BIOSULPHIDOGENIC HYDROLYSIS OF LIGNIN AND LIGNIN MODEL COMPOUNDS

by

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Abstract

Lignin degradation under biosulphidogenic conditions has not been extensively reported in the literature. Although aerobic degradation of lignin is well documented, anaerobic biodegradation has focused mainly on methanogenic systems with biosulphidogenic systems receiving less attention. Sulphate reducing bacteria are known to generate moderately high levels of both sulphide and alkalinity at room temperatures, and these conditions draw some comparison with the Kraft pulping process. In the Kraft pulping process, lignin is degraded chemically at ±170°C under high sulphide and alkaline conditions and may provide a model for understanding biosulphidogenic lignin degrading activity. The aim of this study was to investigate the biosulphidogenic hydrolysis of lignin within the context of the chemical and biological conditions generated by a mixed sulphate reducing bacteria consortia.

The chemical hydrolysis of pine wood powder was investigated with high levels of sulphide and alkalinity at room temperatures and results showed the release of wood aromatic compounds and wood extractives as analysed by high performance liquid chromatography. Similar studies with extractive free depectinated wood showed release of aromatic compounds 20 to 40% that of untreated wood hydrolysis experiments. The aromatic compounds released from depectinated wood could be ascribed directly to the hydrolysis of lignin since the extractable compounds have been removed.

Bioreactor studies with a mixed sulphate reducing consortia and pine wood powder (both untreated and depectinated) resulted in the generation of comparable levels of sulphide and alkalinity used in the chemical hydrolysis studies. Aromatic compound yields were between 20 to 50% of the chemical hydrolysis studies. This fluctuation may have been due to the utilization of these aromatic compounds as electron donors by the sulphate reducing consortia as evidenced by the high rate of sulphate reduction in both the untreated and depectinated wood bioreactors.

Biodegradation of lignin model compounds was investigated in order to elucidate lignin degradation mechanisms. Both mono-aromatic and dimeric lignin model compounds were used as electron donors and carbon sources for the mixed sulphate reducing consortia. Biodegradation and mass spectrometer analysis of mono-aromatic compounds, ferulic acid and ferulic acid ethyl ester resulted in the production of intermediates such as catechol, cyclohexane carboxylic acid and adipic acid. These intermediates were also observed in the degradation of dimeric ferulic acid. Biodegradation of salicin resulted in the production of salicyl alcohol, ortho-cresol and acetate. Biodegradation of benzylic ether resulted in the production of vanillin and acetate as end products.

The results of these studies provide evidence for a biosulphidogenic hydrolysis of lignin, and also the utilisation of lignin-derived aromatic compounds as electron donor sources, by a mixed sulphate reducing consortia.

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List of Abbreviations

AMD = Acid Mine Drainage

EBG = Environmental Biotechnology Group

EPS = Extracellular Polysaccharide

GC = Gas Chromatography

HPLC = High Performance Liquid Chromatography

ISTD = Internal Standard

MS = Mass Spectrometer

NMR = Nuclear magnetic Resonance

PDA = Photo Diode Array

RT = Retention time

SRB = Sulphate Reducing Bacteria

TLC = Thin Layer Chromatography

VFA = Volatile Fatty Acids

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Chapter 1

Lignin Hydrolysis in Biosulphidogenic Environments

1.1. Introduction

Lignin is the second most abundant carbon source in the world, after cellulose, and little is known about the anaerobic biodegradation of this biopolymer, especially under sulphate reducing conditions (Pareek *et al.,* 1998). Lignocellulose represents a huge depository of renewable raw material for use in new biotechnological processes (Buswell and Odier, 1987). The presence of lignin is apparently one of the most important factors affecting the biodegradability of lignocellulosic materials. According to many investigators, lignin forms a three dimensional complex structure with cellulose and hemicellulose and thus renders them less accessible to biodegradation (Kim *et al.,* 1997).

Lignocellulose constitutes ± 89% (see table 1.1.) of the dry weight of wood (Crawford, 1981). The holocellulose fraction, which includes both cellulose and hemicellulose, is the largest component (± 78%), while estimates of 15 to 30% lignin have been made for various hardwood (e.g. Birch) and softwood (Pine) trees (Sarkanen *et al.,* 1971).

Table 1.1. Characteristics of softwood and hardwood fibres (Crawford, 1981).

	Softwood	**Hardwood**
Cellulose content	42% (+/- 2%)	45% (+/- 2%)
Lignin content	28% (+/- 3%)	20% (+/- 4%)
Extractives content	3% (+/- 2%)	5% (+/- 3%)

Aerobic microbial communities are the primary lignocellulose decomposers in most natural systems and progress has been made in understanding the aerobic biodegradation mechanisms (Powell *et al.*, 2001). However, recent studies have shown that the contribution of strictly anaerobic microorganisms to the turn over of lignocellulose-derived carbon has probably been underestimated and merits re-evaluation (Kim, 1997).

Figure 1.1 depicts microbially mediated transformations, and both aerobic and anaerobic processes are involved in the global cycling of lignocellulose derived carbon.

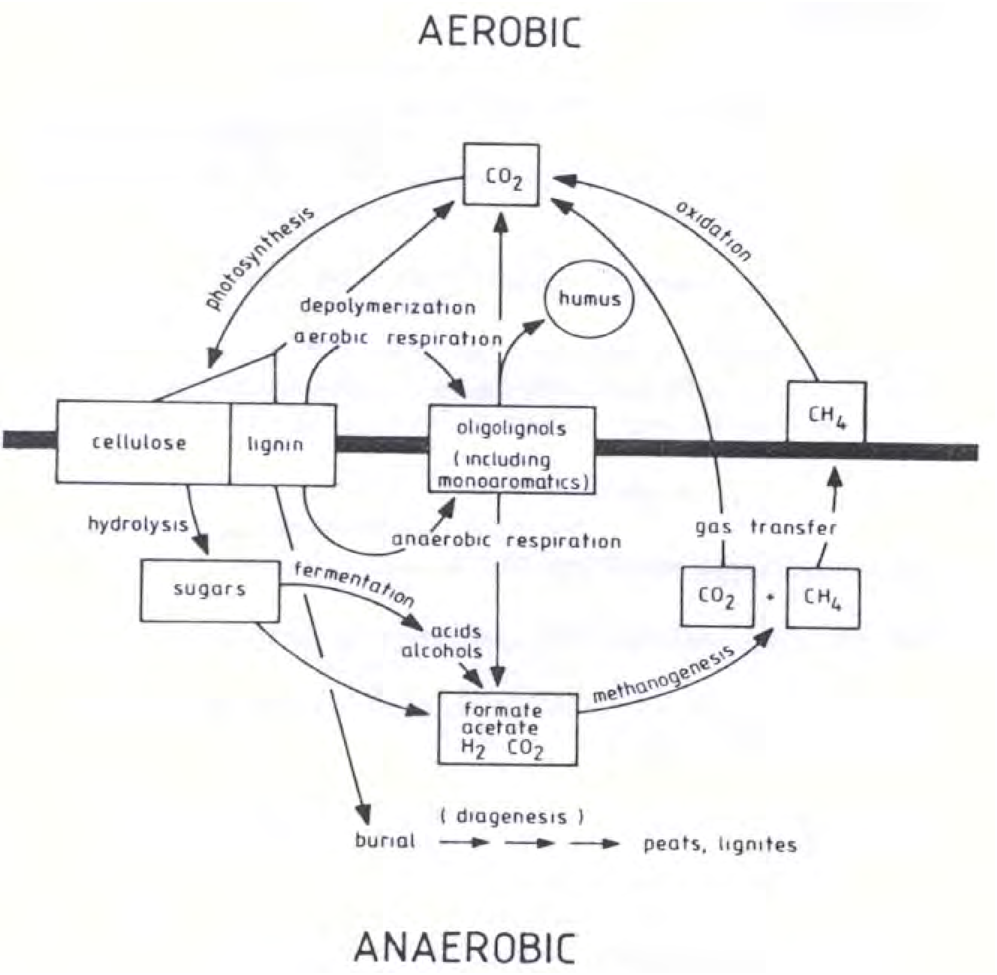


Figure 1.1. Biological cycling of lignocellulose-derived carbon (Hearly, 1979).

Apart from the environmental significance of understanding the cycling of lignocellulose-derived carbon, large quantities of lignocellulose are utilised in forestry industries for timber and paper production. Lignocellulose is a major component of municipal solid waste, and it has been used as a substrate in methane production processes (Young, 1984). Lignocellulose has also been used as an electron source in biotechnological processes such as bioremediation of sulphate rich mine water.

1.2. Lignocellulose as a substrate for sulphate reducing bacteria in the treatment of acid mine drainage

Acid mine drainage (AMD) refers to the water leaching from the mining areas with high levels of sulphate and heavy metals. The major biological approach to the treatment of AMD involves the use of sulphate reduction with the production of sulphide and the precipitation of insoluble metals sulphides (MeS). This takes place according to equation (1) and (2) (Christensen *et al*., 1996)

$$2CH_2O + SO_4^{2-} + H^+ \longrightarrow 2CO_2 + 2H_2O + HS^- \qquad (1)$$

$$Me^{2+} + HS^- \longrightarrow MeS + H^+ \qquad (2)$$

According to equation (1) and (2), these bacteria require an electron donor in order to reduce sulphate. Numerous studies have been carried out with different electron donors such as propionate (Uberoi and Battacharya, 1995), benzoate (Gibson, 1990), tannery effluent and algal biomass (Rose *et al.,* 1998) and primary sewage sludge (Whittington-Jones, 2000).

Various lignocellulosic materials have been tested as electron donors and these include straw and hay (Bechard *et al.,* 1994), spent mushroom compost (Dvorak *et al.,* 1992) and sawdust (Wakao *et al.,* 1979). The electron donor which supports sulphidogenesis for a prolonged period is preferred. Active sulphidogenesis was observed with straw and sawdust when easily assimilable electron donors such as sucrose (Bechard *et al.,* 1994), peptone, lactate and pyruvate (Wakao *et al.,* 1979) were supplemented, while spent mushroom compost supported sulphidogenesis without any supplement (Dvorak *et al.,* 1992)

Chang *et al.,* (2000) tested different organic waste materials as carbon sources for sulphate reducing bacteria (SRB) in treating AMD. These included oak chips, organic rich soil, spent oak chips, spent mushroom compost and sludge from waste paper recycling plant. The study showed good sulphidogenesis in all reactors including that with oak chips, but sludge from waste paper, spent mushroom compost and spent oak chips supported better sulphidogenesis than oak chips and organic rich soil. The less active sulphidogenesis in the reactor with organic rich soil shows that the readily degradable organic content is lower in the organic rich soil than the others. Spent oak chip is the residue of aerobic fungal digestion of oak chips, but the former supported better sulphidogenesis than the latter. Solvent extractibles of oak chips inhibited the growth of sulphidogens. This showed that raw oak contains inhibitors for sulphidogens, probably resins, which can be degraded by aerobic microbial growth as in spent oak chips, spent mushroom compost and during pulping as in sludge from waste paper recycling plant. The good sulphidogenesis in the reactor with oak chips after 20 days of operation showed that the inhibitor is inactivated during initial anaerobic microbial metabolism (Tavendale *et al.,* 1997). This might be the reason why straw and sawdust needed the supplements of easily digestible nutrients. Based on these facts it has been concluded that biologically (spent mushroom compost and spent oak chips) or chemically treated (sludge waste from paper) biomass is a good electron donor for sulphidogenesis to treat AMD and that easily assimilable electron donor should be supplemented to support sulphidogenesis in early stage when raw biomass is used.

1.3. Innovation fund project and the passive treatment of acid mine drainage in South Africa

The passive treatment of AMD in South African mines has been the focus of a major innovation fund grant involving Department of Arts Culture Science and Technology (DACTS), Pulles Howard and de-Lange (PHD), an environmental consulting firm and the Rhodes University Environmental Biotechnology Group (EBG). The project involved the use of biological sulphate reduction process with lignocellulosic material as the readily available carbon source.

Lignin biodegradation under biosulphidogenic conditions is not fully understood and it is thought to be the rate-limiting step in wastewater treatment (Crawford, 1981). Research by Rhodes University Environmental Biotechnology Group focused on the elimination of the rate-limiting step in biosulphidogenic degradation of lignocellulose. Pareek *et al.*, (1998) has demonstrated that degradation of lignocellulose under sulphidogenic conditions is more than twice that under methanogenic conditions. Work by Whittington-Jones (2000) has also showed that there is an increased hydrolysis of phenolic compounds from the sewage sludge under biosulphidogenic conditions.

1.4. Lignocellulose structure

In order to understand the biodegradation of lignin under biosulphidogenic conditions, it is important to define lignocellulose in terms of its structural components and the known biodegradation mechanisms under anaerobic conditions. Lignocellulose refers to lignin, cellulose and hemicellulose as the structural components of plants.

1.4.1. Cellulose

Cellulose is the most abundant natural organic compound on earth and is located in plant cell walls (Crawford, 1981). It is an unbranched polymer of several thousand D-glucose units joined via β-1,4-glycosidic linkages and is insoluble in water (figure 1.2). Cellobiose is a disaccharide produced upon partial hydrolysis of cellulose and contains the β-1,4-bond, while complete hydrolysis results in the release of glucose (Crawford, 1981).

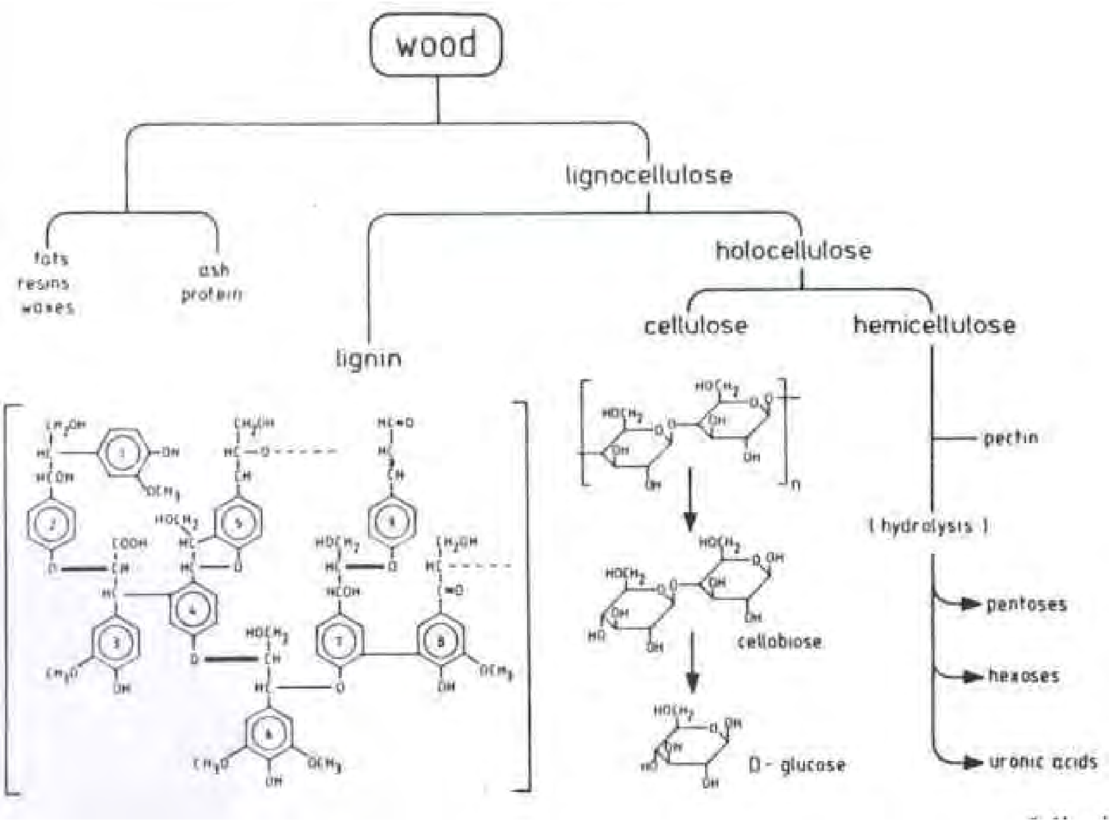


Figure 1.2. Major components of wood, including the chemical structures of lignin (model), cellulose, and its hydrolysis products, cellulose and D-glucose (Sarkanen and Ludwig, 1971).

1.4.2. Hemicellulose

Hemicellulose is rather an ill defined group of polysaccharides occurring in plants. They are not considered cellulose derivatives, but rather, are polymers of D-glucose linked by β-1,4-bonds (Conn and Stumpf, 1972) with branched structures of L-arabinose, uronic acids, and hexoses such as D-glucose, D-mannose, and D-galactose (Browning, 1973). Pectin is sometimes considered to be a type of hemicellulose (Herric and Hergert, 1977). It consists of rhamnose and α-1,4-linked D-galacturonic acid residues whose carboxylic acids groups may be methoxylated (Schink, 1984). While young plants have relatively high pectin contents (12 to 34%) (Jarvis, 1984), it is a minor component of mature plants and wood (1 to 5%) (Cote', 1977).

1.4.3. Lignin

Lignin is a structural polymer of vascular plants that performs functions essential to their survival (Pareek *et al.*, 2001). In industrial and domestic wastes it represents a problem for biological waste disposal due to its recalcitrance to biodegradation. Perhaps most importantly for the beneficial exploitation of lignocellulosics is the intimate association of lignin with cellulose and hemicellulose in woody plant tissues, which prevents efficient conversion of the polysaccharides to useful products (Buswell and Odier, 1987).

Although significant quantities of lignin containing plant materials enter the anaerobic zones and are believed to be the rate limiting components in methane production processes, most studies have considered lignocellulose degradation under aerobic conditions by fungi (Kawai *et al.,* 1995). The role of bacterial action is well established in the degradation of lignin model compounds, chemically depolymerised lignins, and at least to some extent of natural and synthetic lignins (Buswell and Odier, 1987). Although it seems apparent that bacteria participate in lignin degradation by mineralising low molecular weight fragments present in lignocellulose samples, released by abiotic means, or by fungal degradation, their role as primary lignin degraders is not clear. One of the most active bacteria in degrading synthetic lignins is *Xanthomonas* sp. strain99 isolated from soil (Kern *et al.*, 1987) and *Streptomyces viridosporus* (Crawford *et al.,* 1983). Mono-aromatic lignin derivatives have been shown to degrade under methanogenic conditions. The first evidence was provided by Boruff and Buswell (1934), who reported that 54% of lignin in cornstalks was converted to CO_2 and CH_4 after 600 days of incubation. The ability of the SRB consortia to degrade lignin has not been rigorously established.

Lignin is an aromatic polymer of phenylpropane subunits and a major component of vascular tissues in terrestrial plants. It is formed by the random coupling of radical species, which arise from the peroxidase-mediated dehydrogenation of coniferyl, sinapyl and coumaryl alcohols (figure 1.3a). Polymerisation results in a heterogeneous phenylpropanoid structure (figure 1.3b) in which the basic subunits are linked together by several types of inter-unit carbon-carbon and β-aryl ether bonds (Buswell and Odier, 1987).

The three monomeric precursors are present in variable proportions, depending on several factors including the plant species, type of tissue, the exact location of the lignin within the cell wall, and photo-period. Gymnosperm lignin (guaiacyl lignin) contains predominantly coniferyl-type structures, while lignin in angiosperms (guaiacyl/syringyl lignin) is comprised of approximately equal amounts of coniferyl and syringyl units with smaller quantities of p-coumaryl alcohol derived units. Grass lignins are a mixture of all three monomers with relatively high amounts of p-coumaryl alcohols and also contain ester linked ferulic and p-coumaric acids (Buswell and Odier, 1987).

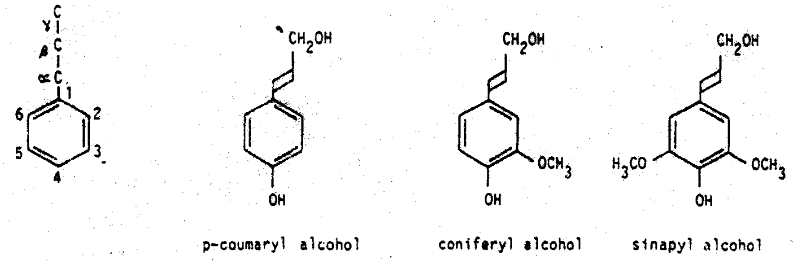


Figure 1.3(a). Cinnamyl alcohol precursors of lignin (Buswell and Odier, 1987).

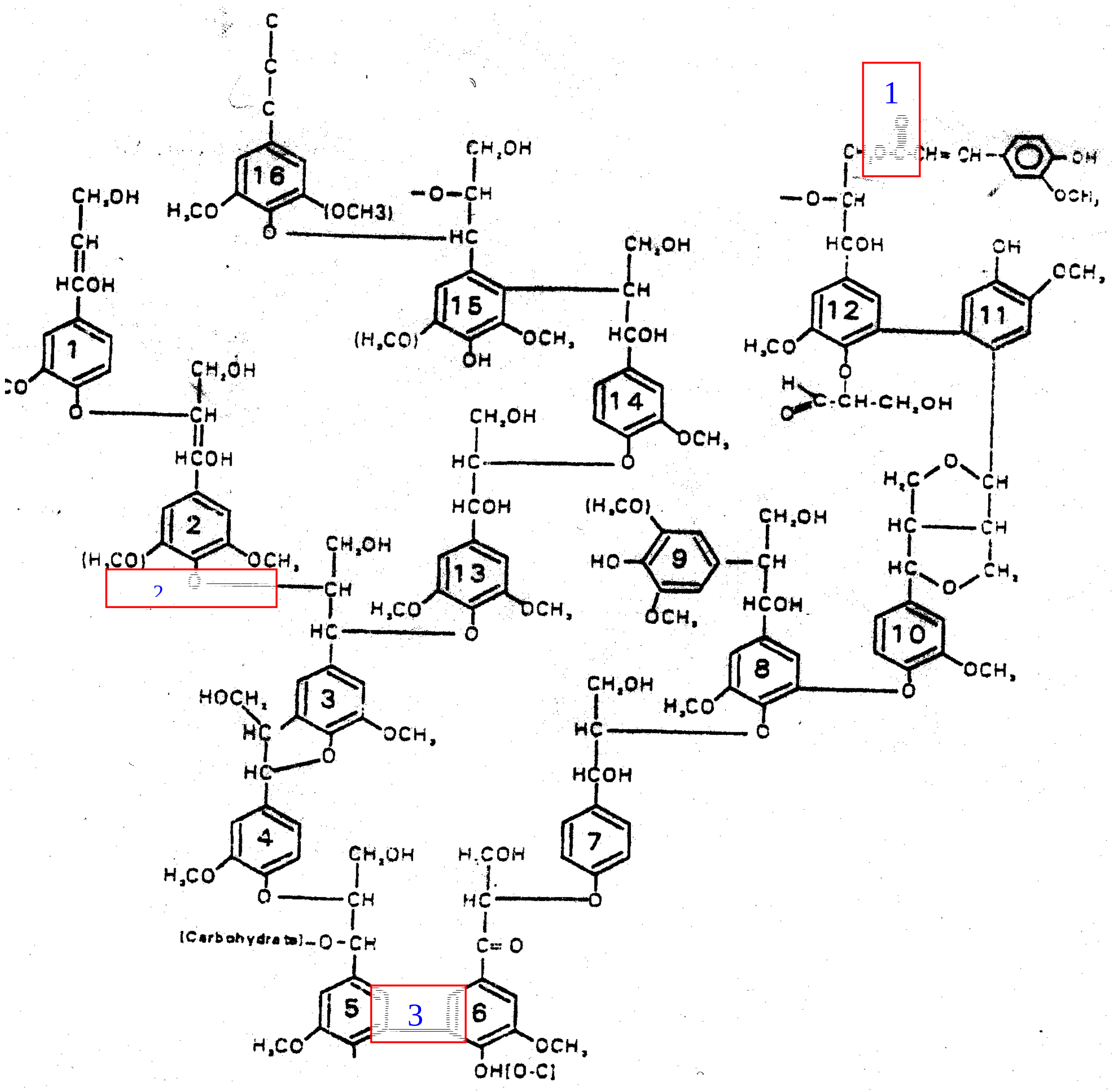


Figure 1.3(b). Model structure of softwood lignin, showing different functional groups (Buswell and Odier, 1987). 1 = Ester bond, 2 = Ether bond and 3 = C-C bond

1.5. Lignin degrading microorganisms

Fungi and bacteria are known to degrade lignin, while higher life forms such as termites and other xylophagic insects are reputed to digest lignin, this ability is probably conferred through certain elements in their intestinal microflora (French *et al.*, 1975). Fungi growing on living or dead trees which degrade wood polymers, including the lignin component are classified into three specific decay groups: white-rot, brown-rot, and soft-rot fungi. In addition, various soil fungi, e.g. *Fusarium* are reported to degrade lignin (Higutchi *et al.*, 1980).

1.5.1. Model compounds used in lignin biodegradation studies

Methods based on various molecular models possessing structural characteristics found in the lignin polymer have also been widely used in biodegradation studies. Although the choice of relevant models is not easy, given the complex structure of the polymer, identification of metabolites arising from dimeric compounds of β-O-aryl ether type have proven extremely important (Kawai *et al.,* 1995). Structures of the β-O-aryl ether type represent the most common inter-monomeric linkage found in lignin, whereas β-1 diarylpropane and phenylcoumaran dimers are useful models for some types of C-C inter-monomeric bonds. Although such dimers are low molecular weight compounds, it is clear in some cases that parts of the lignolytic system are directly involved in their metabolism. The significance of studies using monomeric structures in elucidating the initial reactions involved in lignin breakdown is less evident. For example, although vanillic acid (3-methoxy-4-hydroxybenzoic acid) is a known intermediate in the degradation of lignin, the degradation of this monomer is catalysed by enzymes not associated with the primary attack on the polymer (Ander *et al.,* 1980).

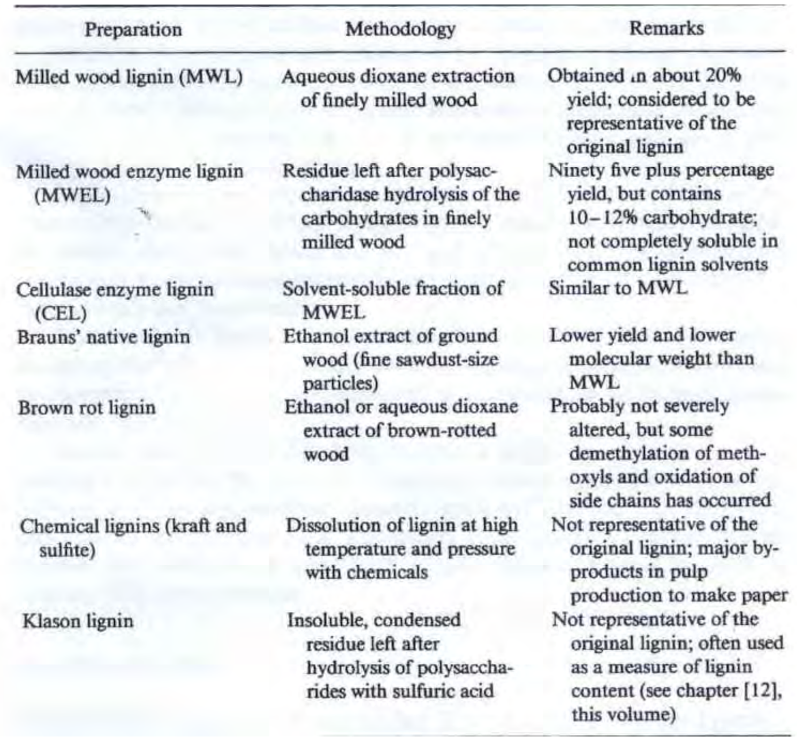
1.5.2. Polymeric substrates used in lignin biodegradation studies

Using classical microbiological methods for studying lignin biodegradation has been made difficult by the macromolecular properties and structural characteristics of this polymer. The problems encountered are linked to the difficulty in obtaining the pure and unmodified preparations and the development of suitable assay systems (Buswell and Odier, 1987).

Lignin cannot be extracted from plant cell wall unless its structure has been modified to some extent either by physical (milling) or chemical treatments. This often raises difficulties when data obtained using extracted lignin material are extrapolated to include native lignin in plant tissues. Various methods have been developed, resulting in different types of extracted lignin ranging from those preparations with only minor modifications (milled wood lignin) to those with major modifications (Kirk and Obst, 1988).

The properties of several extracted lignin preparations and their relevance to lignin biodegradation studies are listed in table 1.2. Synthesis of a dehydroxy-polymer (DHP) resembling lignin can be achieved by oxidative polymerisation of coniferyl, sinapyl, and p-coumaryl alcohol using the commercially available horseradish peroxidase and hydrogen peroxide (Kirk and Brunow, 1988). Various ^{14}C-labeled DHPs, prepared from coniferyl alcohol labelled in the methoxy, the propyl side chain, or the aromatic ring, are widely used in lignin biodegradation studies. However, this method has a drawback arising from the absence of a standard protocol for the preparation of DHP, which occasionally makes it difficult to compare results obtained using the material prepared in different laboratories (Temp *et al.*, 1998)

Table 1.2. Lignin isolation methods (Kirk and Obst, 1988)



Preparation	Methodology	Remarks
Milled wood lignin (MWL)	Aqueous dioxane extraction of finely milled wood	Obtained in about 20% yield; considered to be representative of the original lignin
Milled wood enzyme lignin (MWEL)	Residue left after polysaccharidase hydrolysis of the carbohydrates in finely milled wood	Ninety five plus percentage yield, but contains 10–12% carbohydrate; not completely soluble in common lignin solvents
Cellulase enzyme lignin (CEL)	Solvent-soluble fraction of MWEL	Similar to MWL
Brauns' native lignin	Ethanol extract of ground wood (fine sawdust-size particles)	Lower yield and lower molecular weight than MWL
Brown rot lignin	Ethanol or aqueous dioxane extract of brown-rotted wood	Probably not severely altered, but some demethylation of methoxyls and oxidation of side chains has occurred
Chemical lignins (kraft and sulfite)	Dissolution of lignin at high temperature and pressure with chemicals	Not representative of the original lignin; major by-products in pulp production to make paper
Klason lignin	Insoluble, condensed residue left after hydrolysis of polysaccharides with sulfuric acid	Not representative of the original lignin; often used as a measure of lignin content (see chapter [12], this volume)

In addition to synthetic lignins, ^{14}C-labeled natural lignins have also been prepared by administering a labelled precursor in lignin biosynthesis (L-phenylalanine, ferulic acid, cinnamic acid) to lignifying plants. Careful work-up procedures are necessary to ensure the removal of ^{14}C-labeled low molecular weight contaminants (this also applies to DHPs), as well as protein when phenylalanine is used as a precursor (Temp *et al.,* 1998).

Provided such precautions are observed, measurement of $^{14}CO_2$ released from ^{14}C-labeled lignins by microbial cultures provides a simple, rapid, and unequivocal assay of lignin biodegradation and has become the method of choice. It does not however provide information about individual steps in the overall degradation process. Use of ^{13}C-enriched DHP and ^{13}C-NMR spectroscopy has also facilitated analysis of macromolecular changes, which occur in lignin as a result of microbial attack (Ellwardt *et al.,* 1981).

1.6. Anaerobic degradation of aromatic compounds

Since polymeric lignin is composed of aromatic subunits, its microbial degradation requires an understanding of the mechanism by which aromatic rings are metabolised. Until recently, catabolism of aromatic compounds was thought to be a strictly aerobic process and compounds such as benzene, toluene and xylene are known to be degraded easily by aerobic micro-organisms (Lovley *et al.,* 1995). Recently, Burland *et al.* (1999) has demonstrated that benzene is oxidised to carbon dioxide under nitrate reducing conditions in enrichment cultures developed from soil and ground water microcosms. To confirm that benzene was mineralised to CO_2, cultures were incubated with [^{14}C]benzene and approximately 92 to 95% of the initial benzene added was recovered as $^{14}CO_2$.

Lovley *et al.* (1995) also demonstrated [^{14}C]benzene oxidation coupled to sulphate reduction. Addition of molybdate, an inhibitor of sulphate reduction inhibited benzene uptake and production of $^{14}CO_2$. The stoichiometry of benzene uptake and sulphate reduction was consistent with the hypothesis that sulphate was the principal electron acceptor for benzene oxidation. There are several potential pathways for anaerobic metabolism of benzene, including hydroxylation to produce phenol (Grbic-Galic and Vogel, 1987), carboxylation to produce benzoate, and the initial reduction of the ring to form cyclohexane. None of these potential intermediates was produced and this includes acetate. These results suggest that benzene may be directly oxidised to carbon dioxide and this is consistent with anaerobic oxidation of toluene (Rabus *et al.*, 1993).

Anaerobic naphthalene degradation by an SRB consortium has been studied by Meckenstock *et al.* (2000). The intermediates were reported to be 2 methylnaphthalene, 1-and 2-naphthoic acid, phenyl acetic acid, benzoic acid, cyclohexane carboxylic acid and cyclohex-1-ene carboxylic acid with sulphate as an electron acceptor.

