THE DEGRADATION OF LIGNOCELLULOSE IN A BIOLOGICALLY-GENERATED SULPHIDIC ENVIRONMENT

A thesis submitted in fulfilment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

of

RHODES UNIVERSITY

By

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November 2004

Abstract

South Africa is renowned for its mining industry. The period over which the polluted waters from the existing and abandoned mines will require treatment has driven research into the development of passive treatment systems. These waters are characterised by a low pH, high concentrations of heavy metals, high levels of sulphate salts and low concentrations of organic material. The biological treatment of these waters has been a subject of increasing focus as an alternative to physico-chemical treatment. The utilisation of lignocellulose as a carbon source has been restricted by the amount of reducing equivalents available within the lignocellulose matrix. After a few months of near 100 % sulphate reduction, it was found that although there was a large fraction of lignin and cellulose remaining, sulphate reduction was reduced to less than 20 %. The present study demonstrated that lignocellulose can be utilised as a carbon source for sulphate reduction. It was established that lignocellulose degradation was enhanced under biosulphidogenic conditions and that lignin could be degraded by a sulphate reducing microbial consortium. It was established using lignin model compounds synthesized in our laboratory, that the bonds within the lignin polymer can be cleaved within the sulphidic environment. The presence of cellulolytic enzymes, using CMCase as a marker enzyme, was detected within the sulphate reducing microbial consortium.

Based on the results obtained a descriptive model was formulated for the degradation of lignocellulose under biosulphidogenic conditions. It was determined that the initial reduction in sulphate observed using lignocellulose as a carbon source was due to the easily extractable components. The degradation of which resulted in the production of sulphide, which aided in the degradation of lignin, allowing greater access to cellulose. Once the easily extractable material is exhausted, the cycle is halted, unless the sulphide production can be maintained. This is the focus of an ongoing project, testing the hypothesis that an easy to assimilate carbon source added after exhaustion of the easily extractable material, can maintain the sulphide production.

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µl	Microlitre
µm	Micrometre
µmol/ml/h	micro
^{1}H-NMR	Hydrogen nuclear magnetic resonance
AMD	Acid mine drainage
ARD	Acid rock drainage
bp	Boiling point
$C0_2$	Carbon dioxide
$CaCl_2$	Calcium chloride
CBM	Cellulose binding module
CBH	Cellobiohydrolase
$CdCl_3$	Deuterated Chloroform
CH_4	Methane
CMC	Carboxymethyl cellulose
CMCase	Carboxy methyl cellulase
d.H_20	Distilled water
DACST	Department of Arts, Culture, Science and Technology
DCM	Dichloromethane
DMS0	Dimethyl sulfoxide
DNS	Dinitro salicylic acid
DPBR	Degrading packed bed reactor
DWAF	Department of Water Affairs and Forestry
EBG	Environmental Biotechnology Group, now known as the Environmental Biotechnology Research Unit (EBRU)
EF–hand	Calcium-binding motif of calcium binding proteins
GC-MS	Gas chromatography-mass spectroscopy
H_2S	Dihydrogen sulphide
HPLC	High performance liquid chromatography
HRT	Hydraulic retention time
HS^-	Hydrogen sulphide
Hz	Hertz
IC	Ion chromatography
IF	Innovation Fund
KH_2P0_4	Potassium dihydrogen phosphate
l	Litre
LSRU	Large sulphate reducing unit
M	Molar
Mg/l	Milligram per litre
Mg/l/d	Milligram per litre per day
Mg/l/h	Milligram per litre per hour
$MgS0_4$	Magnesium sulphate
MHz	Megahertz
min	Minute
ml/min	Millilitre per minute
mM	millimolar
mmol	Millimole

MPP	Methane Producing Prokaryotes
MPN	Most probable number
Na-citrate	Sodium citrate
$NaS0_4$	Sodium sulphate
NH_4Cl	Ammonium chloride
nm	Nanometre
PDA	Photo-diode array
PHD	Pulles, Howard and de Lange
PVC	Poly(vinyl chloride)
RPM	Revs per minute
RT	Room temperature
rt	Retention time
$S0_4$	Sulphate
SRP	Sulphate reducing prokaryotes
SRU	Sulphate reducing unit
SSRU	Small sulphate reducing unit
TEM	Transmission Electron Microscopy
TFA	Trifluoro acetate
UK	United Kingdom
USA	United States of America
UV/Vis	Ultraviolet/visible
VCC	Vryheid coronation colliery
VFA	Volatile fatty acid
w/v	Weight per volume
WRC	Water Research Commission
ZnAc	Zinc acetate

Acknowledgements

I would like to take this opportunity to thank the following persons, without whom this thesis would not have been possible:

My supervisor, Prof. Peter Rose, for allowing me the opportunity to work on such a challenging project and for his enthusiasm and financial support for the duration of the study.

My co-supervisor, Dr Brett Pletschke, for his encouragement on the numerous trips to Port Elizabeth.

Dr K. S. Nagabhushana for his encouragement and stimulating conversation.

My fellow post-graduate students, Doug, Clark, Michelle, Anna, Mzi, Neil and Jennifer from our research group. My thanks also to other students within the department, especially Fritha, Michael, Vuyo and Delia. Also the Metals Lab and especially Dr Burgess for the long chats.

The Innovation Fund and the Water Research Commission for funding this research programme.

Pulles, Howard and de Lange (PHD) for their support.

Rhodes University for granting me the privilege of studying at such a prestigious organisation.

My family for their support through all these years of study.

Special thanks to my wife, Tania, for her unfailing support, encouragement and patience through 11 years of study.

Chapter 1

General Introduction

1.1 Acid mine drainage

Acid mine drainage (AMD) wastewater, also known as acid rock drainage (ARD), originates from mining activities where pyritic minerals have been exposed to water and oxygen. Both chemical and bacterially-mediated reactions occur (Lovell, 1983; Johnson, 1995; Cocos *et al.*, 2002), and the various steps involved in its formation may be summarised as follows (Johnson and Hallberg, 2003):

$$FeS_2 + 3.5O_2 + H_2O \rightarrow Fe^{2+} + 2SO_4^{2-} + 2H^+ \qquad (1)$$

AMD is usually characterised by a low pH, high concentrations of dissolved heavy metals and high levels of sulphate salts. However, not all AMD is acidic and some mine drainage waters may contain high concentrations of metals at near neutral pH values (Harris and Ragusa, 2000; Younger, 2001). The wide variation in AMD composition found at different sites is shown in Table 1 and sulphate concentrations may vary from 151 mg/l to 108 000 mg/l, with pH varying from 0.5 to 6.5 (Bell *et al.*, 2001; Johnson and Hallberg, 2003).

Iron is the characteristic, but not the only, metal found in AMD and occurs in either the ferric or ferrous form, depending on the pH and oxygen status of the water. This is responsible for the characteristic orange to red colours observed in AMD flows. Other metals that commonly occur in AMD include aluminium, copper, zinc and manganese. The concentration of organic material within these streams is also generally low (<20 mg/l), presenting a problem for unaugmented bioremediation of these wastewaters (Wittman and Förstner, 1976, 1977; Johnson and Halberg, 2003).

Table 1.1: A comparison of mine drainage waters from various sites (From Johnson and Halberg, 2003).

	Storwartz Mine, Norway	Ynysarwed, Wales	Wheal Jane, England	Parys mine, Wales	Rio Tinto, Spain	Iron Mountain, USA	Middelburg Colliery, South Africa
pH	6.5	6.2	3.4	2.5	2.2	0.5-1	2.95
Sulphate (mg/l)	151	464	400	1 550	10 000	20-108 000	1 730
Iron (mg/l)	1.6	160	290	650	2 300	13-19 000	140

1.1.1 Environmental impacts of AMD

The acidity and dissolved heavy metal content in AMD-polluted streams may have devastating effects on aquatic as well as terrestrial ecosystems, reducing both species diversity and total biomass in these systems (Elliot *et al.*, 1998). There are an estimated 17 000 km of streams in the U.S.A. affected by AMD, as well as more than 700 km of streams in the UK (Younger, 1997; Elliott *et al.*, 1998).

While a comprehensive survey of the full impact of AMD on the South African public water system remains to be done, the nature of environmental impacts of AMD experienced may be illustrated by a case study of the Middelburg Colliery near Witbank, Gauteng (Bell *et al.,* 2001). The mine was decommissioned in 1947, and in 1996 was still discharging a water with sulphate levels in excess of 1 000 mg/l sulphate and pH <3 (Table 2). Water seeping from the mine with pH values of ~2.6 formed a stream that entered the Blesbokspruit River and resulted in reducing an upstream pH >7 to a pH of ~3.2. Sulphate concentrations were between 128 and 2250 mg/l, demonstrating typical AMD contamination and vegetation in the immediate surroundings of the mine showed adverse effects, with an area of about 3 ha that had been nearly completely denuded. With the exception of individual species of algae no aquatic life was present in the seepage area (Bell *et al.,* 2001).

Table 1.2: Chemical composition of AMD from an abandoned mine in the Witbank Coalfield, South Africa (From Bell *et al.*, 2001)

Determinand	May 1990	March 1991	Feb. 1994	Aug. 1996	SA guidelines (1996)*
pH	2.3	2.0	2.8	2.95	6-9
Sulphate	2361	1692	2897	1730	0-200

*(DWAF, 1996).

Slimes dams, which are composed of the extraction process residuals, also contribute to the overall problem. Since many of these dumps have not been disturbed for many decades, and over the course of time have been exposed to oxygenated water, AMD production has occurred with waters percolating through the mine dumps now entering the rivers and streams along the Witwatersrand. Surface waters upstream of the mining region have a low concentration of metals and pH values near neutral. Within and downstream of the mining areas of the Witwatersrand the pH is lowered to between 5 and 6 and a marked increase in heavy metals and sulphates is observed. Upstream of the mining region the concentration of sulphate is 300 mg/l, and within the region it rises to >600 mg/l. Animal and plant life in streams in the area are also affected (Naicker *et al.*, 2003).

1.2 Treatment of AMD

Both physico-chemical and biological methods for the treatment of AMD have been investigated and the subject has been comprehensively reviewed by Gazea *et al.* (1996) and Hulshoff-Pol *et al.* (2001).

Physico-chemical treatment processes include chemical precipitation, electrochemical treatments, reverse osmosis, filtration, ion exchange and adsorption. In addition to high operational costs, these technologies create secondary problems, such as metal-containing sludges and waste concentrates that may be difficult and expensive to dispose. These secondary problems are not particular to active processes but may also be encountered in passive treatment processes (Volesky, 2000; Younger *et al.*, 2002). Biological treatment options have been a subject of increasing focus providing a sustainable alternative to chemical treatment and have been considered as both active and passive systems (Gazea *et al.*, 1996; Harris and Ragusa, 2000; Kaksonen *et al.*, 2003). These are differentiated on the basis of the operational and management controls required, with passive systems running without continuous energy and operator inputs. A passive treatment process utilises naturally available resources, such as topography, microbial metabolic processes and chemical energy, it also requires infrequent maintenance to be successful for its operating life (Van der Merwe, 2002). Passive treatment in the South African context is the remediation of AMD in terms of removing the sulphate content, heavy metals and neutralising the water. In order for a passive system to be successful in the treatment of sulphate-rich waters, the following criteria have to be met:

- There needs to be a carbon source;
- The carbon source has to be accessible to the sulphate reducing prokaryotes (SRP);
- The carbon should be sufficient to reduce the influent sulphate load.

Aspects of passive system applications will be considered here (Dill *et al*., 2001).

Scale-up implementation of passive treatment operations has been attempted in several countries and has been reviewed by Pulles *et al.* (2001). One of the largest pilot studies is that undertaken at Wheal Jane in Cornwall, UK, and illustrates several features of the passive treatment approach (Hamilton *et al*., 1999, Younger *et al.*, 2005). The accidental discharge in 1992 of ~30 000 m^3 AMD into the Carnon River from a mine tailings dam at Wheal Jane, lead to the selection of passive treatment technology, given the advantages of this approach over the predicted time–scales over which treatment would be required. The design consisted of aerobic reedbeds, an anaerobic cell and a rock filter (repeated through 3 separate systems containing the same treatment units). The aerobic reedbed was planted with *Phragmites australis*, *Typha latifolia* (in equal proportions) and some *Seirpus*. The depth of the reedbed was determined by the water depth requirements of the plant species, and surface area by taking into account the pH and influent load (in terms of iron) of the minewater. The anaerobic cell was packed with 95 % (w/v) sawdust and 5 % (w/v) hay, and then inoculated with cattle slurry. The rock filter used 75 mm granite cobbles, and rock berms were installed at 10 m intervals along its length. Hamilton *et al.* (1999) have reported that treatment was successful in removing metals and reducing sulphate (sulphate removed in a range of 0.01-0.04 mol/m^3).

1.2.1 South African experience with passive systems

South Africa has an extensive mining industry and the long time period over which the polluted waters of the large number of existing decommissioned and abandoned mines in South Africa will require treatment has driven interest in the development of the passive treatment approach (Van der Merwe, 2002).

Pulles, Howard and de Lange (PHD), an environmental consulting firm, have been leaders in this initiative and have undertaken three phases of research into the passive treatment of AMD (Heath, 2000). The first was funded by the Water Research Commission (WRC) where AMD from the Western Areas Gold Mine and Arnot Colliery was treated using an integrated passive treatment strategy. It was found that sulphate reduction rates were highly variable, with a high initial efficiency followed by a decline and severely reduced performance with time (Heath, 2000).

The second phase, funded by Anglo Coal, was undertaken at Vryheid Coronation Colliery (VCC). A pilot plant (consisting of four parts) was constructed and operated for two years (Figure 1.1):

- Four small sulphate reducing units (SSRU) arranged in series.
- Two large sulphate reducing units (LSRU).
- Organic substrate testing units.
- An aerobic wetland and oxidation cascade to operate in the final polishing of remaining contaminants.

The units received AMD from splitter boxes where waters from the West and South Adits of VCC were mixed in a 1:1 ratio. After treatment in the sulphate reducing units, the water was subjected to a polishing step to remove residual nutrients and contaminants. The polishing step consisted of an aerobic wetland and oxidation cascade. After treatment at the oxidation cascade the water was discharged into a naturally occurring stream. The entire plant was constructed on a gentle slope, with the splitter boxes at the top of the slope and the oxidation cascade at the bottom (Molwantwa, 2004). The SSRUs were packed with hay, sawdust and wood chips. Chicken litter, manure and sewage sludge served as the source of SRP. The LSRUs were packed in a similar manner, but were ~8 times the size of the SSRUs.

The VCC pilot plant was found to be effective in neutralising the acid rich waters and bicarbonate alkalinity was produced raising the pH from 2.5 to ~7. Metals were also effectively removed. However, while sulphate was reduced to sulphide in the SRUs, this was re-oxidised in the aerobic wetland resulting in no net sulphate removal (van der Merwe, 2002). Again it was observed that for the first 6-9 months of operation, high rates of sulphate reduction were obtained (up to 100 % of the influent sulphate removed). After this period, sulphate removal declined to < 30 % of the influent sulphate (Pulles *et al.*, 2001). This problem has been encountered in other lignocellulose packed bed systems (Pulles pers com,

2001) and it was concluded that a detailed understanding of carbon source utilisation was required in order to deal with this issue. It was assumed, however, that the initial performance could be related to the release of easily soluble materials from the lignocellulose packing. Once these materials have been exhausted, leaving only the core lignocellulose structure, the reduced availability of organic carbon results in reduced sulphate reduction (Rose pers com, 2001).

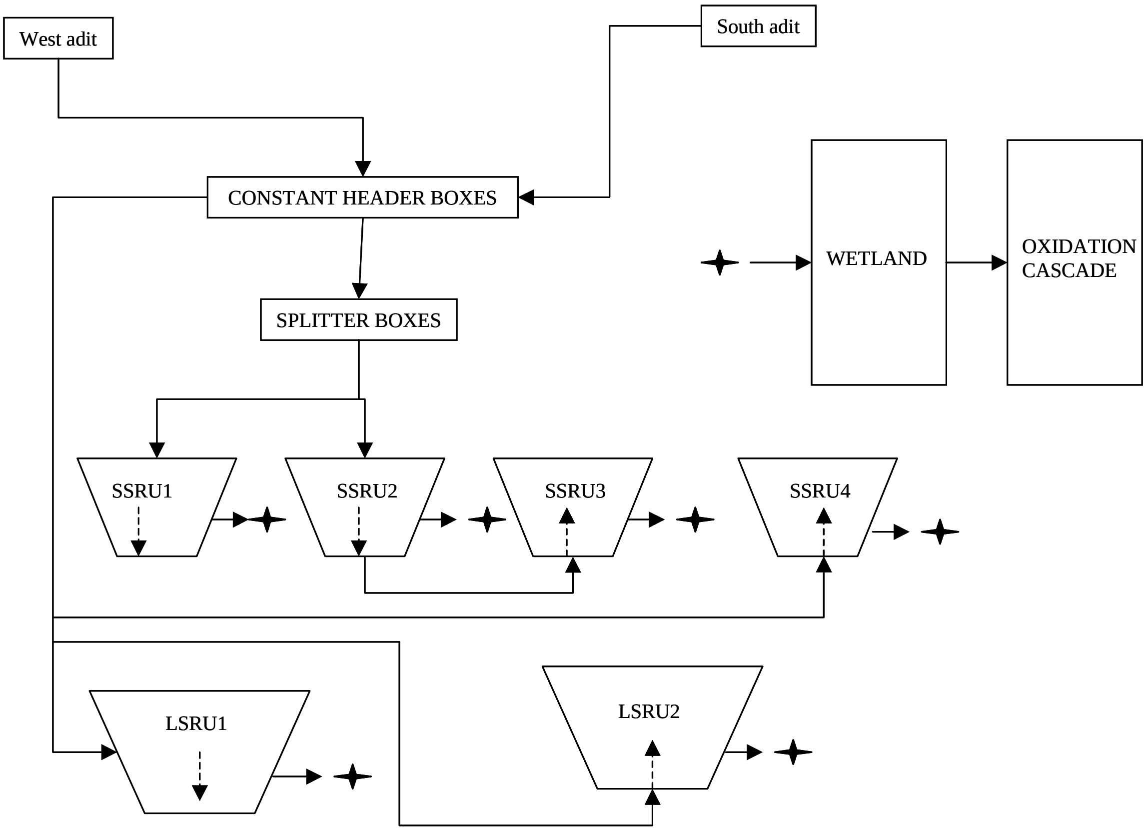


Figure 1.1: Configuration of the pilot plant at Vryheid Coronation Colliery. The solid arrows indicate the direction of flow. The dashed arrows indicate the direction of flow within the reactor. SSRU = small sulphate reducing unit, LSRU = large sulphate reducing unit. ✧ Indicates flow towards the wetland (From Molwantwa *et al.*, 2003).

In the third phase of the investigation of Passive systems a Department of Arts, Culture, Science and Technology (DACST), now known as the Department of Science and Technology (DST), Innovation Fund (IF) study was undertaken in an attempt to answer aspects of the questions concerning organic carbon utilisation in these systems and on the basis of their findings to address the overall issues of process performance. The study reported here deals with an aspect of the IF project programme.

1.3 Electron donor sources used in sulphate reduction

Sulphate reducing prokaryotes (SRP) are a diverse group of obligate anaerobic microorganisms with different morphologies and physiology. They reduce sulphate to sulphide by a process known as dissimilatory sulphate reduction, in which sulphur is not assimilated into the cell, but is rather utilised as the terminal electron acceptor for the oxidation of organic substrates. The summary chemical equation for the reduction of sulphate may be given as (Hansford, 2004):

$$CH_2O_n + SO_4^{2-} \rightarrow H_2S + {}_nCO_2 + {}_nH_2O \qquad (2)$$

Generally the electron donors utilised by SRP are low molecular weight fermentation products derived from the degradation of protein, carbohydrates and other components of biomass (Gibson, 1990). Sulphate-reducing bacteria have the ability potentially to oxidise completely the principal organic end products of bacterial metabolism in anaerobic environments. Recent work has shown that complex organic polymers including lignocellulose may be degraded under sulphidogenic conditions (Pareek *et al.*, 1998, Whittington-Jones, 2000, Whiteley *et al.*, 2002, Whiteley *et al.*, 2003 [a], Whiteley *et al.,* 2003 [b]).

1.3.1 Lignocellulose as a carbon source

A complex organic carbon source that has and still is being explored, as an alternative carbon source for SRP, is lignocellulose. Different types of lignocellulose have been tested including hay and straw, spent mushroom compost, sawdust, oak chips, paper and peat (Wakao *et al.*, 1979; Eger and Lapakko, 1988; Dvorak *et al.*, 1992; Bechard *et al.*, 1994; Pareek *et al.*, 1998; Chang *et al.*, 2000).

The potential of lignocellulose as an alternative carbon source for sulphate reduction was recognised as early as 1969. Whilst working on an acidic stream in southeastern Ohio, Tuttle *et al.* (1969) noticed that the physico-chemical characteristics of the water changed when it passed through a porous dam composed of wood dust. The water above the wood dust dam had a greater concentration of sulphate than the water below it. In addition the water below the dam had a greater concentration of anaerobic microorganisms than the water above the dam. This suggests that the wood dust provided a carbon source for growth of the microorganisms. The pH of the water below the dam was 3.38, compared to 2.84 of the water above the dam. There was also a decrease in sulphate concentration from

8.765 µmol/ml to 6.1 µmol/ml. These observations suggest that SRPs are responsible for these chemical changes in the water quality. This observation was confirmed by the cell counts of 876 MPN/100 ml found below the dam, compared to none found above the dam. This was one of the first reports demonstrating that lignocellulose may be used as a carbon source for the treatment of AMD by SRP (Tuttle *et al.*, 1969).

Under anaerobic conditions it is generally accepted that lignocellulose is a recalcitrant molecule. Evidence of this would be the archaeological finds of intact wood from submerged environments that are hundreds of years old. However, various studies comparing the degradation of (ligno) cellulosic material under sulphidogenic and methanogenic conditions have shown that under sulphate reducing conditions the hydrolysis of this material is greater than under methanogenic conditions (Kim *et al.*, 1997, Pareek *et al.*, 1998). Ligno-cellulosic material such as straw and sawdust have been shown to support bacterial growth in the treatment of AMD, with the addition of low molecular compounds such as sucrose, peptone, lactate and pyruvate (Chang *et al.*, 2000).

1.3.2 Lignocellulose

In terrestrial environments cellulose is the most abundant biopolymer found. Photosynthetic fixation of CO_2 produces more than 10^{11} tonnes per annum of plant material globally, with almost 50 % of it being cellulose (Leschine, 1995). Lignocellulose accounts for a major part of terrestrial global biomass, and its degradation is essential for the operation of the terrestrial global carbon cycle. Figure 1.2 shows microbially mediated biotransformations involved in the global cycling of lignocellulose, in both aerobic and anaerobic processes. The majority of available lignin is not susceptible to anaerobic degradation and through diagenesis forms peats and lignites, eventually forming coal deposits (Madikane, 2002). The lignin, cellulose and hemicellulose in lignocellulosic material form a complex 3-dimensional structure, which renders cellulose and hemicellulose less accessible to microbial attack due to the physical barrier imparted by lignin (Pareek *et al.*, 1998).

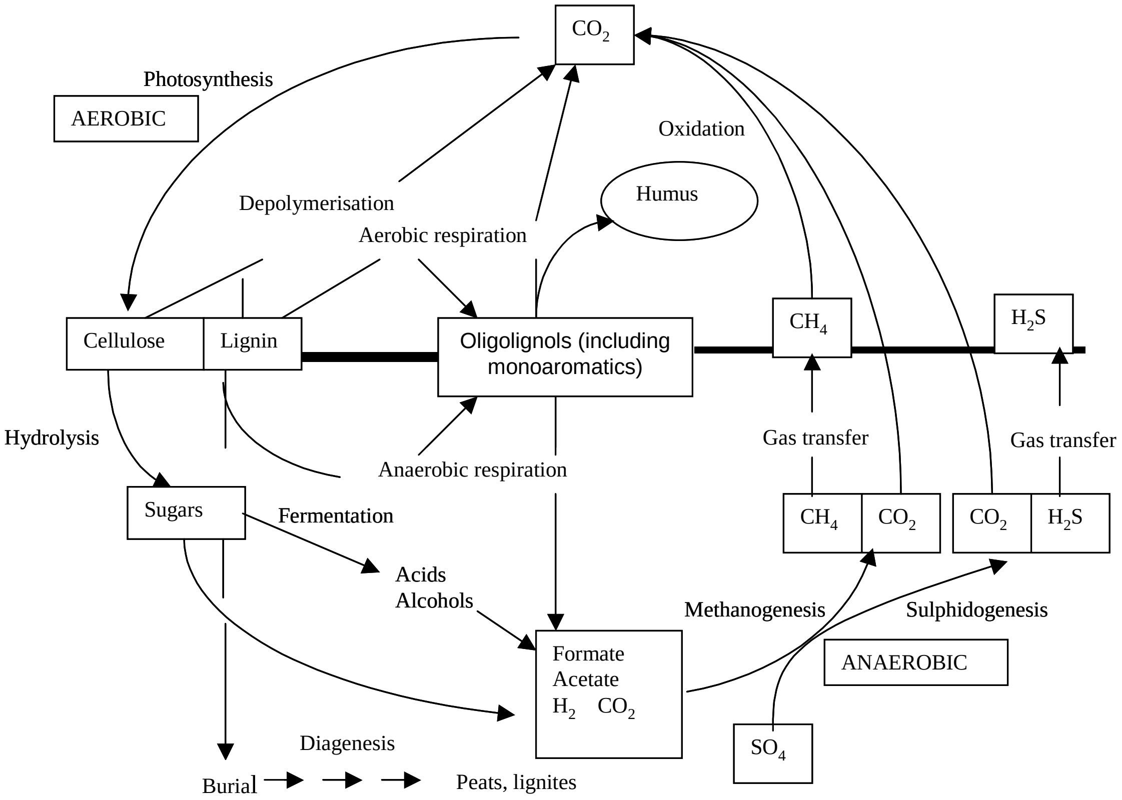
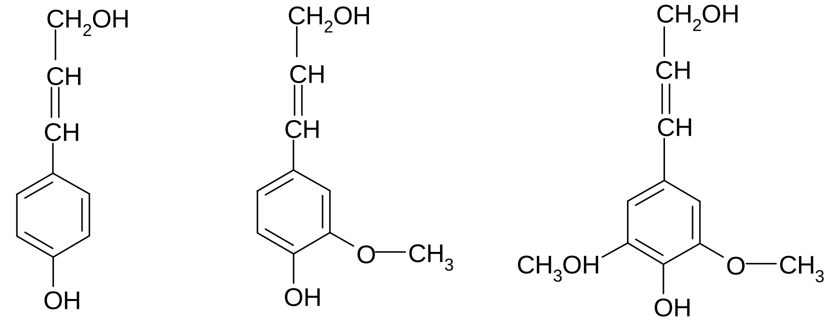


Figure 1.2: The biological cycling of lignocellulose-derived carbon (From: Madikane, 2002).

1.3.3 Structure of lignin

Lignin is the aromatic polymer found in plants that confers rigidity to the cell walls and, in addition, makes the plant resistant to microbial and enzyme attack and mechanical stresses. The synthesis of lignin in vascular plants takes place via a branching sequence of reactions during which CO_2 is first converted to shikimic acid. Shikimic acid is then converted through several steps to prephenic acid, where the pathway branches to yield L-tyrosine and L-phenylalanine. These amino acids are ultimately converted to three phenylpropanoid compounds that comprise the building blocks of lignin: sinapyl alcohol, coniferyl alcohol and *p*-coumaryl alcohol (Figure 1.3).



p-Coumaryl Alcohol

Coniferyl Alcohol

Sinapyl Alcohol

Figure 1.3: Primary building blocks of lignin (Crawford and Crawford, 1988).

The formation of the polymer takes place via a free radical-mediated reaction that is generally initiated by peroxidases, coupling substituted *p*-hydroxycinnamyl alcohol derivatives. The most prevalent linkage formed by the free radical coupling, during synthesis, are the β-*O*-4 linkages, which is an ether linkage between the C-2 of an arylpropane side chain and the *para* position of another lignin monomer.

The differences in lignin composition of different wood types lie in the monomeric make-up of the material. Softwoods contain predominantly guaiacyl residues; hardwoods have both guaiacyl and syringyl residues, while grasses contain mostly *p*-hydroxy-phenyl residues in addition to guaiacyl and syringyl residues. Grasses have another unique feature in that they contain coumaric and ferulic acid bound to the hydroxyl groups of lignin via an ester bond (Wood and Saddler, 1988, Crawford and Crawford, 1988, Deobald and Crawford, 1997).

1.3.4 Structure of cellulose

Cellulose is normally found embedded in a matrix of other polymers; including hemicelluloses, pectin and proteins. Hemicellulose, which is found in close association with celluloses, has to be removed before the cellulose in plant cells can be effectively degraded by cellulolytic bacteria (Leschine, 1995).

The celluloses found in wood and cotton are linear polymers of *D*-anhydro-glucopyranose units linked by β-1,4-glycosidic bonds (Figure 1.4). The number of glucose units per molecule ranges from 15 to 14 000. Polymers of ß-glucose can be represented as a series of rigid pyranose rings in the chair conformation, connected by an oxygen atom bridging two carbon atoms (glycosidic bonds), about which there is a free rotation. The general assumption is that cellulose molecules are deposited in an anti-parallel arrangement. From figure 1.4 it can be seen that cellulose has one reducing end and one non-reducing end (Cowling, 1975).

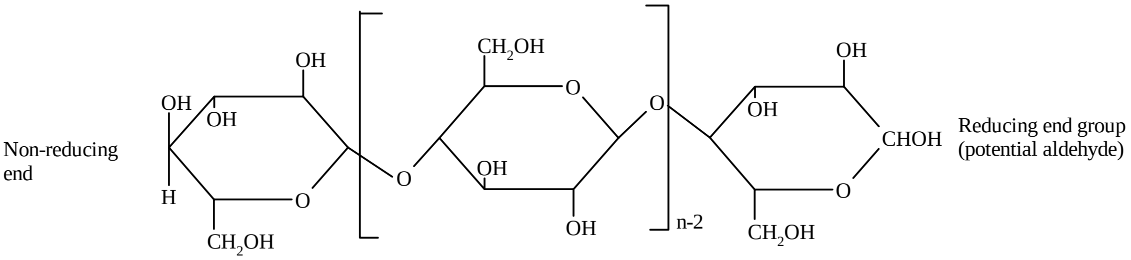


Figure 1.4: Structure of cellulose, a 1,4-O-(β-D-glucopyranoside) polymer, Haworth form (MacDonald and Franklin, 1969).

Cellulose molecules exist in a highly organised state in the form of fibrillar elements, which are in turn organised to form the various cell walls of a fibre. The highly crystalline state of cellulose is a result of extensive hydrogen bonding and van der Waals forces. The cellulose molecules associate to form insoluble fibrils where the chains are held together by hydrogen bonds. It has further been deduced that the hydrogen-bonding network consists of inter- and intra-molecular bonds between successive and adjacent glucose residues and it is thought that the intramolecular bonds maintain the rigidity of the cellulose chain. The molecules are orientated in parallel, with reducing ends of the glucan chains located at the same end of a microfibril. The molecules form highly ordered crystalline domains interspersed by more disordered, amorphous regions. Native cellulose is 60-90 % crystalline (MacDonald & Franklin, 1969; Teunissen & Op den Camp, 1993; Leschine, 1995).

Cellobiose is a disaccharide obtained by the partial hydrolysis of cellulose and consists of two *D*-glucopyranoses joined by a 1,4′-β-glycoside bond. It is a reducing sugar because the anomeric carbon on the right-hand glucopyranose unit has hemiacetal groups (Figure 1.5), which are found in equilibrium with aldehyde forms that can reduce Tollen’s or Fehlings’ reagent (McMurry, 1996).

The bonds within cellulose can be broken enzymatically by cellulases, either endoglucanases or exoglucanases (cellobiohydrolases). The exoglucanases hydrolyse the cellulose polymer from one end, whereas the endoglucanases randomly hydrolyse the chain (Aristidou and Penttilä, 2000).

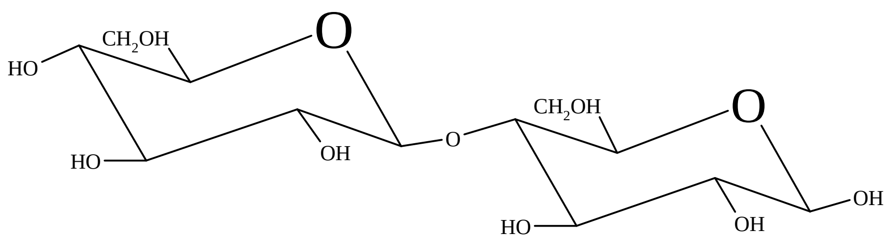


Figure 1.5: Structure of cellobiose, a 1, 4’-β-glycoside (McMurry, 1996).

1.3.5 Hemicellulose structure

The hemicellulose found in wood consists of relatively short, mainly branched heteropolymers of glucose, xylose, galactose, mannose and arabinose as well as uronic acids of glucose and galactose linked by β1-3, β1-6 and β1-4 glycosidic bonds. The degree of polymerisation of hemicelluloses seldom exceeds 200 units (Cowling, 1975).

Hardwood hemicelluloses are rich in xylan polymers with small amounts of glucomannan, and softwood hemicelluloses are rich in galactoglucomannan polymers and contain significant amounts of xylan polymers. Sulphite pulping cleaves most of the acetyl and arabinofuranose groups from xylan hemicelluloses, resulting in methylglucuronoxylan residues, with a marked reduction in the degree of polymerisation. Galactoglucomannan and galactose are not expected in sulphite pulp, due to their being degraded under the acidic conditions. *Kraft* pulping cleaves methylglucuronic acids from the xylan hemicelluloses, resulting in arabinoxylan for softwoods and xylan for hardwoods and prehydrolysed softwoods. *Kraft* pulping results in less reduction in the degree of polymerisation than sulphite pulping (MacDonald & Franklin, 1969).

Hemicelluloses are similar to cellulose in terms of chemical structure and therefore exhibit a similar pattern of chemical reactions and degradations. The rate of reaction of hemicellulose is greater than that for cellulose because of its amorphous nature, allowing it to be more accessible to hydrolysis, and because it is located in the outer regions of the fibre (MacDonald & Franklin, 1969).

The arabinofuranosyl side chains of hemicellulose can be esterified by aromatic acids such as ferulic and ρ-coumaric acids, and are thought to play a role in the lignin-hemicellulose cross links involving ether linkages (Béguin and Aubert, 1994). Ferulic acid has been found to be bound by ester linkages to carbohydrates and core lignin, and guaiacyl units are often crosslinked by ether and carbon-carbon bonds (Akin and Benner, 1988). No direct proof has been shown for the existence of chemical bonds between lignin and carbohydrate, but there is a large amount of evidence indicating that there is such a chemical bond. The fact that one is unable to separate polyphenol from carbohydrate using gel filtration suggests the existence of covalent linkages and further indirect evidence was shown in later work where it was found that these complexes precipitated at low pH values (Conchie *et al.*, 1988). It is known that in grasses there may be a covalent linkage between carbohydrates and phenolic acids via ester linkages, and to lignin through either ester or ether linkages (Figure 1.6). It has also been proposed that the bifunctional phenolic acids could be cross-bridging molecules between lignin and polysaccharide structures (Iiyama *et al.*, 1990).

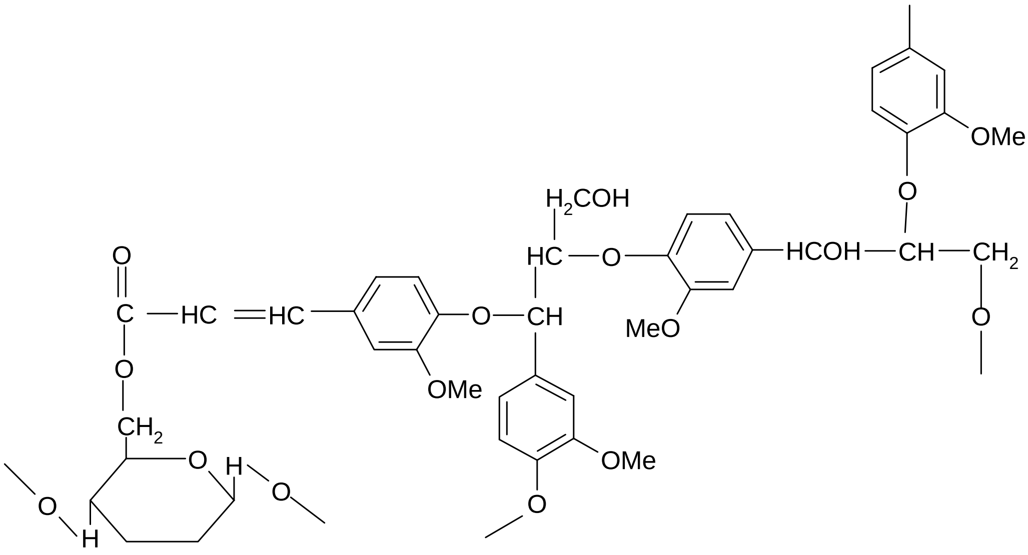


Figure 1.6: Proposed model for the lignin-carbohydrate complex (Iiyama *et al.*, 1990).

1.3.6 Degradation of lignocellulose

It is known that the degradation of polymeric molecules by microorganisms is initially very different from that of small molecular weight compounds. The reason for this difference is that many small molecules can be transported across the cell membrane, whereas polymeric molecules have to be hydrolysed by one or more enzymes to smaller sub-units before transport can occur. This phenomenon is the rate-limiting step in the degradation of polymeric molecules (Haldane and Logan, 1994; San Pedro *et al.*, 1994; Vavilin *et al.*, 1996).

The primary degraders of lignocellulose in terrestrial environments are the basidiomycetes, which degrade the structural components of lignocellulosic material. The secondary degraders are the bacteria, protozoa and insects that further degrade the wood to its final breakdown products. Benner *et al.* (1985) found that acidic pH (~3.9) had a significant effect on the biodegradation rates of lignocellulose. The cellulose component was found to have a 2-6 times greater rate of degradation at pH 8 than at pH 4, whilst the lignin component had an equal rate of degradation at both pH values. It is proposed that pH may serve to partially uncouple lignin and cellulose degradation indicated by the increase in cellulose biodegradation compared to lignin with the change in pH from acidic to alkaline conditions (Benner *et al.*, 1985; Jordan, 2001).

Evidence from different studies with *Phaenerochaete chrysosporium* has shown that coniferyl lignin (softwood lignin) is more resistant to degradation than syringyl lignin (hardwood lignin), a study by Akin and Rigsby (1987) with anaerobic rumen fungi showed similar results. This study further demonstrated that ruminal fungi are capable of colonizing lignin-containing tissue; this suggests that these fungi may be more tolerant of phenolic compounds that are known to be toxic to ruminal bacteria. The only known mechanism for the biological cleavage of the ether and carbon-carbon bonds within the lignin polymer is by the oxidative attack of lignin peroxidases, manganese peroxidases and laccases, which are non-specific extra-cellular enzymes. These enzymes are strictly dependant on molecular oxygen to produce hydrogen peroxide as a co-substrate. The bacterial species that have been reported to perform this function are the *Streptomyces*, *Nocardia* and *Pseudomonas*. Even the biodegradation of lignin-related molecules such as the 3,4-substituted benzenoids or phenylpropanoids is amongst the most challenging processes in the anaerobic degradation of naturally occurring aromatic compounds (Breen and Singleton, 1999, Brune *et al.*, 1995, Tuomela *et al.*, 2000).

Different species of white-rot fungi produce these enzymes in different combinations. Wood is degraded by bacteria under extreme environmental conditions, such as wood saturated with water, almost anaerobic conditions, or wood with a high extractive content. The rate of degradation, however, is very slow. Several genera of bacteria are able to degrade single-ring aromatic compounds (including *Streptomyces*, *Nocardia* and *Pseudomonas*), and their role may be significant in consuming the small molecular weight compounds that are produced by fungi (Tuomela *et al.*, 2000).

Wooden objects are often discovered intact in waterlogged environments at archeological sites, even after centuries of burial (Björdal *et al.*, 2000). Fungal attack is excluded in the anaerobic environments, and these organisms are responsible for wood decay in the presence of oxygen. Waterlogged wood appears to be intact but on closer inspection it can be seen that there have been considerable changes to the wood tissue. One of the alterations to the wood material is the decrease in the cellulose:lignin ratio. These changes have always been attributed to chemical hydrolysis by components within the soil or by water during the long storage and it was thought that little or no degradation occurred. Recently, it has been established that under near anaerobic conditions this decay is caused by erosion bacteria (Björdal *et al.*, 2000). Bacteria capable of degrading wood are often referred to as either erosion or tunneling bacteria. It has been suggested that bacteria responsible for the degradation of wood in waterlogged environments have a wide pH tolerance and that tunnelling bacteria prefer alkaline conditions. It is believed that the cellulolytic and pectinolytic enzyme systems of bacteria play an important role in the structural changes of wood. Furthermore, it has been suggested that these bacteria begin degradation by colonizing the ray cells of the wood tissue. The bacterial cellulases open up the crystalline arrangement of the cellulose allowing for the diffusion of fungal cellulolytic enzymes. It has been proposed that during the initial phase of lignocellulose degradation it is most likely that a succession of bacterial attack occurs prior to colonization by fungi (Benner *et al.*, 1985; Clausen, 1996).

