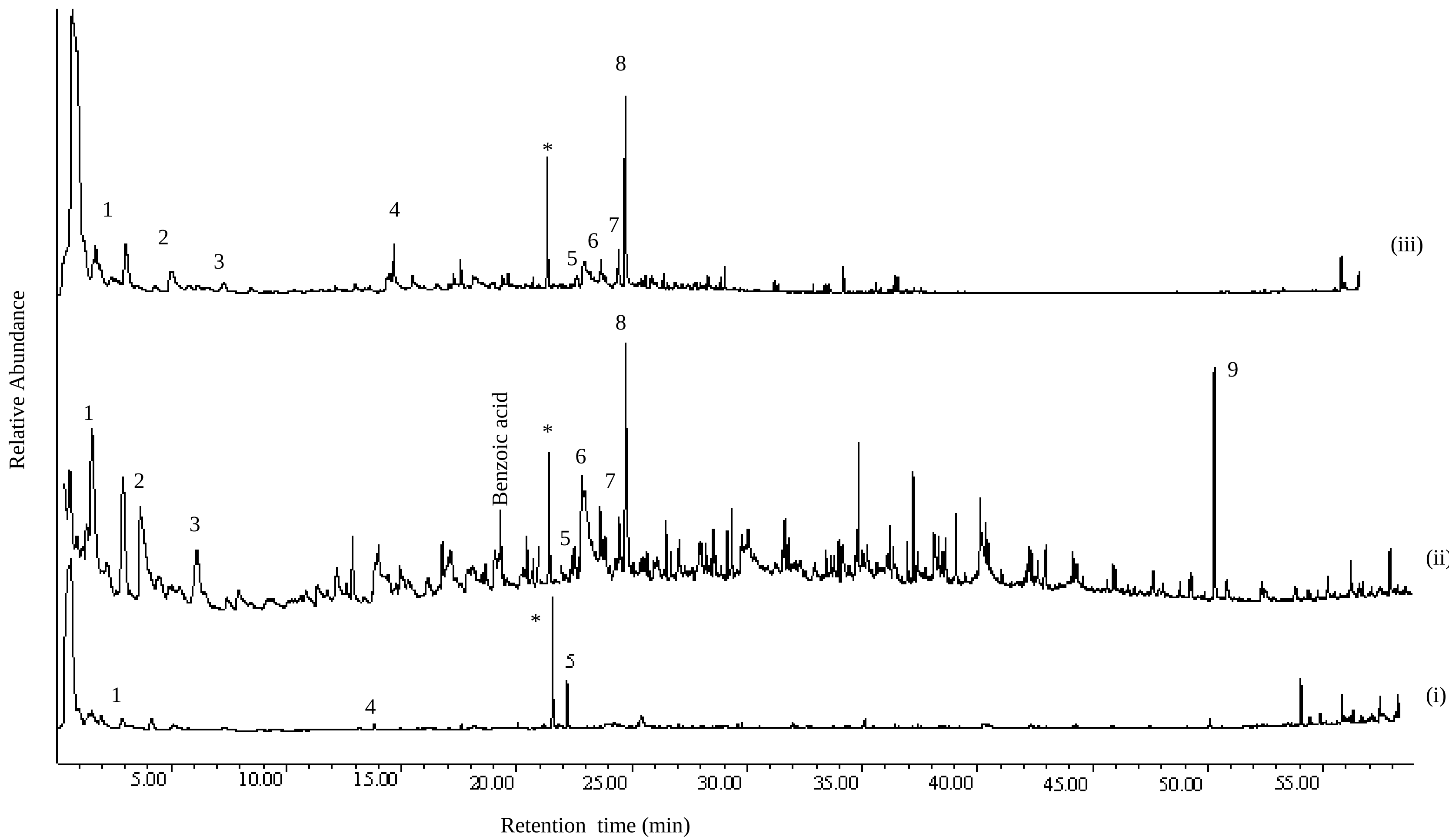


Figure 4-1 The total ion chromatogram for the combined biologically oxidized coal plus grass reactor inoculated with the adapted methanogenic culture. (i) Time day 0, (ii) 21 days (iii) 49 days. * = Internal standard (poly(ter-butylstyrene)). See Table 4-1 for peak and structure identification.

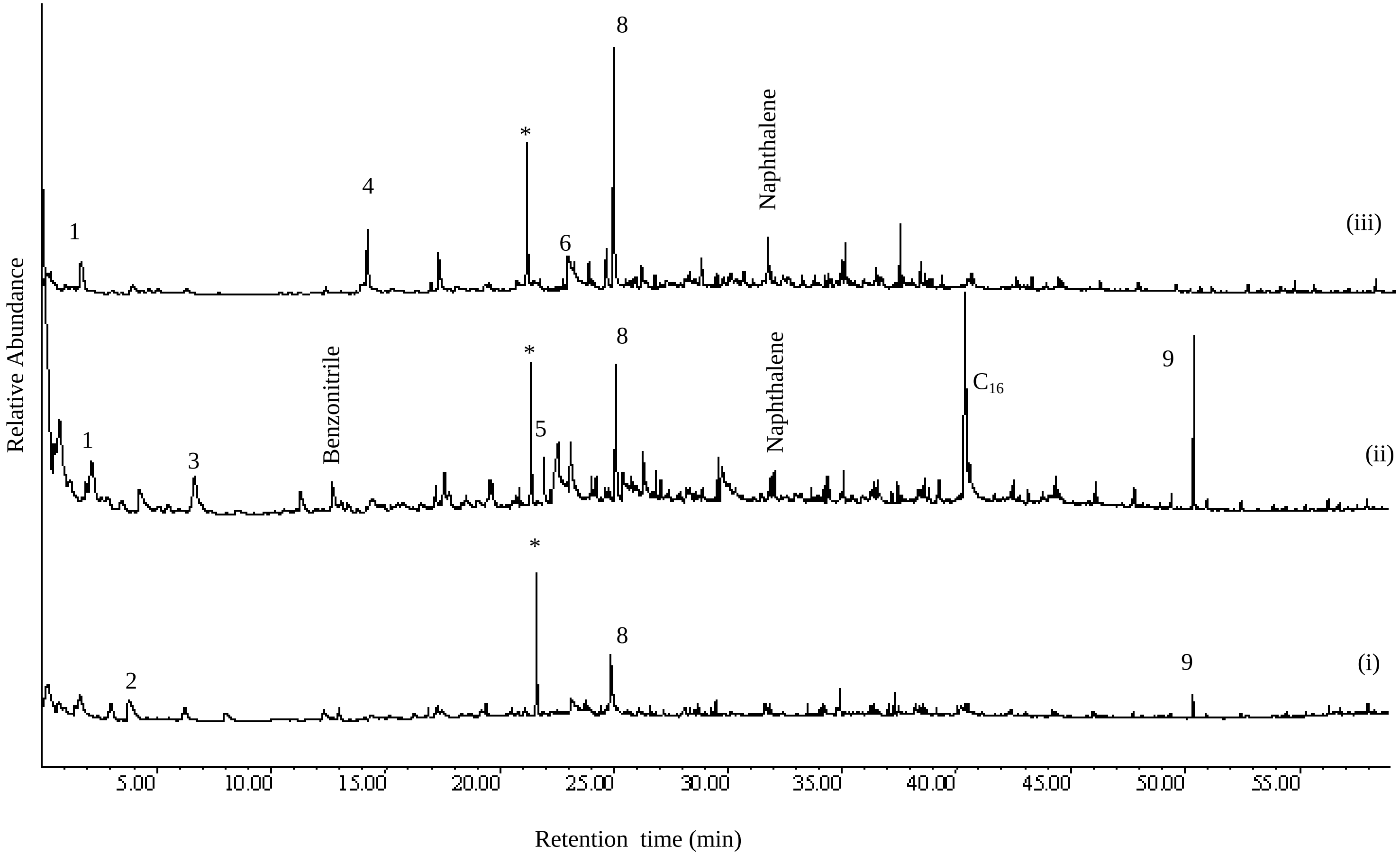


Figure 4-2 The total ion chromatogram for the biologically oxidized coal control reactor inoculated with the adapted methanogenic culture. (i) Time day 0, (ii) 21 days (iii) 49 days. * = Internal standard (poly(ter-butylstyrene)). See Table 4-1 for peak and structure identification.

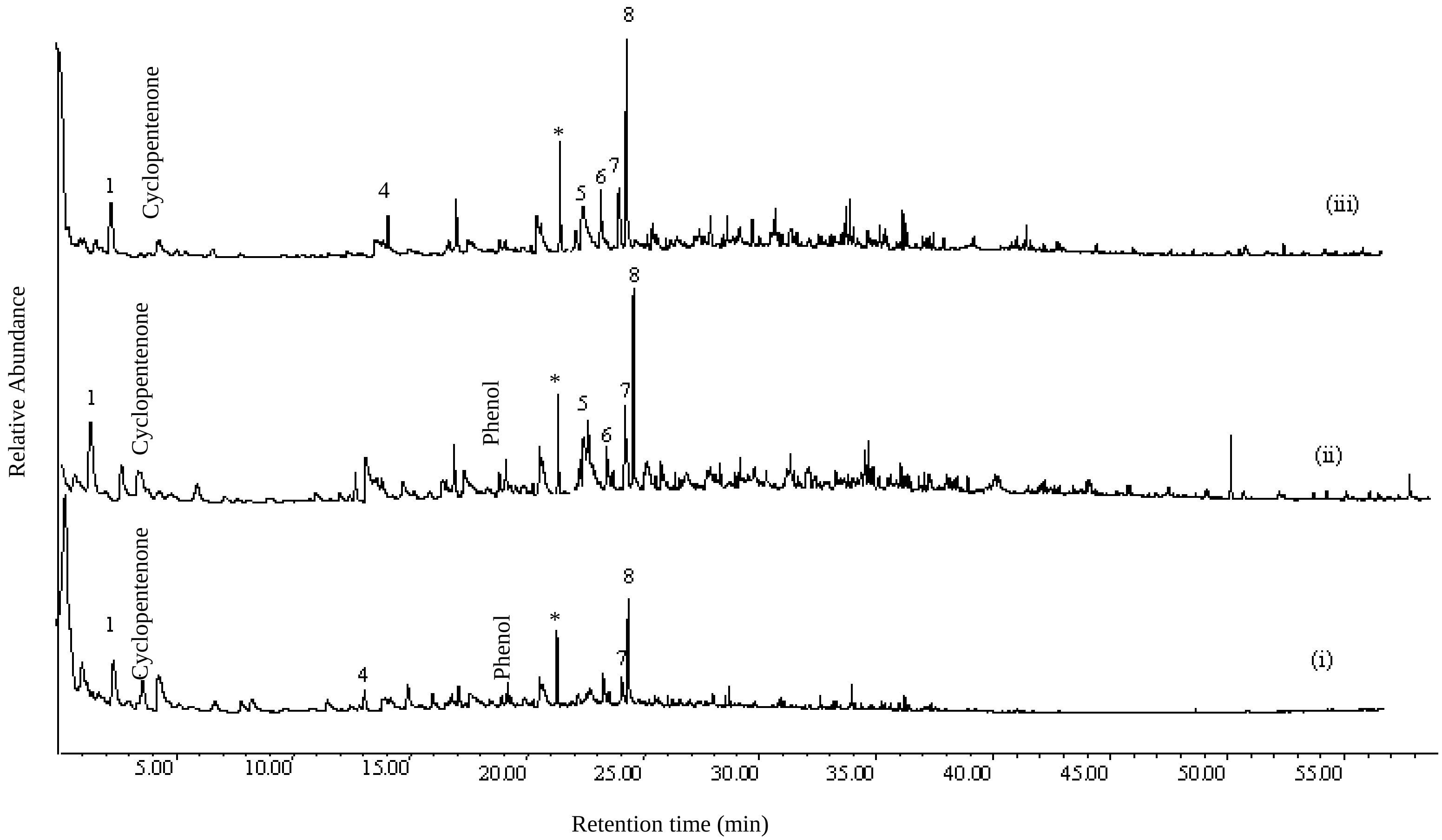


Figure 4-3 The total ion chromatogram for the grass control reactor inoculated with the adapted methanogenic culture. (i) Time day 0, (ii) 21 days (iii) 49 days. * = Internal standard (poly(ter-butylstyrene)). See Table 4-1 for peak and structure identification.

4.3.2 Pyrolysis gas chromatography mass spectroscopy of geologically oxidized coal substrates

The Py-GCMS reaction products of the methanogenic degradation of GOC with and without grass co-substrate is presented in Figure 4-4 and Figure 4-5 (See Figure 4-3 for the grass control). In the combined GOC+G substrate reactor, aromatic benzene and phenolic precursor compounds that included toluene, furfural and styrene were observed at the start of the experiment. Over time, these precursor compounds increased in intensity whilst other new compounds appeared after 21 days. After 49 days, most of the peaks had decreased in intensity or were not detected suggesting consumption by the methanogenic consortium (Figure 4-4). Aliphatic long chain precursor compounds also appeared as the study progressed over time. A similar trend was observed in the GOC control, where a limited number of compounds such as furfural, benzene, pentanedinitrile and phenol were observed throughout the experiment (Figure 4-5). Furfural was detected at the start of the experiment in the GOC control reactor and then was not detected for the remainder of the experiment, (Figure 4-5).

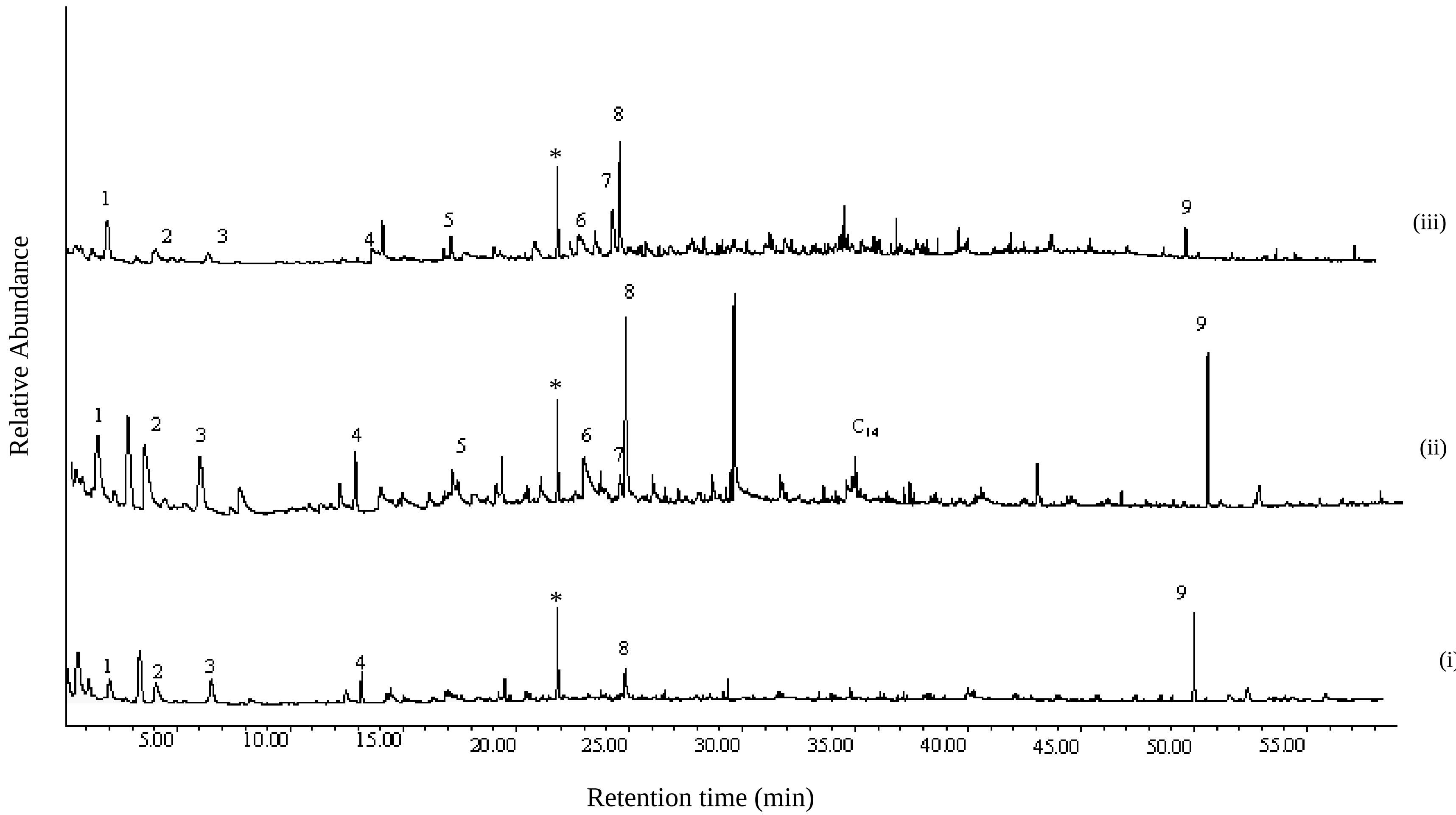


Figure 4-4 The total ion chromatogram for the combined geologically oxidized coal plus grass reactor inoculated with the adapted methanogenic culture. (i) Time day 0, (ii) 21 days (iii) 49 days. * = Internal standard (poly(ter-butylstyrene)). See Table 4-1 for peak and structure identification.

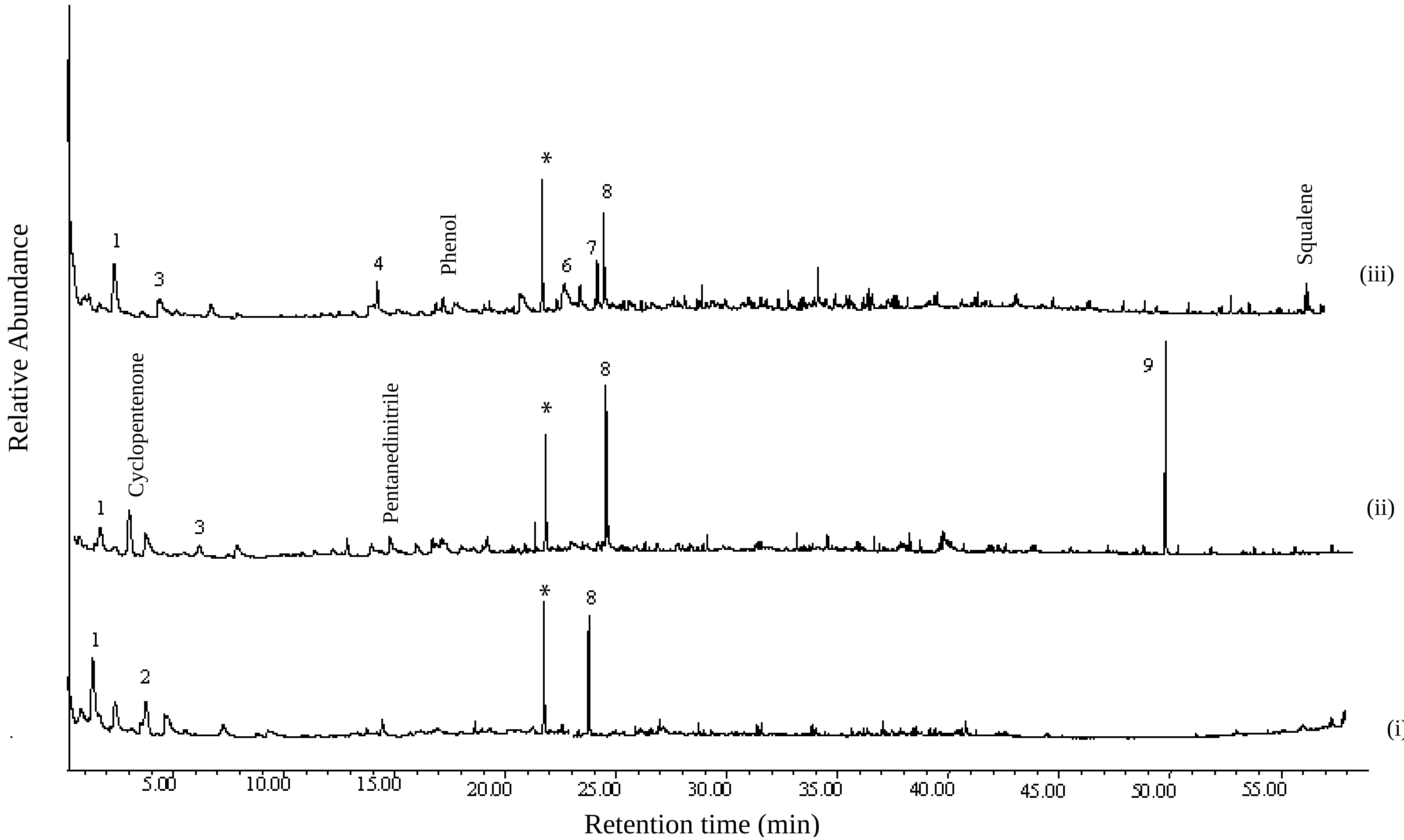


Figure 4-5 The total ion chromatogram for the geologically oxidized coal control reactor inoculated with the adapted methanogenic culture. (i) Time day 0, (ii) 21 days (iii) 49 days. * = Internal standard (poly(ter-butylstyrene)). See Table 4-1 for peak and structure identification.

4.3.3 Comparison of combined substrate products

The pyrolysates observed from the combined reactors showed the presence of precursor molecules that had the following functional groups: hydroxyl (–OH) in phenols; carboxyl (–COOH) in carboxylic acids, carbonyl (–C=O), aldehydes, amides, quinones, esters, and nitro ($-NO_2$). Compounds such as indole, naphthalene, toluene and styrene have been reported as breakdown products from coal and lignin compounds and were observed in both combined reactors and could have originated from either the grass or the coal (Figure 4-1 and Figure 4-4) (Broholm *et al.*, 1999; Zhang *et al.*, 2000; Chang *et al.*, 2003; Safinowski *et al.*, 2006). Nevertheless, both coal and grass are derived from, and/or contain, lignin and cellulose polysaccharides, although the levels of modification are different (Mansfield *et al.*, 1999; Pareek *et al.*, 2000). Naphthalene was only detected after week three in all the reactors. This could indicate that it is one of the intermediate products not readily broken down. A trend of variable increase and decrease of some pyrolysate products suggests a possible cyclic utilization of the compounds. Methoxy vinyl phenol appeared to follow such a cyclic pattern of production and consumption, and this could reflect changes in the rate of macromolecular hydrolysis followed by rapid consumption of smaller molecular weight co-substrates derived from this process.

The appearance of long chain aliphatic molecules in both coal substrates (Figure 4-1 and Figure 4-4) was also noted, as they were not present at the start of the experiment. These could have been derived from the microbial biomass populations in the biogas reactors or possibly appeared as the intermediate products of aromatic hydrocarbon degradation (Fang *et al.*, 2006; Safinowski *et al.*, 2006).

4.3.3.1 Coal derived compounds

Coal derived compounds were mainly aromatic and the pyrolysate products observed here resemble the general make up of coal structures (Figure 4-2 and Figure 4-5). They include benzene, naphthalene, toluene, styrene (Rockne and Strand, 2001; Safinowski *et al.*, 2006; Demirbas, 2007). Humic acid, which is one of the main constituents of coal, can be oxidatively degraded to yield low molecular weight compounds and carbon dioxide (Said-Pullicino and Gigliotti, 2007), which yielded the observed compounds (Figure 4-2 and Figure 4-5).

4.3.3.2 Grass derived compounds

In the grass control reactor, the appearance of several pyrolysate products throughout the experiment was noted. Figure 4-3 shows the production and consumption of pyrolysate compounds over time. Methylated phenols followed a cyclic pattern, in which they appeared at the beginning of the experiment, and thereafter not detected, possibly indicating consumption during the experiment, and re-appeared once again at the end of the experiment when methane production had ceased. Carboxylic acid based pyrolysates, similar to furan-carboxylic acid, only appeared at the start of the experiment, and were not detected thereafter. These precursor molecules could represent intermediate products in the anaerobic degradation of aromatic compounds to generate VFA. The breakdown of polycyclic aromatic compounds such as naphthalene involves reduction and carboxylation reactions (Zhang *et al.*, 2000; Safinowski *et al.*, 2006), therefore the behavior of these precursor compounds in the biogas reactors could be due to these on-going reactions.

Lignin and cellulose are complex phenylpropane polymers found in grass and coal, which are easily broken down into phenol and its derivatives by pyrolysis (Jin *et al.*, 2007). The anaerobic degradation of these polymers could have introduced several phenolic compounds into the medium (Figure 4-3), such as phenol, methoxy-phenol, dimethoxy phenol, ethyl phenol, vinyl phenol and phenyl methyl phenol (Rozzi and Remigi, 2004; Jin *et al.*, 2007). These pyrolysates indicate a pathway for the breakdown of the grass and coal (Rozzi and Remigi, 2004; Jin *et al.*, 2007). A gradual build up of vinyl phenol was observed over time, with the highest peaks being seen after 49 days (Figure 4-3), which corresponded to the termination of gas production. This may suggest an inhibitory effect of phenolic compounds on the anaerobic bacteria in the system. Jin et al (2007) reported the inhibition of cellulose degradation by phenol, methoxy-4-methylphenol, 1,4-benzenediol, and 2,6-Di-tert-butyl-4-methylphenol. They based their experiments on the yield of acetic acid in the reaction mixtures and reported that the more stable 2,6-Di-tert-butyl-4-methylphenol, had a greater inhibitory effect than the others due to its stronger resistance to oxidative decomposition.

4.3.3.3 Trend analysis of selected compounds

In addition to the observed appearance and disappearance of pyrolysate compounds during the methanogenic degradation of coal substrate with and without grass, a number of peaks were found to be specific to either BOC (Figure 4-1 and Figure 4-2) or GOC (Figure 4-4 and Figure

4-5) or the grass co-substrate (Figure 4-3). It was noted that reactors containing both oxidized HC substrates produced benzene-derived products such as benzoic acid and benzonitrile, while reactors that contained the grass co-substrate generated phenol-related (methyl vinyl phenol and methoxy phenol) in addition to benzene derived compounds observed in the coal reactors. This observation may relate to the origin of the macromolecular structures of the coal substrates from plant material (Thomas, 2002). In order to predict the metabolic pathways occurring in the methanogenic reactors, semi-quantification of phenol, furfural, limonene and cyclopentenone pyrolysate compounds is further discussed below.

4.3.3.3.1 Phenolic compounds

Semi-quantitative results of selected pyrolysates such as 2-methoxy-4-vinyl phenol in both oxidized HC substrates showed a cyclic pattern of production that was followed by depletion and then accumulation again, and was coupled to the termination of gas production. In the combined BOC+G reactor, 0.048 µg of 2-methoxy-4-vinyl phenol, increased ~ 2-fold to 0.102 µg after 7 days (Figure 4-6). This was followed by a utilization period over 14 days, where it was not detected and reappeared at 0.016 µg after 28 days and increased 3.5-fold to 0.054 µg by the end of the experiment.

In the combined GOC+G reactor, the initial 2-methoxy phenol concentration (0.079 µg) was higher and increased 4-fold to 0.344 µg over 7 days suggesting hydrolysis of the macromolecules of the two substrates (Figure 4-6). As the experiment progressed there was no indication of this pyrolysate being formed in the combined GOC+G reactor. This may have been due to its utilization resulting in undetectable levels. This corresponded to the highest average gas production (Figure 3-12) and CH_4 to CO_2 ratio (Figure 3-13) during the experiment. At the end of the experiment 0.101 µg of methoxy phenol was detected suggesting either reduced utilization of this compound or possibly end- product inhibition of the culture. Both grass (0.027 µg) and GOC (0.046 µg) separately contained minimal concentrations of this pyrolysate at the start of the experiment suggesting that production of this compound occurred during the experiment but effectively only where coal and grass were present together (Figure 4-6).

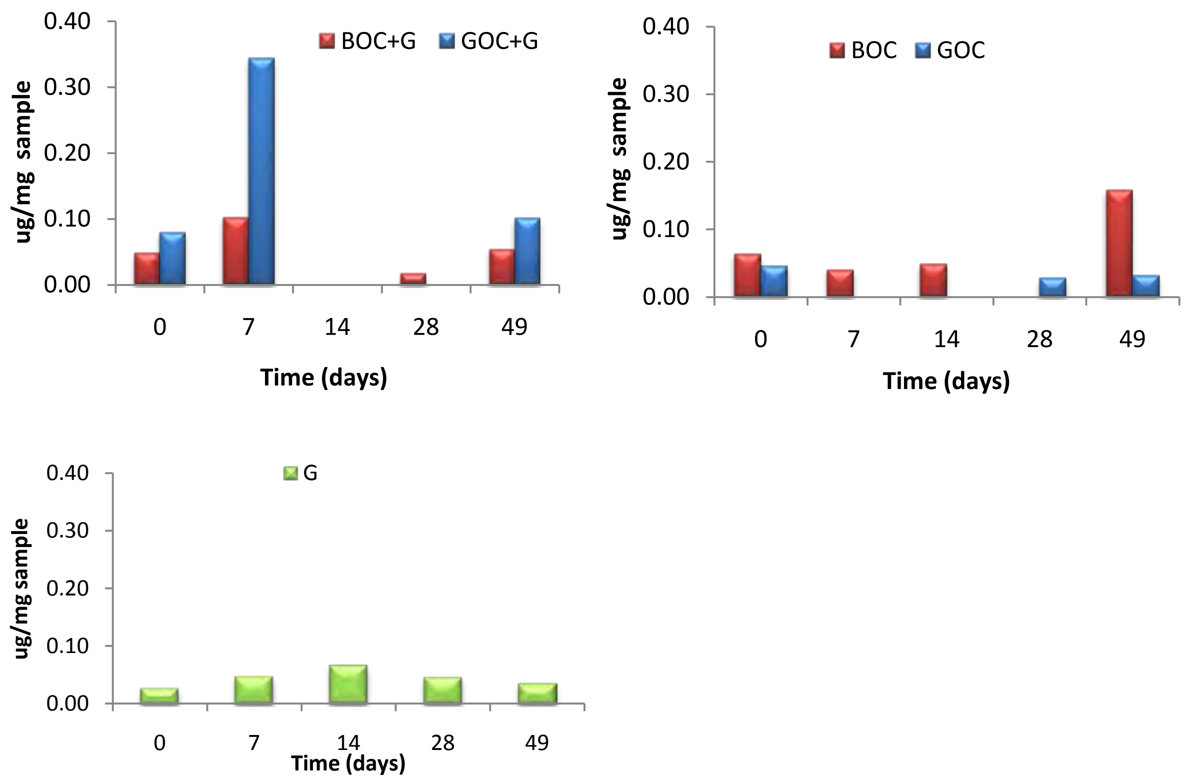


Figure 4-6 Semi quantitative analysis of 2-methoxy-4-vinyl phenol pyrolysates detected in the combined substrate reactors and the respective controls.

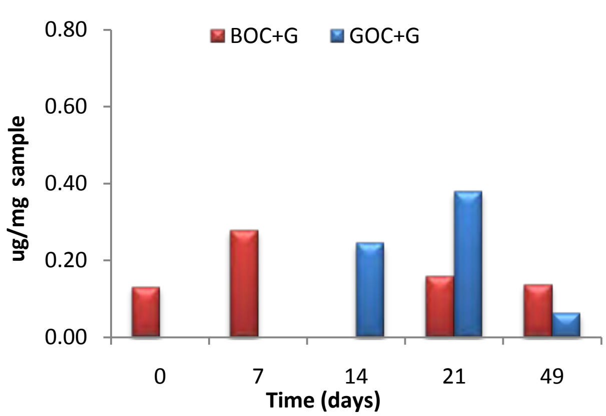
The observed increase in the concentration of 2-methoxy phenol in both combined coal plus grass substrate reactors but not in the respective controls throughout the experiment indicated the possible role of grass as a true co-substrate in the anaerobic degradation of the coal substrate. This could have supplied the necessary compounds required to initiate hydrolysis of the larger lignin based complex structures that make up the coal and grass, thereby resulting in production of the methylated phenols observed during the experiment (Kindzierski *et al.*, 1991; Boopathy, 1997; Fang *et al.*, 2004). Once produced, the 2-methoxy phenol precursor was rapidly utilized probably to generate the substrates required for methane production.

4.3.3.3.2 Furfural

Figure 4-7 shows the appearance and disappearance of furfural in the both combined coal and grass substrates and their respective controls. In the combined BOC+ G, the furfural concentration peak at 0.28 µg after 7 days, and was not detected after 14 days, after which 0.16 µg was detected at day 21. By day 49, the concentration had decreased to 0.14 µg. A lower concentration of furfural was observed in the BOC control alone, where only 0.07 µg was detected at day 0, and increased ~ 3 –fold to peak at 0.2 µg after 14 days. Thereafter, it decreased substantially by 80% to 0.04 µg after 21 days, and was not detected for the remainder of the experiment (Figure 4-7).

In the combined GOC+G, furfural was only detected after 14 days (0.24 µg), and then it increased by 58% to 0.38 µg after 21 days (Figure 4-7). By the end of the experiment, the furfural concentration had decreased by 84% to 0.06 µg. In the control GOC reactor, furfural was only detected after 7 days (0.62 µg) after which it disappeared from the system, until the end of experiment when it was detected at a higher concentration (0.70 µg). Furfural was not detected in the grass control alone throughout the study, indicating that is originated from the coal substrates.

The biological weathering and oxidation processes in the SHCB system may have contributed to the production of this furfural in the BOC therefore leading to its detection in the early stages of the experiment. While in the GOC reactors, it was only detected after contact with the methanogenic consortium as result of the coal breakdown (Figure 4-7). In the combined substrate reactors, the furfural followed a production and consumption in the presence of the co-substrate, where there was more consumption than production. This was not observed in the control reactors, where its breakdown was not as efficient as in the combined substrate rectors. This may indicate the inability of the methanogenic consortium to effectively degrade this compound in the absence of a co-substrate. This strongly suggests that the grass functioned as a true co-substrate in the utilization of furfural by the methanogenic consortium.



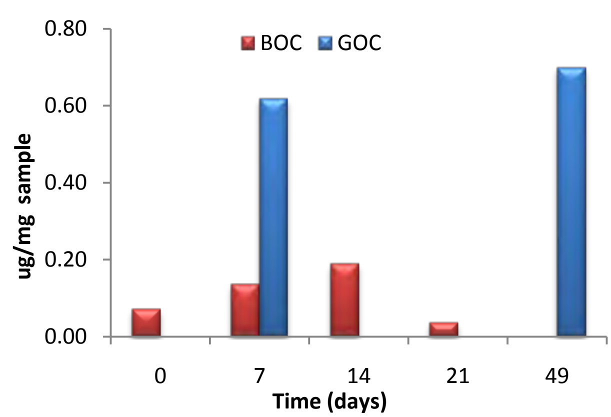


Figure 4-7 Semi quantitative analysis of furfural pyrolysates detected in the combined substrate reactors and the respective controls.

4.3.3.3.3 Benzene

Benzene was detected throughout the experiment in the BOC reactor, it may have followed a production and consumption pattern, and was not detected after day 21. However in the combined BOC+G reactor it is only found at day 14.

Pyrolysates like benzonitrile, benzyl nitrile only appeared at day 21. This may suggest that degradation of coal follows a nitration pathway as proposed by Alvarez *et al.* (2003) and Kulkarni and Chaudhari (2007). This may imply that the microbial consortium degrades the coal by inserting nitrogen into the molecules as observed in the appearance of nitrated compounds such as pyridine in the BOC and GOC reactors, which only appears at day 21 of the experiment (Figure 4-2and Figure 4-5).

4.3.3.3.4 Limonene

Limonene was one of the pyrolysis products observed in all the reactors that had grass and demonstrated changes in concentration during the study. At the start of the combined BOC reactor experiment, the concentration was 0.051 µg (Figure 4-8). This disappeared during the experiment, only to reappear at about twice the concentration (0.115 µg) at day 49. However, in the combined GOC reactor, the concentration at the end of the experiment had decreased to 0.024 µg. While it was not detected during the experiment, the utilization rate of this precursor compound could have been faster than the rate of accumulation, only to slow down again possibly due to inhibition of the culture or exhaustion of substrates necessary for its breakdown. The grass control followed the same trend as the combined BOC, except for the appearance at day 28 (0.06 µg) and a much higher concentration (0.149 µg) at day 49.