1.6.1. Mono-aromatic cleavage during anaerobic photo-metabolism, and nitrate reducing conditions

Proctor and Scher (1960), proposed an oxidative pathway for the observed degradation of benzoate during photosynthetic growth of *Rhodopsuedomonas palustris*. They suggested that an unknown oxidant, believed to be generated during photosynthesis, was biochemically equivalent to molecular oxygen. However, Dutton and Evans (1968) showed that this organism could not grow on benzoate if incubated in air, nor could any enzyme of the aerobic pathway be detected during anaerobic growth. Based on these results they proposed an anaerobic pathway whereby the aromatic ring is reduced prior to cleavage. This reduction was thought to be catalysed by reductive enzymes coupled to a low redox potential component of the light induced electron transport system (ferredoxin).

Based on mixed culture studies, Oshima (1965) proposed that during ring cleavage of both hydroxy-benzoate and proto-catechuate, the oxygen atom of nitrate (NO_3^-) behaved as if it were O_2. Later investigations with *Psuedomonas sp.* suggests that the aromatic metabolism of benzoate in the obligatory presence of nitrate must be different from that of aerobic pathways since no oxygenase enzyme could be detected (Taylor *et al.*, 1970). Studies by William and Evans (1973) using an aromatic degrading *Moraxella sp.* which reduces NO_3^- to N_2, resulted in identification of the same metabolic intermediates found during reductive ring cleavage of benzoate by *R. palustris.*

1.6.2. Anaerobic biodegradation of oligolignols

The pathway by which polymeric lignin is degraded is not well understood (Crawford, 1981). In fact, the first report of anaerobic microbial degradation of oligolignols was that of Colberg and Young (1982).

(Crawford, 1976) prepared [^{14}C-Lignin] lignocellulose from Douglas fir. In order to produce oligolignols that could be used as the substrates in methanogenic cultures, the radiolabeled wood was heat treated under alkaline conditions and resulted in the release of water soluble lignin derived fragments (MW <1400). The carbohydrate free oligolignol mixture was then neutralised and preparatively separated into its component molecular sizes by gel permeation chromatography. Similar fractions were pooled together, dried and reconstituted in a defined medium for use as a sole source of carbon in biodegradation studies (Colberg and Young , 1982).

Using sewage sludge as an inoculum, enriched cultures were prepared under strictly anaerobic conditions (Hearly and Young, 1978), and periodically analysed for the formation of radiolabeled gases. As may be seen in figure 1.4, lignin fragments corresponding to molecular weights of 1000 to 1400 were only minimally degraded. However, more than 25% of the original ^{14}C of smaller oligolignol mixture (MW 400 to 1000 with an average MW 600) was recovered as $^{14}CO_2$ and $^{14}CH_4$ after less than 20 days of incubation.

On the basis of these preliminary incubations, as well as corresponding changes in the molecular profiles when the substrates were re-eluted on gel columns. Colberg and Young (1982) postulated that cleavage of intermonomeric bonds must have occurred during anaerobic degradation of the oligolignols. The hypothesis was subsequently confirmed by Zeikus *et al*. (1982) who incubated a ^{14}C labelled synthetic dilignol compound with an anaerobic lake sediment culture. The structure of this compound and results of their study are shown in figure 1.4.

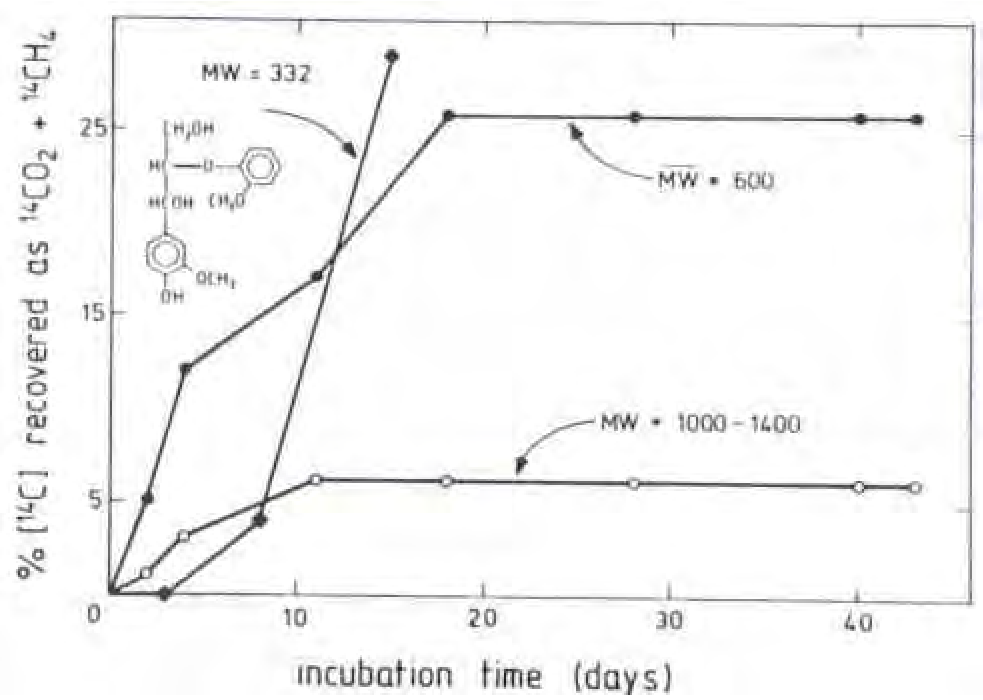


Figure 1.4. Anaerobic biodegradation of two [^{14}C] oligolignol mixtures derived from [^{14}C-Lignin] Douglas fir and a [^{14}C] synthetic dilignol model compound containing the aryl glycerol-β-aryl ether bond (Zeikus *et al.*, 1982).

Since the synthetic dilignol was readily degraded into $^{14}CO_2$ and $^{14}CH_4$, the authors suggested that the β-aryl ether bond doesn’t limit anaerobic mineralisation, but is readily cleaved in the absence of molecular oxygen.

1.6.3. Anaerobic biodegradation of polymeric lignin

Few studies concerning lignin degradation under anaerobic conditions have been reported. The first such a report was that of Boruff and Buswell (1934), who reported a 54% reduction in the lignin content of cornstalks after almost 2 years of anaerobic incubation. However, since they relied on gravimetric analysis to determine lignin weight loss, its difficult to assess the validity of the data. With the introduction of radioisotopic techniques, both the sensitivity and the accuracy of the of lignin biodegradation assays have dramatically increased. Several types of natural ([^{14}C-Lignin] lignocelluloses) and synthetic radiolabeled DHP lignins are now available (Benner *et al.*, 1984).

Colberg and Young (1985) have demonstrated that a mixed bacterial population under strictly anaerobic conditions degrades a mixture of oligolignols to yield the end products of methanogenesis. Colberg and Young (1985) also demonstrated that the oligolignol degradation is enhanced as the molecular size (MW range of 200 to 1400) is reduced in an anaerobic environment. In addition up to 30% of the entire soluble lignin-derived carbon was anaerobically mineralised to carbon dioxide and methane after 43 days of incubation.

1.7. Degradation pathways of lignin-derived monomers under methanogenic conditions.

Methanogenic bacteria are known to form methane from different substrates such as methanol, formate, acetates and utilising hydrogen as an electron donor during CO_2 reduction (Wuhrmann, 1982). The production of methane from more complex substrates therefore depends on the activity of non-methanogenic bacteria in association with methanogens. Consequently, studies of dissimilation of aromatic compounds under methanogenic conditions have relied on microbial consortium (Wuhrmann, 1982). Figure 1.5, merges some of the reported biodegradation reactions by methanogenic consortium involving benzene ring-containing lignin derivatives into a common pathway.

1.7.1. Mixed culture studies with benzoate

Benzoate was used as a model substrate in most studies concerned with aromatic ring catabolism under methanogenic conditions (Clark and Fina, 1952). Using mixed cultures obtained from sewage sludge with no previous exposure to benzoate, Nottingham and Hungate (1969), obtained radioactive methane and CO_2 from uniformly labelled benzoate. Ferry and Wolfe (1976) identified acetate as a key intermediate in the conversion of benzoate to methane. This was soon confirmed by Zehnder *et al*. (1980), who described *Methanothrix sonhgenii*, a non hydrogen oxidising, acetate decarboxylating methanogen.

Based on thermodynamic considerations, Ferry and Wolfe (1976) suggested that a microbial food chain was needed for complete mineralisation of benzoate. The methanogens were postulated to remove metabolic intermediates produced by other members of the consortium (i.e. hydrogen), thereby providing thermodynamically favourable conditions for degradation of aromatic substrates. This implies that ring fission is tightly coupled to product removal and is supported by the work of Mountfort and Bryant (1982).

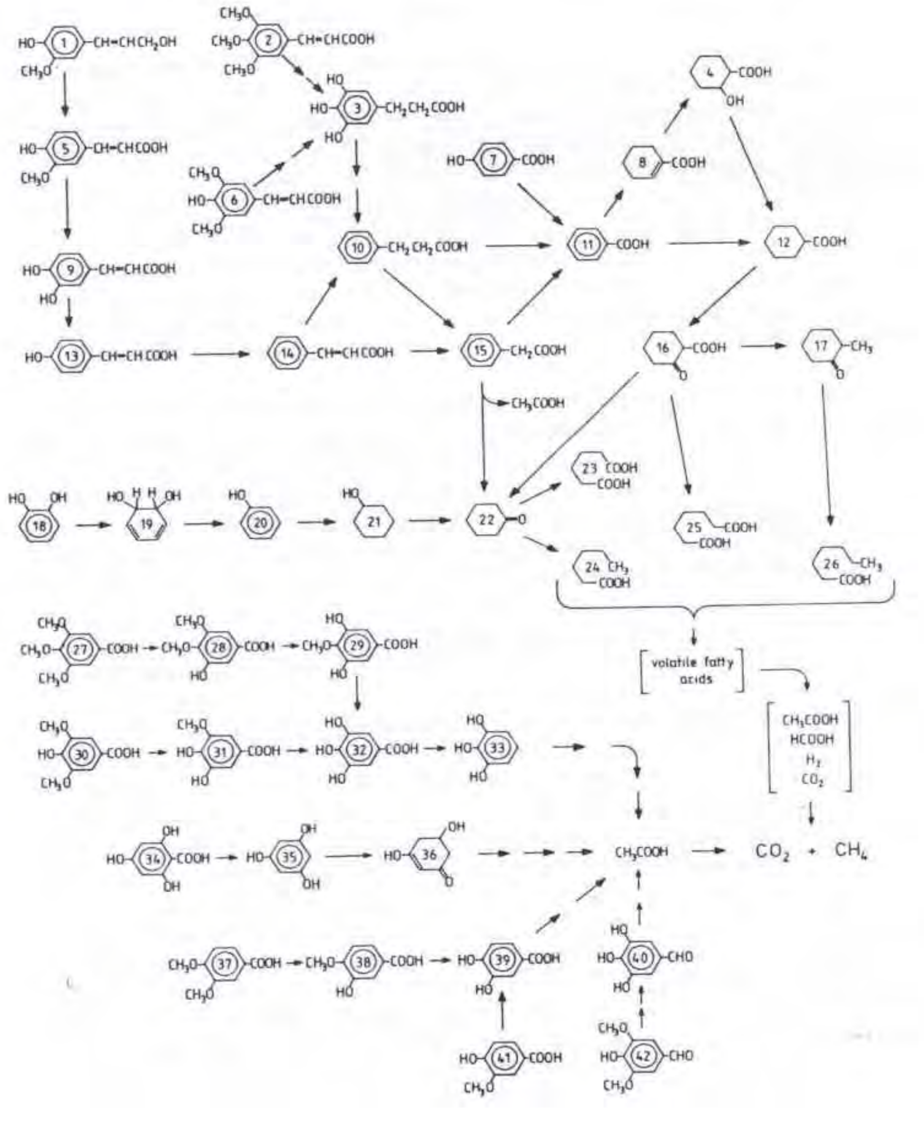


Figure 1.5. Summary pathway merging some reported reactions involving the anaerobic metabolism of lignin derived monoaromatic compounds under methanogenic conditions (HCO_3^- as an electron acceptor). 1=coniferyl alcohol(Grbic'-Galic', 1983); 5=ferulic acid (Akin, 1980); 6=sinapic acid (Bache and Pfenning, 1981); 7=p-hydroxybenzoic acid (Hearly and Young, 1979); 14=cinnamic acid (Grbic'-Galic', 1983); 30=syringic acid (Hearly and Young, 1979) and 41=vanillic acid (Kaiser and Hanselmann, 1982).

They were able to isolate a benzoate utilising bacterium only in co-culture with *Desulfovibrio sp.* that scavanged the hydrogen. However, Grbic′-Galic′ and Young (1983) have shown that it is possible to uncouple methanogenesis and benzoate degradation. 2-Bromoethanesulfonic acid (BESA), a structural analog of coenzyme M (found uniquely in methanogens), acts as a metabolic inhibitor of methane formation by specifically blocking the methyl transfers reaction in the last step of methanogenesis.

Addition of BESA to the active benzoate degrading consortium reduced methane production to 5% of normal levels, but did not affect ring cleavage of benzoate (Young, 1984). Using benzoate enrichment cultures obtained from sewage sludge, Balba and Evans (1977) detected several potential pathway intermediates, including 1-cyclohexene-1-carboxylic acid (figure 1.5) capronic acid, heptanoic acid, propionate and acetate. Similar results were obtained by Keith *et al.* (1978), who studied mixed consortium that had been maintained on benzoate for 10 years. In cultures seeded with anaerobic mud from polluted river, Schlomi *et al.* (1978) did not find cyclohexane carboxylic acid, but did identify two new compounds in the pathway, 2-hydroxycyclohexane carboxylic acid and 2-oxocyclohexane carboxylic acid . Considering the number of different sources used to obtain benzoate degrading cultures, it is not surprising that such a variety of intermediates has been proposed. The sequence of reactions in the anaerobic dissimilation of benzoate under methanogenic conditions however remains the same. That is, reduction of aromatic ring with the formation of cyclohexane derivative, reductive cleavage of the cyclohexane rings yielding aliphatic acids. These in turn are substrates for the members of the consortium (Zehnder, 1978).

1.7.2. Mixed culture studies with other aromatic compounds

None of the substituted mono-aromatic lignin derivatives examined thus far have proven to be resistant to anaerobic biotransformation, although ring cleavage has not always been observed (Bache and Pfenning, 1981). Hearly and Young (1979) extensively surveyed the potential for methanogenic degradation of mono-aromatics other than benzoate, including vanillic acid (figure 1.5), ferulic acid, phenol, catechol, cinnamic acid, protocatechuic acid, p-hydroxybenzoic acid, syringic acid and syringaldehyde. Ring fission was observed in all cases with the resultant production of

CO_2 and CH_4 (Hearly *et al.* 1980) later focussed on the methanogenic degradation of ferulic acid. Based on chromatographic analysis, they proposed cinnamic acid and acetate as major intermediates. Upon inhibition of methane formation by addition of BESA to their mixed cultures, they were able to detect several other compounds including phenyl-propionic acid benzoate, cyclohexane carboxylic acid, butyric acid and propionate. The presence of benzoate, in particular suggested that the ferulic acid pathway might overlap the one already proposed by Evans (1977) for benzoate.

Hearly and Young (1978) showed that both catechol and phenol could be fermented to CO_2 and CH_4 by mixed consortium after a short acclimation period. Balba and Evans (1977) also worked out a degradation scheme for catechol that includes phenol as an intermediate. Catechol is first hydroxylated to phenol through cyclohexadiene-1.2-diol. (figure 1.5). Phenol is then reduced to cyclohexanol, with subsequent formation of cyclohexanone. Ring fission yields adipic acid (figure 1.5), capronic acid, acetate, succinate, and propionate.

1.7.3. Removal of aromatic substituent groups

Since monoaromatic compounds such benzoate, ferulic acid and syringic acid may be stoichiometrically fermented to CO_2 and CH_4, it follows that demethoxylation, decarboxylation, and dehydrogenation reactions are all possible under strictly anaerobic conditions (Hearly *et al.*,1980). Kaiser and Hanselmann (1982) found that when vanillic acid and its analogs were fed to syringic acid adapted cultures, they were converted to catechol with small amounts of acetate and methane, presumably by demethoxylation reactions.

1.8. Chemical degradation of lignin: pulping process

Wood may be chemically delignified, i.e., pulped, with a simple solution of sodium hydroxide. A large body of experimental evidence and many years of commercial experience have shown that the addition of sodium sulphide is beneficial, particularly for the pulping of coniferous species (Clayton, 1969). Addition of sulphide causes the liquor to react faster with lignin, leading to pulps with lower lignin content for a given yield or to a higher yield at a given lignin content.

Figure 1.6(a) shows the relative rates of dissolution of lignin from red spruce wood during 4-kg experimental cooks in both Kraft and soda liquors. The initial effective alkali concentration was 42.0 to 42.6 g/l as NaOH, corresponding to about 1.4 N NaOH in each case, and the Kraft liquor contained 18.5 g/l of sodium sulphide (as Na_2S) corresponding to 30% sulphidity. The amount of lignin removed in a given time is greater with the Kraft liquor at all stages of the cook, but the effect of the sulphide is especially noticeable in the earlier stages of the temperature cycle between 140 and 170 ^{0}C, the maximum temperature. The dissolution of carbohydrate is slightly greater with Kraft liquor because the more rapid removal of lignin with Kraft liquor increases the accessibility and solubility of the wood polysaccharides, an effect which is also observed at 140 ^{0}C . This implies that the sulphide in the Kraft liquor exerts no protective action upon the carbohydrate because of its reducing power. Its role is to increase the rate of delignification (Clayton, 1969).

1.8.1. Model experiments in wood pulping process

The reactions of lignin in alkaline pulping cannot be elucidated fully until the structure of lignin is known with certainty (Clayton, 1969). However, the evidence that lignin in coniferous woods is formed in the same way as the artificial lignins produced by the dehydrogenation polymerisation of the p-hydroxycinnamyl alcohol type (e.g. coniferyl alcohol, Figure 1.3a) is now widely accepted. If lignin can be degraded in hot alkali in the manner indicated by results on model compounds, why does delignification in soda liquor at 140 ^{0}C virtually cease after about one third of the lignin has been dissolved? It is currently assumed that the degradation products condense either themselves or with the residual lignin in the fibres, forming products which are scarcely attacked by alkali at 140 ^{0}C and only slowly attacked at higher temperatures (Clayton, 1969). The reasons for the striking increase in delignification rate when hydrosulphide ions are present has been extensively investigated. One possibility was that the reducing power of the sulphide was responsible, since the redox potential of a Kraft liquor even after the digestion is twice that of the corresponding soda liquor.

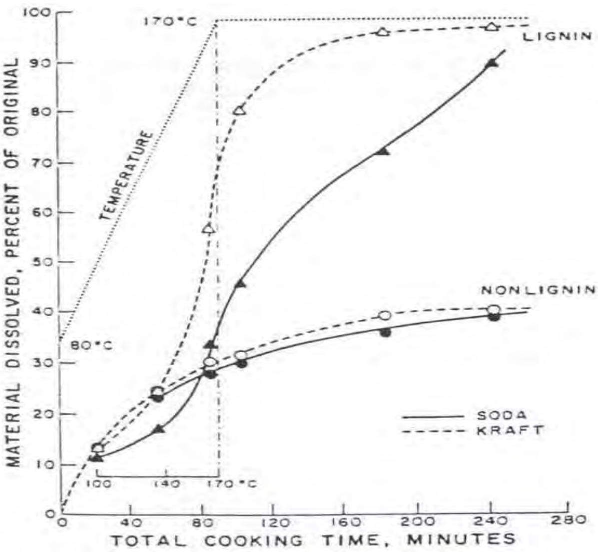


Figure 1.6(a) Removal of lignin and nonlignin by Kraft and soda liquors (Clayton, 1969).

However, if the redox potential of the soda liquor is adjusted to the same value as that of the Kraft liquor by addition of reducing agents other than that of sulphide, e.g., pyrocatechol, delignification is not accelerated. Therefore the effect of sulphide is due not to its reducing properties but rather to a chemical reaction of lignin or its alkaline degradation products, with sulphide or hydrosulphide ion. The β-guaiacylether bonds in model compounds of type I (Figure 1.6b) are split by 2N NaOH at 170 ^{0}C to the extent of about 30%, but in Kraft liquor at the same temperature almost complete cleavage occurs (Clayton, 1969).

In order to explain the difference, Geirer *et al.* (1960) proposed that the splitting in Kraft liquor proceeds by a different mechanism from that pertains in alkali alone. The quinone methide structures such as II (Figure 1.6b) are considered to be the intermediates in each case. On treatment with alkali in the absence of hydrosulphide ions these are partly hydrolysed to yield guiacol ions V (Figure 1.6b) and partly converted to structures such as III, which remain incompletely hydrolysed and thus account for the low yields of guaicol obtained in alkali alone.

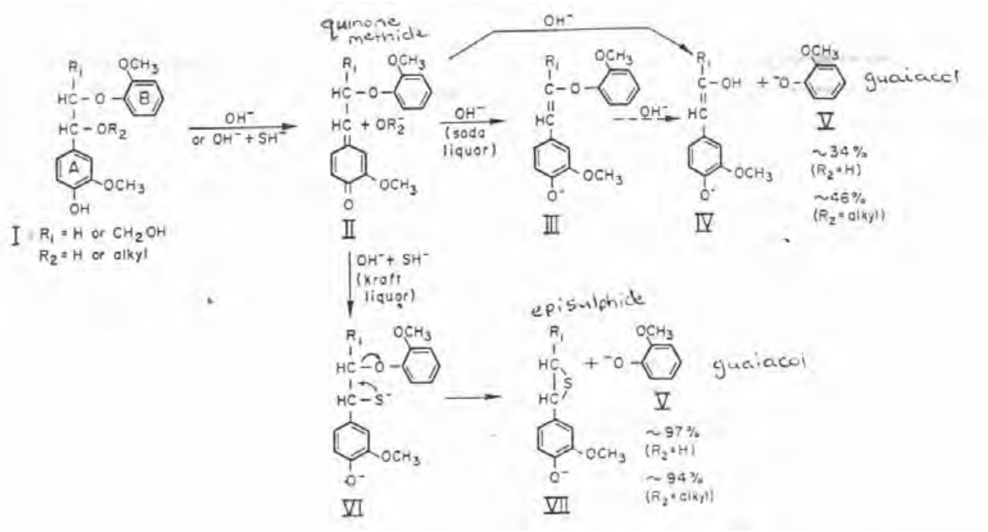


Figure 1.6(b) Cleavage of β-aryl ether linkage in lignin model compounds by soda and Kraft liquors (Clayton, 1969).

In contrast, when hydrosulphide ions, SH^-, are present, as in Kraft liquor, almost complete cleavage occurs in few min at 170 ^{0}C. Model experiments indicate that the initial step is addition of an SH^- ion to the quinone methide II, forming the intermediate VI, which contains a mercaptide ion, attached to the α-carbon atom (Clayton, 1969). This strongly nucleophilic ion then reacts intramolecularly to form an episulphide VII, displacing the guaiacyl ether group attached to the β-carbon. This mechanism is called a "neighbouring group effect."

1.9. Research hypothesis

Although the significance of the various anaerobic mechanisms to turnover of lignin is not fully understood, the following statements can be made in summary:

i) Microbial metabolism of lignin derivatives occurs in the absence of molecular oxygen during anaerobic photo-metabolism, nitrate and sulphate reducing conditions, in microbial consortia where aromatic cleavage is often coupled to methanogenesis, and by individual organisms that are able to cleave and demethoxylate the aromatic ring;

ii) Oligolignols (MW<1400) may be released as intermediate products during lignin degradation by aerobic fungi and bacteria and may be partially degraded to CO_2 and CH_4 by anaerobic microorganisms;

iii) Polymeric lignin is mineralised to CO_2 and CH_4 in anoxic sediments at slow but environmentally significant rates.

Despite recent progress made in understanding the aerobic mechanisms of lignin biodegradation (Crawford, 1981), relatively little attention has been given to its biodegradation under sulphate reducing conditions.

The result of one study, in which no biodegradation of synthetic lignin was observed in a variety of anoxic sediments (Zeikus, 1981) has led to a general belief that lignin is inert in anaerobic habitats. Results from several studies have shown that molecular oxygen is not needed for break down of the β-0-4 bonds present in lignin (Hearly and Young, 1985). This evidence suggest that lignin biodegradation under anaerobic conditions, especially sulphate reducing conditions has been under-estimated and merits re-evaluation.

Based on recent observations and research studies by Pareek *et al*. (1998) and Whittington-Jones (2000), it is proposed that the lignin biopolymer is cleaved under biosulphidogenic conditions with resultant production of lignin derived aromatic compounds that are further utilised as electron sources for the biological sulphate reduction process. In order to use the lignin biopolymer as an electron source for SRB in the treatment of AMD, it is important to understand its biodegradation. Nevertheless, current knowledge concerning biosulphidogenic cleavage of lignin is not adequate and a number of questions have to be answered:

(i) Can lignin be chemically hydrolysed at room temperatures (25 °C to 26 °C) under high sulphide and alkaline conditions?

(ii) What is the biological contribution of the SRB consortia, biogenic sulphide and alkalinity in lignin biodegradation?

(iii) What are the biodegradation mechanisms involved in biosulphidogenic hydrolysis of lignin?

1.10. Research objectives

In order to address various aspects of biosulphidogenic hydrolysis of lignin, the following objectives were identified for this study:

1. To compare the hydrolysis of lignin under chemical and biologically-derived sulphidogenic conditions at room temperature and pressure;
2. To determine the impact of biosulphidogenic microbial consortia on the degradation of model aromatic compounds;
3. To attempt to derive an explanatory model accounting for the biodegradation of lignin under biosulphidogenic conditions.

Chapter 2

The Effects of Sulphide and Alkalinity on Wood Hydrolysis

2.1. Introduction

The abundance of lignocellulose and its potential as a cheap energy source has lead to continued research into improved utilisation of this biopolymer. Lignin is considered to be recalcitrant to anaerobic biodegradation, this impedes the utilisation of cellulose which is the major carbon source in the biosphere (Banks and Humphreys, 1998). It is a polymer of phenyl-propane subunits (figure 1.3) linked together by ether and carbon-carbon bonds, and is highly resistant to biological breakdown (Buswell and Odier, 1987).

In paper industry, lignin is considered as a rate-limiting step in high quality pulp production (Clayton, 1969). During the Kraft process for paper production, lignin is chemically degraded at ±170°C, using 25% Na_2S and 18% NaOH (Clayton, 1969). The common β-aryl ether bonds in lignin are cleaved to yield quinone methide (figure 1.6b) intermediates. The rate of delignification is accelerated in the presence of sulphide, and it is thought that the reaction rate is higher than in the presence of alkalinity alone. In the presence of sulphide, the quinone methide intermediates are converted to episulphide and guaicol compounds. The formation of episulphide is thought to facilitate the pulping process by preventing re-polymerisation of the phenylpropane subunits (Clayton, 1969). Recent attempts in improving the degradation of solid waste revealed that the solubilisation of lignocellulosic waste was greater under sulphate reducing conditions than the conventional methanogenic digestion (Kim *et al.,* 1997). Pareek (1998) also reported that the rate of lignicellulose hydrolysis is enhanced under sulphate reducing conditions.

The effect of sulphide and pH alone at room temperatures and pressure on wood material has not been reported. Recent studies by Whittington Jones (2000) have shown that the rate of release of wood aromatics is enhanced under high sulphide and pH conditions in the treatment of primary sewage sludge.

2.2. Objective

Based on the above-mentioned observations, the chemical degradation of lignin at room temperatures under high sulphide and alkaline conditions was investigated in this study using pine wood powder as the lignocellulose source. The effect of an increase in sulphide and pH at room temperature in the enhancement of the release of wood derived aromatic compounds was investigated. The wood extracts were prepared according to the standard known procedures by Kirk and Bruno (1988).

2.3. Methods

2.3.1. Wood extracts preparation

The different wood extracts were prepared as summarized in figure 2.1 below from dry pine wood powder that was passed through 1000 μm sieve.

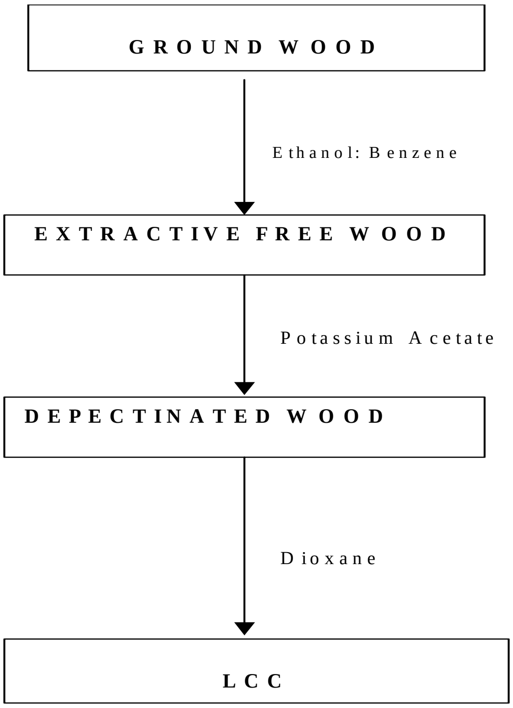


Figure 2.1. Procedure for preparation of different wood extract.

2.3.2. Untreated wood preparation

Pine wood powder was collected from a sawmill, dried and passed through a 1000 μm sieve and used as lignocellulose source for this study. The amount of wood powder, sulphide and alkalinity range is summarised in table 2.1.

2.3.3. Extractive free and depectinated wood preparation

About 100g of wood powder (1000 μm particle size) was extracted with a 1l of ethanol: benzene (1:2, v/v) for 48h. The extracted wood was then dried at room temperature and then depectinated with a 1l of 0.25% (w/v) aqueous potassium acetate at 60 °C for 24h. The dried wood fractions were then used for investigating the effect of pH and sulphide as shown in table 2.1.

Table 2.1. Experimental procedure for investigating the effect of sulphide and alkalinity on wood.

Wood Extract	**Wood / g**	**Na_2S / mg/l**	**pH**
Untreated Wood	100	500	8 - 9
Extractive Free Wood	100	500	8 - 9
Depectinated Wood	100	500	8 - 9

For each wood extract, the experiments were done in 250ml anaerobic flasks (figure 2.2). Each experiment had 5% wood with flask A containing wood in water with 500 mg/l Na_2S and pH 8 – 9, flask B containing wood in water at pH 8 – 9, flask C and D containing wood in water without pH adjustment. The flask reactors were left on an orbital shaker (100 rpm) at 25 ^{0}C during the period of the study and were sampled at regular intervals with pH adjustments on a daily basis using sodium hydroxide. The optimun pH for the release of phenolics was determined experimentally to be pH 8-9, where wood in water was used as a control.

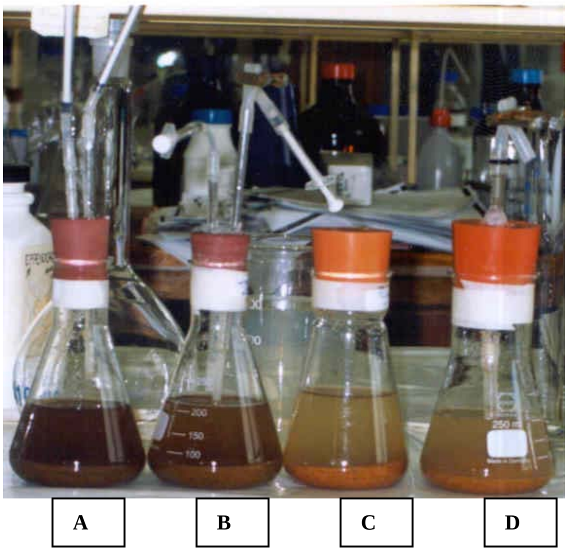


Figure 2.2. Anaerobic flasks used in investigating the effect of alkalinity and sulphide on untreated wood hydrolysis. A=500mg/l sulphide + pH 8 to 9, B= pH 8 to 9, C and D=wood in water as controls.

2.4. Analytical procedure

The pH in the experimental flasks was maintained between pH 8 – 9 with sulphide at 500 mg/l. The release of wood aromatic compounds was analysed by High Performance Liquid Chromatography (HPLC) in triplicate. HPLC analysis was performed using the Beckman HPLC, system gold, module 126 with UV/VIS detector module 166 at 310nm. Waters symmetry C_{18} column (4.6x250mm) 5μm particles size was used for separation of aromatic compounds. Acetonitrile and water (0.1% Acetic acid) at a ratio of 60:40 was used as an eluent at 1ml/minute flow rate. Samples were prepared as described in appendix 4. Naphthalene was used as an internal standard for HPLC quantification and triplicate samples were analysed. The amount of aromatics lost during sampling was estimated by using the internal standard and this gave a relative concentration of wood aromatics released.

2.5. Electron microscopy

Surface modification on wood was investigated by scanning electron microscopy, JSM model 840, at x 500 magnification. The wood samples were prepared by putting small wood powder particles on graphite strips mounted on metallic stubs. The samples were then coated with gold and then viewed for surface modification.