The association of bacteria in wood degradation has been known for the last 50 years, but this has received little attention compared to fungal degradation. Recently wood-degrading bacteria have been classified into four groups depending on their role in decay process:

- Bacteria that affect permeability but cause no loss in strength.
- Bacteria that attack structure.
- Bacteria that work synergistically with other bacteria to degrade wood.
- Bacteria that are passive colonizers that may be antagonistic toward other bacterial populations (Clausen, 1996).

Termites together with their gut microflora make a significant contribution to the mineralization of lignocellulose. The mechanism of cellulose degradation by termites is fairly well understood whereas that of lignin degradation is still incomplete. Breen and Singleton (1999) observed that lignin cannot be degraded under anaerobic conditions, basing their assumption on the fact that the ether and carbon-carbon bonds within the lignin polymer have thus far shown to be cleaved only via an oxidative mechanism.

1.3.7 Anaerobic degradation of lignocellulose

In a study by Björdal *et al.* (2000), the decay pattern of old wooden poles from a maritime Viking site in Stegesund, Sweden, was investigated. These poles were located at a depth of 5m in brackish water and had been covered by clay sediment. Living erosion bacteria were discovered in the inner portions of the wood tissue, but the authors were clearly not able to determine whether the bacteria present were identical to those active 1200 years ago. However, they observed that with an increase in depth, and decrease in oxygen content, there was a commensurate decrease of degradation of the wood tissue. In near anaerobic conditions was found that erosion bacteria were the only organisms responsible for decay.

Anaerobic degradation is the fermentative process whereby anaerobic bacteria produce methane and carbon dioxide from complex material (Novaes, 1986). Six distinct processes have been identified in anaerobic digesters (Figure 1.7):

1. Hydrolysis of biopolymers (proteins, carbohydrates, lipids).
2. Fermentation of amino acids and sugars.
3. Anaerobic oxidation of long chain fatty acids and alcohols.
4. Anaerobic oxidation of intermediary products, such as volatile acids (except acetate).
5. Conversion of acetate to methane by decarboxylation of acetate.
6. Conversion of H_2 to methane (Gujer & Zehnder, 1983).

In the first stage of figure 1.7, complex organic compounds are hydrolysed through enzymes produced by fermentative bacteria. During the second stage acidogenesis occurs with formation of H_2, CO_2, acetate and higher organic acids due to the activities of fermentative bacteria. The third stage involves acetogenesis and the organic acids produced are converted to H_2 and acetate by acetogenic bacteria. In addition a part of the available H_2 and the CO_2 is converted to acetate by the homoacetogenic bacteria. In the fourth stage the methanogenic bacteria reduce the CO_2 and decarboxylate the acetate to form methane (Novaes, 1986).

Fermentative bacteria are responsible for hydrolysis and acidogenesis. They produce enzymes that are liberated into the medium to hydrolyse organic compounds such as cellulose, hemicellulose and others into smaller molecular material that is transported to the interior of the cells and fermented into a variety of products such as ethanol, butyrate, acetate, etc. In general these bacteria need CO_2 as an electron acceptor and an organic acid as carbon source, ammonia as nitrogen source, cysteine or sulphide as sulphur sources and mineral salts, such as the sodium salts. At low partial pressures of hydrogen the formation of organic compounds such as acetate, CO_2 and H_2 is thermodynamically favoured. Hydrogen producing acetogenic bacteria catabolize propionate and other organic acids larger than acetate, alcohols and certain aromatic compounds into acetate and CO_2. It has been shown that lactate is completely degraded to CH_4 and CO_2 by the syntrophic association of *Desulfovibrio* spp. and *Methanosarcina barkeri* in the absence of sulphate (Novaes, 1986).

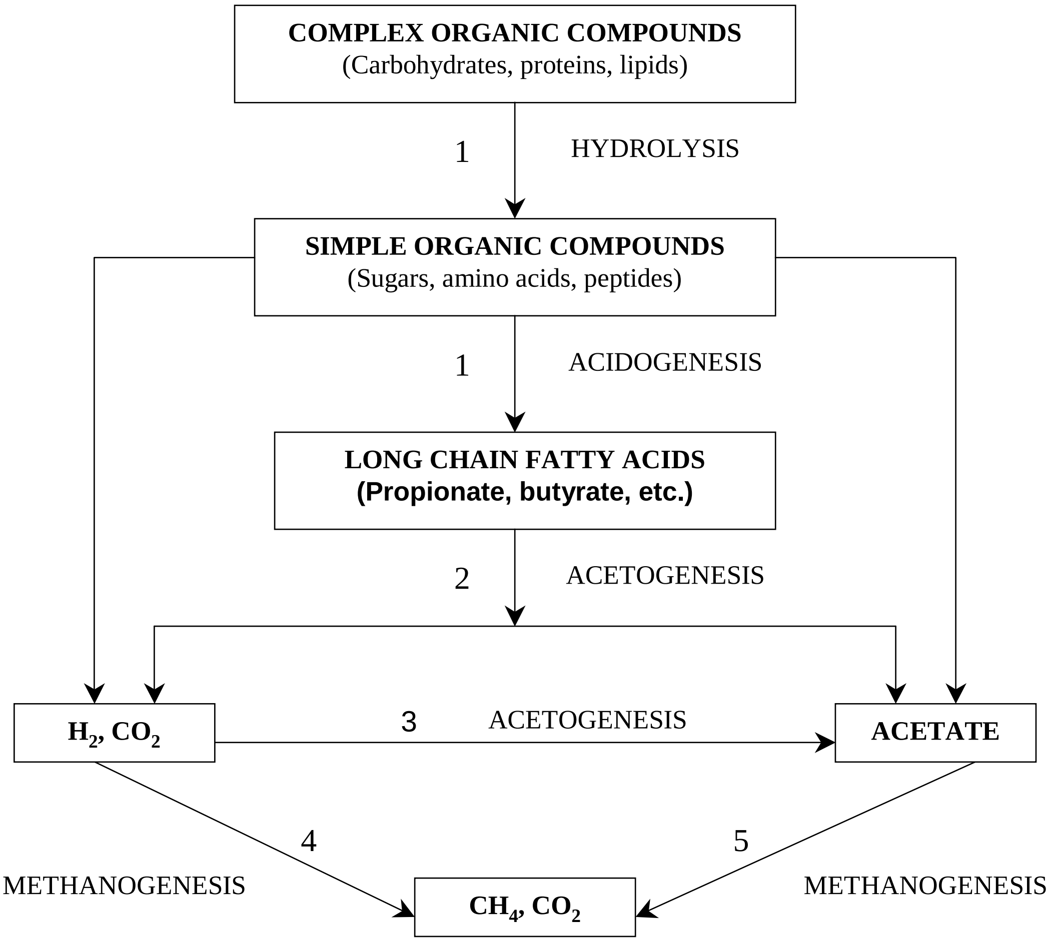


Figure 1.7: Metabolic steps and microbial groups involved in anaerobic digestion: 1) Fermentative bacteria; 2) H_2-producing acetogenic bacteria; 3) H_2-consuming acetogenic or homoacetogenic bacteria; 4) CO_2-reducing methanogenic bacteria; 5) Acetoclastic methanogenic bacteria (Novaes, 1986).

Lignin has been found to be degraded in the highly alkaline gastrointestinal tracts of higher termites. It was demonstrated that lignin-related aromatic substances were degraded in anoxic sediments of natural aquatic ecosystems. These included molecules containing β-aryl ether bonds, in fact the presence of the inter-unit ether linkage did not limit the biodegradation of the molecule. Analysis of fungal decay of lignin suggests that the molecule is degraded by highly oxidative biologically mediated polymer erosion rather than catabolic degradation by enzymes. In light of this, lignin appears to be recalcitrant in anoxic environments because of the lack of an oxidant species required for its depolymerisation (Zeikus *et al.*, 1982).

Under the appropriate conditions it has been shown that gaseous sulphur dioxide can disrupt the lignin-carbohydrate association without selective removal of either constituent. In addition, lignin can be depolymerised during sulphur dioxide treatment and converted to soluble products (Millet & Baker, 1975). The results of Cinq-Mars and Howell (1977) suggested that the potentially rate-limiting steps in the conversion of cellulose to methane by anaerobic digestion might be alleviated by:

- Conversion of cellulose to soluble reducing sugars prior to digestion.
- Availability of reducing sugars to allow more rapid generation of microbial populations.
- More rapid transfer of dissolved products from liquid to gas phase.

Degradation of cellulose may proceed in three stages;
- Initial colonisation.
- Initial degradation by the extracellular enzymes of the primary colonisers, resulting in a loss in tensile strength.
- Proliferation of primary colonisers and other secondary colonisers, resulting in increased enzyme activity and complete degradation (Chew *et al.*, 2001).

The complete degradation of polysaccharides requires a large number of hydrolytic enzymes, and it has been shown that the enzymes produced by microbes are specific for the different linkages within the molecule. For the degradative products not to be released into solution the polysaccharide would have to be at the cell surface, while a suite of hydrolytic enzymes cleaved the molecule into small enough fragments to be transported across the membrane without any loss of intermediates (Haldane and Logan, 1994). Cellulolytic microbes in general are unable to utilise proteins or lipids as energy sources for growth, and primarily degrade carbohydrates. Anaerobic bacteria are even more specialised and grow only on cellulose or its hydrolysis products. They generally do not grow on soluble carbohydrates based on sugars other than glucose. This specialization probably results from the production of the specialised systems for cellulose hydrolysis, and the significant metabolic input devoted to the synthesis of these enzymes. Other nutrients required for growth of cellulolytic bacteria include nitrogen, phosphorus, sulphur and macro- and micro-minerals (Lynd *et al.*, 2002).

Anaerobic microorganisms produce acetic acid and carbon dioxide from the fermentation of hydrolysis products from the cleavage of cellulose, but individual species vary with respect to the reduced products formed. *Clostridia* species and *Ruminococcus albus* produce ethanol and H_2 as the major end products, with acetyl CoA as a branch point. Lactate, however, is not a major fermentation end product of cellulolytic bacteria (Lynd *et al.*, 2002). Anaerobic digestion normally results in high concentrations of fermentation acids, leading to low pHs. Clostridia are generally restricted to near neutral pHs. It has, however, been found that a low pH decreases cell density but not cellulose degradation (results were for continuous and batch culture) (Desvaux *et al.*, 2001).

1.3.8 Cellulolytic enzymes

A distinct difference between aerobic and anaerobic microorganisms is the strategy employed to utilise cellulose. While aerobic microorganisms produce large amounts of individual enzymes that have a strong synergy in the hydrolysis of cellulose, the majority of anaerobes use a multi-enzyme complex, typified by the cellulosome of C*lostridia thermocellum* (Béguin and Aubert, 1994, Lynd *et al.*, 2002). Table 3 shows the morphological features of celllulolytic bacteria.

The crystalline arrangement of the cellulose molecule is of great importance in its hydrolysis. It is a homogenous structure, with the component microfibrils packed so tightly that not even water can penetrate it. This would be a potential rate-limiting step to its enzymatic cleavage, except that the cellulose fibre itself displays heterogeneity. Cellulose has amorphous (less crystalline), crystalline and other irregularities that allow for partial hydration when placed in an aqueous medium. Some of these irregularities are large enough to allow for penetration by cellulolytic enzymes and anaerobic bacteria have developed strategies for enhancing this opportunity (Leschine, 1995; Lynd *et al.*, 2002).

Table 1.3: Morphological features of cellulolytic bacteria (Lynd *et al.*, 2002)

Genus	Representative Species[a]	Gram reaction	Morphology	Growth T°[b]	Resting state	Motility	Features of cellulases
Acetovibrio	*D. cellulolyticus*	-	Curved rod	Meso	None	Nonmotile	Complexed
Anearocellum	*D. thermophilum*	+	Rod	Thermo	None	Flagellar	Noncomplexed cell free
Butyrivibrio	*B. fibrisolvens*	+	Curved rod	Meso	None	Flagellar	Noncomplexed
Caldicellulosiruptor	*C. saccharolyticum*	-	Rod	Thermo	None	Flagellar	Noncomplexed Cell free
Clostridium	*C. thermocellum, C. cellulolyticum*	+	Rod	Thermo, meso	Endospore	Flagellar	Complexed Mostly cell bound
Eubacterium	*E. cellulosolvens*	+	Rod	Meso	None	Nonmotile	
Fervidobacterium	*F. islandicum*	-	Rod	Thermo	None	Flagellar	
Fibrobacter	*F. succinogenes*	-	Rod	Meso	None	Nonmotile	Complexed Cell bound
Halocella	*H. cellulolytica*	-	Rod	Meso	None	Flagellar	Noncomplexed Cell free
Ruminococcus	*R. albus, R. flavefaciens*	+	Coccus	Meso	None	Nonmotile	Complexed Cell bound
Spirochaeta	*S. thermophila*	+	Spiral	Thermo	None		Noncomplexed Cell free
Thermotoga	*T. neapolitana*	-	Rod	Thermo			

[a] Not all the indicated species are cellulolytic.

[b] Meso = mesophilic, Thermo = thermophilic

Although the mechanism of cellulase catalysis by anaerobic bacteria is poorly defined, and is fundamentally different to that of most aerobic fungal and bacterial systems, the fungal system is the best studied and may, nevertheless, be used as a model to understand how anaerobic cellulases might work. The three main activities associated with fungal cellulase systems are: endoglucanases, which hydrolyse β-1,4-bonds randomly within the cellulose molecule, producing reducing and non-reducing ends; exoglucanases, which cleave cellobiose from the non-reducing ends of the polymer and β-glucosidases, which hydrolyse cellobiose producing glucose monomers. All these components act synergistically in the hydrolysis of crystalline cellulose. This synergism has been explained by the attack of endoglucanases on amorphous cellulose, forming sites for exoglucanases to hydrolyse cellobiose units from the more crystalline cellulose. Then the β-glucosidases prevent the accumulation of cellobiose by hydrolysing it, as exoglucanase is inhibited by an accumulation of cellobiose (Figure 1.8) (Leschine, 1995).

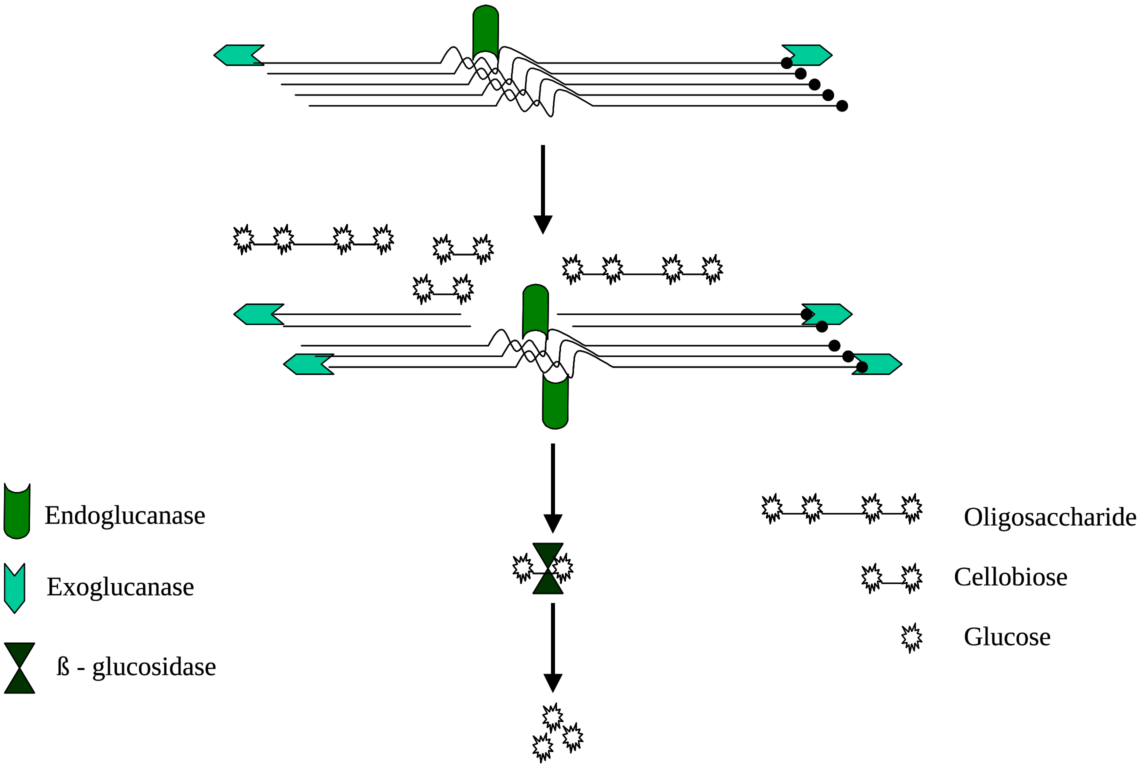


Figure 1.8: Schematic representation of the synergistic interactions leading to hydrolysis of cellulose by non-complexed cellulase systems typical of aerobic microorganisms. The straight lines with circles at the ends represent the cellulose molecule (Adapted from Lynd *et al.*, 2002).

Four different forms of synergy have been noted:

1. Endo-exo synergy, between exoglucanases and endoglucanases.
2. Exo-exo synergy, between exoglucanases from both ends of cellulose.
3. Synergy between β-glucosidases and exoglucanases to remove cellobiose.
4. Intramolecular synergy between the cellulose-binding modules CBMs and the catalytic domains (Lynd *et al.*, 2002).

Another feature of cellulase enzymes is the modular structure of the enzyme that includes both the catalytic and CBMs. The CBM is responsible for binding to the surface of cellulose, possibly to allow for hydrolysis by bringing the catalytic domain close to the substrate. The CBM is of particular importance for the exoglucanases, especially for the initiation as well as the process of hydrolysis.

1.3.9 The cellulosome

Anaerobic microbes have had to develop alternative mechanisms of cellulose attack, because, unlike the fungi, they do not possess penetrative hyphae. One of these is the cellulosome, which is a stable multi-enzyme complex that attaches to the substrate by means of a dockerin domain (Lynd *et al.*, 2002). Lynd *et al*. (2002) speculated that because anaerobic microorganisms have to conserve energy, and have only limited energy available for cellulose hydrolysis, this may have led to the evolution of the cellulosome structure, which brings the bacterium close to the substrate (Figure 1.9). This close proximity in turn allows for efficient enzymatic depolymerisation and uptake of hydrolysis products. Evidence for cellulosomes has been confirmed in other non-clostridial anaerobic bacteria, anaerobic fungi and rumen bacteria (Table 4) (Bayer *et al.*, 1998).

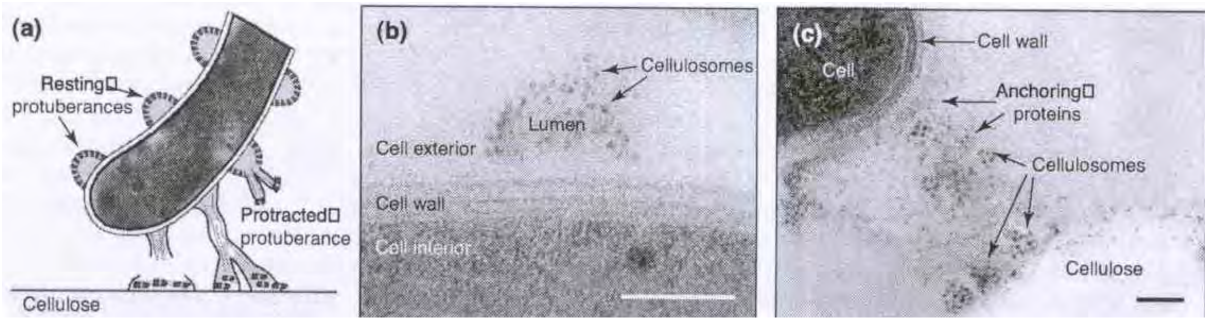


Figure 1.9: Ultrastructure of cell surface of *Clostridium thermocellum*. A) Diagrammatic representation of a bacterium bound to cellulose. B) TEM of a polycellulosomal protuberance. C) TEM of an extended polycellulosomal protrusion. The cellulosome is associated with cellulose and is connected to the bacterium through an extended fibrous material, believed to be comprised of anchoring proteins. Scale bars = 100 nm (Shoham *et al.*, 1999).

The cellulosome is a protuberance that is found on the cell wall of bacteria growing on cellulose. It is a stable multi-enzyme complex and has been studied in detail in different clostridia and *Ruminococcus* species (Lynd *et al.*, 2002, Shwarz, 2001). Although the architecture is similar the composition of the various components differs between different species. The cellulosome structure was elucidated through a combination of biochemical, ultra-structural, immunochemical and molecular biology techniques (Figure 1.10). It consists of a noncatalytic scaffoldin protein (CipA) that includes nine cohesins, four hydrophilic modules (X-modules) and a family III cellulose-binding module (CBM). There are 22 catalytic modules that associate with the cohesins of CipA through dockerin moieties to form the cellulosome. Nine of these modules exhibit endoglucanase activity (CelA, CelB, CelD, CelE, CelF, CelG, CelH, CelN and CelP), four have exoglucanase activity (CbhA, CelK, CelO and CelS), five have hemicellulase activity (XynA, XynB, XynV, XynY and XynZ), one shows chitinase activity (ManA) and there is one with lichenase activity (LicB) (Table 5). The assembly of the cellulosome, including composition, is still poorly understood. However, CelS, a processive exoglucanase, is always present. It has an affinity for microcrystalline or amorphous cellulose (not CMC, though) and produces cellobiose as its major hydrolysis product and cellotriose as the minor. Cellobiose is a natural strong inhibitor of CelS. The major endoglucanase associated with cellulosome is CelA. Cellulosomes are large complexes varying in size from 2-16 MDa and are very stable molecules. They are extensively glycosylated (6-13 % carbohydrate content). Glycosylation is thought to protect the protein complex against protease attack (Lynd *et al.*, 2002). Both genetic and biochemical evidence has revealed that the components of the cellulosome are very strongly bound. This interaction is mediated by a non-catalytic, duplicated segment of 22 amino acid residues that is conserved within all enzymes of the cellulosome (Shwarz, 2001). This duplicated segment bears resemblance to the EF-hand motif of calcium binding proteins. Although the structure of this segment has not been elucidated, the homology with the EF-hand motif is suggestive of a similar fold (helix-loop-helix) (Shoham *et al.*, 1999).

Table 1.4: Evidence for cellulosomes in cellulolytic microorganisms (Bayer *et al.*, 1998)

Organism	**Cellulosome signature sequence Protein**	**Biochemical evidence**
Anaerobic bacteria		
Clostridium thermocellum	Complete scaffoldin Cell surface anchoring proteins Dockerin – containing enzymes	Isolated cellulosome Immunochemical evidence Ulrastructural evidence Cellulosome – related sugars
C. cellulovorans	Complete scaffoldin Dockerin – containing enzymes	Isolated cellulosome
C. cellulolyticum	Complete scaffoldin Dockerin – containing enzymes	Isolated cellulosome
C. josui	Complete scaffoldin Dockerin – containing enzymes	Isolated cellulosome
C. papyrosolvens	Partial scaffoldin	Isolated cellulosome Ultrastructural evidence
Bacteroides cellulosolvens	Incomplete scaffoldin or Cell – surface anchoring proteins	Isolated cellulosome Immunochemical cross – reactivity Cellulosome – related sugars
Acetivibrio cellulolyticus	Complete scaffoldin Cell – surface anchoring proteins	High molecular weight complexes Immunochemical cross – reactivity
Ruminococus flavefaciens	Dockerin – containing enzymes	High molecular weight complexes
R. albus	Dockerin – containing enzymes	Cellulosome – related sugars
Anaerobic fungi		
Neocallimastix patriciarum	Dockerin – containing enzymes	High molecular weight complexes Blotting experiment
Piromyces	Dockerin – containing enzymes	Blotting experiments
Orpinomyces	Dockerin – containing enzymes	High molecular weight complexes

The advantage of the cellulosome is that when one structural type of cellulose is depleted at the adsorptive site, hydrolysis is not stopped because of the presence in the complex of other enzymes with different specificities (Shwarz, 2001).

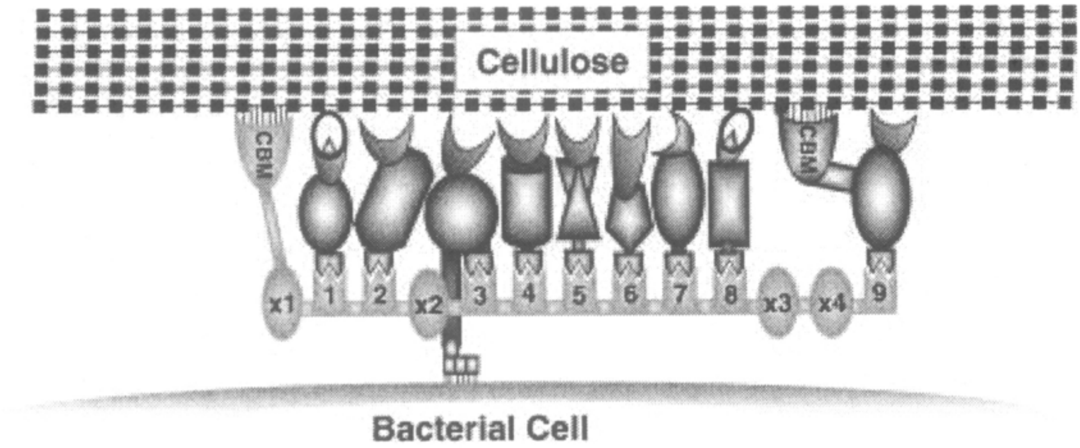


Figure 1.10: Schematic representation of cellulosome. Light gray objects = scaffoldin, dark gray objects = enzyme components, open bowls = endoglucanases, ellipsoids = exoglucanases, CBM = carbohydrate-binding module, x1-x4 = X-modules/hydrophilic module and 1-9 = cohesions (Schwarz, 2001).

It is interesting to note that *C. thermocellum* can only utilise cellulose despite the wealth of non-cellulolytic enzymes available within the complex. These include xylanase subunits containing carbohydrate esterases that are capable of hydrolysing acetyl or feruloyl groups from hemicellulose. It follows that this abundance of non-cellulolytic enzymes can allow for the removal or detachment of plant cell polymers (lignin and hemicellulose) from cellulose (Shoham *et al.*, 1999).

Table 1.5: Cellulosomal components of *C. thermocellum*. Localisation determined in the cellulosome is *in italics*. CBH (cellobiohydrolase), NR / R (processively active from the non–reducing / reducing end) (Adapted from Shwarz, 2001).

Cellulosomal components	Molecular mass (kDa)	Function
CipA[a]	197	Scaffoldin
CelJ	178	Cellulase
CbhA	138	CBH (NR)
XynY	120 111	Xylanase Endoglucanase
CelH	102	Endoglucanase
CelK[a]	101	CBH (NR)
XynZ	92	Xylanase
CelE	90	Endoglucanase
CelS[a]	83	Exoglucanase (R)
CelF	82	Endoglucanase
CelN	82	Endoglucanase
CelO	75	CBH (R)
XynA, XynU	74	Xylanase
CelD	72	Endoglucanase
XynC	70	Xylanase
ManA	67	Mannanase
CelB	64	Endoglucanase
CelG	63	Endoglucanase
CelP	62 58	Unknown Endoglucanase
ChiA	55	Chitinase
CelA[a]	53	Endoglucanase
XynB, XynV	50 47	Xylanase Mannanase
LicB	38	Lichenase

[a] Major components in the cellulosome

1.3.9.1 Other enzymatic activities associated with the cellulosome

Arabinoxylan is one of major hemicellulose molecules; within its backbone is found arabinose. Arabinose can bond to ferulic acid and *p*-coumaric acid through ester linkages. Feruloyl esterases hydrolyse the ester linkage between the arabinose and ferulic acid, and may release lignin from hemicellulose, thus aiding in the degradation of plant cell walls. These esterases have been found in both bacteria and fungi. Blum *et al*. (2000)

demonstrated that two domains of the cellulosome are bifunctional, having both xylanase and feruloyl esterase activities.

1.3.9.2 Regulation of cellulolytic enzymes

In fungi such as *Trichoderma reesei* the production of enzymes for the hydrolysis of complex substrates are induced in the presence of the substrate, but is suppressed in the presence of the breakdown products such as glucose. In anaerobic systems, similar to that for *C. thermocellum*, formation of the cellulosome occurs under carbon-limiting conditions (Lynd *et al.*, 2002). There are conflicting reports as to whether induction is important. It is presumed that cellulolytic production is down-regulated through catabolite repression. It has also been observed that the composition of the complex may be influenced by the carbon source used. When grown on cellulose, CelS is the prominent exoglucanase formed but not when grown on cellobiose. *C. cellulovorans* produces cellulosome on cellulose, but not on CMC or soluble sugars. The organism does, however, display high cellulase activity and transcription of cellulase genes when grown on CMC or cellobiose. These results suggest that cellulolytic enzymes may be produced on soluble substrates, but that assembly of the cellulosome requires some sort of ‘triggering’ mechanism from the presence of insoluble cellulose (Lynd *et al.*, 2002).

It is known that the various components of the cellulosome are secreted outside the bacterium and possess leader peptides that are cleaved during export. The entire complex is then assembled outside the cell (Shoham *et al.*, 1999). The genes encoding the enzymes are found on the chromosome for both bacteria and fungi. For the fungi the genes are randomly distributed over the genome, with each one having its own transcription regulatory elements. Within the bacteria the cellulase genes have been found clustered or randomly distributed on the genome (Lynd *et al.*, 2002). The expression of certain cellulosomal genes of *C. thermocellum* appears to be constitutive and do not involve an induction mechanism (Shoham *et al.*, 1999).

1.3.10 *Anaerobic hydrolysis under sulphidogenic conditions*

Anaerobic microorganisms possessing the cellulosome are normally found existing in consortia with other bacteria. Complex communities of interacting microorganisms, mediate the decomposition of cellulose in decaying plants. The hydrolysis products of cellulose are available as carbon and energy sources for other microorganisms that coexist with cellulolytic microbes (Figure 1.11). In anaerobic environments with inorganic electron acceptors (NO_3, Fe (III), and SO_4) cellulose is degraded to form CH_4, CO_2, and H_2O. From figure 1.11 it can be seen that the fermentative bacteria produce CO_2, H_2, organic acids (acetate, propionate and butyrate) and alcohols. The H_2 is immediately consumed by the methanogens or homoacetogens. The syntrophic bacteria ferment the fatty acids (propionate, butyrate) or alcohols to produce acetate, CO_2 and H_2. Fermentation of fatty acids is normally the rate-limiting step in the anaerobic degradation of cellulose because of the slow growth of the syntrophic bacteria. Within the marine environment SO_4 is in large supply and SRP are able to out-compete methanogenic bacteria for H_2. There is thus a large production of H_2S as a product of SO_4 reduction, during anaerobic degradation of cellulose in marine ecosystems (Leschine, 1995).

Khan and Trottier (1978) found that the removal of sulphate depressed cellulose degradation by more than 80 % and reduced methane formation from cellulose. In addition, they found that at high concentrations of sulphate, the extent of inhibition of cellulose degradation and methanogenesis depended on the nature of the inorganic sulphur. At 9 mM the inhibitory effect on cellulose degradation increased in the following order: sulphate < thiosulphate < sulphite < sulphide. In a study comparing hydrolysis of lignocellulose (in the form of paper and newspaper) under methanogenic and sulphidogenic conditions, it was determined that hydrolysis under sulphidogenic conditions was faster than under methanogenic conditions (Pareek *et al.*, 1998). Pareek *et al.* (2001) showed that a model compound containing the β-*O*-4 linkage found in the lignin polymer could be cleaved under sulphidogenic conditions. In the presence of an additional carbon source such as Avicel the degradation of the model compound was complete in 9 days less than if the compound was degraded on its own. This result provides evidence for the degradation of lignin under sulphidogenic conditions.

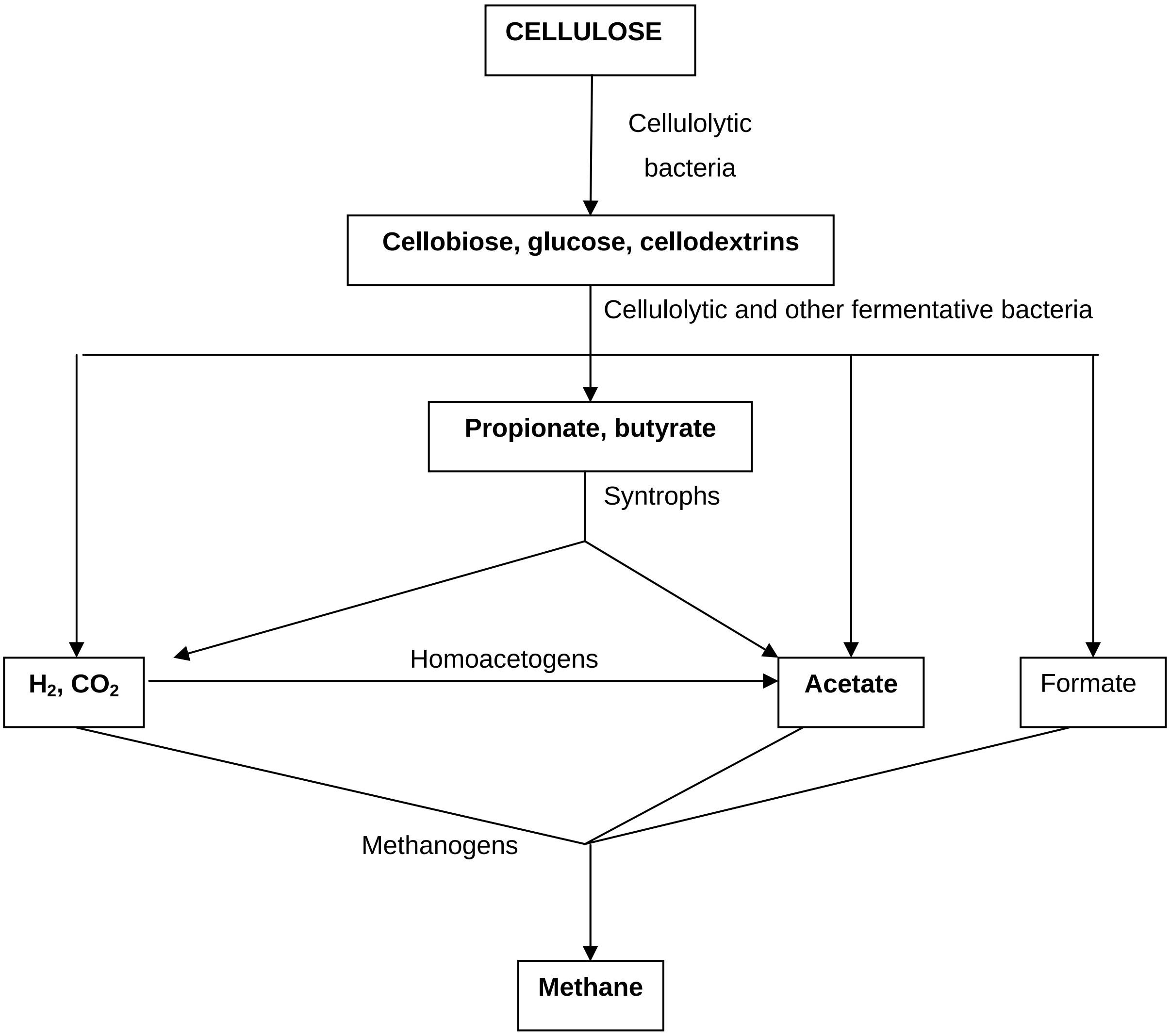


Figure 1.11: Schematic representation of anaerobic cellulose degradation by microbial communities. In environments where nitrate, Mn (IV), Fe (III) or sulphate is present the final products of fermentation may differ (Leschine, 1995).

1.4 Lignocellulose in AMD Passive Treatment

As noted previously, lignocellulosic materials have been investigated extensively as a potential carbon source for sulphate reduction and AMD treatment (Wakao *et al.*, 1979; Eger and Lapakko, 1988; Dvorak *et al.*, 1992; Bechard *et al.*, 1994; Pareek *et al.*, 1998; Chang *et al.*, 2000). These applications have, however, largely remained at bench-scale or as small experimental units. Full-scale development and rollout of the technology has not occurred to date. Lignocellulose packed reactor's performance is observed to deteriorate rapidly after a period of time of several months, and settles at a level, which is generally inappropriate for the long term treatment that is required for sulphate reduction (Pulles *et al.*, 2001).

A suggested reason for this observed sulphate reduction profile is that the soluble and readily extractable organic materials have been exhausted, leaving the more refractory lignocellulose complex relatively unaffected (Pulles *et al.*, 2001). Chang *et al.* (2000) have suggested that resinous compounds within woody tissue could be responsible for the observed profile of sulphate reduction. It is suggested that these resins inhibit the bacterial metabolism.

Given the growing need for efficient low-cost passive treatment technology, as increasing numbers of mines in South Africa face closure, a multi-disciplinary research initiative was supported by the South African Department of Arts, Culture, Science and Technology (DACST). The objective of the programme was to investigate the question, among other matters, constraining the full-scale application of the technology. PHD and researchers in the passive treatment field were appointed to lead the consortium of researchers. The Environmental Biotechnology Group (EBG) at Rhodes University (now known as the Environmental Biotechnology Research Unit), as members of the consortium, undertook to investigate processes underpinning the degradation of lignocellulose in biosulphidogenic environments, and to focus specifically on the potential application of findings to the problem of premature collapse of lignocellulose packed bed reactor systems.

1.5 Research hypothesis

The investigation of the problem was undertaken by a number of EBG researchers who collaborated on several related aspects of the issues involved. This research initiative was predicated by the following research hypothesis:

1. Readily extractable soluble compounds present in woody tissue are responsible for the greater part of sulphate reduction observed in the early stages of biosulphidogenic degradation;
2. Nevertheless, the core lignocellulose component of woody tissue itself may be degraded by sulphate reducing anaerobic microbial consortia;
3. The products used as an electron donor and carbon source in dissimilatory sulphate reduction;
4. The cleavage of the core lignocellulose structure is driven by both inorganic chemical reactions and biochemical effects, generated by the microbial consortium.

1.5.1 Research objectives

In order to address the various aspects of biosulphidogenic hydrolysis of lignocellulose, the following research objectives were identified for the study reported here,

- To determine whether readily extractable compounds in woody tissue are utilized in sulphate reduction;
- To investigate the degradation of the lignocellulose complex under chemical and biologically-derived sulphidogenic conditions;
- To determine whether sulphate reducing bacterial consortia are capable of degrading the lignin component of the complex;
- To investigate cellulase activity within the sulphate reducing microbial consortium;
- To derive a descriptive model explaining the degradation of lignocellulose under biosulphidogenic conditions and to consider application of the findings to the development of lignocellulose-based passive treatment systems for remediation of AMD.

Chapter 2

Degradation of lignocellulose in a chemically-generated sulphidic environment

2.1 Introduction

In ongoing research in the conversion of lignocellulose to valuable products, such as ethanol, the lignin component of the lignocellulose complex has been recognised as one of the primary rate-limiting steps in its degradation (Zeikus *et al.*, 1982, Breen and Singleton, 1999). Lignin has been considered to be recalcitrant to anaerobic biodegradation, and this has been related to the incomplete degradation of wood tissue within these environments, resulting in the formation of peat and coal deposits in anoxic sediments. Lignin, thus, provides a barrier to the utilisation of the cellulose component of the lignocellulose complex, and to overcome this limitation on the availability of cellulose in a range of process applications, various pre-treatments have been used. These include acid hydrolysis, steam treatment, use of dilute solutions of alkali and hydrogen peroxide (Zeikus, 1982; Benner *at al.*, 1984; Curreli *et al.*, 1997).

A study by Benner *et al.* (1985), demonstrated that acidic conditions lower the degradation rate of lignocellulosic compounds compared to more neutral environments. A separate study by Kim *et al.* (1997) showed that the bio-degradation of organic matter under sulphidogenic conditions was superior to methanogenic conditions. In this particular study the rates of Avicel, filter paper and unprinted newspaper degradation were investigated, with both the rate and percentage solubilization of the material being higher under sulphidogenic conditions (Kim *et al.*, 1997). Pareek *et al.* (1998) compared lignocellulose degradation under methanogenic and sulphidogenic conditions and found that hydrolysis rates under sulphidogenic conditions were more rapid than for methanogenic conditions.

The effects of pH and sulphide treatment of pine-wood powder have been reported for the enhanced release of aromatic compounds (Madikane, 2002). Whittington-Jones (2000) had demonstrated that the release of aromatic compounds including the hydrolysis of complex carbohydrates is enhanced during biological sulphate reduction.