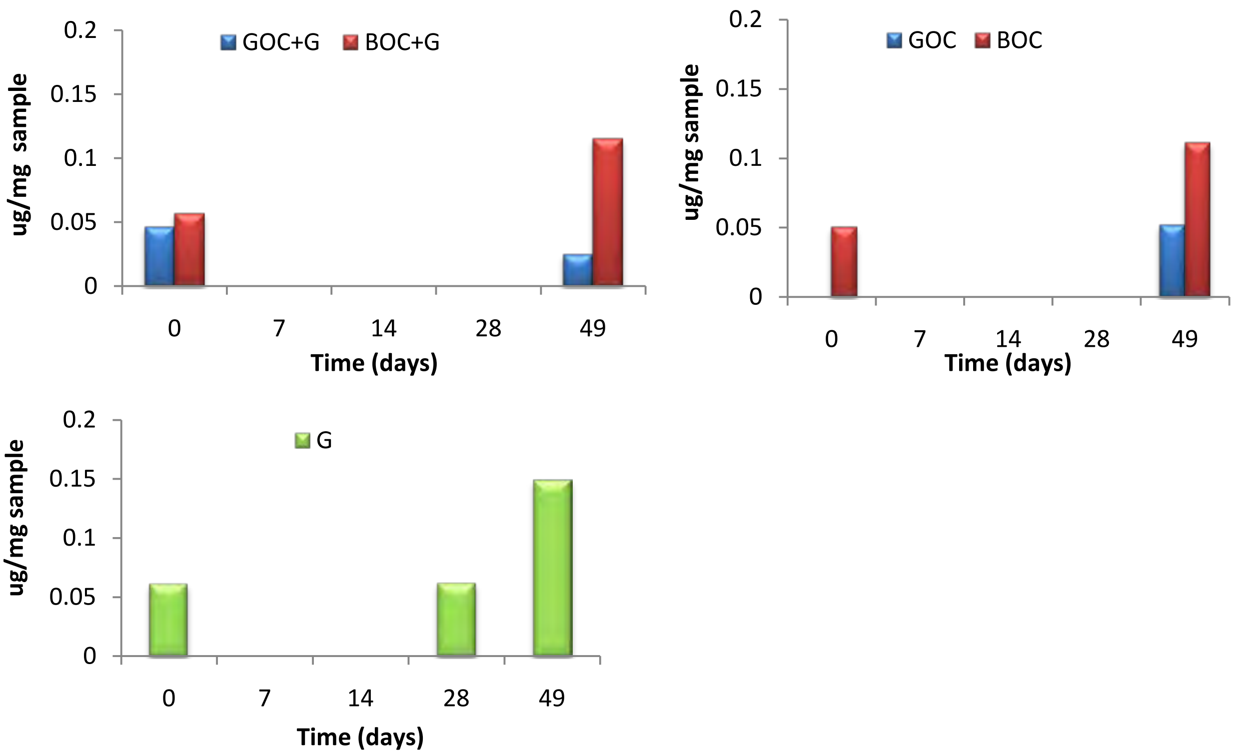


Figure 4-8 Semi quantitative analysis of limonene pyrolysates detected in the combined substrate reactors and the respective controls.

While coal is also primarily of plant origin, the processes involved in its formation probably modifies compounds such as limonene so that it is not observed. Limonene falls under a large and diversified class of hydrocarbons known as terpenes (Vanamala *et al.*, 2007). These terpenes are produced by a wide range of plants and have been suggested to induce defense systems to protect plant tissues by acting as signaling molecules (Flamini *et al.*, 2007; Yamasaki *et al.*, 2007). Limonene is also the major component of essential oils extracted from the rind of citrus fruits (Yamasaki *et al.*, 2007). These reports point to the origin of this compound to be the grass.

4.3.3.3.5 Cyclopentenone

Semi-quantitative analysis of the cyclopentenone pyrolysate is presented in Figure 4-9. Low initial concentrations of this pyrolysate were observed at day 0, in the combined BOC+G reactor (0.028 µg) and GOC+G (0.056 µg). After 7 days, the concentration increased 3-fold to 0.09 µg in the combined BOC+G reactor. Thereafter it was not detected in the reactor suggesting utilization until it reappeared at 0.01 µg after 28 days and then increased 3-fold again to 0.03 µg. This may indicate a cyclic pattern of accumulation and utilization. In the combined GOC+G substrate reactor (Figure 4-9), cyclopentenone production increased over time and peaked at 0.12 µg after 28 days, and then disappeared from the system until the reaction was terminated after 48 days. This may suggest an active production period that was followed by utilization as observed in the combined BOC+G substrate reactor. The trends for combined BOC+G and GOC+G substrates observed towards the end of the experiment corresponded with the decline in gas production as the co-substrate had been exhausted, and therefore could have become the rate-limiting step in the methanogenesis process. It must be noted that at peak gas production (Figure 3-5 and Figure 3-12) during week 2 and 3, consumption of this compound was apparently high.

Figure 4-9 indicates that there was no production of cyclopentenone during the experiment from the coal substrates except for the 0.01 µg detected in the GOC control reactor after 49 days was which could have been an unexpected contaminant. In the grass control reactor, the amount of cyclopentenone peaked after 7 days. Thereafter, it largely disappeared from the system suggesting utilization by the methanogenic consortium. This indicates that the precursor compound of cyclopentenone was derived from the grass. Guo *et al.* (2003) reported cyclopentenone as being a common pyrolysate of aquatic organic matter and cited that it is a product of polycarboxylic compounds. It has also been reported that cyclopentenones together with furans indicate the presence of carbohydrates (Çoban-Yildiz *et al.*, 2000; Guo *et al.*, 2003) and similarly Van Heemst *et al.* (1996) identified cyclopentenones and furans as polysaccharide products. In another study Çoban-Yildiz *et al.* (2000) also reported that cyclopentenones are pyrolysis products of carbohydrates. The results obtained in this study correspond to these findings and suggest that the grass functioned as a true co-substrate by providing carbohydrates for the initial phase of coal breakdown hydrolysis in the anaerobic digestion process.

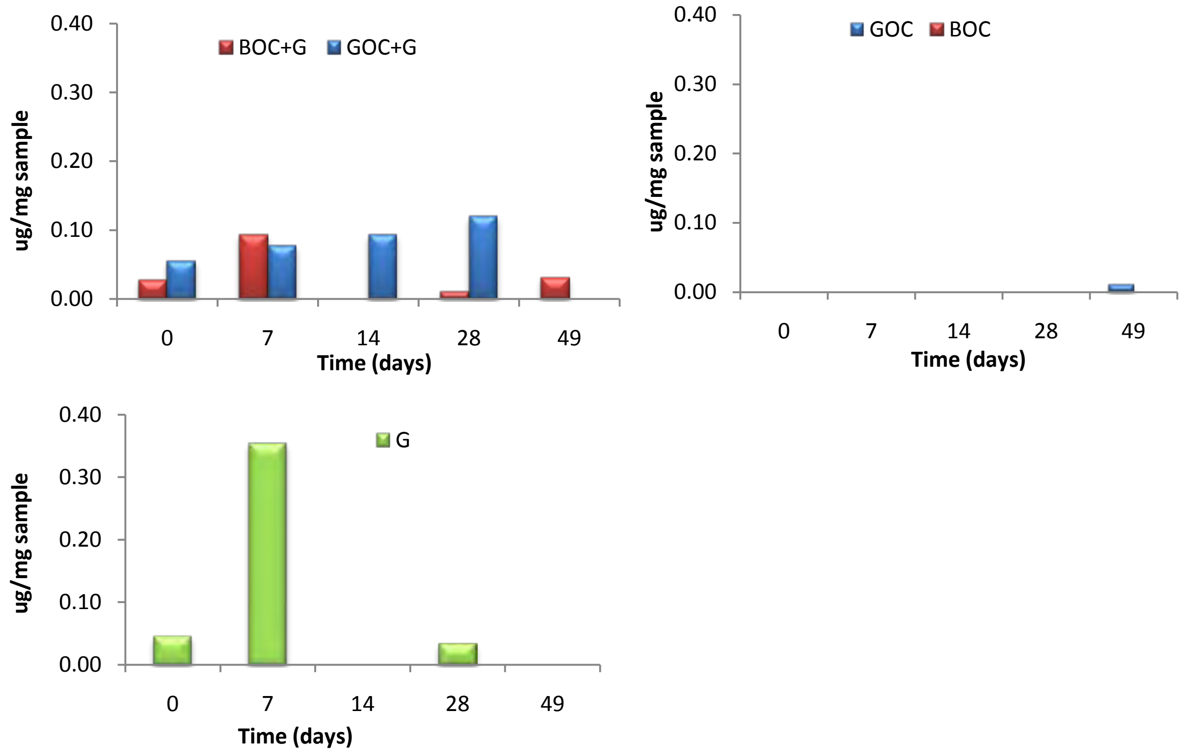


Figure 4-9 Semi quantitative analysis of cyclopentenone pyrolysates detected in the combined substrate reactors and the respective controls.

The semi-quantification of selected pyrolysates discussed above provided an indication of the hydrolytic degradation of the oxidized HC substrates in the presence and absence of the grass co-substrate. The observed trends suggest an accumulation and consumption pattern occurring in the methanogenic system, although some of these compounds may have led to inhibition of gas production. Whilst recognizing that these compounds are derivatized pyrolysates of more complex molecules in the system, a more detailed investigation is needed to confirm their actual presence in the system, in the forms reported here. In light of these observations on the hydrolytic step, a detailed investigation of the acidogenic components of the anaerobic digestion process was undertaken to interrogate aspects of the breakdown of the coal structures.

4.3.4 Volatile fatty acid profile

4.3.4.1 Biologically oxidized coal plus grass

The results presented in Figure 4-10 show the production and consumption of VFA in the reactor with combined BOC+G substrate over a 37 day reaction period. Both acetic acid (474 µM) and butyric acid (129 µM) were observed to be present in the reaction medium. After 18 days, the acetic acid had decreased by 77% to 105 µM, while a ~ 2.4-fold increase in the butyric acid to 307 µM was observed over the same period. At this stage the appearance of formic acid (83 µM), propionic acid (650 µM), and caprionic acid (29 µM) was noted. After 37 days neither, acetic nor butyric, valeric and caprionic acid could be detected in the reactor. The VFAs remaining in the reactor were formic acid (40 µM) and propionic acid (43 µM) with heptanoic acid (32 µM) as the only long chain fatty acid observed. By this stage, gas production in the reactor had ceased (see Figure 3-7).

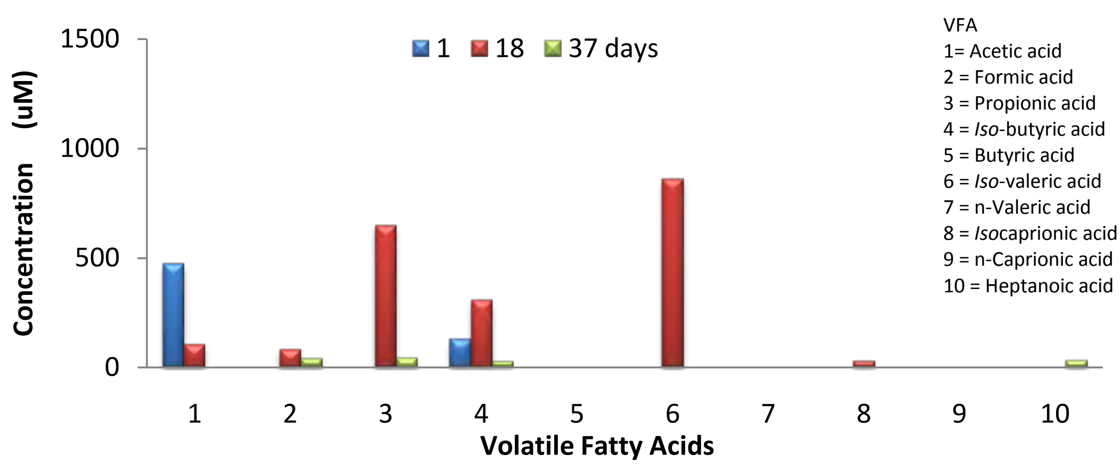


Figure 4-10 Volatile fatty acid concentration profile in the combined biologically oxidized coal plus grass substrate reactor.

The degradation of acetic acid in the first 18 days of the experiment in the combined BOC with grass reactor is consistent with active methanogenesis, and it corresponds to the highest daily gas production rate (Figure 3-5), with a CH_4:CO_2 ratio of ~ 22:78% over the same period (Figure 3-6). The absence of acetic acid at day 37 either suggests elevated conversion to methane or inhibition of the acetogenic stage by the presence of other VFAs. Acetate is the main precursor for methane production during anaerobic degradation of complex organic matter (Karakashev *et al.*, 2006). The two mechanisms of acetate metabolism namely acetoclastic and syntrophic acetate oxidation determine the production of methane in anaerobic systems and depend on the reaction kinetics (Garcia *et al.*, 2000; Karakashev *et al.*, 2006). The former pathway is driven by faster growing *Methanosarcinaceae* and generally has a higher threshold for acetic acid. The syntrophic oxidation of acetate is the main pathway in the presence of inhibitors such as ammonia and other VFAs. In kinetic studies of VFA anaerobic degradation performed in batch reactors, Boltes *et al.* (2008) reported that the accumulation of propionic acid resulted in inhibition of the acetogenesis process. This can be correlated to the results presented in the current study, where the production of the propionic acid corresponded to the termination of gas production in the reactor.

The increased concentration of propionic acid (650 µM) after 18 days in the reactors with combined BOC+G substrate suggests the hydrolysis of macro-molecules leading to the production of *iso*-valeric acid (861 µM), which was then oxidized to propionic acid. The propionic acid could then have been consumed more slowly until the end of the experiment as observed by its reduced concentration (43 µM) after 37 days.

4.3.4.2 Biologically oxidized coal control reactor

Figure 4-11 shows the VFA profile of the BOC control reactor in the absence of grass. The acetic acid concentration at day 0 was double that in the combined substrate reactor (1193 µM) which indicates BOC as the larger source of the initial observed acetate. This then decreased by 70% after 18 days, and was not detected after 37 days by which time gas production had ceased (Figure 3-7). Propionic acid (72 µM), and butyric acid (185 µM) were also detected at the beginning of the experiment, but increased by 38% and 15% respectively after 18 days suggesting VFA generation from the hydrolysis of intermediate metabolites derived from hydrolysis of BOC. Valeric acid (44 µM) was only detected in the reactor at the beginning of the experiment. After 37 days the concentrations of both propionic and butyric acid had decreased by 85% and 87% to 15 and 26 µM respectively, suggesting a similar method of reaction control exercised by propionic acid as noted in the combined BOC+G substrate reactor.

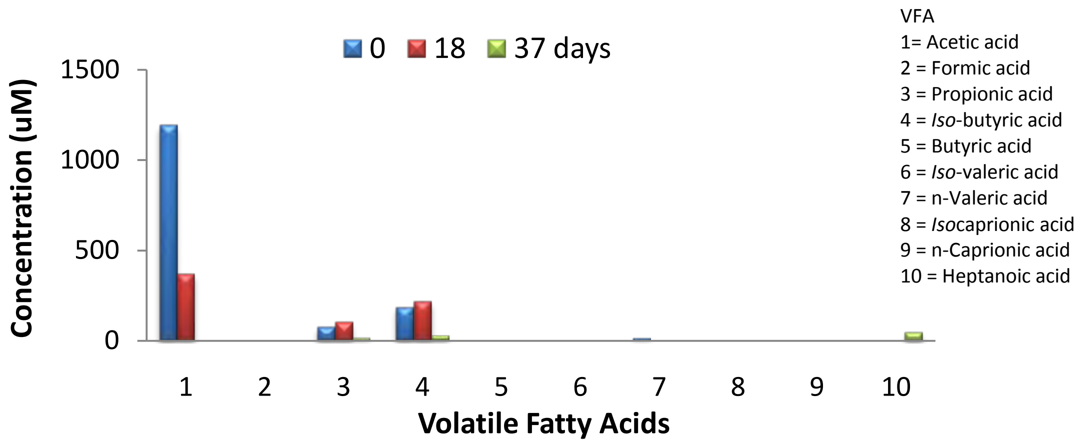


Figure 4-11 Volatile fatty acid concentration profile in the biologically oxidized coal control reactor.

4.3.4.3 Grass control reactor

Figure 4-12 shows the VFA profile observed in the grass control reactor and confirms BOC as the larger source of the initial acetate. The predominant utilization of acetic acid observed throughout the experiment suggests that the acetoclastic pathway was primarily used for methane production. The decline in acetic acid correlates with the reduced gas production rates observed over the course of the study (Figure 3-7). This suggests reduced production of acetic acid from the grass substrate. Propionic acid (59 µM) and butyric acid (107 µM) (which increased ~ 3-fold from 36 µM at day 0) were observed after 18 days of the experiment, suggesting degradation of lignocellulosic macro-molecular compounds in the grass. Heptanoic acid (119 µM) was only observed after 37 days and was not detected in the early stages of the experiment.

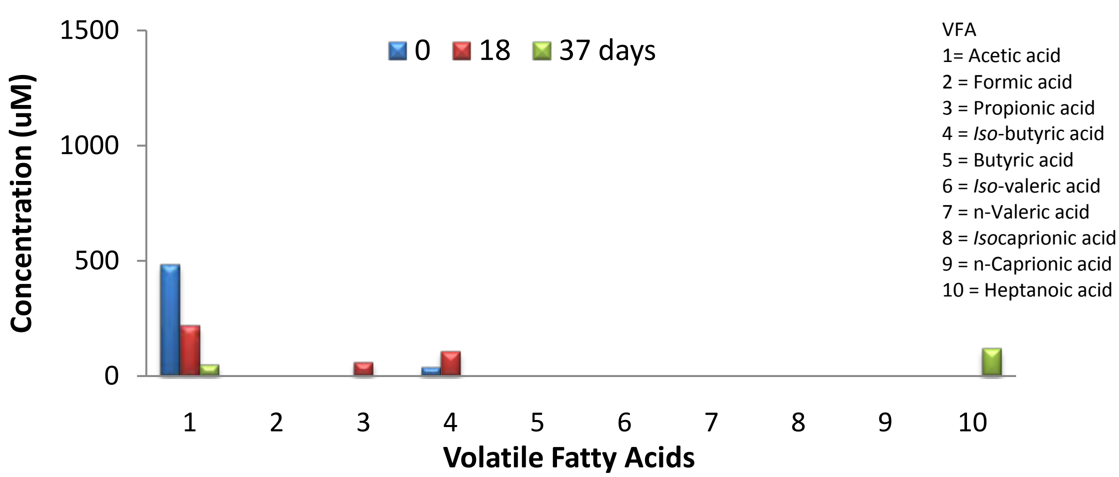


Figure 4-12 Volatile fatty acid concentration profile in the undigested grass control reactor.

4.3.4.4 Comparison of biologically oxidized coal and grass reactors

In the reactor with combined BOC+G substrate, 70% of the profiled VFAs were observed while, only 30% of the VFAs (acetic acid, formic acid, propionic acid, and butyric acid) appeared in both BOC and grass control reactors separately. A total VFA concentration of 2782 µM was measured in the combined BOC+G grass substrate reactor over the course of the study, while 2231 µM and 1075 µM were measured in the BOC and grass control reactors, respectively.

The combined effect of the coal and grass substrates on gas production, demonstrates the importance of a co-substrate in anaerobic processes. The addition of a grass substrate to facilitate degradation of a complex coal substrate was used to enhance process efficiency thereby improving gas production. According to Lou *et al.* (2008), a co-substrate can be described as a compound that is used by microorganisms to degrade non-growth supporting substrates into available substrates. In this study, the grass appeared to function as true co-substrate by providing co-metabolites that were used for the breakdown of complex coal substrates, which constituted the rate-limiting step in the BOC control (Van Wageningen *et al.*, 2006).

It is important to note that amounts of VFA sufficient for methanogenesis vary with the microbial diversity in the anaerobic process. Laloui-Carpentier *et al.* (2006) followed microbial populations using molecular techniques in municipal landfill leacheates. They found that incubations with acetate resulted in dominance of species belonging to the family *Methanosarcinaceae* which favored acetoclastic methanogenesis. Ulrich and Bower (2008) reported that the acetic acid concentrations in methanogenic environments are often maintained at low levels (3 – 7 µM are common in highly methanogenic lake sediments) due to its active consumption by the methanogens.

The acetic acid observed in Figure 4-11 most likely resulted in the elevated gas production recorded in both the combined substrate and the BOC control reactors (Figure 3-5). This further supports the observation that most of the gas produced was derived from the BOC and not the grass. The highest concentration of acetic acid observed at the start of the BOC control reactor (Figure 4-12) may be related to the process of substrate formation in the SHCB (Igbinigie, 2008; Mukasa-Mugerwa, 2008; Rose *et al.*, 2008), where the coal has been previously exposed to microbial rhizosphere/plant interaction which generated LMOs that included acetic acid. Kuzyakov *et al.* (2007) and Paterson *et al.* (2007) have investigated the decomposition of organic matter into LMOs by microbial populations in the rhizosphere. They demonstrated that the release of root exudate components into the rhizosphere induced a significant increase in the decomposition of litter, which led to the release of soluble organic compounds that include sugars, carboxylic acids, and amino acids. In the same manner, the SHCB appears to degrade and oxidizes the coal into readily available LMOs that were used by the methanogenic consortium.

The observed trend in the production of formic acid during the study suggests that it was derived from the combined hydrolysis of BOC and grass in presence of readily available acetic acid for methane generation. When the acetic acid became exhausted, the culture turned to formic acid for methane generation. It has been reported that, formic acid in the presence of H_2 can induce synthesis of acetate, but the reaction is dependent on the pH of the system (Laloui-Carpentier *et al.*, 2006). Therefore, the observed decline in formic acid in this study could also have been the result of homoacetogenic metabolism (Garcia *et al.,* 2000; Laloui-Carpentier *et al.,* 2006). However, in both BOC and grass control reactors, formic acid was not produced, this further supports the idea that the presence of grass acted as a co-substrate an enhanced degradation of the BOC.

An important aspect to note is that valeric acid, which was the predominant VFA in the combined substrate reactor did not occur in the control reactors, and only in very low concentration in the BOC control reactor. This indicates that the source of the VFA in the combined reactors was BOC, but was only released in large amounts in the presence of grass. This provides further indication that the grass functioned as a true co-substrate in the system investigated.

4.3.5 Geologically oxidized coal

4.3.5.1 Geologically oxidized coal plus grass

Figure 4-13 shows the production and consumption of VFAs in the combined GOC+G substrate reactor. Although a greater range of VFAs were observed in the GOC+G at day 0 compared to the BOC system, the VFAs occurred at reduced concentrations except for 634 µM of *iso*-valeric acid that was measured over the same period. *Iso*-valeric acid concentration increased to 1561 µM over 18 days but was then reduced to undetectable levels by day 37. The 2.5 fold increase in *iso*-valeric acid suggests active hydrolysis of larger organic complexes in the GOC but not in the grass (Figure 4-12). The simultaneous increase in propionic acid concentration to 536 µM after 37 days suggests that the *iso*-valeric acid could have been further metabolized to propionic acid.

The *iso*-valeric acid could also have been isomerized into *n*-valeric acid which was observed at an increased concentration towards the end of the experiment (647 µM).

The oxidized valeric acid can be accounted for by the cumulative amount of 536 µM propionic acid produced during the experiment and the remaining *n*-valeric acid of 647 µM which was more difficult to oxidize than its *iso*- form (Lens *et al.*, 1996; Gallert and Winter, 2008). Caprionic acid was initially measured as its *n*- isomer (41 µM) but its *iso*- form was detected towards the end of the experiment at 337 µM suggesting possible isomerization into its stable form. The concentration of heptanoic acid only increased slightly from 60 – 90 µM towards the end of the study. However, it was below detection limits after 18 days.

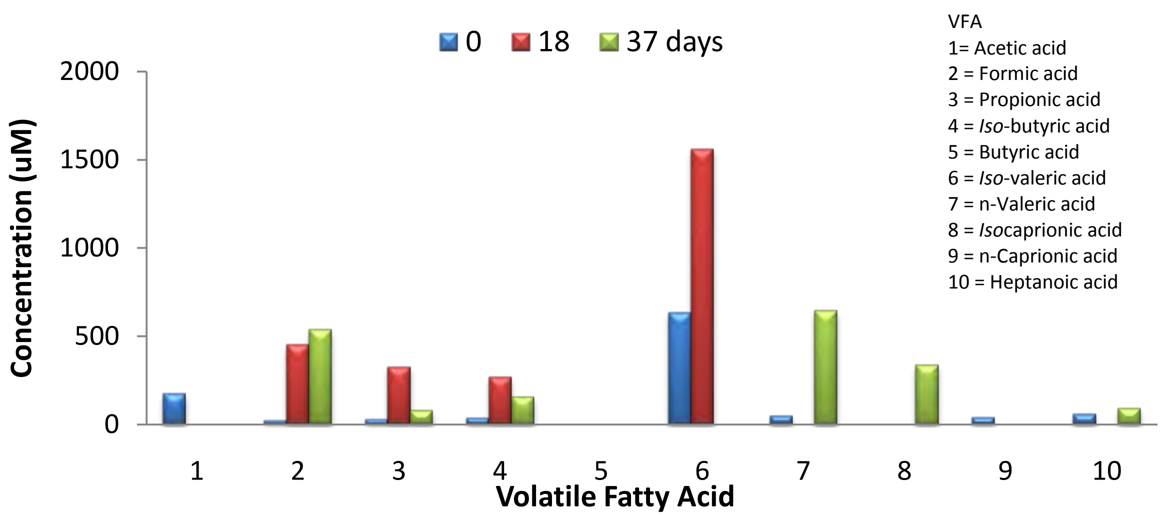


Figure 4-13 Volatile fatty acid concentration profile in the combined geologically oxidized coal plus grass reactor.

The valerate/propionate VFA profile in the combined GOC+G substrate reactor may be compared to a report by Gallert and Winter (2008) who indicated that in the anaerobic digestion of a biowaste suspension, the oxidation of valerate resulted in an increase of propionic acid, which was then degraded after 6 days. They also reported maximal degradation of propionic acid in the fourth week of their study with an overall production of methanogenic activity of 60% after 6 weeks (Gallert and Winter, 2008).

In the GOC control reactor, an initial acetic acid concentration of 131 µM was measured at day 0 (Figure 4-14). Formic (112 µM) and butyric acid (114 µM) were also initially observed and were degraded over time (Figure 4-14). These VFA did not accumulate over the course of the study suggesting either consumption or limited hydrolyzing capacity of the microbial system. While *iso*-valeric was produced up to 702 µM at day 18, there was no further oxidation of this VFA to propionic acid as observed in the combined reactors (Figure 4-13 and Figure 4-10). This was likely due to the absence of a co-substrate to facilitate hydrolysis of the coal substrate. Interestingly neither *iso*-valeric VFA nor its *n*- isomer was detected after day 37. This could mean that the culture had degraded the valeric acid into intermediate products for methane gas. However, the rate of methane production had been substantially reduced at this stage, thereby suggesting the presence of other mechanisms in the system other than methanogenesis (Lens *et al.*, 1996; Gallert and Winter, 2008).

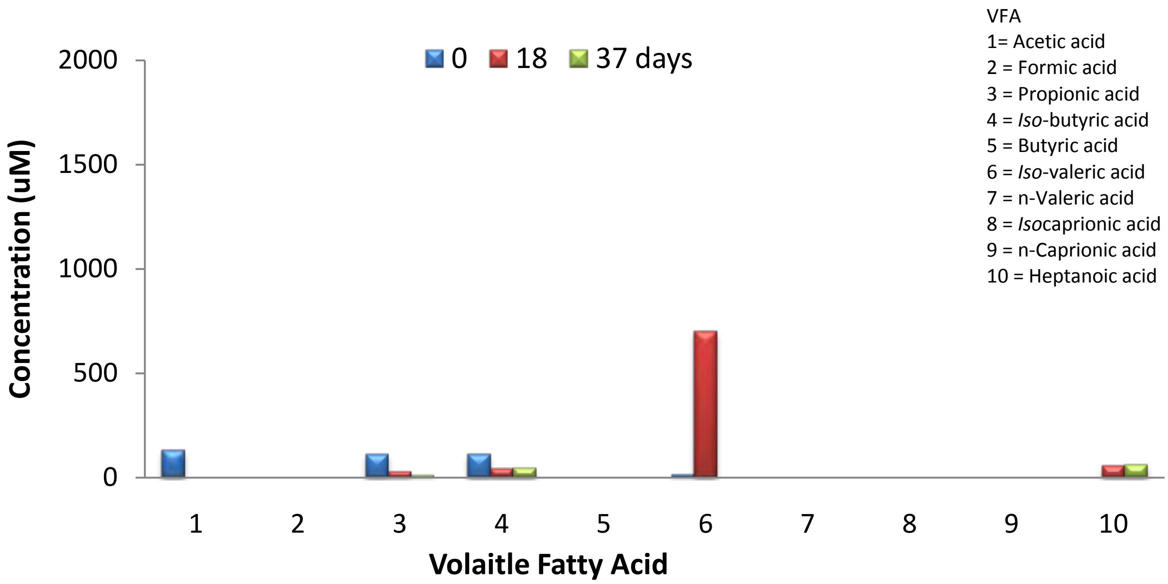


Figure 4-14 Volatile fatty acid concentration in the geologically oxidized coal control reactors.

4.3.5.2 Comparison of geologically oxidized coal and grass reactors

A comparison of the VFA profile in the combined GOC+G substrate reactor, and the respective GOC and grass controls alone showed a similar trend observed in the BOC studies. In the combined GOC+G substrate reactor, 90% of the profiled VFA were observed during the study, whilst in the reactors containing GOC and grass controls only, 50% and 30% of the profiled VFA were observed respectively. The total VFA concentration in the combined GOC+G substrate reactor (5512 µM) was 129% higher than the respective GOC (1322 µM) and grass (1075) controls alone. This strongly suggests a co-substrate effect. In the combined substrate reactor, complete degradation of the VFA was not observed with short chain formic, and *iso*-butyric acid, including long chain valeric and caprionic acids. This was not observed in both the

GOC and grass control reactors, as the generated VFA were not detected after 37 days, except for the residual concentrations of propionic and *iso*-butyric acid including the long chain heptanoic acid.

However, these VFA have not been reported to be inhibitory towards methanogenesis, instead many studies have reported reduction of methanogenic activity by accumulation of propionic acid (Omil *et al.*, 1996; Siegert and Banks, 2005; Gallert and Winter, 2008; Wang *et al.*, 2009). In this study, propionic acid followed an accumulation and consumption cycle which suggests that the action of other inhibitors or the exhaustion of readily available substrates may have resulted in termination of methanogenic activity, rather than it being due to propionate inhibition.

The production of long chain molecules such as heptanoic acid could have resulted from the degradation pathway of aromatic compounds in the GOC. Lepine *et al*. (1996) reported that the degradation pathway of aromatic compounds under methanogenic conditions produced medium long chain fatty acids which were terminally degraded into CH_4 and CO_2. Benzoic acid was transformed through 1-cyclohexene carboxylic acid into heptanoic acid and finally into acetic acid and methane (Lepine F *et al.*, 1996). The GOC control reactor showed an initial acetic acid concentration of 131 µM which was less than the grass control (Figure 4-12) or the BOC control (Figure 4-11). Minimal concentrations of formic (112 µM) and butyric acid (114 µM) were also observed and were degraded over time (Figure 4-14). There was no accumulation of these VFA over the same period suggesting a limited hydrolyzing capacity of the methanogenic culture. Whilst *iso*-valeric production of 702 µM at day 18 was noted, there was no further oxidation of this VFA to propionic acid as observed in the combined reactors (Figure 4-13 and Figure 4-10). A possible explanation was the absence of the co-substrate supporting coal hydrolysis. Interestingly neither *iso*-valeric VFA nor its *n*- isomer was detected after day 37 indicating that the culture fully utilized the valeric acid isomer for methane gas production. Valeric acid is not a preferred substrate for methanogenesis (Lens *et al.*, 1996; Gallert and Winter, 2008) and its consumption occurred during reduced gas production.

Nozhevnikova *et al.* (2000) and Kotsyurbenko *et al.* (2004) have noted that *iso*-butyric acid is an important breakdown product of the anaerobic degradation of organic matter, and was present (Figure 4-10) in the reaction medium (129 µM). Prior breakdown of the BOC in SHCB could have enabled the anaerobic degradation of the BOC to yield *iso*-butyric, which was not observed in the GOC substrate. The highest concentration observed for this VFA was 306 µM, which was higher than the concentration observed in the GOC reactors. This may indicate that it was derived from the coal and not the grass since equal amounts of the co-substrate were added in both reactors. After 37 days, the concentration of *iso*-butyric acid (27 µM) was lower than propionic acid (43 µM, Figure 4-10). This could have been caused by the preferential degradation of *iso*-butyric acid over propionic acid as reported by Nozhevnikova *et al.* (2000). These workers found that addition of butyric acid and propionic acid to an anaerobic reactor resulted in preferential metabolism of the former over the latter, leading to accumulation of acetate and hydrogen (Nozhevnikova *et al.*, 2000).

4.3.6 Comparison of combined coal substrates

Comparative analysis of the VFA profiles in the combined BOC+G and GOC+G reactors showed that 7of the 10 profiled VFA were produced in the former reactor, while 9 of the 10 profiled VFA was observed in the GOC+G reactor. In addition, the total concentration of VFAs in the combined GOC+G substrate reactor was ~ double that in the combined BOC+G reactor. These VFA trends can be correlated to the total gas production and methane yields reported in the previous chapter, where GOC was found to yield more methane than BOC. Furthermore, the VFA profiles and gas production in the two coal substrates may inform the feasibility of the use of these substrates to serve as carbon sources for methane production.

The absence of co-substrate in all control reactors became the limiting factor that resulted in low gas production rates (Figure 3-7 and Figure 3-12) being observed, apparently due to insufficient VFA as observed in Figure 4-10 to Figure 4-12.

4.3.7 Prediction of substrate breakdown pathway

The results presented in this chapter can be used to predict the degradation pathway for both coal substrates and grass. As noted earlier, the anaerobic fermentation of organic matter comprises four steps; hydrolysis of the degradable fraction of the particulate organic matter, acidogenesis of metabolic intermediates into C3 and C4 compounds, acetogenesis of the intermediary products of acidogenesis into VFAs such as acetic acid, carbon dioxide and hydrogen, and finally methanogenesis using carbon dioxide as a terminal electron acceptor for the oxidation of acetic acid and hydrogen (Fang *et al.*, 2004; Buendía *et al.*, 2009; Wang *et al.*, 2009).

The microbial culture developed in this study enabled the hydrolytic cleavage of the macromolecular matrices of the substrates and produced precursor compounds (identified through Py-GCMS), for synthesis of VFAs, that were observed during the study. The methanogenic degradation of phenols and related compounds has been reported to occur via two different pathways; the benzoate into benzoyl-CoA pathway which occurs at ambient and mesophilic temperatures, and the caproate pathway which occurs at thermophilic temperatures (Fang *et al.*, 2004; Fang *et al.*, 2006; Fezzani and Cheikh, 2009). The appearance of benzoic precursor compounds in the Py-GCMS results can be used to predict that the methanogenic degradation of the oxidized HC substrates with and without the grass as observed in this study occurred via the benzoic pathway at 30^{o}C.

The breakdown of phenolic compounds under anaerobic conditions has been reported to follow a reductive pathway. Berry and co-workers (1987) reported that the major intermediates produced by aromatic-degrading methanogenic consortia included butyrate, propionate and acetate along with CO_2 and H_2 (Berry *et al.*, 1987). The end of the experiment was characterized by low methane gas production and VFA concentration and the appearance of the 2-methoxy phenol. This precursor compound could have become inhibitory thereby reducing VFA formation and methane gas production. The toxicity of phenols to anaerobic digestion has been investigated by several researchers who have reported on its production, degradation and inhibition in these systems (VanDenHeuvel and Beeftink, 1988; Fang *et al.*, 2004; Fang *et al.*, 2006; Jin *et al.*, 2007; Kasai *et al.*, 2007).