2.6. Results

The effect of alkalinity and sulphide on the release of wood aromatic compounds on different wood preparations was analysed as shown below.

2.6.1. Untreated wood

The release of wood aromatics from untreated wood was monitored over time as shown in figure 2.3 to 2.5. Using naphthalene as an internal standard, the relative concentrations of the wood aromatics were estimated from naphthalene standard curve.

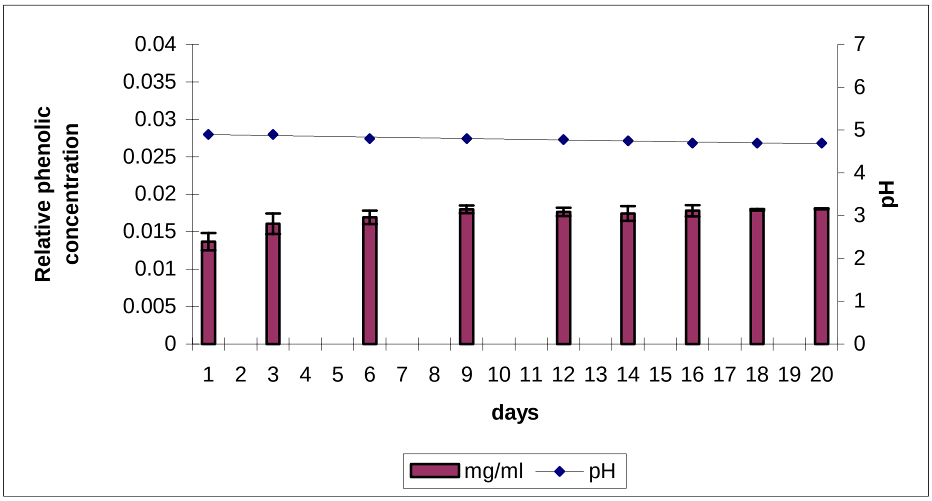


Figure 2.3. Flask studies of untreated wood in water. The graph shows the relative levels of aromatics released from wood in water without pH adjustment. The bars indicate the standard error of the samples analysed in triplicate.

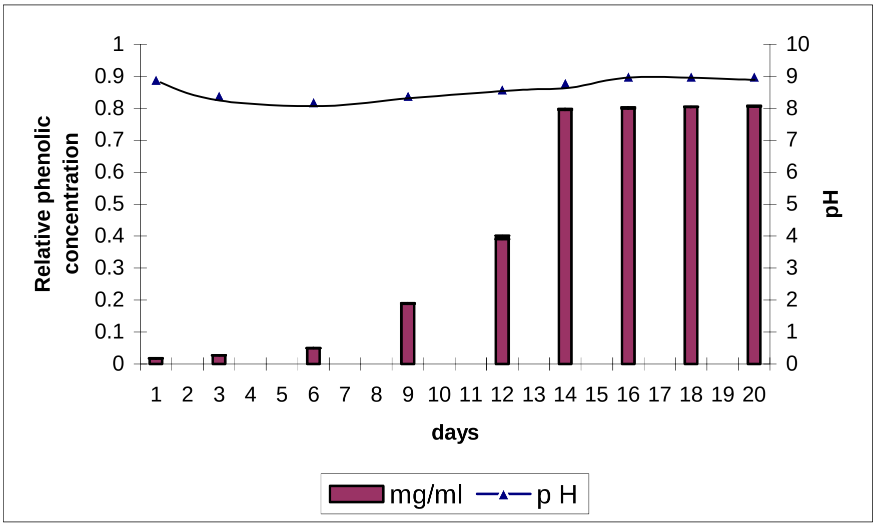


Figure 2.4. Flask studies of untreated wood at pH 8 to 9. The graph shows the release of wood aromatics under conditions of elavated pH.

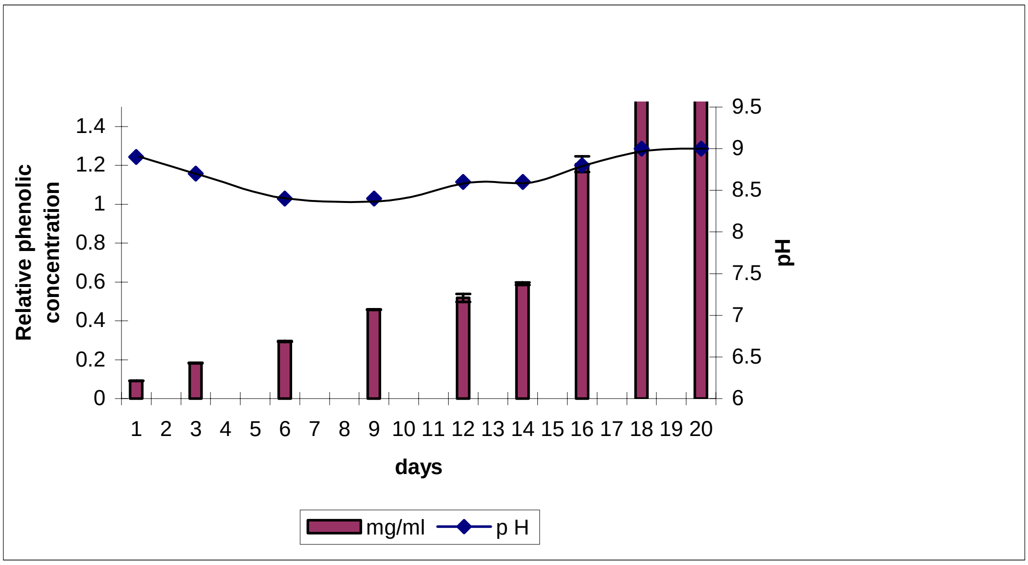


Figure 2.5. Flask studies of untreated wood at pH 8 – 9 and 500 mg/l sulphide. The graph shows the effect of both sulphide and pH on the release of wood aromatics.

The results from figure 2.3 to 2.5 show an increased release of wood aromatic compounds as a result of an increase in alkalinity and sulphide levels.

2.6.2 Extractive free wood

Wood powder was extracted with ethanol:benzene (1:2) in order to remove the extractives (figure 2.6) for further investigation of the effect of both pH and sulphide on wood hydrolysis. Figure 2.6 shows the release of wood extractives after treatment of wood powder with ethanol benzene. The extractive released at retention time less than 10 min are relatively polar in nature. Figure 2.7 to 2.9 show the effect of pH and sulphide on hydrolysis of extractive free wood.

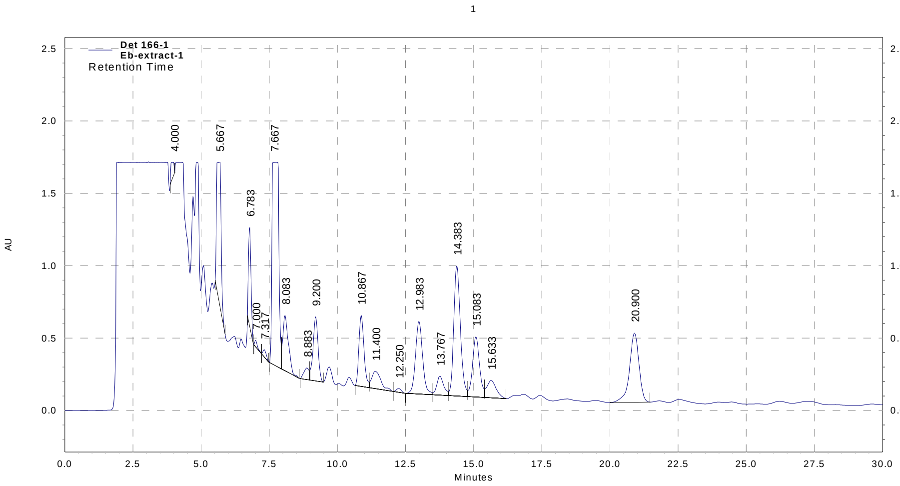


Figure 2.6. HPLC analysis of wood extractives from the treatment of wood powder with ethanol: benzene (1:2).

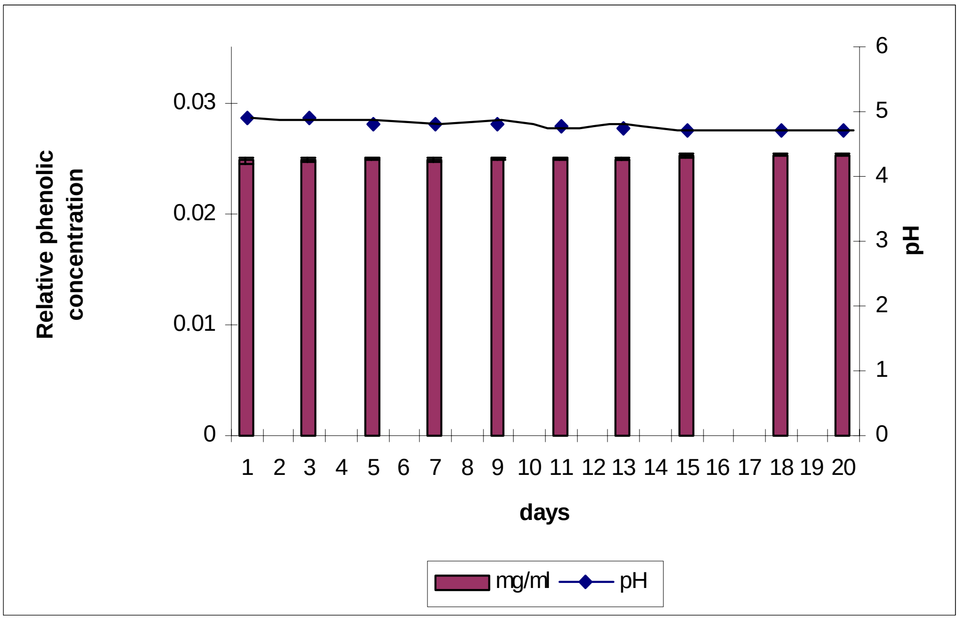


Figure 2.7. Flask studies of extractive free wood in water and the relative levels of wood aromatics released.

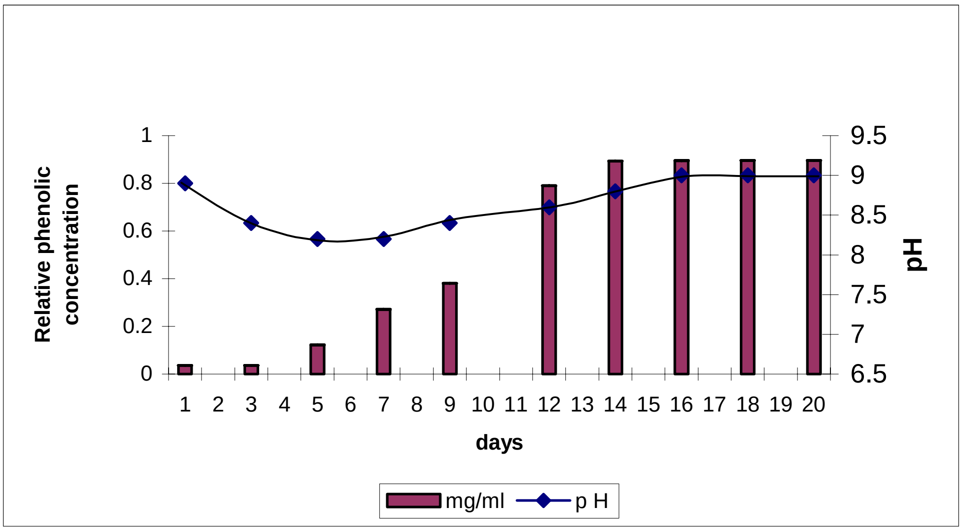


Figure 2.8. The effect of pH on the release of aromatics from extractive free wood.

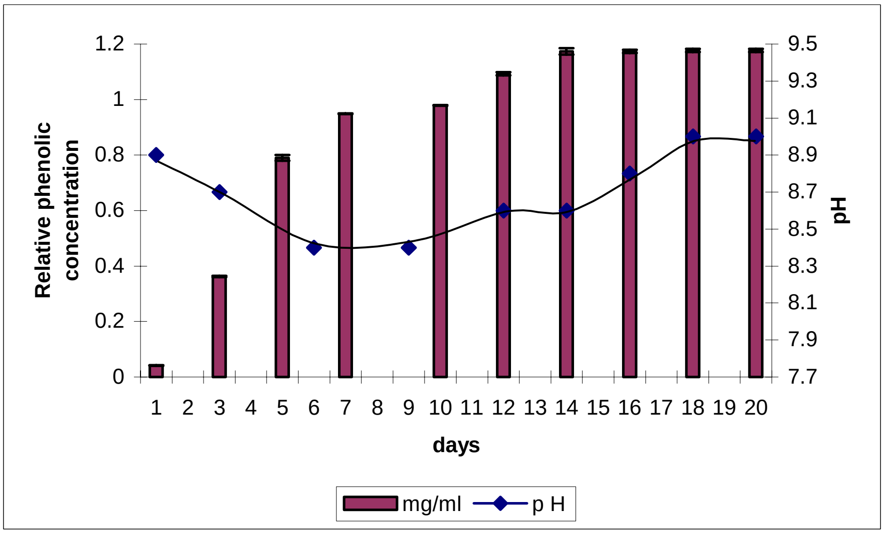


Figure 2.9. The effect of both sulphide and pH on the release of aromatics from extractive free wood.

The results of aromatic release from extractive free wood show that at elevated pH and sulphide, there is an increased release of aromatic compounds.

2.6.3. Depectinated wood

Extractive free wood was treated with potassium acetate in order to remove pectin. Figure 2.10 to 2.12 report the results of effects of sulphide and alkalinity on depectinated wood.

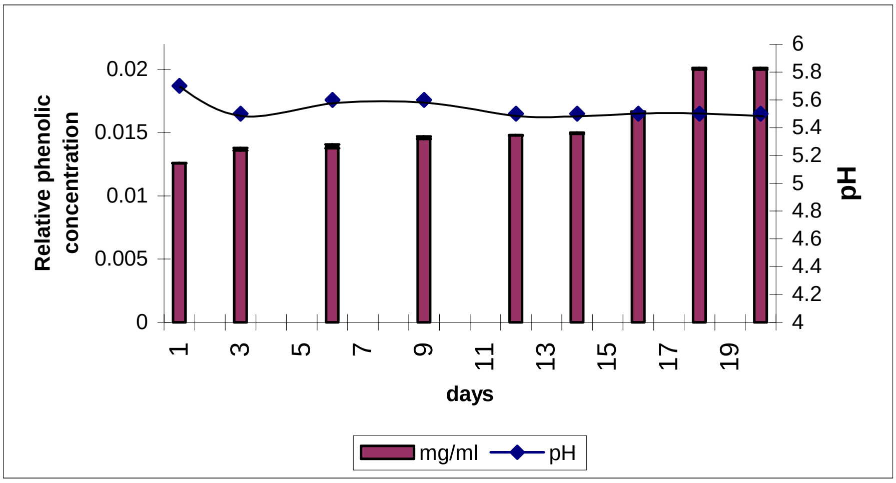


Figure 2.10. Flask studies of depectinated wood in water.

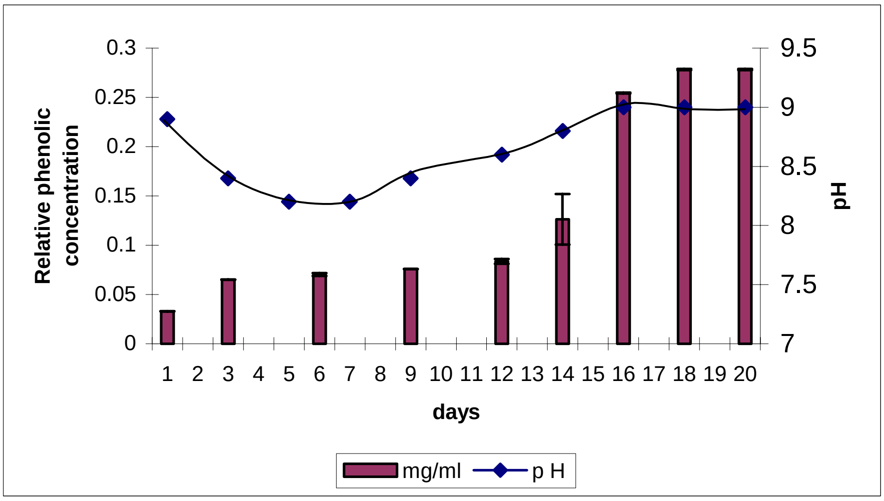


Figure 2.11. Flask studies showing the effect of pH on the release of aromatics from depectinated wood.

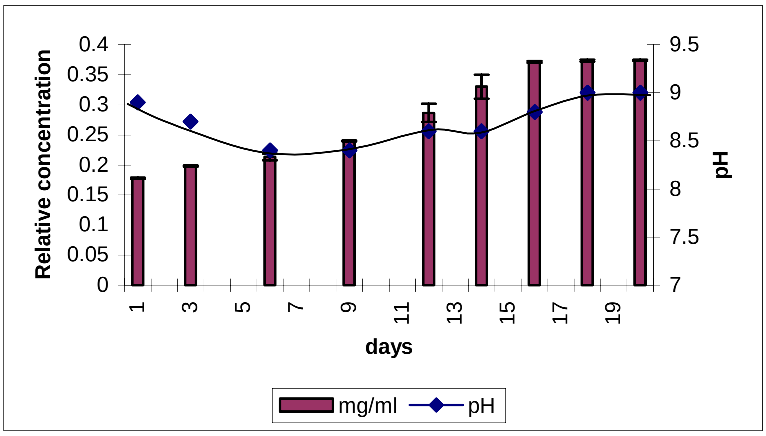


Figure 2.12. The effect of both sulphide and pH on the release of aromatics from depectinated wood.

The results (figure 2.10 - 2.12) show a relative increase in the release of wood aromatic compounds with increase in alkalinity and sulphide levels.

2.6.4. Scanning electron microscopy

The surface modification on wood structure was studied by scanning electron microscopy as shown in figure 2.1.3 below. The results show an increasing exposure of the underlying wood tissue as a result of elevated sulphide and alkalinity levels

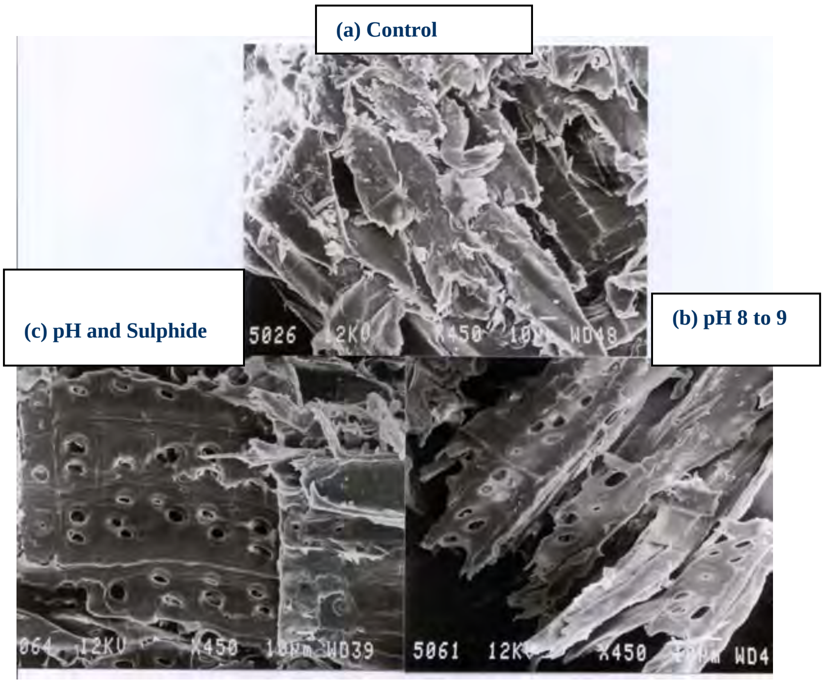


Figure 2.1.3. Effect of pH and sulphide on surface modification of untreated wood; (a) wood in water, (b) wood at pH 8 to 9 and (c) wood at pH 8 to 9 with sulphide.

2.7. Discussion

During Kraft pulping at elevated temperatures, lignin is hydrolysed under alkaline conditions by a combination of high pH and sulphide concentration (Clayton, 1969). Both these are also products of microbial sulphate reduction. The alkali results in swelling of wood and is also responsible for degradation of lignin during Kraft pulping. Lignin phenols produced during the alkaline hydrolysis process are soluble and can be utilized by bacteria (Zeikus *et al.,* 1982). Sulphide is thought to act by preventing the repolymerisation of the hydrolysed aromatic monomers (Clayton, 1969). The effect of elevated pH and sulphide on wood hydrolysis at room temperatures has apparently not been thoroughly investigated.

This study has revealed that, at room temperature there is an increased release of wood aromatics on exposure to elevated pH and sulphide and in the absence of microbial activity. This is also evidenced by the use of different wood preparations; untreated, extractive free and depectinated wood powder. Extractives are the compounds present in wood that can be solubilised by organic solvents and include volatile oils, wood resins, fats, waxes, tannins and lignans. Softwoods are known to have high levels of these extractives (table1.1).

The untreated wood experiments (figure 2.3 – 2.5) showed that the combination of both sulphide and pH enhances the release of wood aromatics compared to wood in water alone. Untreated wood has high levels of extractives as shown by HPLC analysis of ethanol:benzene (1:2) extract (figure 2.6). The increased release of aromatics from untreated wood could be due to the presence of high levels of the extractives and other wood aromatics present. Most of these aromatics are soluble in these basic conditions. Alkalinity modifies the wood structure resulting in wood swelling and an increased release in the amount of soluble aromatics.

Treatment of wood by ethanol:benzene (1:2) removes most of the extractive fraction making wood more susceptible to the effects of high pH and sulphide. Extractive free wood also shows results comparable to untreated wood and shows that the extractives do not necessarily impede the release and accessibility of wood in this particular case (figure 2.7 to 2.9). The removal of these extractives allows more accessibility to lignin and cellulose.

Depectination of extractive free wood is the final step in ensuring the total removal of residual extractives and pectin. Depectination is done at slightly basic conditions and this ensures that pectin and most of the soluble aromatics are removed from wood. Effects of sulphide and pH on depectinated wood gives more information on the hydrolysis of lignin at room temperatures. The low levels of aromatics (≈30% of untreated) released into solution from depectinated wood could be due to the loss of extractives. Hence, the level of these aromatics is lower compared to both untreated wood and extractive free wood (figure 2.10 – 2.12).

Surface modifications on wood structure were observed by scanning electron microscopy (figure 2.6). Both elevated pH and sulphide appear to clear the surface of wood and exposing more wood tissue. This results in the release of wood extractives and wood aromatics that are soluble in basic solution.

2.8. Conclusion

- The release of aromatic compounds from wood is enhanced at pH 8 to 9 with an increase in sulphide concentration around 500 mg/l. This observation although performed at room temperatures resembles the basic outcome of the Kraft pulping process.
- Both aromatics and wood extractives are readily soluble in alkaline medium and they can be utilized as carbon source by SRB in the treatment of acid mine drainage.
- Scanning electron microscopy on wood shows that both high pH and sulphide modify the surface of the wood resulting in wood apparently becoming more porous and brittle. This may accelerate the release of extractives and aromatics.
- The results seem to support the proposed hypothesis that sulphide and high pH at room temperatures accelerates the release of wood aromatics.

- The conditions used in this study (sulphide and alkalinity) are comparable to those generated by SRB. These observations raise one major question concerning the lignin degradation under biosulphidogenic conditions: - Does lignin biodegradation depend only on the chemical environment created by SRB activity or is an additional biological contribution involved?

Chapter 3

Biosulphidogenic Degradation of Wood and Lignin

3.1. Introduction

The SRB consortium is known to produce sulphide and alkalinity from biological sulphate reduction process. In chapter 2, it has been demonstrated that the combination of sulphide and alkalinity at room temperatures accelerates the release of extractives and wood aromatics. In the presence of sulphate reducers, these extractives and wood aromatics can be used as carbon and electron donor sources. The SRB consortium is also known to degrade aromatic compound such phenol (Bak and Widdel, 1986), xylene (Harms *et al.,* 1999) and naphthalene (Meckenstock *et al.,* 2000).

3.2. Objective

Several questions need to be addressed concerning the biosulphidogenic degradation of lignin:

(i) Does sulphide and alkalinity generated by the SRB consortia affect the cleavage of lignin and the release of wood aromatic compounds in a manner similar to chemical addition of these components?

(ii) Do additional biological effects occur in lignin biodegradation in the presence of an SRB consortium?

3.3. Methods

The pine wood extracts used in these studies were prepared as shown previously in figure 2.1.

3.3.1. Untreated wood preparation and bioreactor studies

Small-scale anaerobic bioreactors (figure 3.1) were used in the study and were inoculated with 10% actively growing culture of an SRB consortium from Rhodes Biosure Process pilot plant at the EBG experimental field station in Grahamstown.

About 5% wood powder was used for all biodegradation studies. Initially, the inoculum was supplemented with lactate in order to establish an SRB population. The bioreactor was connected to the 10% zinc acetate trap in order to precipitate excess sulphide as zinc sulphide (figure 3.1).

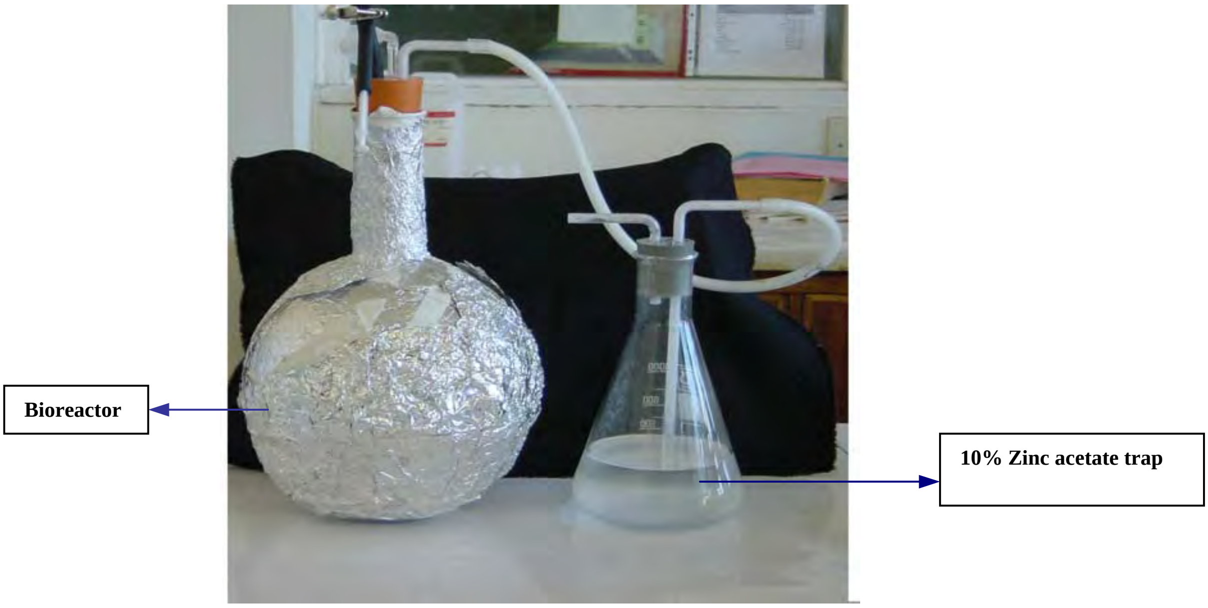


Figure 3.1 Small-scale bioreactor set up used in the studies on biosulphidogenic degradation of lignin.

Subcultures were inoculated with 10% volume of the inoculum supplemented with mineral media (Appendix) in 2 to 5 l flask reactors connected to 10% Zinc Acetate to trap hydrogen sulphide. The subcultures contained 5% wood powder as a sole carbon source and mineral media with ±2000mg/l sulphate at pH 7.5.

3.3.2. Depectinated wood preparation and bioreactor studies

About 100g of wood powder (1000μm particle size) was extracted with a 1l of ethanol:benzene (1:2, v/v) for 48h. The extracted wood was then dried at room temperature and then depectinated with a 1l of 0.25% (w/v) aqueous potassium acetate at 60°C for 24h. 5% dried depectinated wood was used for bioreactor studies as described for untreated wood experiments. Depectinated wood is extractive free and represents the lignin carbohydrate complex.

3.3.3. Lignin isolation and bioreactor studies

Depectinated wood the preparation of which has been described (100g) was used for lignin isolation. About a 1l of dioxane:water (96:4 v/v) was used to extract 100g of depectinated wood powder stirred at room temperature for 48hours. The residue was then re-extracted with the same solvent. The extracts were combined and then freeze dried to give a crude lignin powder.

3.4. Analytical procedure

Sulphate concentration was monitored as an indicator of biological sulphate reduction on HPLC. Wood aromatics were also analysed on HPLC to study the biodegradation of wood and the release of both the extractives and wood aromatics.

3.4.1. Sulphate analysis

Sulphate was analysed by Waters HPLC system, model 510 equipped with Waters Conductivity Detector, model 430. PRP-X100 anion column (150x4.1mm) with particle size of 10µm was used for sulphate separation. 4mM p-Hydroxybenzoic acid with 2.5% methanol at pH 8.5 was used as an eluent at 1.5ml/minute flow rate.

3.4.2 Aromatic compounds analysis

Aromatics were analysed using Beckman HPLC, system Gold, module 126, with Photo-diode Array Detector (PDA), module 168. Waters Symmetry C_{18} column (4.6x250mm) 5µm particles size was used for separation of aromatics. Acetonitrile, water and acetic acid ratio 60:39.9:0.1 was used as an eluent at 1ml/minute flow rate.

3.4.3 Qualitative analysis of volatile fatty acids

Production of volatile fatty acids was analysed by GC (Hewlett Packard 6980 series) equipped with a flame ionization detector (FID). Samples were prepared as described in appendix 4. The time program was 40 °C (1min), 5 °C/min and 250 °C (10min). Commercial standards such as acetic acid, valeric acid and lactic acid were used for identification purposes.

3.5. Results

In this study only the untreated and depectinated wood have been considered since extractive free wood did not show a major difference to the untreated in terms of aromatic compounds released.

3.5.1. Sulphide analysis in wood bioreactors

The levels of hydrogen sulphide in all bioreactors were analysed for a period of 60 days (Appendix 2). Sulphidogenic conditions were comparable to those reported in chapter 2.

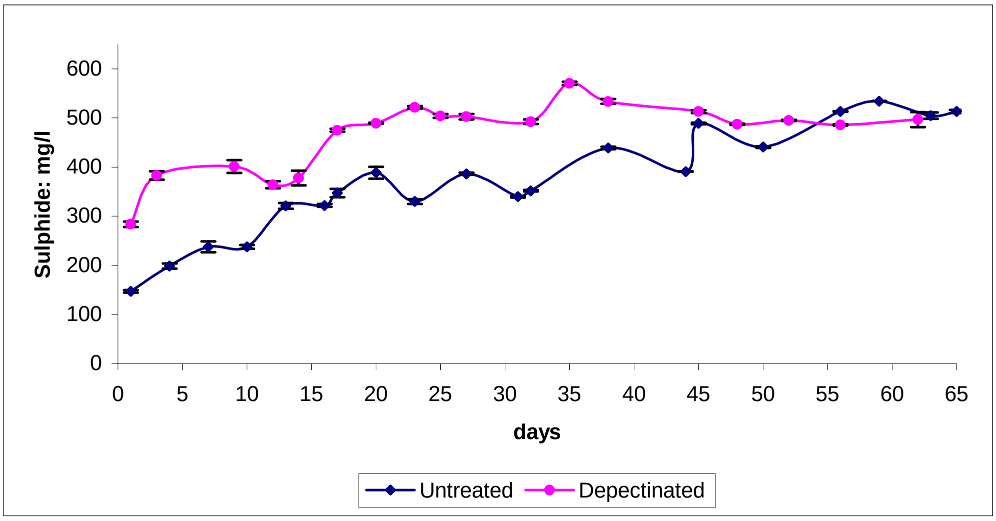


Figure 3.2. Sulphide level in untreated and depectinated wood bioreactors in the presence of the mixed sulphate reducing consortia.

3.5.2. Sulphate analysis in wood bioreactors

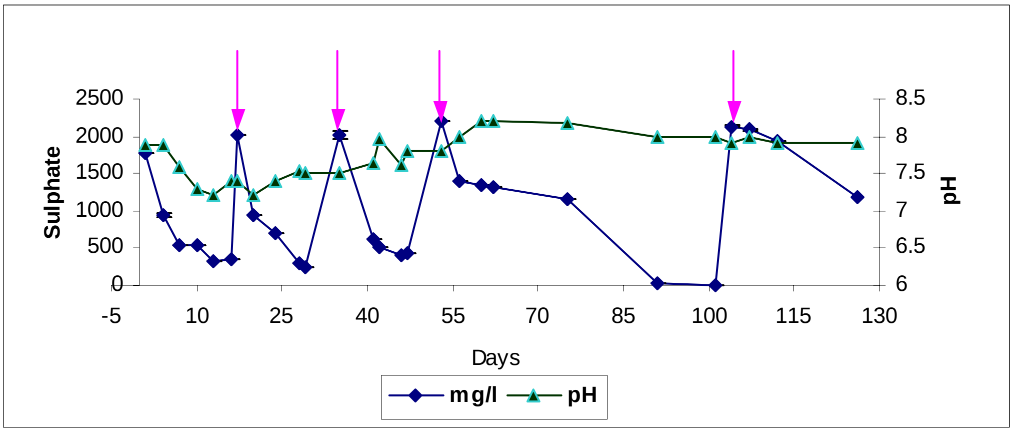


Figure 3.3. Sulphate and pH analysis in untreated wood bioreactor with the sulphate reducing consortia. Arrows indicate spiking with additional sulphate.