What was not clear from these studies was whether the mild alkaline sulphidic conditions generated by sulphate reducing consortia are active in the lignocellulose degradation process alone, or whether additional biological effects are involved. The abiotic studies reported here were undertaken to investigate the influence, on the lignocellulose complex, of the non-biological components of the bio-sulphidogenic process, where wood tissue forms the electron donor and carbon source. Questions that arose from these studies include:

- Does the alkaline sulphidic environment enhance the degradation of the woody tissue?
- Does this environment act only to enhance the release of soluble extractives, or does it also enhance degradation of the lignocellulose complex?
- Do biologically generated alkaline sulphidic conditions produce similar effects in wood tissue?
- Does the biologically generated alkaline sulphidic environment in any way provide an additional effect in woody tissue generally and on the lignocellulose complex in particular?

2.2 Objective

The objective of the study reported here was to determine whether the sulphide and alkaline conditions, prevalent in biosulphidogenic systems, has any effect on the release of the constituents of the wood powder in the absence of microbiologically induced sulphidogenesis, at the standard conditions encountered within sulphidogenic bioreactors. The response of both whole wood powder and various extractions of wood powder were investigated to determine on which components of wood tissue the sulphidogenic effects might be active.

2.3 Methods

2.3.1 Preparation of wood material

Dried untreated *Pinus* sp. sawdust was collected from a sawmill in Grahamstown, passed through a 1 000 µm sieve, and wood extracts were prepared as outlined in figure 2.1.

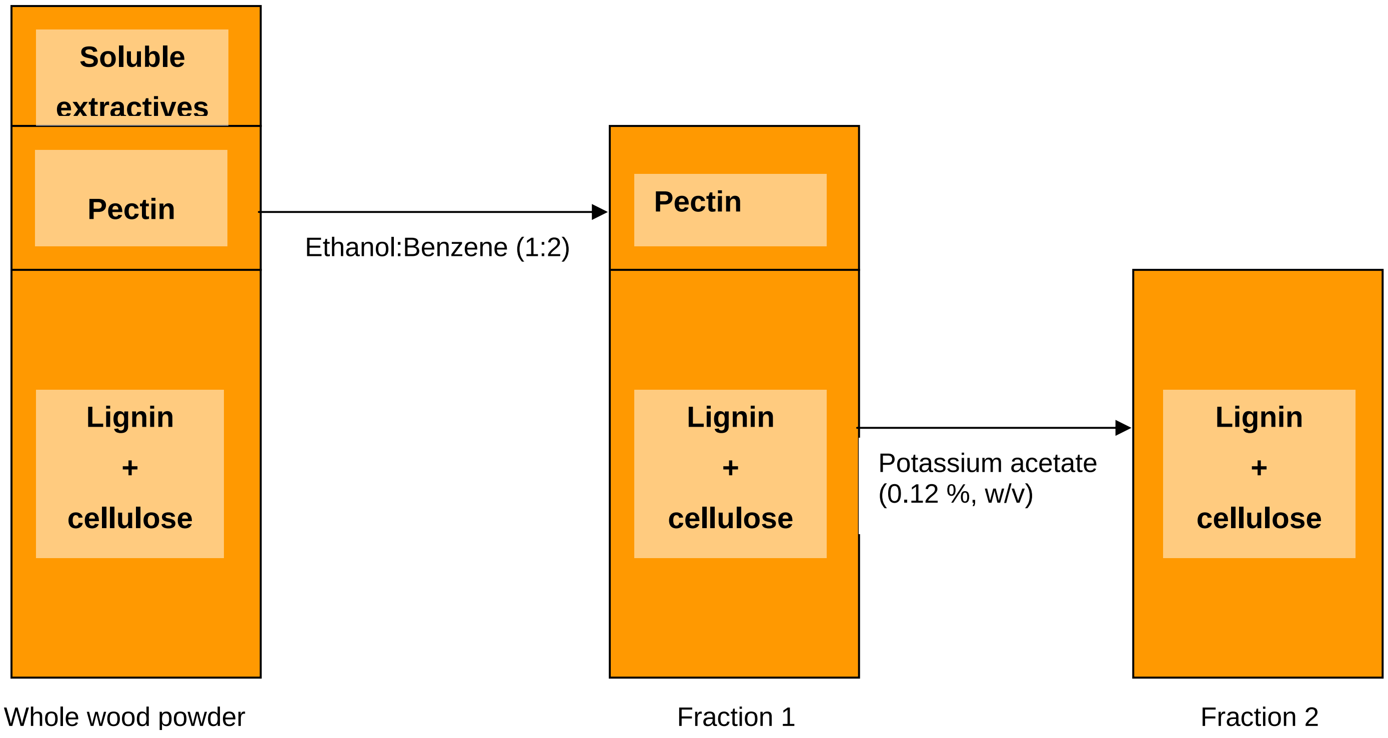


Figure 2.1: Procedures for the preparation of extracts of *Pinus* sp wood powder used in this study. Soluble extractives were removed with ethanol:benzene to give fraction 1, pectin was removed using potassium acetate to give fraction 2.

To remove the easily extractable material from the dried wood powder, it was subjected to extraction in ethanol:benzene (1:2, v/v) for 48 hours with continual stirring. Residual solids were then filtered on a Buchner funnel through Whatman No. 1 filter paper, the residue was washed with benzene followed by ethanol to remove any remaining soluble organic compounds. The treated material was then dried at room temperature (RT), and this material was termed fraction 1.

Depectinated wood powder was produced by treating the fraction 1 with potassium acetate (0.12 %, w/v) for 24 hours at 60° C with continual stirring. The residue was then filtered as before and allowed to dry at RT. The residue was termed fraction 2, and corresponds to the lignocellulose complex, the core structure of wood tissue.

2.3.2 Experimental set-up

Degradation studies were performed in triplicate in 250 ml Erlenmeyer flasks for each of the extracts. Each flask contained 5 % (w/v) of the various wood powder preparations; with flask 1 containing tap water with pH adjustment to 8.5 with 1M NaOH and flask 2 contained tap water with addition Na_2S to final concentration of 500 mg/l as sulphide (Merck). This concentration of sulphide was chosen to determine the gross effect of sulphide on the wood material. All flasks were sparged with nitrogen and sealed to maintain an anaerobic atmosphere. The flasks were placed on an orbital shaker (100 rpm) (Labcon) at 25° C for the period of the study.

In order to evaluate the effects of sulphide and an alkaline environment on the degradation process, where pH adjustment was not performed on a daily basis, a second study was performed on whole wood powder alone using 0.2 M phosphate buffer at pH 8. All the liquid in the experimental flasks was removed and replaced with fresh buffered solution every 7 days to maintain sulphide levels at 300mg/l and to prevent an accumulation of the end products of lignocellulose breakdown. Flasks were incubated at 30 °C for comparison with biotic studies, where the optimum temperature for SRB growth was applied. The same amount of wood powder was used as before, but the experiments were carried out in 500 ml buffer in 1 litre Erlenmeyer flasks. The flasks were incubated on an orbital shaker at 100 rpm. Flask 1 contained 0.2 M phosphate buffer (pH 8), without adjustment after the addition of the wood powder. Flask 2 contained 0.2 M phosphate buffer (pH 8), with adjustment of the pH with 1M NaOH after addition of the wood powder. Flask 3 contained 0.2 M phosphate buffer (pH 8) containing Na_2S in a final concentration of 300 mg/l as sulphide (Merck). All flasks were sparged with nitrogen gas to maintain anaerobic conditions.

The phosphate buffer was made up as follows:

- $NaH_2PO_4.2H_2O$ (31 g) (Merck) in 1 litre of distilled water (d.H_2O) (Solution A)
- Na_2HPO_4 (28.39 g) (Merck) in 1 litre of d.H_2O (Solution B)
- To produce the required buffer 2.6 ml solution A was added to 47.3 ml solution B and made up to 100 ml.

2.3.3 Measurement of reducing sugars

Reducing sugars released from the wood tissue were measured using the method adapted from Wood and Bhat (1988). The sample (100 μl) of was added to 150 μl dinitrosalicyclic acid (DNS) reagent (see below) and heated at 100 °C for 10 min. Distilled water (1 ml) was added to the reaction vessel and the resulting solution's absorbance was read at 540 nm on a Beckman DU® 530 Life Science UV/Vis spectrophotometer. Absorbance values after subtraction of the reagent blank were then translated into glucose equivalents using a glucose standard curve.

The DNS reagent was made up as follows (Wood and Bhat, 1988):

Dinitrosalicyclic acid (Sigma)	10 g
Phenol (Sigma)	2 g
Sodium sulphite (Sigma)	0.5 g
Rochelle salt (Merck)	200 g

Components were dissolved in 500 ml of a 2 % (w/v) NaOH (Merck) solution, and once dissolved, diluted to 1 000 ml with d.H_2O.

2.3.4 Measurement of aromatic compounds

2.3.4.1 The Box method

The release of aromatic compounds was followed by measuring total phenol in the reacted media with an adaptation of the method by Box (1983), in which Folin-Ciocalteau reagent (Merck), and colour change occurring in the presence of reducing substances, may be monitored at 750 nm (Beckman DU® 530 Life Science UV/Vis). The colour development is due to the transfer of electrons at alkaline pH values that reduce the phosphomolybdic/phosphotungstic acid complexes to form chromogens that can be detected spectrophotometrically. Ferulic acid (Sigma) as a monomer of lignin, was used to monitor the appearance of lignin-derived (or wood-derived) aromatic compounds. A standard curve was constructed using ferulic acid.

Prior to analysis sulphide was removed from solution to prevent interference by acidification to pH <5, then sparged with nitrogen gas for 10 min (Box, 1983).

2.3.4.2 Reverse-phase high performance liquid chromatography

A Beckman System Gold HPLC, module 126, with detection by a Beckman Photo-diode array (PDA), module 168 was used together with a Waters Symmetry C_{18} column (4.6 x 250 mm) with 5-µm particle size. The mobile phase was composed of acetonitrile (HPLC-grade, Merck) and water in the ratio 60:40 with the water containing 0.1 % acetic acid (HPLC-grade, Sigma). Naphthalene (Sigma) was used as the internal standard. The flow rate was set at 1 ml/min.

2.3.5 Statistics

Statistics was performed using MSExcel. The significance of the results obtained was determined using a student’s t-test, assuming unequal variances.

2.3.6 Definition of abiotic

For the purpose of this study, abiotic was defined as having no known living organisms in the flasks. Aerobic organisms were excluded by maintaining an anaerobic atmosphere (sparging with nitrogen). SRP and methanogen producing bacteria (MPB) were excluded by having a concentration of sulphide (500 mg/l) present that was toxic to the organisms (Hansford, 2004). Any fluctuations observed in reducing sugar levels may be attributed to the non-sterility of the whole wood powder, the reason for this is because autoclaving would lead to ultra-structural changes to the wood tissue, and there may thus be residual microbial activity consuming released carbohydrates.

2.4 Results

2.4.1 Sulphide-induced release of reducing sugars and aromatic compounds in an unbuffered system

2.4.1.1 Whole pine-wood powder

The release of reducing sugars and aromatic compounds from whole pine-wood powder was measured in the presence and absence of sulphide. This experiment was undertaken to investigate the release of the extractive components from the whole wood powder in alkaline sulphidic environment.

2.4.1.1.1 Release of reducing sugars

Figure 2.2 and 2.3 show the release of reducing sugars from whole pine-wood powder. With no sulphide present (Figure 2.2) reducing sugars were not detectable on day 1, by day 4 40.53 mg/l was measured which decreased to 33.49 mg/l by day 8. In the presence of sulphide (Figure 2.3) a rapid increase in reducing sugar content was observed and then followed by a slow decrease, from 258 mg/l on day 0 to 128 mg/l by day 8. There was 6.7 times more reducing sugars released in the presence of 500 mg/l sulphide at pH 8.5, than without. The rate of reducing sugar release without sulphide was highest on day 4, with 1.7 mg/l/h released compared to the highest release by day 3 of 6.1 mg/l/h in the presence of sulphide. These results show that the presence of sulphide significantly enhances the release of reducing sugars from whole pine-wood powder ($t = 7.39$, $p < 0.05$).

The acidic nature of the wood material is evident in the flask containing no sulphide (Figure 2.2). Although the pH was adjusted on a daily basis to 8.5, it can be seen that only by day 8 did the pH start remaining close to 8.5. In contrast the flask containing sulphide (Figure 2.3) maintained a more stable pH (~8.5 ±0.1).

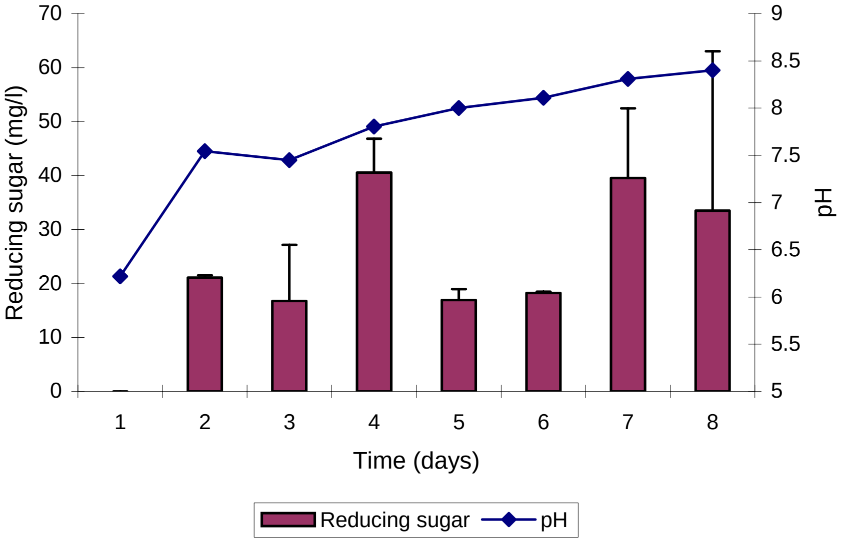


Figure 2.2: Flask study of the release of reducing sugars from whole pine-wood powder without sulphide. Error bars indicate the standard error of the samples analysed in triplicate.

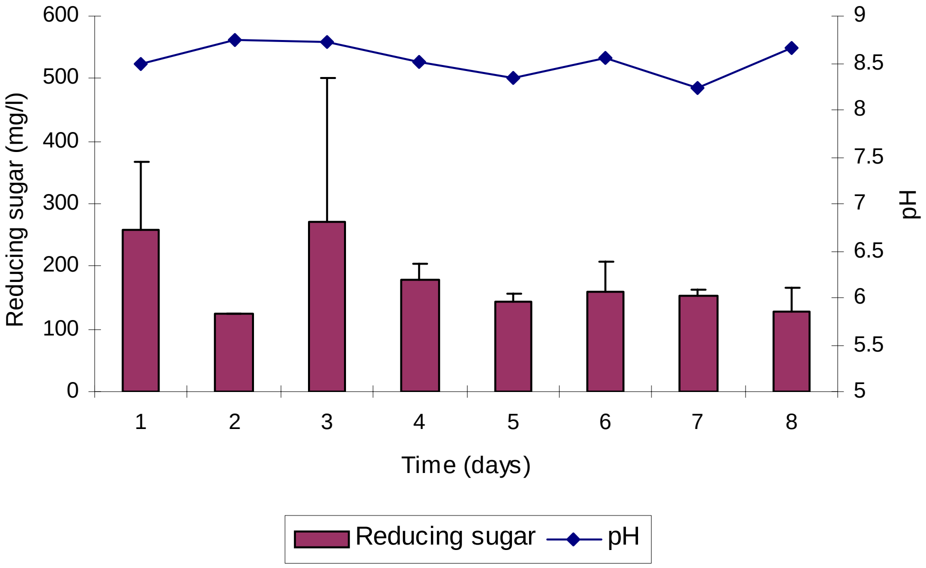


Figure 2.3: Flask study of the release of reducing sugars from whole pine-wood powder in the presence of 500 mg/l sulphide. Error bars indicate the standard error of the samples analysed in triplicate.

2.4.1.1.2 Release of aromatic compounds

The chemically induced release of wood aromatic compounds was studied collaboratively in the EBG laboratories together with M. Madikane, and aspects of those results have been reported as part of his Masters thesis (Madikane, 2002). Figure 2.4 shows the maximal release of aromatic compounds from whole pine-wood powder of 806 mg/l without addition of sulphide. Figure 2.5 shows that the release of wood aromatic compounds in the presence of sulphide is two-fold higher (1570 mg/l). In both cases the release of aromatic compounds increased gradually with time until the release of aromatic compounds start levelling off. When no sulphide is present this is reached on day 14 (796 mg/l). In the presence of sulphide the release reached a maximum by day 18 at 1 568 mg/l (Figure 2.5). With no sulphide present the rate of aromatic compound release increased steadily from 0.7 mg/l/h on day 1 to 8.4 mg/l/h on day 14. In the presence of sulphide the rate of release was much greater, with 3.8 mg/l/h being released on day 1, and increasing to 12.8 mg/l/h on day 16.

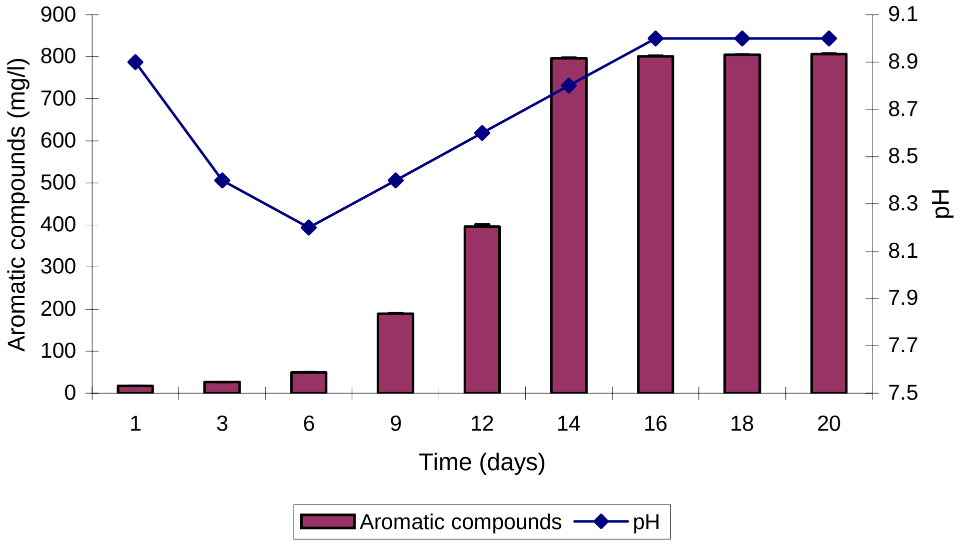


Figure 2.4: Flask study of the release of aromatic compounds from whole pine-wood powder without sulphide. Error bars indicate the standard error of the samples analysed in triplicate.

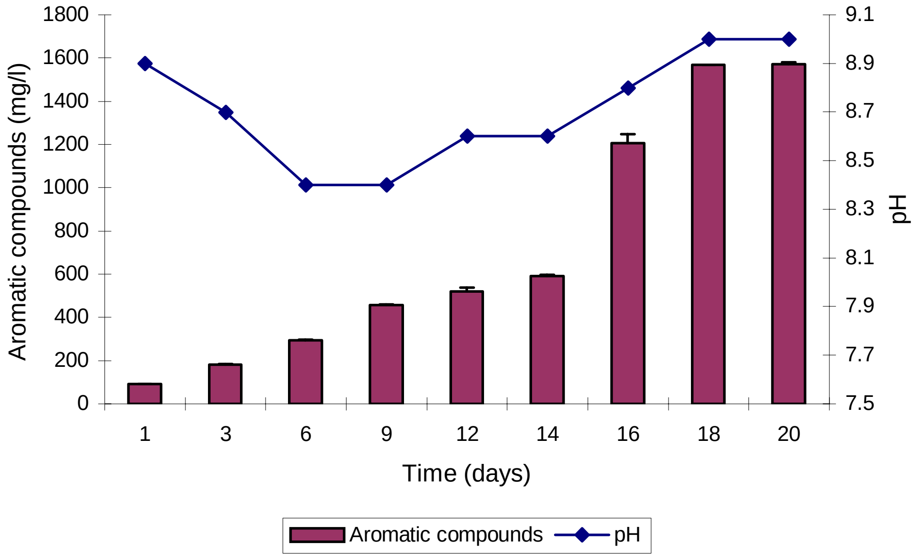


Figure 2.5: Flask study of the release of aromatic compounds from whole pine-wood powder in the presence of 500 mg/l sulphide. Error bars indicate the standard error of the samples analysed in triplicate.

The initial decrease in pH observed in the presence and absence of sulphide can be attributed to the acidic nature of the extractive components in the whole pine-wood powder.

2.4.1.2 Pine-wood extract-Fraction 1

Fraction 1 of the wood powder was prepared to remove the extractive component of the wood material and to investigate whether the release of reducing sugars and aromatic compounds observed in the previous study with untreated wood were derived only from this component of the wood material or possibly also due to the mobilisation of other components.

2.4.1.2.1 Release of reducing sugars

Figure 2.6 and 2.7 show the release of reducing sugars from Fraction 1. With no sulphide present (Figure 2.6) the level of reducing sugars increased from 23.2 mg/l to 28.1 mg/l by day 4, where it remained for the duration of the study (±1.02 mg/l). In comparison to the whole wood powder slightly less reducing sugars were released (10 mg/l less) from Fraction 1. In the presence of sulphide (Figure 2.7) reducing sugar content increased from 71.4 mg/l on day 2 to 94.8 mg/l by day 8. This represents an increase of 3.1 times more reducing sugars released in the presence of 500 mg/l sulphide at pH 8.5, than without.

The rate of reducing sugar release without sulphide was greatest on day 2, with 0.9 mg/l/h released. In the presence of sulphide the greatest release was also on day 2, with 1.9 mg/l/h being released. Compared to whole wood the rates of release of reducing sugars are substantially lower for Fraction 1 (3.2 times lower). However, in this case too the presence of sulphide had a significant effect on the release of reducing sugars from Fraction 1 (t = 5.36, p <0.05).

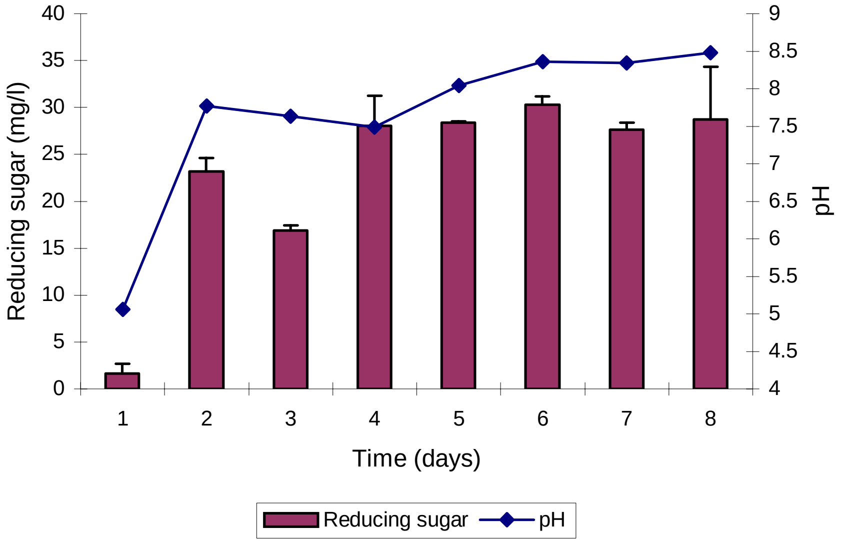


Figure 2.6: Flask study of the release of reducing sugar from Fraction 1 without sulphide. Error bars indicate the standard error of the samples analysed in triplicate.

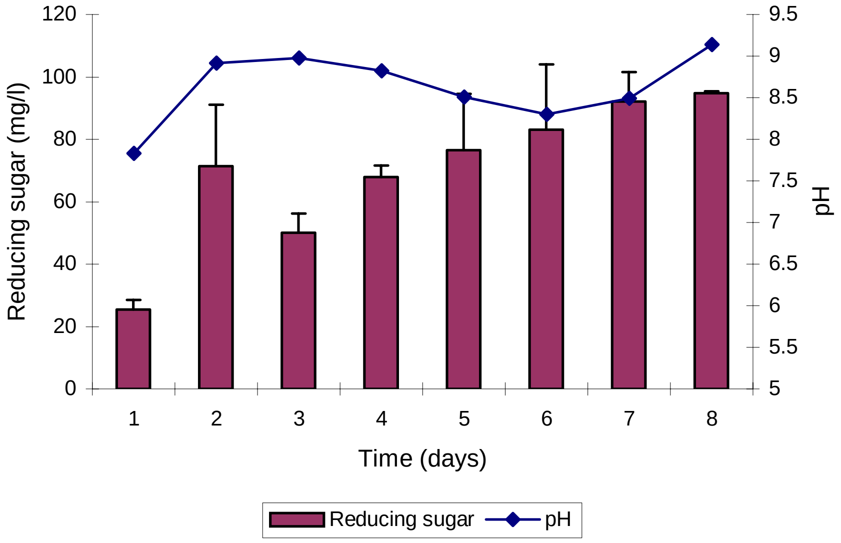


Figure 2.7: Flask study of the release of reducing sugar from Fraction 1 in the presence of 500 mg/l sulphide. Error bars indicate the standard error of the samples analysed in triplicate.

2.4.1.2.2 *Release of aromatic compounds*

Figures 2.8 and 2.9 show that even though the soluble extractives have been removed, a substantial source of aromatic compounds remains available. The flask containing no sulphide showed a gradual increase in the release of aromatic compounds from day 1 to day 12 (36.2 mg/l-790 mg/l). In the presence of sulphide there was a more rapid increase in the release of aromatic compounds, from 27.7 mg/l on day 1 to 1 093 mg/l on day 12.

Rates of release of aromatic compounds were greater in the presence of sulphide for the first 5 days of the experiment (8.9 mg/l/h on day 5), compared to 1.8 mg/l/h on day 5 without any sulphide. However, without any sulphide present there was a greater rate of aromatic compound release on day 12 (5.7 mg/l/h) compared to day 12 in the presence of sulphide (2.4 mg/l/h). The effect of sulphide on the release of aromatic compounds from Fraction 1 at 1.3 times the non-sulphidic treatment was lower than that observed with whole wood.

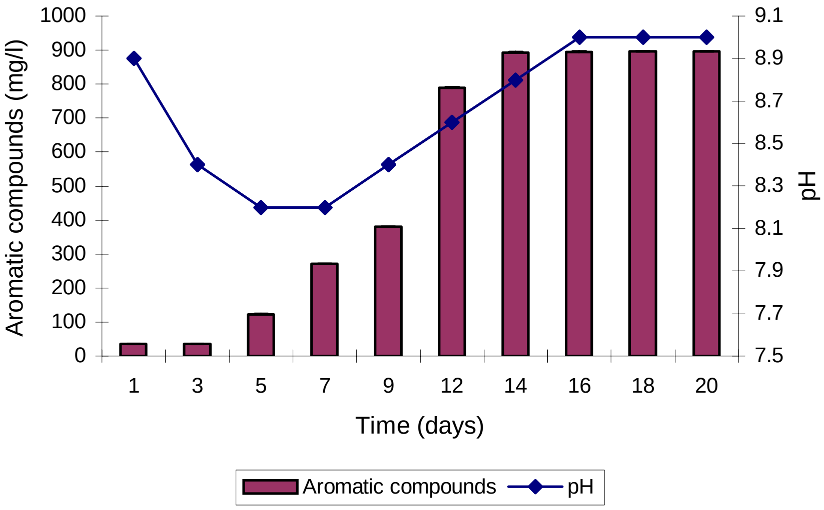


Figure 2.8: Flask study of the release of aromatic compounds from Fraction 1 without sulphide. Error bars indicate the standard error of the samples analysed in triplicate.

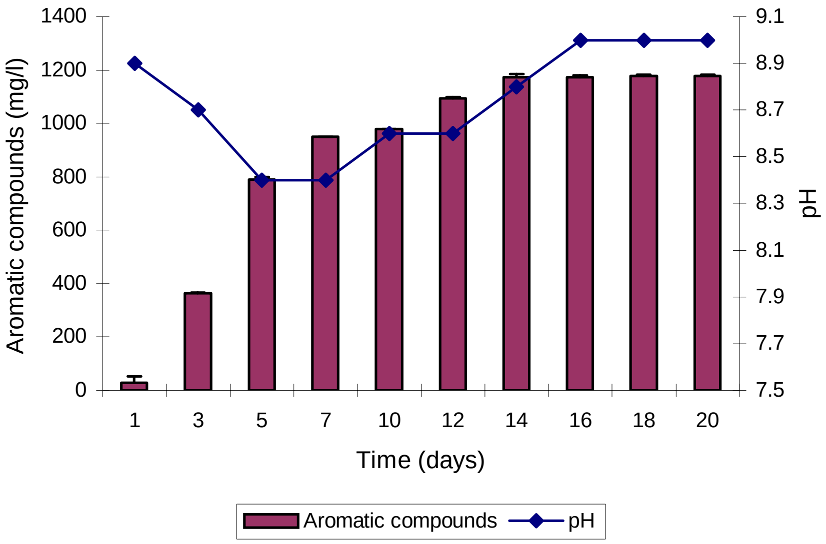


Figure 2.9: Flask study of the release of aromatic compounds from Fraction 1 in the presence of 500 mg/l sulphide. Error bars indicate the standard error of the samples analysed in triplicate.

2.4.1.3 Pine-wood extract-Fraction 2

The extractive-free pine-wood powder (Fraction 1) was further depectinated to give Fraction 2. After depectination all the wood components are removed with the exception of hemicellulose, cellulose and lignin. This experiment was undertaken to investigate the effect of the alkaline sulphidic environment on the extraction of reducing sugars and aromatic compounds from the core lignocellulose fraction of the wood tissue.

2.4.1.3.1 Release of reducing sugars

Figures 2.10 and 2.11 show the release of reducing sugars from Fraction 2. Without sulphide the release of reducing sugars had levelled off after the day 3 at an average of 22 mg/l (± 2.9 mg/l) (Figure 2.10). In the presence of sulphide there was a continued increase in the concentration of reducing sugars released to a maximum of 105 mg/l by day 7 (Figure 2.11), which represents an increase of 3.7 times more reducing sugars released from Fraction 2. The increase in the release of reducing sugars in the presence of sulphide compared to the non-sulphide treatment was significant ($t = 5.9$, $p < 0.05$).

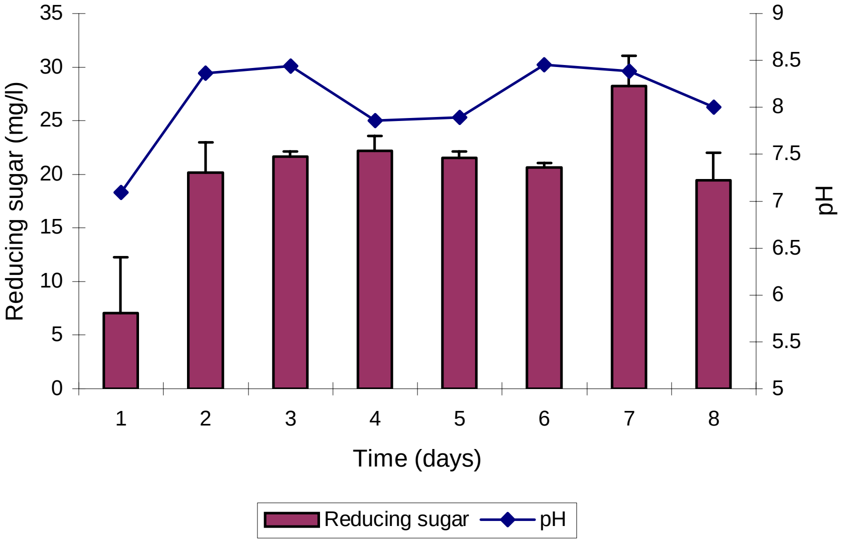


Figure 2.10: Flask study of the release of reducing sugars from Fraction 2 without sulphide. Error bars indicate the standard error of the samples analysed in triplicate.

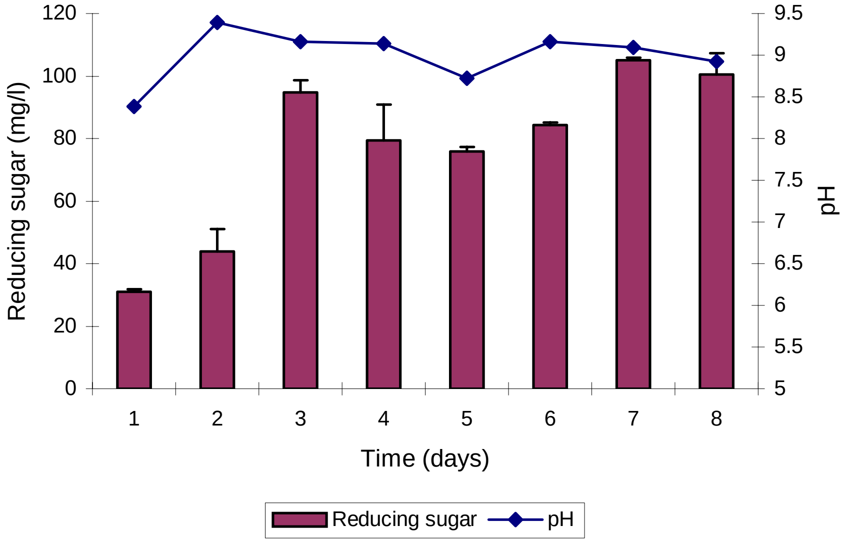


Figure 2.11: Flask study of the release of reducing sugars from Fraction 2 in the presence of 500 mg/l sulphide. Error bars indicate the standard error of the samples analysed in triplicate.

With no sulphide present the highest rate of release for reducing sugars was 0.55 mg/l/h on day 2. In the presence of sulphide, the rate of release was greater, reaching 2.12 mg/l/h on day 3.

2.4.1.3.2 Release of aromatic compounds

Without sulphide the release of aromatic compounds reached a maximum of 278.3 mg/l on day 18 (Figure 2.12). In the presence of sulphide a maximum release of 373.3 mg/l was attained on day 18 (Figure 2.13), which is 1.3 times greater than when no sulphide was present. The rate of release of aromatic compounds from Fraction 2 without sulphide was 1.4 mg/l/h, and in the presence of sulphide it was 7.4 mg/l/h. Fraction 2 was the only fraction where the presence of sulphide was shown to have a significant effect on the release of aromatic compounds ($t = 3.37$, $p < 0.05$).

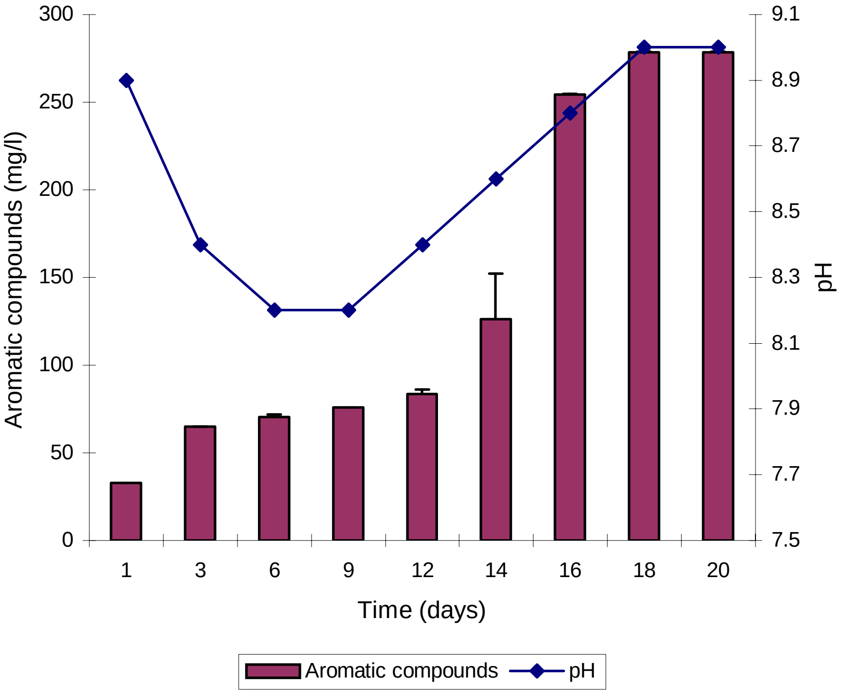


Figure 2.12: Flask study of the release of aromatic compounds from Fraction 2 without sulphide. Error bars indicate the standard error of the samples analysed in triplicate.

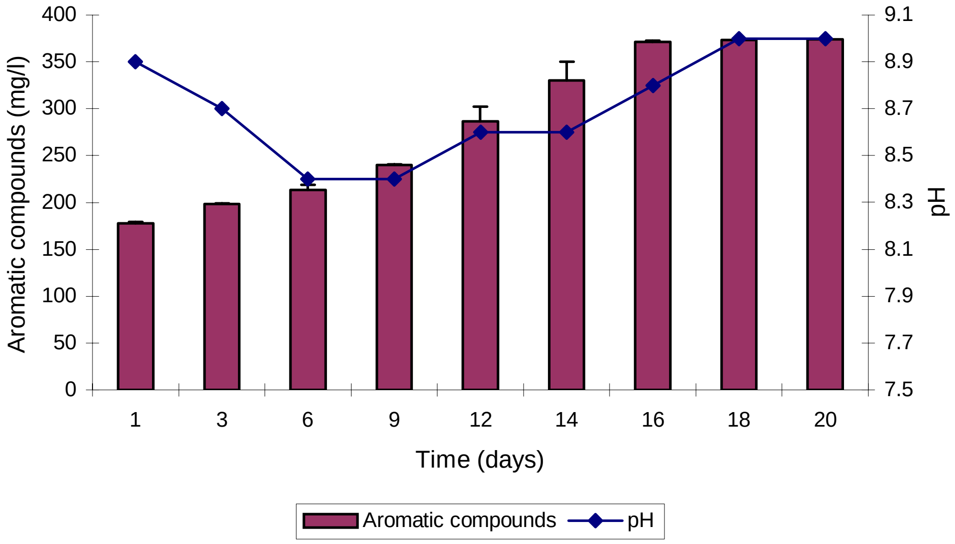


Figure 2.13: Flask study of the release of aromatic compounds from Fraction 2 in the presence of 500 mg/l sulphide. Error bars indicate the standard error of the samples analysed in triplicate.

2.4.2 Release of reducing sugars and aromatic compounds in a buffered system

From the experiments shown previously it was found that the pH did not remain stable in the presence of wood tissue and had to be continuously corrected to maintain stable conditions. In follow-up repeat experiments a buffered solution was used in order to simulate the buffering environment maintained within a biological system. Three regimes were employed, Flask 1 contained phosphate buffer with no pH adjustment after adding whole wood powder, Flask 2 contained phosphate buffer and the pH was adjusted to 8 after addition of wood powder, and Flask 3 contained phosphate buffer and sulphide. On the sampling days all of the solution was removed for analysis and replaced with fresh buffer, this was done to determine the cumulative production of released compounds without possible feed back inhibition effects.

2.4.2.1 The release of reducing sugars

Flask 1 and Flask 2 showed initial release of reducing sugar of 3.5 mg/l and 5.7 mg/l. Flask 3, containing sulphide, released 19.3 mg/l on day 1, an increase of 13.7 mg/l. All of the flasks demonstrated very low release of reducing sugars till day 30, and by day 59 no further reducing sugars were detected (Figure 2.14). All three flasks had their greatest long term release of reducing sugars on day 44, with flasks 1 and 2 having an identical rate of 0.058 mg/l/h, and Flask 3 having a rate of 0.036 mg/l/h. Cumulatively more reducing sugars were released from whole wood in the presence of sulphide than without, with Flask 1 compared to Flask 3 (Figure 2.15).

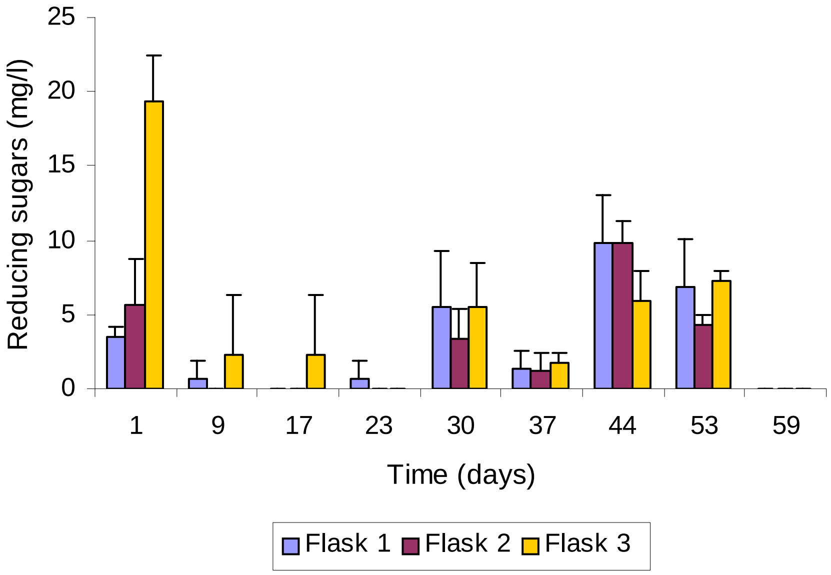


Figure 2.14: Release of reducing sugars over time. Error bars indicate the standard error of the samples analysed in triplicate. Flask 1=phosphate buffer without sulphide and no pH adjustment, flask 2=phosphate buffer with no sulphide and pH adjustment to pH 8 and flask 3=phosphate buffer with 300 mg/l sulphide.