Previous studies have reported that conversion of VFAs to methane varies in the order of acetic acid > ethanol > butyric acid > propionic acid (Ren *et al.*, 2003; Wang *et al.*, 2009), According to Wang *et al.* (2009), all longer chain length VFAs used for methane production are initially degraded to acetic acid, and their conversion rates may vary in the order ethanol > butyric acid > propionic acid. This can be correlated to the results reported here, where there was an initial presence of acetic acid in the reactors, and once that became exhausted; the other VFAs were also degraded into acetic acid, as observed by the reduction in concentration of valeric acid, butyric acid and propionic acid. All these reactions are fermentative and may involve CO_2 evolution. The available acetic acid was then utilized for methane production, until it became exhausted or the presence of inhibiting compounds such as propionic acid became apparent at elevated concentrations or, coal breakdown products such as the methoxy-phenols.

The Py-GCMS results and VFA profiles presented here confirm the findings mentioned earlier in section 3.3.4, where GOC substrates provided more substrates for methane gas production than BOC substrates in the presence of the grass co-substrate. In the BOC substrates, the available substrates would have supported fermentation reactions but were not sufficient to sustain methane production.

4.4 Conclusions

The following conclusions maybe drawn from this study:

- The methanogenic culture developed was shown to be capable of generating the vital VFAs from the metabolism of coal and its co-substrate grass which were utilized in the production of methane and CO_2 gas;
- The oxidized HC and grass controls produced less VFA and gas than the combined coal and grass substrates for both BOC and GOC;
- The grass appeared to function as a true co-substrate enhancing coal biodegradation;
- GOC performed as a better substrate than BOC in methane gas production;
- While the accumulation of propionate may have slowed down the methanogenic process, it was apparently not responsible for terminating gas production;
- The generation of phenolic compounds from the biodegradation of coal substrates may account for the ultimate inhibition of gas production rather than substrate exhaustion.

If oxidized HC is to be successfully used as a substrate for methane gas production, a detailed account of this proposed inhibition effect would need to be taken. Strategies to counter the problem may include either washing out of the phenol compounds from the methanogenic system in order to maintain an ongoing process, or shift the partly digested substrate to downstream processes, which could be capable of degrading the inhibiting intermediate products. Furthermore, the observation that GOC substrate produced better methane yields than BOC led to decision to adopt the GOC as the coal substrate of choice for the remainder of the experiments in this study.

CHAPTER FIVE

OXIDIZED HARD COAL AS A SUBSTRATE FOR SULFATE REDUCTION 1: FEASIBILITY STUDY

5. INTRODUCTION

The viability of an anaerobic process for utilizing oxidized HC, with carbon dioxide used as the terminal electron acceptor, resulted in the demonstration of energy recovery from waste coal in the form of methane. However, the full potential of the process was limited probably by the build-up of inhibitory intermediate metabolites, in the form of aromatic compounds and long chain fatty acids. The question then arose whether an alternative terminal electron acceptor such as sulfate would enable further utilization of these substrates either as a downstream process as a follow-on to methane production or as a stand-alone unit of operation for sulfate removal in AMD treatment. It seemed important to establish this point within the broader objective of investigating the bioprocess potential of oxidized HC as a substrate.

The use of sulfate as a terminal electron acceptor offers a route for the further degradation and utilization of intermediate metabolic products, which are inhibitory to methanogenesis. This is primarily due to the more robust nature of Sulfate reducing bacterial (SRB) consortia, and their ability to generate sulfide from an elaborate electron transport system which has been convincingly linked to the cleavage of aromatic compounds (Rothermich *et al.*, 2002; Chang *et al.*, 2003; Christensen *et al.*, 2004). The possibility of utilizing coal as a substrate for sulfate reduction could revolutionize biological processes for the treatment of AMD.

The environmental problems arising from AMD have been comprehensively reviewed by Luptakova and Kusnierova (2005), Kalin *et al*. (2006) and Mayes *et al*. (2008a). AMD arises from the oxidation of metal sulfides, mainly pyrite and marcasite, on exposure to air during and following mining operations. It is characterized by low pH and elevated levels of heavy metal complexes, most notably iron and salts such as sulfates and chlorides (Johnson and Hallberg, 2005; Costa *et al.*, 2008). AMD is derived from both chemical oxidation and biological processes (Burgess and Stuetz, 2002).

Several approaches to the treatment of AMD have been extensively reviewed in the literature (Johnson and Hallberg, 2005; Costa *et al.*, 2008). Active treatment technologies that have been used include conventional chemical addition of alkaline compounds (with varying cost and effectiveness) such as limestone, carbonate salts, NaOH, and magnesium hydroxide that serve to raise the pH, and accelerate the rate of chemical oxidation of the ferrous ion (Johnson and Hallberg, 2005).

Biological remediation strategies that offer alternative and economically attractive option for AMD treatment have also been reported (Johnson and Hallberg, 2005; Kalin *et al.*, 2006; Mayes *et al.*, 2008b). Aerobic wetlands, anaerobic or compost wetlands, vertical flow wetlands, AMD treatment ponds, bioreactors and permeable reactive barriers are some of the passive bioprocesses that have been widely investigated (Kalin *et al.*, 2006; Batty and Younger, 2007; Prasad and Henry, 2009). According to Johnson and Hallberg (2005), bioremediation of AMD relies on the ability of certain microbial populations to generate alkalinity and immobilize metals, thereby essentially reversing the reactions responsible for the genesis of AMD.

SRB use sulfate ions as terminal electron acceptors for the metabolism of organic substrates such as lactate to generate sulfide ions or free H_2S (Elliott *et al.*, 1998) as shown in the following equations (Jong and Parry, 2006).

$$2CH_3CHOHCOOH + SO_4^{-2} \rightarrow 2CH_3COOH + H_2S + 2HCO_3^- \quad (1)$$

$$CH_3COOH + SO_4^{-2} \rightarrow H_2S + 2HCO_3^- \quad (2)$$

$$2CH_3CHOHCOOH + 3SO_4^{-2} \rightarrow 3H_2S + 6HCO_3^- \quad (3)$$

$$M^{2+} + H_2S \rightarrow MS_{(s)} + 2H^+ \quad (4)$$

This process has been effectively used in the bioremediation of acid mine drainage by removing metals and sulfate from solution (Alvarez-Puebla and Garrido, 2005; Jong and Parry, 2006; Neba and Rose, 2006). The sulfide ions and carbonates generated increase the pH of the solution, and readily react with most dissolved metals to form insoluble metal sulfide precipitates.

Although these bioprocesses are feasible, the availability of an efficient, cost effective substrate has hindered the commercial viability and development of these processes (Neba and Rose, 2006). In this context, the Rhodes BioSURE Process® developed at EBRU provided a solution, by utilizing Primary sewage sludge (PS) as the sole carbon source for Biological sulfate reduction (BSR) (Rose *et al.*, 2004). Following successful bench-scale studies of the enhanced hydrolysis of particulate matter from PS to small molecular weight fatty acids (Whittington-Jones *et al.*, 2002), the process was then scaled up to a 40 m^3 pilot plant treating an AMD stream with a sulfate load of ~ 2000 mgl^{-1}. The pilot plant was configured as a multi-stage process consisting of three unit operations; the hydrolysis unit where PS was hydrolyzed into low molecular weight fractions, the sulfate reduction unit where AMD was fed from the top, and a final polishing unit for cleaning-up the final effluent.

However, PS is not available in appropriate quantities at all mining sites and the need for other low cost electron donors is required. To the best knowledge of this author, the work presented in this section is a first report of the investigation of oxidized HC used as a substrate for BSR. In AMD bio-treatment, sulfate reduction is the core process that is accompanied by pH neutralization and heavy metal removal. Downstream removal of the residual sulfide from the final treated water would then be required as a polishing step. Only the feasibility of the core sulfate reduction process utilizing oxidized HC as the electron donor and carbon source was considered in this study. Given the relative advantage of the GOC over the BOC observed in the previous chapter, it was decided to base these studies on the GOC substrate.

5.1 Objectives

The objectives of this study were to:

- Develop an SRB culture adapted to acidic conditions in the presence of GOC;
- Investigate the feasibility of the adapted SRB culture to use an oxidized HC substrate (GOC) as the sole electron donor and carbon source for sulfate reduction;
- Evaluate the role of the GOC-fed SRB system in the neutralization of acidic media.

5.2 Materials and Methods

5.2.1 Source of microorganisms and culture preparation

A modified Postgate's basal medium B (Postgate, 1984 and Atlas, 1993) for the cultivation of sulfate reducing bacteria was mixed in tap water and formulated to include in solution A: lactate (0.5% v/v), Na_2 SO_4 (0.3%), NH_4Cl (0.1%), KH_2PO_4 (0.05%), $CaCl_2$ (0.02%),

$MnSO_4.4H_2O$ (0.002%), $Na_3C_3H_5O(CO_2)_3$ (0.01). Solution B contained ascorbic acid (1.0%) and sodium thioglycholate (1.0%). All medium materials were purchased from Merck, South Africa. The two solutions were sterilized at 121^{o}C for 15 min, cooled and then 10% of solution B was mixed into 0.99 L of solution A to make up 1 L of medium. This medium was used as a basal medium for all subsequent experiments reported in this chapter. A 10% inoculum derived from section 3.3.1 was used to grow the SRB in a stock cell generator.

5.2.1.1 Adaptation of sulfate reducing bacteria culture to low pH conditions

Adaptation of the SRB cultures to acidic conditions (pH 3 – 5) was performed to investigate the feasibility of the SRB to utilize oxidized HC, as carbon and electron donor respectively, in acidic environments anticipated in AMD treatment (Costa *et al.*, 2008; Prasad and Henry, 2009).

A series of anaerobic reactors were set up to adapt the consortium of SRB to acidic conditions. The medium in different reactors was adjusted to pH values of pH 3.0, pH 4.0 and pH 5.0 using 2 M H_2SO_4 or NaOH. GOC (0.2% w/v) and lactate (0.5%) were used as electron donor and carbon sources. As in the methanogenic study, initial amenability tests showed that a co-substrate would be required to enable GOC breakdown by a sulfidogenic consortium. The reactors were sealed with a rubber bung and purged with N_2:CO_2 (80:20%) (Afrox, South Africa) for 15 min and agitated on a rotary shaker (100 rpm, Labcon-3100u) at 30^{o}C. The SRB cultures were allowed to raise the pH to 8.5, after which they were sub-cultured into new medium at pH 3.0, 4.0 and 5.0. Continuous sub-culturing was performed until the cultures were able to increase pH from acidic conditions. These cultures were then used for all subsequent studies in this section. Sulfide generation and pH increase were measured daily and were used as primary indicators for bacterial activity. The SOC was measured weekly to determine depletion, and new feed was added to replenish substrate used.

5.2.2 Oxidized hard coal as carbon source for sulfate reduction

Batch studies on the ability of GOC to serve as carbon source for sulfate reduction were conducted in 1 L round flat-bottomed anaerobic flasks. The pH of the medium was adjusted to pH 7.5 using 1 M NaOH and 1 M H_2SO_4 (Merck, South Africa), since most sulfate reducing bacteria are neutrophiles (Pikuta *et al.*, 2000). Different concentrations of oxidized HC (0.2 – 1.0% and 2.5 – 10%, w/v) in separate flasks reactors were supplemented with a reduced lactate co-substrate (0.05%, w/v) to formulate a molar carbon to sulfate ratio of 4:1, and added to the basal medium described in section 5.2.1. The flasks were incubated on a rotary shaker (25 ºC, 120 rpm). A control reactor without lactate was also set up to determine effect of a co-substrate on sulfate reduction. A zinc acetate 10% (w/v) trap was attached to the flasks to capture the sulfide gas produced from the reactor, as zinc sulfide. The total sulfide produced over time was calculated as the sum of the soluble sulfide in the reactor and that trapped in the zinc sulfide.

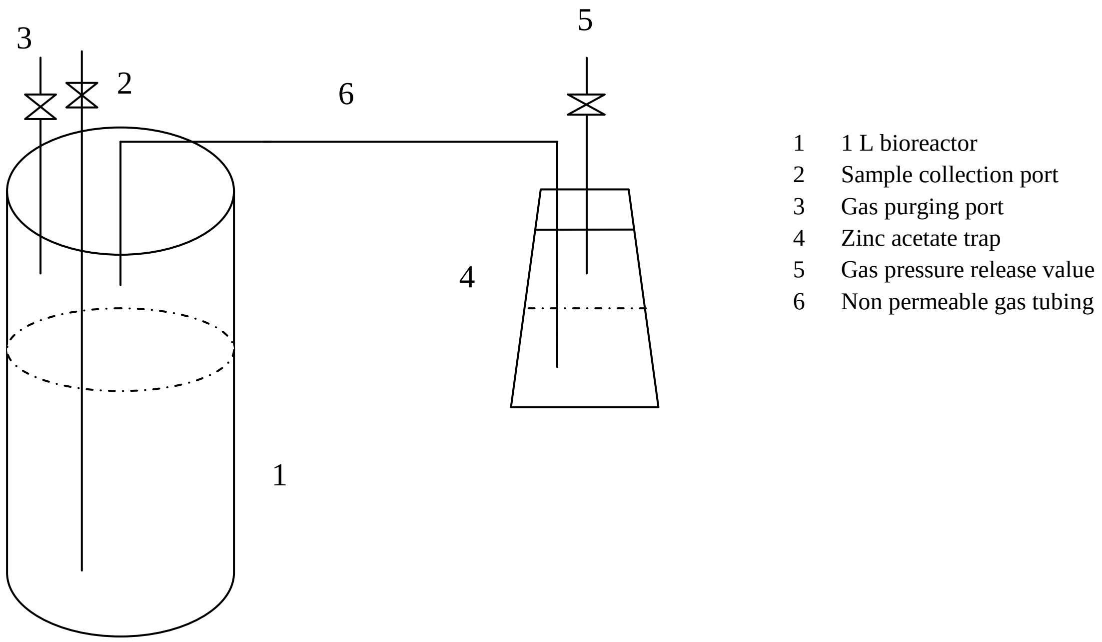


Figure 5-1 Schematic diagram of the continuously agitated flask reactor used in the investigation of pH and sulfate reduction using geologically oxidized coal as a carbon source in a batch study.

5.2.3 Analyses

5.2.3.1 Sulfide analysis

A Merck® spectroquant test kit was used to determine the sulfide concentration of the samples (Catalogue no. 1.14779.0001, Merck®, South Africa). Two mL samples were collected from the reactor in test tubes containing 100 µl of 0.1 M zinc acetate solution and 100 µl of 6 M NaOH to prevent sulfide from escaping. Concentrated samples were diluted using dH_2O. Equivalent samples were also collected from the zinc acetate trap, vortexed and diluted with dH_2O, before assaying for sulfide. The total sulfide obtained from the reactor flask and zinc acetate sulfide trap (Figure 5-1) was used for this analysis. Distilled water was used as a blank.

The amount of sulfate reduced in the reactors was determined by using the stoichiometric calculation of the molar mass of sulfate: sulfide ratio of 3:1 (Ristow and Hansford, 2001):

Molecular mass SO^{2-}_4: Molecular mass HS^-

94: 32

3: 1

5.2.3.2 Humic acid analysis

The formation of humic acid was monitored using the procedure previously described in section 2.2.3.

5.2.3.3 Soluble organic carbon analysis

The SOC was determined by TOC measurement as previously described in section 2.2.6.3

5.3 Results and discussion

5.3.1 Adaptation studies

The adaptation of SRB to acidic conditions was performed in continuously agitated flask reactors (CAFR) at three pH values (pH 3.0, pH 4.0 and pH 5.0) (Table 5-1). In the first adaptation experiments, the SRB consortium cultured in pH 3.0 medium only managed to increase the pH to ~ pH 3.5 after 16 days. Average sulfide concentration in the same reactor decreased by 36% from 54 to 34.2 mg.L^{-1} over same period. In the SRB culture started at pH 4.0, the pH increased to ~ pH 6.5 over 16 days, while the average sulfide concentration increased ~ 8.2 fold from 106 to 895 mg.L^{-1} over the same period. The SRB cultured in pH 5.0 medium raised the pH to ~ pH 6.0 over 16 days, while the sulfide concentration increased ~ 7-fold from 90 to 612 mg.L^{-1}.

Table 5-1 Average pH and sulfide changes in SRB batch reactors for adaptation to acidic conditions in the presence of oxidized hard coal and lactate co-substrate.

Days	pH 3.0 reactor		pH 4.0 reactor		pH 5.0 reactor	
	pH	sulfide (mg/L)	pH	sulfide (mg/L)	pH	sulfide (mg/L)
0-4	3.12	53.8	4.1	109.2	5.3	89.9
5-8	3.3	33.4	4.4	55.6	5.6	296.2
9-12	3.4	43.2	5.5	425.1	5.7	635.8
13-16	3.5	34.2	6.5	894.6	5.9	612.4

At this point, these results suggested that pH 3.0 was too low to support SRB growth. The SRB grown in pH 4.0 medium initially demonstrated ability to generate alkalinity. Unexpectedly, the SRB cultured at pH 5.0, did not raise the pH to alkaline levels. This could have been due to exhaustion of readily available substrates from the oxidized HC plus lactate substrate. The adaptation of microbial cultures to extreme conditions is highly variable and previous studies have reported extended lag phases, which are dependent on the culture conditions (Akram and Stuckey, 2008).

In the second adaptation phase, new medium was introduced into all three cultures, and the pH was adjusted back to the respective values. The SRB re-cultured in pH 3.0 medium raised the pH to ~ pH 3.7 after 5 days, and remained constant throughout the experiment (Table 5-2). Interestingly, a ~ 3-fold increase in the sulfide concentration to 147 $mg.L^{-1}$ was observed over 17 days. The SRB grown in pH 4.0 medium, increased the pH by 75% to an average of 7 by the end of the experiment, while ~ 11-fold increase in sulfide production to 1097 $mg.L^{-1}$ over 17 days was noted. In pH 5.0 medium, a pH increase to pH 7.8 was recorded while the sulfide production showed ~ 20-fold increase over 17 days (Table 5-2).

Table 5-2 Average pH and sulfide changes of SRB cultured in second batch experiments to adapt to acidic conditions in the presence of oxidized hard coal and lactate co-substrate.

Days	pH 3.0 reactor		pH 4.0 reactor		pH 5.0 reactor	
	pH	sulfide (mg/L)	pH	sulfide (mg/L)	pH	sulfide (mg/L)
0-4	3.5	50.2	4.7	96.2	5.7	77.8
5-8	3.7	103.0	6.0	445.0	6.6	264.8
9-12	3.7	135.3	6.6	824.4	7.1	924.4
13-17	3.6	147.0	7.0	1097.0	7.8	1519.3

The results presented in Table 5-2 show the ability of SRB to adapt to acidic concentrations as reported in literature (Laborda *et al.*, 1999; García *et al.*, 2001; Boshoff *et al.*, 2004; Luptakova and Kusnierova, 2005). Although the SRB in pH 3.0 medium was unable to generate alkalinity over the experimental period in the both phases (Table 5-1 and Table 5-2), the production of sulfide in the second adaptation phase confirmed active performance of the SRB culture, which could possibly have increased the pH to alkaline levels, had the incubation time been extended. In pH 4.0 medium, the SRB were more adapted to the medium and generated more alkalinity and sulfide than the first batch experiments (Table 5-2). In pH 5.0 medium, the second adaptation phase (Table 5-2) showed increased alkalinity and sulfide production. The above procedure provided SRB cultures adapted to grow in acidic medium that could be used in the subsequent studies.

5.3.2 Oxidized hard coal batch reactor studies

5.3.2.1 Effect of oxidized hard coal concentration on pH

The ability of mixed SRB consortia to grow and utilize different oxidized HC concentrations was investigated and compared (Figure 5-2). The SRB cultured in 0.2% oxidized HC raised the pH gradually to ~ pH 8.0 after 10 days and then dropped to ~ pH 6.5 after 15 days. At higher concentrations of oxidized HC (0.4 – 1.0%), the pH increased after day 1 and dropped to ~ pH 7.2 for SRB in 0.4 and 0.6% , while in the flasks with 1.0% oxidized HC the pH dropped to pH 7.0 after day 3 (Figure 5-2). The control reactor, which contained lactate as the sole carbon source, followed the same trend as the lower oxidized HC concentrations. The pH then started to increase in these reactors to reach pH 8.0 ± 0.2 after 10 days. Afterwards the pH in all reactors dropped below pH 6.5 after 15 days and continued to decrease until the study was stopped after 19 days (Figure 5-2).

The pH results presented in Figure 5-2 indicated that SRB raised the pH in the reactors via generation of alkalinity in the conversion of sulfate to sulfide (McCartney and Oleszkiewicz, 1991; Costa *et al.*, 2008). The initial drop in pH observed in the first 3 days in the higher concentrations of oxidized HC (0.4 – 1.0%) may be attributed to the release of humic substance into solution, which was probably concomitantly linked to the lag phase of SRB growth. This trend was not observed in the lactate control reactor flask and 0.2% oxidized HC, which provides further evidence to support the possibility that the pH drop was due to the increased concentration of the coal in the medium. The reactor with 1.0% oxidized HC showed the highest drop in pH to below pH 7.0 at day 3.

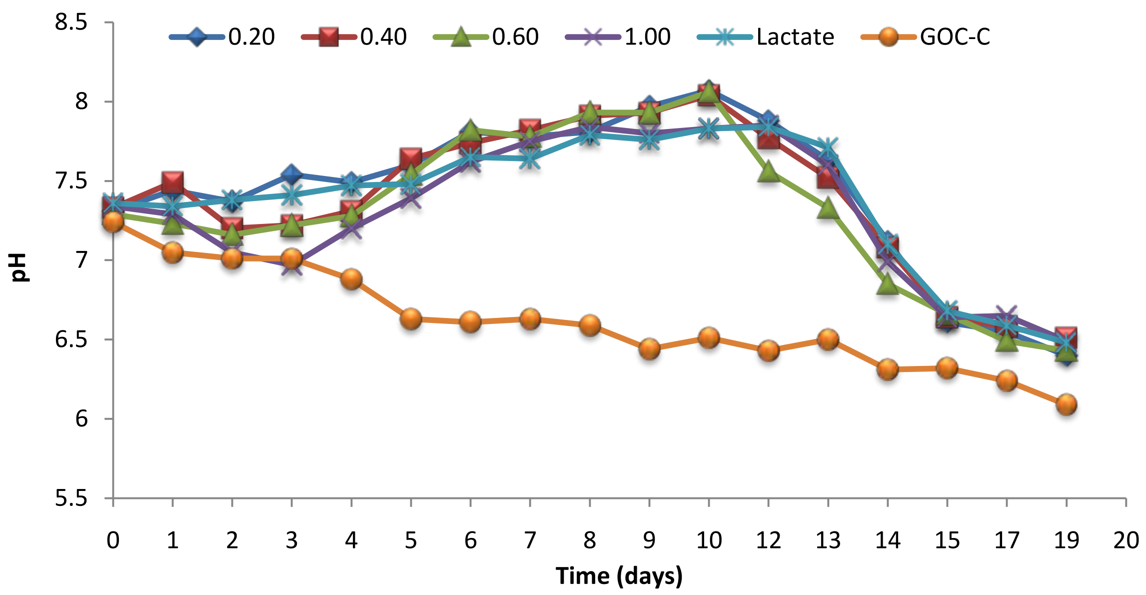


Figure 5-2 Changes in pH by adapted sulfate reducing bacteria cultured in 1 L continuously agitated flask reactor batch studies using increasing concentrations of oxidized hard coal (0.2 – 1.0%, w/v) and reduced lactate co-substrate. GOC-C is the oxidized hard coal substrate (0.2%) control in the absence of lactate.

Over time the SRB in all the flasks were able to generate alkalinity and increase the pH to ~ pH 8.0. Between day 8 and 12, the pH in all the reactors was ~ pH 8 .0 ± 0.3. These results concurred with the maximal sulfide production in the oxidized HC substrate reactors (Figure 5-3). The decrease in pH observed in all reactors containing the coal substrates at day 10 is a likely indication of the exhaustion of lactate in the reactors, although the pH in the lactate control only begins to decrease after 14 days. A probable reason could be that, the SRB in the flasks containing the oxidized HC substrates used some of the lactate to degrade the coal, thereby leading to faster depletion. However, in the lactate control flask the SRB only utilized the lactate for cell growth and sulfide production. The work reported here is comparable to the studies conducted by Moosa and Harrison (2006), where up to 90% of sulfate in the medium was reduced to sulfide, while 40 – 66% of the sulfate was reduced at lower pH values of pH 6.0 – 7.0.

5.3.2.2 Effect of oxidized hard coal concentration on sulfide production

Batch studies on different oxidized HC concentrations were conducted to evaluate the growth and reduction of sulfate in the presence of a lactate co-substrate. Development of a characteristic black color signifying iron sulfide, was observed in all reactors as the experiment progressed up to day 15. In Figure 5-3, a lag phase was observed in the experimental and the control reactors after day 1, where sulfide production was below 10 mgl^{-1}. This phase was extended to day 2 in the flasks containing the oxidized HC, while in the control reactor there was ~ 4-fold increase in sulfide to 37 mgl^{-1} (Figure 5-3). In the control flask, an exponential increase in sulfide production to 118 mgl^{-1} was observed after day 3, and was followed by a stationary phase between days 4 and 9. Thereafter, sulfide production increased gradually to a maximum of 284 mgl^{-1} after 19 days when the experiment was terminated. In the experimental reactors containing 0.2 and 0.4% oxidized HC, an exponential increase in sulfide production was not observed after the lag phase, although there was a 52% and 98% increase to 86 and 53 mgl^{-1} between day 3 and 4 respectively. The production of sulfide continued to increase steadily and peaked at 153 mgl^{-1} and 107 mgl^{-1}, for 0.2% and 0.4% oxidized HC flasks after 17 days (Figure 5-3).

In the SRB cultured in 0.6 and 1.0%, sulfide production remained below 50 mgl^{-1} until day 6 for 0.6% oxidized HC, whilst the SRB in 1.0% oxidized HC achieved 52 mgl^{-1} after 5 days. Sulfide concentration in the reactors containing 0.6% oxidized HC decreased slightly to 42 mgl^{-1} after day 9 and then increased again to a maximum of 158 mgl^{-1} after day 15. Thereafter, the black color which is normally associated with iron-sulfide formation gradually faded away into a grayish color after 19 days. Similar observations were noted in the reactors containing 1.0% oxidized HC, in which sulfide production increased gradually to 133 mgl^{-1} after 19 days (Figure 5-3).

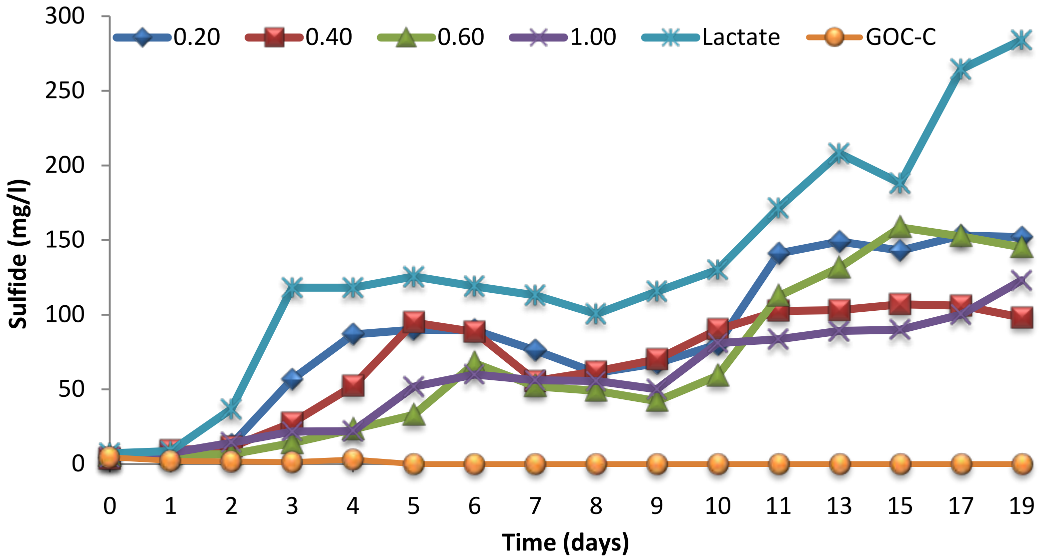


Figure 5-3 The production of sulfide by adapted sulfate reducing bacteria cultured in 1 L continuously agitated flask reactor batch studies using increasing concentrations of oxidized hard coal (0.2 – 1.0%, w/v) and reduced lactate co-substrate. GOC-C is the oxidized hard coal substrate (0.2%) control in the absence of lactate.

5.3.2.3 Carbon utilization

Figure 5-4 shows the utilization SOC by SRB in different oxidized HC concentrations. The amount of SOC in the experimental reactors at the start of the study was variable due to the solubility of the different oxidized HC concentrations. Results presented in Chapter 2 demonstrated that the amount of SOC in water was minimal and did not increase proportionally with an increased oxidized HC concentration. A substrate utilization trend over time was observed in the experimental reactors including the lactate control reactors. In the reactors containing 0.2 – 0.6% oxidized HC, the SRB utilized 51% SOC for 0.2% oxidized HC, 49% for 0.4 oxidized HC and 46% for 0.6 and 1.0% oxidized HC after day 10, while in the control reactor flask 63% of the SOC had been removed from solution over the same period (Figure 5-4). Thereafter, the utilization rates decreased for the lower oxidized HC concentration reactors (0.2 – 0.6%) between day 10 and 16. At day 20, 81% of the SOC in 0.2% oxidized HC, while 83% of the SOC in 0.4% oxidized HC and 80% in the 0.6% oxidized HC reactors had been utilized.

The SOC utilization in the 1.0% oxidized HC reactors continued to decrease to 65% after day 14. This was followed by an increase in the SOC concentration to 369 mgl^{-1} at day 16, which could have been derived from the biodegradation of the oxidized HC. After 20 days, ~ 78% of the SOC in the 1.0% oxidized HC reactors had been removed from solution. Similarly, in the lactate control reactor flask 87% of the SOC (695 mgl^{-1}) had been removed from solution over the same period (Figure 5-4).

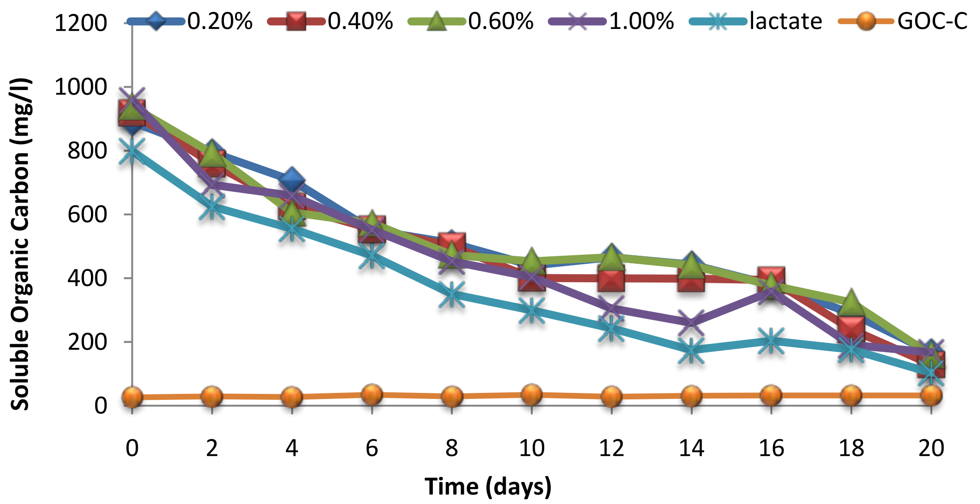


Figure 5-4 Soluble organic carbon utilization by adapted sulfate reducing bacteria inoculated in different oxidized hard coal concentrations.

The observed sulfide, pH and SOC trends shown above are compatible with growth characteristic of SRB (Luptakova and Kusnierova, 2005; Moosa and Harrison, 2006; Velasco *et al.*, 2008). While there was an overall increase in sulfide concentration and decrease in the SOC, the presence of the oxidized HC in the reactors appeared to inhibit sulfide production and SOC utilization over time as observed in Figure 5-3 and Figure 5-4. Instead of enhancing microbial activity, the addition of oxidized HC could have affected the metabolic activities of SRB by binding to the sterically active sites of the enzyme systems. Inhibition of enzyme systems in the presence of coal has been reported by Catcheside and Ralph (1999). They reported that the action of lignin peroxidase and manganese peroxidase was inhibited by the non-specific binding of enzymes by hydrophilic and hydrophobic groups in solubilized low rank coal (Ralph and Catcheside, 1994). The absence of oxidized HC in the lactate control reactor resulted in higher sulfide production (Figure 5-3) although the rate of carbon utilization was similar in all reactors, thereby supporting the theory of inhibition by the coal.

Table 5-3 shows the influence of lactate as SOC:SO_4 ratio (*R*) based on stoichiometric calculations of the molar mass sulfate: sulfide ratio of 3:1 (Ristow and Hansford, 2001). According to Vela *et al* (2002), the SOC:SO_4 ratio is a widely used parameter in regulating BSR. Ideally, effluents with *R* of 0.67 contain sufficient sulfate to completely remove the organic matter via sulfate reduction, based on the assumption that all soluble carbon can be utilized by SRB, such as acetate (Vela *et al.*, 2002). Based on the amount of sulfate and lactate present in the batch study reported here, where lactate was the readily available carbon source, the minimum molar SOC:SO_4 ratio was calculated to be 0.63 (equation 3). The *R* in all reactors was higher than the minimal 0.63. Based on the *R* (0.8) in the lactate control reactor, it became apparent that the lactate was substantially contributing to sulfate reduction in the GOC reactors, and it was not possible to establish the contribution of the coal towards sulfate reduction. An increase in the GOC (0.2 – 1.0%) concentration did not increase sulfate reduction, but reduced the production of sulfide (Figure 5-3). Spiking the reactors with lactate resumed sulfate reduction, and confirmed exhaustion of lactate as the source of carbon.

Although the results are not presented here, SRB growth and activity was completely inhibited when cultured in higher concentrations of oxidized HC (2.5 – 10%).

Table 5-3 Stoichiometric ratio of sulfate to soluble organic carbon removed from solution during the oxidized hard coal batch studies.

Sample	Lactate removed (mgl^{-1})	Total SO_4 reduced (mgl^{-1})	Lactate: SO_4ratio (*R*)
0.2% GOC	695	459	1.6
0.4% GOC	695	321	2.1
0.6% GOC	695	474	1.5
1.0% GOC	695	369	1.8
Lactate	695	852	0.8

* - GOC= geologically oxidized HC

5.4 Conclusions

The following conclusions can be drawn from these studies:

- A preliminary feasibility has been shown for sulfate reduction supported by an oxidized HC –lactate mixed substrate;
- Sulfate reduction occurs in the presence of lactate and a low concentration of oxidized HC (0.2%);
- BSR from oxidized HC is dependent on the presence of a co-substrate;
- Increases in the oxidized HC are accompanied by reduction in SRB activity;
- It cannot be established from these studies whether lactate functions in the system as a co-substrate enabling oxidized HC breakdown;
- Potential use of the oxidized HC as a substrate in bioprocess treatment of AMD has been indicated and may be a first report.

Further bioprocess development for AMD treatment, based on the observations of batch BSR supported by a mixed oxidized HC-lactate substrate in a batch system, would require a continuously operated reaction environment, in which the SRB are allowed to adapt. Such a process would eliminate accumulation of potential inhibitors such as aromatic coal breakdown products and metal sulfides, by continuously purging of the system. This would not only provide operational advantage, but also add fundamental knowledge on the effective development and scale-up of a bioprocess for AMD treatment.

The function of the co-substrate within the system would also need to be more clearly understood, perhaps by reducing the co-substrate concentration to enable the SRB to adapt to the GOC as a substrate.