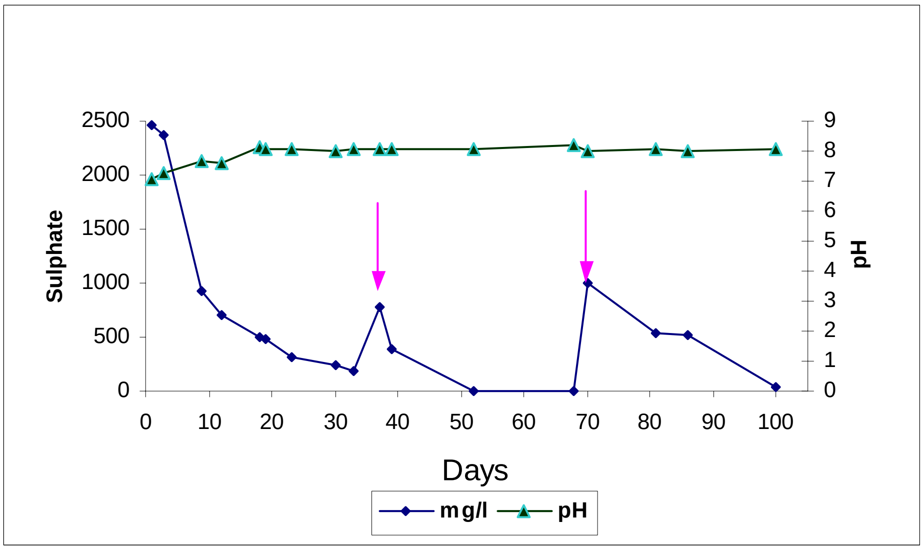


Figure 3.4. Sulphate and pH analysis in depectinated wood bioreactor with sulphate reducing consortium. Arrows indicate the spiking with additional sulphate.

Table 3.1.

The reduction rate of sulphate in untreated wood bioreactor. The standard deviation is shown brackets.

Day	Initial sulphate (mg /l)	Final sulphate (mg/l)	Sulphate utilised (mg/l)	% Sulphate reduction	Rate (mg/l/day)
1 - 16	1780 (0)	342 (2.8)	1438	80.8	95.9
17 - 29	2022 (1.7)	240 (1.3)	1782	88.1	145
35 - 47	2020.9 (4.6)	436.4 (1.2)	1584.5	78.4	132
53 - 91	2200.9 (1.1)	0	2200	100 (1.08)	57.8
104 - 126	2127.65 (13.1)	1176.95 (4.6)	950.7	45.7	43.2

Average reduction rate = 94.78 mg/l/day of sulphate

Table 3.2.

The reduction rate of sulphate in depectinated wood bioreactor.

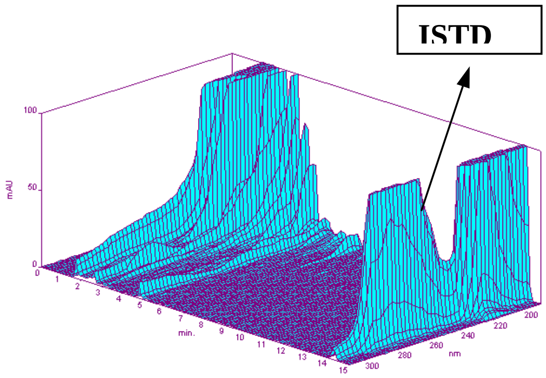
Day	Initial sulphate (mg /l)	Final sulphate (mg/l)	Sulphate utilised (mg/l)	% Sulphate reduction	Rate (mg/l/day)
1 - 33	2455.26 (1.8)	64.44 (2.3)	2390.82	97.4	74
37 - 58	776.67 (2.5)	0	776.67	100	37
70 - 100	1000 (6.0)	29.89 (1.6)	970.11	97	32

Average reduction rate = 47.67 mg/l/day of sulphate

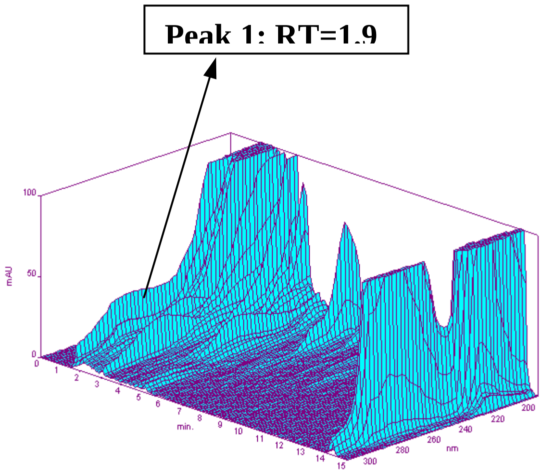
3.5.3. The release of aromatic and aliphatic compounds from untreated wood.

The PDA chromatograms below show the release of wood aromatics and aliphatic compounds in the presence of SRB consortia. The samples were scanned from a 190nm to 310nm. Peak 1, with the retention time of 1.9 min was used to estimate the amount of wood aromatics released at 310nm. Naphthalene was used as an internal standard to estimate the relative concentrations of aromatics and eluted at 14 min.

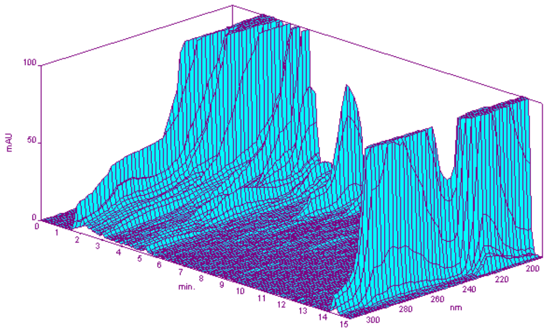
Day-1



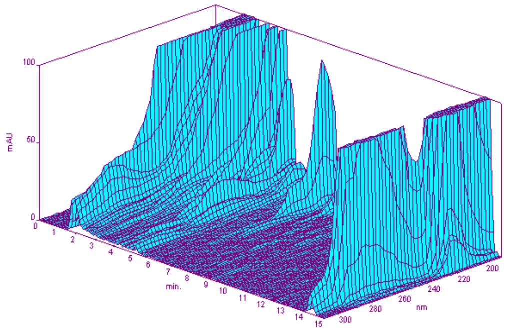
Day-4



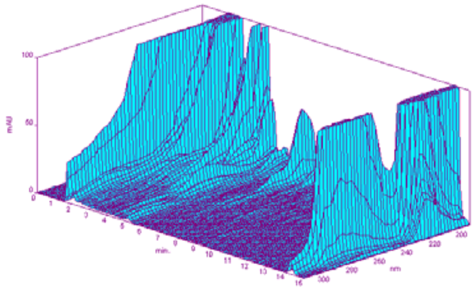
Day 7



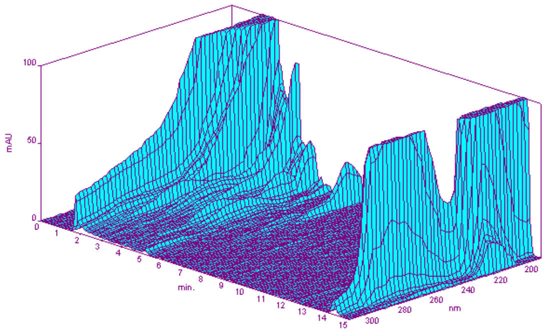
Day 10



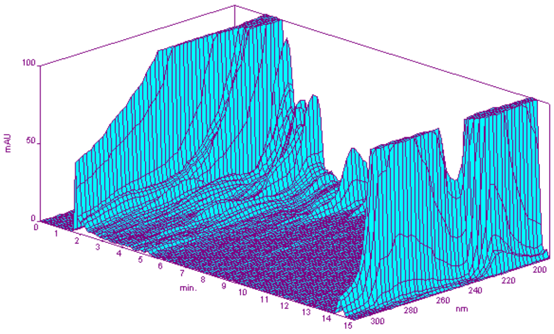
Day 13



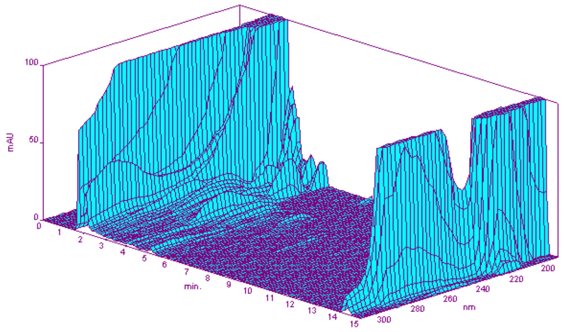
Day 20



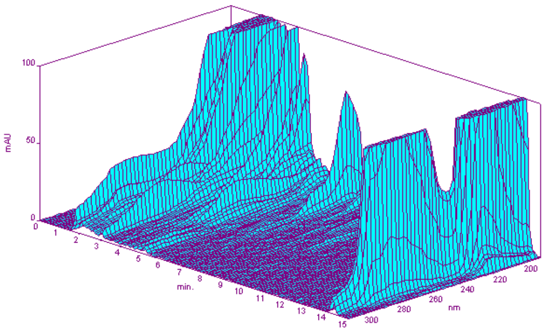
Day 27



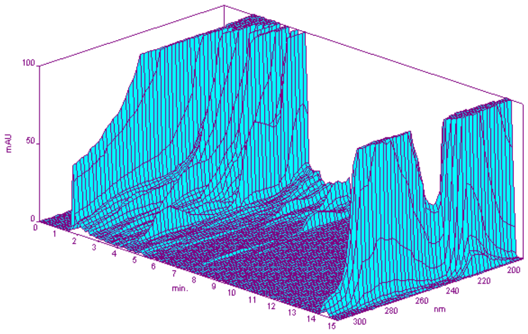
Day 31



Day 35



Day 38



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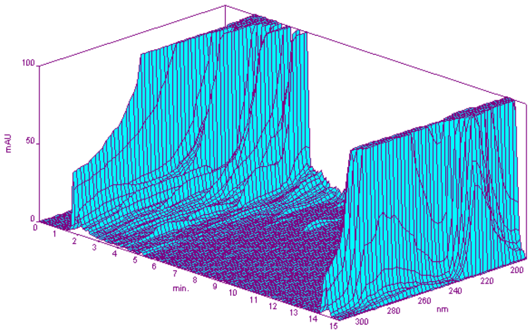
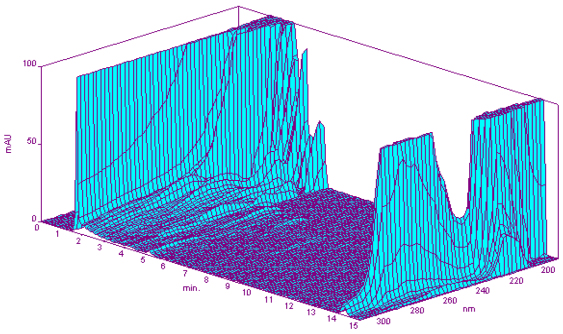
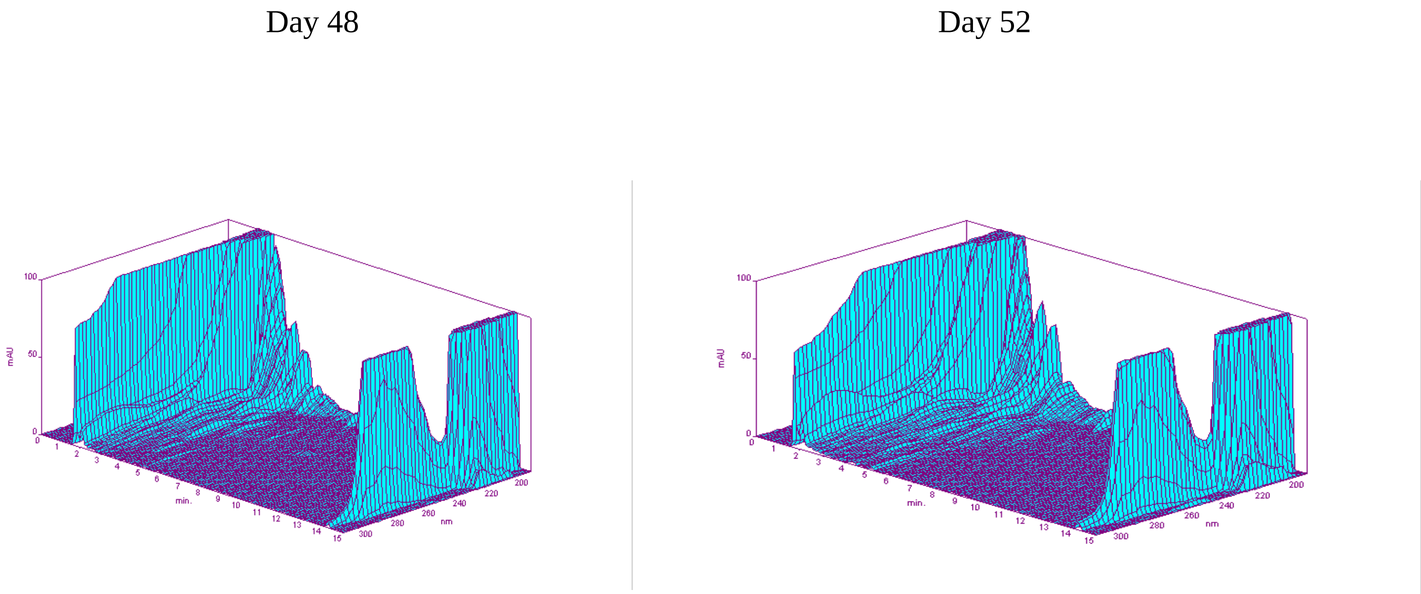
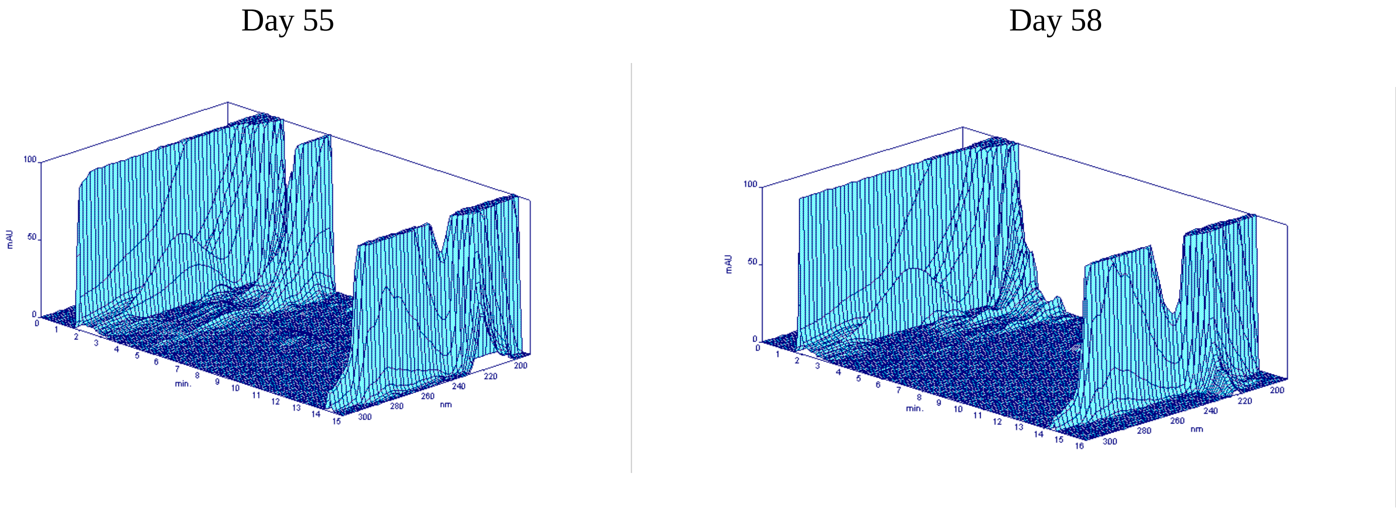


Day 44




Day 48

Day 52


Day 55

Day 58

Figure 3.5. The release of aromatic and aliphatic compounds from untreated wood in the presence of a mixed sulphate reducing consortia was followed over a period of 58 days. Individual phenolics from day 1 to day 58 are shown here.

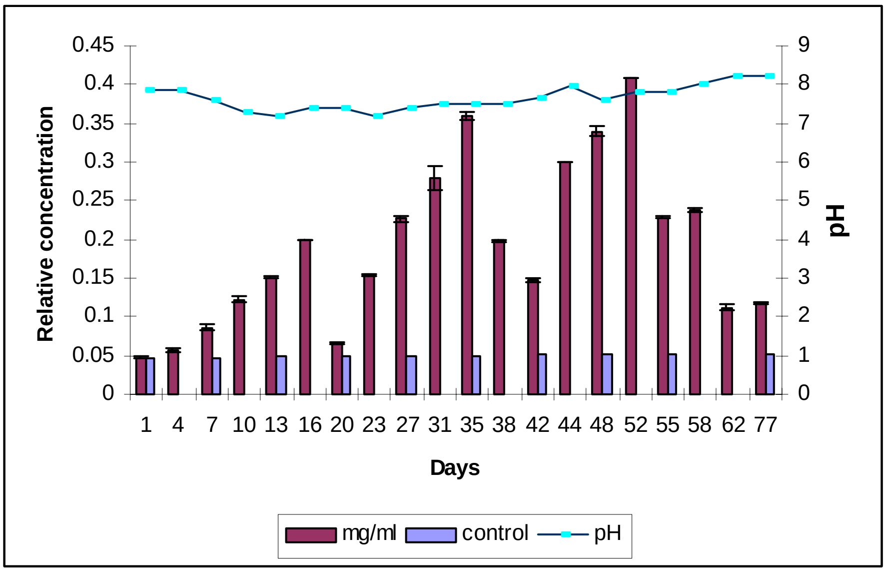
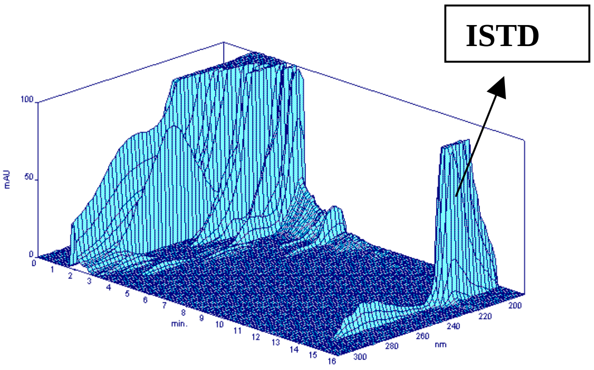


Figure 3.6. The relative concentration of wood aromatics (RT=1.9) in the untreated wood bioreactor exposed to the sulphate reducing consortia. Naphthalene was used as an internal standard to estimate the relative concentration of wood aromatics at 310nm.

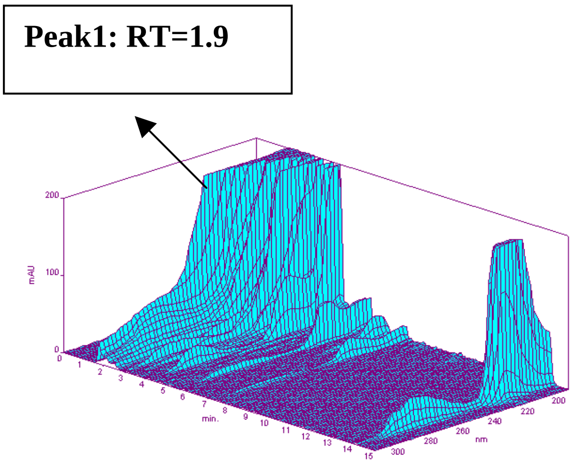
3.5.4. The release of aromatic and aliphatic compounds from depectinated wood.

The PDA chromatograms below show the release of aromatic and aliphatic compounds from depectinated wood in the presence of a mixed SRB consortia. Naphthalene was used as an internal standard and it eluted at 14 min.

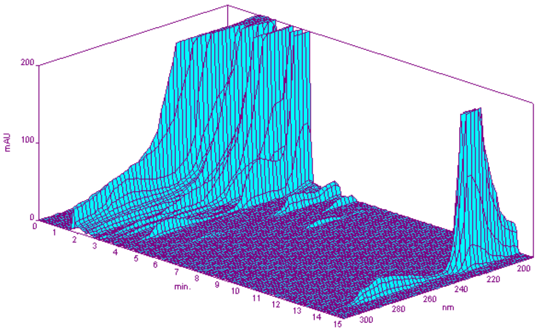
Day 1



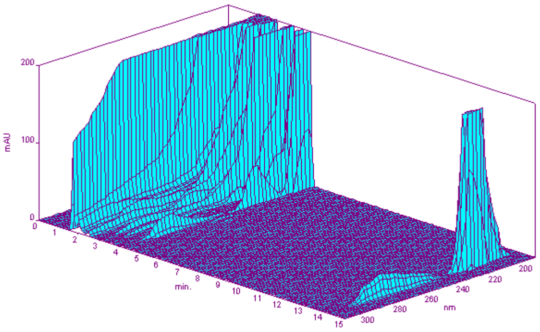
Day 3



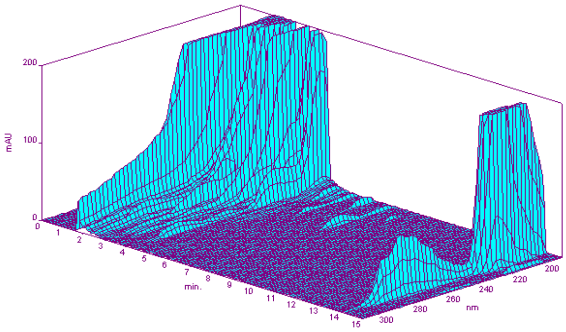
Day 9



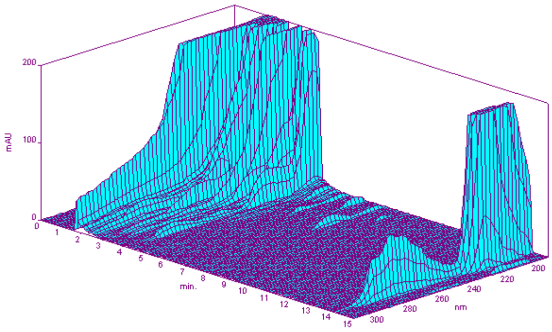
Day 13



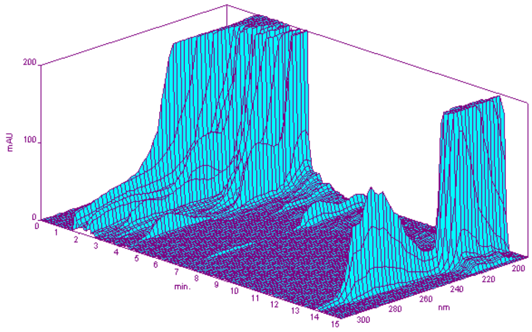
Day 17



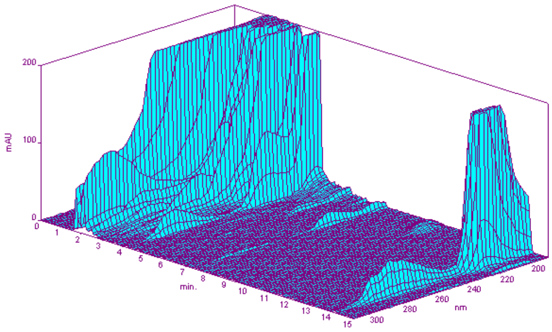
Day 19

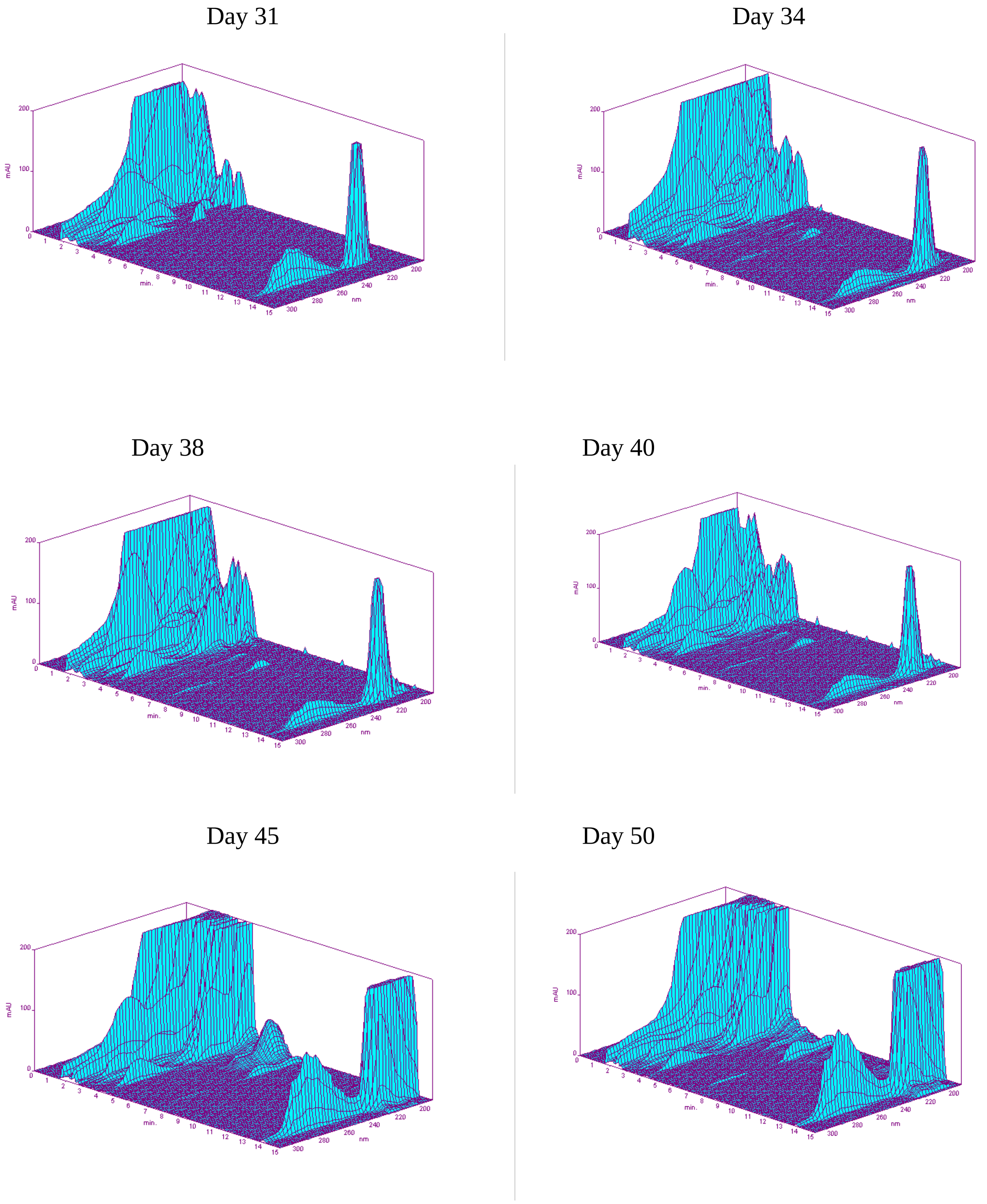


Day 22



Day 24




Day 31

Day 34

Day 38

Day 40

Day 45

Day 50

Figure 3.7. The release of aromatic and aliphatic compounds from depectinated wood in the presence of a mixed sulphate reducing consortia followed over a period of 50 days.

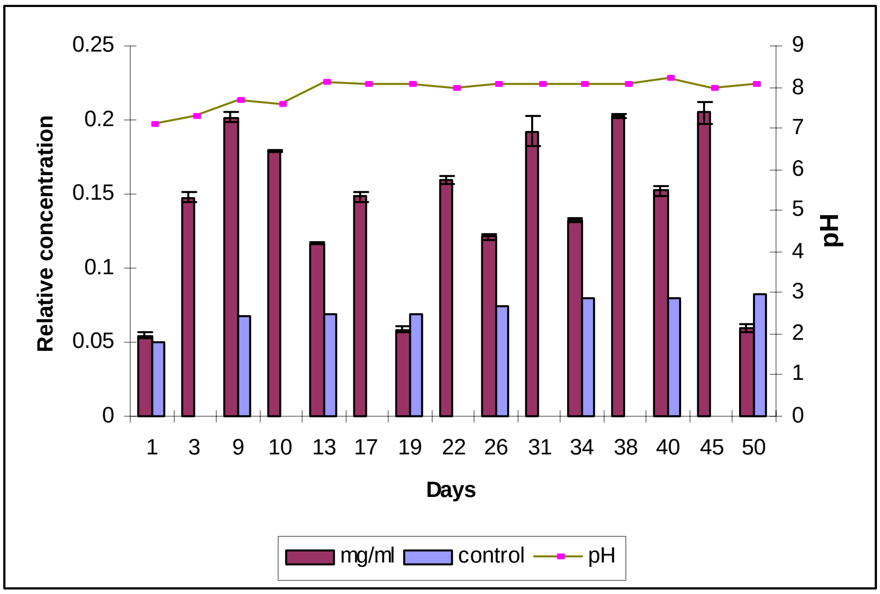


Figure 3.8. The relative concentration of wood aromatics (RT=1.9) in the depectinated wood bioreactor. Naphthalene was used as an internal standard to estimate the relative concentration of wood phenols at 310nm.

3.5.5. Lignin biodegradation under sulphate reducing conditions.

Lignin was isolated from depectinated wood using dioxane as described in the methods. About 1000mg/l lignin powder was used as a substrate for mixed SRB consortia. Sulphate and pH were analysed as shown in figure 3.9. The release of lignin aromatics was analysed using PDA as shown below.

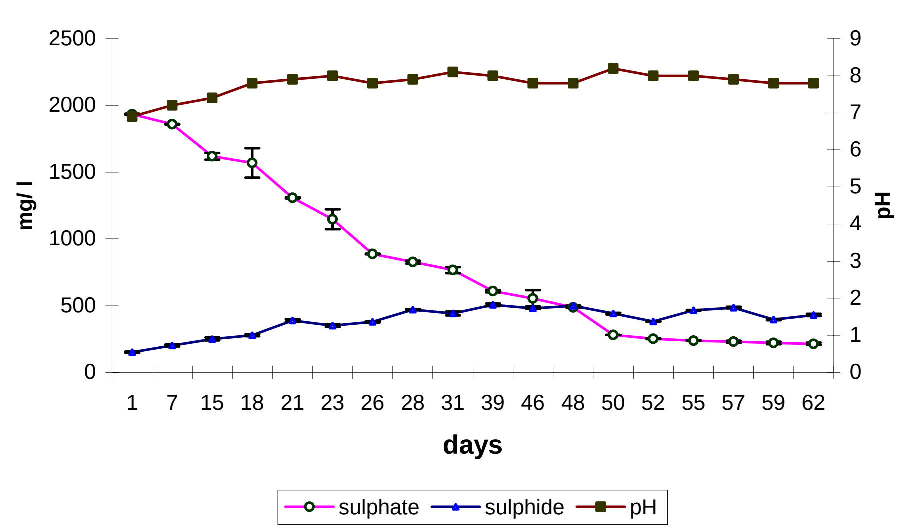
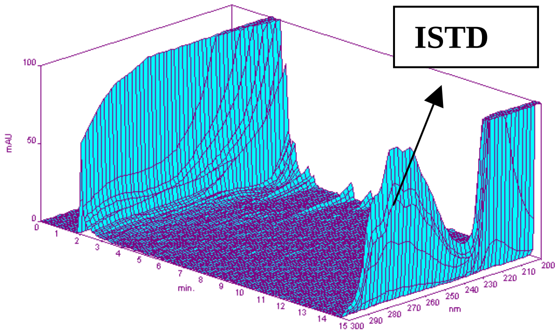


Figure 3.9. Sulphate reduction by a mixed sulphate reducing consortia using lignin as an electron donor and carbon source.

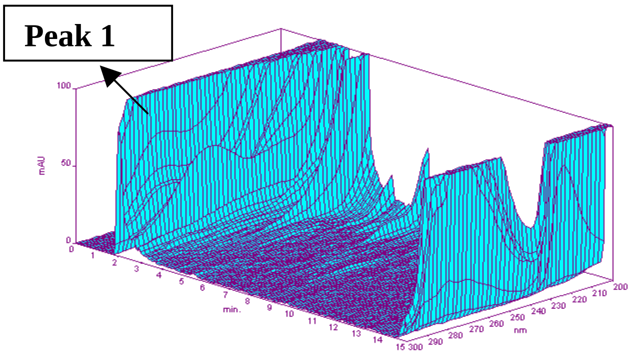
3.5.6. Release of aromatic compounds from lignin bioreactor

The PDA chromatograms below show the release of aromatic and aliphatic compounds from lignin in the presence of a mixed SRB consortia.