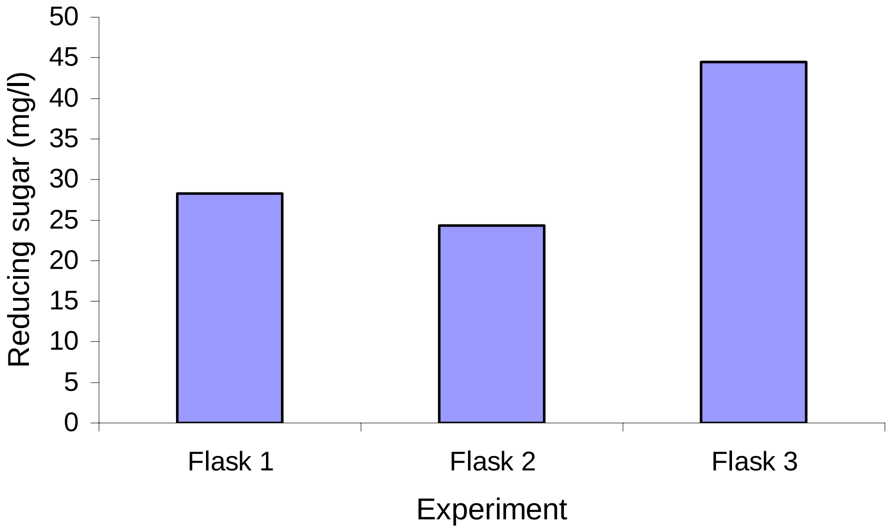


Figure 2.15: The cumulative release of reducing sugars from whole wood in a phosphate buffered medium. Flask 1 = phosphate buffer without sulphide and no pH adjustment, flask 2 = phosphate buffer with no sulphide and pH adjustment to pH 8 and flask 3 = phosphate buffer with 300 mg/l sulphide.

2.4.2.2 The release of aromatic compounds

Flasks 1 and 2, without any sulphide, showed a steady decline in the release of aromatic compounds after the initial release of 51.03 mg/l and 59.6 mg/l, respectively on day 1 (Figure 2.16). In the presence of sulphide, the release of aromatic compounds increased to 161.5 mg/l on day 23, from 68.9 mg/l on day 1. Thereafter, the release of aromatic compounds decreased to 33.9 mg/l on day 59 (Figure 2.16). Sulphide significantly enhanced the release of aromatic compounds ($t = 4.01$, $p < 0.05$). Cumulatively Flask 3 had 3.8 times the release of aromatic compounds than Flask 1, and 3.5 times the release of Flask 2, with 853.7 mg/l, 227.7 mg/l and 245.2 mg/l released respectively (Figure 2.17).

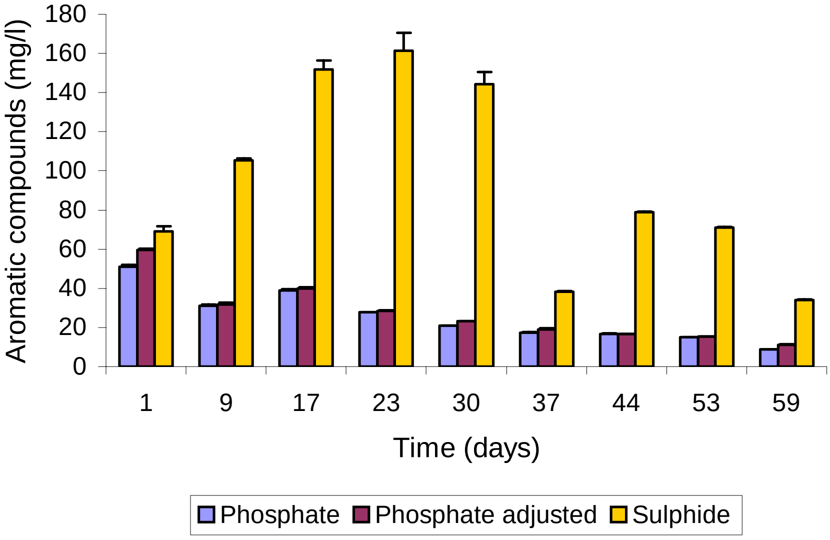


Figure 2.16: Release of aromatic compounds over time. Error bars indicate the standard error of the samples analysed in triplicate. Flask 1=phosphate buffer without sulphide and no pH adjustment, flask 2=phosphate buffer with no sulphide and pH adjustment to pH 8 and flask 3=phosphate buffer with 300 mg/l sulphide.

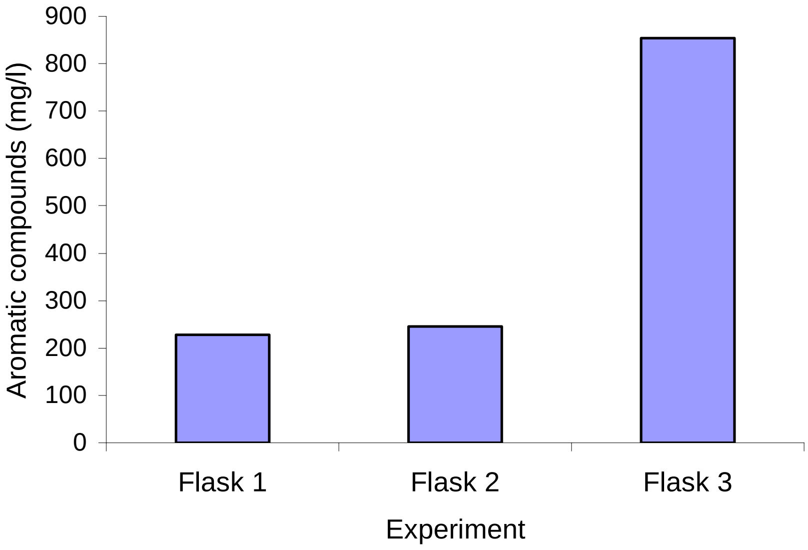


Figure 2.17: The cumulative release of aromatic compounds from whole wood in a phosphate buffered medium. Flask 1 = phosphate buffer without sulphide and no pH adjustment, Flask 2 = phosphate buffer with no sulphide and pH adjustment to pH 8 and Flask 3 = phosphate buffer with 300 mg/l sulphide.

2.5 Discussion

This study has demonstrated that in an alkaline environment, in the presence of hydrogen sulphide, an increased release of reducing sugars as well as aromatic compounds may be observed. This is particularly evident for whole pine-wood powder (Figure 2.2), where it was observed that there is a 6.7 times greater release of reducing sugars in the presence of sulphide than without (Table 2.1). The results suggest that high values for the release of reducing sugars from the whole pine–wood powder may be related to the extractive materials that are soluble in aqueous solutions, and include free sugars and polysaccharides (starch, galactans and gums), (Freeman, 1944).

The rate of release of reducing sugars without sulphide present was lower with each progressive fraction, with whole wood having the greatest release (0.99 mg/l/h), followed by Fraction 1 (0.9 mg/l/h) and with Fraction 2 having the lowest rate of release (0.55 mg/l/h). In the presence of sulphide whole wood had the greatest release of reducing sugars (6.12 mg/l/h), followed by Fraction 2 (2.12 mg/l/h), with Fraction 1 having the lowest rate of release (1.91 mg/l/h) (Table 2.2). The extraction process removes the easily available reducing sugar but not all, considering the decrease in sugar release without sulphide only decreased by 10.2 mg/l for fraction 1 and 12.3 mg/l for fraction 2.

The results suggest that sulphide enhances the release of reducing sugars, mainly from whole wood powder, and less so from fractions 1 and 2. The rates of release from the other two wood fractions were also much slower than for whole wood powder.

Table 2.1: A comparison of the release of reducing sugars and aromatic compounds from whole wood, Fraction 1, Fraction 2 and whole wood in a buffered medium.

	Release of reducing sugars (mg/l)			Release of aromatic compounds (mg/l)		
	- Sulphide	+ Sulphide	Δ	- Sulphide	+ Sulphide	Δ
Whole wood	40.53	270.91	6.7	806.33	1 570.33	2
Fraction 1	30.3	94.82	3.1	896	1 176.67	1.3
Fraction 2	28.23	105.03	3.7	278.33	374	1.3
Buffered#	28.28	44.49	1.6	227.67	853.73	3.8

= Flask 1 and flask 3 were compared

The results suggest that sulphide does enhance the release of aromatic compounds from wood tissue, and from the lignocellulose component itself (Fraction 2). Again it was observed that the majority of the release was from whole wood powder. Aromatic compounds were released to a similar extent in whole wood and Fraction 1, but was 3.2 times reduced in Fraction 2. The recalcitrance of fraction 2 is evident from the low rate of release (1.37 mg/l/h), without sulphide. In the presence of sulphide, however, the rate of release was 7.42 mg/l/h, nearly the same as for whole wood (8.35 mg/l/h).

Table 2.2: A comparison of rates of release of reducing sugars and aromatic compounds from whole wood, Fraction 1, Fraction 2 and whole wood in a buffered medium.

	Release of reducing sugars (mg/l/h)		Release of aromatic compounds (mg/l/h)	
	- Sulphide	+ Sulphide	- Sulphide	+ Sulphide
Whole wood	0.99	6.12	8.35	12.83
Fraction 1	0.9	1.91	5.69	8.9
Fraction 2	0.55	2.12	1.37	7.42
Buffered#	0.058	0.036	0.2	1.12

= Flask 1 and flask 3 were compared

In the buffered system less cumulative release of the two components was observed, but the trend was similar to that of the unbuffered system. Sulphide enhanced the release of reducing sugars by 1.6 times, and aromatic compound release by 3.8 times (Table 2.1). The buffered system, however, had an opposite result to that of the unbuffered systems, in terms of rates of reducing sugar release (Table 2.2).

It was observed that by day 59 of the study there were no detectable reducing sugars in any of the flasks (Figure 2.14), indicating that the limit of chemical hydrolysis had been reached. On day 30 there was an increase in reducing sugars from approximately 0 mg/l to greater than 5 mg/l. It was also observed that when the release of wood aromatics (Figure 2.16) was at its greatest (days 17 to 23), the release of reducing sugars was at its lowest. As the release of wood aromatics decreased the release of reducing sugars increased, days 23 to 30 and days 37 to 44 (Figure 2.14 and 2.16). This could be due to the possible separation of the aromatic fraction from the carbohydrate fraction (Sun and Cheng, 2002).

Figure 2.16 shows that sulphide does, in fact, have an enhanced effect on the release of wood aromatics for a prolonged period. Initially the release of wood aromatics was similar for all three treatments, but by day 9 there can already be observed a marked enhancement of wood aromatic release, that continued to the end of the study. Dilute alkali pre-treatments to remove lignin for the utilisation of cellulose have shown that it leads to a swelling of the wood material as well as a separation of the linkages between lignin and hemicellulose (Sun and Cheng, 2002). This swelling of the lignocellulose material also leads to an increase in the release of soluble aromatics, which is observed by the very high concentration of phenol from day 17 to day 30 (Figure 2.16). The decrease in wood aromatics after day 30 is possibly due to the exhaustion of soluble aromatics and the slow hydrolysis rate of lignin.

The results provide an indication of what may happen in wood packed reactors over the short term. Most of the readily extractable reducing sugars and aromatic compounds are recovered from whole wood in the short term. Fraction 1, with the easily extractable components removed, showed a reduction in the amounts of reducing sugars recovered, but the recovery of aromatic compounds were similar, but greater than whole wood. This indicates that most of the readily extractable aromatic compounds are recovered from whole wood as well as Fraction 1. Fraction 2, representing the lignocellulose component, showed a similar recovery of reducing sugars as Fraction 1, indicating that most of the reducing sugars are recovered from whole wood. Although the recovery of aromatic compounds was 3.2 times less than for Fraction 1, the enhancement of recovery was similar. This suggests that in the short term, the extractable material is rapidly utilised, leading to efficient sulphate reduction, but that once these components have been exhausted, the rates of release slow down, and in turn the sulphate reduction decreases (Figure 2.18).

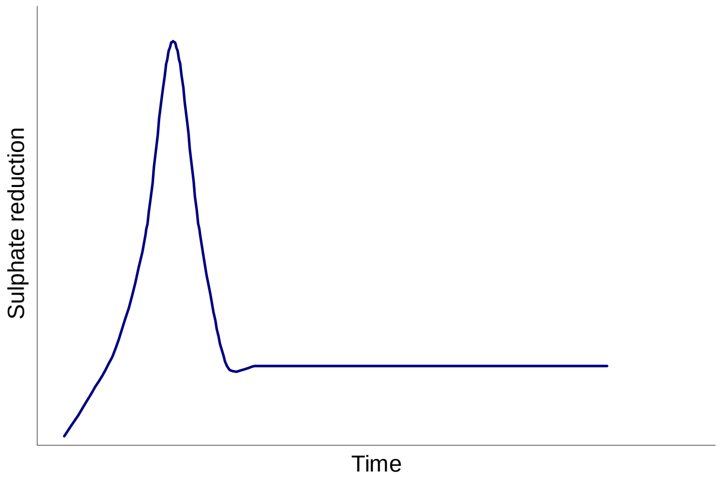


Figure 2.18: Diagrammatic representation of what happens in reactors with lignocellulose material. Initially sulphate reduction is very good, it then reaches a peak and starts declining to levels that are not effective for treatment of AMD, butr are appropriate for desulphurisation (Pulles pers com, 2003).

Lignin is closely associated with the carbohydrate polymers, cellulose and hemicellulose, and is recognised as the being the most resistant to biodegradation, it is thus the rate-limiting step in the biodegradation of lignocellulose (Benner *et al*., 1985, Pareek *et al.*, 1998). It is thus important for biodegradation to be successful that either the lignin component is partially hydrolysed, allowing greater access to the preferred carbohydrate fraction, or is dissociated from the carbohydrate fraction. Benner *et al.* (1985) found that a change in pH had no significant effect on the biodegradation of the lignin component but did affect the biodegradation rate of the cellulosic component. It was suggested that a more alkaline pH (7.8) served to uncouple lignin and carbohydrate degradation, based on the observation that lowering the pH from 7.8 to 4.0 decreased the degradation of the cellulosic component without affecting the lignin fraction. Pareek *et al.* (1998) demonstrated that under sulphidogenic conditions there was a greater mineralization of lignocellulose than under methanogenic conditions. The effect of sulphate reduction on cellulose degradation was illustrated in an experiment where the removal of sulphate led to an 80 % reduction in cellulose degradation (Khan and Trottier, 1978). In the present study it was determined in a purely chemical environment that sulphide, combined with an alkaline pH, has a significant effect on both the carbohydrate and aromatic component of pine-wood powder. In the buffered experiment it was shown that as the aromatic release decreased, the release of reducing sugars increased, possibly due the greater availability of the polysaccharide component, suggesting that the lignin and cellulose components had been uncoupled.

2.6 Conclusions

The results obtained for an abiotic system provides an indication of events occurring in wood-packed sulphate reducing biological reactors treating sulphate-rich wastewaters:

- Sulphide and alkalinity enhance the release of reducing sugars and aromatic compound from wood material compared to water treatment on its own.
- There is an early response to the release of reducing sugars and aromatic compounds, with reducing sugars mainly derived from whole wood and aromatic compounds from whole wood and Fraction 1 (extractive-free pine-wood powder).
- Fraction 2, representing the lignocellulose component, had a lower recovery of reducing sugars and aromatic compounds, compared to whole wood and fraction 1, but sulphide and an alkaline environment was still observed to enhance the release of these components.

The question now is whether a similar response may be noted in biological systems where sulphide and alkalinity is microbially generated.

Chapter 3

Degradation of lignocellulose in a biologically-generated sulphidic environment

3.1 Introduction

Although it has been suggested that lignocellulose is not degraded in anaerobic environments (Björdal *et al.*, 2000), Tuttle as early as 1969 had shown that after minewater passed through a bed of sawdust sulphate was reduced in the water (Tuttle *et al.*, 1969). In 1985 Benner *et al*. found that in wetland ecosystems alkaline conditions are necessary for the optimal degradation of cellulose. These findings were confirmed by Pareek *et al*. (1998) who investigated the degradation of newspaper in sulphate reducing conditions. It has been shown in the studies reported in chapter 2, that the extraction of reducing sugars and aromatic compounds from pine wood powder is significantly enhanced in the presence of a chemically-generated alkaline sulphidic environment. It seems likely that the observed enhanced release of these components would also occur where the alkaline sulphidic environment had been generated biologically by sulphate reducing microbial consortia. What is uncertain, however, is whether the microbial activity in such systems makes any additional contribution to the wood tissue degradation, over and above the generation of the alkaline sulphidic conditions. Another question that needs to be answered relates to the observed pattern of lignocellulose utilization and whether the collapse of packed-bed reactor systems treating AMD may be related to exhaustion of the easily extractable components of wood tissue. What is the performance of the microbial consortium under these conditions and does the core lignocellulose complex remain unaffected after exhaustion of the easily extractable fraction?

The following studies were undertaken to investigate the possible contribution of the sulphate reducing microbial consortia in the anaerobic degradation of wood tissue.

3.2 Methods

Bench-scale flask reactors operated in batch and continuous mode were used and inoculated with an actively growing culture of a sulphate reducing microbial consortium from the Rhodes Biosure Process®, and supplemented with sulphate reducing microbial consortia from various sources including PHD at Robertsville in Johannesburg, South Africa. Scaled up column reactors were set up to simulate, under controlled conditions, the vertical flow regimes occurring in the lignocellulose packed-bed reactors used in AMD treatment (Figure 1.13). Three column reactors were used and in addition to the study described here, the molecular microbial ecology of the system was investigated by Anna Clarke (PhD in progress, Rhodes University). Aspects of these findings will be noted.

3.2.1 Flask batch reactor

In the wood powder degradation studies (prepared as previously described in 2.3.1), 5 % whole wood powder (w/v) was used in mineral medium containing no other carbon source and 2000 mg/l sulphate at pH 7.5. All flasks were connected to zinc acetate (5 % ZnAc, w/v) to collect H_2S. The reactor was covered with foil to prevent the growth of photosynthetic bacteria and incubated at 30 °C on a Labcon shaker at 90 rpm.

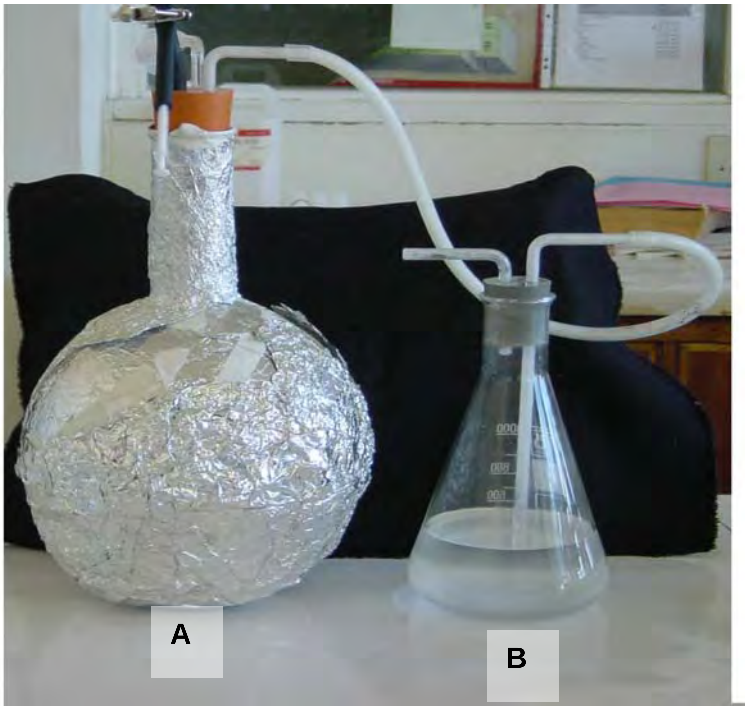


Figure 3.1: Representative reactor set up for degradation studies. A) Bioreactor with 5 % (w/v) wood powder in Postgate's minimal media. B) ZnAc trap.

3.2.2 Bench-scale continuous reactor

The bench-scale continuous reactor was inoculated with 1 000 ml of an actively growing culture from the flask batch reactor. This was centrifuged at 3 840 xg for 10 min at RT (Beckman Model J2-21 Centrifuge), then resuspended in 800 ml Postgate's media. Whole pine-wood powder (80 g) was added to this inoculum. The reactor was not fed for 7 days to allow for colonisation of the wood powder to take place before the continuous feed regime commenced. Figure 3.2 presents a diagrammatic representation of the experimental set-up of the continuous reactor system.

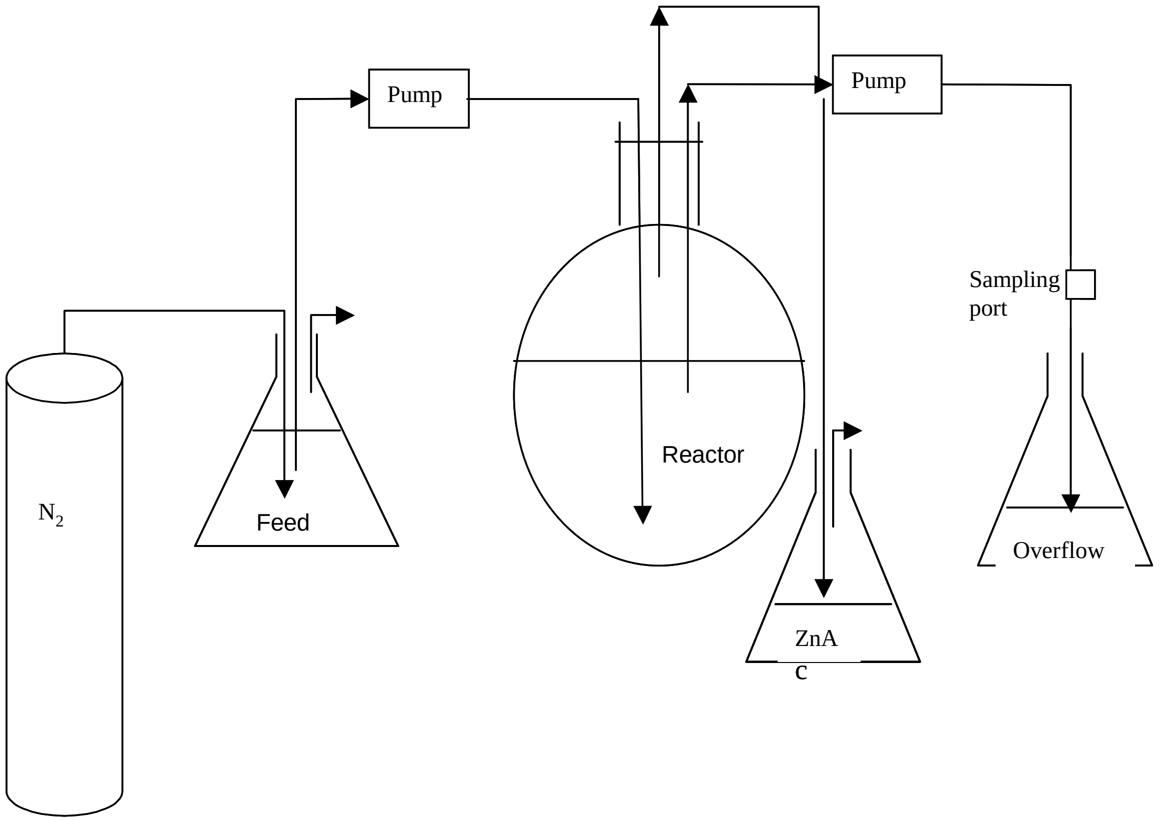


Figure 3.2: Diagrammatic representation of the continuous reactor system used to study the biodegradation of wood powder. Nitrogen was used to maintain an anaerobic atmosphere in the feed vessel.

3.2.3 Packed-bed column reactors

The packed bed column reactor were constructed of polyvinyl chloride (PVC) piping with a volume of 62 L (Figure 3.3) and set up at the Robertsville Laboratories of PHD in Gauteng. They were packed with Buffels grass, wattle chips and begasse as the carbon source and inoculated with 3 L of sewage sludge obtained from Daspoort, Gauteng. Figures 3.4 and 3.5 show the packing of the reactor. Previous experiments had shown that a mixed carbon source (including wood chips, begasse, Buffels grass and sewage sludge) was more effective at reducing sulphate than a single carbon source (Pulles pers com, 2001).

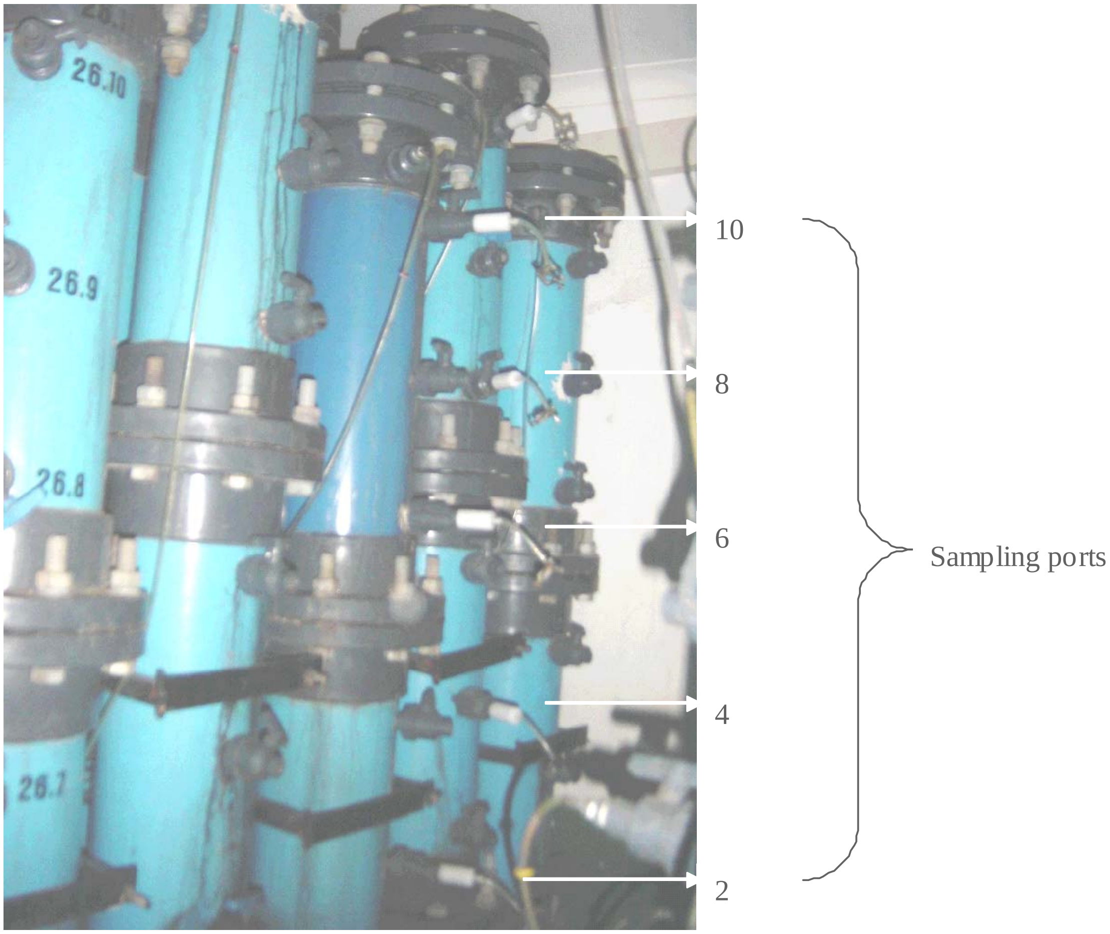


Figure 3.3: Packed-bed column reactors set up in the Robertsville laboratories. Ten sampling ports were arranged along the length of the reactor for the depth profile studies.

The reactors were operated at a hydraulic retention time of 96 h (based on tracer studies) and monitored for 378 days. The reactor was allowed to incubate for 7 days before it was operated, to allow for bacterial colonisation of the packing material. A synthetic AMD was used. Molasses (350 ml of a 1 % stock solution), as an easily assimilated carbon source, was fed on day 81 when it was assumed that the stage of easily extractive exhaustion had been reached.

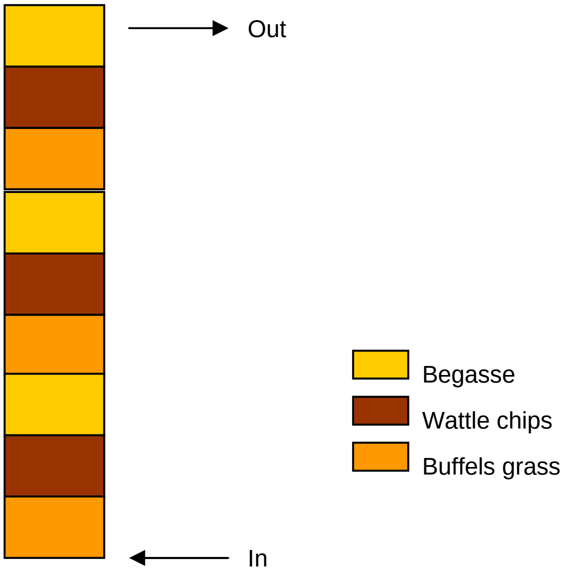


Figure 3.4: Schematic diagram of the packing of the column reactors.

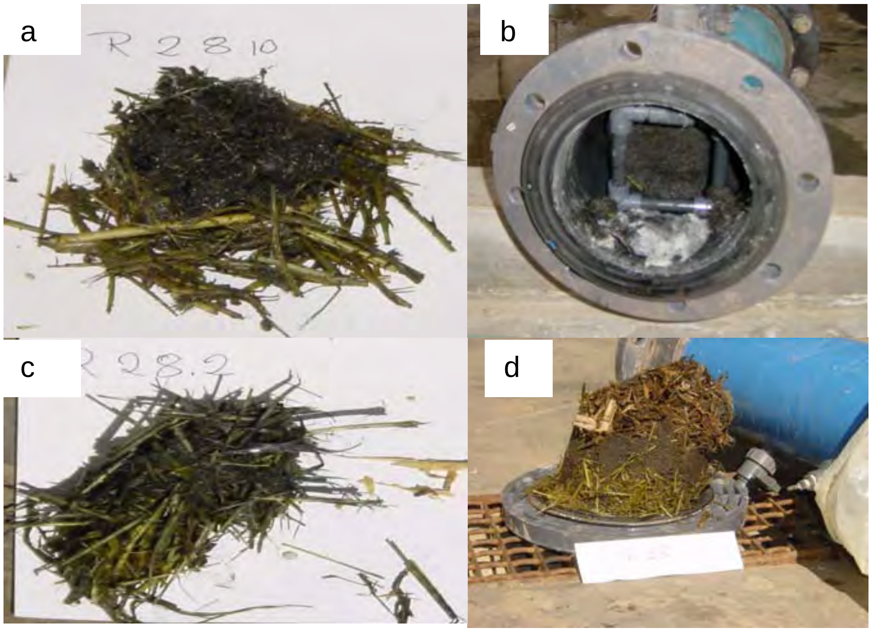


Figure 3.5: Unpacking the packed-bed column reactor. a) The Buffels grass at port 10 in the column, b) the top of the packed-bed column, c) the Buffels grass at port 2 in the column and d) the bottom of the reactor.

3.2.4 Growth medium

The universal medium was made up as follows in 1 L (Atlas, 1993):

Solution A:	NH_4Cl	1 g
	$NaSO_4$	1 g
	$CaCl_2.2H_2O$	1 g
	$MgSO_4$	2 g
	Na-citrate.$2H_2O$	0.3 g

Solution A was made up to 980 ml with d.H_2O and the pH adjusted to 7.5 prior to autoclaving.

Solution B:	KH_2PO_4	2.5 g

Solution B was made up in 100 ml and 10 ml was added to solution A after autoclaving.

Solution C:	Na-thioglycolate (Sigma)	0.1 g
	Ascorbic acid	0.1 g

Solution C was made up in 100 ml and 10 ml were added to solution A after autoclaving. No carbon source was added to the medium.

3.2.5 Sulphate analysis

Sulphate concentrations for the flask batch reactor and the packed-bed column reactor were determined using ion chromatography (IC) with a Hamilton PRP-X100 column (15mm x 4.1mm). The mobile phase was 4mM p-hydroxybenzoic acid, 2.5% methanol, pH 8.5. The flow rate was 1 ml/min maintained with a Waters 510 pump and detection was with a Waters 430 conductivity detector. Prior to chromatography, samples were cleaned by mixing in a 1:1 ratio of sample:ZnAc (10.44 %) to allow for removal of sulphide, followed by filtration through 0.45μm nylon filters (to remove particulates) then passing it through a 25 mg C_{18} Isolute® solid phase extraction column to bind contaminating organics (Rein, 2002). Sulphate concentrations were determined using a sulphate standard and calculated from a sulphate standard curve.

The Hach® Sulfaver 4 method 8051 was utilized for sulphate determination for the continuous bench-scale reactor. The principle is that the sulphate ions in solution react with barium ions and form a precipitate; the samples are then analyzed at 450 nm. The turbidity formed is proportional to the amount of sulphate present in the sample (Hach® technical manual). The protocol as outlined in the technical manual was followed.

3.2.6 Sulphide analysis

The principle of the sulphide analysis method involved reaction of hydrogen sulphide (HS^-) with ρ-phenylene diamine in an acidic medium to form 3,7-bis(dimethylamino)phenothiazin-5-iumchloride (methylene blue), with ferric chloride as catalyst (Quentin and Pachmayr, 1961).

This was carried out as follows:

- Place 100 μl solution C into a test tube.
- Add sample diluted 100x with d.H_2O (total volume of 5 ml).
- Add 500 μl solution A and 500 μl solution B and mix.
- Allow to stand for 60 min. and then read at 670 nm.

3.2.7 Reducing sugar and aromatic compound determinations

Reducing sugars were determined according to the method outlined in 2.3.3. The determination of aromatic compounds was performed as outlined in 2.3.4.

3.3 Results

3.3.1 Flask Batch Reactor study

In the flask batch reactor study inoculated with the sulphate reducing microbial consortium sulphate was reduced to zero in 27 days by the sulphate reducing microbial consortium (Figure 3.6). After spiking with an additional 1 750 mg/l sulphate on day 36, this was reduced to 187.1 mg/l in a further 46 days. The rate of sulphate reduction changed from 0.4 mg/g wood/day for the first 18 days to 0.031 mg/g wood/day between days 18 and 27. After spiking on day 36 the rate of sulphate reduction was observed to be 0.2 mg/g wood/day between days 36 and 56, and the pH stabilised around pH 8.5. Sulphide increased from 286.3 mg/l on day 27 to 534.3 mg/l by day 59 (Figure 3.7) thus establishing comparable conditions to the abiotic study.

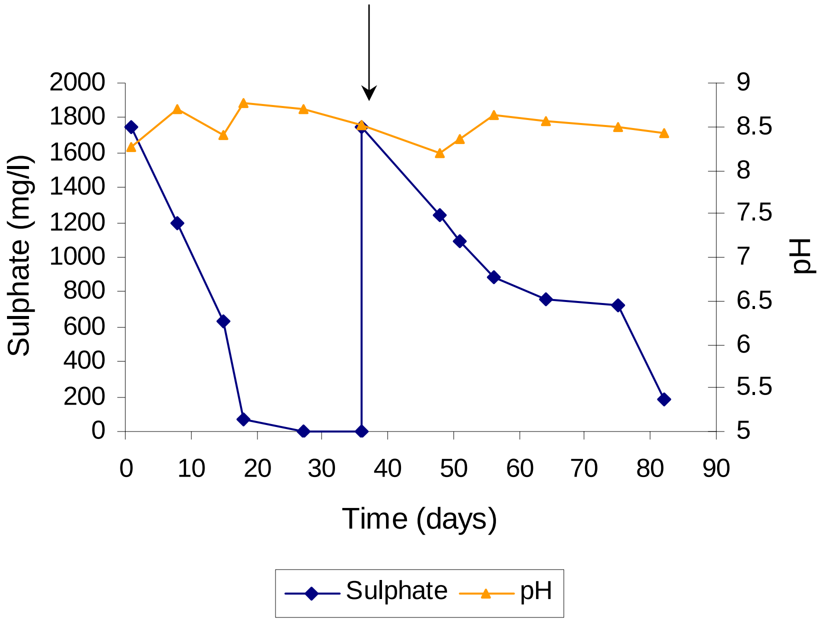


Figure 3.6: Sulphate and pH profile in the flask batch reactor over the 82 days of the wood biodegradation study. The arrow indicates spiking with additional sulphate on day 36.

Figure 3.8 shows production and consumption of reducing sugars within the batch reactor with a peak around day 18 and again at day 58 and 72, and may be related to the activity of the microbial consortium. This pattern coincides with peaks in sulphide production at the same time (Figure 3.7) and after a period of 10-20 days following sulphate addition at T_0 and T_{36}.

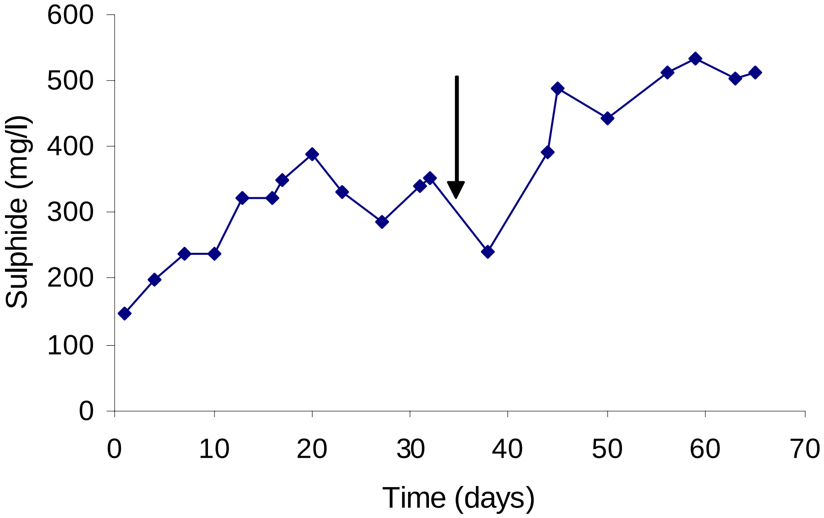


Figure 3.7: Sulphide production over time in the flask batch reactor over 82 days of the wood biodegradation study. The arrow indicates spiking with additional sulphate on day 36.

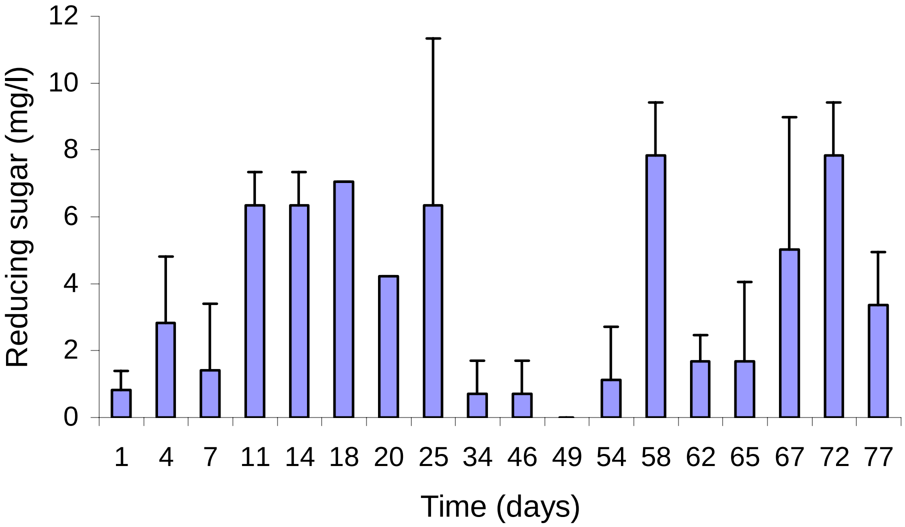


Figure 3.8: The release of reducing sugar with time in the batch reactor.

Release of aromatic compounds from whole pine-wood powder, showed similar patterns of production and consumption with peaks on days 16, 35 and 52 (Figure 3.9), which either coincided with or preceded the reducing sugar peaks. As in the abiotic study the amount of aromatic compounds released is far greater than the reducing sugars. The levels of aromatic compounds released only reached 26 % of that of the abiotic study (Figure 2.8), which may be accounted for by microbial consumption.