CHAPTER SIX

OXIDIZED HARD COAL AS SUBSTRATE FOR SULFATE REDUCTION 2: OPERATION IN A CONTINUOUS BIOPROCESS ENVIRONMENT

6. INTRODUCTION

Process development using oxidized HC as substrate for BSR would require some form of continuous process for cost-effective treatment of coal mine wastewaters. Continuous bioreactor design offers several advantages over batch designs which include a higher degree of process control via regulation of substrates and product, continuous removal of possible inhibitors and waste products such as sulfide from the bioreactor, higher product quality and yield, and tighter operational controls. However, there are inherent disadvantages such as possible washout of the original microbial strains or their being overtaken by faster growing strains (Vega *et al.*, 1990; Williams, 2002; Jong and Parry, 2006).

Reactor design configurations that have been extensively used for continuous commercial bioprocess applications include Continuously stirred tank reactors (CSTR) and column packed bed reactor designs (Jong and Parry, 2006; Akram and Stuckey, 2008; Costa *et al.*, 2008). The former has advantages that include removal of potential reaction inhibitors and discharge of solids from the reactor. However, it also has disadvantages that include expensive reaction vessel, high energy inputs and high operational costs. Column-type packed bed reactors, present practical applicability in the coal mining industry, where large volumes of water are to be treated and the void created in the coal extraction operations can be used for the construction of a reactor vessel *in-situ*. These could be filled with crushed coal as packing material on an impermeable membrane. The fixed bed could be used to immobilize the biocatalyst, thereby increasing productivity and output (Elliott *et al.*, 1998; Scott *et al.*, 1998). In addition, the energy requirements are lower when compared to the CSTR concept. Potential problems likely to be encountered include blockage of the reactor due to accumulation of sludge, although this may be reversed by purging the reactor (Kalin *et al.*, 2006). The aim of this study was to investigate the operation of a continuous Up-flow anaerobic packed bed column reactor (UAPB) using oxidized HC as a substrate for the treatment of AMD.

6.1 Objectives

The objectives of the study reported here were to:

- Investigate the performance of BSR in a continuously operated fixed bed reactor configuration using an oxidized HC-lactate mixed substrate as an electron donor and carbon source;
- Determine whether lactate functions as a true co-substrate in the system.

6.2 Materials and Methods

6.2.1 Up-flow anaerobic packed bed bioreactor

Anaerobic packed bed column bioreactors (120 x 800 mm) with a void volume of 2.6 ± 0.1 L were set up to investigate a continuously operated process. The bioreactors contained a porous layer 50 mm above the base of the column, to allow the liquid component to pass while retaining the coal matrix in the bioreactor. A layer of glass beads (20 mm diameter) was placed on top of the permeable mesh layer to prevent the smaller coal particles from clogging the pores of the mesh and allowing efficient flow of the liquid (Figure 6-1).

The bioreactor bed was made up of discard roof coal (bituminous) obtained from Klein Kopje Colliery (Mpumalanga, South Africa). The coal was crushed into fragments with a diameter of 1 ± 0.5 mm and washed with dH_2O until a clear rinsate was obtained. The coal in the bioreactor was then soaked in dH_2O to remove air pockets from the interstitial spaces of the coal in the bioreactors. The dH_2O was drained and then, nitrogen gas was pumped continuously through a sterile filter (0.45 micron) into the bioreactor at 0.8 $mL.min^{-1}$ to maintain anaerobic conditions.

6.2.1.1 Medium formulation

A modified Postgate's medium A adapted from Atlas (1993) was used to prepare the feed for the UAPB. The medium was formulated to include: GOC 0.2% (w/v), lactate 0.5%, Na_2SO_4 0.3%, $MgSO_4.7H_2O$ 0.006% (final sulfate concentration was 2500 $mg.L^{-1}$), sodium citrate 0.006%, KNO_3 $CaCl_2$ 0.02%, NaCl 0.02% and medium B (10% v/v), which was made up of sodium thioglycolate 1.0% and ascorbic acid 1.0% in 100 mL dH_2O. The medium was autoclaved before addition of the oxidized HC to prevent modification during autoclaving. The pH was adjusted to pH 4.0 using 2 M H_2SO_4 or NaOH. The feed reservoir was continuously agitated on a magnetic stirrer and kept anaerobic by purging the headspace with nitrogen gas. The feed was pumped from the bottom into the bioreactor at 0.8 $mL.min^{-1}$ and the effluent collected into a settling tank (Figure 6-1). A zinc acetate trap was connected to the effluent settling tank to capture sulfide. Three sampling ports were distributed every 220 mm along the length of the column and an effluent sampling port located at top of the reactor.

6.2.1.2 Operation

The UAPB (Figure 6-1) were held in a constant environment laboratory at 30^{o}C. The bioreactors were inoculated with 450 mL of mixed SRB inoculum derived from the stock culture adapted to low pH, as described in section 5.2.1. An oxidized HC enriched feed was pumped from the bottom of the reactor at 0.8 mL.min^{-1} and re-circulated for 48 h (Figure 6-1) to facilitate adhesion of the SRB onto the coal packed surface. The circulation loop was closed off and new feed was continuously pumped from the bottom of the bioreactors at 0.5 mL.min-^{1}. The effluent was collected daily and analyzed for sulfide and pH. SOC analysis was undertaken every second day.

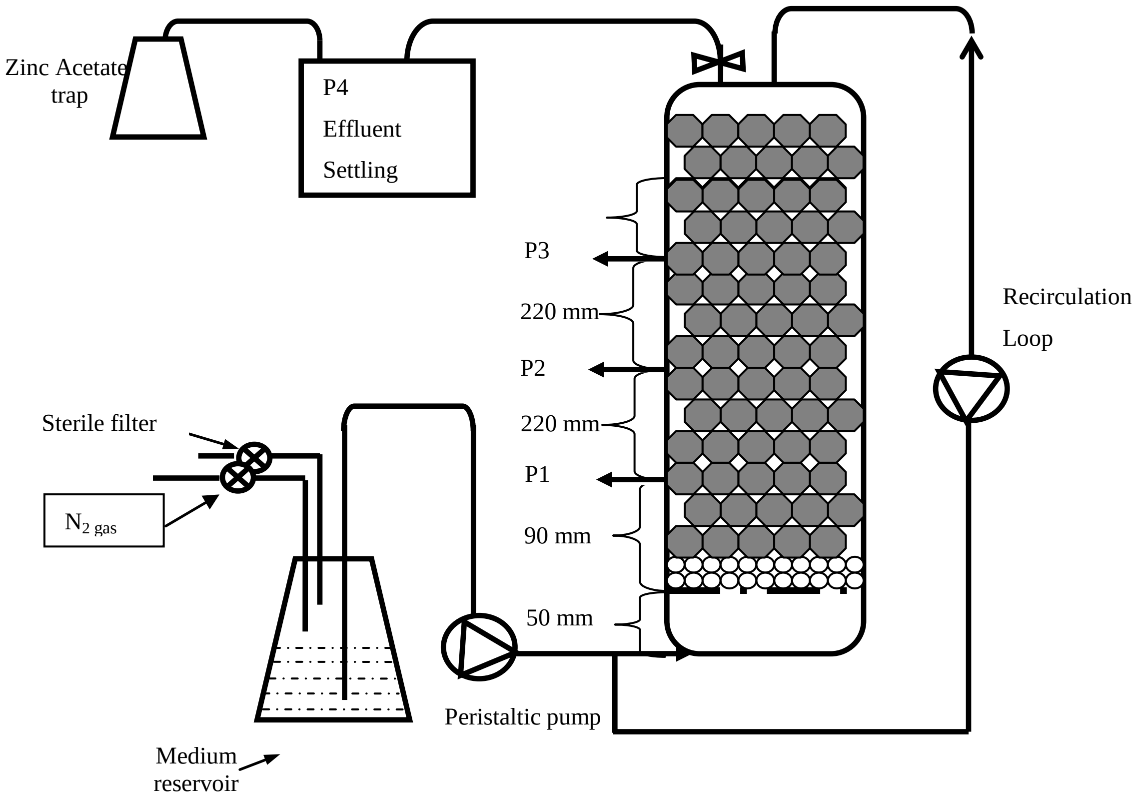


Figure 6-1 Schematic diagram of the up-flow anaerobic packed bed reactor used in the biological sulfate reduction studies using geologically oxidized hard coal and lactate as substrate, showing three sampling ports distributed along the height of the reactor and port 4 (P4) flowing into an effluent settling tank. Sulfide was trapped in the zinc acetate trap connected to the effluent settling tank.

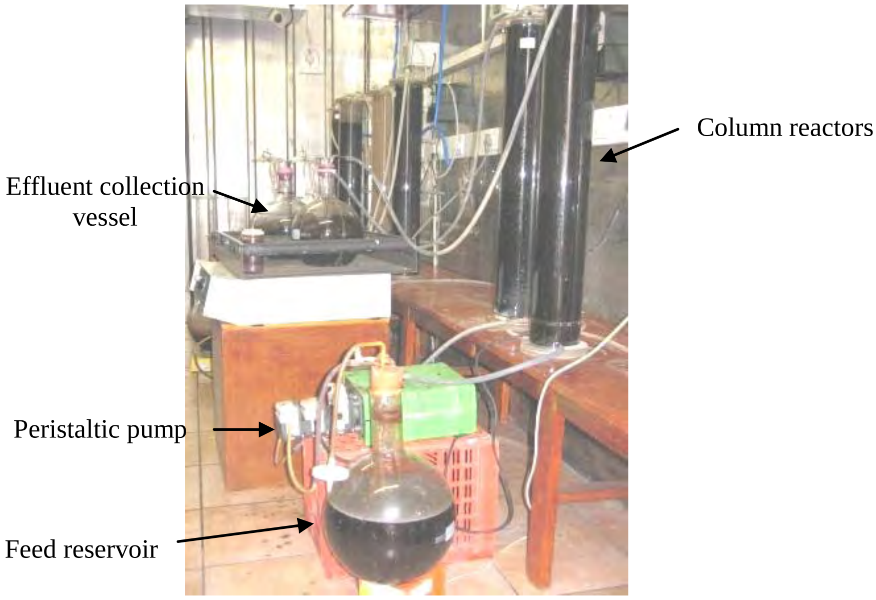


Figure 6-2 Experimental set up of the up-flow anaerobic packed bed bioreactor packed with bituminous roof coal and fed with an oxidized hard coal-lactate enriched medium.

6.2.2 Humic acid studies

HA was prepared as described in section 2.2.3 and was used to investigate its utilization as a carbon source for sulfate reduction in the presence of a lactate co-substrate. The HA (0.2%, v/v) was included into the enriched medium as described in section 6.2.1.1.

6.2.3 Analysis

6.2.3.1 pH analysis

The pH was monitored as previously described in section 2.2.6.1.

6.2.3.2 Sulfide analysis

The production of sulfide was monitored using the procedure previously described in section 5.2.3.1.

6.2.3.3 Humic acid analysis

The formation of humic acid was monitored using the procedure previously described in section 2.2.3.

6.2.3.4 Soluble organic carbon analysis

The soluble carbon was determined by TOC measurement as previously described in section 2.2.6.3.

6.2.3.5 Statistical analysis

STATISTICA, version 8.0 software (StatSoft, Inc. 2008) was utilized for statistical analysis.

6.2.4 Experimental set up

Column bioreactors similar to those described in section 6.2.1 were used for this study. Quartz sand (Sparrow Pools, Grahamstown, South Africa) was used as packing material in one bioreactor while HC was used in the other reactor as shown in Figure 6-3. The bioreactors were operated as previously described in section 6.2.1.2. Un-inoculated controls containing both sand and coal in separate reactors were set up and operated concurrently with the experimental reactors. Lactate substrate was removed from the feed medium of the control reactors to minimize bacterial contamination.

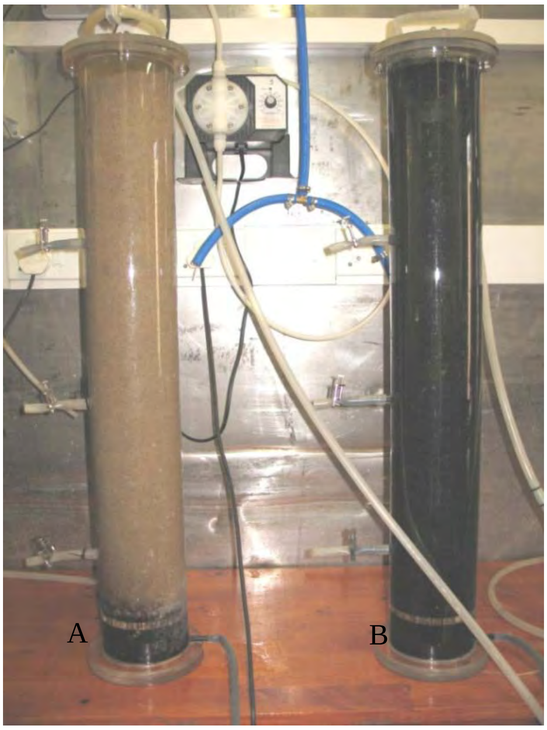


Figure 6-3 Experimental set up of the up-flow anaerobic packed bioreactors used in the investigation of humic acid as the carbon source for sulfate reduction in the presence of a co-substrate. Sand packing (A) and roof coal packing (B).

6.2.4.1 The role of lactate co-substrate on sulfate reduction

The function of lactate as a co-substrate in BSR with HA as a carbon source was investigated. The study was performed using a SRB culture that were in log growth phase. During the study, lactate concentration in the feed was progressively reduced until the lactate was completely removed from the medium. Two volume changes were allowed for steady state conditions to be established between each change in feed.

6.2.5 Bioprocess design – biological extraction of humic acid

One problem that arose was the blocking of the up flow packed bed reactor by the particulate matter in coal. By taking advantage of the alkalinity generated from sulfide generation, and using it to solubilize humic substances, generated from oxidation of the coal (Avena and Wilkinson, 2002), this solved the problem, and provided a means to large scale production of the alkaline soluble humic substances, briefly described as follows.

6.2.5.1 Extraction of humic acid from oxidized hard coal

The biological extraction of HA from the oxidized HC was performed in 5 L flasks using the alkaline effluent from the sand and coal UAPB reactors separately (Figure 6-4). The effluent from the bioreactors was collected into 5 L flask reactors containing 1.0% (w/v) oxidized HC. The flasks were filled from the bottom-up, at the same flow rate (0.8 $mL.min^{-1}$) at which the feed was being pumped into the respective UAPB reactors. The reactors were agitated on a rotary shaker (100 rpm, Labcon-3100u) at 30^{o}C. The flasks were sealed with a rubber bung and purged with N_2:CO_2 (80:20%, Afrox, South Africa) for 15 min. Water – bubble traps were used to prevent air return into the reactors as they filled. After collecting 4.5 L over 4 days, the water – bubble trap was removed and replaced with a zinc trap to capture sulfide. The flasks were purged again with N_2:CO_2 (80:20%) and agitated at the same rotary speed under anaerobic conditions for 12 days. Samples were collected for analysis of HA, pH, SOC and sulfide.

Figure 6-4 Experimental set up of the flask reactors for external extraction of HA from oxidized hard coal using alkaline effluent collected from coal and filter sand reactors.

6.3 Results and Discussion

6.3.1 Culture adaptation

The adaptation to acidic conditions of SRB cultures to be used in subsequent studies was undertaken in a UAPB reactor that was fed with an oxidized HC (0.2% w/v) enriched medium (pH ~ 4.0) over a period of 60 days (Figure 6-5). A rapid increase in pH from pH 4.0 to pH 6.8 and pH 6.9 was recorded at P1 and P4 respectively, between day 2 and 4 (Figure 6-5). As the reaction progressed, the effluent pH (P4) increased to pH 9.1, while the pH at P1 peaked at pH 8.4. SRB generally grow optimally in the range pH 6 – 8 (Maree *et al.*, 2004; Moosa and Harrison, 2006; Valdes *et al.*, 2006). Thereafter, the pH fluctuated between pH 6.1 and pH 7.2 at P1; and between pH 7.2 and pH 8.8 at P4 (Figure 6-5). After 60 days, the pH at P1 and P4 decreased to pH 5.1 and pH 7.1 at which point the experiment was terminated.

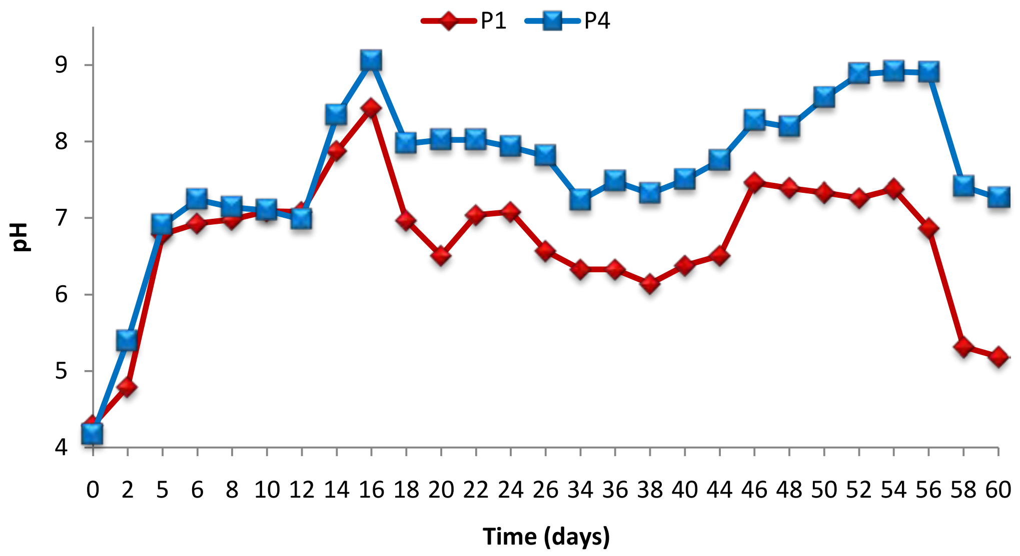


Figure 6-5 pH changes during the start-up and adaptation of sulfate reducing bacteria in an up flow anaerobic packed bed reactor fed with an enriched basal medium containing oxidized hard coal and lactate co-substrate. P1 = First sampling port 140 mm from the feed inlet; P4 = effluent from the reactor.

Figure 6-6 shows production of sulfide by the SRB in the UAPB which occurred in three phases. In the first phase which may be referred to as the lag phase, the rates of sulfide production were low at both P1 and P4, of which, P1 took over 38 days while P4 took only 16 days before sulfide production increased substantially. In the second phase, the rate of sulfide production increased rapidly to 202 $mg.L^{-1}. day^{-1}$ at P1, while a higher rate of 533 $mg.L^{-1}. day^{-1}$ was observed at P4 between day 18 and 46. Thereafter, sulfide production increased to 850 $mg.L^{-1}$and 927 $mg.L^{-1}$ at P1 and P4 respectively after 48 days, before entering steady state production of sulfide for the remainder of the experiment. Maximum sulfide production was observed during this phase Figure 6-6.

These results provided evidence of SRB growth in the presence of acidic oxidized HC and AMD for biotransformation and remediation, although an acclimatization period was required along the length of the column. The substantial increase in sulfide concentration observed at day 16 (Figure 6-6) corresponded with the highest pH (Figure 6-5), thereby demonstrating the ability of the SRB to neutralize the acidic medium. Although, the highest pH at P1 was also measured at day 16, the proximity of this sampling port to the influent could have masked the efficiency of BSR until by SRB until such a time that the microbial population had acclimatized to the system.

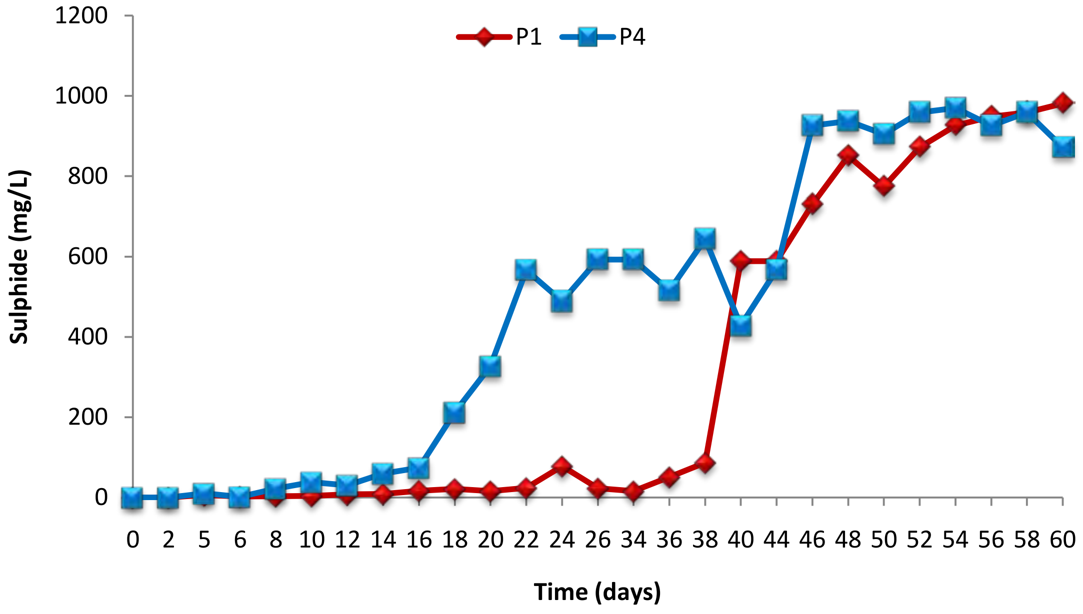


Figure 6-6 Sulfide changes during the start-up and adaptation of sulfate reducing bacteria in an up flow anaerobic packed bed reactor fed with an enriched basal medium containing oxidized hard coal and lactate co-substrate. P1 = First sampling port 140 mm from the feed inlet; P4 = effluent from the reactor.

6.3.2 Effect of oxidized hard coal concentration

Coal packed UAPB reactors were seeded with the adapted SRB and were continuously fed with various loadings of oxidized HC (0.2 – 0.8%) in separate reactors over time. Reactor performance was monitored using sulfide production and generation of alkalinity over time.

However, the operation of the packed bed-type reactor operation was hindered by the clogging effects of the oxidized HC feed, ultimately leading to reactor failure. This was observed even at lower feed concentrations. Efforts to purge the reactors by pressure back washing were only moderately successful. The results presented below reports one of the experiments conducted using the oxidized HC and illustrates the performance of the adapted SRB in the HC packed UAPB system.

6.3.2.1 pH changes

The changes in pH with increasing addition of oxidized HC feed are presented in Figure 6-7. Although the pH of the influent feed was set at pH 4.0, the adapted SRB culture was able to raise the pH to ~ pH 6.0 at P1 (140 mm from the feed inlet port) on day 1, whilst, an increase to pH 7.2 was recorded at P4 over the same period. The pH increased gradually over time and peaked at pH 7.5 at P1 after 10 days, and after addition of 0.4% oxidized HC into the reservoir medium. At P4, the pH peaked at pH 9.0 after 20 days, after increasing the oxidized

HC to 0.6%. At higher coal loading, the pH dropped at both P1 and P4, and fluctuated between pH 5.6 ± 0.4 and pH 7.2 ± 0.2 respectively (Figure 6-7).

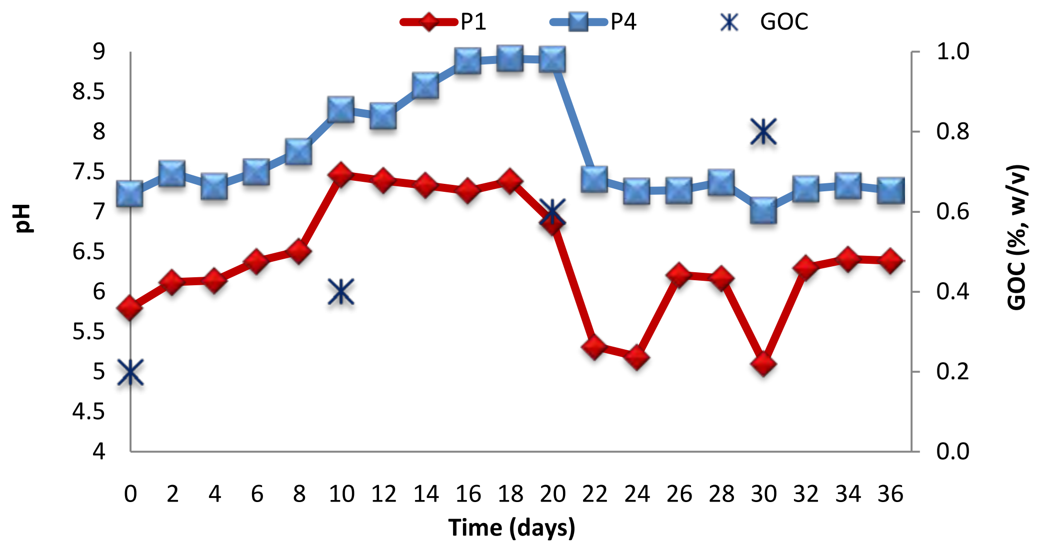


Figure 6-7 Changes in pH with increasing addition of oxidized hard coal in an up flow anaerobic packed bed reactor cultured with adapted sulfate reducing bacteria. P1 = First sampling port; P4 = effluent from the reactor; GOC = geologically oxidized coal. * = shows the incremental addition of the oxidized hard coal substrate over time.

The results presented above demonstrate the ability of the adapted SRB to generate alkalinity from sulfide production, and suggests that the full length of the UAPB may not be necessary to achieve this. In a comparable study, Jong and Parry (2006) found that loading at pH 4.0 resulted in failure of their system.

6.3.2.2 Sulfide production

Figure 6-8 shows sulfide production by adapted SRB in a coal packed UAPB with increasing oxidized HC concentration. The appearance of a black precipitate in the effluent setting tank was indicative of sulfide production. A ~ 5.6-fold and ~ 2.4-fold increase in sulfide concentration was observed between day 0 and 4, at P1 and P4 respectively, as the SRB adjusted to the acidic medium enriched with 0.2% oxidized HC feed (Figure 6-8). This was followed by a rapid increase in sulfide production at P1 to 850 $mg.L^{-1}$ at a rate of 97 $mg.L^{-1}$ per day, between day 4 and 12.

Similarly at P4, a rapid increase in the sulfide concentration at a rate of 106 $mg.L^{-1}$ per day to 937 $mg.L^{-1}$ was measured over the same period. Thereafter, sulfide concentration was constant between days 10 to 28 and was not affected by the increasing oxidized HC concentration from 0.2 – 0.6%. The sulfide concentration at P1 and P4 was similar between day 16 and day 30 (~920 mg.L^{-1}) suggesting steady state conditions. However, the addition of 0.8% oxidized HC at day 30 resulted in ~ 22% reduction in sulfide concentration at both P1 and P4, probably as the culture adjusted to the new feed. However, 4 days after the addition of the 0.8% oxidized HC feed, the reactor became blocked and resulted in termination of the study.

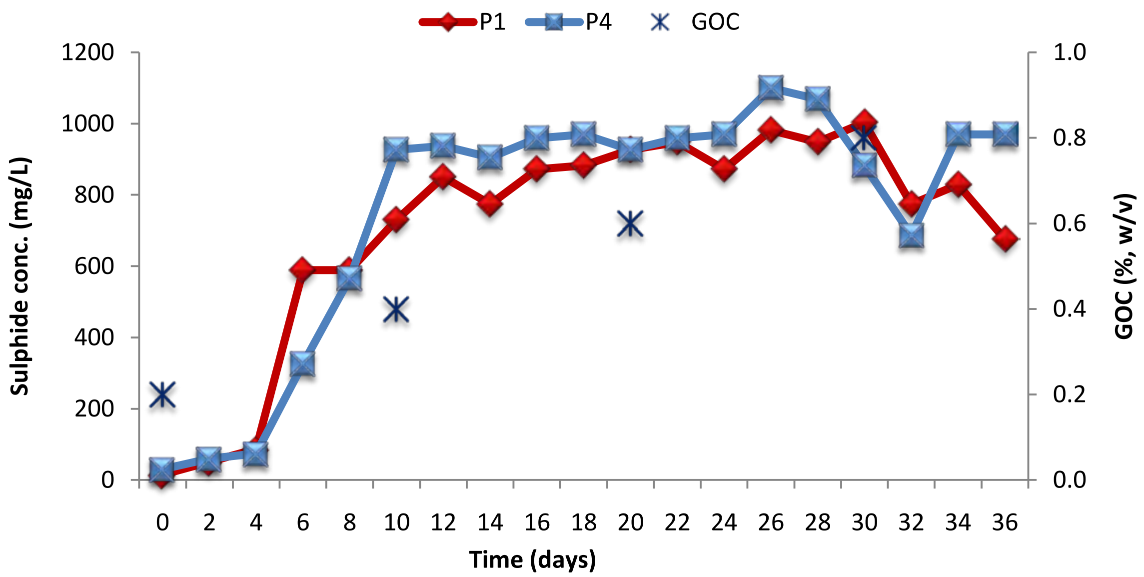


Figure 6-8 Sulfide production in an up-flow packed bed reactor using the continuous feed of increasing oxidized hard coal concentrations. P1 = First sampling port; P4 = effluent from the reactor. GOC = geologically oxidized coal. * = shows the incremental addition of the oxidized hard coal substrate over time.

Table 6-1 shows the lactate fraction in the feed removed from solution and related to SO_4 reduced in the UAPB reactor and expressed as the C:SO_4 ratio *(R)*. It was assumed that all the lactate was consumed given that reactivity resumed when the reactor was spiked. Vela *et al*. (2002) and Velasco *et al*.,(2008) have noted a stoichiometric *R* of 0.67 for defined substrates such as acetate and ethanol respectively, and the stoichiometric ratio for lactate was calculated to be 0.63. During the addition of 0.2% GOC, an *R* of 0.93 was measured over 8 days. When the GOC concentration was increased to 0.4%, the *R* decreased by 77% to 0.21, and remained constant even after increasing the GOC concentration (0.6 – 0.8%) until the experiment was stopped due to blockage.

Table 6-1 Stoichiometric ratio of soluble organic carbon to sulfate ratio for lactate utilized in the presence of oxidized hard coal substrate in up flow anaerobic coal packed bed reactor studies.

Feed	C in lactate feed (mgl^{-1})	Total SO_4 reduced (mgl^{-1})	lactate C:SO_4 (*R*)
0.2% GOC	2940	3175	0.93
0.4% GOC	2940	14094	0.21
0.6% GOC	2940	15072	0.20
0.8% GOC	2206*	10527	0.21

* - less lactate due to blockage

At the start the experiment, the *R* (0.93) which was higher than the minimum calculated ratio (0.63) for lactate suggests two factors. Firstly, a lag phase as the SRB adapted to the medium and is noted by a low sulfide production (Figure 6-8). Secondly, it may suggest that the lactate is functioning as the main substrate for sulfate reduction and it was not possible to determine the contribution of the oxidized HC towards sulfate reduction.

Subsequent increase in the GOC concentration (0.4 – 0.8%) was accompanied by an increase in sulfate reduction, thereby reducing the *R* (0.21) substantially by 66% to < 0.63. Since lactate in the feed could have only contributed to 33% of the required carbon for optimal sulfate reduction, the difference (67%) therefore must have been derived from another carbon source, which was the oxidized HC. It was then further assumed that a large amount of oxidized HC would be degraded in order to yield VFA required to achieve a minimum *R* of 0.63.

In summary, although an initial adaptation of the SRB to the acidic medium was observed at the start of the study, the gradual increase in oxidized HC over time did not appear to affect sulfide production and therefore SRB performance. Rather, sulfide production between P1 and P4 was ~ similar over the column reactor indicating optimal performance of the reactor (Figure 6-8).

6.3.2.3 Release and breakdown of humic acid under sulfidogenic conditions

Figure 6-9 shows the spectrophotometric determination of the extractable HA component derived from oxidized HC at P1 and P4 of the coal packed UAPB. As sulfate reduction efficiency increased at P1, and with addition of up to 0.6% oxidized HC, HA increased ~ 7 fold to 32 $mg.L^{-1}$ and peaked at ~ 35 $mg.L^{-1}$ after 24 days, where it remained constant for the remainder of the experiment. At P4, the HA concentration was initially reduced by 91% between day 0 and 4, before gradually increasing to peak at 12 $mg.L^{-1}$ after 14 days, and remained constant for the remainder of the experiment despite the increase on oxidized HC concentration to 0.6%. Further increase in the coal concentration to 0.8% resulted in a 73% decrease in the humic acid concentration (Figure 6-9).

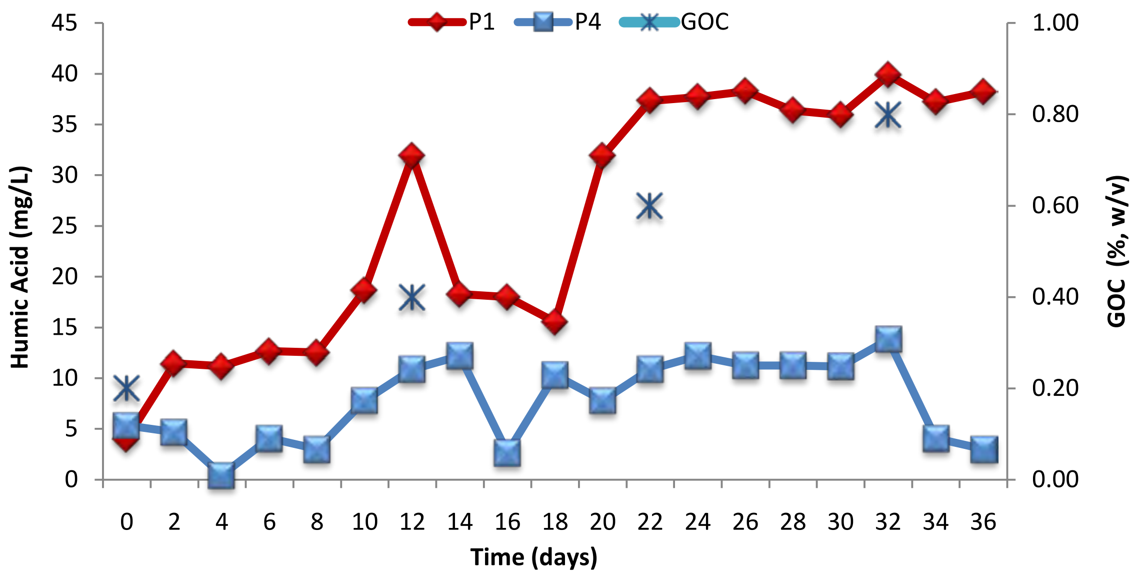


Figure 6-9 Humic acid analysis and percentage addition of oxidized hard coal in an up flow anaerobic packed bed reactor cultured with adapted SRB. P1 = First sampling port; P4 = effluent from the reactor; GOC =geologically oxidized coal. * = shows the incremental addition of the oxidized hard coal substrate over time.

The relatively high HA content at P1 in comparison to the low HA content at P4 presented in Figure 6-9, indicates that the HA may have either been consumed or precipitated in the column. Although HA has been thought to be a microbially inert substrate, the presence of quinone moieties in its structure (Stevenson, 1994; Scott *et al.*, 1998), has indicated its function as a suitable electron acceptor for anaerobic oxidation of substrates such as acetate, lactate and H_2 (Scott *et al.*, 1999; Lovley, 2000; Cervantes *et al.*, 2001). In related studies by Cervantes *et al.* (2001), they reported that *Desulfitobacterium* species were able to utilize lactate or H_2 when soil HA was used as terminal electron acceptors. The HA may also contain functional groups that may serve as electron donors that become reduced and would thus precipitate as a reduced product, or may adsorbed and/or desorbed onto the packing material in the reactor. In addition, the coal itself may have released HA into the solution as the result of biological and chemical interaction by SRB and sulfide respectively. These findings raised the question whether the HA released from the oxidized HC was being utilized as a substrate by the SRB, and formed the basis for the next study.

6.3.3 Humic acid as substrate

To determine if HA was used as a substrate in the UAPB, an inert quartz sand packed up flow anaerobic column reactor was set up as comparison to the coal packed reactor. The oxidized HC in the feed was replaced with an equivalent HA concentration.

6.3.3.1 Sulfide production

In addition to production of sulfide, SRB growth on sand and coal UAPB reactors was observed by the appearance of black patches of iron sulfide on the surfaces of both packing material (although it was not as evident in the coal reactor) (Figure 6-10A). In the un-inoculated reactors, the gradual accumulation of HA was evident in the sand reactor but could not be seen in the coal reactor due to the dark color of the coal (Figure 6-10B).