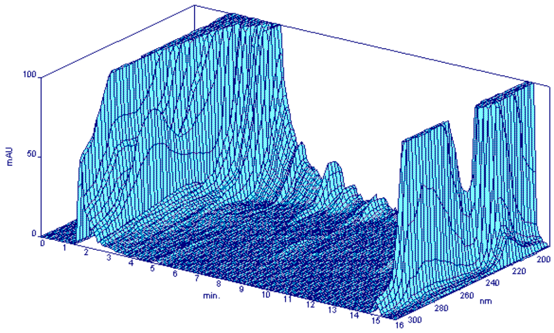
Day 1



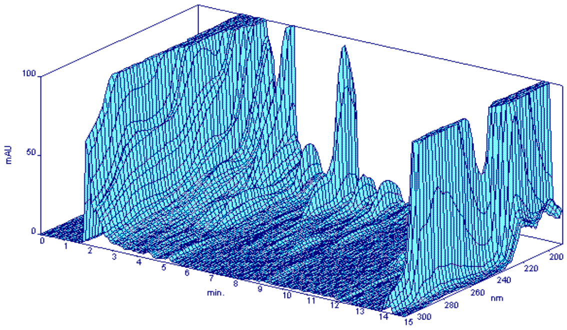
Day 5



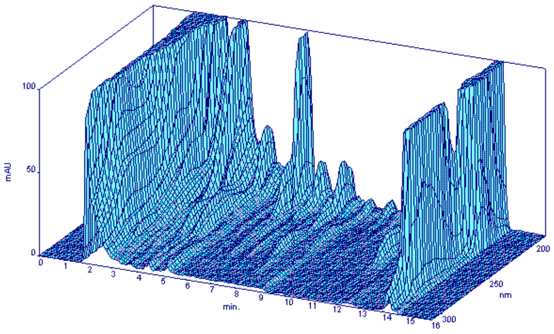
Day 11



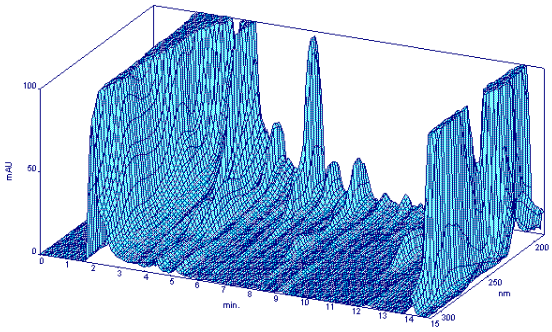
Day 15



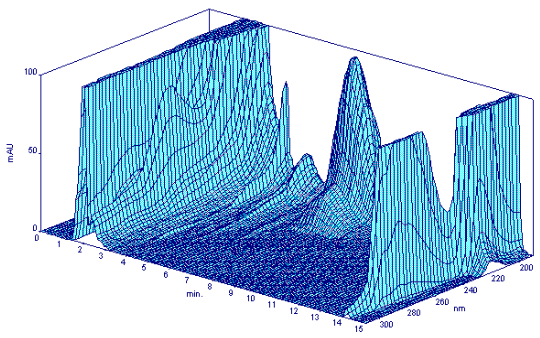
Day 18



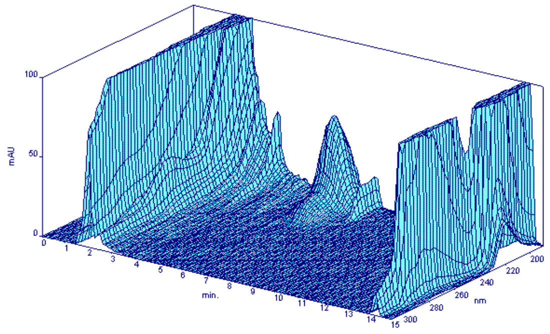
Day 24



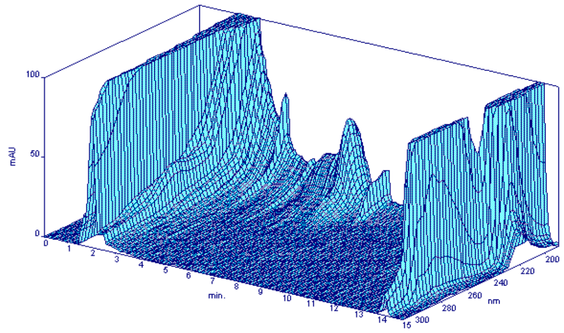
Day 28



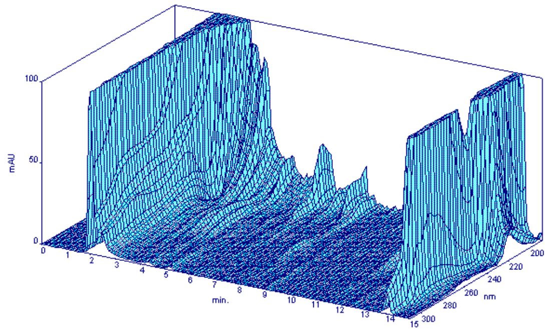
Day 31



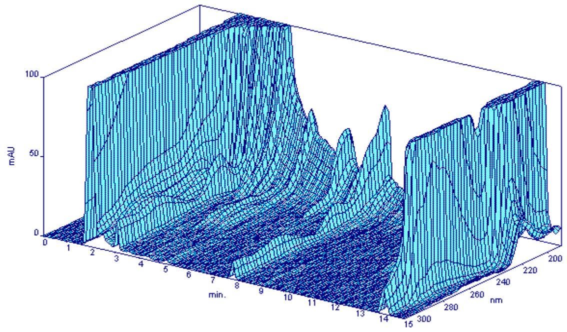
Day 39



Day 47



Day 54



Day 62

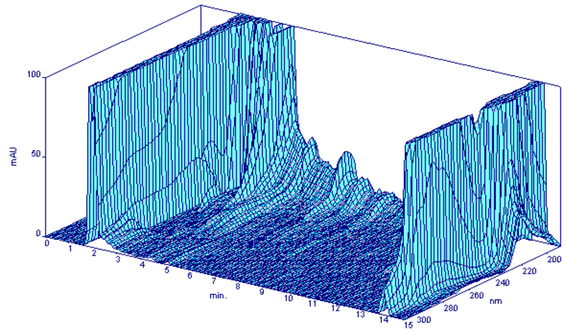


Figure 3.10. The release of aromatic and aliphatic compounds from lignin in the presence of a mixed sulphate reducing consortia over a period of 62 days. Individual results are shown fron day 1 to day 62.

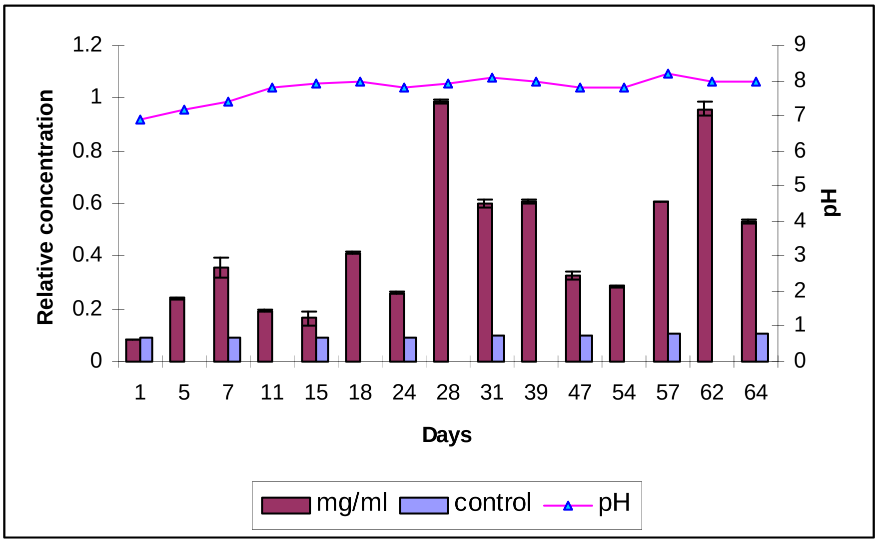


Figure 3.11. The relative concentration of lignin aromatics (RT=1.9min.) from lignin bioreactor. Naphthalene was used as an internal standard to estimate the relative concentration of aromatics at 310nm.

3.5.7. Electron Microscopy Studies

Wood surface colonisation and modification in the presence of a mixed SRB consortia studied by scanning electron microscopy.

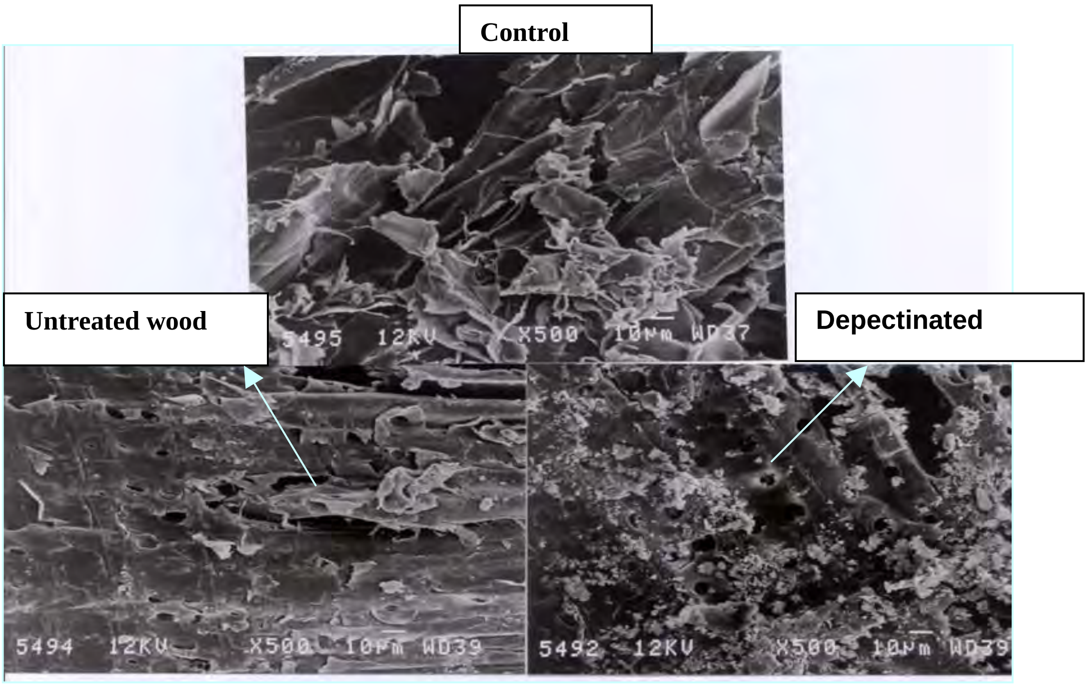
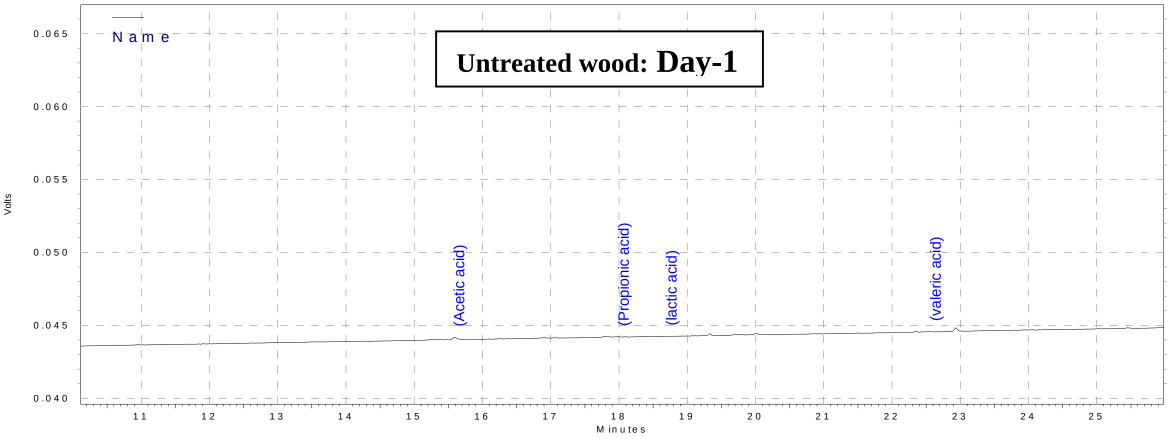
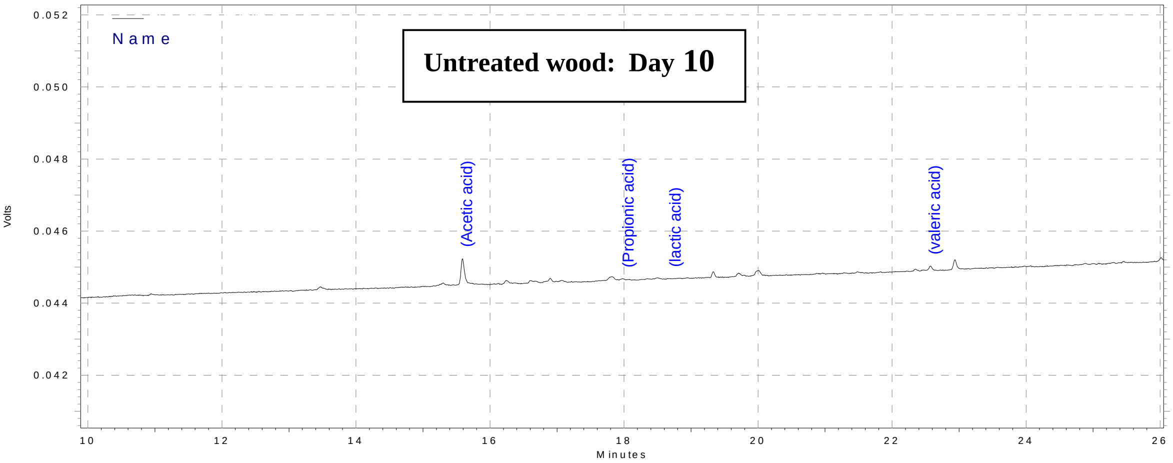


Figure 3.12. Scanning electron microscopy showing surface modification on wood due to the activity of sulphate reducing bacteria at x500 magnification.

3.5.8. Qualitative analysis of volatile fatty acids

The production of volatile fatty acids in the biosulphidogenic bioreactors was qualitatively analysed using GC. Volatile fatty acids are the end products of the metabolic processes and their presence in the bioreactors indicates the utilisation of wood aromatics, lignin and cellulose. Figure 3.13 shows the GC chromatograms for each bioreactor.





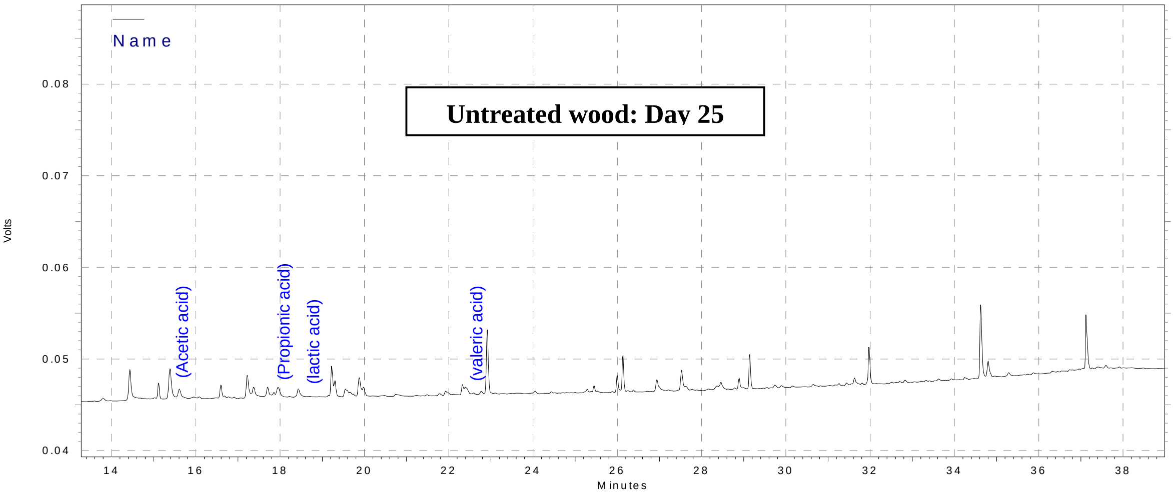
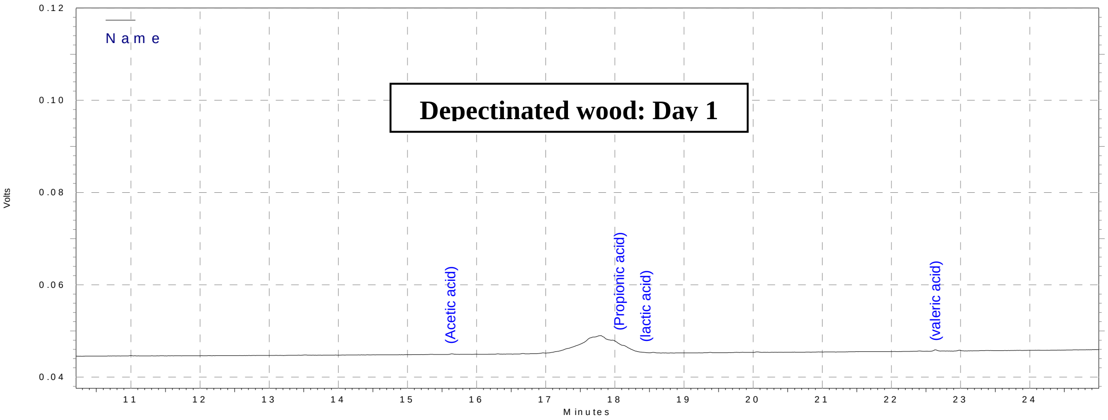
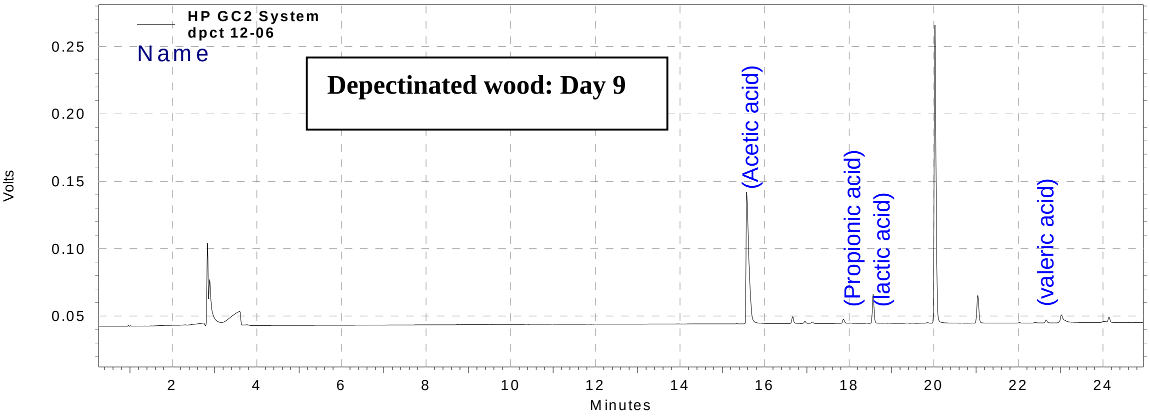


Figure 3.13. Production of volatile fatty acids from untreated wood bioreactor. Commercial standards such as acetic, lactic, propionic and valeric acids were used for identification.





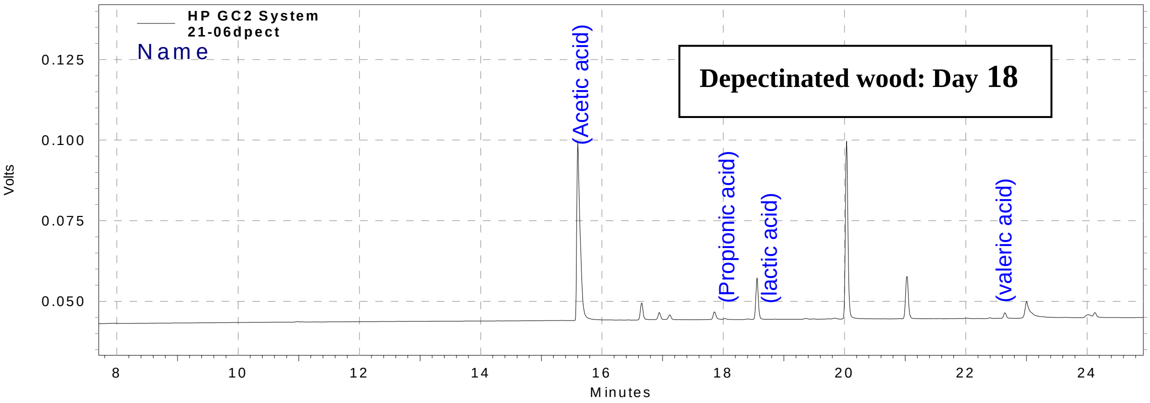
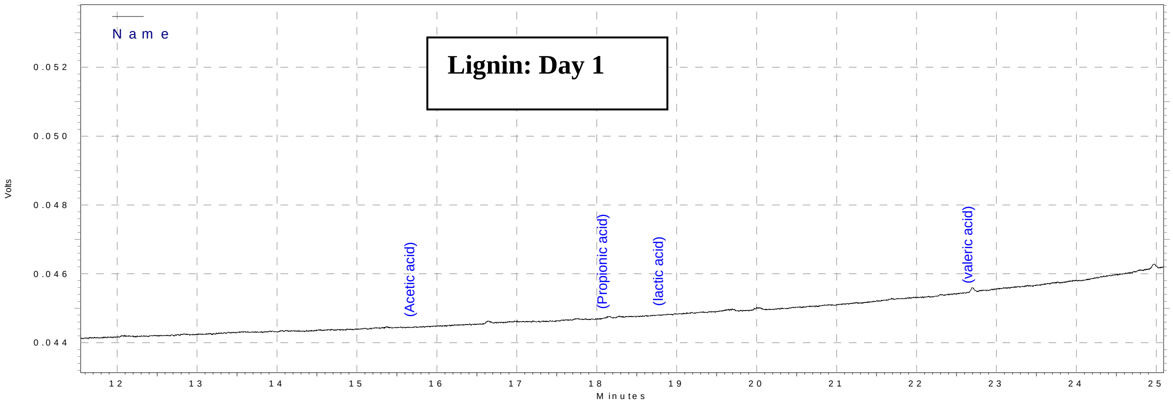
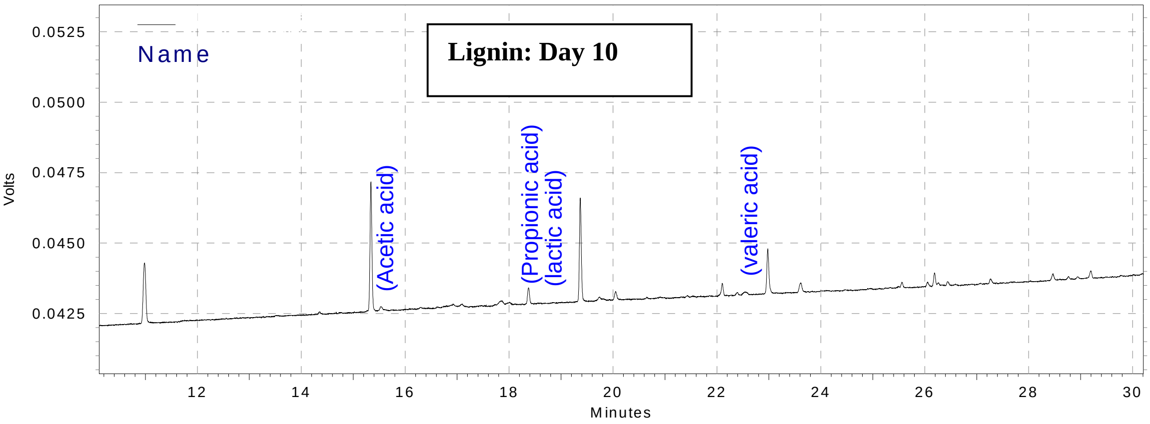


Figure 3.14. Production of volatile fatty acids from depectinated wood bioreactor. Commercial standards such as acetic, lactic, propionic and valeric acids were used for identification.





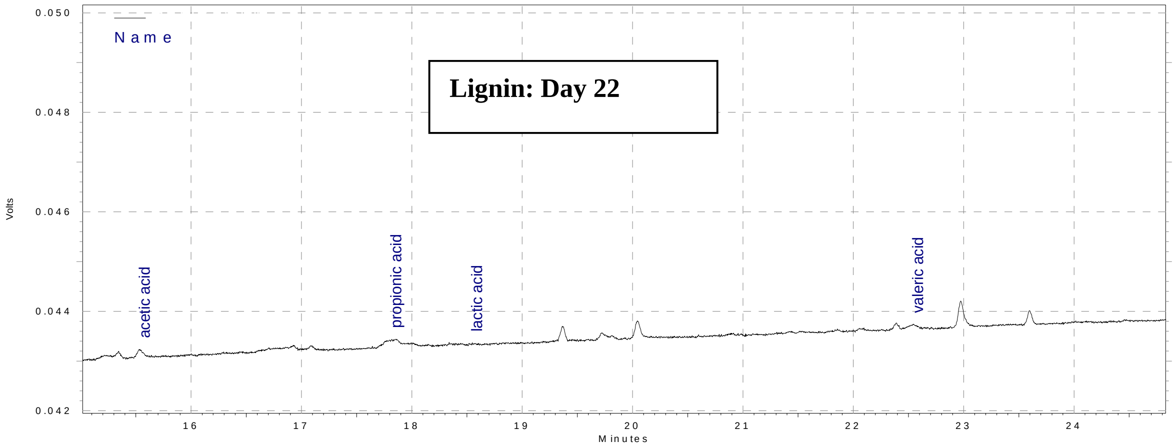


Figure 3.15. Production of volatile fatty acids from lignin bioreactor. Commercial standards such as acetic, lactic, propionic and valeric acids were used for identification.

3.6. Discussion

It had been demonstrated in chapter 2 that both alkalinity and sulphide under abiotic conditions result in the modification of wood surface thereby accelerating the release of wood extractives and soluble aromatics. The wood bioreactors incorporating mixed SRB consortia show a significant reduction in sulphate concentrations (figure 3.2 and table 3.1.). This indicates the utilization of these extractives and wood aromatics by mixed SRB consortia as electron donor sources. These soluble extractives include volatile oils, wood resins, fats, waxes, pectin, tannins and lignans. Softwoods are known to have high levels of these extractives (Burnes *et al.*, 2000).

The untreated wood bioreactor showed significant release of wood aromatics with a maximum release of about 40% of the chemical hydrolysis experiment in chapter 2. Sulphide generation in the wood bioreactors is comparable to the chemical hydrolysis experiments, the biogenic sulphide levels detected were between 300 and 500mg/l after 10 days. The sulphide graphs only indicate the approximate levels since most of sulphide is lost as a gas during sampling and also by diffusion from the bioreactors. The availability of extractives such as pectins, which are carbohydrate in nature, also serve as readily available carbon and electron source for SRB. This accelerates the sulphate reduction process as shown by an increased average sulphate reduction rate of about 94mg/l/day.

Depectination results in lignin and cellulose becoming more accessible to biodegradation by a mixed SRB consortia because of the removal of pectin and wood extractives. The production of aromatics from depectinated wood is also probably due to the breakdown and utilization of lignin by the consortia. The average sulphate reduction rate for depectinated wood is almost half (47.67mg/l/day) that of untreated wood. This is due to the fact that the readily available extractives such as pectins and simple sugars have been removed. Nevertheless, the results show a significant reduction in sulphate concentrations.

PDA is an informative tool in monitoring the release of organic compounds from wood. It gives an absorption scan in the electromagnetic regions relevant to our study in order to investigate release and utilization of aliphatic (volatile fatty acids) compounds (190 to 220nm), aromatic compounds (254 nm), aromatics (280nm) and lignin-derived aromatics (310nm). The PDA for both untreated and depectinated wood indicates that there is an increased production of aliphatic compounds and this indicates the utilization of phenolics and wood aromatics to produce these aliphatic compounds. The production of these aromatics is likely due to the activity of SRB consortia and their production of both alkalinity and sulphide. This is supported by rapid rates of sulphate reduction in untreated, depectinated and lignin bioreactors.

Lignin bioreactor showed high levels of aromatic release and this also corresponds to the increased rate of sulphate reduction in this bioreactor. This implies that the mixed SRB consortia can utilize lignin to produce aromatics, which can be further degraded to produce more aliphatic compounds as shown on the lignin PDA traces.

Electron microscopy studies on the wood bioreactors indicate the surface modification, which was reported in part I, as a result of sulphide and pH effect. The pH in bioreactor is also maintained at around pH 8 due to the increased production of alkalinity by SRB consortia. The scanning electron microscopy of depectinated wood shows EPS production, which indicates colonization of wood by bacteria.

The production of volatile fatty acids is an indication of biodegradation in the bioreactors. These fatty acids have been observed in the PDA data since they absorb in the region of 190 to 220nm. Qualitative analysis on GC identifies specific fatty acids using commercial standards as shown in figure 3.13 – 3.15. Acetic acid appears to be the major metabolic product in all the bioreactors.

The production of these volatile fatty acids in the untreated wood bioreactor indicates the biodegradation of wood extractives, aromatics and pectin by a mixed SRB consortium. The depectinated wood bioreactor also has high levels of these volatile fatty acids and this indicated the utilisation of the residual extractives, lignin and cellulose. Lignin bioreactor shows the utilisation of lignin and the biodegradation of lignin aromatics to produce volatile fatty acids as shown on the PDA and GC data.

Our investigation has shown that a mixed SRB consortia can degrade wood aromatics, extractives and pectin as electron sources during sulphate reduction. This indicates that during the biodegradation of lignin biopolymer, SRB consortia degrades lignin to produce aromatics, which are further degraded into volatile fatty acids. The use of different wood fractions for our studies gives an in-depth understanding of the biodegradation of lignin under sulphate reducing conditions. It is known that chemical degradation of lignin (Kraft process) requires high temperature, pH and high sulphide concentrations. Also, anaerobic biodegradation under sulphate reducing conditions has not been thoroughly investigated. Our investigation has shown that lignin is biodegradable under sulphate reducing conditions, the production of both alkalinity and sulphide modifies the wood surface resulting in the release of wood extractives, aromatics and pectins for biodegradation by a mixed SRB cosortium.

2.7. Conclusion

The biological contribution of a mixed SRB consortia in lignin hydrolysis has been demonstrated with the following conclusions:

- Biological sulphate reduction resulted in the production of alkalinity and sulphidity levels comparable to the chemical hydrolysis process.
- There is a significant production of aromatics in wood bioreactors, although, yielding only between 20 to 40% of the chemical hydrolysis. The lignin bioreactor indicates very high levels of aromatics release as observed in the

chemical hydrolysis experiments. There is also a significant consumption of lignin derived aromatics as indicated by their fluctuating levels and the corresponding sulphate reduction. This is an indication that wood and lignin are hydrolysed under biosulphidogenic conditions.

- The production of aliphatics as shown by PDA analysis is an indication of utilisation of extractives, aromatic and phenolic compounds from wood and lignin.

- The mechanism of the chemical effect of both sulphide and alkalinity, although occurring at room temperatures, may be loosely compared to the Kraft process. The biological contribution of the SRB consortia includes the maintainance of alkaline and sulphidogenic conditions, the production of enzymes essential for cleavage of aromatic compounds and the utilisation of these aromatic compounds as electron sources.

- If these aromatic compounds are utilised as electron and carbon sources by the SRB consortia, it is very important to elucidate the degradation mechanism of their cleavage and this will give an additional understanding of lignin biodegradation under biosulphidogenic conditions.

Chapter 4

Biodegradation of Mono-Aromatic Lignin Model Compounds under Biosulphidogenic Conditions

4.1. Introduction

Lignin is the biopolymer formed by free radical polymerisation of the phenyl-propane subunits. The degradation of simple model compounds, which are derivatives of lignin subunits, have been used to follow the biodegradation process by aerobic fungi (Buswell *et al*., 1987). The biodegradation pathway for lignin and model compounds by mixed SRB consortia has not been thoroughly investigated.

4.2. Objective

The purpose of this study was to investigate the susceptibility of chemical bonds to cleavage by mixed SRB consortia. The biodegradation of wood and lignin by a mixed SRB consortia has been demonstrated in chapter 3. In order to establish the pathway for the biodegradation of lignin biopolymer, a number model compounds similar to lignin in its chemical nature were considered. Ferulic acid (figure 4.1), a relatively stable molecule having close resemblance to the lignin monomeric molecules (coumaryl, coniferyl and syringyl alcohols) was the first choice. The ferulic acid ethyl ester was considered because it provided the opportunity to study the cleavage of ester linkages found in grass lignin, and because it could enable the characterisation of intermediates in the degradation of ferulic acid type molecules.