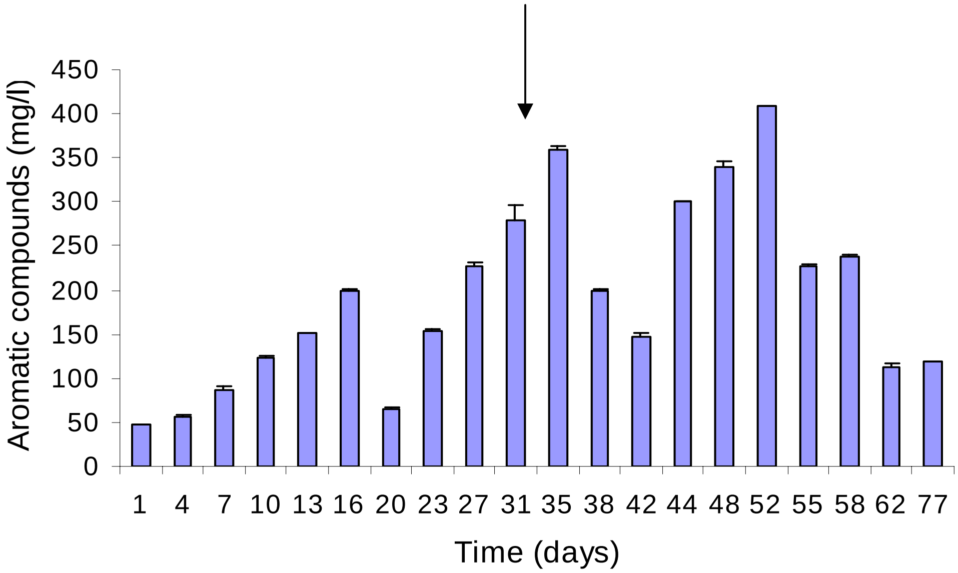


Figure 3.9: The release of aromatic compounds from the flask batch reactor. The arrow indicates spiking with additional sulphate.

In order to determine the actual production of reducing sugars and aromatic compounds to enable a comparison with the abiotic study the following calculation was made:

1. Products consumed were estimated from the value of sulphate reduction on the basis of a 1:1 sulphate:acetate reduction:consumption ratio,
2. The VFA produced was calculated as acetate equivalents on the basis of a 1:3 glucose/phenol:acetate ratio,
3. Total reducing sugars and aromatic compounds measured were summed and the estimated consumed fraction was added to this value,
4. The combined total reducing sugars and aromatic compounds were then compared to that produced in the abiotic study.

It needs to be borne in mind that the calculated value for products consumed does not take into account the products used by the organisms for cell structure and repair. The result is summarised in Table 3.1.

Table 3.1: Comparison of the release of organic compounds from the buffered abiotic test and the flask batch study. Organic compounds refers to the combined values of reducing sugars and aromatic compounds. All values are in mg/l.

	Abiotic release of organic compounds	Biological release of organic compounds*
Days 1-18	349.9	765.7
Days 36-56	203.3	532.7
Total	553.2	1 298.4

* = Sum of the measured and calculated release of organic compounds

It can be seen in Table 3.1 that the release of organic compounds in biologically produced sulphidic environments is 2.3 times greater than in the presence of chemically produced sulphidic environments.

3.3.2 Bench-scale continuous reactor

The bench-scale continuous reactor had a hydraulic retention time (HRT) of 2.32 days and a flow rate of 9 ml/h. The reactor was monitored for sulphate reduction (Figure 3.9); reducing sugar release (Figure 3.11) and aromatic compound release (Figure 3.12) for 80 days. Given the attachment period allowed day 1 corresponds to day 7 after inoculation.

Figure 3.10 shows that the rate of sulphate removal for the first 12 days was 0.35 mg/g wood/day, and then fluctuated between 0.5 and 2.4 mg/g wood/day. The reactor showed a cyclic pattern of sulphate reduction with maximal sulphate reduction at days 15, 29, 47, 52 and 75 (Figure 3.10).

Figure 3.11 shows that after 19 days the continuous reactor reached a neutral pH (pH 7.0) and then operated between pH 6.5 and 7.

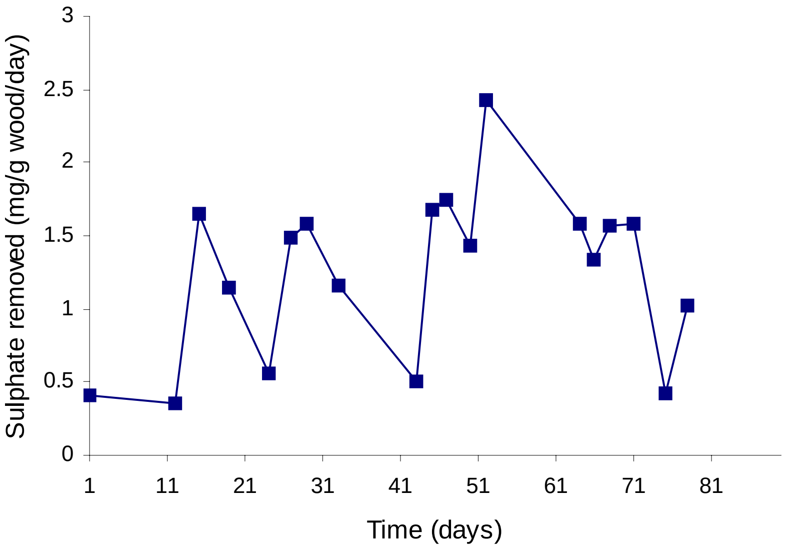


Figure 3.10: The rate of sulphate removal over time.

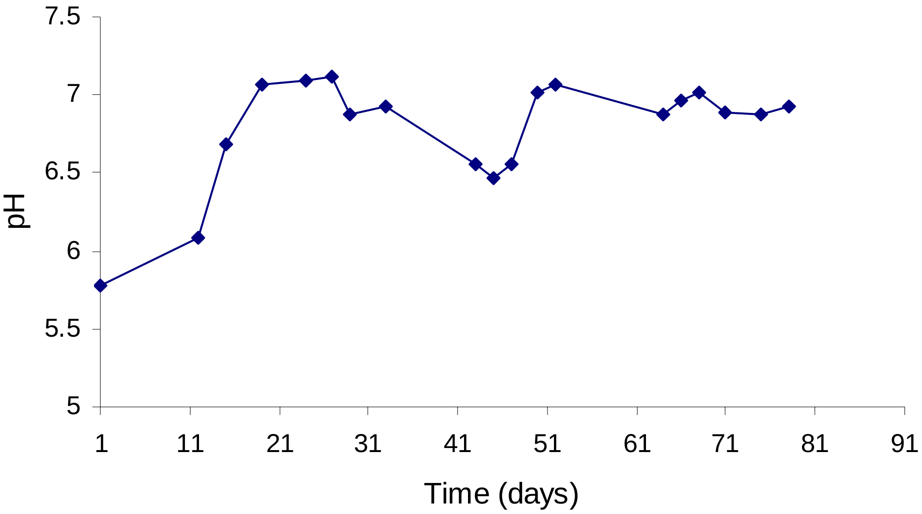


Figure 3.11: pH in the bench-scale continuous reactor.

Initially high levels of reducing sugars (306 mg/l) accumulated (Figure 3.12), which was followed by consumption, until by day 24 production and consumption remained in balance. From figure 3.11, it can be seen that the increase in pH of the reactor from 5.8 to ~7 over the same period, corresponds to the consumption of reducing sugars and may be related to sulphate reduction and the production of alkalinity within the system. Small peaks at days 27 and 66 suggest a pattern of release possibly comparable to that of observed in the batch study.

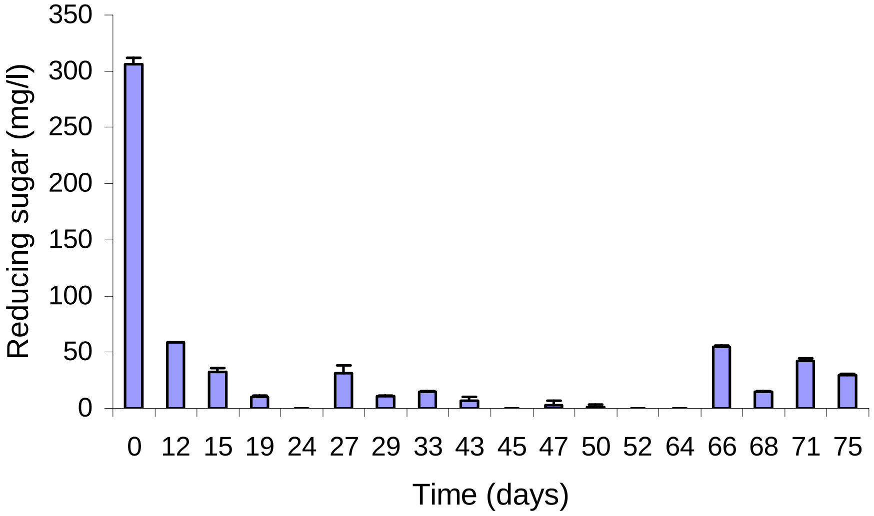


Figure 3.12: Release of reducing sugars over time from the bench-scale continuous reactor.

As with the reducing sugars there was a high initial release of aromatic compounds followed by consumption with production and consumption remaining in balance thereafter (Figure 3.13). Here again a pattern may be seen with peaks at days 33 and 75.

Again it was observed that in the presence of a microbial consortium there was a 1.8 times greater release of organic products from the wood matrix than in the presence of sulphide alone (Table 3.2).

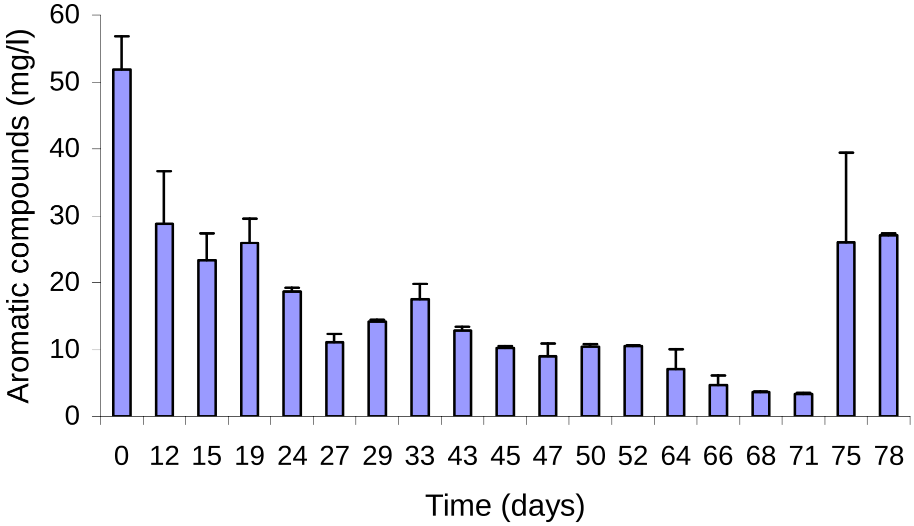


Figure 3.13: Release of aromatic compounds from the bench-scale continuous reactor.

Table 3.2: Comparison of the release of organic compounds from the buffered abiotic test and the bench-scale continuous reactor study. Organic compounds refers to the combined values of reducing sugars and aromatic compounds. All values are in mg/l.

	Abiotic release of organic compounds	Biological release of organic compounds
Days 1-18	349.9	660.0
Days 36-56	203.3	312.2
Total	553.2	972.2

* = Sum of the measured and calculated release of organic compounds

On comparing the rate of sulphate reduction with the detection of reducing sugars and wood aromatics it can be observed that the rate of sulphate reduction corresponds to the increase or decrease in either product. Over the first 18 days the release of organic compounds was 660.0 mg/l and the rate of sulphate reduction increased from 0.406 mg/g wood/day to 1.2 mg/g wood/day from day 1 to 19. It was observed that when the rate of reducing sugars decreased, there was an increase in the rate of aromatic compound production (Figure 3.14). It was observed that on day 66 there was a substantial increase in the rate of release of reducing sugar s compared to aromatic compounds, it is possible that at this time there was an uncoupling of lignin and cellulose allowing greater access to cellulose.

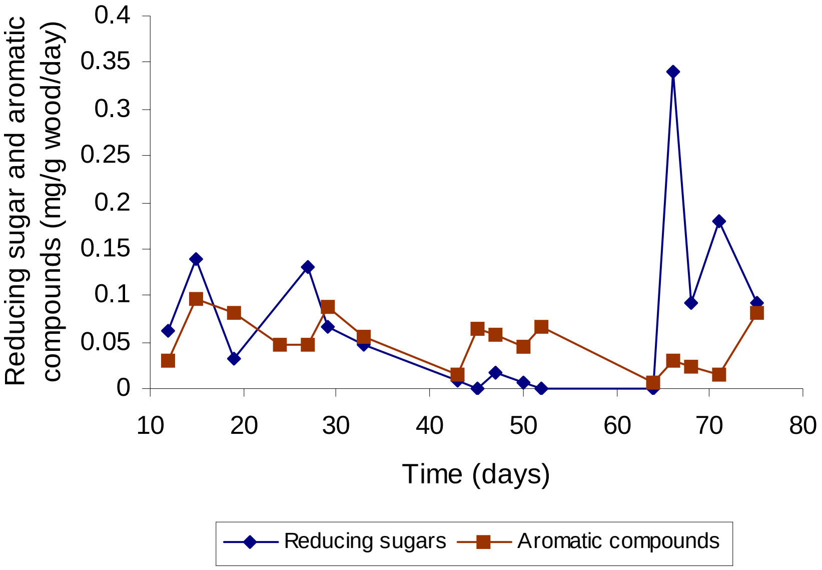


Figure 3.14: Comparison of the rates of measured reducing sugar and aromatic compounds release from the bench-scale continuous reactor.

3.3.3 Packed-bed Column Reactors

The column reactors were also continuously operated and obtained operational sulphate removal from day 39 onward, with a sulphate removal rate of 43.2 mg/l/day (Figure 3.15) and continued to increase till day 97 (212.6 mg/l/d), whereafter a decrease in reduction was observed. On day 200 the system had a shock loading of 13 484 mg/l sulphate as well as 9 184 mg/l sulphate on day 207. Even though the system was shocked with such a large amount of sulphate, it still managed to reduce the levels of sulphate to 1 800 mg/l. After the shock loading the rate of sulphate reduction was 419 mg/l/d (± 612 mg/l/d) between days 200 to 277. From day 280 the HRT was reduced from 96 h to 48 h, resulting in a decrease in the rate of sulphate reduction to 309 mg/l/d (± 161 mg/l/d)

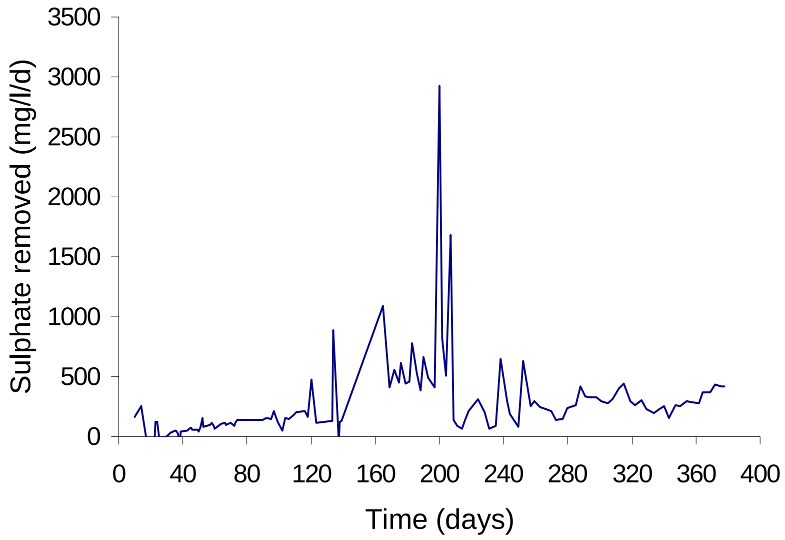


Figure 3.15: Rate of sulphate removal in the packed-bed column reactor over time.

Sulphide production was between 157.2 mg/l and 271.4 mg/l (± 55.4) for the duration of the study (Figure 3.16).

The pilot reactor was also periodically sampled through its depth; this was to determine the performance of the reactor across its profile. After 31 days the reactor was reducing sulphate at a rate of 62.9 mg/l/d, with the best performance, in terms of sulphate reduction, observed on day 113, with 155.6 mg/l/d sulphate being removed. The percentage of sulphate removed from the synthetic AMD feed on day 31, amounted to 12.6 % compared to 28.4 % by day 113 (Table 3.3). From day 113 to day 273, 25.6 % (± 2.61) of the influent sulphate was removed by the pilot reactor.

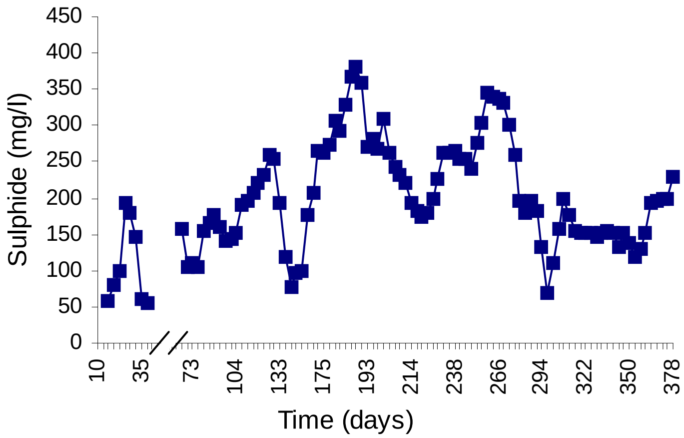


Figure 3.16: Sulphide production for the packed-bed column reactor over the trial period of 376 days. The break in the x-axis indicates a period when no data was collected.

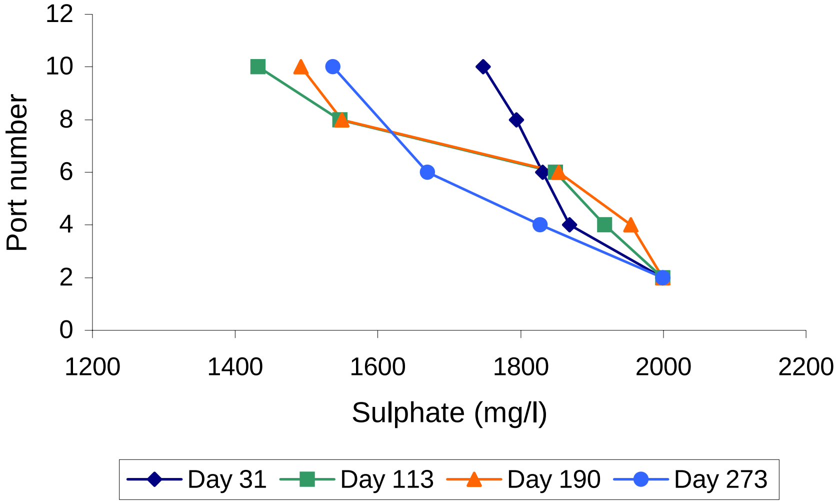


Figure 3.17: Profile of sulphate reduction across the depth of the packed-bed column reactor.

Table 3.3: Sulphate reduction at each port of the packed-bed column reactor, expressed as percentage sulphate removed.

Time (days)	Port number	% Removal
31	4	6.5
	6	8.4
	8	10.3
	10	12.6
113	4	4.1
	6	7.5
	8	22.7
	10	28.4
190	4	2.3
	6	7.3
	8	22.5
	10	25.4
273	4	8.6
	6	16.5
	10#	23.2

#: 7.97 % sulphate was removed between ports 6 and 10; no data was available for port 8.

Figure 3.18 shows how the column reactor changed with respect to pH over time. It can be seen in the early stages that there was an acidifying effect, probably due to fermentation reactions. Looking at figure 3.20 it can be seen that there is a low amount of reducing sugars present, these are probably being fermented to volatile fatty acids (VFAs), causing the low pH. Day 113 shows an alkalising effect up to port 6, from where acidification occurs again. Figure 3.21 shows that from port 8 there is a marked increase in aromatic content, causing the decrease in pH. Days 190-273 show an alkalising effect through the depth of the reactor (Figure 3.18). Reducing sugar content except for day 190 was low and aromatic content for this period was also low.

Comparing sulphate reduction through the depth of the column reactor to the pH of the effluent, it was observed that the greater the sulphate reduction the higher the pH of the effluent (Figure 3.19)

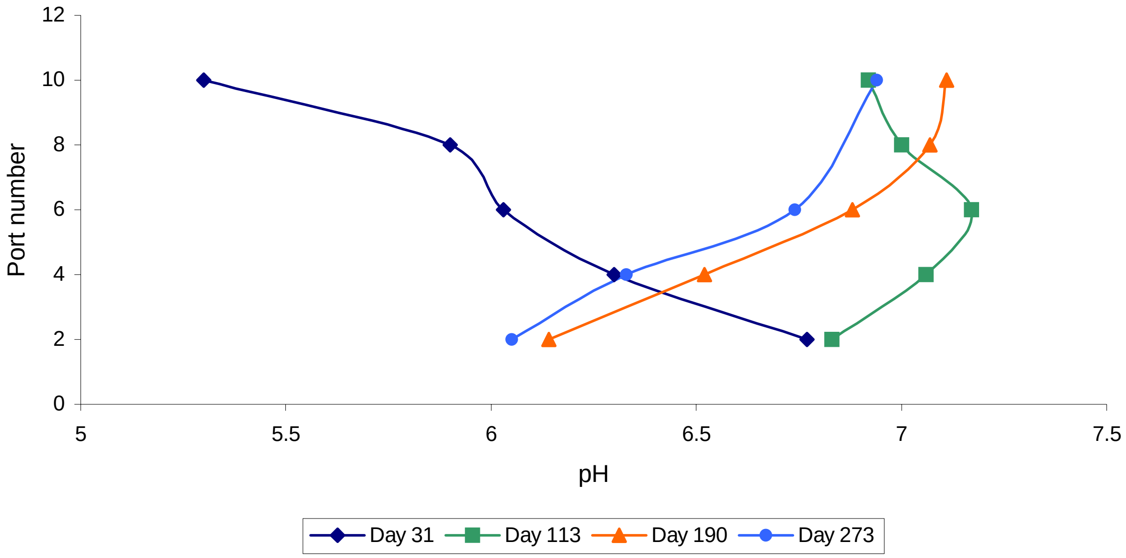


Figure 3.18: Profile of pH across the depth of the packed-bed column reactor. The dependent and independent variables have been interposed to the change over depth to be visualised against the y-axis.

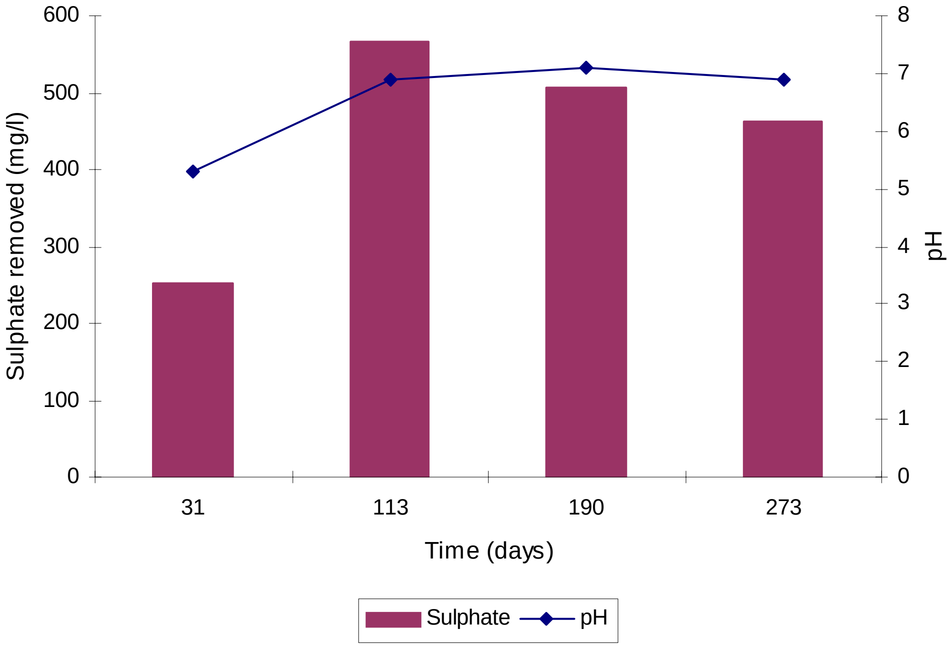


Figure 3.19: Comparison of sulphate removed and pH on the days of sampling the depth of the column reactor.

Figure 3.21 shows that there was an increase in aromatic compounds up the column (upflow regime) indicating that the process of lignocellulose hydrolysis was accelerated.

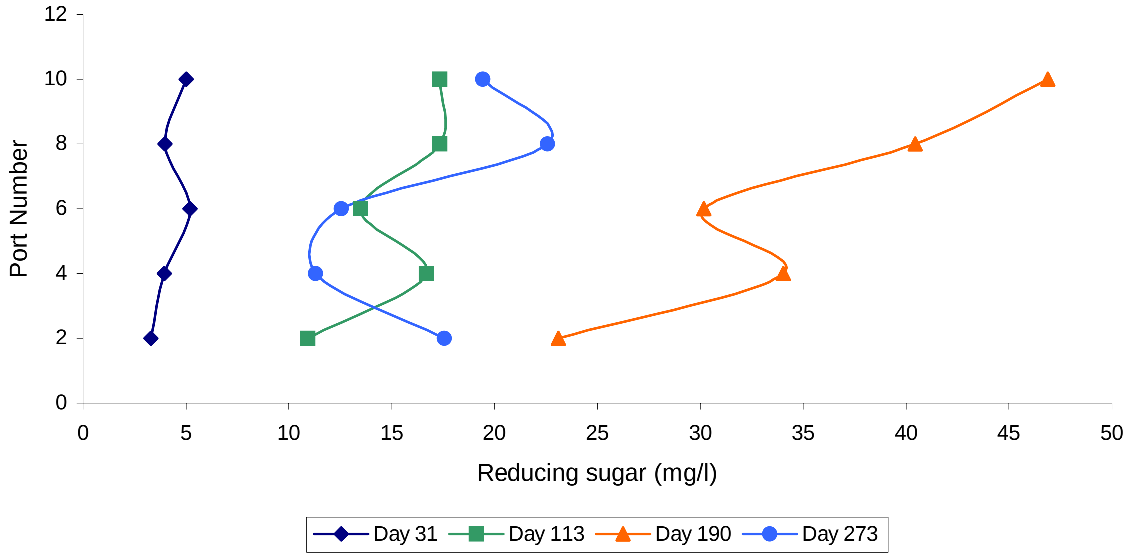


Figure 3.20: Profile of reducing sugar across the depth of the packed-bed column reactor. The dependent and independent variables have been interposed to the change over depth to be visualised against the y-axis.

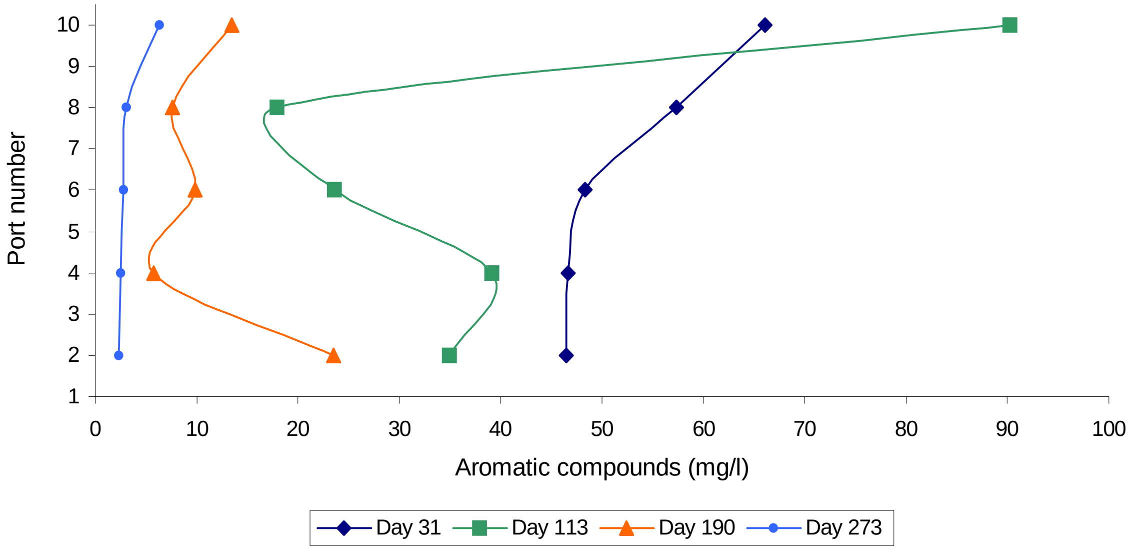


Figure 3.21: Profile of aromatic compounds across the depth of the packed-bed column reactor. The dependent and independent variables have been interposed to the change over depth to be visualised against the y-axis.

In order to compare the release of reducing sugars to wood aromatics, port 10 of the pilot reactor was used. Comparing the release of reducing sugars to wood aromatics it was observed that wood aromatics was at its greatest up to day 113, thereafter it decreased. The release of reducing sugars showed an inverse effect, with it being low up to day 113, when it increased to levels higher than for aromatic compounds (Figure 3.21).

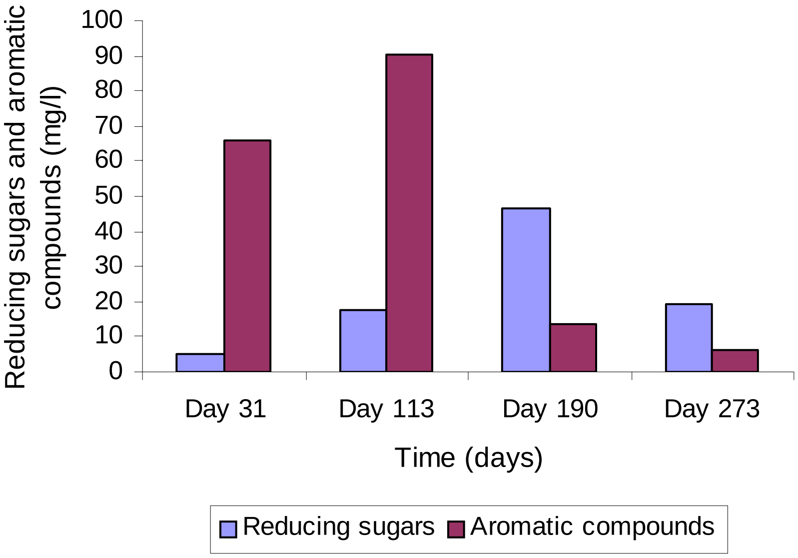


Figure 3.22: Comparison of reducing sugar and aromatic compound release for the packed-bed column reactor at port 10.

Table 3.4: Comparison of the release of organic compounds from the buffered abiotic test and the packed-bed column reactor study. Organic compounds refers to the combined values of reducing sugars and aromatic compounds. All values are mg/l.

	Abiotic release of organic compounds	Biological release of organic compounds*
Day 31	660.9	1 201.3

* = Sum of the measured and calculated release of organic compounds, assuming an average release of 155 mg/l per HRT (hours).

Table 3.4 again shows that in the presence of biologically produced sulphide there is a greater release (1.8 times) of organic compounds than in the presence of a chemically generated sulphidic environment.

3.4 Discussion

It was decided to use pine-wood powder for the flask and lab-based studies, in order to elucidate a mechanism of hydrolysis and utilisation under biosulphidogenic conditions of wood material under defined conditions without the introduction of too many variables.

Comparing the rates of organic compounds released from the buffered abiotic experiment to the studies reported in this chapter, there was a greater rate of release in the presence of the microbial consortia. Between days 1-18 19.4 mg/l/d organic compounds were released by the chemically produced sulphidic environment, the flask study released 42.5 mg/l/d and the bench-scale continuous reactor released 36.7 mg/l/d over the same period. The packed-bed column reactors released 38.8 mg/l/d organic compounds over 31 days compared to 21.3 mg/l/d in the buffered abiotic system. The packed-bed column reactor study is one of the longest spatial studies undertaken, Colberg and Young (1985) did a 43 day study on the soluble fraction of lignocellulose, Clarkson and Xiao (2000) did a 300 day study on the production of biogas from the anaerobic conversion of paper, Kim *et al.* (1997) followed the degradation of filter paper, cellulose and newspaper for 30 days and Benner *et al.* (1985) did a 25 day study on lignocellulose biodegradation rates in two separate wetland samples. The packed-bed reactor study was undertaken for 378 days.

Table 3.5 summarises the release of organic compounds in all the systems tested. The table shows that in the presence of a microbial consortium there is an enhanced release of organic compounds. The flask study showed the greatest release followed by the packed-bed column reactors. The bench-scale continuous reactor had the weakest performance in terms of compounds released.

Table 3.5: Comparative release of organic compounds from the abiotic and biological treatments of whole pine-wood powder. All values are in mg/l.

	Abiotic	Flask study	Bench-scale continuous reactor	Packed-bed column reactor
Total release of organic compounds (mg/l)	553.2	1 298.4	972.2	1 201.3

The results suggest that there is a definite effect exerted by the microbial consortium on the degradation of the wood material, compared to the abiotic system. In the abiotic studies, it was shown that aromatic compounds were released to a greater extent than reducing sugars from whole wood, which was used as the substrate for the studies presented here. In both the presence and absence of sulphide the release of aromatic compounds was greater (Figure 2.7 and 2.8) than for reducing sugars (Figure 2.5 and 2.6). For the flask batch reactor this result was also obtained (Figure 3.8 and 3.9). The bench-scale continuous reactor had a greater release of reducing sugars, but the release of wood aromatics was more constant (Figure 3.12 and 3.13). The packed-bed column reactor had the greatest release of aromatics prior to the greatest release of reducing sugars (Figure 3.19 and 3.20), with minimal release of wood aromatics when the release of reducing sugars was at its greatest. According to Miyajima *et al.* (1997) there are three main components of vascular plants that are susceptible to mineralization:

- Simple organic molecules that are soluble in an aqueous medium, and is the most readily degradable component.
- The non-lignified cellulose and hemicellulose polymers that can be degraded anaerobically, but at much slower rates than for the previous component.
- The least degradable components are the lignified cellulosics and lignin.

Based on the results obtained it is proposed that in order for the bacterial consortium to utilise lignocellulose as a carbon source, the aromatic content has to be consumed (i.e. lignin), or removed from the carbohydrate fraction, allowing for access to the cellulose fraction. Looking at the results comparatively, it could be proposed that either there are aromatic compounds that are inhibitory to reducing sugar release, or that a feedback inhibition may operate and that these compounds are washed out in the continuous system. This is particularly evident when the rates of sulphate reduction are compared; the batch reactor had a maximal reduction of 0.37 mg/g wood/day compared to 1.4 mg/g wood/day for the continuous reactor (Figure 3.10).

The results suggest that sulphide and alkalinity enhance the release of aromatic compounds from the wood matrix, and this stimulates the sulphate reducing microbial consortium to degrade the cellulosic fraction of the wood matrix. It is suggested that the addition of an easily available carbon source stimulates sulphate reduction, increasing sulphide and alkalinity, releasing aromatic compounds that are metabolised by the consortium, to produce VFAs. This in turn stimulates more sulphate reduction, and the alkalinity produced opens up the lignocellulose molecule allowing for enzymatic attack on the cellulose. This concept is explored further in the final chapter where the degrading packed-bed reactor concept is put forward.

The results of the abiotic and the biotic studies seem to indicate that the lignocellulose complex of the pine-wood tissue was degraded in the presence of sulphide and alkalinity. However, the question posed by Breen and Singleton (1999) remains, whether the lignocellulose complex can, in fact, be degraded in an anaerobic environment. The studies reported in the following two chapters were undertaken to test this point.

Questions that are brought to light by the results obtained in this chapter are:

1. Does sulphide and alkalinity enhance lignin degradation?
2. Is there an enzymatic degradation of lignin?

3.5 Conclusions

The results provide a provisional indication of lignocellulose hydrolysis in anaerobic biosulphidogenic environments:

- Biologically generated sulphide had a greater effect on lignocellulose than the chemically produced sulphide alone.
- The pilot study demonstrated the importance of the removal or consumption of the aromatic portion of lignocellulose to facilitate access to the cellulose portion.

The results obtained suggest that lignin is hydrolysed before, or that it is uncoupled from the cellulose component. The easily extractable aromatic component contributes to the initial sulphidogenesis that takes place. The conclusion that the biological effect is greater than the chemical effect suggests that there are other factors to consider, such as enzymatic attack on lignocellulose. Two questions were raised and are dealt with in the following two chapters:

1. Is the sulphate reducing microbial consortium capable of cleaving lignin and utilise it as an electron donor and carbon source?
2. Are there cellulolytic enzymes present in the sulphate reducing microbial consortium? Can the consortium utilise cellulose as an electron donor and carbon source?

Chapter 4

Degradation of lignin and lignin model compounds in a biologically generated sulphidic environment

4.1 Introduction

Lignin is a complex polyaromatic polymer formed by the free-radical coupling of substituted *p*-hydroxycinnamyl alcohol derivatives. Although fungi under highly oxidative conditions, utilising hydroxyl radicals and singlet oxygen are able to hydrolyse lignin, the precise mechanisms involved in aerobic cleavage remain obscure (Brune *et al.*, 1995). Lignin has been regarded to be highly resistant to anaerobic degradation and the recalcitrance of lignocellulose degradation is thought to be responsible for the formation of peat and leading ultimately to coal production (Zeikus, 1982, Benner *et al*., 1984, Crawford and Crawford, 1988).

Aerobic studies on the degradation of lignin have demonstrated the release of intermediate and low-molecular weight phenolic compounds and oligo-lignols that are soluble and able to enter the environment. Breen and Singleton (1999) have proposed that lignin cannot be degraded under anaerobic conditions because the ether and carbon-carbon bonds can only be cleaved in systems utilising an oxidative reactive mechanism. However, others have argued that molecular oxygen is not required for the anaerobic cleavage of lignin-derived monomers, and that oligo-lignols could be mineralised under anaerobic conditions (Benner *et al.*, 1984, Colberg and Young, 1985).

Results reported in the previous chapter have provided an indication that lignocellulose derived from pine-wood may be utilised by sulphate reducing microbial consortia, and that the lignocellulose complex was apparently degraded to some extent in these experiments. The question remains whether the lignin molecule itself can actually be degraded in this system or whether residual free aromatic compounds and/or cellulose were responsible for the results observed. Iiyama *et al*. (1990) have demonstrated that lignin in wheat straw is covalently linked to carbohydrates through phenolic acid bridges (Figure 1.6). Although cellulose is readily degraded under anaerobic conditions the lignified cellulose molecule is highly resistant to enzymatic attack, and it has been proposed that if the lignin component could be preferentially removed this would allow greater access to cellulose for microbial degradation (Harper and Lynch, 1981). Since the lignin polymer consists of non-repetitive phenyl-propane units that are randomly linked by various C-C and ether bonds, the study of its degradation has been problematic. Researchers have thus utilised lignin model compounds that possess the structural characteristics of the lignin polymer, in order to elucidate the mechanism of is degradation (Buswell and Odier, 1987, Brune *et al.*, 1995). The degradation of lignin model compounds has not been thoroughly investigated under sulphidogenic conditions compared to methanogenic conditions.

This section will attempt to address the problem of deciding whether lignin can actually be degraded under biosulphidogenic conditions. In this regard both commercially prepared analytical grade lignin (Sigma) and lignin model compounds were used. Collaborative studies with Madikane (2002) will be reported in which the biodegradation of mono-aromatic and dimeric lignin model compounds was investigated under biosulphidogenic conditions.

4.2 Methods

Bench-scale flask reactors operated in batch mode were used and inoculated with an actively growing culture of a sulphate reducing microbial consortium from the batch reactor with pine-wood powder as substrate. The degradation of analytical grade lignin and chemically synthesised lignin model compounds was investigated.

4.2.1 Bench-scale batch reactor-biodegradation of lignin

An inoculum of 100ml was taken from the flask batch reactor used in 3.3.1 and washed to eliminate carry forward of soluble substrate to the experimental system. The inoculum was centrifuged at 9 820 xg for 10 min at room temperature (Beckman Model J2-21 Centrifuge). The pellet was re-suspended in 100 ml of Postgate’s media (section 3.3.1) and used to inoculate duplicate 250 ml flasks with each connected to a zinc acetate trap (Figure 4.1). Flask A contained only analytical grade lignin (Sigma) as substrate with no additional carbon source provided. Flask B contained analytical grade lignin and 60 µl sodium lactate (Saarchem), to determine whether a readily available carbon source was necessary to initiate the biodegradation process. Control flasks were set up containing lignin but no microbial inoculum or lactate.