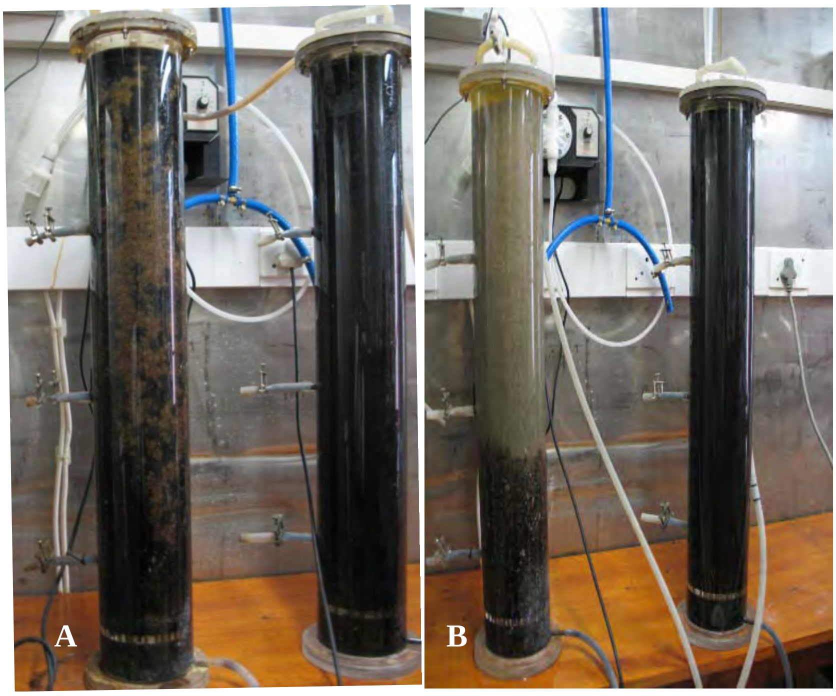


Figure 6-10 A) Sulfate reducing bacteria inoculated sand and coal up-flow anaerobic packed bed reactors. B) Un-inoculated sand and coal up-flow anaerobic packed bed reactors.

Sulfate reduction in sand packed up-flow anaerobic bioreactor was compared to the coal packed bioreactor. Figure 6-11 shows sulfide production in both sand and coal packed up-flow bioreactors. In the sand reactor, sulfide production increased gradually at a daily rate of 252 $mg.L^{-1}.day^{-1}$ over 20 days. A similar rate (250 $mg.L^{-1}.day^{-1}$) of sulfide production was observed in the coal reactor, over 6 days. This was followed by a 64% decrease in the sulfide content to 127 $mg.L^{-1}$ after day 8, and gradually decreased for the remainder of the experiment to 32 $mg.L^{-1}$ (Figure 6-11). The decrease in sulfide concentration observed in the coal reactor could have been due to volatilization, or transformation into an insoluble iron sulfide, or a combination of both (Krohn, 2007; Neba, 2007).

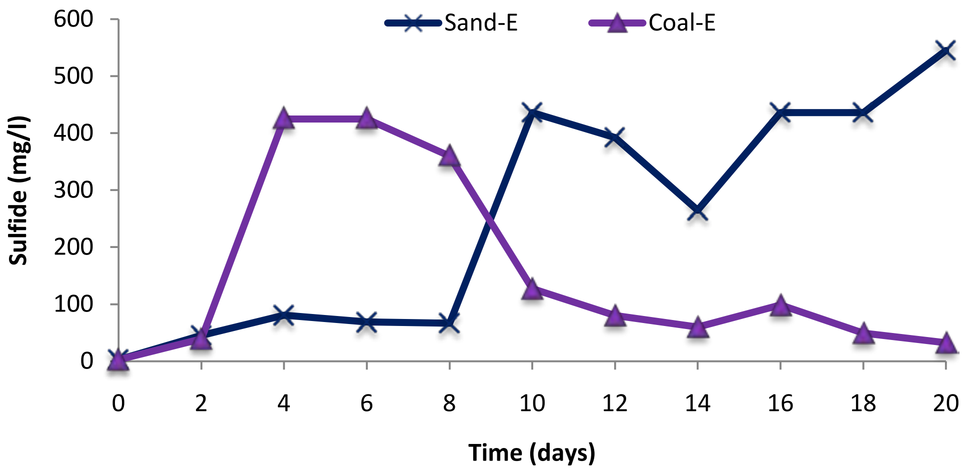


Figure 6-11 Sulfide production measured from the effluent in sand and coal up flow anaerobic packed bed reactors using humic acid and lactate as carbon sources.

6.3.3.2 pH change

Changes in pH in the sand and coal packed up-flow bioreactors were compared to the un-inoculated control reactors (Figure 6-12A-C). In the sand bioreactor, the pH increased from pH 4.0 at both P1 and P4 over time and peaked at pH 6.1 and pH 7.0 after 8 and 14 days respectively (Figure 6-12A). In the coal reactor, there was a rapid increase in the pH from P1 (pH 4.0) to P4 (pH 6.8) at day 0. This was followed by a gradual increase in the pH to pH 5.0, where it fluctuated for the remainder of the experiment, while at P4, a gradual decrease was observed over the same period. Towards the end of the experiment, the pH at P1 and P4 was similar suggesting establishment of steady state conditions in the coal reactor (Figure 6-12B).

In the un-inoculated controls, there was no change in pH in the sand reactor between P1 and P4. However, in the coal reactor, the pH at P4 increased to pH 6.5 from pH 4.0 at P1.

The increase in pH between P1 and P4 in the sand experimental reactor can be attributed to the generation of sulfide by the adapted SRB, and is confirmed by the absence of pH increase in the un-inoculated control. While the pH increase at P4 in the coal reactor can be attributed to the presence of SRB, the coal also appeared to play a role in increasing the pH. However, the presence of the SRB becomes apparent when the pH at P1 in the experimental reactor increases more than the pH at P1 in the control reactor. Similar observations were also noted at P4 (Figure 6-11C). A statistical t-test analysis of the pH in the experimental reactors shows that in the sand reactor there was a significant difference in the pH at P1and P4 ($p < 0.05$, t-value = -2.53, df = 20). A statistical t-test analysis of the difference in pH between P1 and P4 in the coal reactor, was highly significant ($p < 0.01$, t-value = -4.70, df = 20).

These results showed that the coal material raised the pH of the medium in the absence of a biological catalyst. This could be attributed to the release of carbonates and alkalizing substances from the coal. Coal mine water discharged from abandoned Caphouse Colliery, United Kingdom was found to be supersaturated with iron and manganese hydroxides, with a pH ranging from pH 6.7 – 7.9 and a high alkalinity (Kruse and Younger, 2009).

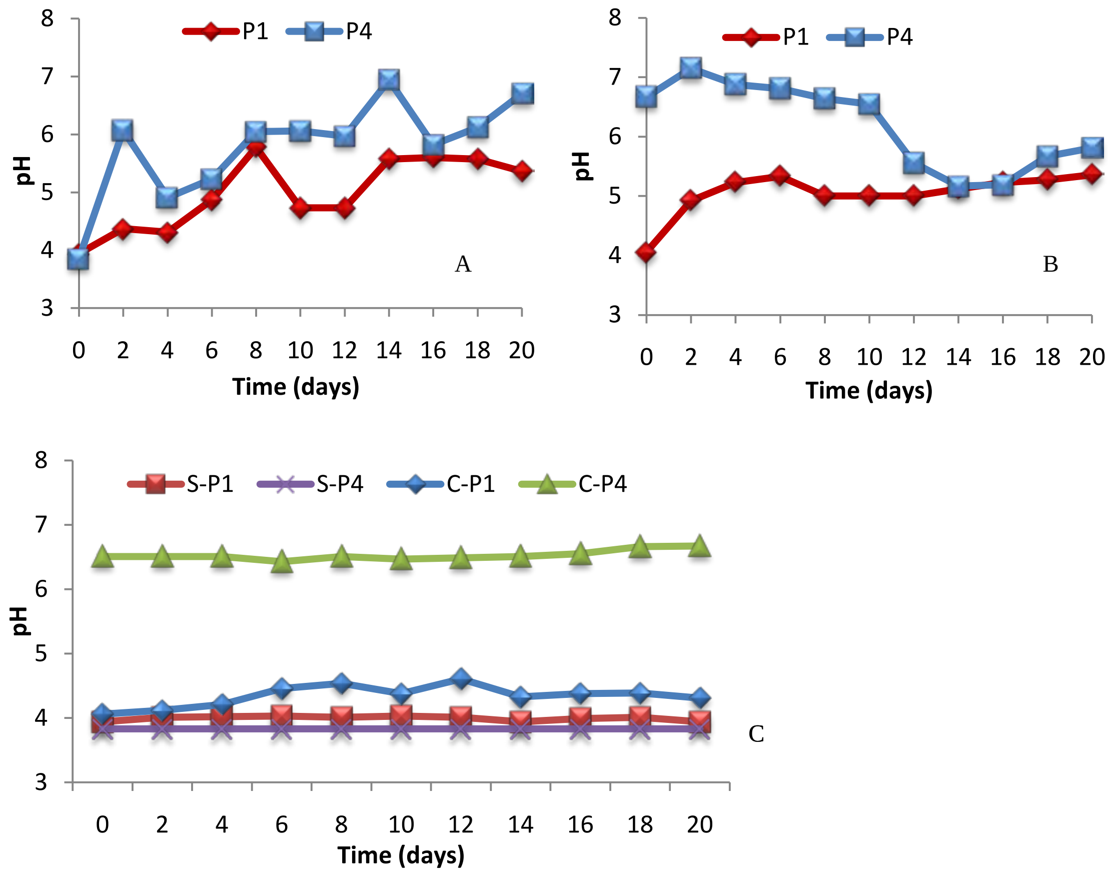


Figure 6-12 pH changes in A - up flow anaerobic sand packed bed reactor and B - up flow anaerobic coal-packed bed reactor, during the investigation of humic degradation by sulfate reducing bacteria. C = un-inoculated control reactors. (S-P = sand sampling port; C-P = coal sampling port).

6.3.3.3 Humic acid profile

Changes in the HA concentration along the column of the sand and coal reactors were investigated to determine the degradation and/or adsorption of the HA in the respective reactors (Figure 6-13A). The influent HA concentration fed into sand and coal reactors, was ~ 55 $mg.L^{-1}$ at day 0. In the sand reactor, the extractable HA measured at P1 increased gradually over time and peaked at 110 $mg.L^{-1}$ after 10 days before dropping by 16% to 97 mg.L^{-1} where it remained for the duration of the experiment. At P4, the HA content decreased initially by 56% to 24 mg.L^{-1}at day 0, before increasing and fluctuating at 20 ± 2 mg.L^{-1} until the end of the experiment (Figure 6-13A). In the coal reactor (Figure 6-13B), the HA at P1 was similar to the influent HA feed (55 mg.L^{-1}) at day 0. A 49% decrease in the HA content was observed after 4 days suggesting increased retention within the system from either adsorption onto the coal surface or biodegradation by the SRB. Thereafter, the HA gradually increased to 54 mg.L^{-1}until the end of the experiment. At P4, in the coal reactor, a low HA content fluctuating ~ 5 mg.L^{-1} was measured throughout the experiment.

In the un-inoculated sand control reactor, there was no substantial increase in the HA concentration at P1 over 20 days. At P4, there was a 54% decrease in the HA content over the course of the study, indicating adsorption of the HA to the sand (Figure 6-13C). In the coal control reactor, the HA content at P1 decreased by 21% to 44 mg.L^{-1} at day 0, and remained constant for the remainder of the study, while an average of 5 mg.L^{-1} HA was measured at P4 over the same period (Figure 6-13C).

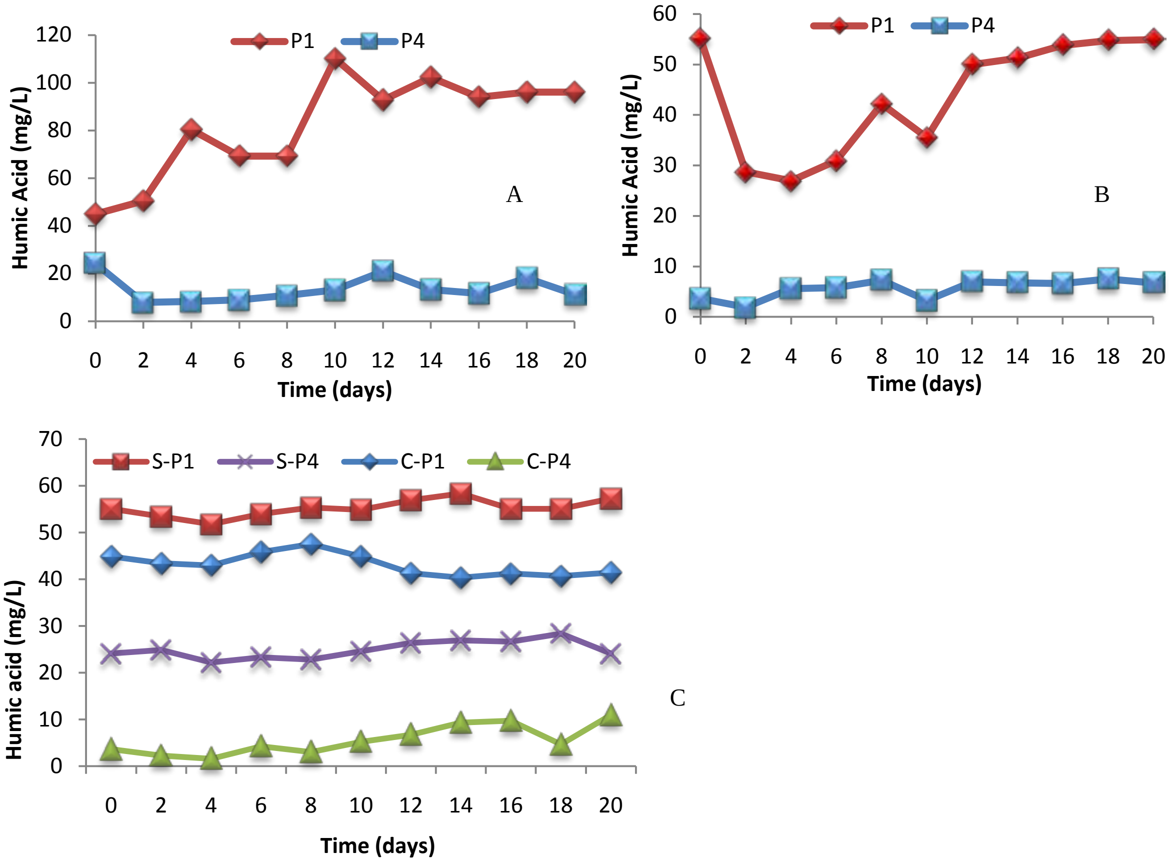


Figure 6-13 Changes in extractable humic acid content in A- up flow anaerobic sand packed bed reactor and B- up flow anaerobic coal packed reactor on the investigation of humic acid degradation by sulfate reducing bacteria. C = un-inoculated control reactors. (S-P = sand sampling port; C-P = coal sampling port).

6.3.3.4 Comparison of sand and coal reactors

A statistical t-test comparison of sulfide concentration showed that there was no significant difference in the production of sulfide ($p > 0.05$, t-value = 1.24, df = 20) and pH ($p > 0.05$, t-value = -1.17, df = 20) between the sand and coal reactors.

A highly significant difference in the retention of HA at P1 between the sand and coal reactors was observed, with the coal reactor retaining 50% more HA than the sand reactor ($p < 0.01$, df = 20). Similarly, the difference in HA at P4 in both sand and coal reactors was highly significant ($p < 0.01$, t-value = 4.61, df = 20). Similar observations were also noted in the un-inoculated controls, where, in the sand reactor, 21% more HA was measured than in the coal reactor at P1, while 78% more HA was eluted in the sand than the coal reactor.

These results suggest that there was a combination involving adsorption of the HA to the packing matrix, as well as its utilization by the SRB. In the absence of SRB, 45% more HA was eluted at P4 in the control sand reactor, than in the experimental reactor, which can be attributed to the presence of the biocatalyst. On the other hand, the coal reactors appear to indicate a larger adsorption effect of the HA on the matrix. There was no significant difference ($p>0.05$, t-value = 0.04, df = 20) in the effluent from the experimental reactors and the un-inoculated control reactor, therefore suggesting that the reduction in HA content in the coal reactor was not due to the presence of a biocatalyst but due to a chemical adsorption effect. Furthermore, these results do not clarify at the point whether the HA or the lactate was primarily responsible for sulfate reduction.

6.3.4 The role of humic acid in sulfide production

The ability of SRB to utilize HA as an electron donor was investigated by successively reducing the lactate co-substrate concentration in the reactor feed to zero. The lactate concentration was adjusted in each case after two reactor volumes had passed allowing establishment of approximately steady state conditions for each concentration of feed.

6.3.4.1 The effect of lactate concentration on pH

The pH profile of the effluent from the sand and coal reactors was not greatly affected by the reduction in lactate concentration until the lactate had been completely removed from the feed (Figure 6-14).

The pH in the coal reactor was higher than the sand reactor as observed in the experimental and control reactors (Figure 6-12). When the lactate had been completely removed from the feed reservoir, the pH in both reactors dropped dramatically. In the sand reactor, after day 16, the pH dropped from pH 6.2 to below 4, while in the coal reactor, the pH dropped from pH 7.2 to pH 4.4 at the end of the experiment. In spite of this, the SRB in the coal reactor were able to keep the pH above 6.0 for 5 days before the system failed. This further suggests that a portion of the alkalinity generated in the coal reactor was derived from the coal packing material.

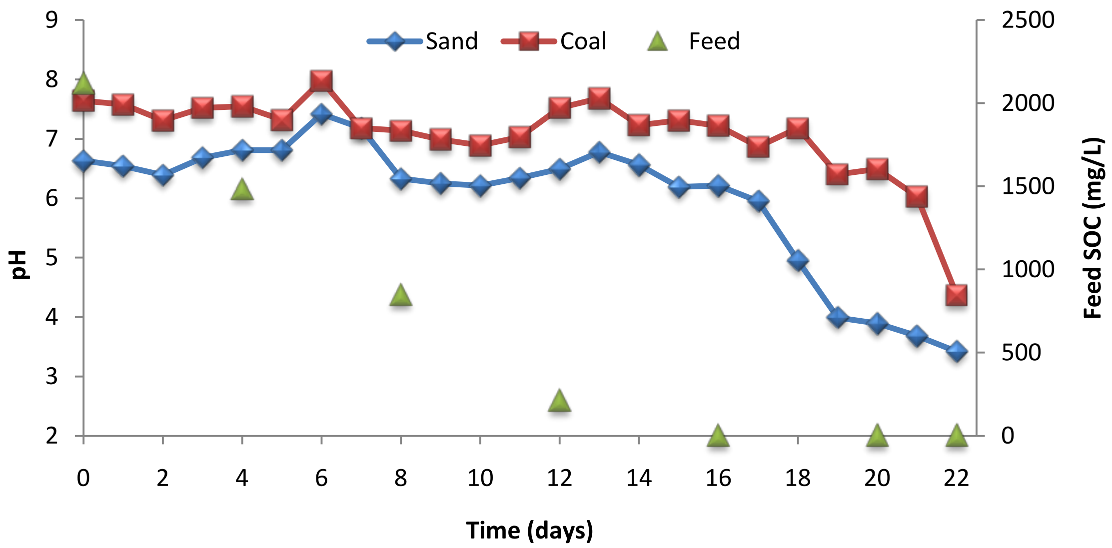


Figure 6-14 pH changes of the effluent from the sand and coal up flow anaerobic packed bed reactors, with gradual reduction in lactate concentration.

6.3.4.2 Sulfide production

Figure 6-15 shows sulfide production in both sand and coal reactors, with progressive reduction of lactate in the feed reservoir. In the sand reactor, the sulfide concentration initially increased by 64% to 948 $mg.L^{-1}$ between day 0 and 2, as 100% lactate was fed into the reactor. This was followed by rapid decrease in sulfide content to 621 $mg.L^{-1}$ after 4 days. Reduction of the lactate by 30% in the medium, at day 4, did not reduce the sulfide concentration, instead it increased by 18% to 730 $mg.L^{-1}$.

Further reduction of the lactate to 40%, resulted in an 18% decrease in sulfide concentration to 610 $mg.L^{-1}$, which continued to decline to 560 $mg.L^{-1}$ after 10 days. Subsequent reduction of lactate to 10% resulted in a 64% decline in sulfide concentration to 127 $mg.L^{-1}$ at day 14. The complete removal of lactate in the feed reservoir after 16 days resulted in an 88% decrease in sulfide to 17 $mg.L^{-1}$ and failure of the reactor after 22 days (Figure 6-15).

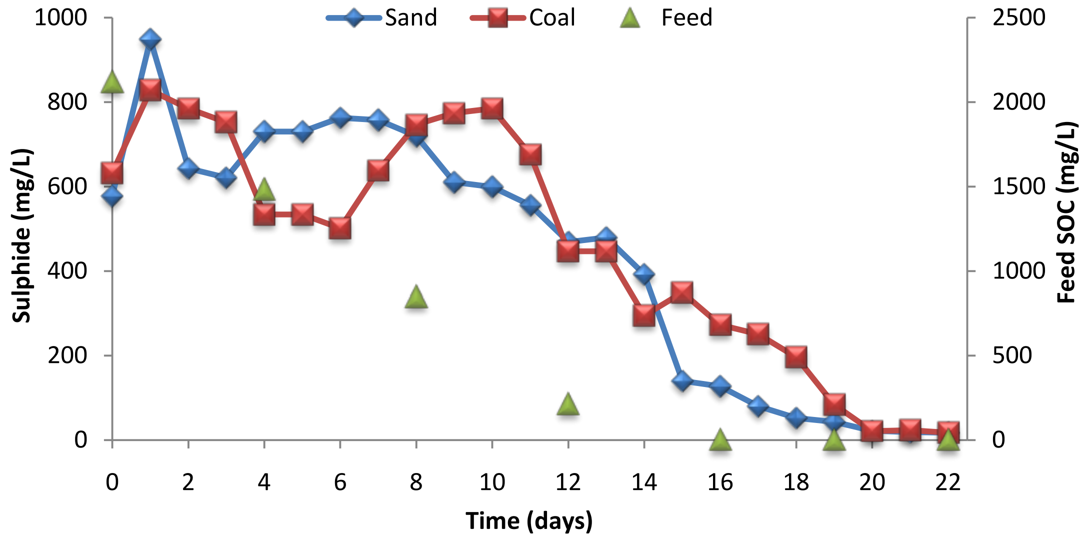


Figure 6-15 Sulfide concentration of the effluent from the sand and coal up flow anaerobic packed bed reactors, with gradual reduction in lactate concentration.

Sulfide production in the coal reactor followed a similar trend to the sand reactor as lactate concentration was reduced over time. Reduction of lactate to 70% resulted in a 29% decrease in sulfide production after 4 days. However, when lactate was reduced to 40%, a 49% increase in sulfide concentration (785 $mg.L^{-1}$) was observed at day 10.

As previously noted in section 5.3.2.2, a stoichiometric *R* of 0.63 is considered to be ideal for complete conversion of sulfate to sulfide using a lactate substrate (Vela *et al*., 2008; Velasco *et al*., 2008). The *R*s presented in Table 6-2 and Table 6-3 are below this threshold, and therefore demonstrate that the lactate alone was not entirely responsible for the sulfate reduction in both reactors. The difference between the lactate used and the minimum additional carbon that would be required to account for the observed sulfate reduction suggests the use of HA as an electron donor substrate. Since only small concentrations of the lactate are required to facilitate the utilization of HA as an electron donor, this would indicate that the lactate functioned in this system as a true co-substrate.

Table 6-2 Stoichiometric ratio of soluble organic carbon to sulfate ratio for lactate utilized in the presence of humic acid substrate in a up flow anaerobic sand packed bed reactor studies.

Lactate Conc.	Total lactate C added ($mg.L^{-1}$)	Total SO_4 reduced ($mg.L^{-1}$)	lactate C:SO_4 ratio (*R*)
100%	1471	8731	0.17
70%	1030	8944	0.12
40%	588	7456	0.08
10%	147	4442	0.13
0%	0	1080	

Table 6-3 Stoichiometric ratio of soluble organic carbon to sulfate ratio for lactate utilized in the presence of humic acid substrate in up flow anaerobic coal packed bed reactor studies.

Lactate Conc.	Total lactate added ($mg.L^{-1}$)	Total SO_4 reduced ($mg.L^{-1}$)	lactate C:SO_4 ratio (*R*)
100%	1471	8992	0.16
70%	1030	6622	0.16
40%	588	8944	0.07
10%	147	4610	0.03
0%	0	2592	

This study has demonstrated that the HA fraction derived from oxidized HC, together with the addition of a co-substrate is able to provide a satisfactory electron donor for sulfate reduction. This was initially not suspected as previous reports have pointed to its function as an electron acceptor in sulfate reducing systems (Scott *et al.*, 1998; Cervantes *et al.*, 2001). The use of the alkaline HA extract also circumvents the problems of blockage in packed bed reactor designs where these are loaded with a particulate feed.

However, it has been shown that the co-substrate play a crucial role in the process if only required in low concentrations. Clearly, lactate could not play this role in extensive use of the process to treat large volumes of the AMD in the field.

6.4 Biological extraction of humic acid for sulfate reduction

Having shown that particulate coal substrates cause blockage problems in the UAPB reactors, the system was run with HA chemically derived from coal, which solved the problem. It was also shown that a co-substrate was crucial for sulfate reduction. However, for potential bioprocess application of the process, one would require sufficient HA amounts not derived chemically. Based on the chemical alkaline fractionation of oxidized HC section 2.2.3 and the generation of alkalinity by SRB during sulfate reduction (section 5.3.2.1), it was decided to develop a bioprocess model that used the alkalinity generated from the sulfidogenic process in a UAPB to extract the HA (Figure 6-16). This would serve as the electron donor and carbon source in conjunction with a co-substrate for BSR in remediation of AMD.

In this bioprocess, BSR using coal derived HA with an inexpensive co-substrate as carbon sources and electron donors, and sulfate ions from AMD as terminal electron acceptors would generate an alkaline effluent (without the metal sulfides) that would be pumped into an anaerobic continuously stirred reactor containing oxidized HC (Figure 6-16). The reactor would be agitated and incubated until sufficient amounts of HA were extracted from the oxidized HC into solution. A separation step would remove the residual particulate matter from the soluble HA in a settling tank. A portion of the extracted HA would be pumped back into the UAPB to drive BSR and generation of alkalinity (Figure 6-16), while the other portion would be used for downstream process of interest such as rehabilitation of mined land.

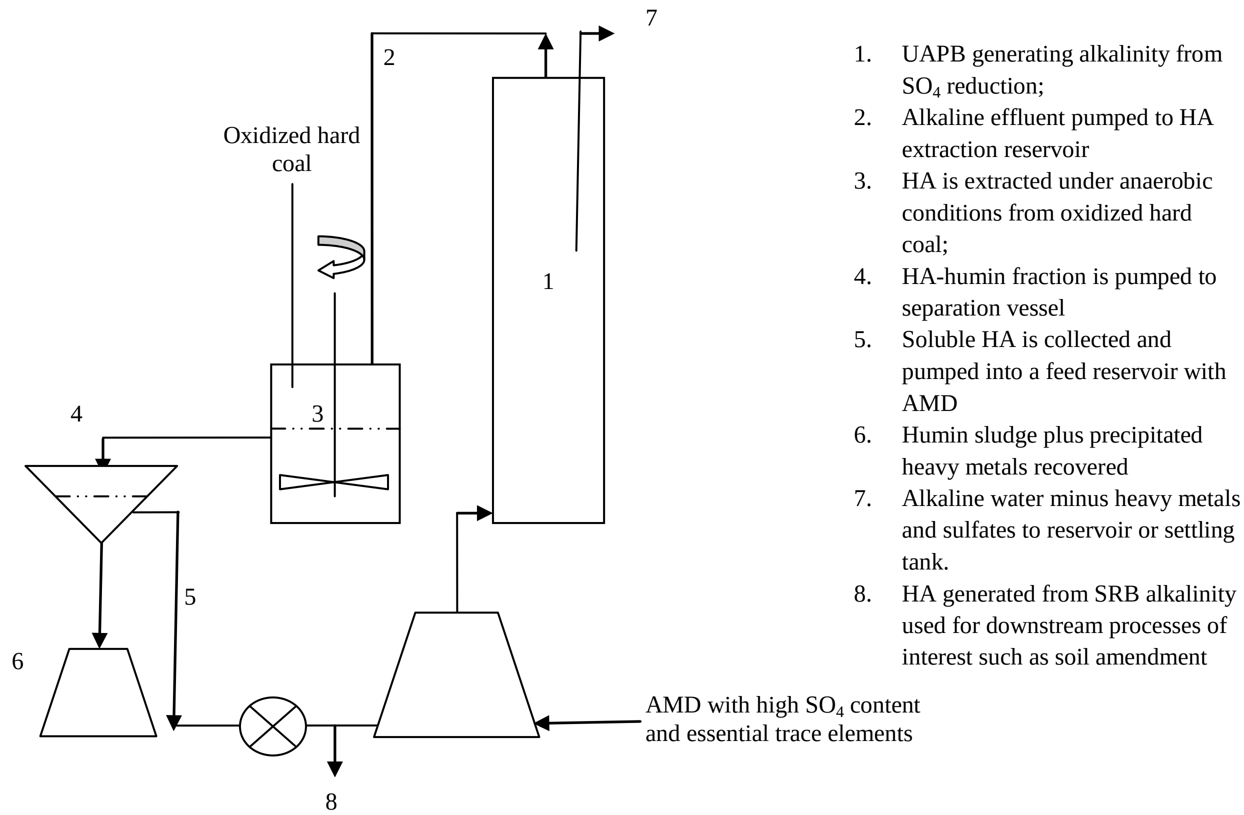


Figure 6-16 Schematic flow diagram of the proposed biological extraction of humic acid from oxidized hard coal using alkalinity generated from sulfate reduction in an up flow anaerobic packed bed reactor.

Figure 6-17 shows the extraction of HA from oxidized HC using an alkaline effluent generated during BSR in a sand packed UAPB reactor. An increase in pH was observed during the study, although there was an initial drop at the beginning from pH 7.6 to pH 6.1 on day 1. By maintaining anaerobic conditions in the flask, the residual bacteria from the UAPB reactors were able to maintain the alkalinity of the effluent feed. Preliminary tests during this study, where the extraction process was aerobic did not yield soluble HA from the coal and a decrease in the pH was also observed. Therefore, anaerobic conditions were required to extract HA from oxidized HC.

As previously described in section 2.3.3, the total extractable HA from oxidized HC substrates was ~ 35% (w/v). In the first 3 days, while the alkaline effluent from the UAPB was being added into a reactor containing oxidized HC, the extractable HA content increased ~ 3-fold to 26%, where it remained constant until day 6. Thereafter, the HA content increased by ~ 1.8 fold to 48% after 8 days, and increased to ~ 50% after 12 days when the experiment was stopped (Figure 6-17).

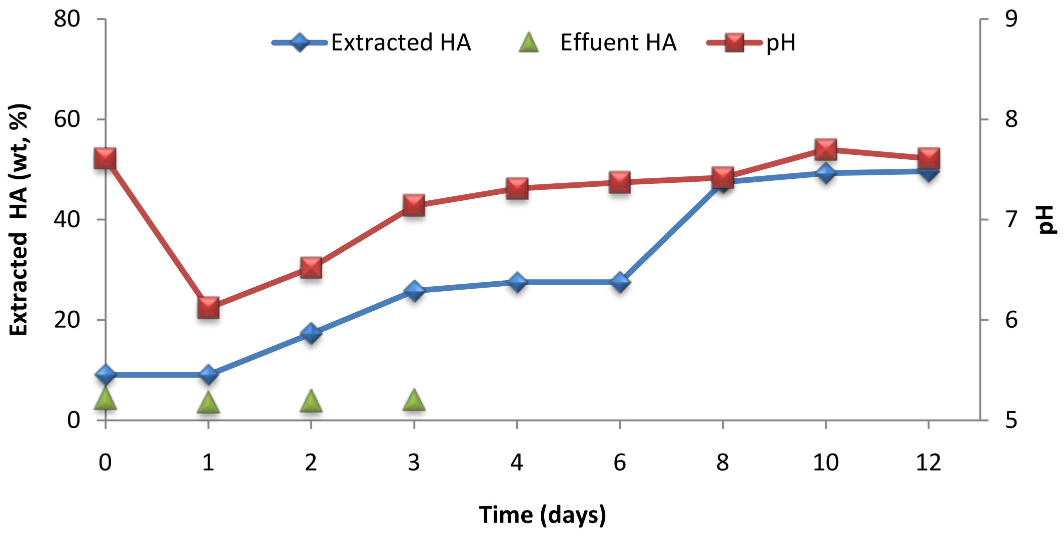


Figure 6-17 pH changes and extraction of humic acid from 1.0% oxidized hard coal in 5 L reactors using effluent from a sand packed up-flow sulfate reducing bacteria reactor. ▲ = indicates the addition of an alkaline effluent from the sulfate reducing bacteria reactor into the extraction reactor containing the oxidized hard coal.

In contrast to the sand effluent, the pH in the coal reactor was higher than the sand effluent with the highest pH of 8.1 being measured after 4 days of the study. The pH then gradually decreased to pH 7.7 by the end of the experiment on day 12.

Similar trends were observed with effluent from the coal packed UAPB (Figure 6-18). However, the HA yield was ~ 3 times lower than the sand effluent, with a maximum extractable HA concentration (19%) being achieved after day 8 of the experiment.

An intriguing observation in which the sand packed UAPB reactor yielded a higher extractable HA concentration than the coal packed UAPB reactor, despite a higher pH (± pH 8.0) in the coal packed UAPB effluent suggests the hindrance of the extraction process by the presence of the coal. However, this would require further interrogation for optimization of this bioprocess.

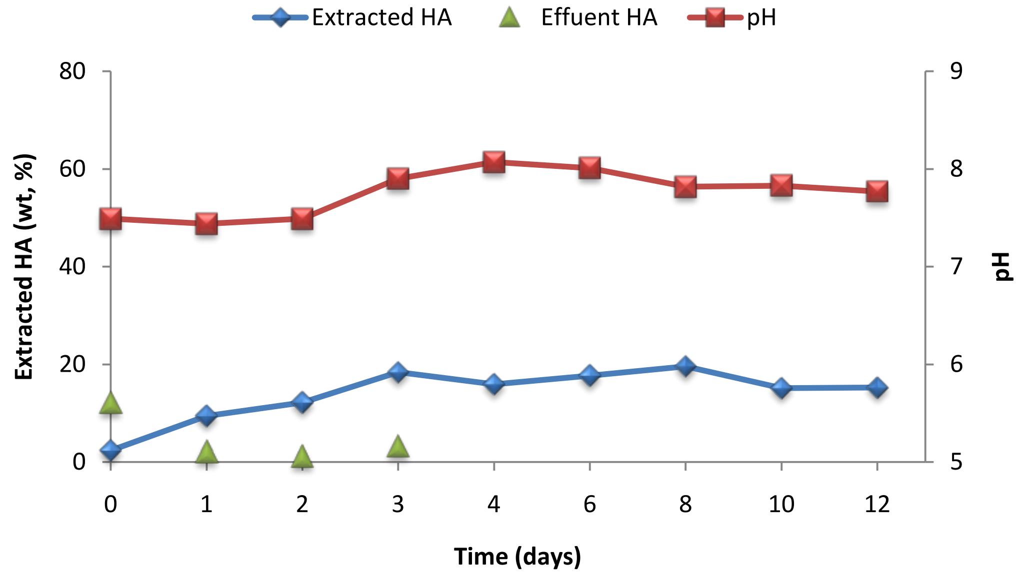


Figure 6-18 pH changes and extraction of humic acid from 1.0% oxidized hard coal in 5 L reactors using effluent from a coal packed up-flow sulfate reducing bacteria reactor. ▲ = indicates the addition of an alkaline effluent from the sulfate reducing bacteria reactor into the extraction reactor containing the oxidized hard coal.