4.3. Methods

Commercially available ferulic acid was purchased from Sigma-Aldrich and Merck respectively. Ferulic acid ethyl ester was synthesized from the starting materials as described below.

4.3.1. Synthesis of ferulic acid ethyl ester

Ferulic acid (900 mg, 4.62 mmol) was dissolved in 20 ml in absolute ethanol and 1-2 drops of conc. sulphuric acid was added and the whole mixture was refluxed for 3 hours (Li and Lundquist, 1997). The resulting solution was treated with saturated solution of sodium bicarbonate to remove unreacted ferulic acid and extracted with ethyl acetate. The organic layer was separated, dried over anhydrous sodium sulphate and evaporated at reduced pressure to get ferulic acid ethyl ester in 964 mg (93% yield). The isolated ester was characterized by TLC using ethyl acetate-hexane (1:3) as mobile phase (R_f 0.13). The GC parameters were 50 °C (1min), 10 °C/min and 250 °C (10 min). Further characterization was done by GC-MS: (r t 19.18 min.) 222 (M^+), 194, 177, 161, 150 (bp), 135, 117, 105, 89, 77, 63, 51, and ^{1}H NMR: (400MHz, $CDCl_3$) 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).

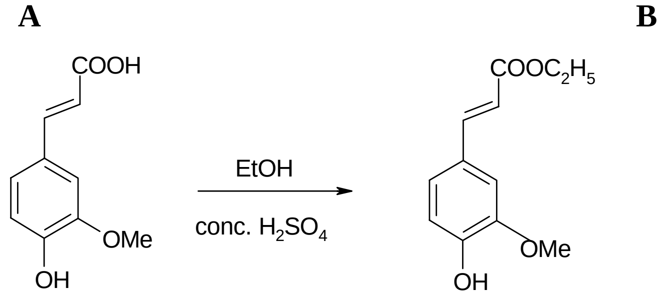


Figure 4.1. Synthesis of ferulic acid ester. **A**=Ferulic acid and **B**=Ferulic acid ester.

4.3.2. Bacterial growth and model compounds bioreactors

The SRB consortia used in this study was from the same wood degrading culture used in chapter 3. Bioreactors (250ml) were inoculated with 50% volume of bacterial culture and were connected to 10% Zinc Acetate to trap hydrogen sulphide as shown in figure 4.2. The subcultures were supplemented with model compounds as the sole carbon sources with mineral media (Appendix 6) containing ±2000 mg/l sulphate at pH 7.5.

Figure 4.2. Small-scale bioreactor to study the biodegradation of lignin model compounds. The bioreactor was connected to the zinc acetate trap to precipitate excess hydrogen sulphide produced by a mixed sulphate reducing consortia.

4.4.Analytical procedure

4.4.1. Sulphate and model compounds analysis

Sulphate was analysed by Waters HPLC system as previously described in chapter 3. The biodegradation products from model compounds were also analysed using Beckman HPLC System Gold as previously described.

4.4.2. Gas Chromatography and Mass-spectrometry

Sample preparation: Since the GC-MS study was to qualitatively assess the product formed during bacterial degradation the internal standard was not used. The sample preparation followed the procedure of treating 2.5 ml of the test sample with 250 µl of dichloromethane and 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. 1 µl of this dry dichloromethane solution was injected. All the 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^{o}C(1 min)-10^{o}C/min.-250^{o}C(10 min.). From the splitting pattern and comparison with standard compounds, products of degradation were characterized. Volatile fatty acids were analysed by GC (Hewlett Packard 6980 series) instrument equipped with a Flame Ionization Detector (FID).

4.4.3. Test for catechols

Catechol derivatives were qualitatively tested by treating the sample with an aqueous sodium carbonate solution in order to test the initial bacterial degradation reaction of ferulic acid and ferulic acid ethyl ester. Catechols develop typical brown coloration when treated with sodium carbonate (1M) solution (figure 4.6).

4.4.4. Qualitative analysis of acetates by photodiode-array

Acetates were expected to be the end products of model compounds biodegradation by SRB. This has already been observed in wood and lignin experiments where PDA data showed the production of aliphatic compounds.

4.5. Results

The control experiments showed that the model compounds investigated were stable room temperature in the uninoculated mineral media.

4.5.1. Ferulic acid biodegradation

Ferulic acid (analysis at 310nm) was biodegraded rapidly and the aromaticity was completely lost in 25 days (figure 4.3). No changes were observed in the control experiment. The molecule was utilized without any lag period. The HPLC chromatograms below (figure 4.3 to 4.5) show biodegradation of ferulic acid, the appearance of intermediate and final biodegradation compounds. Sulphate reduction with concomitant degradation of ferulic acid was evidenced as shown in figure 4.4. below.

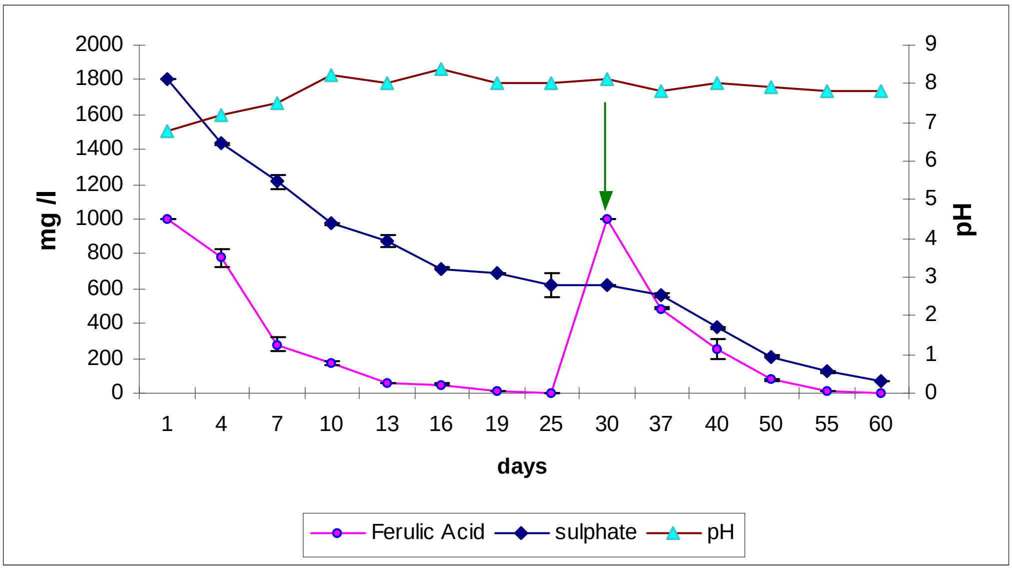


Figure 4.3. Biodegradation of ferulic acid by a sulphate reducing consortia with corresponding pH and sulphate reduction data. The arrow indicates spiking with additional ferulic acid`.

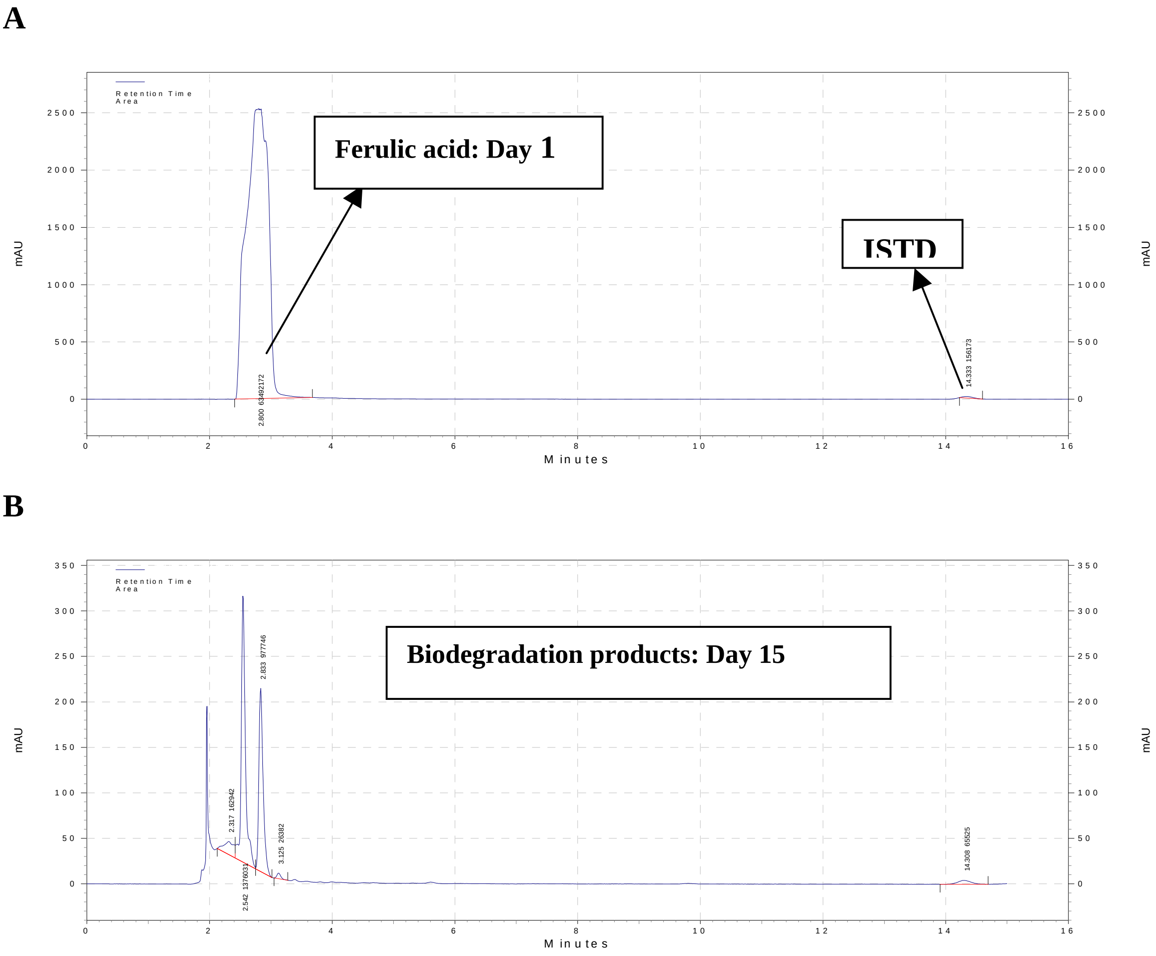
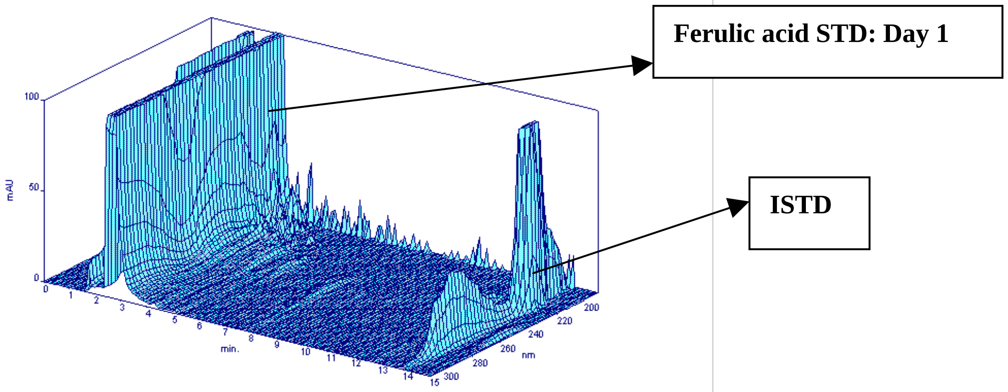


Figure 4.4. The results of two-dimensional HPLC analysis of ferulic acid and its biodegradation products. **A**=Initial ferulic acid concentration (day 1) and **B**=Production of intermediates on day 15.

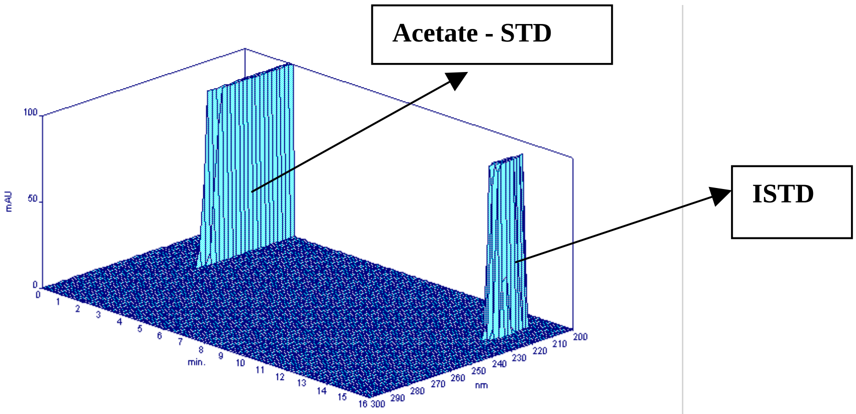
C



Ferulic acid STD: Day 1

ISTD

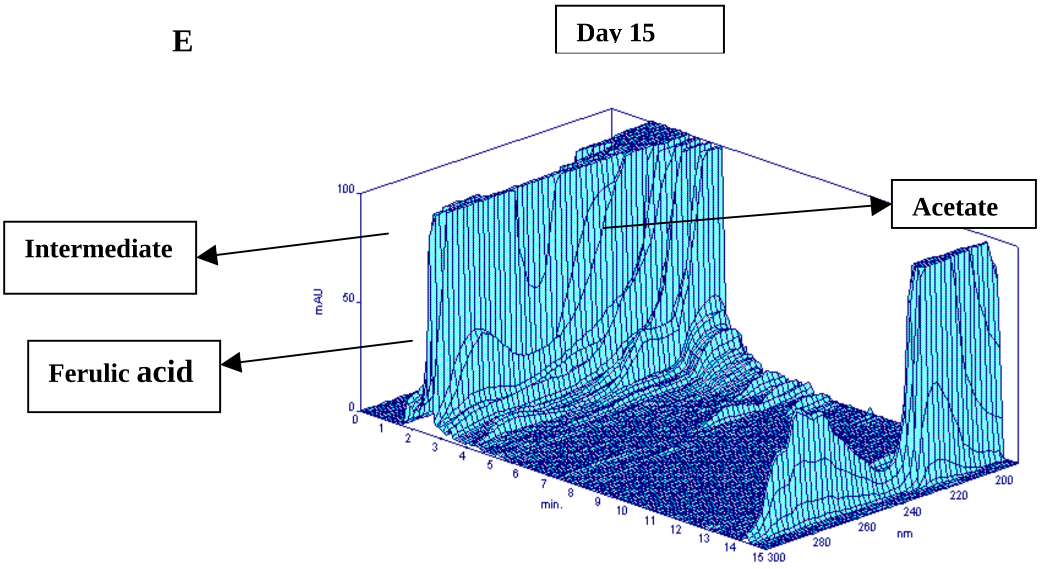
D



Acetate - STD

ISTD

E



Day 15

Intermediate

Ferulic acid

Acetate

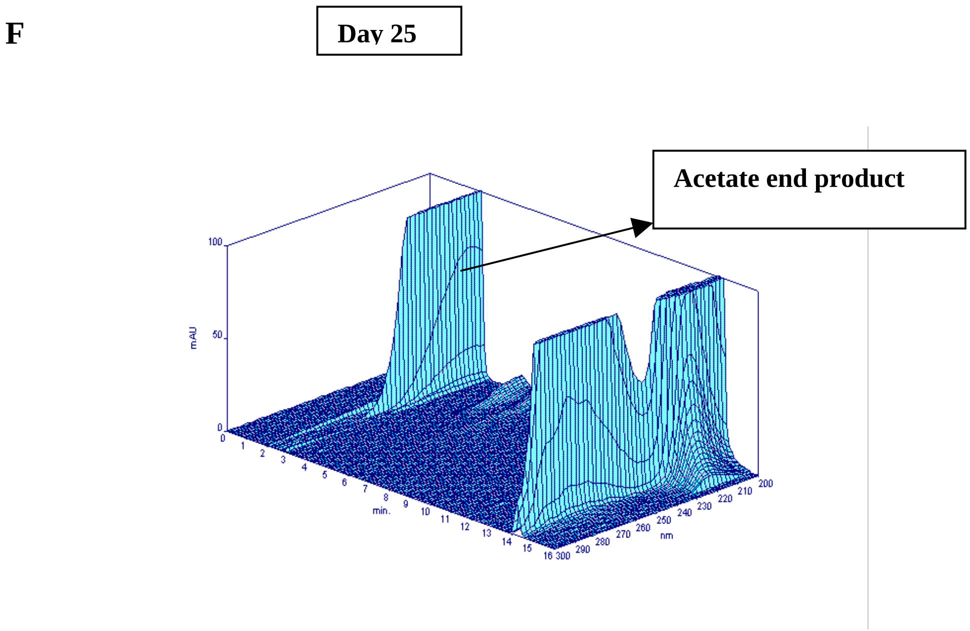


Figure 4.5. Biodegradation of ferulic acid analysed by three-dimensional HPLC. **C** = Ferulic acid (day 1), **D** Acetate standard for qualitative analysis in the ferulic acid bioreactor, **E** = PDA data on day 18 and **F** = Acetate as one of the end products of ferulic acid biodegradation.

The results in figure 4.3 to 4.5 show the biodegradation of ferulic acid by a mixed SRB consortia and the appearance of the intermediates such as acetates. Figure 4.6 show the production of a catechol intermediate from ferulic acid.

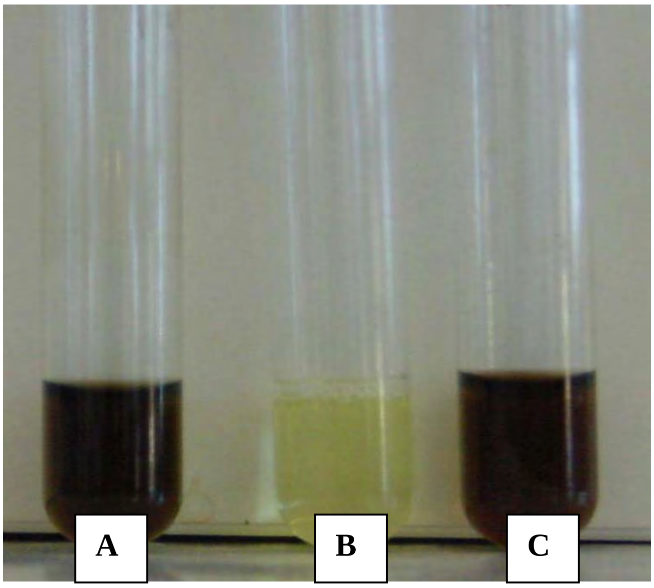


Figure 4.6. Chemical test for catechol production. **A** = catechol standard, **B** = Ferulic acid control. **C** = Biodegradation product from the ferulic acid bioreactor.

The degradation products of the ferulic acid study were extracted from the bioreactor in dichloromethane and analysed on GC–MS in order to elucidate lignin biodegradation. The results in figure 4.7 show the production of cyclohexane carboxylic acid and adipic acid from ferulic acid biodegradation.

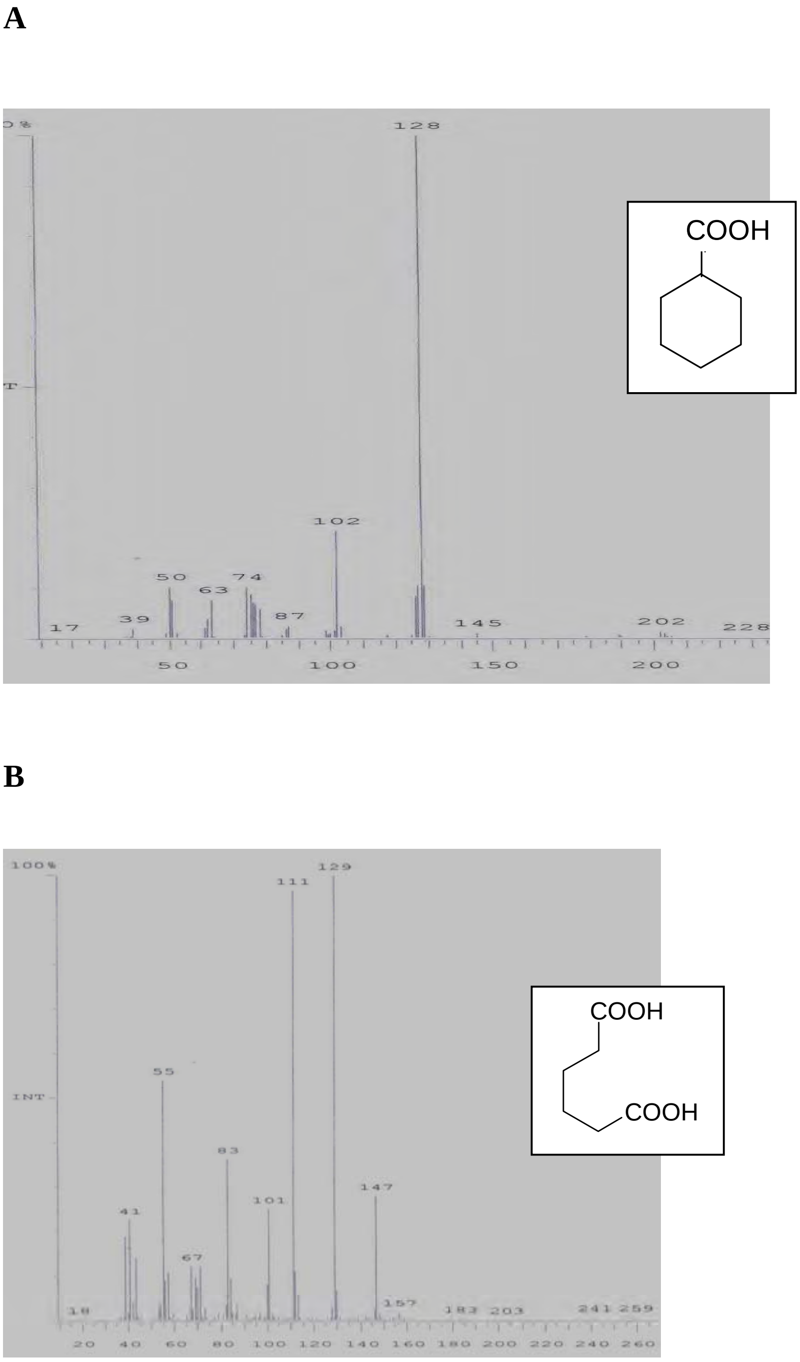


Figure 4.7. GC-MS chromatograms showing intermediate products from the ferulic acid bioreactor. **A** = Cyclohexane carboxylic acid and **B** = Adipic acid.

4.5.2. Biodegradation ferulic acid ethyl ester

The ferulic acid ethyl ester was biodegraded rapidly and the aromaticity was completely lost within 20 days (figure 4.8). No changes were observed in the control experiment. Figures 4.8 to 4.10 show the biodegrdation of ferulic acid ethyl ester and intermediate products in day 10.

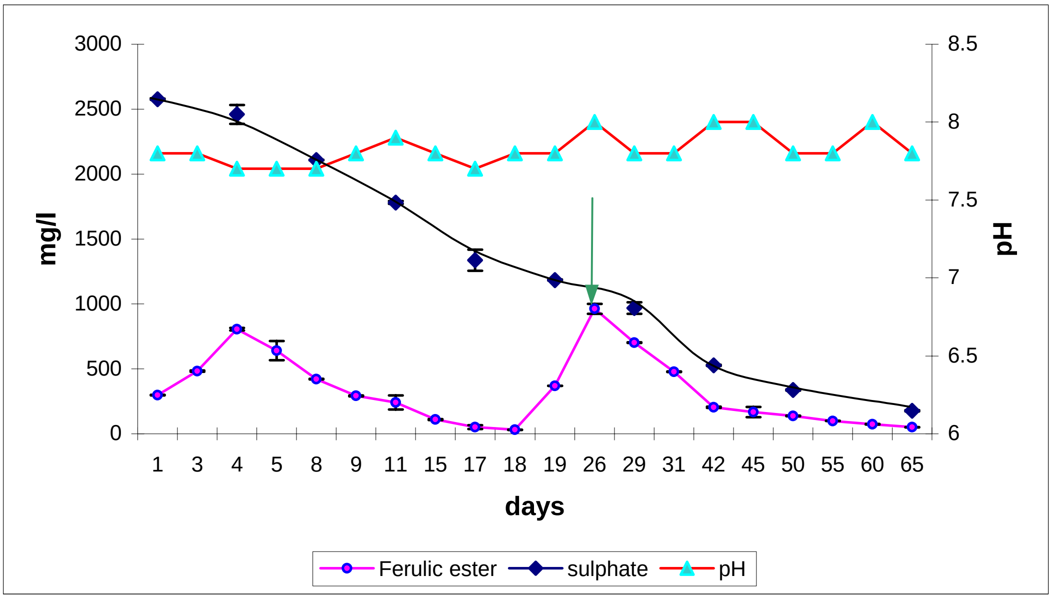
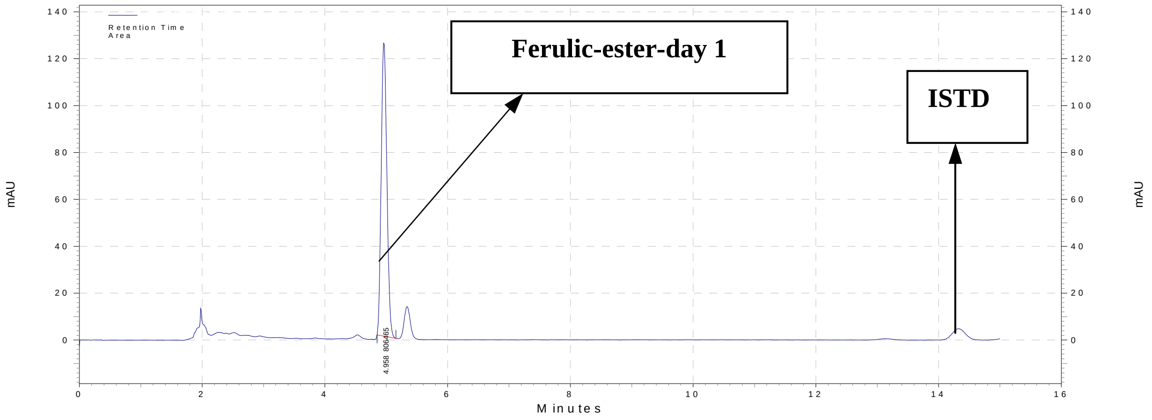


Figure 4.8. Biodegradation of ferulic acid ethyl ester by the sulphate reducing consortia with corresponding pH and sulphate reduction data. Ferulic acid ethyl ester is not soluble in the mineral media hence it slowly distributes in aqueous phase. The arrow indicates spiking with additional ferulic acid ethyl ester.

A



B

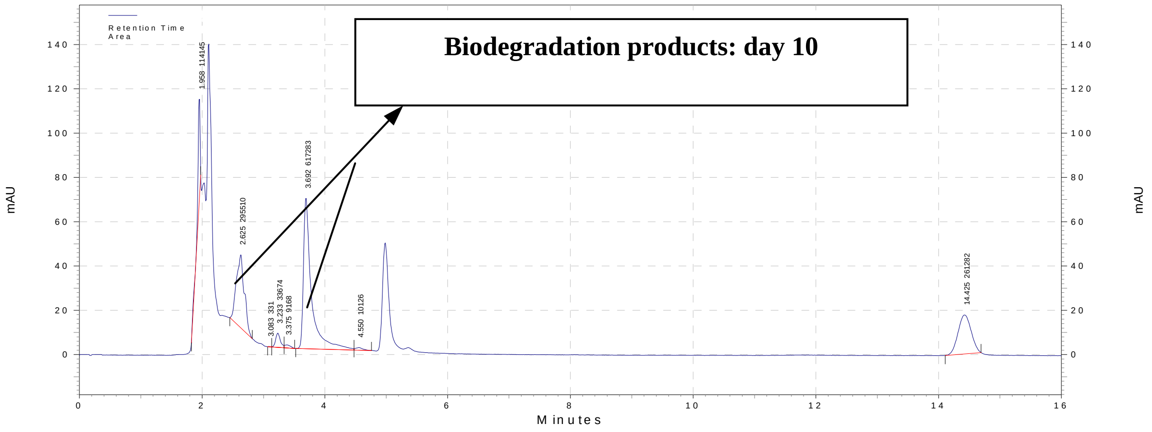
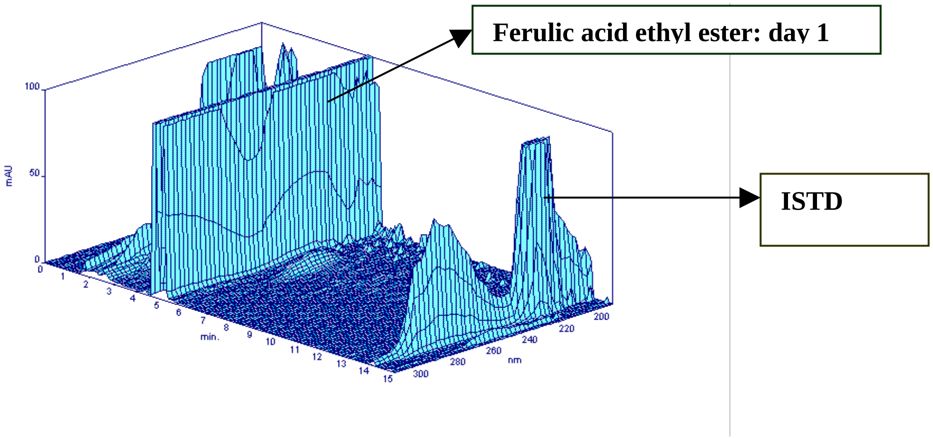
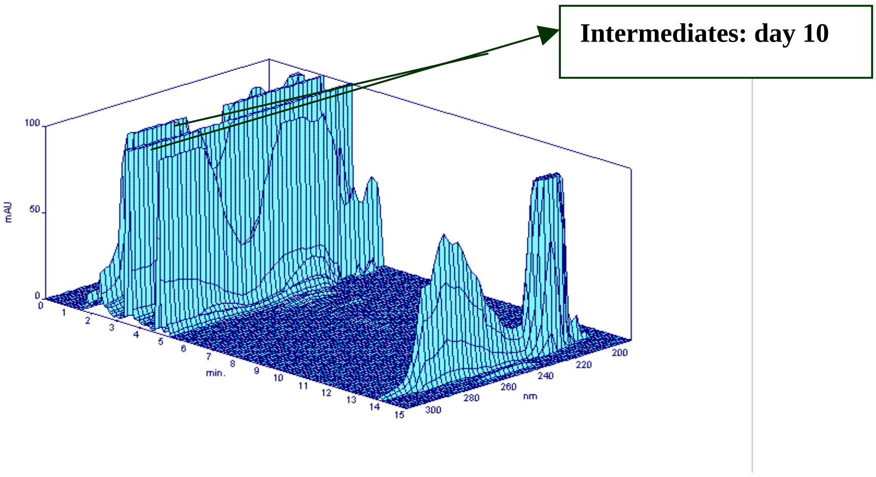


Figure 4.9. Two dimensional HPLC showing the biodegradation of ferulic acid and production intermediate.