4.2.2 Bench-scale batch reactor-biodegradation of lignin model compounds

The same experimental set-up and bacterial culture as applied in the lignin biodegradation study was used for the model compound study. The model compounds chosen were ferulic acid, which bears close resemblance to the monomers of lignin (Figure 1.3), ferulic acid ethyl ester, to determine if ester linkages could be cleaved under biosulphidogenic conditions, and to characterise the intermediates of ferulic acid degradation. The ferulic acid dimer was investigated to determine whether the carbon-carbon bonds could be cleaved under the experimental conditions, benzyl vanillin was investigated to determine the susceptibility of ether linkages to cleavage under bio-sulphidic conditions. Salicin was used to investigate whether the sulphate reducing microbial consortium was capable of separating the phenol component from the carbohydrate component. The Postgate medium was supplemented with the various model compounds (1 000 mg/l) as the sole carbon sources.

Figure 4.1: Batch reactor (left) connected to a zinc acetate sulphide trap (right) used to study the biodegradation of lignin and lignin model compounds. Flasks were covered with foil to prevent photosynthetic growth.

All flasks were sparged with nitrogen to maintain anaerobic conditions. Samples were drawn by syringe, while maintaining a continuous stream of nitrogen gas, to prevent entry of oxygen.

4.2.3 Synthesis of lignin model compounds

Synthesis of the model compounds was carried out in collaboration with Dr Nagabhushana, a postdoctoral fellow in the Environmental Biotechnology Group. Ferulic acid and vanillin were sourced from Sigma Aldrich as the monomers of the synthesised model compounds, with salicin obtained from Merck.

4.2.3.1 Synthesis of ferulic acid ethyl ester

Synthesis of the ferulic acid ethyl ester, to investigate the cleavage of ester linkages, was carried out according to the method of Li and Lundquist (1997). Ferulic acid (4.62 mmol), 900 mg, was dissolved in 20 ml absolute ethanol. Concenrated sulphuric acid (1-2 drops) of was added and the whole mixture was refluxed for 3 hours (Figure 4.2). The solution was then treated with a saturated solution of sodium bicarbonate in order to remove any unreacted ferulic acid followed by further extraction with ethyl acetate. After organic separation and drying over anhydrous sodium sulphate the solution was evaporated at reduced pressure to get 964 mg (93% yield) ferulic acid ethyl ester. The isolated ester was characterized by GC-MS: (rt = 19.18 min.) M^+ 222, 194, 177, 161, 150 (bp), 135, 117, 105, 89, 77, 63, 51, and ^{1}H NMR: (400 MHz, $CDCl_3$) δ 0.90 (3H, t, -CH2-CH3, J = 6 Hz), 1.50 (1H, s, br, -OH), 3.90 (3H, s, -OCH3), 4.23 (2H, q, -CH2-CH3, J = 6 Hz), 6.249 (1H, d, 1'-H, J = 16 Hz), 6.89 (1H, d, 5-H), 7.01 (1H, s, 3-H), 7.050 (1H, d, 6-H), 7.575 (1H, d, 2'-H, J = 16 Hz).

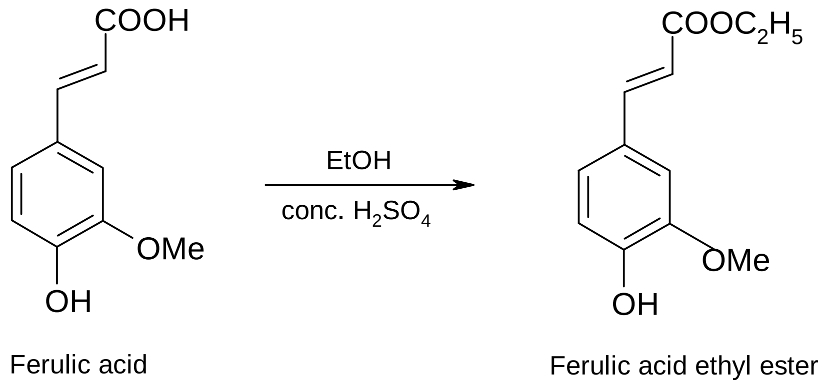


Figure 4.2: Synthesis of ferulic acid ethyl ester.

4.2.3.2 Synthesis of the ferulic acid dimer

Synthesis of the ferulic acid ethyl ester, to investigate the cleavage of carbon-carbon bonds, was carried out according to the method of Li and Lundquist (1997). 950 mg of ferulic acid (4.87 mmol), was suspended in 10 ml trifluoro-acetic acid (TFA) and stirred for 24 h at 50 °C in a round-bottomed flask (Figure 4.3). After a few hours a precipitate was observed (850 mg, 89 % yield). The precipitate was removed and washed with cold ethanol, followed by recrystallisation in ethanol. The crystal obtained had a melting point of 247 °C. The resulting crystal was characterised by ^{1}H NMR (400 MHz, deuterated DMSO) δ 2.33 (1H, dd, H-8a, J = 16, 8.4 Hz), 2.53 (1H, dd, H-8b, J = 16, 6.5 Hz), 3.36 (1H, dd, J = 9.5, 8.1 Hz, H-2), 3.76 (1H, ddd, J = 8.4, 8.1, 6.5 Hz, 1-H), 4.40 (1H, d, J = 9.5 Hz, H-3), 3.70 (3H, s, -OCH3), 3.71 (3H, s, -OCH3), 6.20 (1H, s, H-4), 6.56 (1H, dd, J = 7.6, 1.7 Hz, H-6’), 6.69 (1H, d, J = 1.7 Hz, H-2’), 6.70 (1H, d, J = 7.6 Hz, H-5’), 6.81 (1H, s, H-7).

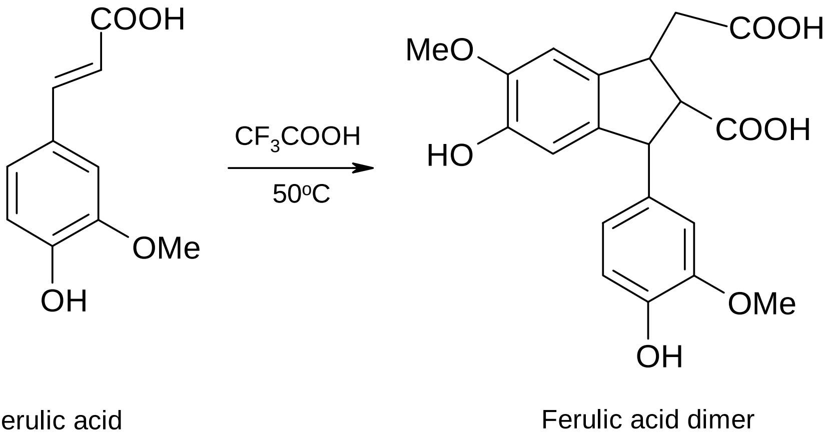


Figure 4.3: Synthesis of ferulic acid dimer.

4.2.3.3 Synthesis of benzyl vanillin

Synthesis of benzyl vanillin, to investigate the cleavage of ether linkages, was by the method of Nakatsubo *et al.* (1975). Vanillin (9.8 mmol), 1.5 g, was placed in a round-bottomed flask with 100 mg KOH dissolved in 10 ml of absolute ethanol. Benzyl bromide (2 ml) was added stepwise over 15 min and the mixture was then stirred at RT for 72 h. After cooling the mixture was extracted with ethyl acetate and washed with water to remove any inorganics. The organic fraction was evaporated and column purified using silica gel. The mobile phase used was ethyl acetate: hexane (1:5). Fractions that contained the compound were pooled and evaporated to obtain the benzyl vanillin crystals (1.7 g, 71.1 % yield) with a melting point of 61 °C. The purified compound was characterised by GC-MS: 242 (M+), 91 (bp), 77, 65, 51, 39 and ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.9(3H, s, OCH_3), 5.2 (2H, s, CH_2), 6.9 (1H, d, Ar-H), 9.5 (1H, s, CHO).

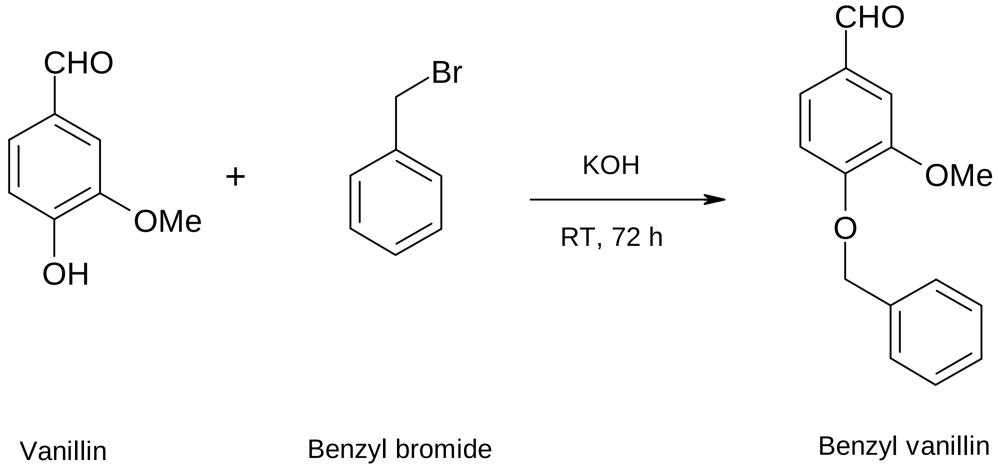


Figure 4.4: Synthesis of benzyl vanillin.

4.2.4 Sulphate analysis

Sulphate analyses for the lignin reactors were performed as outlined in 3.2.5 for the Hach protocol. Sulphate analysis for the lignin model compounds was by the HPLC method outlined in 3.2.5.

4.2.5 Analysis of aromatic compounds and lignin model compounds

Analysis of aromatic compounds was carried out according to the method outlined in 2.3.4.2. The same method was applied to analyse the lignin model compounds. The model compounds concentrations were determined from standard curves analysed on reverse phase-HPLC.

4.2.6 GC-mass spectrometry

GC-mass spectrometry was used to quantify the product formed during bacterial degradation of the lignin model compounds. The sample was prepared by treating 2.5 ml of the test sample with 250 µl of dichloromethane (DCM) followed by stirring the resulting biphasic solution on a vortex for 10 seconds. The organic layer was passed through a thin layer of anhydrous Na_2SO_4. Dry DCM solution (1 µl) was injected into the instrument. All GC-MS analyses were performed using a GCQ Mattson instrument equipped with a capillary column (J and W Scientific) with helium as the carrier gas. The temperature programme was 50 ^oC (1 min)-10 ^oC/min-250 ^oC (10 min). Products were characterised from their resultant splitting patterns, which were compared to spectrographs of standard compounds. An internal standard was not used.

4.3 Results

4.3.1 Biodegradation of lignin under sulphidogenic conditions

An analytical grade commercial lignin was used to investigate whether the adapted sulphate reducing microbial consortium was utilising lignin as its sole carbon source (Flask A) or whether a readily available carbon source, such as lactate, was required to either initiate or enhance lignin degradation (Flask B). In Flask A reduction was observed with sulphate reduced by 594.16 mg/l in 21 days. With the additional source of carbon in the form of lactate (Flask B), sulphate was reduced by only 466.9 mg/l over the same period (Figure 4.5). The additional carbon source apparently, had no significant effect in enhancing the reduction of sulphate in the presence of lignin as the main carbon substrate ($t = 1.01$, $p < 0.05$). The pH increased for both sets of flasks, which is an indicator of sulphate reduction. However, the lower sulphate reduction measured in Flask B was surprising, considering an elevation in pH of 8.54 compared to 7.11 in Flask A (Figure 4.5). The higher pH in Flask B could also be due to the increased production of alkalinity in the form of carbonate.

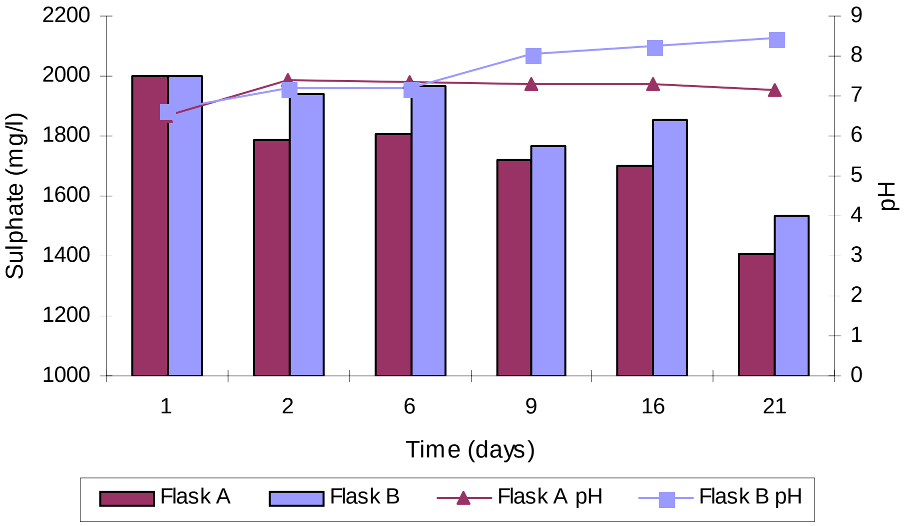


Figure 4.5: Sulphate concentration and pH for the lignin degrading bioreactors. Flask A had only commercial lignin as its carbon source and Flask B had lactate as an additional carbon source.

Release of aromatic compounds showed the opposite effect with a maximum release of 70.3 mg/l in Flask B which was significantly higher than the 47.9 mg/l in Flask A (Figure 4.6) (t=5.77, p <0.05). So although less sulphate was reduced (carbon consumed), the addition of the readily available carbon source appears to have stimulated lignin breakdown.

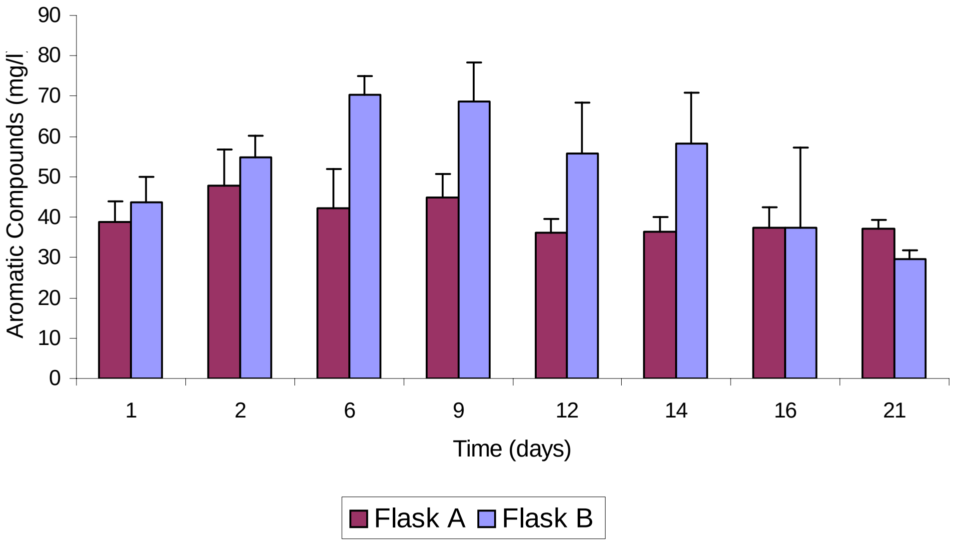


Figure 4.6: The production of aromatic compounds from Flasks A and B with lignin as substrate.

4.3.2 The biodegradation of lignin model compounds under sulphidogenic conditions

The lignin model compound study was undertaken to confirm that cleavage of the lignin molecule under anaerobic biosulphidogenic conditions could have occurred in the previous biodegradation studies. In addition the study also provided an indication of the type of chemical bonds that are susceptible to cleavage under the specified conditions.

4.3.2.1 Biodegradation of ferulic acid

Ferulic acid, added to the adapted sulphate reducing microbial consortium, was degraded completely within 25 days, and after addition of a further 1 000 mg/l ferulic acid on day 30, was again completely degraded within 25 days (Figure 4.7). Over the corresponding period sulphate was reduced from 1 800 mg/l to less than 100 mg/l, with ferulic acid as the sole carbon source (Figure 4.7). The rate of sulphate reduction changed over the course of the experiment from 58.5 mg/l/d up to day 19 to 6.25 mg/l/d up to day 30. When the additional ferulic acid was added it increased again to 18.56 mg/l/d to day 60.

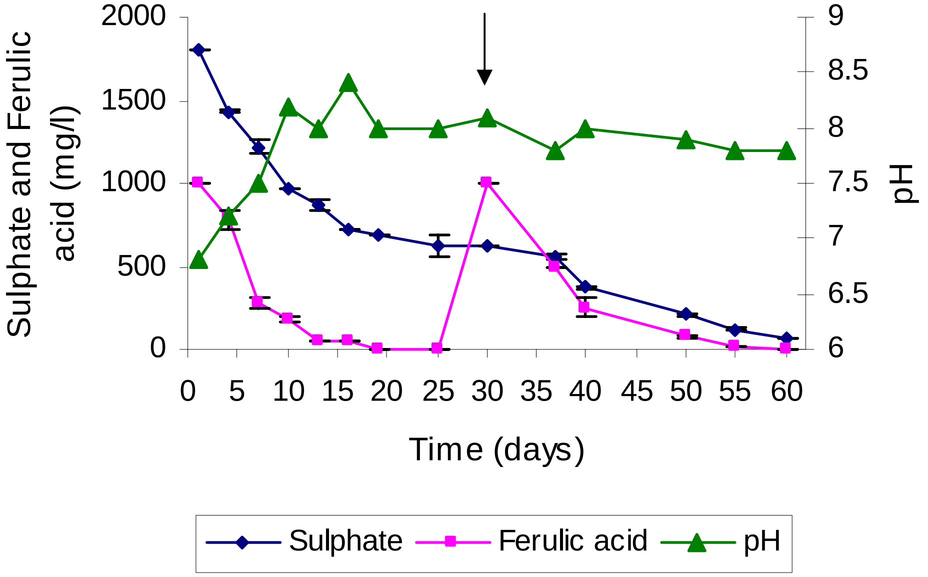


Figure 4.7: Biodegradation of ferulic acid under biosulphidogenic conditions. pH and sulphate concentrations are also shown. The arrow indicates spiking of the reactor with an additional 1 000 mg/l ferulic acid.

The pH in the reactor rose from 6.8 to 8 by day 10, indicating active sulphate reduction. After this it stabilised for the remainder of the study (Figure 4.7).

The GC-MS study showed that after 14 days the demethylation of the *O*-methyl ether unit occurred, resulting in the formation of a catechol derivative. The degradation of the catechol derivative was apparently rapid and resulted in the production of cyclohexane carboxylic acid and adipic acid (Figure 4.8). A proposed degradation pathway for ferulic acid in this system is shown in figure 4.9 and it seems reasonable to assume that adipic acid could have been converted to acetate before its consumption by the sulphate reducing microbial consortium.

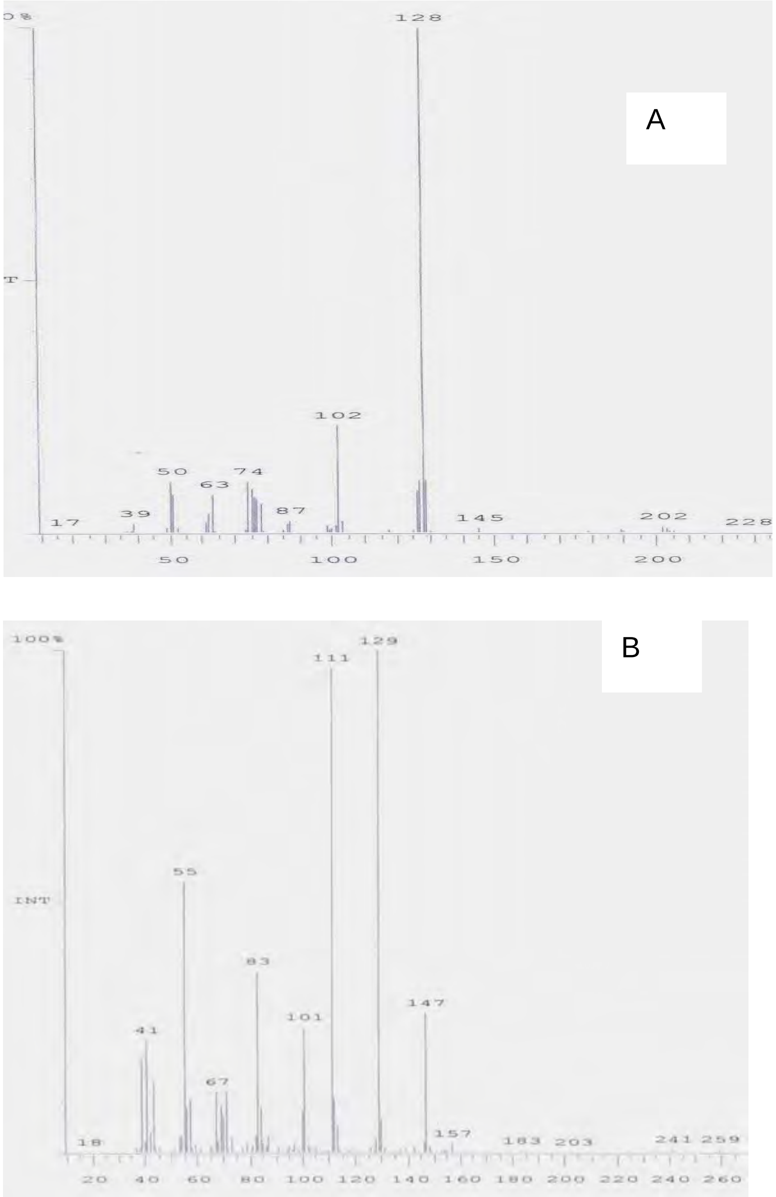


Figure 4.8: GC-MS of the intermediate products from ferulic acid biodegradation. A = cyclohexane carboxylic acid and B = adipic acid.

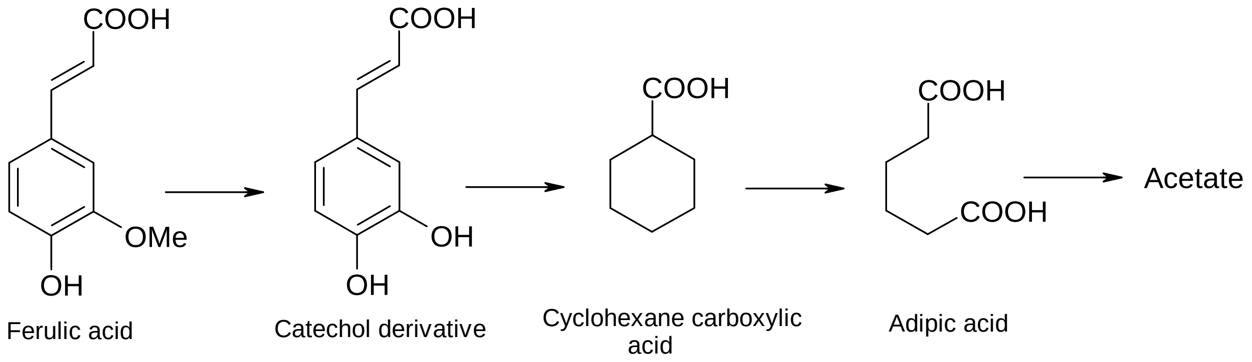


Figure 4.9: Proposed pathway for the degradation of ferulic acid under biosulphidogenic conditions.

4.3.2.2 Biodegradation of ferulic acid ethyl ester

Ferulic acid ethyl ester is not easily soluble in an aqueous medium, and was slowly distributed through the medium, as indicated by the increase in concentration of the compound from 299 mg/l to 807 mg/l between days 1 and 4. This effect was also seen after spiking at day 19 where the concentration increased from 370 mg/l to 963 mg/l between days 19 and 26 (Figure 4.10). The ferulic acid ethyl ester as sole carbon source was fully degraded within 20 days, and after spiking with an additional 1 000 mg/l of the compound, it was degraded within further 16 days. Sulphate was reduced from 2 576.5 mg/l to 177 mg/l at a rate of 36.92 mg/l/day over the 65 days of the experiment. The pH stabilised within 10 days to between 7.8 and 8 (Figure 4.10).

After 10 days the first intermediate was observed and was shown to be a demethylated catechol derivative (Figure 4.11). This catechol derivative was further degraded to cyclohexane carboxylic acid and adipic acid (Figure 4.12) with the final product assumed to be acetate by day 16 (Figure 4.13).

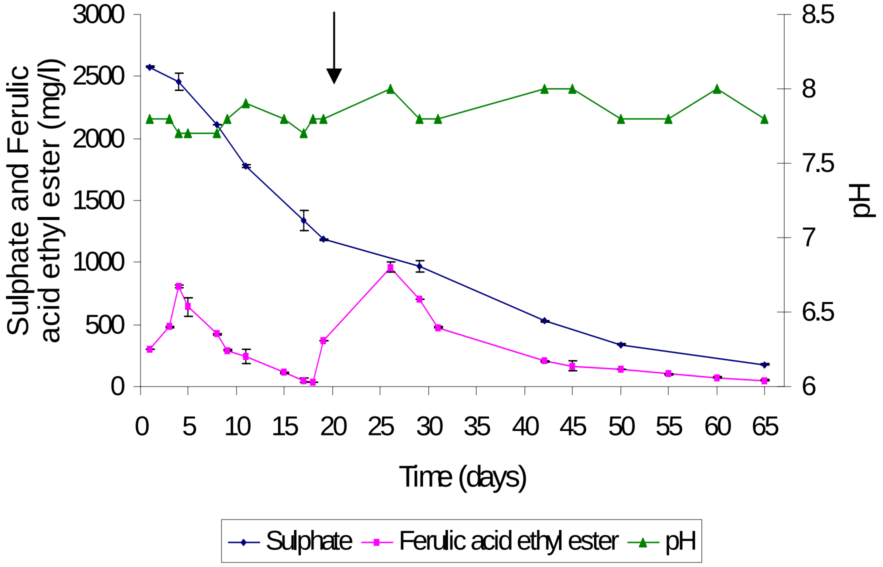


Figure 4.10: Biodegradation of ferulic acid ethyl ester under biosulphidogenic conditions, showing sulphate reduction and pH values. The arrow indicates spiking with an additional 1 000 mg/l ferulic acid ethyl ester at day 19.

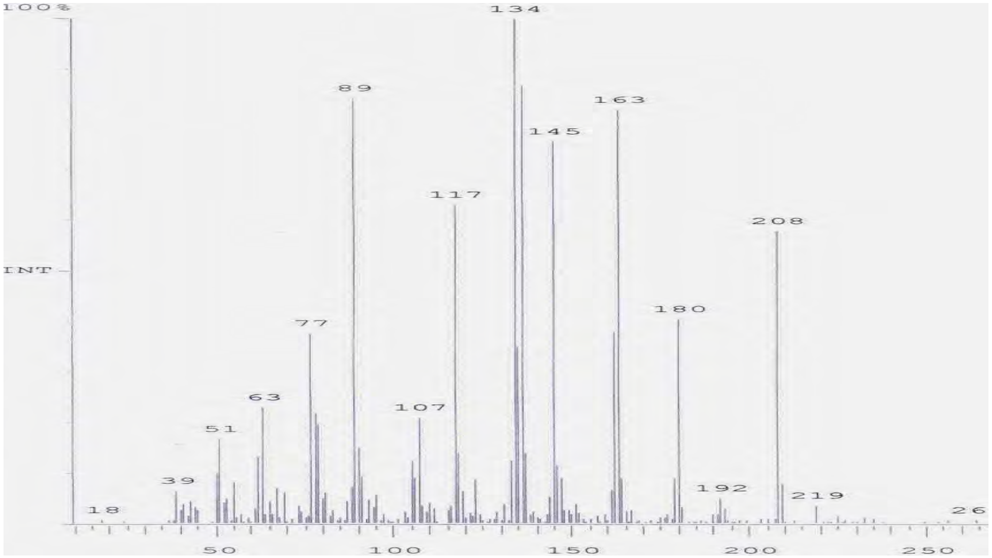


Figure 4.11: GC-MS of catechol, the first intermediate, observed after 10 days.

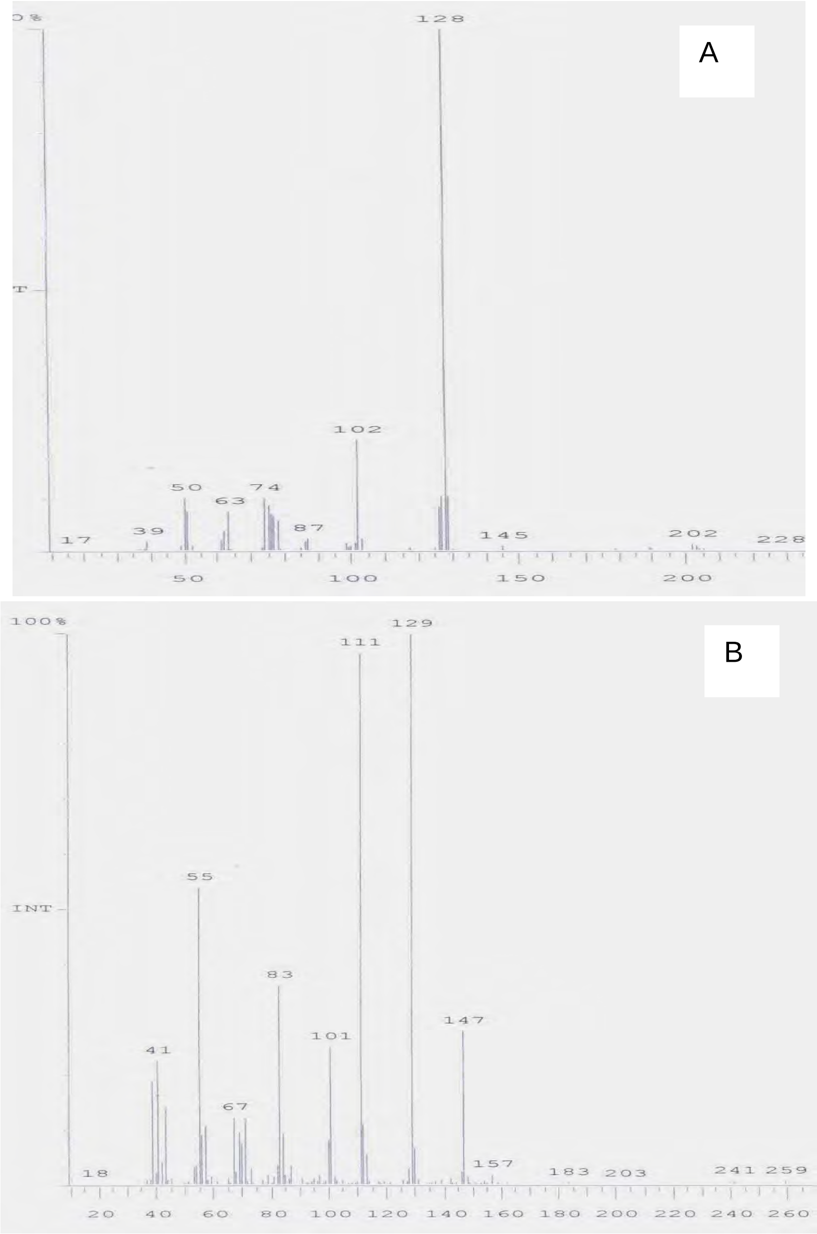


Figure 4.12: GC-MS of degradative products from the biodegradation of ferulic acid ethyl ester, observed between days 10 and 16. A = cyclohexane carboxylic acid and B = adipic acid.

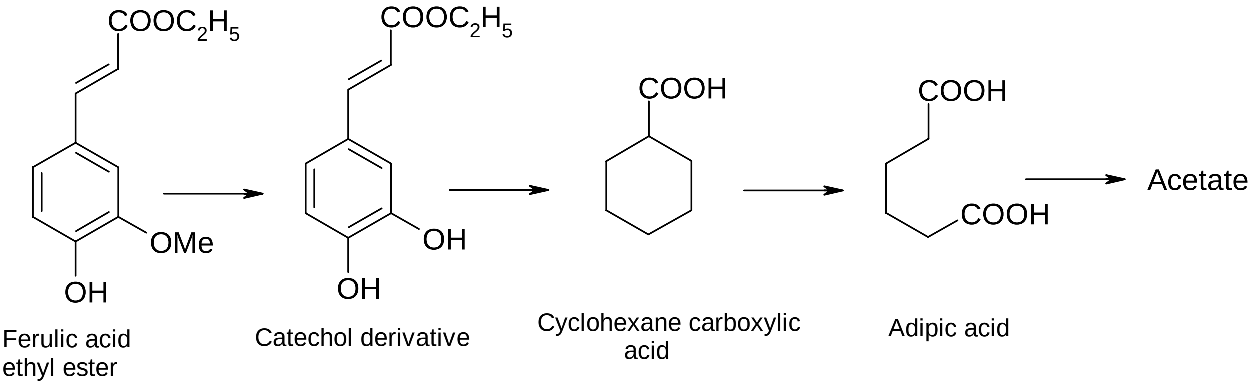


Figure 4.13: Proposed pathway for the degradation of ferulic acid ethyl ester under biosulphidogenic conditions.

4.3.2.3 Biodegradation of the ferulic acid dimer

The degradation of the ferulic acid dimer was undertaken to investigate the cleavage of carbon-carbon bonds within the lignin polymer. The ferulic acid dimer was fully degraded within 50 days (Figure 4.14), and sulphate was reduced from 2 561 mg/l to 612.8 mg/l at a rate of 38.96 mg/l/day over the 50 days of the experiment (Figure 4.14). The pH stabilised by day 25 to between 8.2 and 8.5 (Figure 4.14)

By day 10 an intermediate was observed corresponding to cyclohexane carboxylic acid (Figure 4.15 A). This was followed by degradation to adipic acid (Figure 4.15 B) and finally to acetate by day 30 (Figure 4.16).

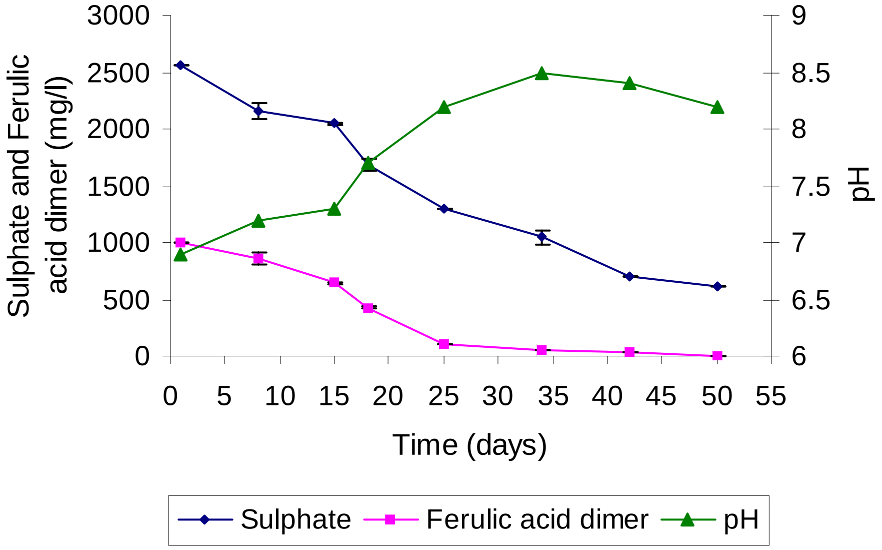


Figure 4.14: Biodegradation of ferulic acid dimer under biosulphidogenic conditions. Sulphate and pH concentrations are shown.

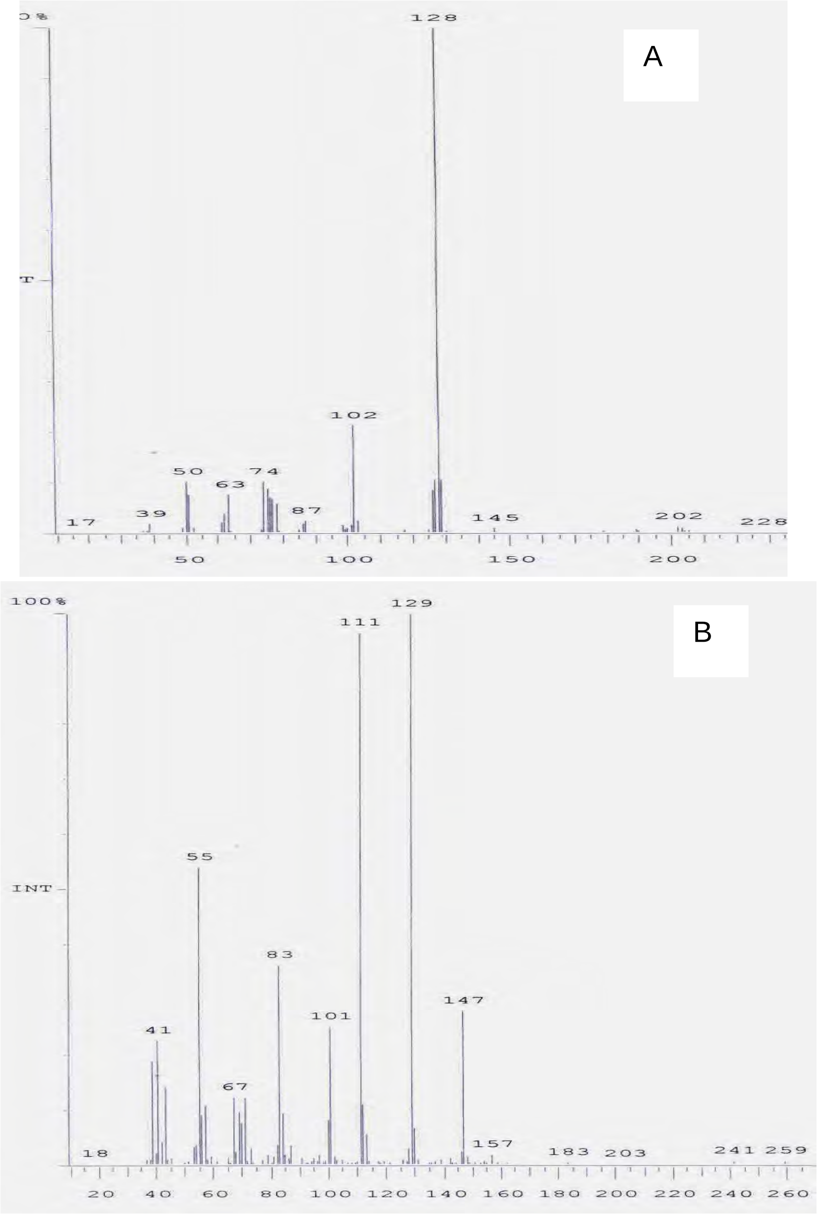


Figure 4.15: GC-MS of degradative products from the biodegradation of the ferulic acid dimer observed between days 10 and 30. A = cyclohexane carboxylic acid and B = adipic acid.

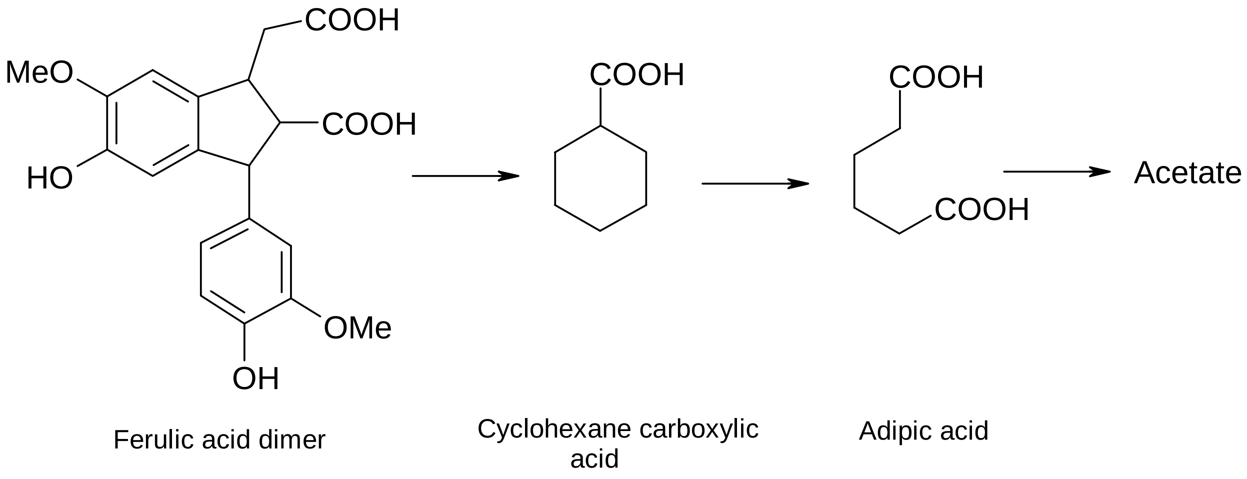


Figure 4.16: Proposed pathway for the degradation of the ferulic acid dimer under biosulphidogenic conditions.