6.5 Conclusions

The following conclusions can be drawn from these results:

- Sulfate reduction occurs in continuously operated UAPB reactors utilizing oxidized HC as a substrate;
- The particulate nature of the feed causes blockage and ultimately failure of the UAPB;
- The alkalinity generated in the sulfate reduction operation can be used for the extraction of HA from oxidized HC and provides an effective electron donor in the presence of lactate functioning as a true co-substrate;
- The lactate C: SO_4 ratio showed that in continuous systems, a portion of the C used in sulfate reduction was derived from coal;
- Continuous systems are also able to overcome limitations of sulfate reduction observed in the batch studies.

The use of a HA extract as a more refined substrate eliminates particulates associated with blockages of the UAPB. However, a problem that still remains is the essential requirement of a co-substrate and clearly the use of lactate in large scale process applications could not be envisioned. Viability of the process potential of this system will thus be substantially influenced by the availability of alternative inexpensive source of co-substrates. Where possible these should be readily available and/or generated on or close to the mining site. One such co-substrate is investigated in the following chapter.

CHAPTER SEVEN

PRODUCTION OF FUNGAL BIOMASS AS A CO-SUBSTRATE FOR SULFATE REDUCTION

7. INTRODUCTION

Previous studies showed that the use of a co-substrate was an essential requirement for the degradation of oxidized HC by anaerobic microbial consortia using either carbon dioxide or sulfate as terminal electron acceptors. Both grass and lactate had been used as sources of co-substrate for laboratory studies. However, the cost implication would be prohibitive and fatally impact on attempts to scale up the sulfate reduction unit operation in particular for application in the mining environment. Although grass had been shown to function as an effective co-substrate in methane production, it could become limiting in the large stacked heap leach type reactors envisaged. A need was thus identified to develop a low cost co-substrate in which the use of oxidized HC as a substrate on site would present substantial advantage.

Fungi have been shown to solubilize low rank coals as well as oxidized HC substrates (Silva-Stenico *et al.,* 2007; Igbinigie, 2008). The production of fungal biomass has been extensively used in a wide range of industrial applications that include the production of extracellular enzymes, hormones, specialty chemicals and biomass (Tari *et al.*, 2007). More relevant to this study is the large-scale production of fungal biomass using an inexpensive and widely distributed substrate as a carbon source. GOC constitutes such a substrate, although fungal biosolubilization of coal has been shown to produce limited methane yields (Johnson *et al.*, 1994; Volkwein *et al.*, 1994; Panow *et al.*, 1997). In solubilization studies, Igbinigie (2008) had reported that the new isolate *N. fischeri* is capable of rapidly generating large volumes of fungal biomass in an oxidized HC-enriched medium. As in the studies already reported here, Igbinigie also found that effective biodegradation of HC was dependent on the presence of an easily metabolized co-substrate. Glutamic acid was used as a co-substrate in this process.

Glutamic acid is an abundant low cost substrate that can be easily derived from the fermentation of protein rich effluents such as swine manure and palm wine waste by *Bacillus subtilis* and *Brevibacterium lactofermentum,* respectively (Das *et al.*, 1995; Chen *et al.*, 2005).

The nature of the substrate and targeted products are critical in determining bioreactor configuration (Williams, 2002) and the choice of reactor provided an important focus in this study. Two reactor configurations, namely Packed bed reactors (PBR) and CSTR were investigated for their feasibility to generate fungal biomass and solubilize oxidized HC.

These studies were undertaken to investigate the feasibility of generating large volume fungal biomass for use as a low cost co-substrate in methane production and sulfate reduction using oxidized HC as a substrate.

7.1 Objectives

The objectives of the study reported in this chapter were to:

PART A

- Investigate fungal biomass production using *N. fischeri* grown on GOC;
- Compare solid state and stirred liquid culture reactor configurations in the production of biomass.

PART B

- Investigate the use of fungal biomass as an electron donor and carbon source for sulfate reduction.

PART A

7.2 Materials and Methods

7.2.1 Inoculum development

Inoculum preparation was performed by scraping and washing off *N. fischeri* spores in sterile distilled water from the surface of cultured Potato dextrose agar (PDA) plates. The spore suspension was collected into a Schott bottle containing 100 mL of distilled water and mixed thoroughly. The washing was repeated until a spore count of ~ 1.8×10^9 spores.mL^{-1} was attained. The spore count was determined using a haemocytometer (Neubauer, Merck, South Africa). The spore suspension was used as the inoculum in the reactor studies.

Twenty mL of *N. fischeri* spore suspension (~1.8 x 10^9 spores.mL^{-1}) was then transferred into 1 L of Potato dextrose broth (PDB) in a conical flask (Sigma, South Africa), and placed on a rotary shaker (120 rpm, 30^{o}C). The spores were allowed to develop into pellets and harvested during the exponential growth phase after 2 days. The biomass was harvested by filtration through a sterile 36 µm nylon mash and washed several times with sterile distilled water until a clear rinsate was observed. The pellets were then aseptically transferred into the respective experimental reactors containing prepared medium. For larger bioreactor studies, 100 mL of spore suspension was transferred into 5 L reactor flasks and pre-grown for 2 days before aseptic addition to the bioreactors. Dry fungal pellet biomass was determined gravimetrically on samples by drying duplicate inoculum for 24 h at 60^{o}C.

7.2.2 Biodegradation of pre-treated hard coal in packed bed reactors

The PBR reactor design was used to investigate the growth of fungal spores on the surface of Chemically oxidized coal (COC), since previous work on this substrate had been reported (Igbinigie, 2008). Furthermore, GOC was found to disintegrate when stacked into a column reactor. This would also enable modification of the coal matrix thereby stimulating the production of secondary metabolites from COC substrate. It was intended to expand this to GOC and then to BOC at a later stage of the investigation. The fungal biomass and the solubilized coal (and products) would be used as substrates for the bioprocesses described in chapter 3 to 6.

7.2.2.1 Hard coal preparation

Hard coal (300 g) was mechanically crushed into 50 ± 10 mm fragments to increase surface area and treated with 1 N HNO_3 in 2 L conical flasks on a rotary flask (140 rpm) for 24 h at 25^{o}C. The resultant oxidized coal was thoroughly washed with dH_2O until pH 5.0 ± 0.1 was achieved.

7.2.2.2 Reactor set up

A 30 x 3 cm tank reactor with a working bed of 25 cm was set up as shown in Figure 7-1. The reactor contained a wire mesh and glass balls (Merck, South Africa) at the cone to prevent the coal from clogging the effluent outlet during perfusion. The reactor was aerated from the bottom with humidified air passed through sterilized air filters as illustrated in Figure 7-1 . The feed was perfused from the top of the reactor and effluent was collected in a continuously stirred reservoir flask, which was then re-circulated back into the reactor though a peristaltic pump at a flow rate of 5 mL.min^{-1}.

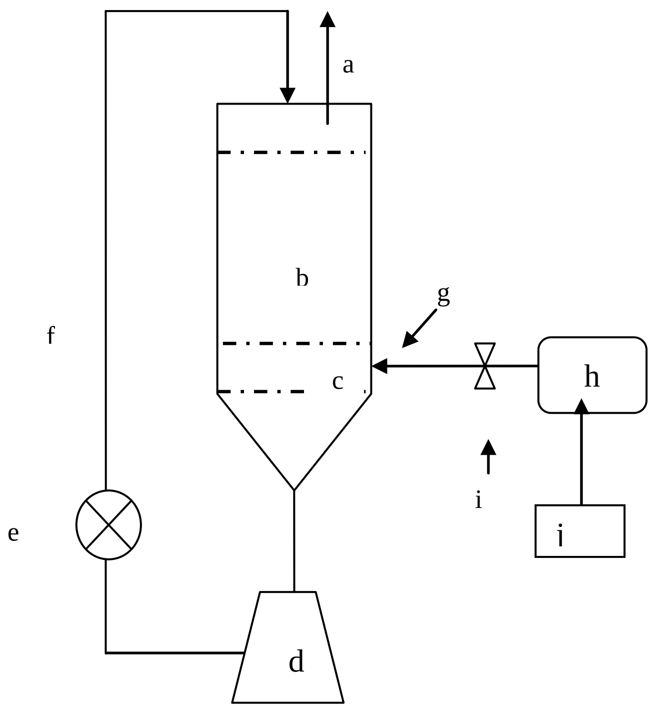


Figure 7-1 Experimental set up of perfusion reactors packed with HNO_3 acid pretreated hard coal.

7.2.2.3 Media formulation

A saline medium adapted from Igbinigie (2008) was prepared in 1 L sterile dH_2O and included: $NaNO_3$ (0.3%, w/v), K_2HPO_4 (0.31%), KH_2PO_4 (1.27%), $MgSO_4.7H_2O$ (0.05%), KCl_3 (0.05%). The pH was adjusted to 5.0 using 1 M NaOH and 1 M HCl (Merck, South Africa). Three experimental reactors and two control reactors were set up (Table 7-1).

Table 7-1 Experimental set up used to investigate the role of glutamic acid in the solubilization of pre-treated hard coal in perfusion reactors.

Reactor	PBR1	PBR2*	PBR 3	PBR 4	PBR 5
HC (HNO_3 treated)	√	√	√	√	√
Glutamic acid	√	√	-	-	√
Saline solution	√	√	√	√	-
N. fischeri	√	√	√	-	-

* - pH maintained at 5.0 with 1 N HCl.

7.2.2.4 Perfusion reactor operation

Distilled water was circulated though all the reactors for 24 h to wash off residual HNO_3 and carbon from the acid pre-treatment of the coal. A 4 ml concentrate of *N. fischeri* spores in 200 ml of saline medium was used to inoculate the experimental reactors. The fungus was allowed to establish on the surface of the coal for 48 h with continuous circulation of the respective feeds before perfusion of new medium was started at 10 $mL.min^{-1}$. Continuous aeration of sterile air prevented the reactor from clogging. The experiment was conducted at 30^oC. Samples were collected daily for glutamic acid utilization and every second day for SOC to monitor the breakdown of coal. Humic acid formation from degradation of HC was also monitored spectrophotometrically at 450 nm.

The fungal growth rate was determined by washing off the biomass from the coal, filtering through a 36 µm mesh, followed by oven drying at 60^oC for 24 h. The biomass was weighed and used to calculate the growth rate.

7.2.3 Flask studies

Although the chemical partitioning studies section 2.2.3 were conducted with 1.0% (w/v) GOC, it was observed in the biological studies, that concentrations greater than 1.0% inhibited the aerobic fungal activity. Therefore, all biological studies reported here were conducted with the lower concentrations up to 1.0%.

N. fischeri spores were inoculated into 5 L of PDB and incubated in flasks on a rotary shaker (30º C, 120 rpm, 48 h). The liquid shake culture conditions resulted in the growth of fungal pellets measuring ~ 8 mm in diameter, with a sponge-like texture, which collapsed when removed from the submerged liquid cultures. The fungal pellets formed were harvested by filtering through a sterile 36 µm mesh and excess PDB was washed off with sterile dH_2O. A pre-determined wet weight of *N. fischeri* pellets were inoculated into 1 L experimental flasks containing an enrichment medium that included glutamic acid 0.2% (w/v), NH_4NO_3 (0.3%), $MgSO_4.7H_2O$ (0.05%), and KCl_3 (0.0 5%). The medium was added into separate flasks together with different concentrations of GOC (0.2 – 1.0%, w/v). The flasks were incubated on a rotary shaker (30 ºC, 120 rpm, 24 h), and were monitored for pH and clearance of GOC from the enrichment medium. Fakoussa and Frost (1999) used visual observation of growth as an indicator for reactor performance. SOC was used to validate clearance of the coal substrate from the medium. It must be noted that glutamic acid analysis was not performed at this stage but only after optimization of the process in preparation for the inoculation of larger volume bioreactor was involved.

Fungal pellets that had cleared the GOC from solution were fed with additional GOC (0.2%), which was added incrementally until saturation was achieved. The fungal pellets that did not remove GOC from solution had the pH re-adjusted to 5.0 (to minimize bacterial contamination) and were not re-fed. Control reactors containing enrichment medium, with 0.2% GOC alone and, biomass in the absence of GOC were also set up and run concurrently with the experiment. All flask studies were conducted in duplicate.

7.2.4 Bioreactor studies

A 30 L stirred tank bioreactor was set up as illustrated below (Figure 7-2). The medium used was prepared as described in section 7.2.3**,** and included GOC, which was added gradually to maintain a final concentration of 0.2% (w/v). The reactor was sparged with sterile air (10 $mL.min^{-1}$) pumped from the bottom of the reactor through a sintered glass filter. The reactor was agitated continuously (250 rpm) using a motor driven marine-type impeller. The pH of the reactor was set at pH 5.0 to minimize bacterial contamination. All bioreactor studies were conducted at $30^{o}C$ and repeated at least once.

7.2.4.1 Dry cell mass determination

The fungal biomass with coal attached was harvested from the reactors, re-suspended in 0.1M NaOH and agitated for 24 h. The resulting biomass/humin was filtered through a 36 µm mesh to recover the soluble HA. The samples were then dried for 24 h at $60^{o}C$ to a constant weight. The weight of the fungal biomass was calculated by subtracting the weight of the biomass/humin from the dry weight of the biomass/coal.

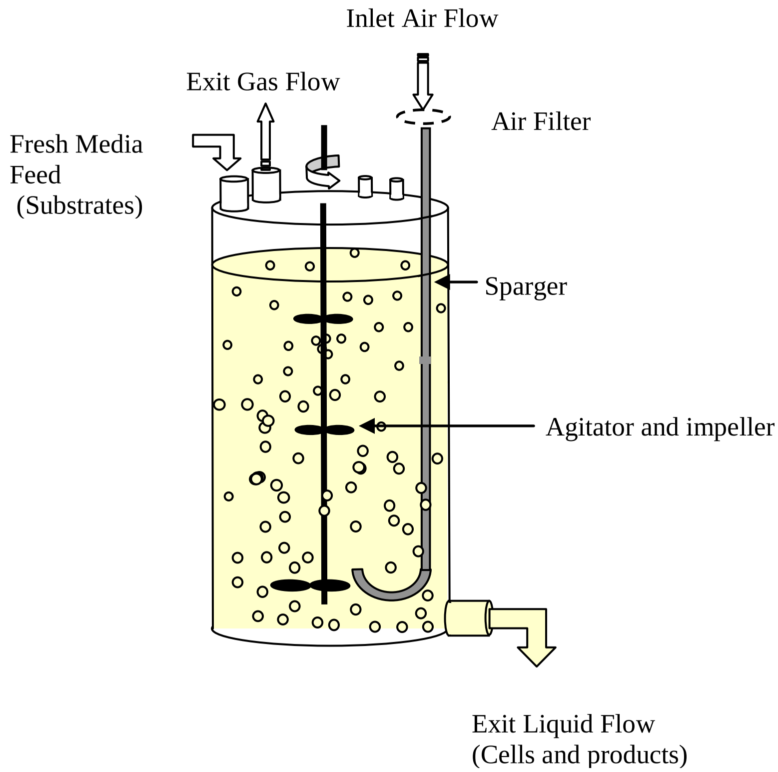


Figure 7-2 Schematic representation of the aerobic continuously stirred tank bioreactors used in the investigation of fungal biomass production and coal degradation.

7.2.5 Analyses

7.2.5.1 Glutamic acid

A modified glutamic acid assay, adapted from Hwang and Ederer (1975) was used in this study. This method is based on the colorimetric changes, in which ninhydrin (originally yellow) reacts with the amino acid and turns deep purple (Hwang and Ederer, 1975). 1 mL of 0.35% (v/v) ninhydrin solution in 96% ethanol was added to a 5 mL sample in a test-tube covered with parafilm to avoid the loss of solvent due to evaporation. The test tubes were heated with gentle stirring at 90^{o}C for 7 min, and then cooled to room temperature. Spectrophotometric analysis was carried out at 570 nm to determine the concentration of glutamic acid against a standard curve.

7.2.5.2 Humic acid analysis

The formation of HA was monitored using the procedure previously described in section 2.2.3.

7.2.5.3 Soluble organic carbon analysis

The SOC was determined by TOC measurement as previously described in section 2.2.6.3.

7.2.5.4 Scanning electron microscopy

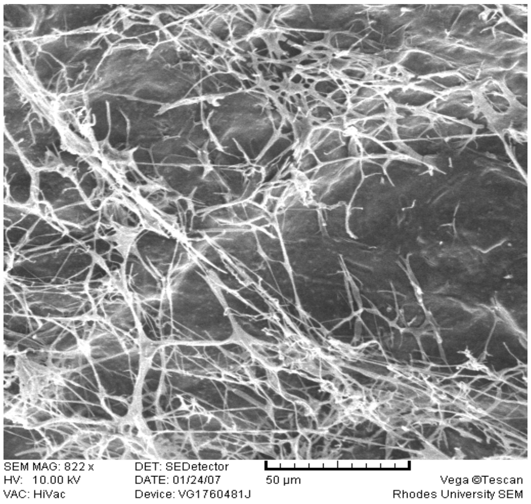
Fungal pellets were harvested from both experimental and control bioreactors and prepared for SEM using a modified method from Cross (2001). Briefly, the samples were fixed overnight in a 2.5% (v/v) glutaraldehyde solution (Merck, South Africa) in 0.1 M pH 7.0-phosphate buffer. The samples were then washed twice with phosphate buffer (0.1 M, pH 7.0, 10 min), dehydrated using an ethanol dilution series (30%, 50%, 70%, 80%, 90%, 100%, with two changes of 100% ethanol; each separated by a 15 min step) and dried (2 h) in a Polaron critical point dryer (Watford England). The dried samples were mounted on 12 mm diameter aluminum stabs, fixed with 12 mm carbon conducting adhesive tabs and then transferred onto a sputtering device (Balzers Union) before gold coating for 160 s at 80-mT pressure at an applied current of 45 mA. Carbon paint was applied on the surface of the gold coating to the aluminum 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.

7.3 Results and Discussion

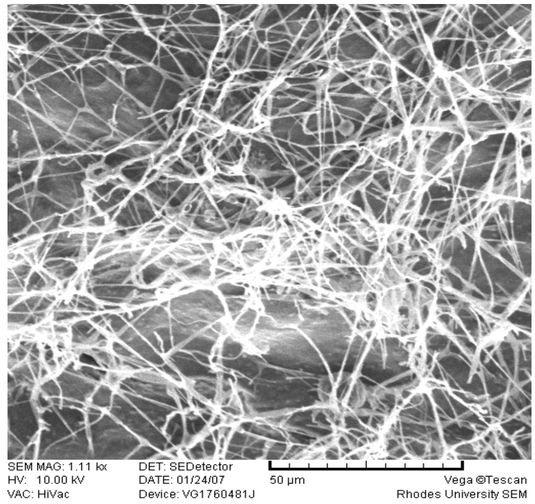
7.3.1 Packed bed reactor studies

7.3.1.1 Growth studies and scanning electron microscopy

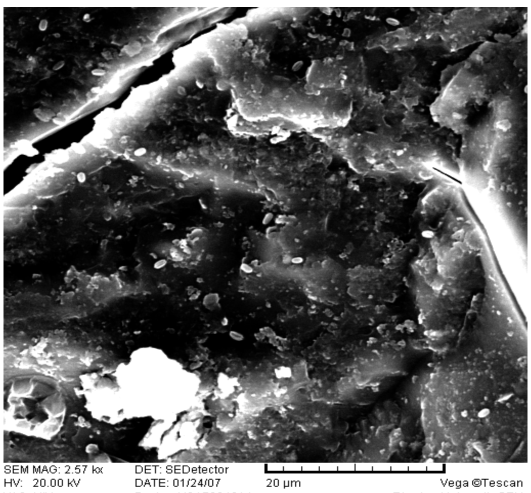
PBR 1 and 2, that were inoculated with *N. fischeri* spores, showed minimal colonization of the HC visually, but upon closer investigation using SEM revealed expansive colonization and penetration of the coal by fungal hyphae (Figure 7-3). The addition of glutamic acid further enhanced the growth and establishment of the fungi on the coal surface in both reactors with an average growth rate of 45 ± 2 mg biomass.L^{-1}.day $^{-1}$. The role of the glutamic acid was confirmed by the lack of fungal hyphal growth on the surface of the coal in PBR 3 control, which had been inoculated with *N. fischeri* but was perfused with a saline medium. No fungal growth was observed in the un-inoculated PBR 4 and 5 controls, which contained saline solution and glutamic acid respectively (Figure 7-3).



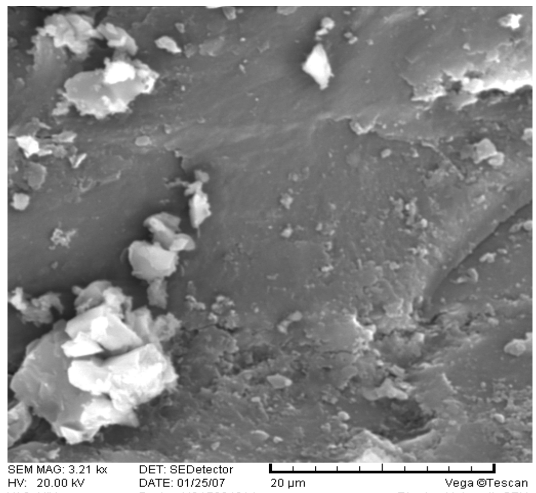
PBR1



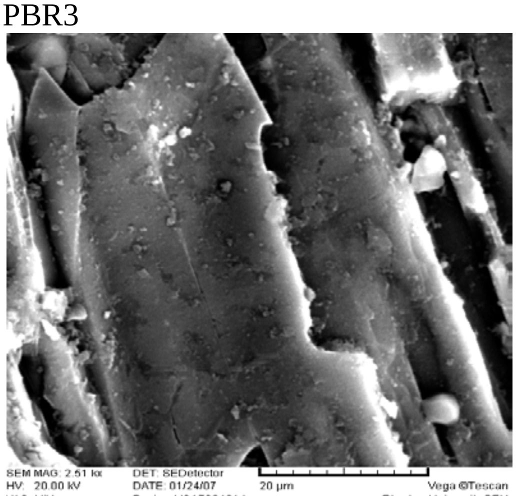
PBR2



PBR3



PBR4



PBR5

Figure 7-3 Scanning electron micrographs of the packed bed reactor 1 – 3 inoculated with *N. fischeri* with different medium configurations and un-inoculated controls 4 and 5.

The establishment of fungal hyphae on the coal surface in the presence of a co-substrate has been reported previously by Laborda *et al.* (1999), where they attributed the formation of extracellular polymer-like structures to the solubilization of coal and nutrient uptake. Although, biomass yields in the PBRs were low, the fungal hyphae demonstrated the ability to partially solubilize the coal.

The growth in observed PBR 1 and 2 was accompanied by a gradual increase in pH which suggested secretion of alkalizing substances, while in the control PBR 4 and 5 the pH dropped from pH 5.0 to ~ pH 4.3 due to a lack of alkalizing substances and, probably due to the residual acid that could have remained un-neutralized during the acid-pretreatment prior to the start of the bioreactors.

7.3.1.2 Carbon analysis

The uptake of glutamic acid and release of SOC in the experimental perfusion bioreactors, was compared against the controls (Figure 7-4). In PBR1, where the pH increased to ~ pH 7.5, a glutamic removal of 98% was observed over 5 days, while a molar carbon removal rate of 93% was observed over the same period. In PBR2, where pH was regulated at pH 5.0, lower glutamic acid and SOC removal rates of 93% and 87%, respectively, were measured over an extended period of 8 days. PBR1 shows the appearance of more carbon in solution as the glutamic acid was utilized, for example at day 2 of the study, a 70% difference in the carbon concentration was observed. However, in the pH regulated PBR2, there was no significant difference in the concentration of the carbon from glutamic acid and SOC in the medium suggesting that the carbon measured as SOC was predominantly glutamic acid ($p<0.05$). After the glutamic acid became exhausted in PBR1 at day 5, the remaining soluble carbon was also removed from solution (Figure 7-4). A biomass yield of ~ 0.07 g biomass.g SOC^{-1} was measured in the experimental reactors (PBR1 and 2) over 14 days.

The control reactors (PBR3 and 4) showed minimal reactivity and demonstrated the critical role of glutamic acid as a co-substrate for coal solubilization. In the un-inoculated PBR5, minimal decrease in the SOC or glutamic acid was noted after 14 days of the study (Figure 7-4).

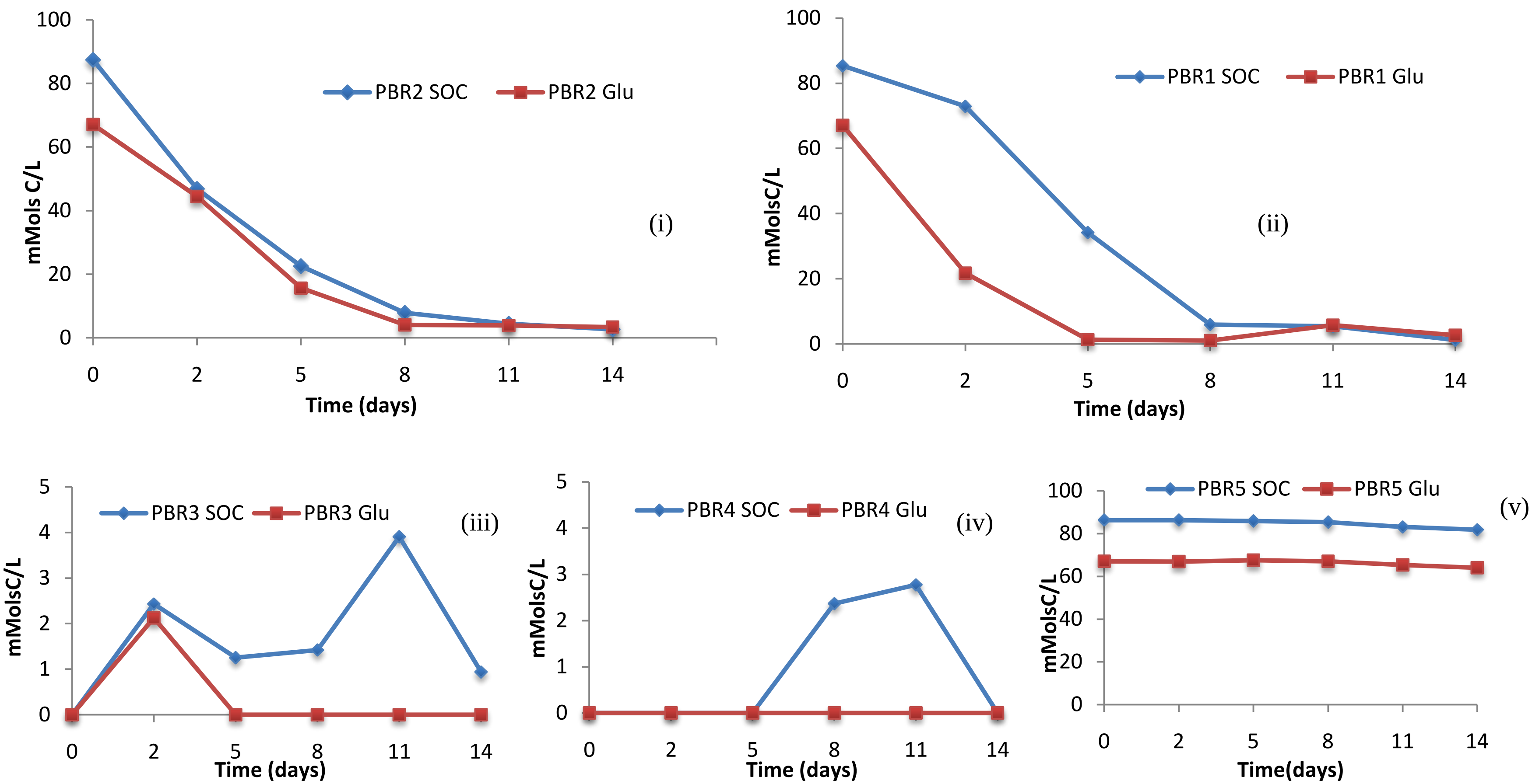


Figure 7-4 Packed bed reactor studies showing the utilization of soluble organic carbon and glutamic acid by *N fischeri* growing on hard coal (i) PBR1 –no pH control (ii) PBR2 – pH maintained at 5.0 with HCl (iii) PBR3 –no glutamic acid (iv) PBR4 – control with no *N fischeri* and glutamic acid (v) PBR5 – control with glutamic acid but no *N fischeri*. PBR = packed bed reactor. Carbon utilization calculated as molsC/l for comparison of glutamic acid and other soluble organics in the reactors.

The carbon results presented in Figure 7-4 shows that pH plays an important role in the metabolism and production of carbon during coal bioconversion. In PBR1, where pH was allowed to increase due to secretion of alkalizing substances (Fakoussa and Frost, 1999; Hölker *et al.*, 1999), release of organic carbon into solution accounted for ~ 70% of the SOC after 2 days of the reaction. On the other hand, in the pH-regulated PBR2 bioreactor, there was no evidence of SOC production, since the production and effect of the alkalizing substances was suppressed by regulating the pH at pH 5.0. The SOC and glutamic acid measured in this study showed a similar trend (Figure 7-4), where there was a preferred consumption of the readily available glutamic acid, followed by minimal consumption of coal derived substrates. After the glutamic acid had been exhausted, the microbial culture shifted to the remaining soluble carbon, which also became exhausted by day 14 in both bioreactors.

Contamination of the perfusion reactors by bacteria cannot be ruled out, since the study was not performed under sterile conditions and may have caused the negligible increase in SOC in the control reactors (PBR3 and 4) and the minimal decrease in glutamic acid in un-inoculated PBR5 observed (Figure 7-4).

These results provided an insight into the role of co-substrate and pH in oxidized HC solubilization. The results further confirm the importance of glutamic acid for growth of fungal species on a coal substrate. According to Hölker *et al.* (1999), coal solubilization by *Trichoderma atroviride* and *Fusarium oxysporum* was strictly dependent on glutamate for its growth. In spite of this, the fixed bed reactor configuration yielded insufficient products for qualitative analysis that would allow closer interrogation of the coal substrate solubilization and/ or oxidation. While there was an apparent indication that HNO_3 oxidized HC could be solubilized, there was insufficient evidence to show the potential of this reactor set up to generate modified products for downstream processes, let alone specialized chemicals. Therefore, it was decided to change the reactor configuration from fixed bed to submerged liquid culture.

7.3.2 Flask studies

7.3.2.1 Growth in liquid culture

The growth of *N. fischeri* using GOC and glutamic acid as C and N sources respectively was investigated and compared to determine the effective range of substrate concentration for optimal biomass generation. This was measured visually by the ability of the fungal biomass to adsorb the GOC and so clear the medium. A light microscopy observation of the surface of the fungal biomass pellets revealed a dark material adsorbed to the surface, which was comparable to GOC

HA (Figure 7-5). In the first GOC substrate feed regime, there was complete adsorption of the GOC in the flasks containing 0.2% GOC after 24 h (Figure 7-6). Successive addition of 0.2% GOC feed aliquots was performed until the biomass became saturated. Four 0.2% GOC batch feeds with a cumulative concentration of 0.8% were added into the 0.2% flask before the biomass became saturated after 120 h (Table 7-2). In the flasks containing 0.4% GOC, the second feed was only adsorbed completely from the medium after 48 h, with no further adsorption after addition of the third feed. The total concentration of GOC that was adsorbed onto the surface of the fungal pellets in the 0.4% flask was thus also 0.8%. Complete adsorption was not observed in the first feed regime of flasks containing 0.6 – 1.0% GOC after 24 h. Extended incubation of up to 72 h did not improve the adsorption rate in the flasks. Instead, bacterial contamination was observed by the appearance of cloudy solution in the flasks (Table 7-2). In the control reactors containing GOC in the absence of fungal biomass, no clearance was observed, instead the substrate was loosely suspended in the medium, and settled to the bottom once agitation was stopped. Again, bacterial contamination was observed by the appearance of a cloudy suspension after 120 h.

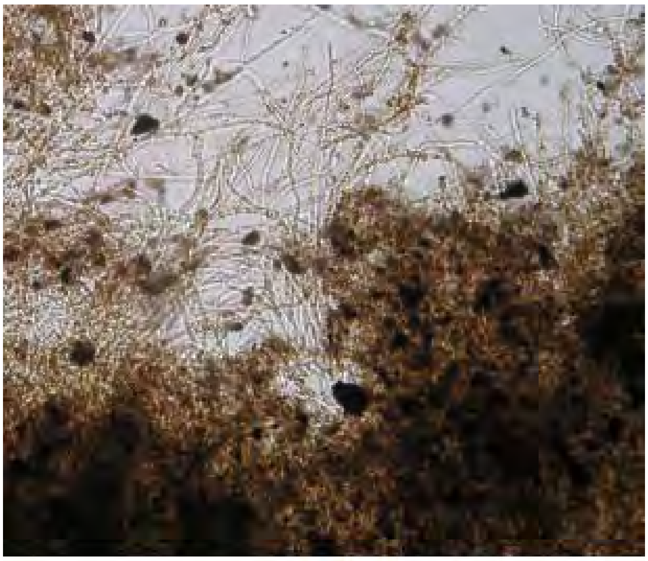


Figure 7-5 Micrograph (x40) of geologically oxidized coal adsorption onto the surface of *N. fischeri* pellets.

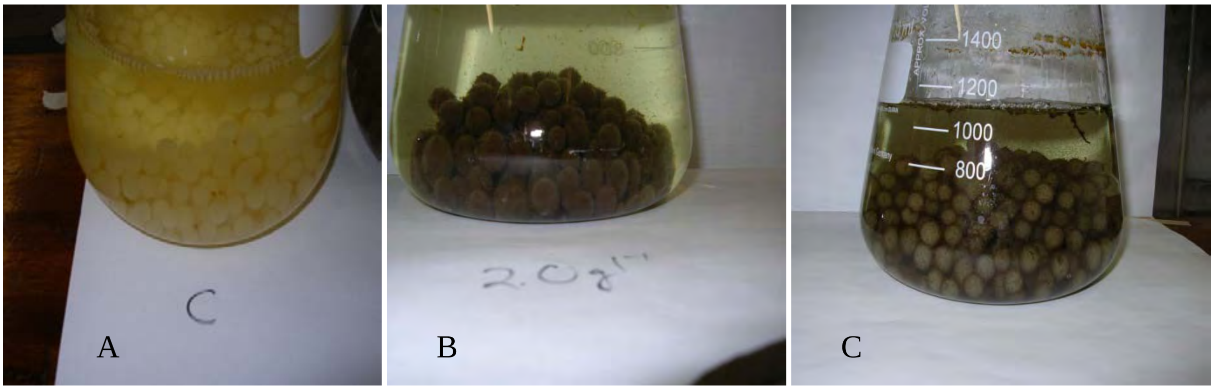


Figure 7-6 ***N. fischeri*** **adsorption of geologically oxidized coal in flask studies. A = control flask with glutamic acid feed only; B = 0.2% geologically oxidized coal and glutamic acid; C = 0.4% geologically oxidized coal and glutamic acid.**

Table 7-2 Visual observation of the ***N. fischeri*** **adsorption with increasing geologically oxidized coal concentration.**

GOC (%, w/v)	Feed Frequency	Visual observation
0.2	1	Complete clearance of suspended GOC from medium after 24 h
	2	Complete clearance of suspended GOC from medium after 24 h
	3	Clearance of suspended GOC but traces of GOC still visible
	4	Partial clearance of GOC and development of a cloudy –grayish color in the medium
0.4	1	Complete clearance of suspended GOC from medium after 24 h
	2	Minimum clearance after 24h but gradually cleared after 48 h
	3	No clearance, appearance of a cloudy grayish color.
0.6	1	Relative clearance of suspended GOC from medium
		Minimum to no clearance of suspended GOC from medium
0.8	1	Relative clearance of suspended GOC from medium
		Minimum to non clearance of suspended GOC from medium
1.0	1	Slight clearance of suspended GOC from medium
		No clearance after 72h of incubation

Igbinigie (2008) had demonstrated that the fungal isolate *N. fischeri* could solubilize 0.2% (w/v) of GOC in a glutamic acid-enriched medium, but the saturation concentration was not determined. The work reported here presents a first attempt to determine the effective GOC concentration for fungal adsorption and/or modification using *N. fischeri*.