C



D



E

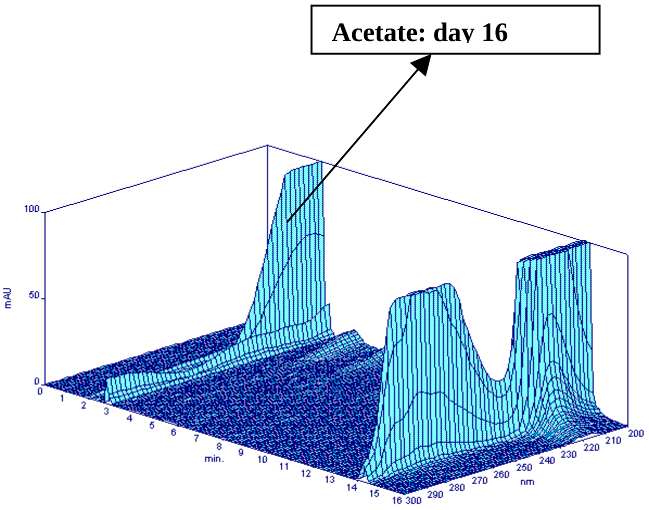
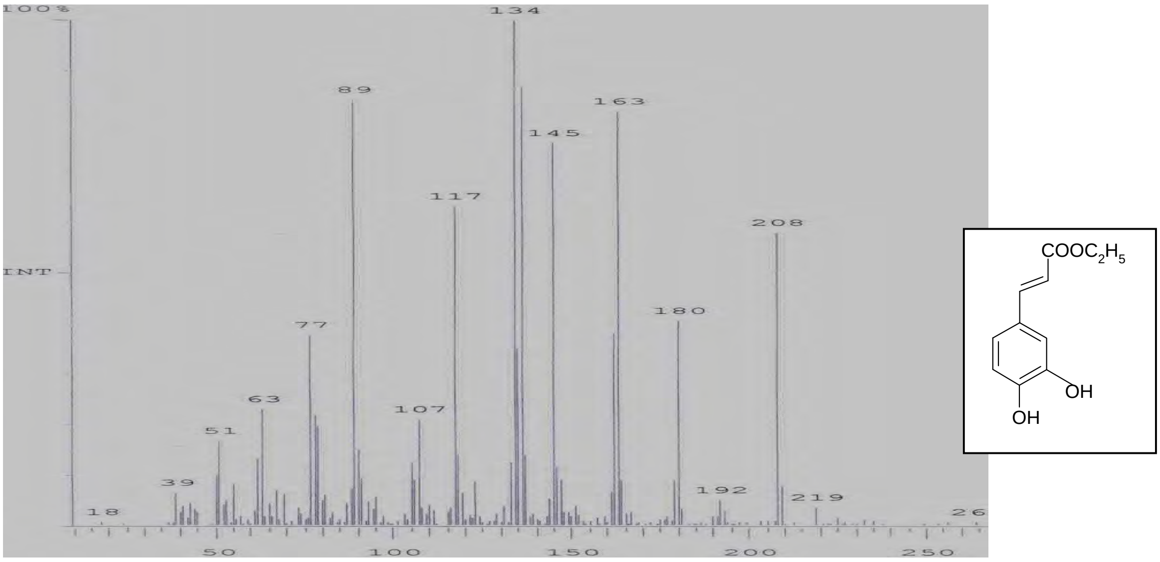


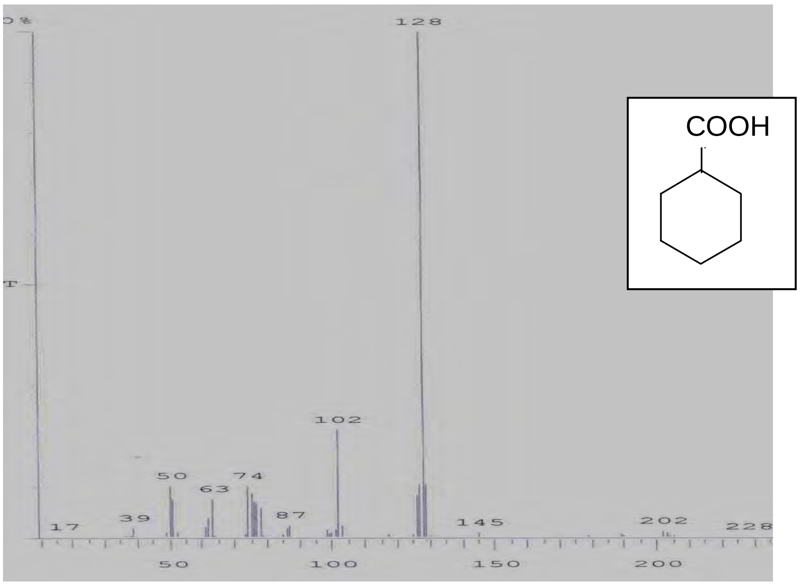
Figure 4.10. Biodegradation of ferulic acid ethyl ester analysed by PDA. C = day 1, B = Production of intermediate on day 10 and E = Acetate as one of the end product.

The biodegradation products were analysed on GC-MS below. The results in figure 4.11 show the production of the catechol unit, cyclohexane carboxylic acid and adipic acid from ferulic acid ethyl ester.

A



B



C

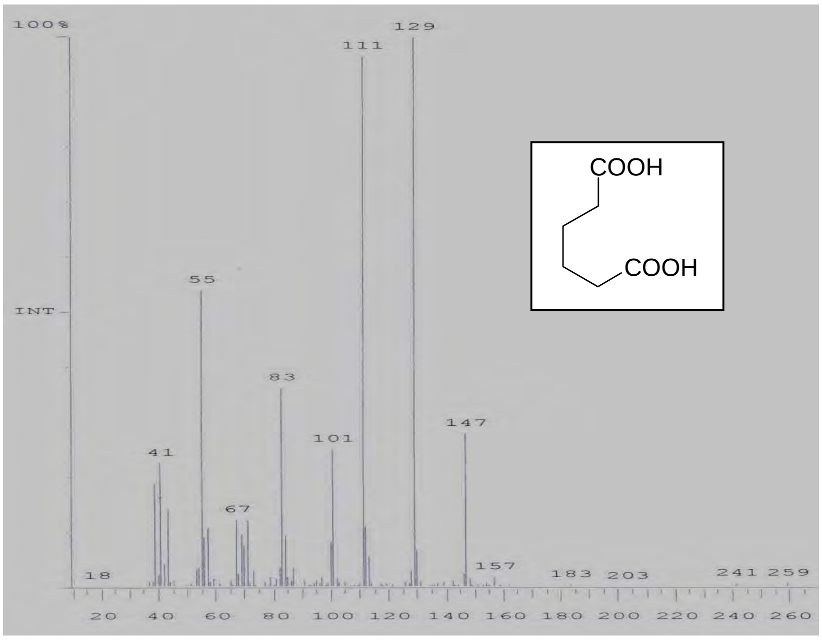


Figure 4.11. GC-MS chromatograms showing intermediate products from the ferulic acid ester bioreactor. **A** =Catechol, **B**= Cyclohexane carboxylic acid and **C** = Adipic acid.

4.6. Discussion

The initial reaction for ferulic acid cleavage (after 14 days) was found to be demethylation of the O-methyl ether resulting in the formation of a catechol derivative. This was observed from the appearance of an additional peak (RT=2.6 min) to the ferulic acid peak (RT=2.8 min). Figure 4.3 indicates the formation of this product along with the presence of unconverted ferulic acid. This product was characterized by GC-MS and chemical test. Formation of this catechol derivative was found to be the driving force for the reaction as catechol can lose aromaticity easily through keto-enol tautomerism, the keto form which is prevalent under basic conditions (prevailing in the SRB consortium media). The degradation of this compound was found to be rapid and the aromaticity seemed to have been lost within 28 days resulting in the formation of aliphatics for which GC-MS results showed the formation of cyclohexane carboxylic acid and adipic acid (Figure 4.7.). Adipic acid was further degraded to acetic acid, which was identified as an end product by PDA analysis in figure 4.5. Ferulic acid ethyl ester, unlike ferulic acid is a relatively neutral molecule and has limited solubility in aqueous medium. The solubility was found to increase with time (Figure 4.8) and immediately degraded through demethylation reaction to form a catechol unit as shown in figure 4.11a. Cyclohexane carboxylic acid and adipic acid (Figure 4.11b to c) were identified as intermediates forming after 10 days in this case too.

Cyclohexane carboxylic acid can be formed by the β-oxidation of the reduced side chain of ferulic acid type molecules and complete reduction of the aromatic nucleus. Ring opening, oxidation followed by decarboxylation of this molecule results in the formation of adipic acid. This implies that the degradation pathways of both ferulic acid and ferulic ethyl ester are similar. Also, in the case of ferulic acid ethyl ester, acetic acid was identified as one of the end products. Figure 4.12 below shows the proposed pathway for biodegradation of both (**A**) ferulic acid and (**B**) ferulic acid ester. Both these model compounds are converted to a catechol unit then cyclohexane carboxylic acid, adipic acid and finally to acetate which is the end product.

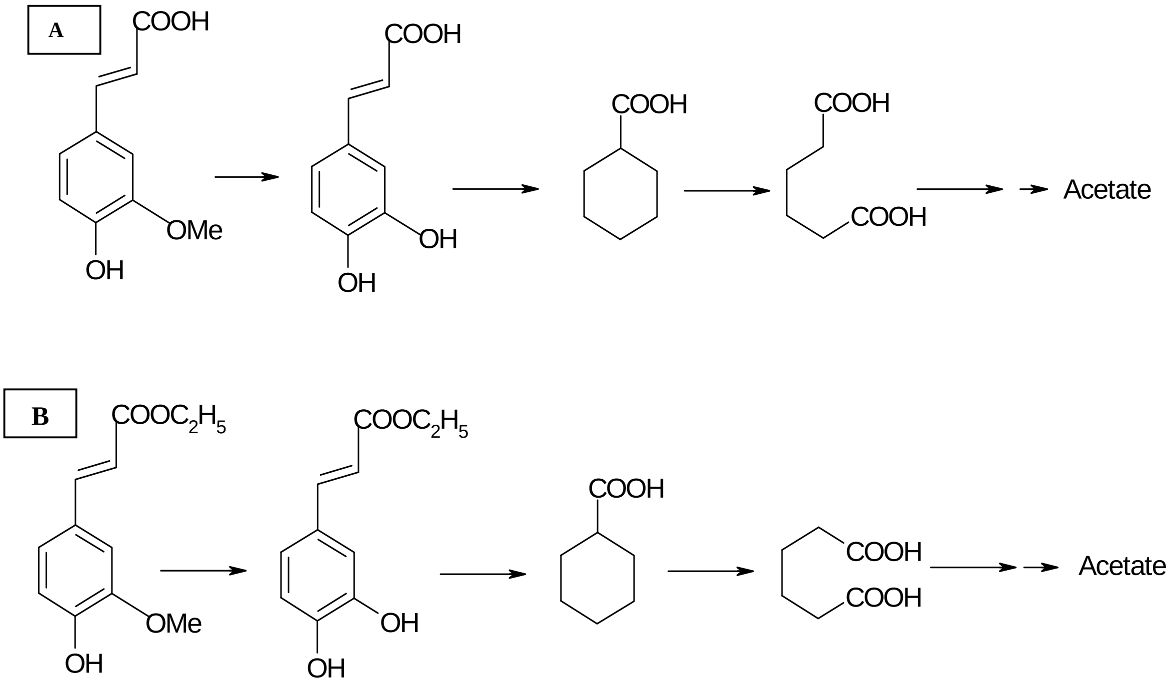


Figure 4.12. The proposed pathway for the biodegradation of **A**=Ferulic acid and **B**=Ferulic acid ethyl aster by the SRB consortia.

4.7. Conclusion

Lignin model compounds studied in this investigation have demonstrated possible mechanisms for lignin biodegradation by the SRB consortia. As a complex biopolymer, lignin biodegradation has proven difficult to study without considering the model compounds that are derivatives of lignin subunits (phenyl propane) (Hearly *et al.,* 1980). The findings in this study suggest that both ferulic acid and ferulic ethyl ester can be degraded by the SRB to produce different intermediates and this has led to the following conclusions:

- Both ferulic acid and ferulic acid ester are representative of monomeric lignin compounds. Their similarities in biodegradation are supportive of the proposed pathway.

- Biodegradation of monomeric lignin derivatives involve the cleavage of the ether bond linking the methyl group to the aromatic ring followed by the reduction of the aromatic ring, ring fission and the production of the aliphatics such as the acetates as shown by PDA analysis.

- Elucidation of lignin biodegradation cannot be conclusive without considering dimeric lignin model compounds. The use of dimeric model compounds that are representative of linkages found in polymeric lignin will give a better understanding of the mechanisms involved in biosulphidogenic degradation of lignin.

Chapter 5

Biodegradation of Dimeric Lignin Model Compounds under Biosulphidogenic Conditions

5.1. Introduction

Dimeric lignin model compounds represent the principal substructure of lignin and have been used successfully to characterize lignin biodegradation under aerobic conditions (Jokela *et al.*, 1985). The results of biodegradation experiments where lignin was used as the substrate are often difficult to interpret, because of the great heterogeneity of the size and the functional groups of the molecules, as observed in chapter 3. This difficulty can be avoided by the use of synthetic model compounds both monomeric and dimeric. Hearly *et al.* (1980) has demonstrated the ability of methanogenic bacteria to degrade these lignin model compounds.

5.2. Objective

Biodegradation of lignin model compounds under sulphate reducing conditions has not been thoroughly investigated compared to methanogenic biodegradation. In this study, biodegradation of dimeric lignin model compounds with ether and C-C bonds, which are prominent in lignin, is investigated. The three dimeric compound structures considered in this study are shown in figure 5.1 below. Since lignin is found to exist in nature with hemicelluloses through a glycosidic linkage, salicin (**A**), a salicyl alcohol linked to glucose unit through a glycosidic bond was considered. A more complex C-C linked ferulic acid dimer (**B**) and benzyl ether (one of the most common ether linkages in lignin) in the form of vanillin benzyl ether (**C**) were chosen. These five molecules, represent almost all chemical bonds that are present in lignin polymer. In addition to the proposed biodegradation pathway of mono-aromatic model compounds in chapter 4, it is necessary to use dimeric compounds which are representative of principal lignin substructure. This will give more conclusive understanding of the lignin degradation mechanism under sulphidogenic conditions.

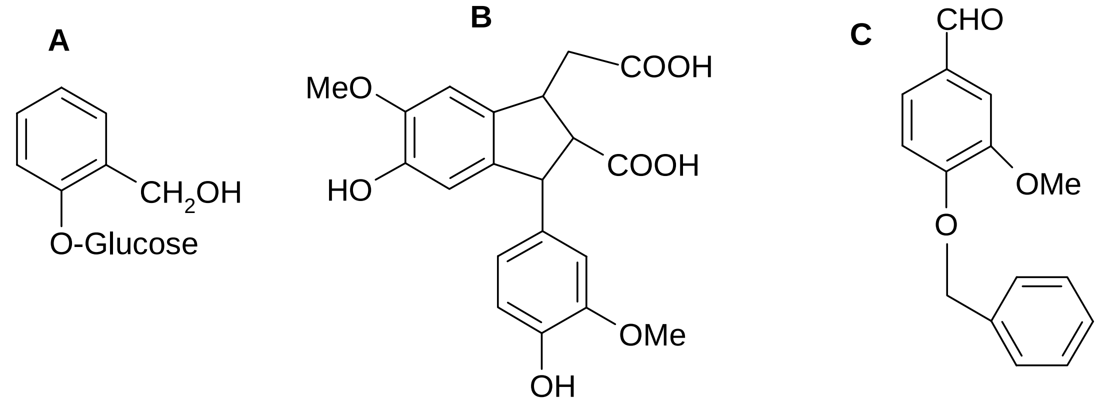


Figure 5.1. Dimeric compounds considered in elucidating lignin biodegradation mechanisms.

5.3. Methods

Commercially available salicin was purchased from Merck. All the other lignin model compounds were synthesized from the starting materials as described below.

5.4. Organic synthesis

5.4.1. Ferulic acid dimer

Ferulic acid (950 mg, 4.87 mmol) was suspended in 10 ml of trifluoro-acetic acid and stirred at 50°C for 24 hours in a flask (Li and Lundquist, 1997). The opaque solution turned pink and a precipitate was formed after few hours. The precipitate obtained (850 mg, 89%yield) was filtered, washed with cold alcohol and recrystallised from alcohol. The solid having m.pt.247°C, had ^{1}HNMR (400MHz, deuterated DMSO) δ 2.33(1H, dd, H-8a, J=16, 8.4Hz), 2.53 (1H, dd, H-8b, J=16, 6.5Hz), 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).

5.4.2. Benzyl vanillin

Vanillin (1.5 g, 9.8 mmol) was placed in a round bottom flask with 100 mg of KOH dissolved in 10 ml of absolute ethanol. Benzyl bromide (2 ml, 1.2 equivalent) was added drop wise over 15 min and the whole reaction mixture stirred for 72 hours at mild reflux temperature (Nakatsubo *et al.*, 1975). The reaction mixture was cooled, extracted with ethyl acetate and washed with water to remove the inorganics. The organic layer was evaporated and purified on silica gel column using ethyl acetate: hexane (1:5) as mobile phase. Fractions having this component were pooled and evaporated to obtain benzyl vanillin as colourless crystals (1.7 g, 71.1% yield) having a melting point of 61°C. Characterization of the product was done using tlc (Rf 0.49, solvent system ethyl acetate hexane 3:7),and GC-MS: 242(M+), 91(bp), 77, 65, 51, 39. ^{1}H NMR (400MHz, $CDCl_3$) δ 3.9(3H, s, OCH_3), 5.2(2H, s, CH_2), 6.9(1H, d, Ar-H), 9.5(1H, s, CHO)

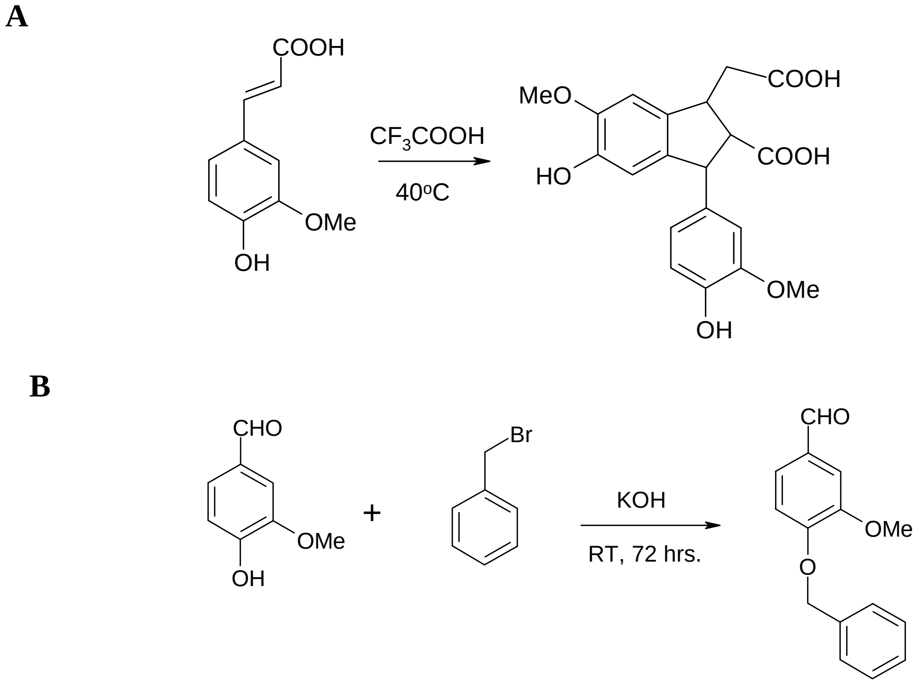


Figure 5.2. Pathway in the synthesis of lignin model compounds. **A** = Ferulic acid dimer and **B** = Benzyl Vanillin ether.

5.5. Bacterial growth and model compounds bioreactors

The SRB consortia used in this study was from the same wood degrading culture studied in chapter 3. Model compounds bioreactors (250ml) were inoculated with 50% volume of bacterial culture (figure 4.2) and connected to 10% Zinc Acetate to precipitate hydrogen sulphide. The subcultures were supplemented with model compounds as the sole carbon sources with mineral media containing ±2000mg/l sulphate at pH 7.5.

5.6. Analytical procedure

5.6.1. Sulphate analysis and model compounds analysis

Sulphate was analysed by Waters HPLC system as previously described in chapter 3. Aromatic compounds and biodegradation products were analysed using Beckman HPLC as described previously. The internal standard, naphthalene was used for quantitation.

5.6.2. Gas Chromatography and Mass Spectrometer

Samples for GC-MS were prepared as previously described in chapter 4.

5.7. Results

The control studies for all dimeric compounds investigated showed that these molecules are stable at room temperature and in SRB uninoculated mineral media.

5.7.1. Biodegradation of salicin

The results reported in figure 5.3 show rapid mineralisation of salicin within 7 days of incubation in the presence of the SRB consortia.

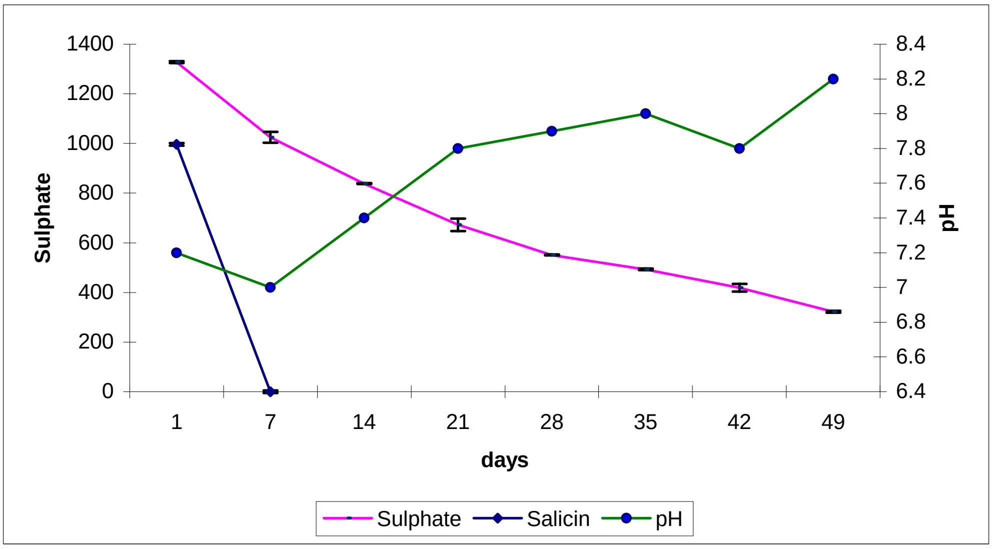
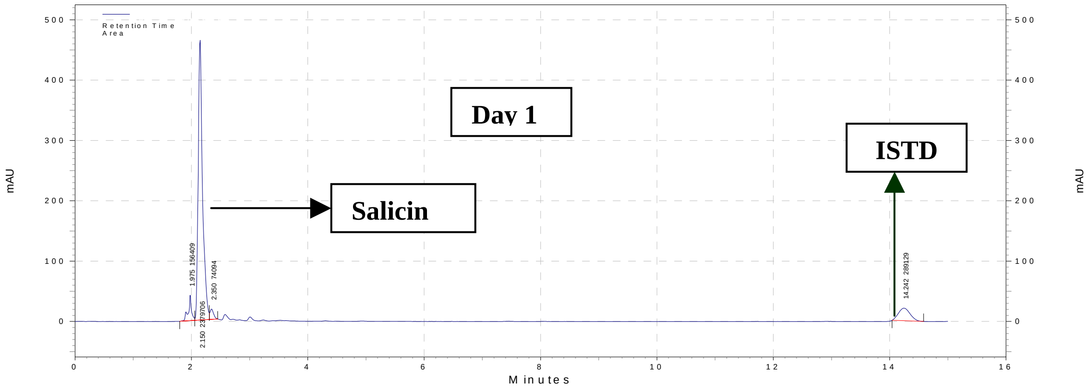


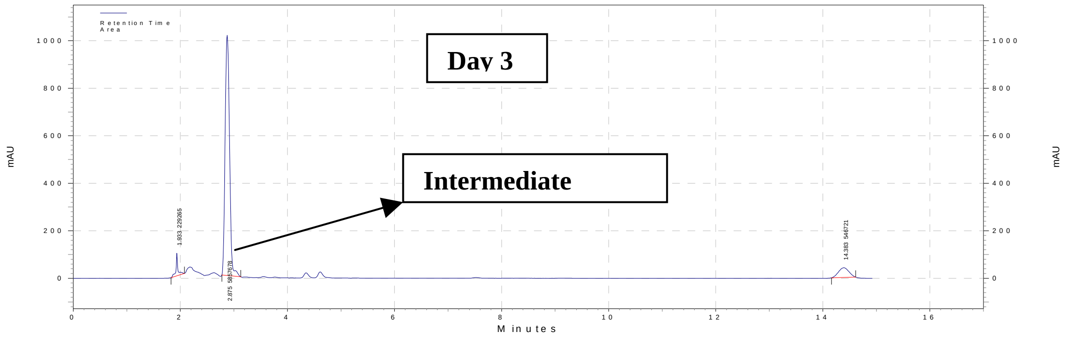
Figure 5.3. Biodegradation of salicin by the sulphate reducing consortia. Analysis of the samples was done in triplicates.

Figures 5.4 and 5.5 report the sequential HPLC traces of salicin degradation study and show the appearance of intermediate products of this process by day 3.

A

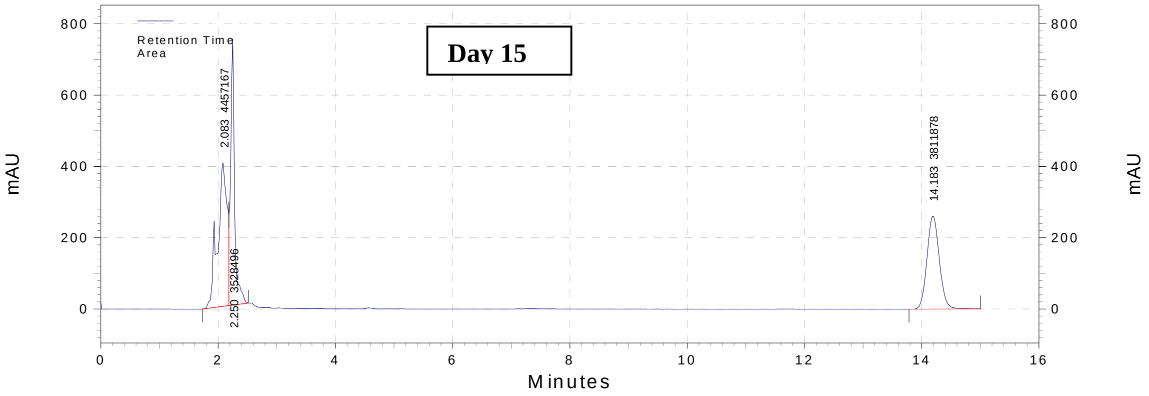


B



t

C



D

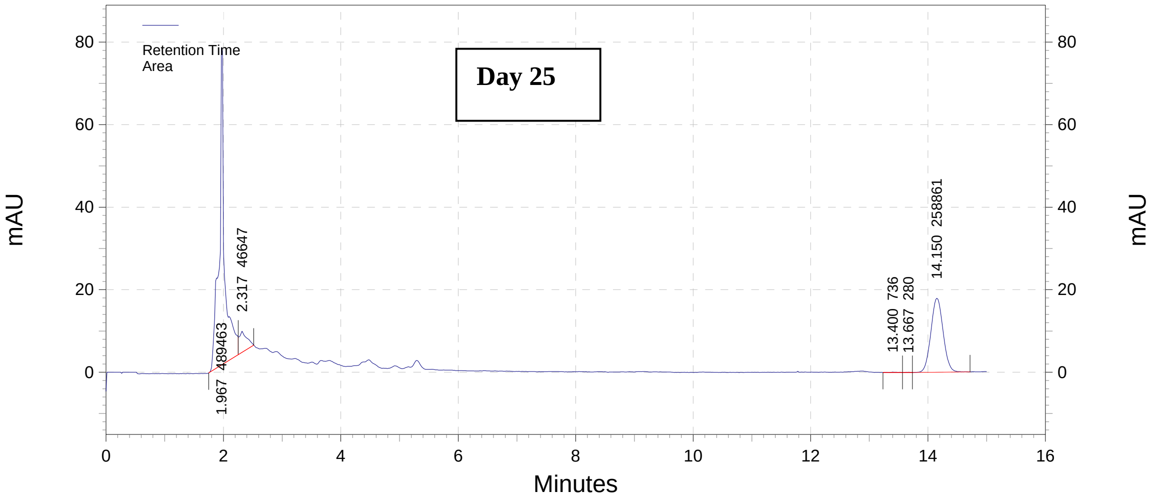
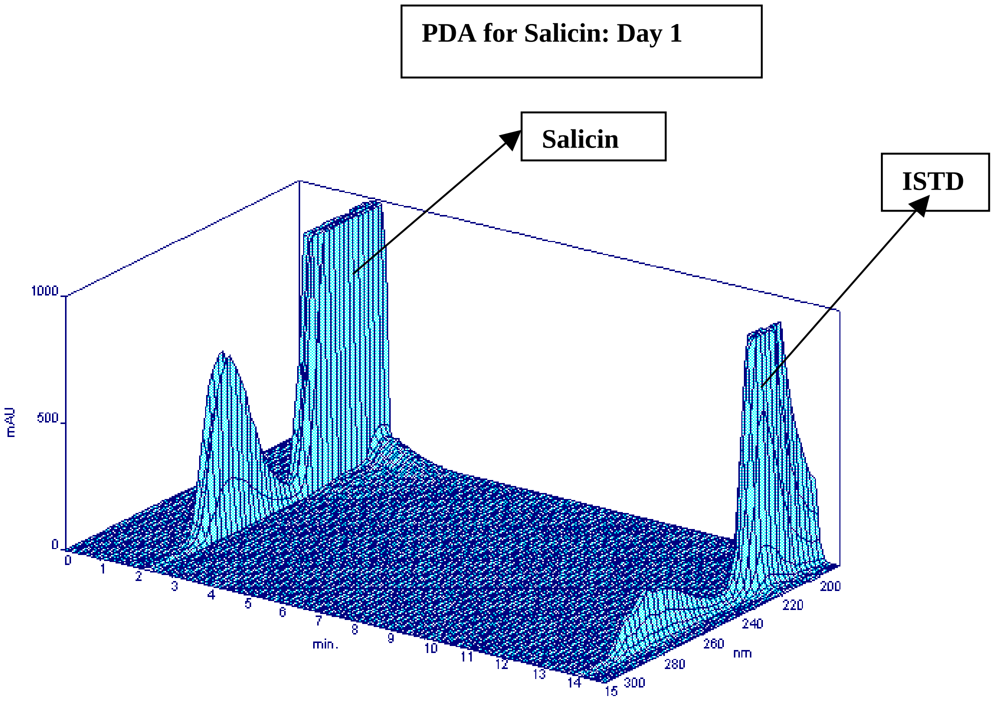


Figure 5.4. Biodegradation of salicin. **A**=day 1, **B**=day 3, **C**=day 15 and **D**=day 25

E



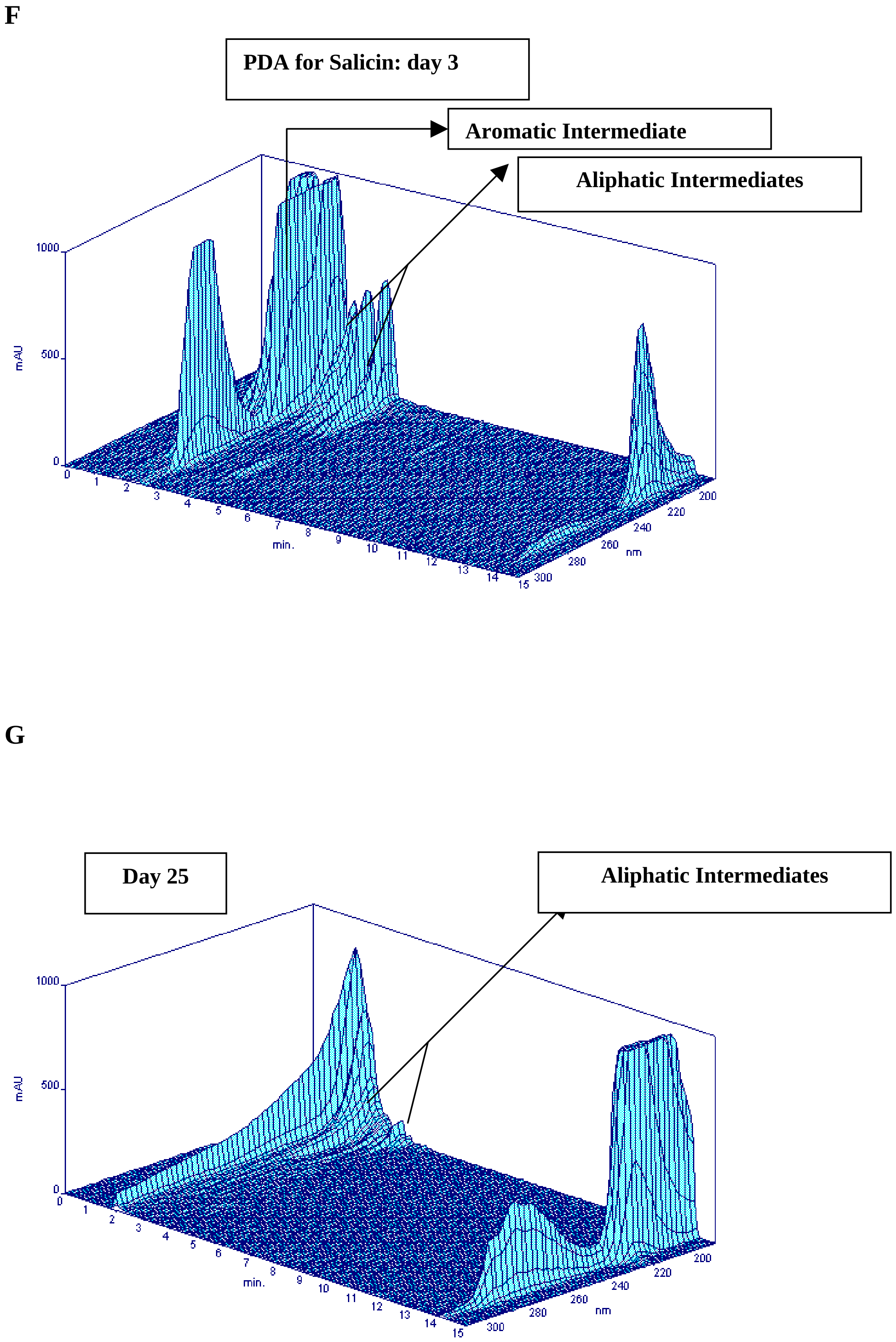


Figure 5.5. HPLC Chromatograms showing the biodegradation of salicin and its intermediates.