4.3.2.4 Biodegradation of benzyl vanillin

The degradation of benzyl vanillin was undertaken to investigate the cleavage of ether linkages within the lignin polymer. Benzyl vanillin is not easily soluble in an aqueous medium, and was slowly distributed through the medium, as indicated by the increase in concentration of the compound from 295 mg/l to 783 mg/l between days 1 and 4 (Figure 4.17). Benzyl vanillin was fully degraded within 33 days (Figure 4.17). The sulphate concentration decreased from 1992.7 mg/l to 340.7 mg/l in 19 days (Figure 4.17), at a rate of 87.0 mg/l/day, from day 19 to 33 sulphate was reduced by a further 222.3 mg/l at a rate of 15.9 mg/l/day. The pH remained stable throughout the study and fluctuated between 7.7 and 8.2 (Figure 4.17).

Degradation products were observed on days 7 and 25, with the first intermediate found to be benzyl vanillyl alcohol (Figure 4.18). This was further cleaved at the ether bond to produce vanillin, which was assumed to be degraded to the final product acetate, on day 33, and then utilised by the sulphate reducing microbial consortium (Figure 4.19).

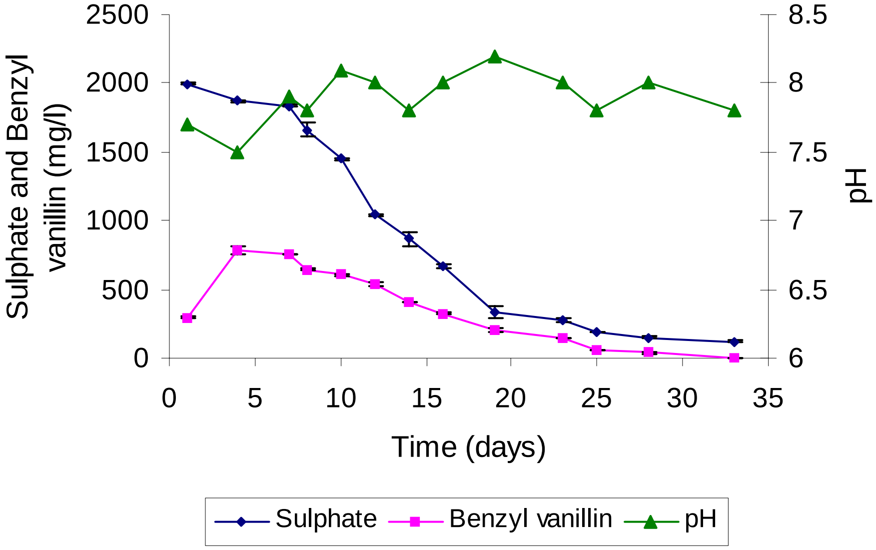


Figure 4.17: Biodegradation of benzyl vanillin under biosulphidogenic conditions. Sulphate and pH concentrations are shown.

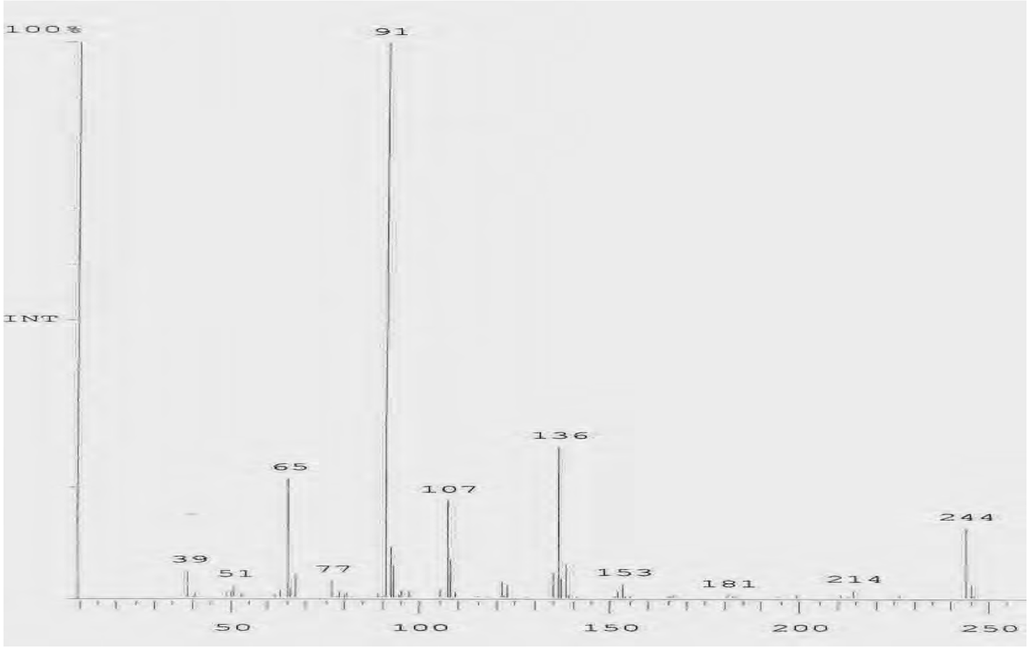


Figure 4.18: GC-MS of benzyl vanillyl alcohol observed on day 7, one of the products of benzyl vanillin degradation under biosulphidogenic conditions.

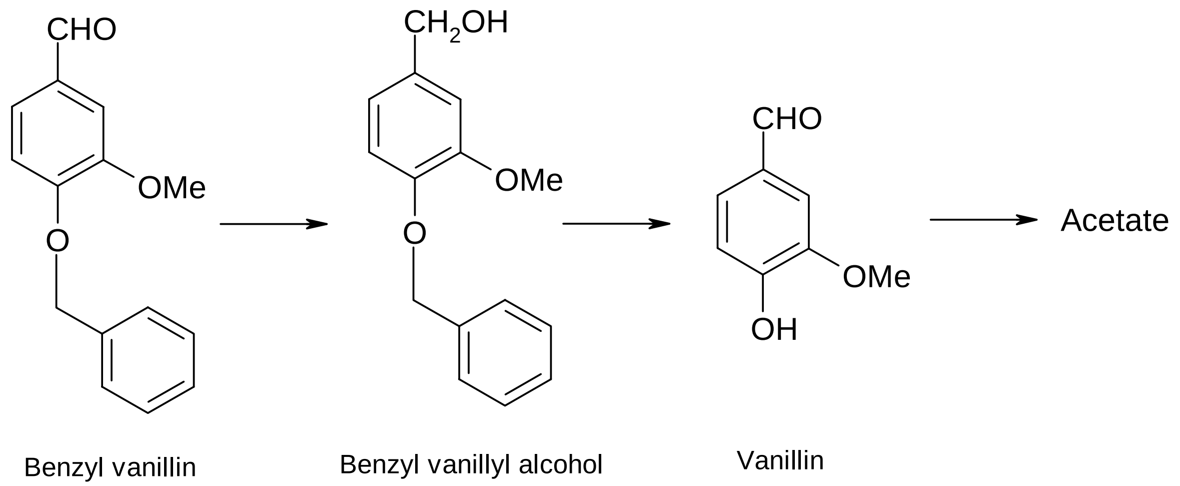


Figure 4.19: Proposed pathway for the degradation of benzyl vanillin under biosulphidogenic conditions.

4.3.2.5 Biodegradation of salicin

The biodegradation of salicin was undertaken to investigate the cleavage of the glycosidic linkage between the aromatic group and the carbohydrate, simulating the lignin-cellulose linkage in the lignocellulose complex. Salicin was fully degraded within 28 days of the study, with rapid degradation within the first 7 days (996.5 to 173.45 mg/l) (Figure 4.20). Sulphate was reduced from 1 327.5 mg/l on day 1 to 322.8 mg/l on day 49 (Figure 4.20), at a rate of 20.9 mg/l/day. The pH took 34 days to reach a pH of 8; it then fluctuated between 7.8 and 8.2 for the duration of the study (Figure 4.20).

The first degradative product, salicyl alcohol, was observed after three days, (Figure 4.21 A). This is the product of the hydrolysis of the glycosidic linkage between the phenol and the glucose moieties. The alcohol group of the salicyl alcohol was further reduced to ortho-cresol as the second intermediate by day 15 (Figure 4.21 B). After a further 30 days the ortho-cresol was degraded again presumably to acetate by day 28 (Figure 4.22).

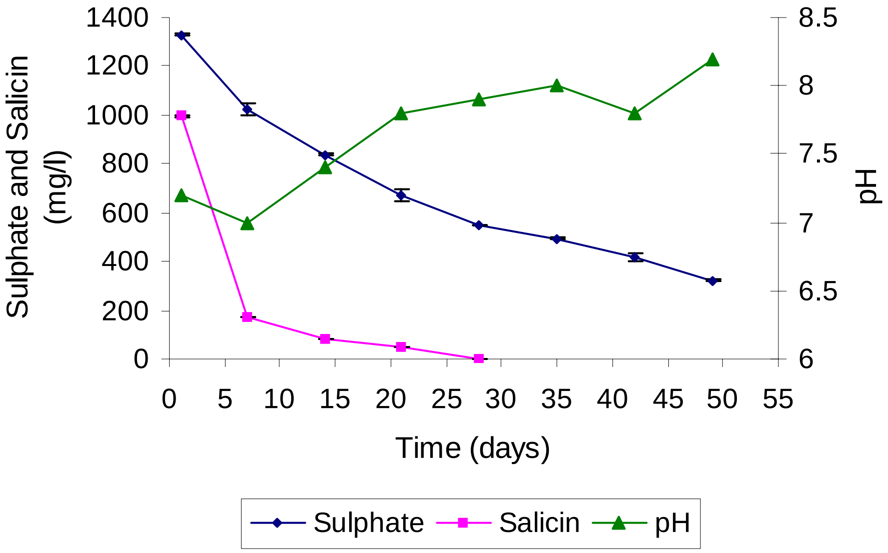


Figure 4.20: Biodegradation of salicin under biosulphidogenic conditions. Sulphate and pH concentrations also provided.

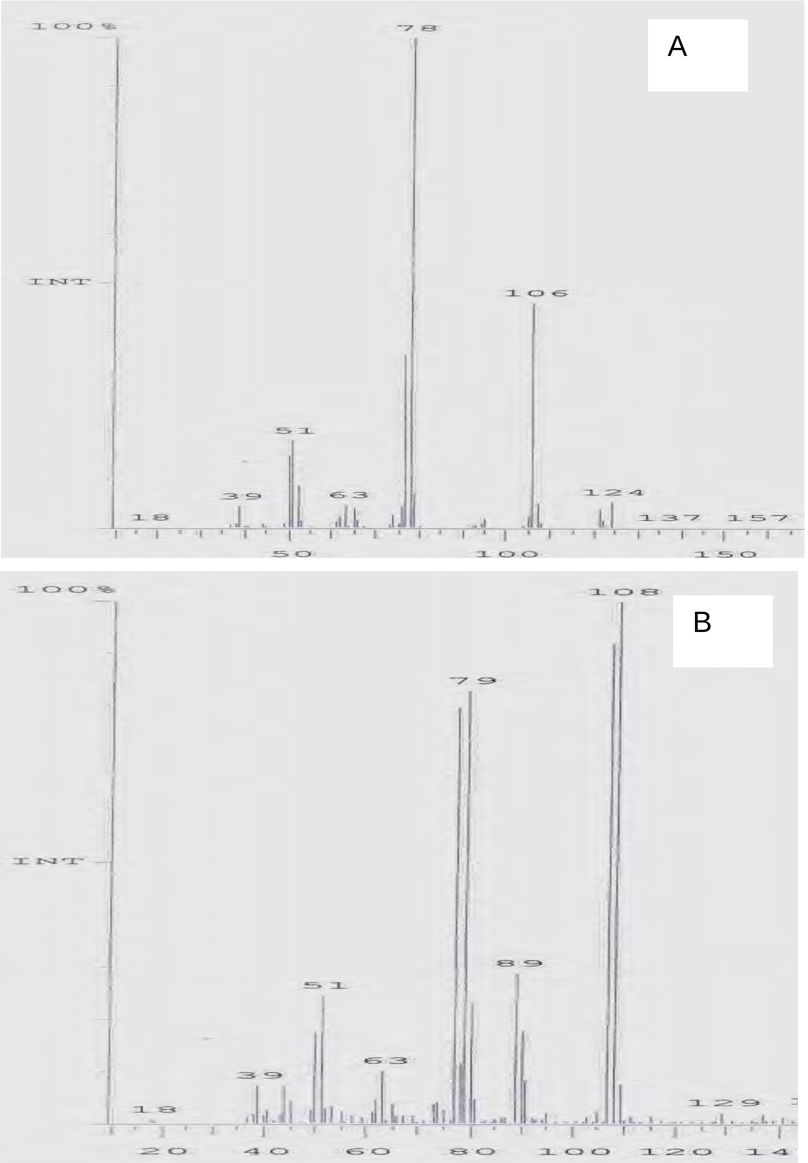


Figure 4.21: GC-MS of the degradative products of salicin. A = salicyl alcohol and B = ortho-cresol.

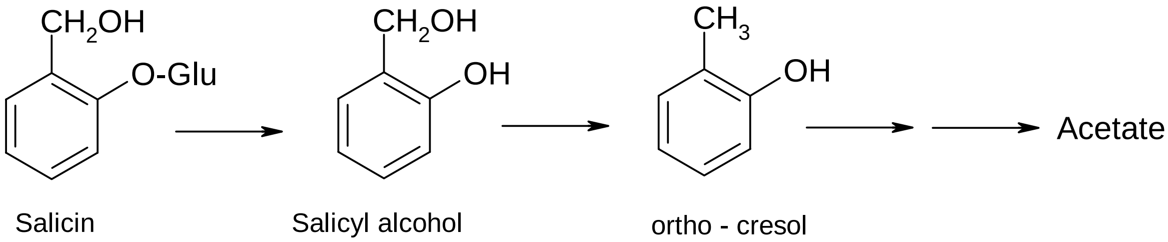


Figure 4.22: Proposed pathway for the degradation of salicin under sulphidogenic conditions.

4.4 Discussion

The results of the studies reported here have shown that aromatic compounds released from lignin, as the sole carbon source, is significantly enhanced by sulphate reducing microbial activity. This effect is significantly enhanced with the addition of lactate as a carbon source that is readily available to SRPs. Although sulphate reduction was not significantly enhanced (it was in fact lower), clearly substantial sulphide and alkalinity generation had occurred in both systems. It was shown that in the presence of lactate there was a significant increase in the release of aromatics from lignin, possibly due to the sulphate reducing microbial consortium being able to utilise the lactate for sulphate reduction, producing sulphide that exerted a chemical enhancement on the release of aromatic compounds from the analytical grade lignin.

Based on these findings the schema may be proposed:

1. The consortium used the available carbon to produce sulphide and an alkaline environment.
2. The sulphide and alkalinity enhances the release of aromatic compounds from lignin.
3. With the addition of lactate the aromatic release rate increases, indicating that the production of sulphide from reduction of sulphate plays a role in the degradation of lignin.

One of the problems encountered during the aerobic cleavage of lignin, is the re-polymerisation of the lignin polymer. During lignin hydrolysis under sulphidogenic conditions it is proposed that sulphide prevents the re-polymerisation reactions. This hypothesis is based on the *Kraft process*, a paper pulping process, which is used as a possible model for elucidating the mechanism operating under the specified conditions. The process involves mercaptation, alkaline hydrolysis and alkaline condensation of lignin. During this process it appears that the sulphide stabilizes the phenolic groups preventing the condensation of lignin, as well as aiding the cleavage of the ether bonds between the phenylpropane units that posses free hydroxyl groups. The products generated by this process are low in molecular weight and rich in phenolic groups due to ether cleavage and the removal of methoxyl groups (Figure 4.23) (Clayton, 1969).

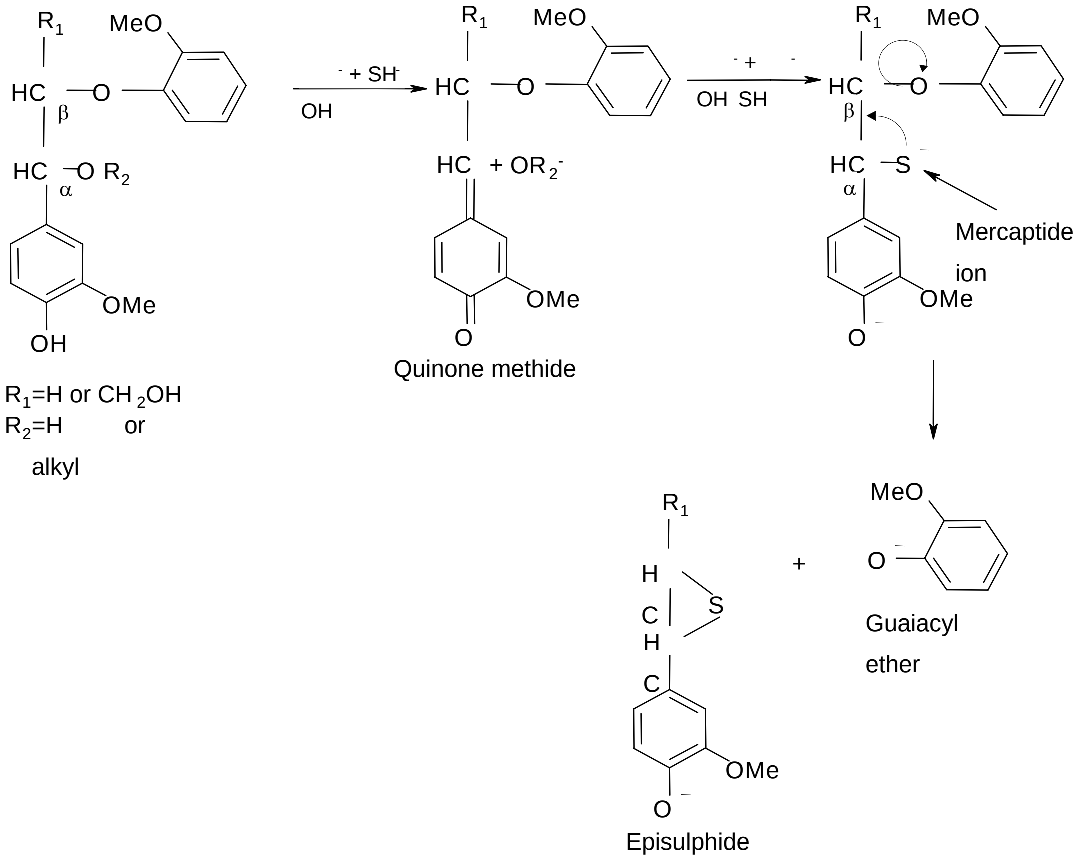


Figure 4.23: Cleavage of the ether linkages in lignin model compounds during the *Kraft process* (Clayton, 1969).

The lignin polymer is too large to enter bacterial cells either by active or passive transport mechanisms; therefore its degradation requires extra-cellular enzymes that are secreted into the surrounding medium. This is a common depolymerisation strategy catalysed by hydrolytic enzymes, however, lignin does not have repetitive linkages that allow enzymes to recognise it, or bonds that are commonly hydrolysed. Ligninolytic enzymes therefore have to have broad specificity, such as the laccases. It has been demonstrated that for the degradation of lignin, singlet oxygen or oxygen radicals are required, to initiate radical chain reactions leading to the production of low-molecular weight phenols. All known enzymes for lignin cleavage are derived from aerobic organisms, mostly fungi. No enzymes have been isolated from anaerobic species that are capable of degrading lignin (Brune, 1998). The products of lignin cleavage by fungi, oligo-lignols, can be degraded under anaerobic conditions, indicating that these bacteria do possess the enzymes necessary for the breakdown of these compounds (Colberg and Young, 1985). It would appear that the sulphate reducing microbial consortium possesses enzymes capable of hydrolysing the complex bonds linking the monomers of lignin; these enzymes would be novel and would require isolation and characterisation.

According to Breen and Singleton (1999), the ether linkages and the carbon-carbon bonds within lignin cannot be degraded anaerobically. Under anaerobic conditions oxygen is not present; this limits the options of the bacteria. The options left are hydration or hydrogenation followed by non-oxidative ring fission. It has been established that the degradation of aromatic compounds, like the model compounds investigated here, is initiated by ring reduction, followed by β-oxidation to produce aliphatic acids (Evans, 1977).

Using synthesized lignin model compounds, cleavage of these compounds was investigated in biosulphidogenic conditions. The degradation of the model compounds demonstrated the ability of the SRP consortium to utilize these compounds as a carbon source and also as electron donors. Table 4.1 shows that benzyl vanillin was the carbon source giving the largest rate of sulphate reduction (87 mg/l/day), and salicin gave the lowest rate of sulphate reduction (20.9 mg/l/day). Since both compounds have ether linkages, it is probable that the ether linkage is not the rate-limiting step. The initial reaction for the cleavage of the ferulic acid monomer was found to be de-methylation of the *O*-methyl ether that resulted in the formation of the catechol derivative. The formation of the catechol derivative was determined to be the driving force of the reaction, as it can lose its aromaticity rapidly through keto-enol tautomerism, of which the keto form is prevalent under alkaline conditions. Cleavage of the ring structure resulted in the formation of aliphatic compounds, cyclohexane carboxylic acid and adipic acid (Figure 4.9). The ferulic acid ethyl ester followed a similar degradative pathway (Figure 4.13), with the production of the catechol derivative being identified as the driving force of the reaction. In summary, the degradation of the lignin monomeric model compounds involves the cleavage of the ether bond that links the methoxyl group to the aromatic ring, reduction of the ring structure, ring fission and the production of aliphatic compounds, resulting in a monomeric end product probably acetate. β-oxidation of ferulic acid-type compounds leads to the formation of cyclohexane carboxylic acid, and reduces the aromatic nucleus completely. Adipic acid is formed by the benzene ring opening and being oxidised, followed by decarboxylation. Since both intermediates were found for ferulic acid, ferulic acid ethyl ester and the ferulic acid dimer, it implies that the biodegradation pathways are similar for these three molecules, except for the formation of the catechol derivative for ferulic acid and ferulic acid ethyl ester.

Table 4.1: Sulphate degradation rates with different lignin model compounds as carbon source.

Model compound	Rate of sulphate reduction (mg/l/day)
Ferulic acid	58.52
Ferulic acid ethyl ester	36.92
Ferulic acid dimer	38.96
Benzyl vanillin	86.95
Salicin	20.93

The degradation of the ferulic acid dimer (Figure 4.16) followed a similar mechanism as that for the monomeric lignin compounds. This compound is representative of the carbon-carbon bonds which together with ether linkages are prevalent within lignin polymer. Benzyl-vanillin ether tested whether the consortium was capable of degrading ether linkages. Under the conditions of the *Kraft process* these linkages can be cleaved. Under the conditions in the reactor, it was found that benzyl-vanillin was initially reduced to produce benzyl-vanillyl alcohol, followed by cleavage of the ether linkage to produce vanillin (Figure 4.19). In addition, the study with salicin (representing the lignin-carbohydrate complex) further supported the finding that ether linkages can be cleaved under biosulphidogenic conditions. Concurrent sulphate reduction with all the model compounds tested indicates that anaerobic conditions prevailed.

Pareek *et al.* (2001), using newspaper as a source of lignocellulose also demonstrated an increase in aromatic compounds. Using a model compound it was determined that under sulphate reducing conditions there was degradation of the model compound (containing a β-*O*-4 linkage), and that the degradation of the compound was enhanced in the presence of an additional source of carbon (avicel and newspaper). In our study the addition of lactate allowed for a 1.5 times enhanced release of lignin-derived aromatic compounds. Comparing the sulphate reduction profile to production of aromatic compounds it was observed that for the first 6 days with a decrease in sulphate there was an increase in aromatic compounds, thereafter when sulphate decreased, levels of aromatic compounds also decreased. This suggests that initially sulphate reduction lead to enhanced mobilisation of lignin, due to the production of hydrogen sulphide and alkalinity. After day 6 it appears that the aromatic compounds were utilised as electron donors for sulphate reduction. In fungal studies it has been demonstrated that an easier to assimilate carbon source (cellulose or glucose) is necessary for lignin degradation to proceed (Pareek *et al*., 2001). In soil microbe studies lignocellulose is degraded at a faster rate than a synthetic lignin, with no attached carbohydrate fraction (Buswell and Odier, 1987). One theory that has been advanced is that a co-substrate is required to provide the energy for the production of the ligninolytic enzymes, and maybe also for the production of effectors of the enzyme system (Buswell and Odier, 1987; Crawford and Crawford, 1988). Under the conditions utilised in this study, it is possible that the additional lactate substrate enhanced the production of sulphide, which in turn aided the chemical mobilisation of the lignin molecule. This would have allowed other bacteria within the consortium to produce enzymes that may be able to degrade the lignin polymer, or products of the cleavage thereof.

4.5 Conclusions

- The SRP consortia demonstrated that it is possible to utilise lignin as a carbon source and an electron donor for sulphate reduction.
- The use of an easily assimilated carbon source enhanced the degradation of lignin.
- The results for the lignin model compounds support the theory that molecular oxygen is not an absolute requirement for the degradation of lignin, and that lignin can be degraded anaerobically.
- It has also been demonstrated with the use of the model compounds that the ether linkages are not a limiting factor in the degradation of lignin, and that the glycosidic linkage between the lignin and carbohydrate fraction can be cleaved, indicating uncoupling of the lignin-cellulose complex under biosulphidogenic conditions.
- The model compounds were degraded by reductive ring cleavage, followed by ring fission and the production of aliphatic compounds.

Chapter 5

Degradation of cellulose in a biologically generated sulphidic environment

5.1 Introduction

In contrast to the aerobic degradation of cellulose, where a single species may be responsible for degradation, in anaerobic systems degradation is mainly effected by mixed populations of microorganisms (Leschine, 1995). Most research on anaerobic bacterial production of cellulases has been focused on *Clostridium thermocellum* and more recently specifically on the occurrence of the cellulosome, a multi-subunit enzyme complex (Béguin *et al.*, 1992).

C. thermocellum is a strict anaerobic gram-positive bacterium with an optimum growth temperature of 60 °C. Its preferred substrates are cellulose and cellobiose, which it ferments to ethanol, acetate, lactate, CO_2 and H_2. Cellulase activity of *C. thermocellum* was compared to that of a fungal species, *T. reesei,* and it was found that the bacterium produced similar yields but with a 50-fold lower concentration of protein (Béguin *et al.*, 1992). The reason for this highly efficient cellulolysis is thought to be the cellulosome, where synergism between the different enzyme components is optimised through the tight regulation of the components, and this is in turn determined by the structure of the complex (Shwarz, 2001).

Research in this Department has shown that alkaline sulphidic conditions enhance the activities of a range of enzymes including proteases, lipases and cellulases (Whiteley *et al.*, 2002; Whiteley *et al*., 2003 [a]). Chapter 2 of the present study had shown that after extraction of the wood powder, leaving only the lignocellulose complex, reducing sugars were still being released in the presence of sulphide. In addition in chapter 3 it was found that reducing sugar release was influenced by biologically effected sulphate reduction. The question remained whether this could be related specifically to cellulase activity or possibly due to some non-specific effects of the system. The objective of the study reported here was to determine whether cellulolytic activity could be observed in a sulphate reducing system and whether this was related to cellulosome production.

5.2 Methods

Two experimental methods were used to determine cellulase activity. The first was a direct assay for CMCase (endoglucanase) activity and the other was an indirect method using inhibitors of CMCase. Bench-scale flask reactors operated in batch mode were used in the same configuration as for the chapter 4 study (Figure 4.1). The sulphate reducing microbial consortium used for the study was prepared by enrichment of the wood powder degrading culture. CMCase was used a marker of cellulolytic activity, as it is known to form part of the cellulosome structure (Lynd *et al.*, 2002)

5.2.1 Inoculum development

Five sulphate reducing microbial consortia were compared for CMCase activity. A culture from the cell generator used to inoculate the laboratory experiments was used (C1), and based on the results obtained in chapter 3 a culture from the packed-bed column reactor was also used (C2). Three other sulphate reducing consortia were used from our laboratory (C3-5).

5.2.2 Growth of the sulphate reducing microbial consortium

In setting up the experimental system the inoculum was prepared by centrifugation at 9 820 xg for 10 min at room temperature (Beckman Model J2-21 Centrifuge), the pellet was then re-suspended in 750 ml of Postgate’s medium (section 3.3.1) in duplicate 1 000 ml flasks with each connected to a zinc acetate trap (Figure 4.1). Pine-wood powder 5 % (w/v) was added as the sole carbon source.

After testing for CMCase activity the culture was used to inoculate two different experimental systems (as per the procedure outlined above) one with only pine-wood powder as carbon source (Reactor W), and the other with pine-wood powder and 1 % (w/v) Avicel (Reactor C) to induce the production of CMCase (Matano *et al*, 1994). The reactors were monitored for 60 days, for the following parameters: CMCase activity, reducing sugar content, pH and sulphide.

5.2.3 Effect of metabolites on CMCase production

This study was undertaken to determine the effect of metabolic end products (feedback inhibition) on the production of CMCase. An inoculum from the flask batch reactor was used, and incubated as outlined above in 250 ml flasks, with differing amounts of acetate and glucose. Reactor G100 had 100 mg/l glucose, reactor G500 contained 500 mg/l glucose, reactor A100 contained 100 mg/l acetate and reactor A500 contained 500 mg/l acetate. The reactors were monitored for 9 days, as the metabolite's effect would decrease with time, due to utilisation by the consortium.

5.2.4 Method for culturing Clostridium

To determine whether *Clostridium* sp. were responsible for the production of observed cellulolytic activity, an isolation of the bacteria was undertaken. Reinforced Clostridium medium (DIFCO) was supplemented with 2 % bacteriological agar (Merck) to prepare poured plates, as well as with CMC. Streaked plates were incubated in anaerobic jars using Anaerocult® A gas packs (Merck). After gram staining and spore staining, selected colonies were picked and colonies on microscopic examination showing endospore formation were selected and inoculated into Reinforced Clostridium broth.

5.2.5 Effect of metabolic end products on production of CMCase in Clostridial cultures

Agar plates containing a reinforced clostridial media were inoculated with culture from the cell generator. The culture was subcultured twice onto fresh plates, prior to inoculating into a Reinforced Clostridium broth containing 1 % Avicel, to induce production of CMCase and the cellulosome of which it is part (Matano *et al.*, 1994). This culture was then used for the study of the effect of metabolites on CMCase activity. The metabolites used were glucose (50, 250 and 500 mg/l), acetate (50, 250 and 500 mg/l) and cellobiose (50, 250 and 500 mg/l). Glucose is a known inhibitor of CMCase (Mosier *et al.*, 1999), but not for CMCase from *Bacillus circulans* (Hakamada *et al.* 2002), acetate inhibits the growth of Clostridium, by decreasing the degree of the proton motive force (Lynd *et al.*, 2002). Cellobiose, if added in excess, inhibits CMCase (Hakamada *et al*., 2002).

5.2.6 CMCase assay

CMCase or endoglucanase activity may be assayed in the presence or absence of exoglucanase using water-soluble derivatised cellulose polymers such as CMC. After the assay period the resultant reducing sugars are determined using the DNS method outlined in 2.3.3 (Wood and Bhat, 1988).

A modified procedure of Lamed and Bayer (1988) was used for the CMCase assay:
- 50µl of a 2 % (w/v) solution of CMC in sodium phosphate buffer (pH 6.0) was added to 50 µl of the enzyme sample in a 1.5 ml Eppendorf tube.
- The enzyme reaction was initiated by placing the tube in a heating block (SMC by Elabtec) at 60° C for 30 min. and terminated by the addition of 150 µl DNS reagent (as made up in 2.3.3).
- Tubes were then mixed and heated at 100° C for 10 min in a heating block.
- 1 ml of d.H_2O was added and the tubes were read at 540 nm.
- The enzyme blank was prepared by adding DNS reagent prior to the 30 min incubation period.
- The reagent blank contained water and DNS reagent.

5.2.7 Determination of reducing sugars and aromatic compounds and sulphate

Reducing sugars were determined according to the method outlined in 2.3.3, and aromatic compounds according to the method outlined in 2.3.4.1. Sulphate was determined according to the Hach method (3.2.5) and sulphide according to the methods outlined in 3.2.6.

5.3 Results

5.3.1 Production of cellulases under biosulphidogenic conditions

Five sulphate reducing microbial cultures were tested for their potential to produce CMCase. In terms of sulphate reducing potential, C1 and C2 also showed the best sulphate reducing potential, removing 960 mg/l and 874 mg/l sulphate, respectively (Figure 5.1). Of the cultures tested C1 and C2 showed the best potential as a source of cellulase, with a maximum activity of 0.65 µmol/ml/h CMCase activity compared to a maximum of 0.40 µmol/ml/h CMCase activity in C1 (Figure 5.2). Comparing sulphate reduction and CMCase activity, C1 was the best performing sulphate reducing microbial consortium with the best CMCase activity, and was selected for use in the rest of the study.

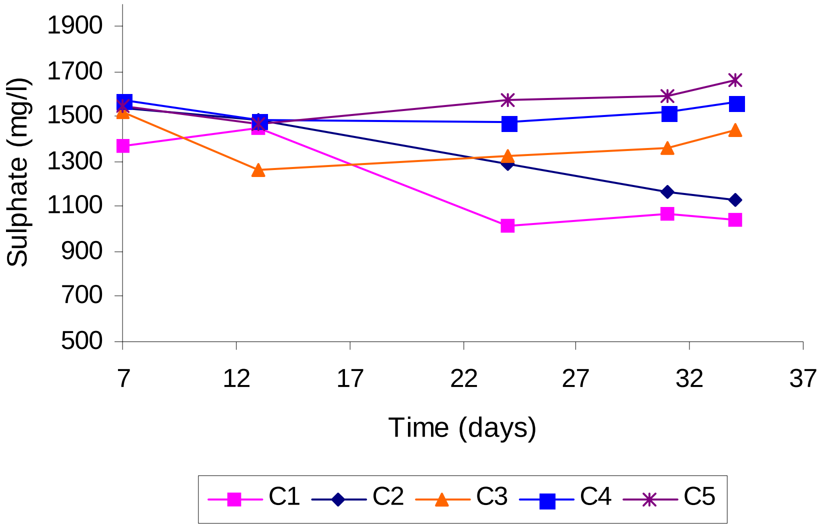


Figure 5.1: Sulphate reduction in the five sulphate reducing microbial consortia.

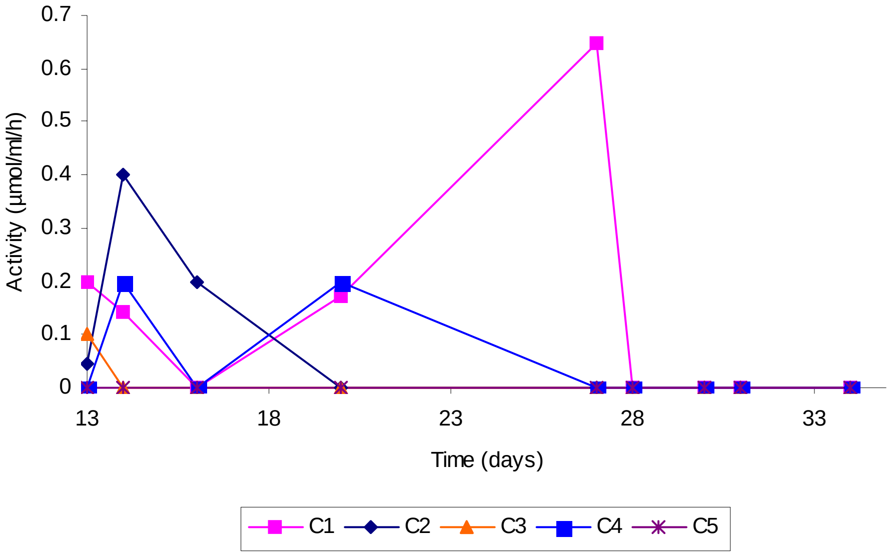


Figure 5.2: Carboxymethylcellulase activity of the five sulphate reducing microbial consortia compared.

A positive correlation was demonstrated between CMCase activity and reducing sugar release in Reactor W, containing pine-wood powder as a sole carbon source. Between day 2 and 12 of the study (Figure 5.3) reducing sugar content decreased from 0.042 mg/ml to 0.0026 mg/ml. Over the same time period CMCase activity was low. CMCase activity started to increase after the reducing sugar content had reached its lowest value. Enzyme activity then increased from 0.028 µmol/ml/h on day 12 to 0.28 µmol/ml/h on day 21 (Figure 5.3) with a positive correlation between sugar release and increasing CMCase activity (P = 0.36). CMCase activity increased from 0.15 µmol/ml/h on day 30 to 0.32 µmol/ml/h on day 37, together with a corresponding increase in reducing sugar content from 0.025 mg/ml on day 30 to 0.042 mg/ml on day 37 (Figure 5.3). This cycle of CMCase activity to reducing sugar release continued for 4 cycles, thereafter it becomes unsynchronised (day 50). Glucose is a known inhibitor of CMCase activity (Mosier *et al.*, 1999), and this is indicated by the increase in reducing sugar content (0.038 mg/l to 0.049 mg/ml) with a corresponding decrease in CMCase activity (0.31 µmol/ml/h to 0.24 µmol/ml/h) between days 52 and 56.

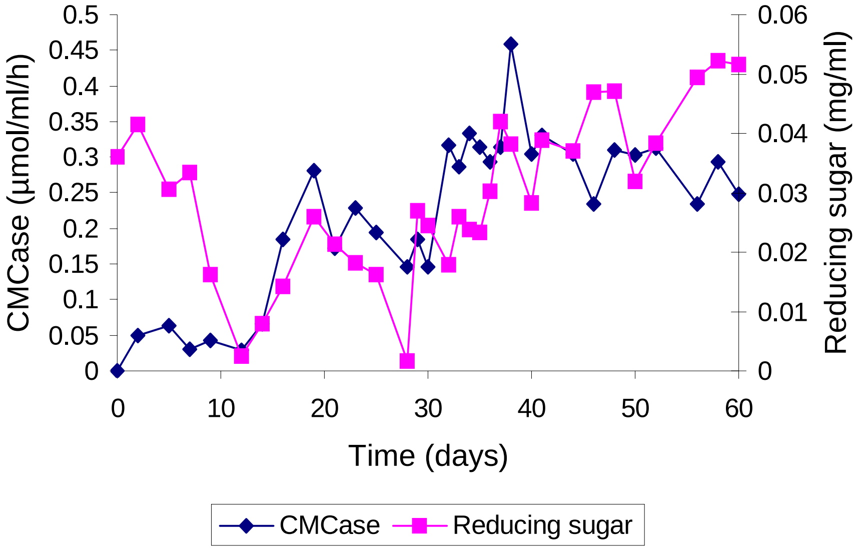


Figure 5.3: Production of CMCase and reducing sugar content in Reactor W, containing pine-wood powder as the sole carbon source.

Aromatic compound release occurring at the same time was measured and increased from 36 mg/l on day 0 to 150 mg/l on day 36 (Figure 5.4). The increase in aromatic compounds between days 1-10 correlated with the decline in reducing sugar over the same period, supporting the previous finding of lignin blocking access to cellulose.

The pH profile of this study is shown in figure 5.5 and after the first 9 days remained constant between pH 6 and 6.4.

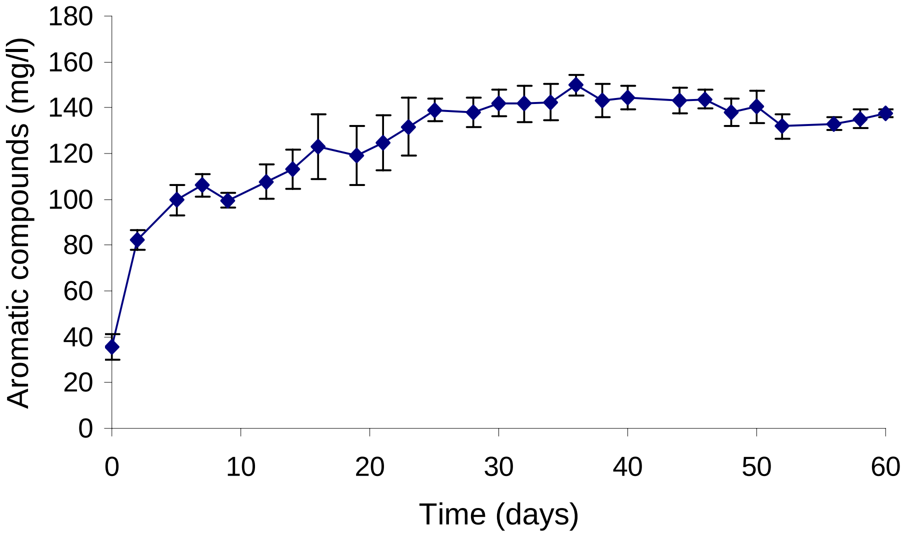


Figure 5.4: Aromatic compounds produced in Reactor W, containing pine-wood powder as the sole carbon source.