The observed trends in adsorption efficiency appear to be dependent on the GOC concentration added to the medium. The lower concentration of 0.2% was rapidly removed from the medium to enable the fungal pellets to adsorb successive feed additions. However, the efficiency of adsorption declined as the GOC concentration increased a saturation of the adsorption capacity on the fungal pellet surface after the fourth feed became apparent. Successive feed additions in the flasks containing 0.4% were not removed from the medium, suggesting inhibition of the adsorption capacity of the pellets. In the 0.8% and 1.0% flasks, there was minimal to no adsorption of the GOC from the medium suggesting immediate saturation of the fungal pellets surface area and inability to mineralize the GOC. While factors such as O_2 mass-transfer and, pellet growth and size are important for the mineralization of recalcitrant aromatic compounds (Michel Jr. *et al.*, 1992; Wu and Yu, 2006), the concentration of the substrate is the most crucial factor as observed in this study. Previous researchers have reported that the net charge of the substrate affects the adsorption as in the study by Antizar-Ladislao and Galil (2004) who reported that adsorption concentration of phenol and chlorophenol was directly correlated to their hydrophobicity (Antizar-Ladislao and Galil, 2004).

GOC is known to contain functionally charged groups, which may have had an impact on the adsorption efficiency of the fungal pellets (Ueda *et al.*, 2004; Zhang *et al.*, 2007). The metabolism of adsorbed organics by fungal pellets of *Phanerochaete chrysosporium* is facilitated by extracellular lignin peroxidases and manganese dependent peroxidases on the surface of the pellets (Michel Jr. *et al.*, 1992; Hofrichter and Fritsche, 1997a; Filley *et al.*, 2000). Production of these enzymes occurs during secondary metabolism and is substantially enhanced by hyperbaric oxygen (Dosoretz and Grethlein, 1991; Michel Jr. *et al.*, 1992). At increased GOC concentration, the saturation of the fungal pellet surface area could have limited oxygen transfer from the medium to the fungal biomass resulting in reduced production of the respective enzymes, and consequently inhibiting those that were present. At the same time, in the lower concentrations of 0.2 and 0.4% the saturation thresholds were not initially achieved and therefore enabled sufficient oxygen transfer and may have allowed increased production of extracellular peroxidases. However, without data to demonstrate activity changes of these enzymes, the interpretation of reduced GOC adsorption should be limited to the saturation threshold of the fungal pellets.

Optimization studies by Fakoussa and Frost (1999) on different HA concentrations using *T. versicolor* showed that 0.05 to 0.12% (wt) was the most suitable concentration for *in-vivo* decolorization during the first 7 days. After 14 days, 0.01 to 0.2% was the most optimal concentration for HA degradation. They also found that exceeding these concentrations resulted in total inhibition of the fungi (Fakoussa and Frost, 1999). The results obtained during this study are comparable to previous findings by Fakoussa and Frost (1999) where the adsorption of GOC was inhibited at concentrations above 0.8%, which contained ~ 0.3% extractable HA.

7.3.2.2 Carbon analysis

The utilization of glutamic acid as a co-substrate for the adsorption and mineralization of GOC was investigated and compared with increasing GOC concentration. The SOC measurement of glutamic acid in the absence of GOC was 856 $mg.l^{-1}$. Addition of GOC (0.2 – 1.0%, w/v) increased the SOC of the respective flasks by between 11% and 37% respectively (Figure 7-7). Initially, ~ 55% of the SOC in the flasks containing 0.2% GOC was removed from solution after 24 h, while 53% was removed from solution in the 0.4% GOC flask. As the GOC concentration increased (0.6 to1.0%), the SOC removal efficiency decreased from 46 to 23% after 24 h. From then onwards, the SOC removal efficiency decreased as GOC was successively increased by 0.2% in the respective flasks over time (Figure 7-7).

In total, 93% of SOC was removed from the solution of a cumulative GOC concentration of 0.8% in the flasks that had started with 0.2%. In the flasks that had started with 0.4% GOC, 83% of the cumulative 0.8% was removed from solution by the fungal biomass over 120 h.

The flasks containing 0.6% GOC having had no further addition of substrate, removed 85% over 120 h. The higher GOC concentrations (0.8% and 1.0%) removed 85% and 84%, respectively over the same period. In the absence of fungal biomass catalyst, there was no SOC removal from solution, although a decrease was observed after 72 h probably due to bacterial contamination.

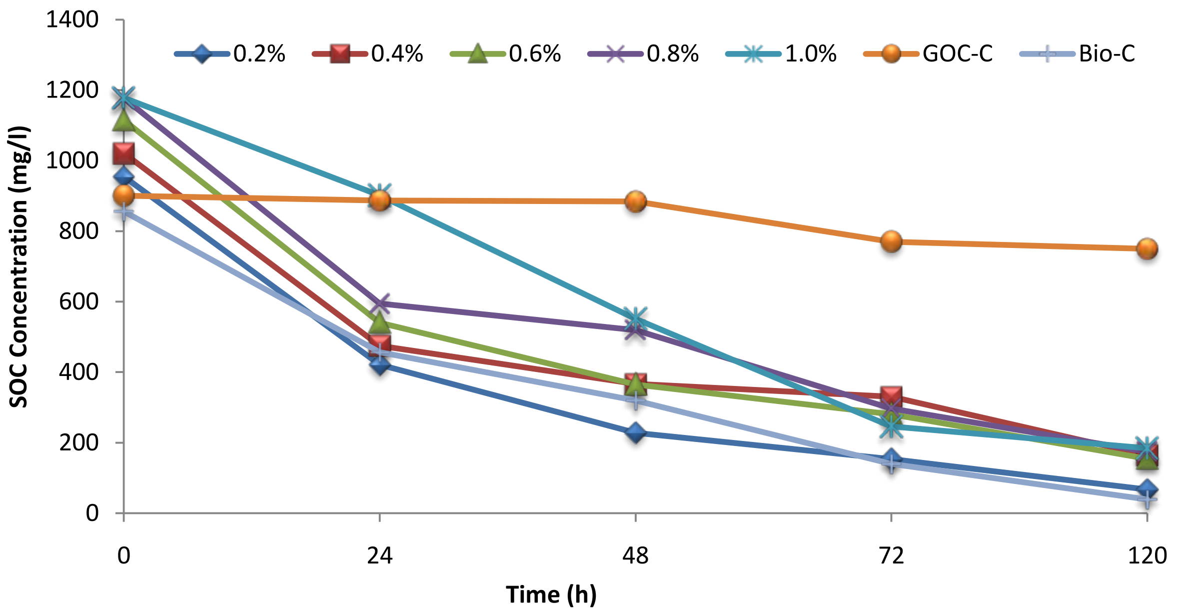


Figure 7-7 Soluble organic carbon utilization by *N. fischeri* pellets in flask reactors containing (0.2 – 1.0%) oxidized hard coal. Incremental batches of 0.2% oxidized hard coal were added into the flasks that showed clearance of the oxidized hard coal from solution. GOC-C – substrate control in the absence of *N. fischeri*. Bio-C- Biomass control in enrichment medium without GOC.

The results presented in Figure 7-7 showed that the efficiency of carbon removal from solution decreased as the starting GOC concentration increased. Flasks containing 0.2% GOC showed comparable trends of SOC utilization with the control fungal biomass reactor that did not contain

GOC. Furthermore, the incremental addition of 0.2% GOC into the reactors, as it was removed from solution did not reduce the rate of carbon utilization by the fungal pellets. However, as the starting GOC concentration was increased to between 0.4 and 1.0%, the efficiency of soluble carbon removal was reduced. A likely explanation could have been the saturation of the fungal pellet surface area by the increased GOC at the start of the reactor, which consequently prevented uptake of the soluble carbon from the medium. This was probably due to inhibition of the fungal active sites by the large molecular weight HS components. Catcheside and Ralph (1999) reported inhibition of lignin peroxidase and manganese peroxidase enzymes from white rot fungi in the presence of coal. They suggested that the amorphous nature of coal and the presence of both hydrophilic and hydrophobic groups led to non-specific binding to proteins thereby inhibiting enzyme action (Ralph and Catcheside, 1994 b).

On the other hand, the addition of smaller concentrations of GOC as it was cleared from solution enabled the uptake of the soluble carbon as well as removal and/or modification of the coal substrate until a saturation concentration was achieved. While SOC removal slowly progressed over time in the reactors containing higher GOC concentrations, the complete inhibition of the fungal pellets may have inhibited the utilization of soluble carbon thereby preventing removal of the GOC from solution and uptake of the soluble carbon. In the absence of the biocatalyst, there was no decrease in the soluble carbon, thereby demonstrating that the fungal biomass pellets were responsible for GOC adsorption and soluble carbon utilization. However, bacterial contamination after 72 h marginally reduced the SOC. Furthermore, in the absence of GOC, the *N. fischeri* pellets utilized the glutamate present in the medium, further confirming their role in soluble carbon utilization in the system.

7.3.2.3 Effect of geologically oxidized coal concentration on pH

Changes in pH were monitored in flasks containing different GOC concentrations (0.2 – 1.0%) to investigate the effect of pH on the adsorption and/or modification of the coal. All experimental reactors flasks with the various concentrations showed increases in the pH in the medium from pH 5.0 to ~ pH 8.0 over 120 h (Figure 7-8A and B). The fungal biomass reactors without GOC raised the medium pH from pH 5.0 to pH 8.0 and the rate of pH increases declined thereafter to reach ~ pH 8.4 after 120 h.

Figure 7-8A and B show that the rate of pH increase was related to the GOC concentration. Flasks containing the lower GOC of 0.2% raised the pH in the medium rapidly to pH 7.5 after 48 h, as the increasing GOC concentration was removed from solution. However, after addition of

0.8% GOC, pH increase stopped after 72 h after which no further addition of GOC occurred. The pH then increased to ~ pH 8.1 after 120 h.

Flask reactors that started with 0.4% GOC showed an upward increase in pH from pH 5.0 to ~ pH 8.0 after 120 h (Figure 7-8A). Interestingly, a decline in pH was not observed when the concentration in the reactors doubled to 0.8% as observed in the 0.2% reactors. A similar trend was observed in the reactors that started with 0.6% GOC, as the pH increased gradually from pH 5.0 to pH 7.8 after 120 h, although no additional coal substrate was introduced into the medium (Figure 7-8B). Fungal pellets that were initially incubated in 0.8 and 1.0% GOC, showed similar pH trends, where the pH increased gradually to ~ pH 7.0 after 48 h before declining marginally and finally increasing to ~ pH 7.9 (Figure 7-8B). In the absence of biocatalyst there was no substantial increase in pH, although after 120 h bacterial contamination may be implicated in the increase of the pH to ~ pH 6.4.

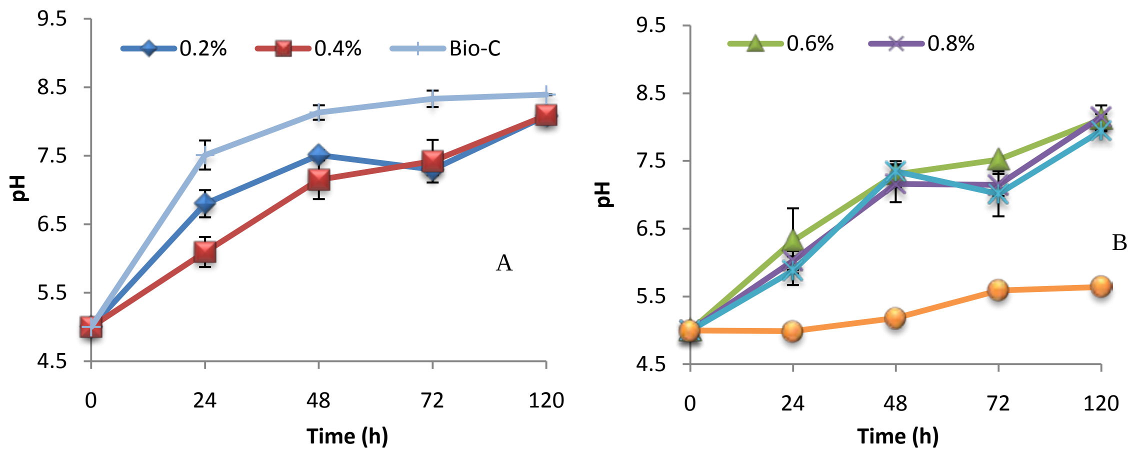


Figure 7-8 pH changes of *N. fischeri* cultures inoculated within different concentrations of geologically oxidized coal A = 0.2 and 0.4%, Bio-C; B = 0.6 – 1.0%, GOC-C= geologically oxidized coal control. Bio-C= Biomass control in enrichment medium without GOC.

The pH results showed that the presence of a fungal biocatalyst in a coal/co-substrate medium results in a pH increase regardless of the coal substrate concentration. This increase in pH is comparable with previous studies on coal biosolubilization that have reported a direct relationship between increase in pH or alkalinity and the extent of coal solubilization (Strandberg and Lewis, 1987; Faison and Lewis, 1990; Hőlker *et al.*, 1997; Hőlker *et al.*, 1999). However, the concentration of the coal substrate affects the rate at which alkalinity is generated in the medium. According to Hofrichter *et al.* (1997a), the coal solubilization process depends mainly on the nitrogen content of the medium, which leads to the release of ammonium ions and other alkaline amines. In this experiment, the increased coal concentration in the medium, which according to

Catcheside and Ralph (1999) can be inhibitory, prevented uptake of the soluble carbon and nitrogen from glutamic acid, thereby reducing the amount of alkalizing substances responsible for raising pH. This may have led to a reduced adsorption potential of the fungal biomass causing saturation of the system as observed in Table 7-2.

The drop in pH observed in the flasks containing 0.2% GOC after 72 h can be attributed to the addition of the acidic GOC which could have neutralized the alkalizing substances. Furthermore, the accumulation of GOC to 0.6%, may have forced the fungi to enter lag phase as a response to the increased concentration. In the pellets incubated in 0.4% GOC a drop in pH was not observed because there was no further addition of GOC after 48 h, and the fungi had acclimatized to the medium with 0.8% GOC although they could not effectively adsorb the GOC from solution (Table 7-2). In the higher GOC concentration (0.6 – 1.0%) flasks it was evident the fungi experienced longer lag phases as observed by the gradual increase in pH over time after addition of only one feed of the respective concentrations. The secretion of alkalizing substances became limited due to the inhibitory effect of the elevated GOC concentration, but as the fungi adapted to the respective environments the pH increased over time (Figure 7-8B).

The experiments undertaken in this study served as a platform for optimizing the coal substrate concentration in preparation for scale-up studies for bulk production of fungal biomass and GOC solubilization and/or modification. From these results, it was decided to use 0.2% GOC for all subsequent aerobic studies as single batch or sequence batch feeds. Observed limitations of the flask reactors such as adaptation time, pellet surface area, optimal aeration of the medium and homogenous mixing of the medium led to the development of a large volume bioreactor that was used for further investigations into GOC bioprocessing using *N. fischeri*.

7.3.3 Continuous stirred tank reactor studies

7.3.3.1 Growth studies

The fungal spores were inoculated into the bioreactor and grew into spherical sponge-like pellets (Figure 7-9) with a maximum diameter of ~ 24 mm before the GOC was introduced into an enriched medium similar to the one reported in section 7.3.2. The fungal pellets were allowed to adapt to the environment for 48 h before introduction of the coal. The bioreactors were operated for 12 days after the acclimatization period. Daily average fungal growth rates of 106 ± 5 mg biomass.L.day^{-1} were recorded in the 30 L reactors, to achieve biomass yields of 1.2 ± 2 g.L^{-1} over 12 days.

Previous studies using different carbon and electron donor sources that include glucose, acetate and rice extracts have reported fungal growth yields ranging from 1.5 – 13 $g.L^{-1}$ using (Al-Taweil *et al.*, 2009; Matar *et al.*, 2009; Potila *et al.*, 2009), which is comparable with the results reported here.

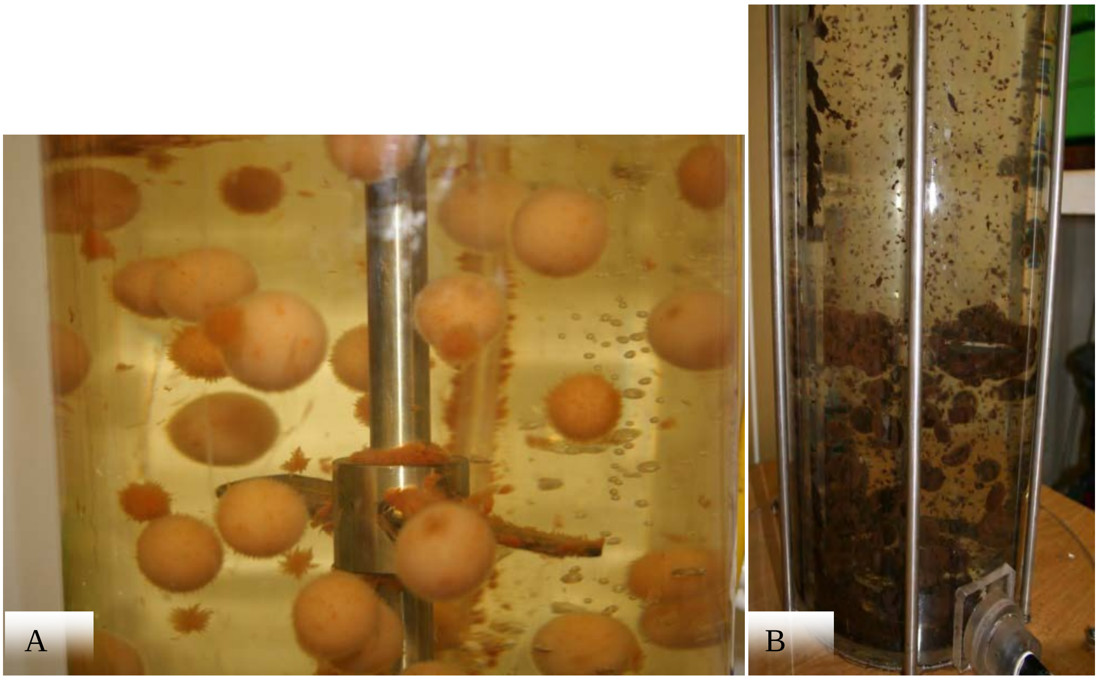


Figure 7-9 A- Growth of *N. fischeri* in enriched glutamic acid medium before addition of the oxidized hard coal and B- 12 days after addition of the oxidized hard coal.

The gradual addition of the GOC ensured that the overall concentration of the coal in the bioreactor did not exceed 0.2% (w/v). Total removal of GOC from solution and adsorption onto the fungal pellets was observed to occur over 6 days, after which the GOC was released back into the medium as shown by the measurement of HA (Figure 7-10). At day 8 the fungal pellets started to disintegrate and released GOC back into solution. Spectrophotometric analysis of the medium supernatant detected ~ 3% of the extractable HA in solution (Figure 7-10). At day 10 the amount of extractable HA had increased to ~ 14%, however, the bulk of the fungal pellets had broken down, and became trapped on the impeller blades. At day 12, the amount of extractable HA in solution had increased to ~ 26% but there were no pellets in the bioreactor (Figure 7-10). The remaining biomass was either trapped on the rotating impellers or settled at the bottom and sidewalls of the reactor (Figure 7-10B). In addition, a cloudy suspension was observed at day 12, which was confirmed to be bacterial contamination by light microscopy.

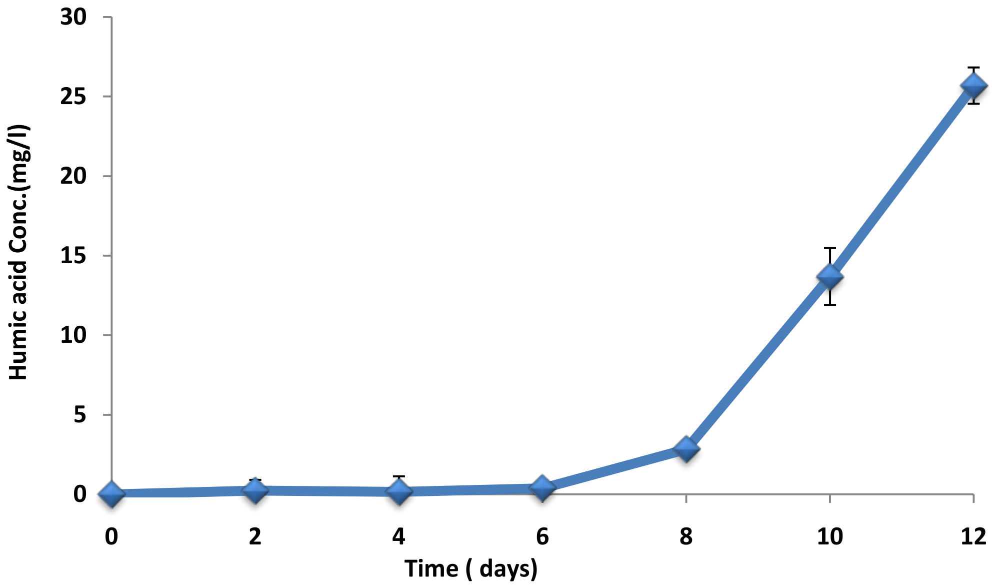


Figure 7-10 Release of extractable humic acid into solution from geologically oxidized coal in a 30 L fungal bioreactor.

The cultivation of *N. fischeri* in a 30 L bioreactor enabled increased growth of pellets thereby increasing the surface area for GOC adsorption and subsequent release of HA from the GOC. The formation of pellets by filamentous fungi such as *P. chrysosporium,* which is similar to *N. fischeri,* typically occurs when they are grown in agitated liquid cultures (Wainwright *et al.*, 1993; Jiménez-Tobon *et al.*, 1997). The pellets are formed by aggregation of spores in the medium immediately before and after germination. This is dependent on several factors such as initial spore concentration and state, pH, temperature and medium composition, which affects the rate of pellet development and structure (Wainwright *et al.*, 1993).

The increased biomass and apparent increase in surface area observed was due to homogenous agitation and efficient aeration of the reactor, which was not observed in the flask studies. According to Jiménez-Tobon *et al* (1997), the formation of pellets is a pre-requisite for the production of secondary metabolites such as alkaloid production by *Claviceps paspali.*

7.3.3.2 pH profile

The pH increased rapidly from pH 5.0 to ~ pH 8.0 over the first 3 days of the experiment (Figure 7-11). Thereafter, it plateaued between day 4 and 7, before gradually declining to pH 6.0 by day 12 when the fungal pellets had completely disintegrated (Figure 7-9B). The initial rapid increase in pH is comparable to the pH changes observed in the optimization flask studies (Figure 7-8). This demonstrates the reproducibility of the process and its potential for scale-up to larger volume bioprocess reactor configurations.

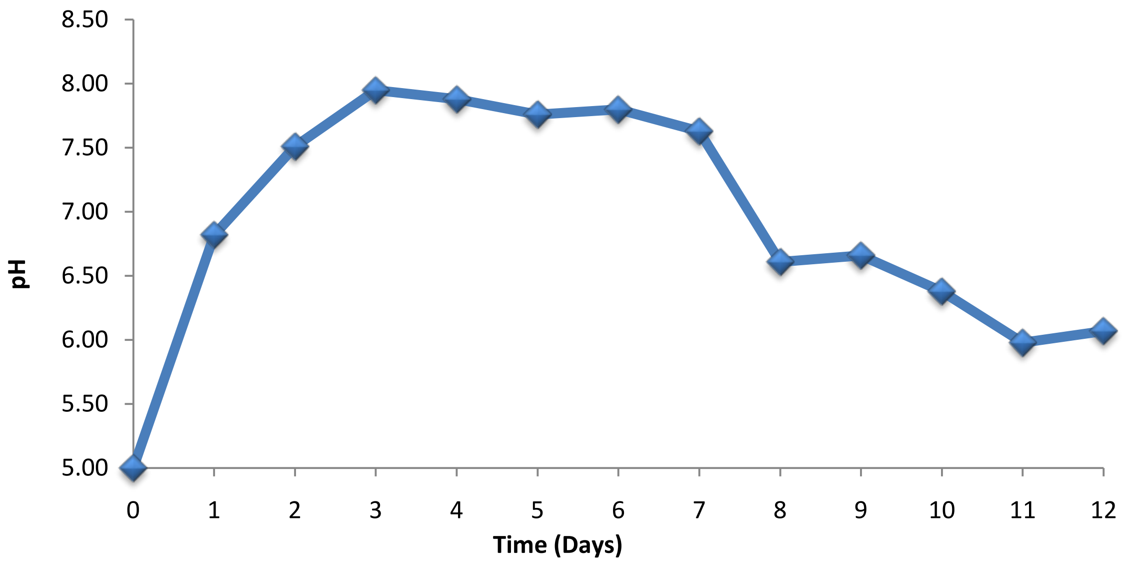


Figure 7-11 Illustration of pH change in the growth of fungal pellets in the 30 L bioreactor.

The secretion of alkalizing substances by the fungal pellets in the presence of GOC resulted in the observed increase in pH between day 0 and 3 (Figure 7-11). This period can be described as the log of fungal growth. The glutamic acid co-substrate in the medium could have been responsible for the increase in pH during fungal growth and coal adsorption and/ or solubilization. The involvement of glutamic acid in nitrogen metabolism, protein and nucleotide synthesis, as well as a substrate for energy metabolism, has been reported (Wice *et al.*, 1981).

These results are comparable to the work reported by Hölker *et al* (1999) on the growth of fungi on glutamate. They reported that the growth of fungi on carboxylic carbon sources resulted in a pH increase due to accumulation of accompanying cations in the medium. However, growth on carbohydrates such as glucose caused the culture to depress the pH (Hölker *et al.*, 1999). The pH in the bioreactor plateaued between days 4 and 7, indicating that an equilibrium had been achieved where the rate of secretion of alkalizing substances was equal to the consumption of the glutamic acid and concomitant adsorption and/or solubilization of coal. This can be correlated to the stationary phase of fungal growth, which is characterized by the production of secondary metabolites, alkalizing substances and extracellular enzymes (Strandberg and Lewis, 1987; Michel Jr. *et al.*, 1992; Jiménez-Tobon *et al.*, 1997).

After day 7, the pH started to drop as the fungal cells entered the autolytic phase of fungal growth thus desorbing the GOC and accompanying HA (Figure 7-10). Autolysis of cells is typically followed by release of proteolytic enzymes that degrade cell material releasing nucleic acid derivatives that can contribute to the lowering of pH (Maukonen *et al.*, 2003). The shearing effect of an impeller for lowering the viscosity of fermentation medium often leads to hyperfragmentation, and fungal cell damage. This results in the secretion of proteases that lower the pH of the medium through production of cations (Jiménez-Tobon *et al.*, 1997; Heo *et al.*, 2004). Furthermore, the gradual addition of GOC-glutamic acid enriched feed to the bioreactor at pH 5.0 may have contributed to the lowering of the pH, more so, due to the acidic nature of the GOC.

7.3.3.3 Carbon analysis

The removal of SOC from solution was monitored to determine the use of the co-substrate glutamic acid and release of extractable HA. Figure 7-12 shows the removal of soluble carbon from both the experimental bioreactor containing GOC and the control bioreactor (Bio-C) over the first six days of the study. A 98% decrease in the soluble carbon was observed in the experimental bioreactor, while a 67% decrease was observed in the control bioreactor over the same period (Figure 7-12). After 8 days of the study, there was gradual release of carbon back into solution as the cells started to rupture. After 12 days, up to 40% of the carbon (Figure 7-12) had been released back into solution due to complete disintegration of the fungal pellets (Figure 7-9b). Carbon removal in the control bioreactor continued until day 10 where it leveled off until the end of the experiment. It was interesting to note that the SOC did not increase after the cells had started to disintegrate around day 8. The carbon that was not released back into solution, in the control bioreactor could have been converted to carbon dioxide during fungal respiration.

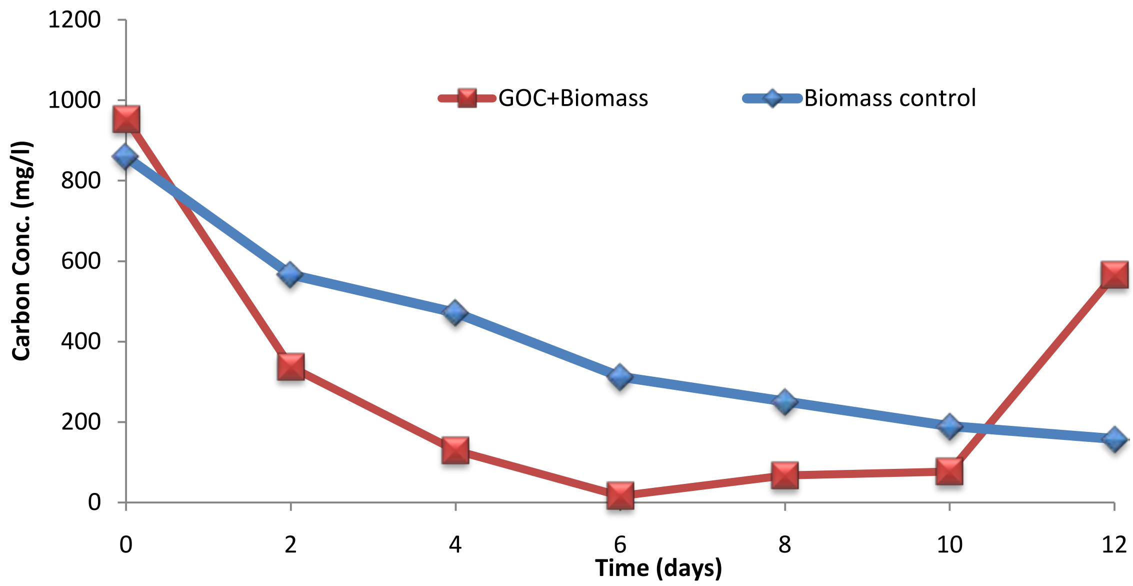


Figure 7-12 The removal of soluble organic carbon from solution in a 30 L continuous stirred tank reactor inoculated with *N. fischeri* biomass.

The consumption of SOC observed (Figure 7-12) corresponded to the active growth of the fungal pellets and pH changes observed in Figure 7-9 and Figure 7-11, respectively. The control bioreactor showed typical substrate utilization in a fed-batch bioreactor set up, during fungal growth (Raposo *et al.*, 2006; Nopharatana *et al.*, 2007). The rate of utilization gradually declined until the carbon was exhausted, and the cells entered into autolytic phase. In the experimental bioreactor, the presence of the GOC could have accelerated the rate of SOC utilization in order to facilitate and maintain the adsorption and solubilization process between day 0 and 6, with a biomass yield of 0.636 g biomass.g SOC^{-1}. When the SOC became exhausted, the cells entered into a decline phase and desorbed the GOC and HA back into solution.

7.3.3.4 Pyrolysis gas chromatography mass spectrometry

The reaction products of the GOC adsorption and/or bioconversion by *N. fischeri* in the bioreactor were investigated using Py-GCMS (Figure 7-13). A qualitative analysis of the pyrolysis products showed the transformation of the GOC after adsorption onto the fungal biomass by appearance and disappearance of pyrolysate products during the study (Figure 7-13). Analysis of the GOC in the absence of the fungal biomass showed the presence of benzene and suspected contaminants such as benzenemethanamine, while the biomass on its own showed the presence of long chain fatty acids. The reaction of the GOC and *N. fischeri* resulted in the appearance of several pyrolysate products summarized in Table 7-3.

A qualitative analysis of the reaction products using Py-GCMS provided evidence supporting the solubilization of coal by the fungal pellets, and suggests mechanisms that may be occurring inside the pellet. The adsorption process facilitated contact of the GOC and the cell walls of the fungal pellets where secretion of extracellular enzymes and alkalizing substances may act in the bioconversion of the GOC. The appearance and disappearance of styrene and benzene derivatives over the course of the reaction may be indicative of the breakdown of the condensed GOC structure to release aromatic compounds related to the humic component of the coal. In addition, the appearance of nitrogenated pyrolysates such as quinoline, indole and imidazole, further highlights the role of alkalizing substances in the coal solubilization mechanism. These results are comparable to previous work by Igbinigie (2008), where the presence of similar nitrogenated aromatic compounds in simulated studies of the accelerated biological weathering process was reported. According to Hofrichter and Fritsche (1997a b), the alkalization effect of the medium results in the extraction of HAs from the macromolecular coal network.

Although these compounds are pyrolysates of more complex molecules in the medium, these results provide more evidence for the observed increase in extractable HA (Figure 7-10). The methyl esters of C_{16-20} saturated and unsaturated fatty acids observed in (Figure 7-13) were probably derived from the fungal biomass, since these peaks were not observed in the GOC control.

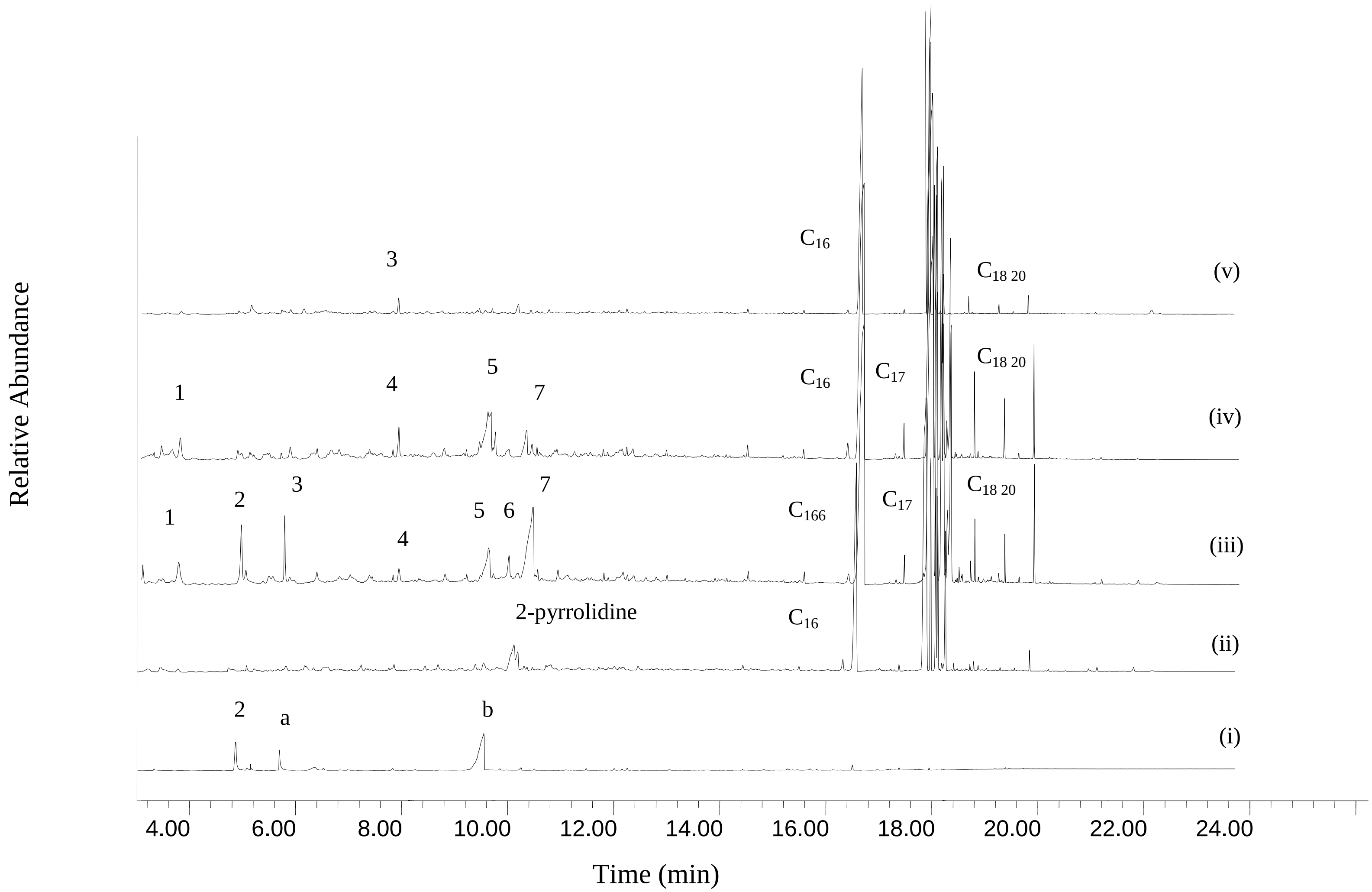
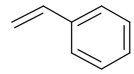
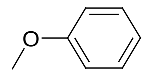
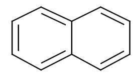
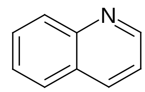
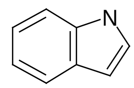
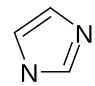
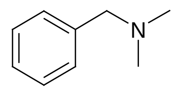
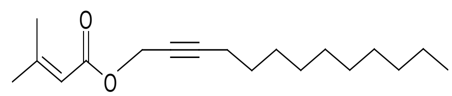


Figure 7-13 The total ion chromatogram for the 30 L bioreactor studies (i) geologically oxidized coal control (ii) *N. fischeri* biomass control (iii) geologically oxidized coal control and fungal biomass after 1 day (iv) 6 days and (v) 12 days. (a and b) suspected contaminants. See Table 7-3 for peak and structure and identification.

Table 7-3 Pyrolysis products and their structures from geologically oxidized coal extracts derived from geologically oxidized coal adsorbed onto the *N. fischeri* pellets.

Peak no.	Compound	Structure
1	Styrene	
2	Benzene-methoxy-methyl	
3	Naphthalene	
4	Quinoline	
5	Indole	
6	Imidazole	
a	Benzenemethanamine	
b	Butanoic acid	

PART B

7.4 Biological sulfate reduction using fungal biomass as a co-substrate

The demonstration that fungal biomass could be grown in large volumes using oxidized HC and a glutamate co-substrate in the previous section, provided a solution for the requirement of a co-substrate in the anaerobic coal bioprocesses investigated in this study. Furthermore, the implications of the biomass as a suitable co-substrate could eliminate the cost implications of a defined substrate such as lactate. It would also avoid the introduction of potential inhibitors from complex but inexpensive substrates such as grass, and would not impact on the process flow in the envisaged reactor design. However, the question that remained was the feasibility of this concept, whether the fungal biomass could actually be used as an electron donor and carbon source for sulfate reduction and methane production in a coal mining environment. In order to conclude this study, a preliminary investigation would be required to demonstrate the ability of a fungal biomass serve as a substrate for sulfate reduction and methane production. Since the main objective of this thesis was a feasibility study of the utilization of HC as bioprocess substrate, the work reported in this section is only a demonstration study. Sulfate reduction was chosen for this preliminary work because of its more robust nature and ability to grow faster than the methanogens. This would be extended to the methanogenic components once the concept was demonstrated.

7.4.1 Materials and methods

7.4.1.1 Preparation of fungal biomass

A pre-determined wet weight of *N. fischeri* pellets was inoculated into 5 L flasks containing a glutamic acid-enriched medium that has previously been described in section 7.2.3. The fungal biomass was harvested by filtration through a 36 µm mesh, followed by several washing steps (dH_2O) until the rinsate became clear. Excess water was squeezed from the biomass before it was weighed, and fed into a sulfidogenic reactor.

7.4.1.2 Sulfidogenic reactor set up

A batch SRB reactor was set up and operated as previously illustrated in Figure 5-1 and in section 6.2.1.2. The reactor flasks contained a basal medium as previously described in section 5.2.1 where the lactate was replaced with an equivalent amount (w/v) of fungal biomass, and was inoculated with an SRB culture developed in section 5.2.1. A control reactor that contained only the basal medium was set up and run concurrently with the experimental reactors. Reactor performance was monitored by generation of sulfide over time.

7.4.1.3 Analysis

Samples for sulfide analysis were collected and analyzed as previously described in section 5.2.4.1.

7.5 Results

Figure 7-14 shows the production of sulfide by SRB using fungal biomass as an electron donor and carbon source. A lag phase was observed at the start of the experiment as the SRB adapted to the feed medium. Thereafter, sulfide production increased ~ 13-fold over time and peaked at 490 $mg.L^{-1}$ corresponding to ~ 1500 $mg.L^{-1}$ of sulfate reduced, until the reaction was terminated due to reactor failure, possibly due to substrate exhaustion. In the control reactor, no sulfide production was observed throughout the study.

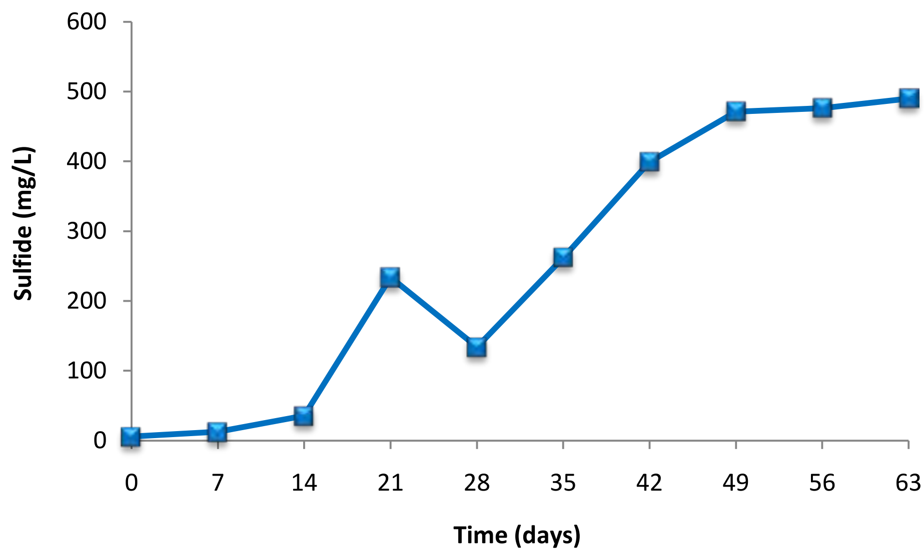


Figure 7-14 The production of sulfide in 1 L batch flask reactors by sulfate reducing bacteria using fungal *N. fischeri* biomass as an electron donor and carbon source.

The results presented above demonstrate that fungal biomass can be used as an electron donor and carbon source for BSR. This was further confirmed by a lack of sulfide generation in the control reactor, in the absence of the fungal biomass. The amount of sulfide produced in this study is comparable to the sulfide generated in the GOC plus lactate reactor (section 5.3.2.2). A maximum sulfide production of 284 mg.L^{-1} was observed after 20 days in the lactate only reactor, while 234 mg.L^{-1} was measured in the fungal biomass only reactor after 21 days.

Therefore, it may be feasible to replace lactate as a co-substrate to oxidized HC for BSR, however, a more detailed investigation of this system would be required to interrogate different factors such as C:SO_4 ratio, and further application in continuous processes such as the UAPB. Since these results demonstrate sulfate reduction, it can be assumed that the fungal biomass can also serve as a co-substrate for methane production using oxidized HC.

7.6 Conclusions

The above studies have demonstrated the potential application of various aerobic reactor configurations in the biosolubilization of oxidized HC using *N. fischeri* as the biocatalyst, from which the following conclusions can be drawn:

Part A

- *N. fischeri* fungal biomass can be grown using GOC as a carbon source;
- The presence of a co-substrate in the form of glutamic acid is necessary for the biosolubilization of oxidized HC. The glutamic acid can be produced commercially using elementary fermentation technologies;
- At optimal GOC concentrations (0.2%) in the bioreactor studies, there was total adsorption of the coal substrate from the medium onto the fungal pellets;
- Higher fungal biomass yields of 0.636 g biomass.g SOC^{-1} for pre-grown inoculum in CSTR and ~ 0.07 g biomass.g SOC^{-1} for spore inoculum in PBR were observed.

Part B

- The use of fungal biomass to support the sulfate reduction using GOC has been demonstrated;
- While the results acquired were of particular interest, it was regretted that more detailed investigations could not be undertaken. Further studies required to optimize the system are ongoing, but the objective to demonstrate the primary feasibility of the process was met.

CHAPTER EIGHT

CONCLUSION AND APPLICATION

8. INTRODUCTION

The rapid development of coal biotechnology, reported during the 1980s and 1990s was focused mainly on elaborating an understanding of the fundamental physico-chemical and biological processes underpinning the biodegradation of coal substrates (Fakoussa, 1981; Hofrichter *et al.*, 1997a; Catcheside and Ralph, 1999; Fakoussa and Hofrichter, 1999; Klein *et al.*, 1999). Although a limited number of process applications emerged from these studies, they had largely been restricted to the use of lignitic coals and generally as very low-volume throughput operations (Hofrichter and Fritsche, 1997a; Hő́lker *et al.*, 2002). Little progress has been demonstrated to date in the biodegradation of HC, nor in the development of process applications based on its use as a substrate. In addition, little work has been reported on attempts to develop applications of HC biotechnology in environmental remediation operations and specifically to the substantial problems of environmental sustainability experienced by the coal mining industry itself.

It has been noted that further development of the coal biotechnology field would be likely to be dependent on, and largely driven by, break-throughs achieved in bioprocess applications of these systems (Ziegler and Van Heek, 1998; Klein *et al.*, 1999). Klein *et al* (1999) have noted that, "the results available to date on microbial conversion of coal do not warrant bioengineering-scale studies, and until there has been some form of breakthrough in this area, no biological system for coal processing will be economically viable".

Accessibility of the substrate to microbial attack, especially in the case of HC, was identified as one of the constraining factors and Igbinigie (2008) and Mukasa-Mugerwa (2008) showed that the oxidation of HC, by both geological and biological processes, could substantially enhance the subsequent biological reactivity of these coals. They were able to demonstrate that an accelerated biological oxidation of HC was achievable in principle in the SHCB process. Since stacked heap bio-mining is an already well established technology in the extraction of minerals (Brierley, 2001; Pradhan *et al.*, 2008), a practical implication of these observations for coal biotechnology could be the provision of a coal substrate accessible in large tonnages and at low cost, thereby providing a basis for further development of coal bioprocessing on an industrial and commercially viable scale. Although they had shown, in principle, that their discoveries could provide a potentially functional substrate, further investigation of the use of oxidized HCs in bioprocess applications has not been reported.

The objective of this study was thus to undertake a preliminary investigation of the feasibility of environmental applications of coal biotechnology based on the use of oxidized HC substrates and, dependent on the results, to inform a feasibility decision-making process that could lead towards bioengineering-scale studies. The work reported here focused on both anaerobic and aerobic processes and investigated systems using carbon dioxide, sulfate and oxygen as terminal electron acceptors.

A preliminary characterization of the oxidized HC substrates was undertaken to determine their respective organic matter composition for subsequent use in process application development. The oxidized HC fractionated readily into hydrophilic (HS), hydrophobic organic matter and the inorganic matter in the coal substrates. However, unmodified HC did not yield appreciable soluble organic matter. The results also showed that unmodified HC could not be fractionated into FA, HA and humin components. Where the HC was exposed to oxidation processes, loss of carbon and incorporation of oxygen was observed, with geological oxidation showing a greater loss of carbon and incorporation of oxygen into the substrate than the biological oxidation process. A simulation of the biological extraction of HA from oxidized HC in a reactor set up revealed that the process was dependent upon pH and time of agitation.

In studies where CO_2 was used as a terminal electron acceptor, production of methane was demonstrated from both BOC and GOC substrates but not from unmodified HC. In this regard, GOC performed better than BOC, and therefore, while optimization of the biological coal oxidation process is still ongoing and additional improvement in its utilization may be forthcoming, further applications investigated in this study were carried out using GOC. Furthermore, the methane yields reported in this study were higher than previous comparable studies that have reported methane production from coal substrates (Panow *et al.*, 1994; Budwill, 2003; Green *et al.*, 2008).

An important observation that emerged was the demonstration of the dependence of the methane generating system on the presence of an effective co-substrate supporting the breakdown of the complex organic structures within in the HC substrate. The use of co-substrates in the biodegradation of complex organic materials has been well described (Buendía *et al.*, 2009; Ko *et al.*, 2009).

A partial elucidation of the breakdown pathways for oxidized HC in the anaerobic bioprocess operation was demonstrated and this highlighted the formation of aromatic intermediate breakdown compounds that could be linked to the production, and subsequent consumption, of VFAs for conversion into methane gas. As a result, it was shown that the probable accumulation of inhibitors within the system in the form of aromatic intermediate breakdown products, rather than substrate exhaustion, led to decline and ultimately failure of the methanogenic reactors after a period of operation as batch processes. The use of a continuous process might enable the wash out of inhibitors, and thereby allow an extension of the methanogenic process. However, this potential would need to be demonstrated and certainly account would need to be taken of the treatment requirements of the effluent from the continuous reactor operation produced in this way.

Sulfate reducing microbial consortia have been shown to be able to undertake the breakdown of certain aromatic compounds and, due to the more robust nature of these systems, may better tolerate or utilize the inhibitory products from the methanogenic reactions (Mayes *et al.*, 2008a). Where environmental remediation work is to be done, it could prove simpler to link the capture of an initial energy recovery product with the downstream use of BSR in an integrated treatment of mining wastewaters with sulfate-related salinity and acidity problems. Since preliminary demonstration of process feasibility was the overall objective of this study it was considered necessary to understand how BSR systems may function in such an application and using oxidized HC as a substrate. The use of GOC as a functional substrate by SRB consortia was demonstrated in the neutralization of acidic media, in both batch and continuous process operations.

Here again, the requirement for a co-substrate was demonstrated in the use of GOC by SRB and lactate was shown to function as a true co-substrate in the sulfidogenic system. However, due to cost considerations, an alternative to lactate as a co-substrate would need to emerge if the process was to function commercially in a real-world bioprocess environment treating large water volumes. A further requirement for a low-cost co-substrate would be its generation using resources largely available within the coal-mining environment itself.

In earlier studies, Igbinigie (2008) had observed the prolific growth of *N. fischeri* on an oxidized HC substrate, albeit in the presence of glutamic acid as co-substrate. It was shown that the production of *N. fischeri* may be upgraded into a bioprocess environment and that large volumes of fungal biomass might be generated in this way. It was also shown, as a preliminary demonstration that the fungal biomass could function as an effective electron donor in sustaining the operation of a sulfate reducing microbial consortium. In this way, the co-substrate dependence of methanogenic and sulfidogenic coal bioprocess applications might be resolved with biomass produced on site. While, this component of the operation remains dependent on glutamate, its inexpensive production by crude fermentation of proteineous waste effluents has been previously described (Das *et al.*, 1995; Tari *et al.*, 2007).

Had time permitted in this study, it would have been desirable to further develop these observations providing indications of anticipated process kinetics, mass balances, substrate loadings, and yield recovery data. This will need to be undertaken as the subject matter of future work.

Despite the provisional nature of certain findings reported here, this study has provided preliminary insights into the feasibility of, and requirements for, the use of oxidized HC as a bioprocess substrate. In certain respects, it has provided an indication that scaled-up bioengineering studies would be warranted, especially in environmental remediation applications. In this regard, it is a first report of this potential and may provide a core enabling technology in the further development of coal biotechnology.

On this basis, the production of an alkaline effluent stream by SRB using GOC as a carbon and electron donor source, coupled to the observation that HA maybe extracted led to the development of a practical bioprocess application for the recovery of the soluble hydrophilic organic matter. Preliminary results demonstrated effective recovery of soluble organic matter, although process optimization is ongoing.

8.1 Process model

Given that the objective of this study was to investigate the bioprocess feasibility of the use of oxidized HC substrates, it was considered important to try to understand how applications of these findings might be linked as integrated unit operations in environmental remediation applications in the coal mining industry.

Problems relating to AMD and land rehabilitation experienced on the mines on which the field studies in the Fungcoal Project were undertaken led to the development of a specific application of the above findings as a conceptual integrated process model. While somewhat speculative, this provides an example of how these findings might be developed in an environmental remediation application. It also provides a framework for focusing the planning of future research programmes in this area.

The concept proposal for the Integrated Coal Bioprocess (ICB) (Figure 8-1) would involve the use of a packed bed reactor, possibly established in a mining void, as the bioreactor environment in which the primary reaction of the GOC and/or BOC could be managed. Where the pit can be covered with a flexible membrane, an initial crop of methane may be recovered. The methane can contribute to the energy requirements of plant operation. The intermediate breakdown products formed in the system are potentially inhibitory to methanogenesis, as noted in Chapters 3 and 4, and would therefore need to be removed. These could be passed into a separate reactor, such as developed in the BioSURE® Process, where sulfate replaces carbon dioxide as the terminal electron acceptor and SRB consortia would further degrade the intermediate compounds as shown in Chapters 5 and 6. In turn, the alkalinity generated from BSR could be fed back to the UAPB where the extraction of soluble HA from the oxidized HC substrates would be effected (see Chapter 6). The extracted HA would in turn provide a feedstock for the AMD treatment reactor. After extraction of HA, and purging the intermediate aromatic compounds from the system, the UAPB could be switched back to a methanogenic process using the remainder of the original substrate with a new feed of oxidized HC and fungal biomass co-substrate.

The BioSURE® process is a well-established AMD treatment operation (Whittington-Jones *et al.*, 2002; Rose *et al.*, 2004; Neba and Rose, 2006) that traditionally uses PS as a carbon source and electron donor for BSR. Where a large supply of PS is not readily available, the system could use HA and fungal biomass as carbon and electron donor sources for sulfate reduction (Cervantes *et al.*, 2001; Rothermich *et al.*, 2002; Safinowski *et al.*, 2006).

Although not discussed at any length in this study, the recovery of large masses of HA may also play an important role in the rehabilitation of open cast mining soils and, in so doing, enable environmentally effective closure of these mines (Avena *et al.*, 1999; Wan and Liu, 2006; Giannouli *et al.*, 2009).

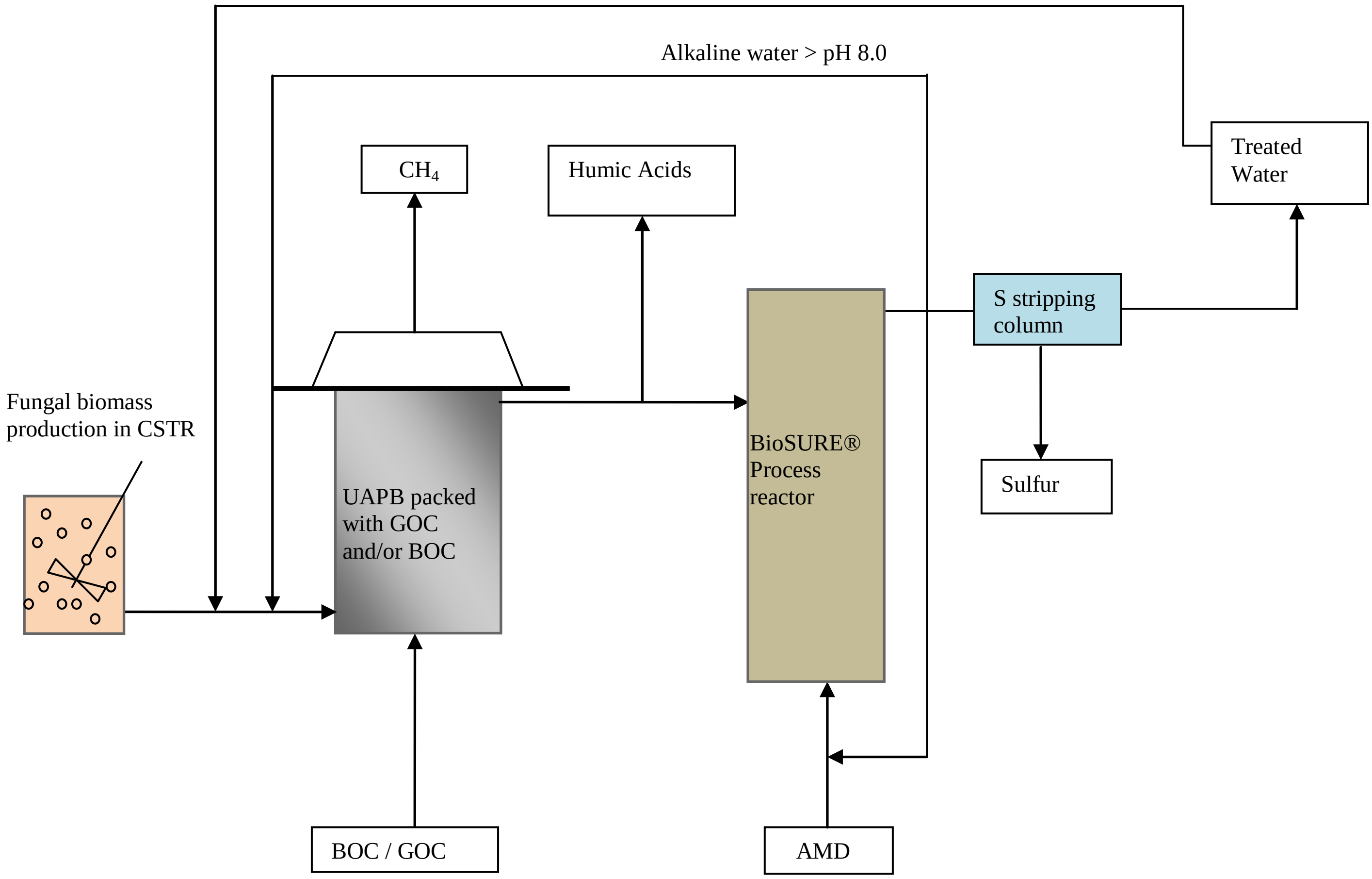


Figure 8-1 Schematic diagram illustrating a proposed Integrated Coal Bioprocess model for the beneficiation and remediation of coal mining wastes. The oxidized hard coal serves as a substrate for methane production and is augmented by a fungal biomass co-substrate in an up flow anaerobic packed bed reactor. Humic acid is extracted from the oxidized hard coal substrate to serve as a carbon source and electron donor for biological sulfate reduction in the BioSURE® process, which is also supplemented with fungal biomass co-substrate. Part of the resulting water stream is pumped back into the up flow anaerobic packed bed reactor to provide an aqueous environment for methanogenesis, and is also channeled towards raising the influent pH of the acid mine drainage fed into the BioSURE® process. Sulfide may be removed from the biological sulfate reduction effluent producing a treated water stream.

8.2 Future work

The main objective of the work reported here, to investigate the feasibility of using oxidized HC substrates as a carbon source and electron donor in bioprocesses operations, was met to some degree with the emergence of largely positive results from the studies reported. In this regard the hypothesis on which this study was based, that oxidized HC can be used as an effective substrate for viable bioprocess applications, has largely been demonstrated. Although such a preliminary demonstration of process feasibility may provide a basis for follow-up bioengineering studies at intermediate scale and, advancing the further development of coal biotechnology, many questions have been raised concerning the mechanisms of these systems and especially in quantifying the rather complex reactions involved. In particular, more detailed process kinetic and mass balance data would be required. While it may rightly be argued that more detailed fundamental studies are required in order to extend an understanding of these factors, it also seems that a case can be made to proceed to preliminary bio-engineering scale-up and process development studies as a specific requirement for advancing the development of coal biotechnology as generally proposed by Klein *et al* (1999). It is suggested that future work should consider managing both approaches in tandem given the important reciprocal feedback that is achievable where fundamental and process development studies are undertaken together.

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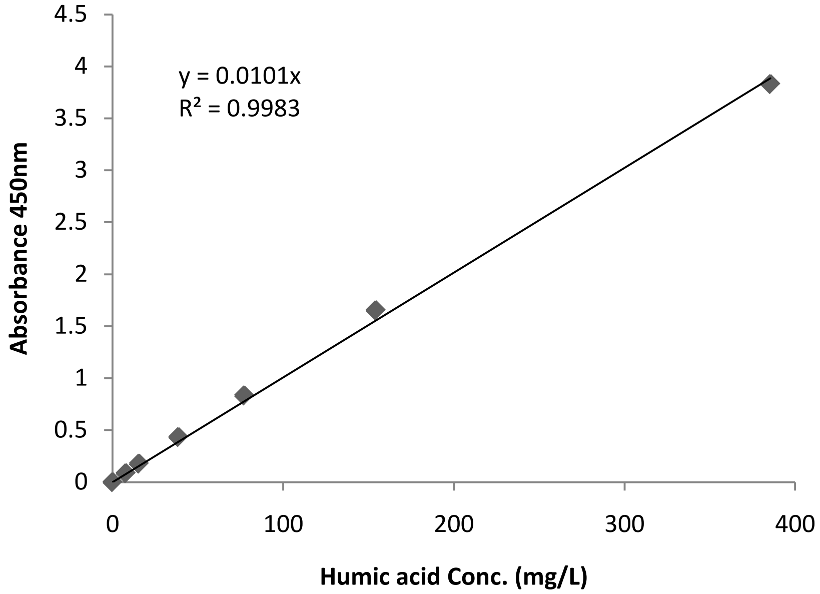
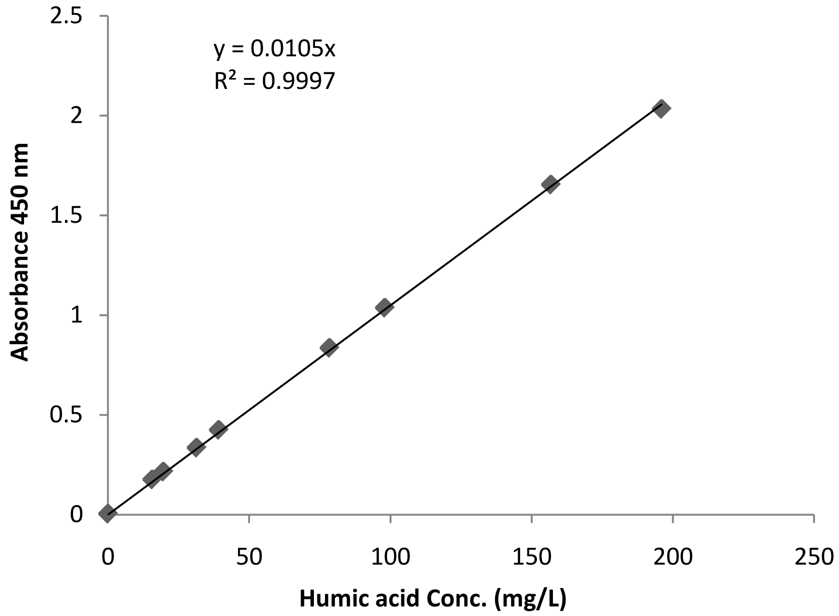
APPENDICES

Appendix 1-A



International humic substances society humic acid standard curve plot. Absorbance values were converted to mass by extrapolation made from the plot.

Appendix 1-B



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

Appendix 1-C

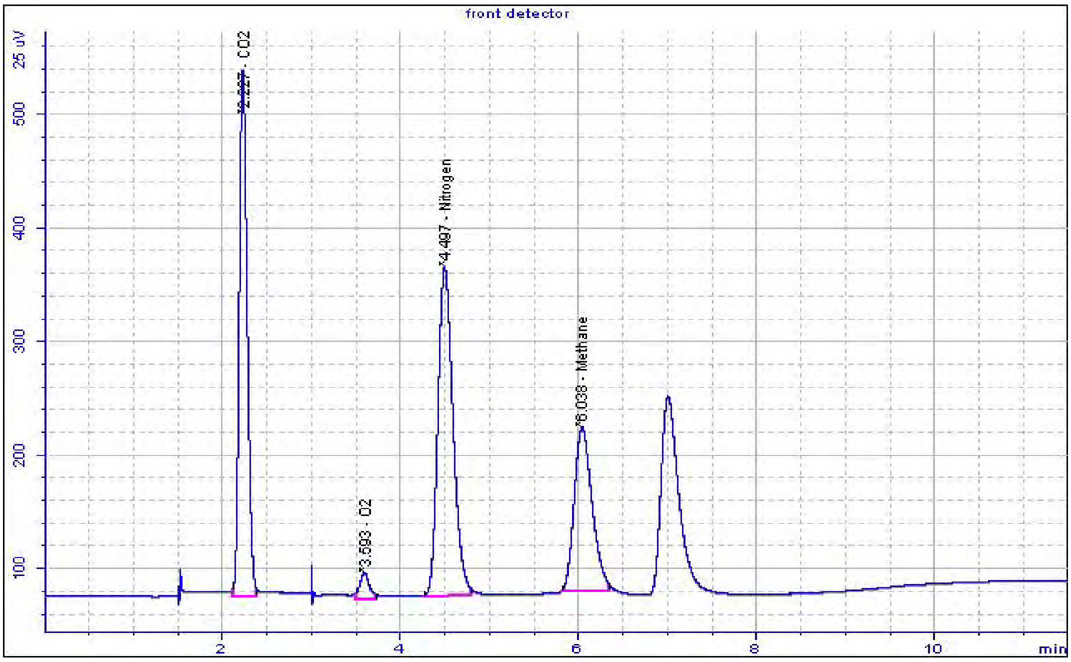
Results indicating dry weight analysis and spectrophotometric measurement of geologically oxidized coal fractionated using three alkaline extraction methods.

	Extraction Method	WC (g)	Humin (wt%)	FA (wt%)	HA (wt%)	HA 450 nm
1	1x 100 ml NaOH 0.1M	2.5	60.1 1.9*	0.2 0.0*	32.1 0.4*	34.9 0.4*
2	3x 35 ml NaOH 0.1M	2.5	57.8 0.2*	0.1 0.0*	32.1 0.3*	35.8 0.4*
3	3x 35 ml NaOH 0.1M	2.5	58.2 0.2*	0.1 0.0*	33.7 0.6*	35.8 0.4*

*= standard deviation (SD)

Appendix 2-A

Gas chromatograph peaks of gas standards used for the identification of gases produced in the methanogenic studies using oxidized HC with and without grass co-substrate.



Gas chromatograph of the Alpha gas standard (Afrox, South Africa) containing 20% H_2S; 20% CO_2; 20% N_2 and 40% CH_4 used to identify the gases produced in the methanogenic studies. Area under curve was used to quantify gas methane gas production.

Appendices 2-B

Fisher LSD test of the total gas production in reactors using biologically oxidized coal substrates. Marked differences are significant at $p < 0.05$.

Reactor	{1}	{2}	{3}
Grass {1}		0.814529	0.033195
BOC {2}	0.814529		0.054786
BOC+G {3}	0.033195	0.054786	

Appendix 2-C

Fisher LSD test of the methane yields in reactors using biologically oxidized coal substrates. Marked differences are significant at $p < 0.05$.

Reactor	{1}	{2}	{3}
Grass {1}		0.015536	0.214508
BOC {2}	0.015536		0.182774
BOC+G {3}	0.182774	0.214508	

Appendix 2-D

Fisher LSD test of the total gas production in reactors using geologically oxidized coal substrates. Marked differences are significant at $p < 0.05$.

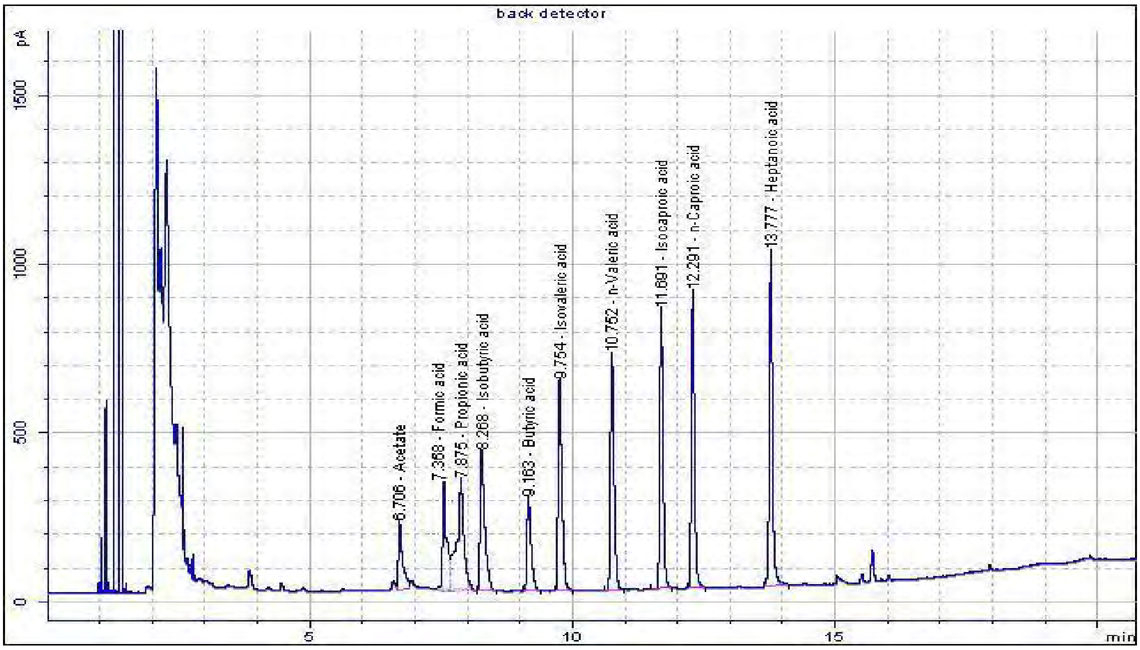
Reactor	{1}	{2}	{3}
Grass {1}		0.006972	0.001
GOC {2}	0.006972		0.001
GOC+G {3}	0.001	0.001	

Appendix 2-E

Fisher LSD test of the methane yields in reactors using biologically oxidized coal substrates. Marked differences are significant at $p < 0.05$.

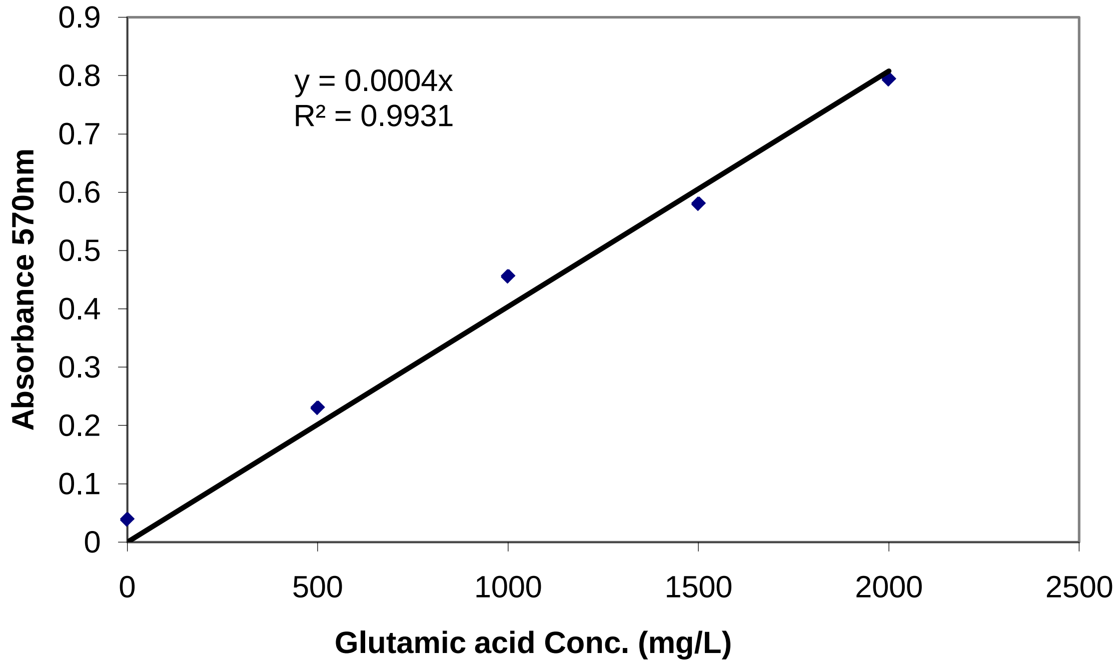
Reactor	{1} Grass	{2} GOC	{3} GOC+G
Grass {1}		0.640643	0.094605
GOC {2}	0.038001		0.038001
GOC+G {3}	0.640643	0.094605	

Appendix 3-A



Volatile fatty acid standard mix containing C1 – C7 acids at 10 mM each in deionized water, and used to identify and quantify volatile fatty acids produced in the methanogenic study.

Appendix 4-A



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