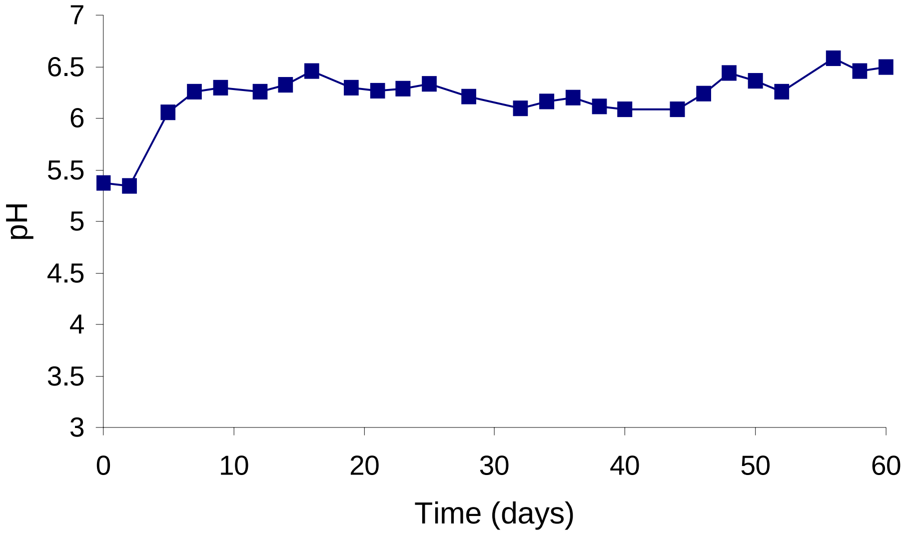


Figure 5.5: Analysis of pH for Reactor W, containing only pine-wood powder.

In Reactor C, which contained Avicel in addition to pine-wood powder, a positive correlation was once again demonstrated between reducing sugar content and CMCase activity (P = 0.476). Again it was found that a cycle of decreasing reducing sugar was followed by an increase in CMCase activity. The maximum concentration of reducing sugar found in Reactor W was 0.052 mg/ml on day 58, Reactor C had a maximum of 0.14 mg/ml reducing sugar on day 48 (Figure 5.6). In terms of CMCase activity Reactor C also showed a 1.3 times greater enzyme activity of 0.58 µmol/ml/h on day 38, compared to 0.46 µmol/ml/h on the same day for Reactor W. Both the reactors took approximately 10 days to acclimatise and produce CMCase activity (Figure 5.3 and 5.6). In addition both Reactor C and W reached their highest enzyme activities on day 38, reactor C with 0.5 µmol/ml/h, and Reactor W with 0.46 µmol/ml/h.

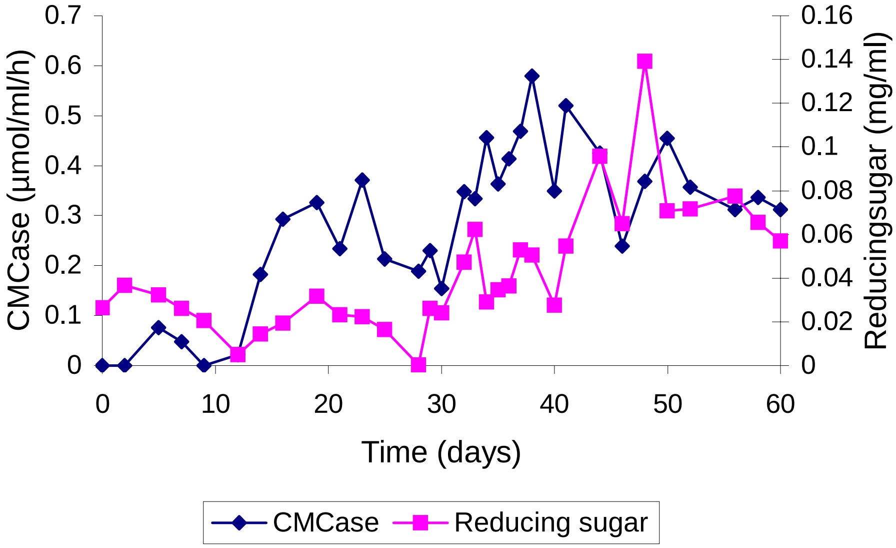


Figure 5.6: Production of CMCase and reducing sugar content in Reactor C, containing pine-wood powder and 1 % (w/v) Avicel.

The concentration of aromatic compounds in Reactor C increased from 23.6 mg/l on day 0 to 133 mg/l on day 7 (Figure 5.7). As was observed for Reactor W, there was an increase in aromatic content for the first 10 days that is correlated to a decrease in reducing sugars for the same period.

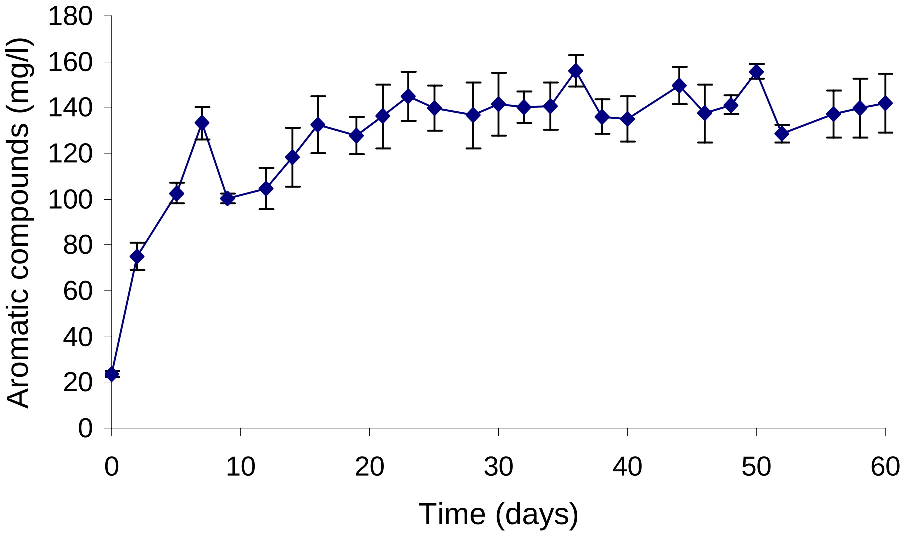


Figure 5.7: Release of aromatic compounds from Reactor C, containing pine-wood powder and 1 % (w/v) Avicel.

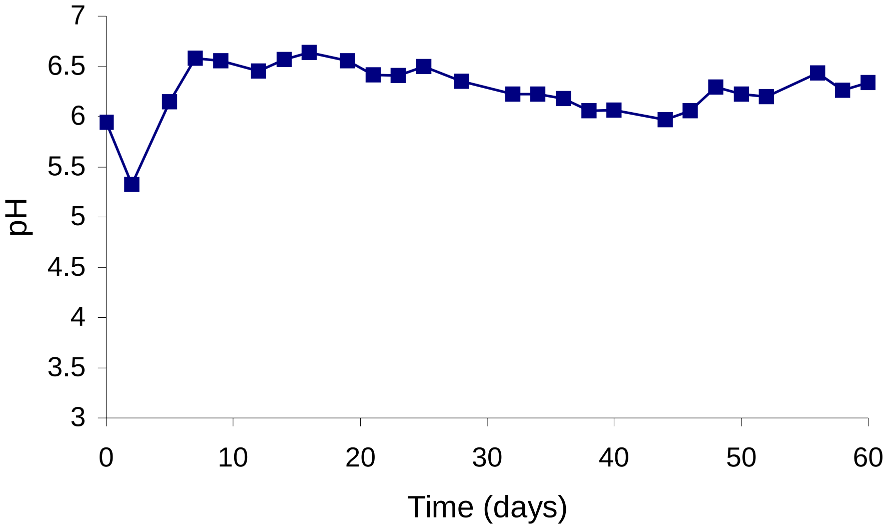


Figure 5.8: Analysis of pH for Reactor C, containing pine-wood powder and 1 % (w/v) Avicel.

The pH of Reactor C increased from 5.3 on day 2 to 6.6 on day 7 (Figure 5.8) and then fluctuated between 6 and 6.6 until the end of the study.

5.3.2 Effect of metabolites on cellulase production under biosulphidogenic conditions

With the addition of excess glucose to the reactor environment results in figure 5.9, indicates that CMCase activity is enhanced over the first 3 days of the experiment, and is then inhibited. G500 demonstrated less inhibition of CMCase activity than G100, with 63.5 % and 23.3 % inhibition on day 5, respectively, which was surprising, considering that it was expected that a greater amount of inhibitor would inhibit the enzyme more effectively. The reducing sugar content of G100 and the control were similar, except for day 7 when G100 exceeded the control. G500 showed a lower reducing sugar content than the control and G100 throughout the period of the study (Figure 5.10).

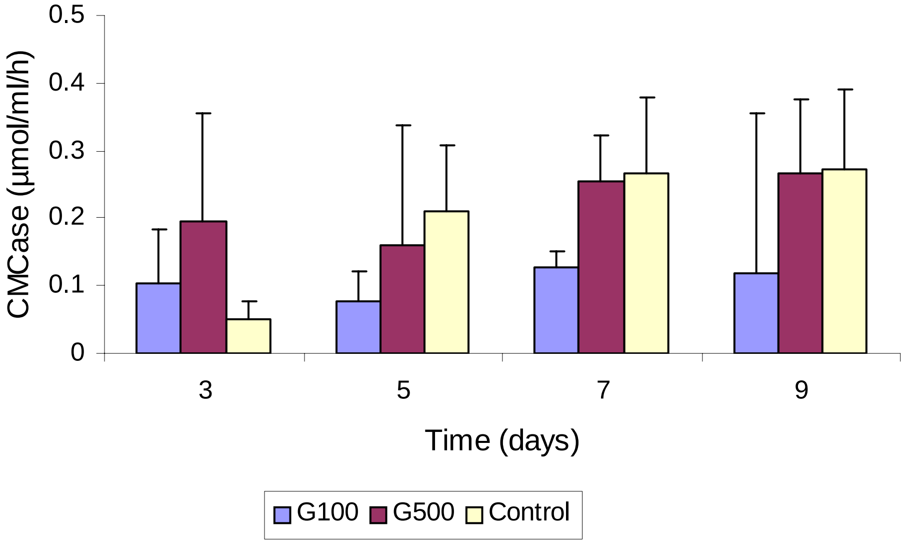


Figure 5.9: CMCase activity in the presence of excess glucose. G100 = 100 mg/l glucose, G500 = 500 mg/l glucose. The control contained only SRP culture and 5 % (w/v) pine-wood powder.

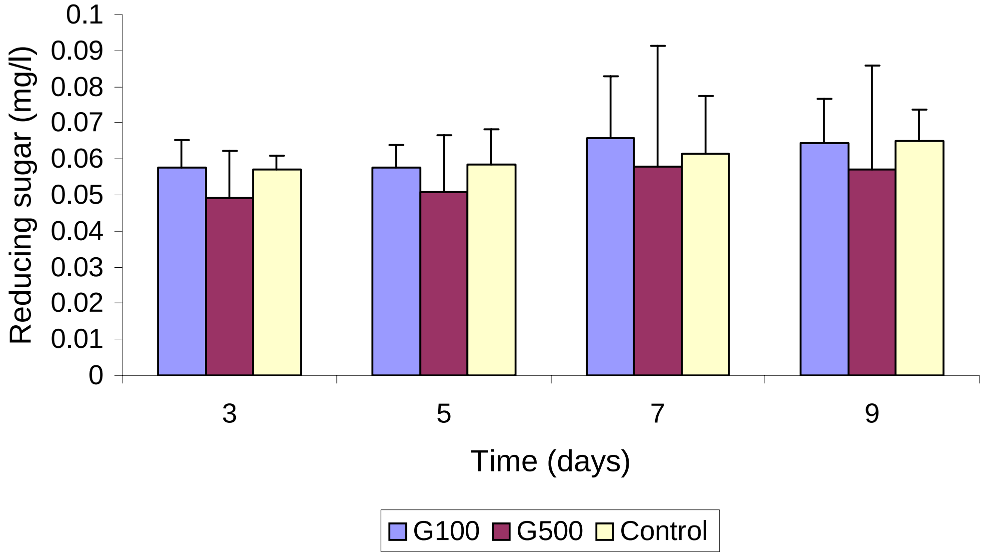


Figure 5.10: Reducing sugar content of reactors in the glucose inhibition study. G100 = 100 mg/l glucose, G500 = 500 mg/l glucose. The control contained only SRP culture and 5 % (w/v) pine-wood powder.

With the addition of acetate to the reaction system A500 appeared to enhance the CMCase activity for the first 7 days of the study. Inhibition was only demonstrated on day 9, with 10.3 % inhibition observed (Figure 5.11). A100 demonstrated an enhancement of activity over the first 3 days, followed by a progressive increase in inhibition over the period of the study, with 76.0 % inhibition by day 9 (Figure 5.11).

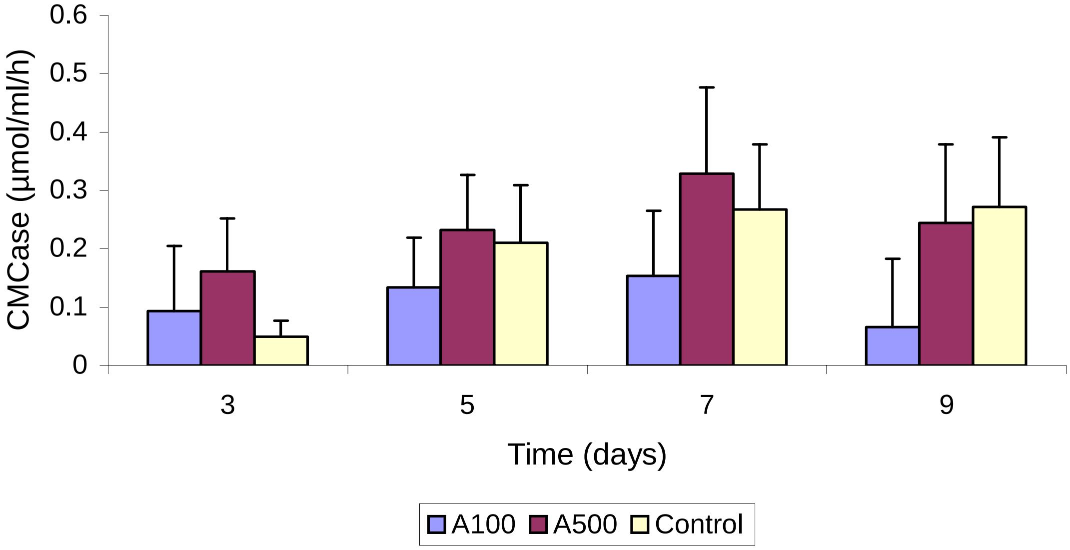


Figure 5.11: CMCase activity in the presence of acetate where 100 and 500 mg/l were added. A100 = 100 mg/l acetate, A500 = 500 mg/l acetate. The control contained only the SRP media and 5 % (w/v) pine-wood powder.

5.3.3 Effect of metabolites on cellulase production in Clostridial cultures-isolated Clostridium culture

After growth in Reinforced Clostridial Broth containing 1 % Avicel, to induce production of cellulolytic enzymes (Matano *et al.*, 1994). Glucose, acetate and cellobiose were added to the Clostridium culture medium. The highest inhibition was obtained with cellobiose (Table 5.1). Plotting the inhibition against cellobiose concentration (Figure 5.12) a direct relationship can be seen is between the concentration of cellobiose and the amount of remaining CMCase activity. Glucose and acetate both demonstrated inhibition at a concentration of 250 mg/l.

Table 5.1: Effect of metabolites on the activity of CMCase over a 48 hour period. Results are for a triplicate study, with the standard deviations shown in brackets.

Metabolite (mg/l)	**CMCase (µmol/ml/h)**		**Inhibition[#]**	
	12 hours	**48 hours**	**12 hours**	**48 hours**
CONTROL	**0.201 (± 0.0591)**	**1.0945 (± 0.353)**	**-**	**-**
Glucose 50	0.211 (± 0.225)	1.665 (± 0.268)	-	-
250	0.13 (± 0.0867)	1.664 (± 0.747)	64.7	-
500	0.291 (± 0.135)	0.882 (± 0.532)	-	80.6
Acetate 50	0.305 (± 0.190)	2.031 (± 0.576)	-	-
250	0.0450 (± 0.0535)	1.461 (± 0.435)	22.4	-
500	0.229 (± 0.317)	2.206 (± 0.317)	-	-
Cellobiose 50	0.144 (± 0.127)	1.191 (± 0.723)	71.6	-
250	0.0852 (± 0.132)	1.539 (± 0.0558)	42.4	-
500	0.0237 (± 0.0580)	2.017 (± 0.640)	11.8	

#: Expressed as the percentage of remaining CMCase activity.

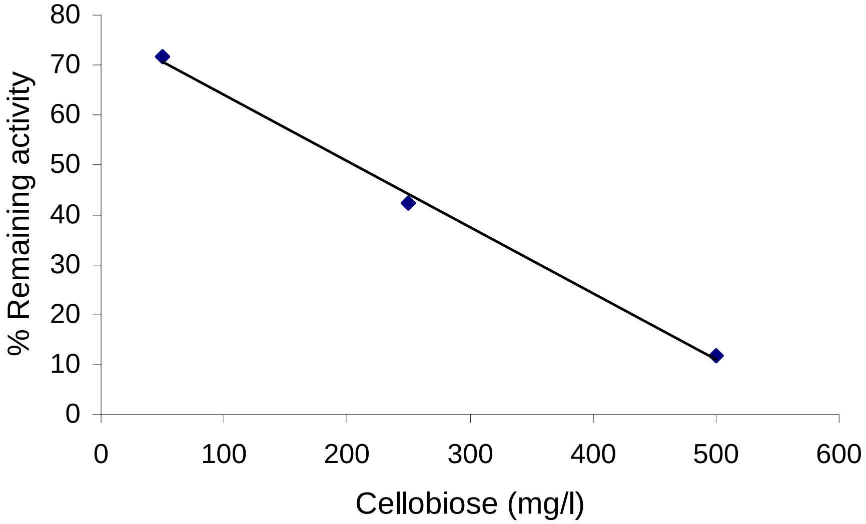


Figure 5.12: Inhibition of CMCase activity by cellobiose (r^2 = 0.99).

5.4 Discussion

The enzyme chosen for this investigation was CMCase (endoglucanase) because of the presence of cellulose in the substrate, and will provide an indication of the role that cellulose digestion plays in the system. CMCase is a well-documented enzyme in studies on the hydrolysis of cellulose. It is also part of the multi-unit cellulosome, found in anaerobic environments. The sulphate reducing microbial consortium used in the previous studies was the culture of choice, but there were four other sulphate reducing cultures in the laboratory, and these were also tested to determine which would be the best culture to use. From the results obtained it was determined that culture C1, from the packed-bed column reactors, was the best culture.

The results obtained indicated the presence of CMCase, and that it can be produced in the sulphate reducing microbial consortia. Avicel was added to induce production of CMCase, as it is known that the presence of crystalline cellulose (Avicel) can induce the production of the complete cellulosome (Matano *et al.*, 1994). The presence of the Avicel did not appear to have a significant effect on the production of CMCase, but Reactor C did have a 1.3 times greater CMCase acitivity than reactor W, as well as a higher production of reducing sugars. The increased production of reducing sugars could have been due to the readily available cellulose present in reactor C.

Lignin restricts the consumption of cellulose within anaerobic environments, and in order for anaerobic organisms to utilise cellulose, they may have developed techniques to get around lignin. *Clostridium* sp. have been shown to have enzyme activities other than cellulolytic, such as feruloyl esterase and cinnamoyl esterase (McSweeney *et al.*, 1999 and Blum *et al.*, 2000). Both these enzymes are capable of cleaving the ester bond between the polysaccharide and lignin components of lignocellulose, which is particularly important in anaerobic environments because of the recalcitrance of lignin. In figures 5.4 and 5.7 it can be seen that aromatic compounds were released, although it is probable that these aromatic compounds were derived from the extractive portion of the wood powder. The release of aromatic compounds was observed to precede the release of reducing sugars (Figures 5.3, 5.4, 5.6 and 5.7). This suggests that the removal of lignin is central to the utilisation of cellulose. A possible reason for not detecting the consumption of aromatic compounds is the possible cleavage of the ester linkages between the lignin and polysaccharide components, allowing for greater access to cellulose by the cellulolytic bacteria for the preferential use of cellulose.

Figure 5.9 and 5.11 show enhanced activities for CMCase over the first 3 days, that decreases to values lower than the control by day 9, with 100 mg/l glucose reducing CMCase activity to 44 % of the control and 100 mg/l acetate reducing it 24 % of the control. Acetate has been shown to inhibit Clostridial growth by reducing the level of transmembrane proton motive force (Lynd *et al.*, 2002). Gehin *et al.* (1996) showed by dialysis that if the metabolic end products are removed there is a 10-fold increase in biomass, in addition it was determined that cellulase activity followed biomass growth, and it can be assumed that there is a correlation between biomass formation and cellulase activity. The experiment also demonstrated that catabolite repression is the mechanism utilised for cellulase regulation (Gehin *et al.*, 1996). The importance of this observation to the system being studied here is that when there is an excess of hydrolysis products the enzyme system is repressed, and is induced when the products have been metabolised. This is observed in the cyclical production of CMCase observed in figures 5.3 and 5.6. A possible reason for the enhanced activities during the first three days could be due to enhanced growth of bacteria, other than Clostridia, within the consortium because of the easily available carbon source. The enhanced growth would have lead to a greater production of enzyme, which is evident in the greater enzymatic activities. This enhanced activity would lead to increased hydrolysis products that would in turn inhibit the CMCase activity at a later stage. This is seen in the decrease in CMCase activity by day 5 of the glucose-supplemented reactors (Figure 5.9) and day 9 of A500 (Figure 5.11).

Within anaerobic environments it is known that there are synergistic interactions among various species. Within communities degrading cellulose, the products of the hydrolysis of cellulose, are made available to other non-cellulolytic organisms as carbon and energy sources. There have been previous investigations on cellulose degradation by co-culturing cellulose degrading bacteria with syntrophic bacteria. It was found that the co-culture in all experiments was more efficient at degrading cellulose than the mono-culture (Leschine, 1995). The effect of sulphide on the activity of CMCase would need to be investigated further, by isolating the enzyme and subjecting it to various concentrations of sulphide. The Environmental Biotechnology Group has looked at the effect of sulphide on various enzymes from sulphate reducing microbial consortia and found that sulphide in biosulphidogenice systems does enhance the activities of certain enzymes (Whiteley *et al.*, 2002; Whiteley *et al.*, 2003 [a,b]).

Production of free glucose was inhibited by the presence of cellobiose in the Clostridial growth media (Table 5.1). Matano *et al*. (1994) also observed this, in an investigation into different growth media for the assembly of cellulosomes from *C. cellulovorans*. CMCase cleaves cellulose to form cellobiose units; it is possible that the product of the reaction, if in excess inhibits the action of the CMCase. CMCase acts in concert with ß-glucosidase, which cleaves cellobiose to free glucose. By 48 hours the inhibition of CMCase had ceased, presumably all the cellobiose had been metabolised, allowing for production of CMCase to proceed. It would appear from this study that inhibition is not due to catabolite repression, as there is still a residual CMCase activity observed, and that maybe feedback inhibition is the mechanism of action, where the product of the reaction inhibits the enzyme, if in excess.

Cellulase production, in the form of CMCase, has been demonstrated within the SRB consortia, and during bacterial growth in Clostridial media, after subculturing. Based on the formation of endospores and gram stain results, it is probable that Clostridia are the source of CMCase within the sulphate reducing microbial consortia used in this study. Clostridia are not the only anaerobic microorganisms to produce the cellulosome, and it is probable that the other known anaerobic species may be present; molecular microbial ecology studies are currently being undertaken to determine the species present within the consortium.

5.5 Conclusions

- Cellulases may be produced within a sulphidogenic reactor with wood powder as a carbon source.
- The addition of avicel did not have a significant effect on the production of CMCase, but did impact on the activity of CMCase.
- Cellulolytic bacteria were cultured in a Clostridial growth medium from the SRB consortia, demonstrating that Clostridia are probably the effective agents within the consortia for the production of cellulolytic enzymes.
- Excess cellobiose is an effective inhibitor of CMCase.
- It would appear that esterases are also present within the system, separating the aromatic component from the carbohydrate component, but this needs further elucidation.

There is a need for further study on the enzymology of this system, particularly the enzymology of lignin uncoupling from cellulose and the hydrolysis of lignin.

Chapter 6

The degradation of lignocellulose in a biologically-generated sulphidic environment and the development of a passive treatment technology for AMD remediation

Passive treatment of AMD, utilising an organic matrix that serves as both a support and electron donor and carbon source for microbial consortia, is limited by the amount of reducing equivalents within the matrix. Once these reducing equivalents have been exhausted, the sulphate reduction within the reactor ceases (Tsukamoto *et al.*, 2004). This has been observed in passive treatment systems utilising lignocellulose as carbon source for the treatment of AMD but with a decrease in sulphate reduction occurring while a major fraction of the lignin and cellulose remains in the system. This is one of the main factors constraining large-scale implementation of this technology (Pulles *et al.*, 2001).

Lignocellulose has been extensively investigated as a potential carbon source for sulphate reduction and AMD treatment (Wakao *et al.*, 1979; Eger and Lapakko, 1988; Dvorak *et al.*, 1992; Bechard *et al.*, 1994; Pareek *et al.*, 1998; Chang *et al.*, 2000). However, it remains unclear as to why the decrease in sulphate reduction occurs, whether the core lignin and cellulose contribute at all to reducing equivalents within the process, or whether this relates to the exhaustion of the easily extractable components of wood tissue, leaving the lignocellulose complex undegraded.

The questions included the following.

- To determine if the readily extractable material identified for the current study, provided the only source of electron donors for sulphate reduction?
- Is the lignocellulose complex degraded in these systems and does it contribute electron donors for sulphate reduction?
- What role do the chemical and biological components of the sulphate reduction system play in the degradation of wood tissue?
- Are lignin and cellulose degraded under biosulphidogenic conditions?

In studies on the chemically generated sulphidic environment it was shown that sulphide and alkalinity contributed to an enhanced release of reducing sugars and aromatic compounds, especially for whole pine-wood powder, where a 6.7 times greater release of reducing sugars was observed, compared to water alone. Aromatic content displayed a similar trend, with a two-fold greater release than in the presence of water alone. The release of both components was lower with each wood treatment, indicating the recalcitrance of lignocellulose to degradation. Using a buffered system, to simulate self-regulating conditions within a biosulphidogenic environment, it was found that there was a greater release of aromatic compounds compared to that of reducing sugars (Table 2.1). The results also suggested a relationship may exist in the release of the aromatic content and the carbohydrate fractions, which is indicated by the rise in reducing sugar content when there was a commensurate decrease in aromatic compound content (Figure 2.15 and 2.17).

In order to determine whether the observed enhancement of soluble product release from wood tissue was also related to the lignocellulose complex, a number of fractions were prepared and release of reducing sugars and aromatic compounds was compared under similar conditions. Although the quantity of reducing sugars and aromatic compounds released from the depectinated and lignocellulose complex samples was lower, sulphide and alkalinity nevertheless still seemed to enhance release of reducing sugars and aromatic compounds in these fractions. This result suggested that the lignocellulose complex may well be degraded as long as sulphide/alkaline conditions were being generated.

In the studies on biologically generated sulphidic conditions it was shown that in the presence of the microbial consortium there is a greater release of reducing sugars and aromatics than under chemically generated sulphidic conditions alone. Biosulphidogenesis enhances the release of lignocellulose, indicated by the 2-fold increase in released organic compounds (Table 3.5). Figure’s 3.8 and 3.9 again suggested a cycle of release and consumption of hydrolysis products in the flask batch reactor. Figure 3.22 shows that the release of reducing sugars is inversely related to the release of aromatic compounds, with aromatic compounds being released first. This phenomenon was noted in all the reactor systems, indicating that lignin has to be removed from the cellulose fraction. The three scenarios selected for biologically generated sulphide (flask batch reactor, bench-scale continuous reactor and upflow packed bed column reactors), demonstrated that the lignocellulose complex in the wood tissue was at least partially degraded and could serve as a carbon source and electron donor for sulphate reducing microbial consortia.

Having shown that the wood material and various of its fractions could be degraded under biosulphidogenic conditions, it was necessary to confirm that the hydrolysis of lignin was possible under these conditions. It had previously been stated that the lignin polymer cannot be degraded anaerobically, and that the carbon-carbon bonds and ether linkages can only be cleaved under aerobic oxidative conditions, utilising single oxygen and hydroxyl radicals

(Brune *et al.*, 1995; Breen and Singleton, 1999). The degradation of analytical grade lignin was studied in the presence and absence of an additional carbon source in the form of lactate, and an increase in degradation products was observed in both. It was found that the additional carbon source did not enhance sulphate reduction; however, it did lead to an increase in pH (Figure 4.5), and a significantly greater release of aromatic compounds (Figure 4.6). The results suggested that lignin can be metabolised in biosulphidogenic systems. This study showed that the mechanism involved may be somewhat akin to the *Kraft process*, suggesting that lignin is the primary target of sulphide and alkalinity within the lignocellulose system.

In order to confirm the observation of biosulphidogenic lignin degradation, a study of model compound breakdown was undertaken. A number of compounds were chosen to represent particular bonds occurring within lignin, and which would have to be cleaved in order for the lignin degradative reactions to proceed. The results of the lignin model compound study suggest that under anaerobic biosulphidogenic conditions the carbon-carbon, glycosidic and ether linkages of lignin can be cleaved and that the observation by Breen and Singleton (1999), that oxidative conditions are necessary for the cleavage of the bonds within lignin, may be incorrect. All the compounds investigated were metabolised by the sulphate reducing microbial consortium to acetate, which is used as an electron donor and carbon source by SRP.

Avicel was used as a model substrate of crystalline cellulose to investigate cellulose degradation by the appearance of CMCase (part of the cellulosome enzyme complex) (Broda *et al.*, 1995). The reason for selecting CMCase (endoglucanase) as an indicator of cellulase activity is that the cellulosome has up to 9 CMCase modules, and would thus be an ideal marker for cellulosome production. Avicel is used to induce the production of the complete cellulosome in Clostridial cultures (Lynd *et al.*, 2002), but in these studies did not show a significant effect in increasing the production of CMCase. It did, however, induce the production of CMCase earlier than without Avicel being present, as well as effecting a higher production of reducing sugars.

The presence of the enzyme system was further investigated with the use of inhibitor studies. Glucose was shown to have more of an inhibitory effect than acetate on the sulphate reducing microbial consortium (44 % compared to 24 % remaining activity compared to the control). The partially isolated Clostridial culture demonstrated that cellobiose was an effective inhibitor of CMCase activity, confirming the observation by Matano *et al.* (1994) who investigated various carbon sources for the assembly of cellulosomes. It was also observed that the inhibition by cellobiose ceased after 48 hours, probably due to the cleavage of cellobiose by β-glucosidase, producing glucose. The results indicate that the sulphate reducing microbial consortium produces cellulolytic enzymes and that they were probably produced by *Clostridium* species. An investigation into the effect of sulphide on the enzyme is required, but it may be proposed that enzyme activity might have been stimulated as shown by Whiteley *et al.*, 2002 and Whiteley *et al.*, 2003 [a,b] using a sewage sludge system.

Results of this study provide evidence that lignocellulose can be degraded in biosulphidogenic environments and the following descriptive model of lignocellulose degradation in biosulphidogenic environments has been proposed to account for events taking place (Figure 6.1). The initial increase in sulphate reduction observed when using lignocellulose material as a carbon source for AMD remediation occurs due to the release of the easily extractable portion of the lignocellulose material. This material is metabolised by the microbial consortium and part of it is used as electron donors by SRP, generating a sulphidic alakaline environment. The release of the easily extractable fraction, followed by its hydrolysis, leads to an increase in hydrogen sulphide and alkalinity. These two factors were shown to enhance the release of reducing sugars and aromatic compounds from wood material, including the lignocellulose core, under chemically generated sulphidic conditions. This creates a positive feedback loop, as the extractables are metabolised, more sulphide and alkalinity is produced leading to increased degradation and swelling of lignocellulose material. This is followed by a chemical, and probably also enzymatic, action on lignin. The removal of lignin is necessary to allow access to cellulose and an inverse relationship exists with sulphide acting on lignin breakdown, exposing cellulose to degradation making reducing sugars available to SRP. Cellulolytic bacteria, such as Clostridia, can then act on cellulose, the hydrolysis products of cellulose degradation, inhibit further enzymatic attack, halting this particular process. However, enough sulphide and alkalinity have been generated to allow for renewed action on the remaining extractable fraction, initiating the process again.

Eventually, however, the extractable fraction will become exhausted and this may lead to winding down of the feedback loop, because there will be less sulphide available, leading to less breakdown of lignin, exposing less cellulose, resulting in less reducing sugars being available, and even less sulphide being released by the SRP.

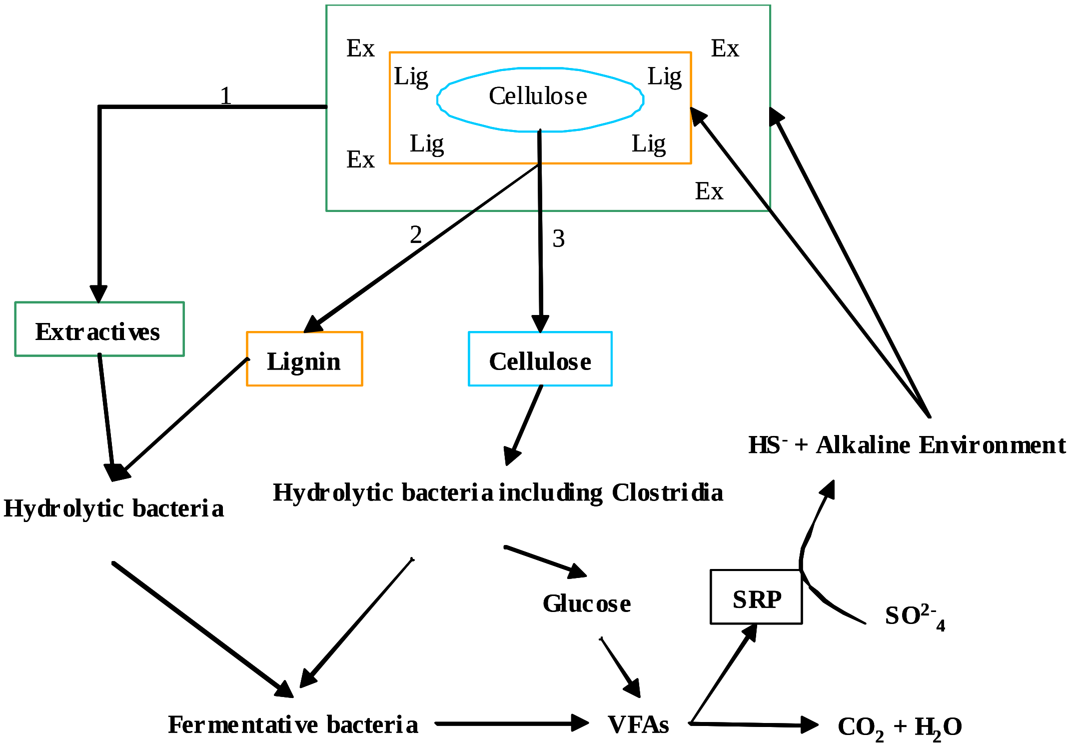


Figure 6.1: Proposed model of lignocellulose degradation under anaerobic biosulphidogenic conditions. 1) Soluble extractives are metabolised to produce VFAs, that are utilised as electron donors for sulphate reduction. 2) The sulphide and alkalinity produced leads to swelling of the lignocellulose matrix, releasing aromatic compounds from lignin. 3) This allows access to cellulose by cellulolytic bacteria (Clostridia). The products from 2 and 3 allow for further production of sulphide and alkalinity. If 1 and 2 are inhibited then the entire process comes to an end, with no sulphate reduction being observed.

The studies reported here were from an ongoing investigation into the microbial ecology of the column reactor at the laboratory of PHD, was undertaken by Clarke (PhD thesis, in progress). The columns provided a linear flow regime and showed spatial segregation of the various components of the process. It was found that fermentative *Clostridia* sp. were prevalent at the inlet of the reactor, phenol degrading species in the middle and cellulolytic species near the outlet of the reactor. These findings seem to support an observation that the degradation of carbohydrate compounds by the Clostridia leads to the production of VFAs through fermentation reactions and consumption of VFAs in the initial sulphate reduction reactions. The sulphide generated here acts on lignin components to enhance the release of aromatic compounds that are utilised by the phenol degrading species, producing acetate and thus more sulphide. The uncoupling/fractionating of the aromatic component from the carbohydrate allows greater access to the cellulose portion. The cellulolytic bacteria hydrolyse cellulose to products that can be fermented to VFAs that can be used as electron donors by SRP to reduce more sulphate. Clearly such a linear flow system is entirely dependent on the readily extractable components. It may be proposed that ongoing activity in such a system will be dependent on supplementing this fraction. This led to the suggestion that application of a small kick-start supplement to lignocellulose packed-bed systems may overcome this problem and maintain the positive feedback loop. An opportunity occurred to test this hypothesis at the AMD treatment pilot plant located at VCC and molasses was used (Coetser, 2004).

The VCC pilot plant was operated from November 1996 to August 2002, and following collapse of performance one of the sulphate-reducing units, SSRU2, was converted to operate with a kick-start supplement. Figure 6.2 compares the performance of SSRU2 during the initial phase of operation, when performance collapsed and with the kick-start supplement. Phase 1 refers to the period when readily extractable carbon was available from lignocellulose and phase 2 to the period when the extractables were exhausted. SSRU2 reduced sulphate leading to the production of sulphide, in both phases sulphate reduction remained similar, but sulphide production decreased in the second phase. The addition of 0.1 % (v/v) molasses to SSRU2 lead to a 47-fold increase in sulphide through the depth of the reactor, compared to a 9.7 times increase during phase 1, this large increase in sulphide production, however, cannot be due to the addition of such a small amount of reducing equivalents. In a similar study by Tsukamoto *et al*. (2004), reducing equivalents in the form of ethanol was added to reduce 50% of the influent sulphate, and it was shown that with removal of the ethanol the sulphate reduction ceased, contrary to the findings of this study, where a minimal amount of reducing equivalents was added but greatly improved sulphate reduction.

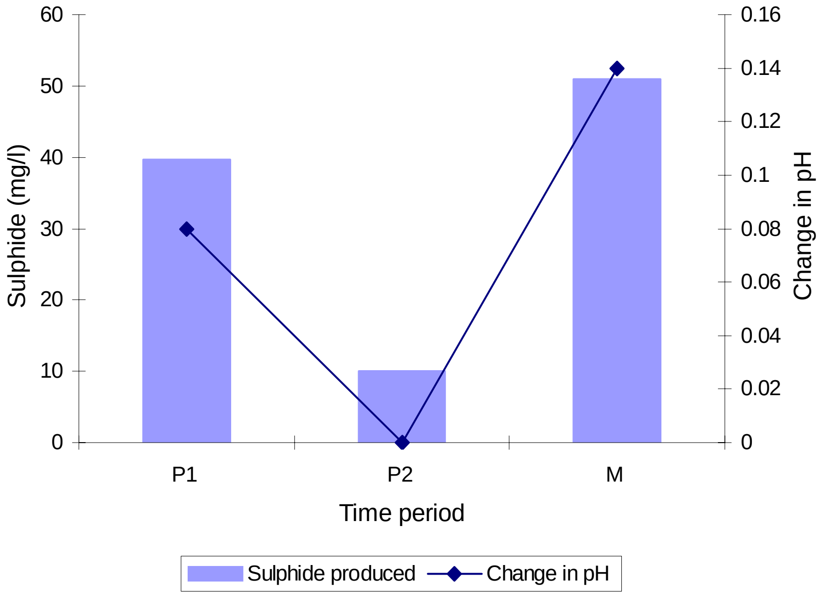


Figure 6.2: Comparison of the sulphide production through SSRU2, and the change in pH through the reactor. P1-Nov 1996-Dec 1998, P2-Jan 1999-Apr 2002, M-supplementation with 0.1 % molasses from Apr 2003.

Based on the VCC study with kick-start supplementation it was proposed that a greater fraction of lignocellulose might be accessed leading to a degradation of all components in the packed bed. This led to conceptualising the degrading Packed Bed Reactor (DPBR), and Coetser (2004) has reported this study.

The study reported here demonstrated that lignocellulose could be utilised as a carbon source and electron donor for sulphate reduction in the treatment of AMD. Some of the findings, however, need to be confirmed through additional studies. The spatial and temporal constraints on the uncoupling of the lignin fraction from the cellulose fraction needs to be investigated further. This uncoupling is of particular importance as it was suggested by the release cycle of aromatic compounds and reducing sugars that lignin has to be removed from cellulose to allow for access to the cellulose by enzymes. In addition, radiotracer studies can be done to map the degradative pathway of lignocellulose within biosulphidogenic environments, to compliment the study done on the lignin model compounds.

The practical implication of this study is that the halt in sulphate reduction due to exhaustion of extractive components may now be prevented. This is of particular relevance to the long term treatment of AMD, where the discharge from mines can continue for decades after closure (Younger, 1997).

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