The results below show the GC-MS analysis of salicin biodegradation products and these results also show that the SRB consortia can use salicin as an electron donor source to produce intermediates such as salicyl alcohol, ortho-cresol and acetate.

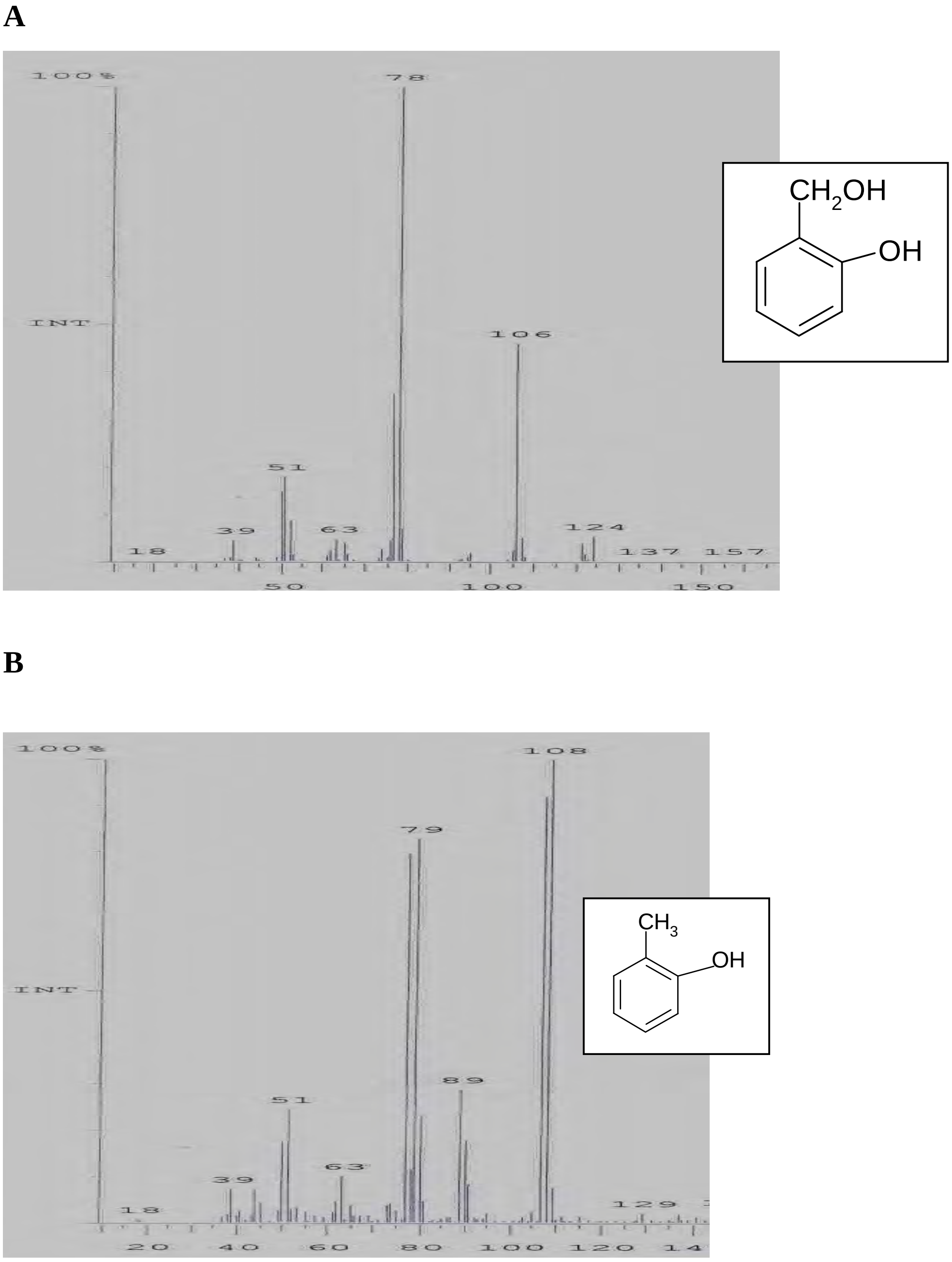


Figure 5.6. GC-MS results for salicin showing the biodegradation products: **A** = Salicyl alcohol and **B** = o-cresol.

5.7.2. Biodegradation of Ferulic acid dimer

The results reported in figure 5.7 show the biodegradation of ferulic acid dimer by the SRB consortia over a period of 50 days.

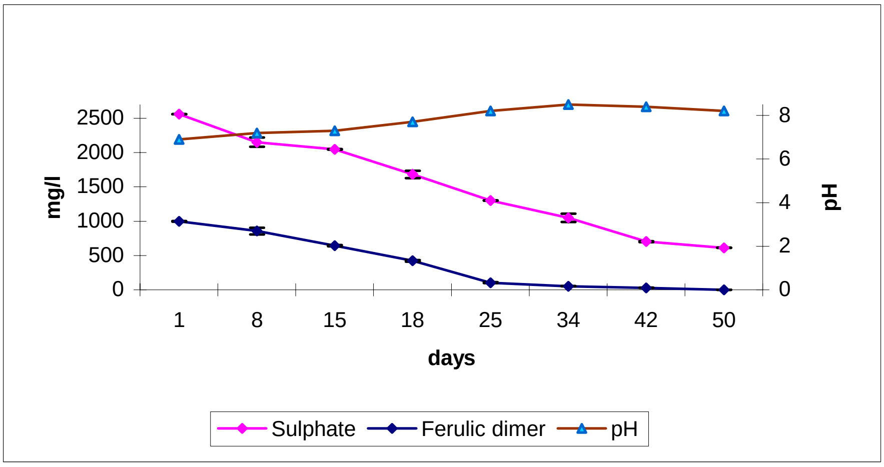
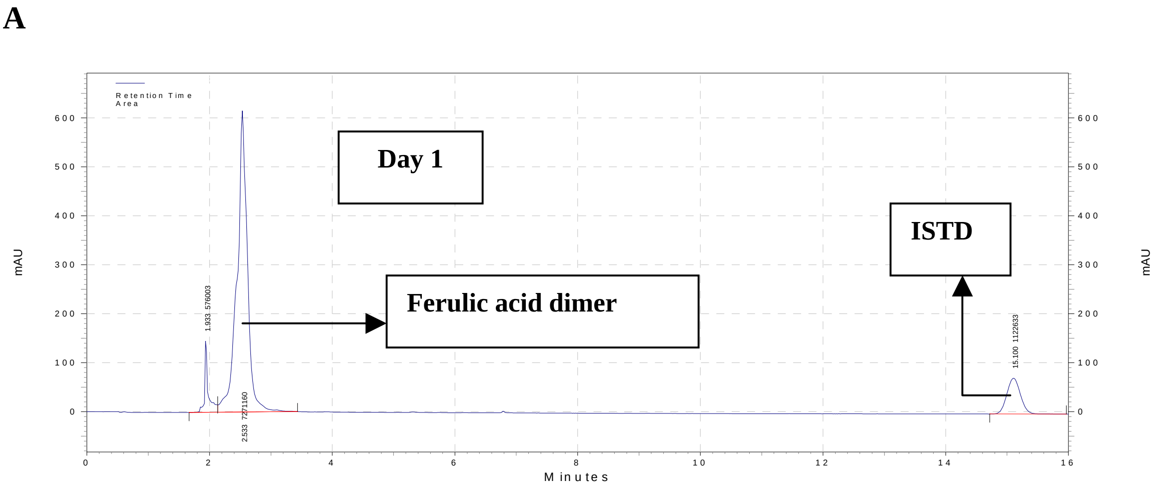


Figure 5.7. Biodegradation of ferulic acid dimer by the sulphate reducing consortia.

Figures 5.8 and 5.9 report the sequential HPLC traces of the ferulic acid dimer biodegradation.



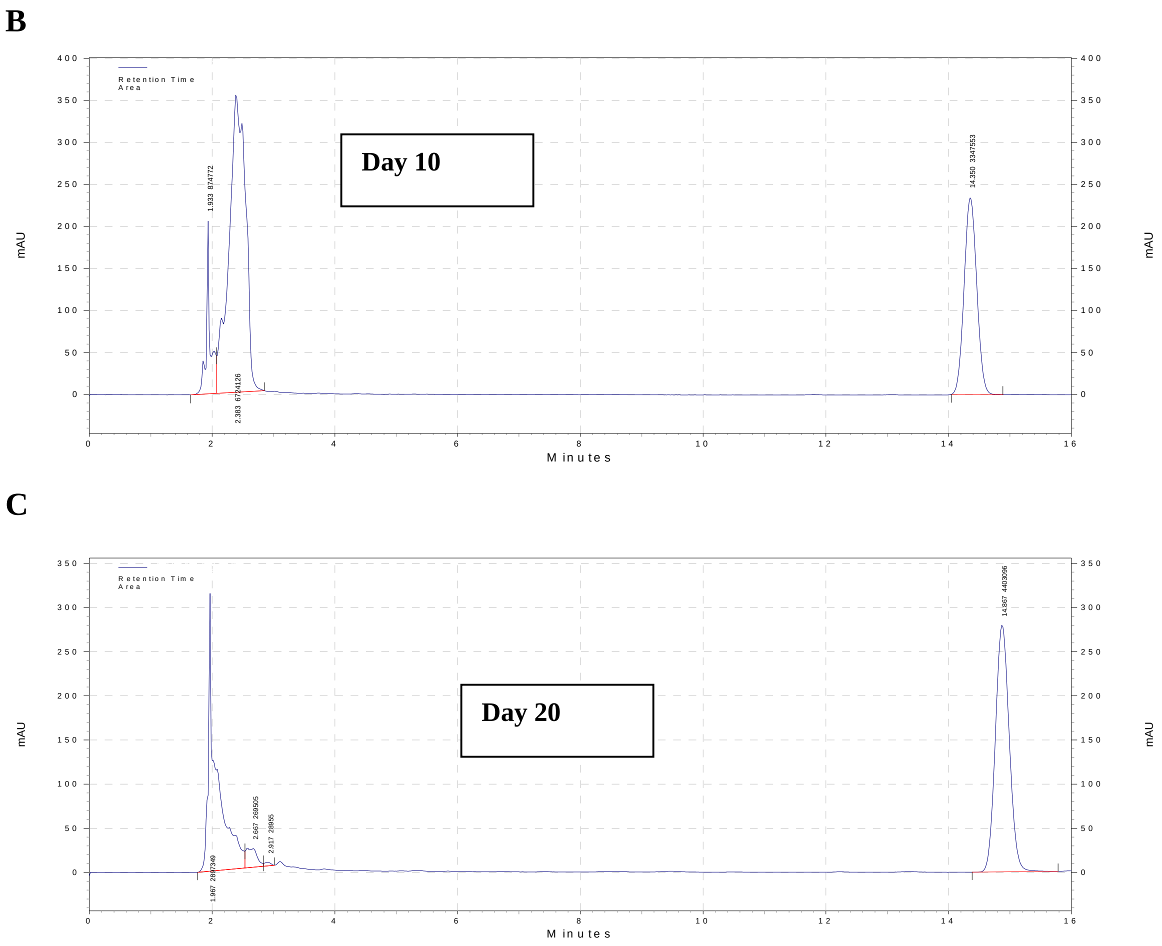
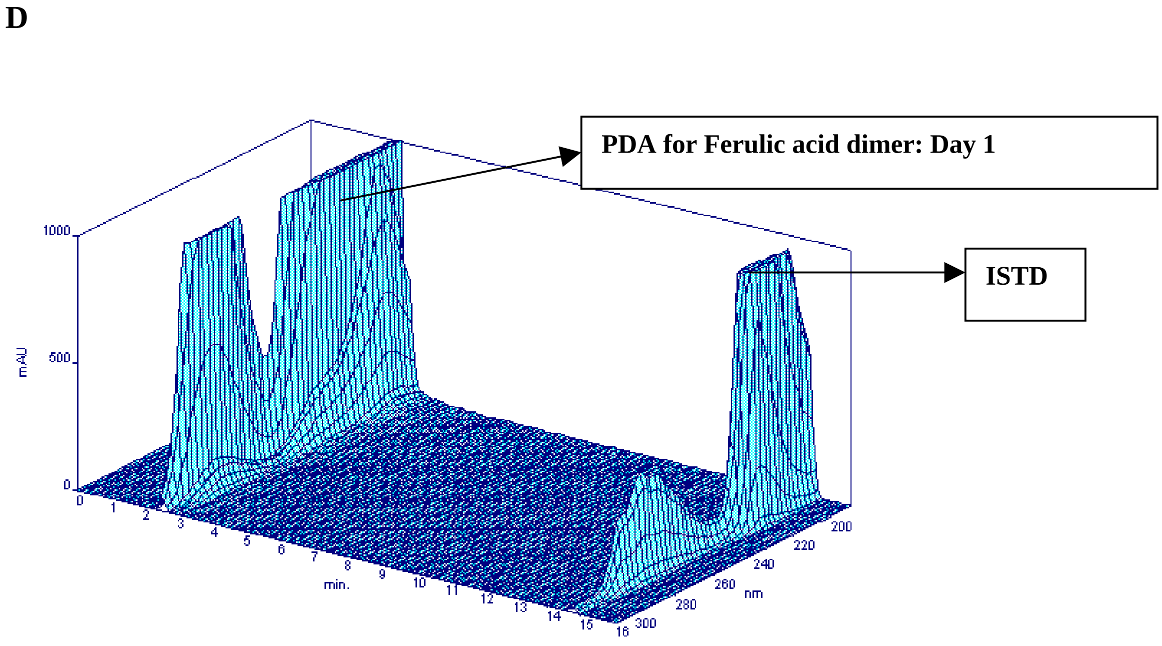


Figure 5.8. Two-dimensional HPLC chromatograms showing the biodegradation of ferulic acid dimer by an sulphate reducing consortia. **A**=day 1, **B**=day 10 and **C**=day 20.



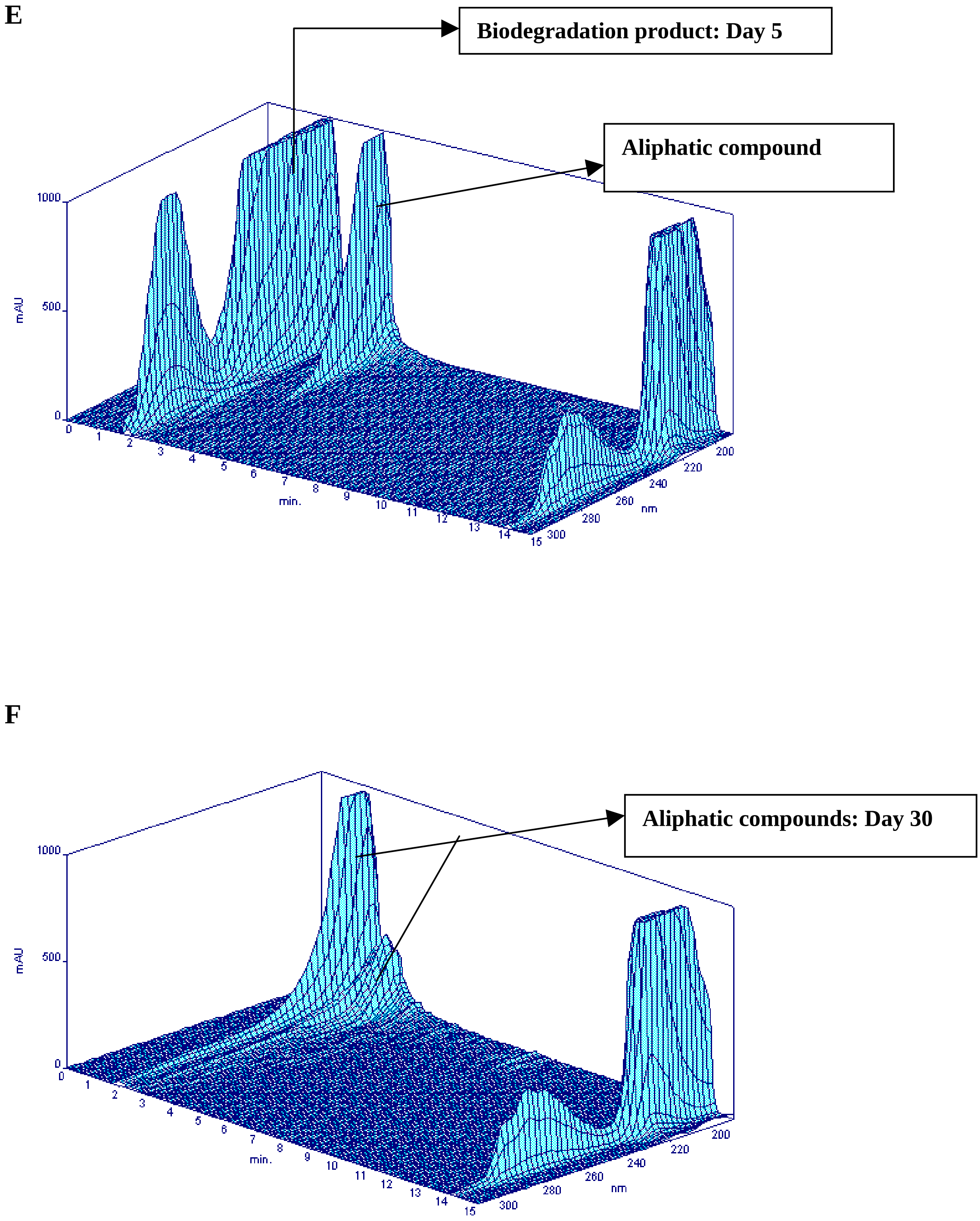


Figure 5.9. PDA chromatograms showing the biodegradation of ferulic acid dimer and its intermediates.

The GC-MS results reported in figure 5.10 show cyclohexane carboxylic acid and adipic acid as the intermediate products of ferulic acid dimer biodegradation by an SRB consortium.

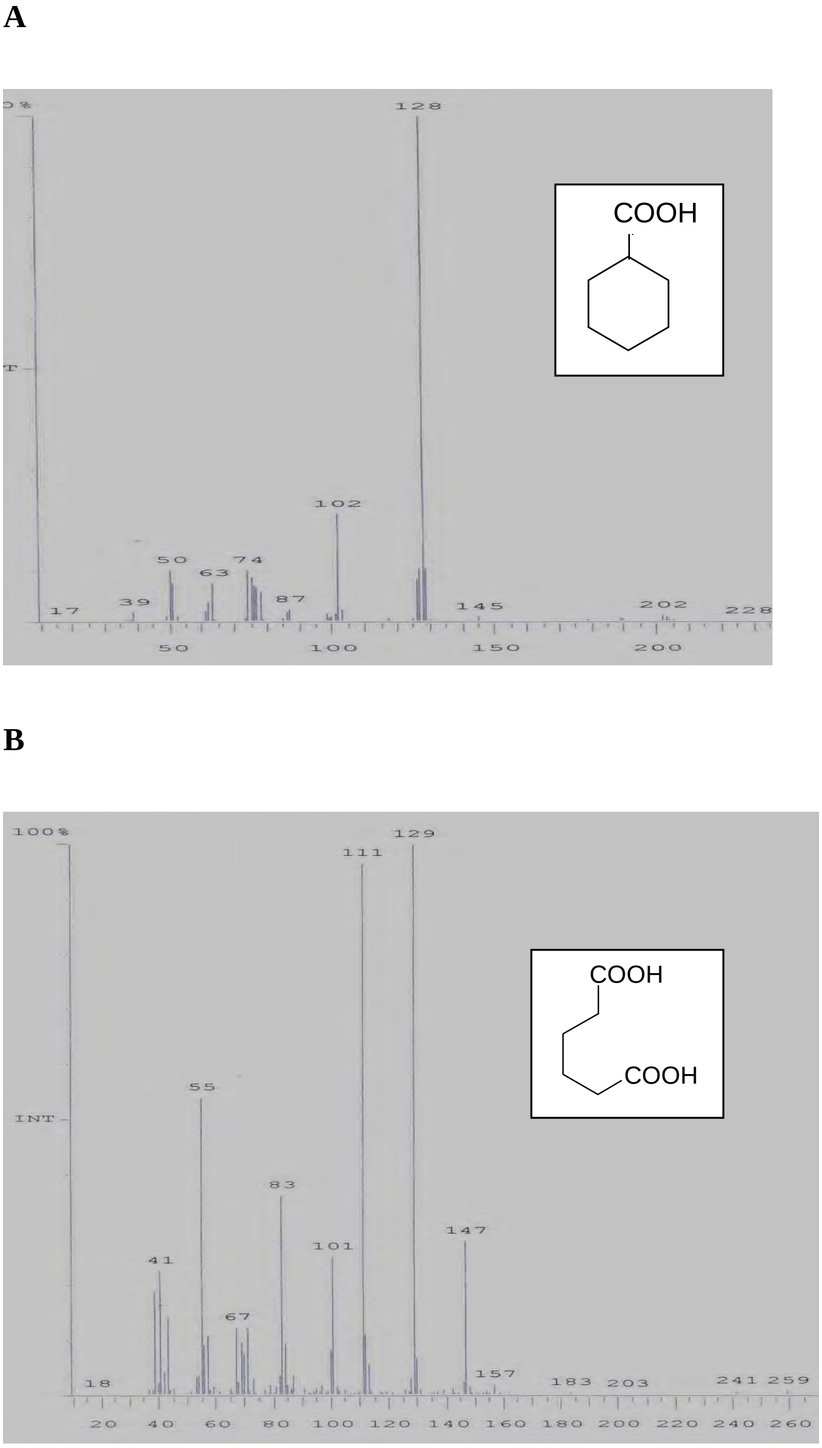


Figure 5.10. GC-MS chromatograms for intermediate products from biodegradation of ferulic acid dimer. **A** = Cyclohexane benzoic acid and **B** = Adipic acid.

5.7.3. Biodegradation of Benzyl-Vanillin ether

The results shown in figure 5.11 report the biodegradation of Benzyl-Vanillin in flask bioreactor studies over a period of 33 days.

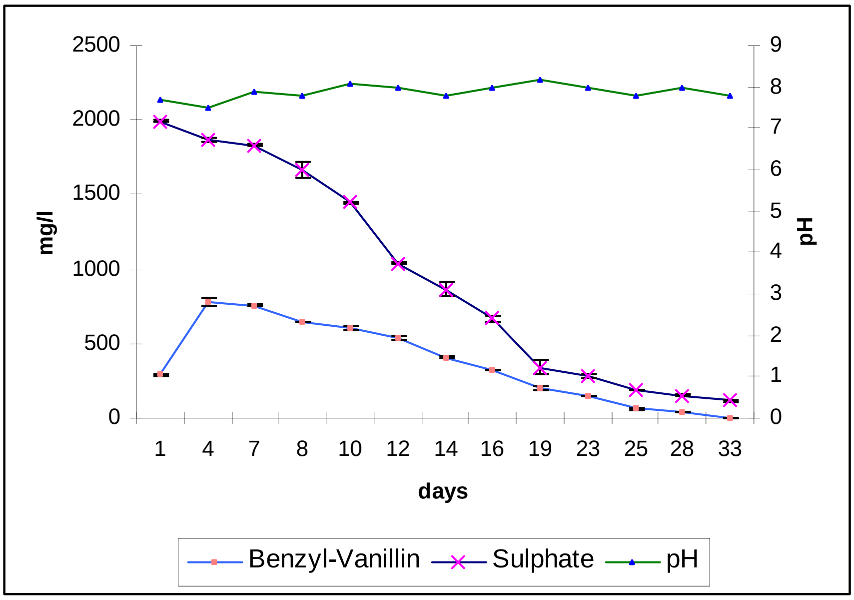
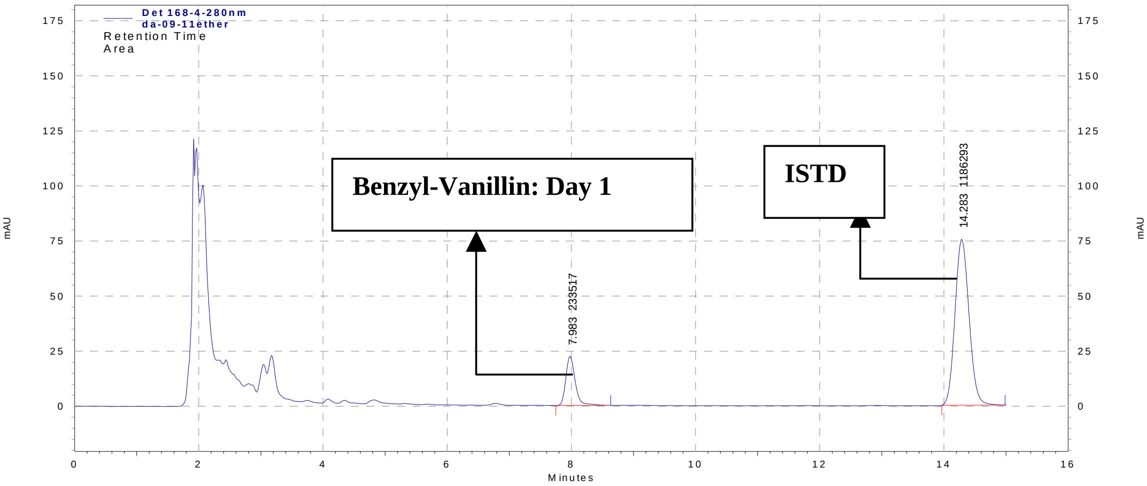


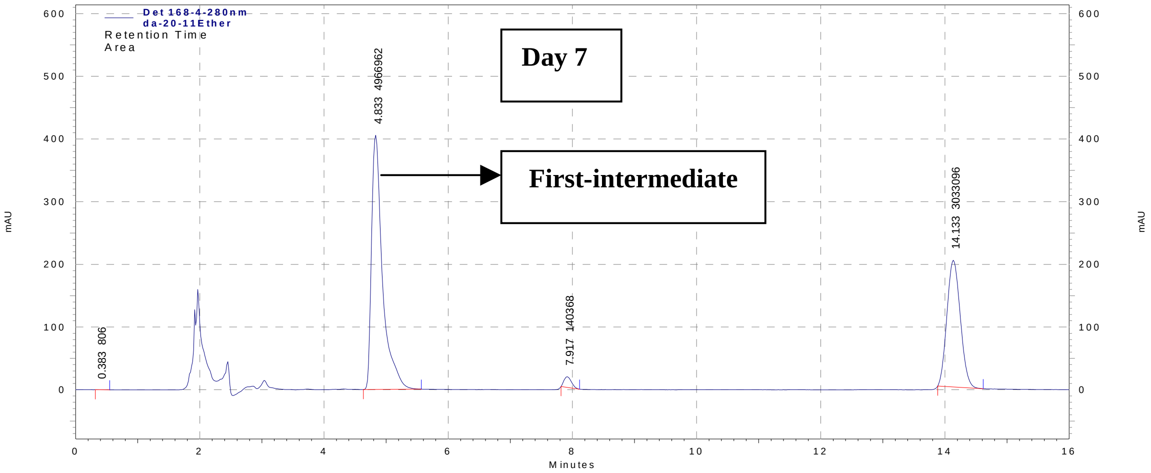
Figure 5.11. Biodegradation of benzyl-vanillin ether by a sulphate reducing consortia. Benzyl-Vanillin is not soluble in aqueous hence the initial concentration is low and it distributes uniformly with time.

Figures 5.12 and 13 report the sequential HPLC traces of the Benzyl-Vanillin ether biodegradation and the appearance of the intermediate products by day 7 and 25.

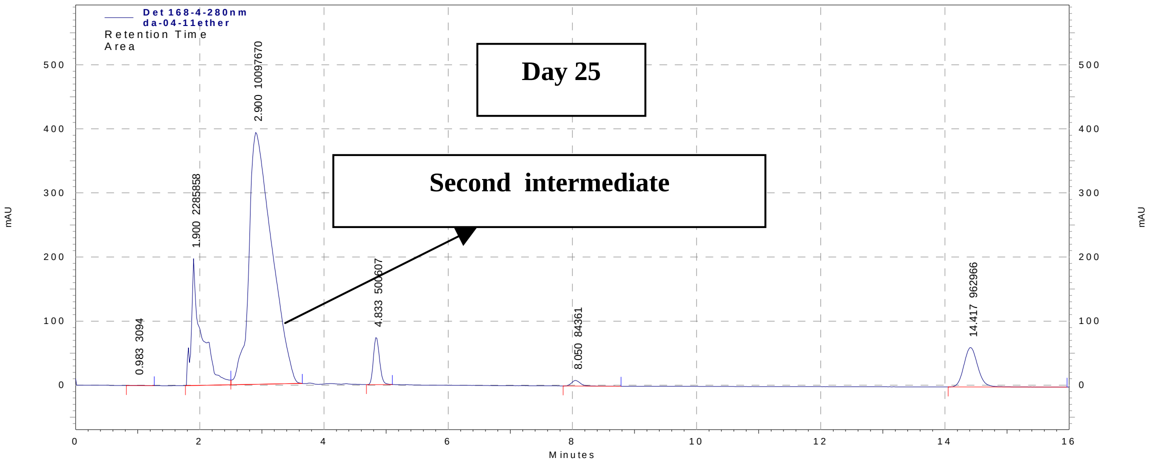
A



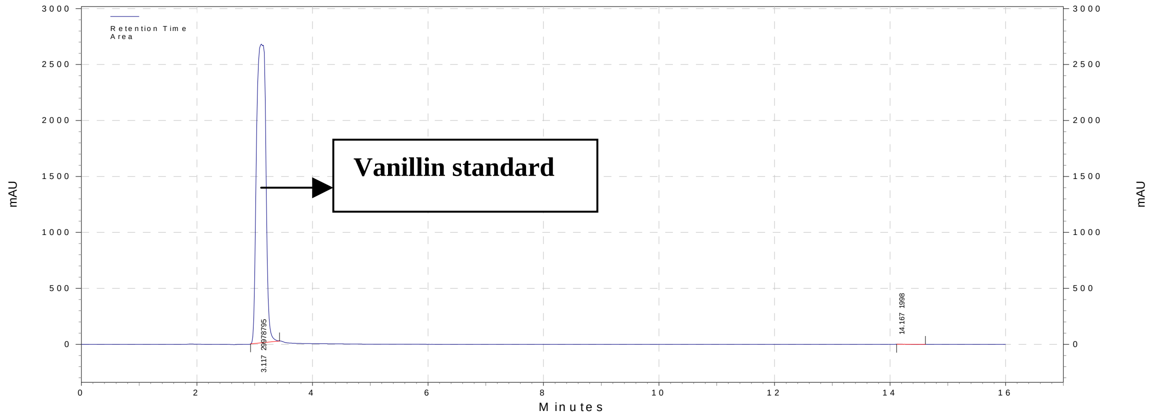
B


Det 168-4-280nm
da-20-11Ether
Retention Time
Area
Day 7
First-intermediate
0.383 806
4.833 4966962
7.917 140368
14.133 3033096
mAU
0
100
200
300
400
500
600
0
2
4
6
8
10
12
14
16
Minutes

C


Det 168-4-280nm
da-04-11ether
Retention Time
Area
Day 25
Second intermediate
0.983 3094
1.900 2285858
2.900 10097670
4.833 500607
8.050 84361
14.417 962966
mAU
0
100
200
300
400
500
0
2
4
6
8
10
12
14
16
Minutes

D


Retention Time
Area
Vanillin standard
3.117 29978795
14.167 1998
mAU
0
500
1000
1500
2000
2500
3000
0
2
4
6
8
10
12
14
16
Minutes

E

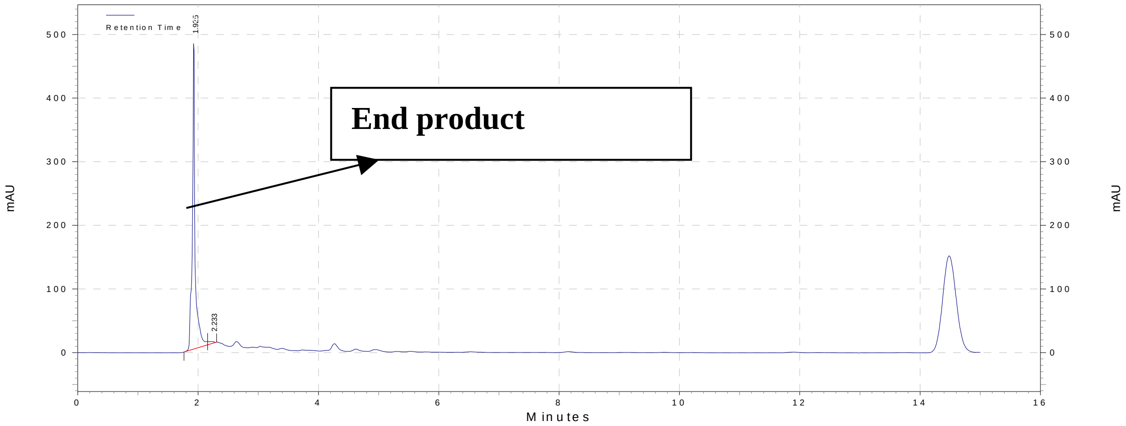
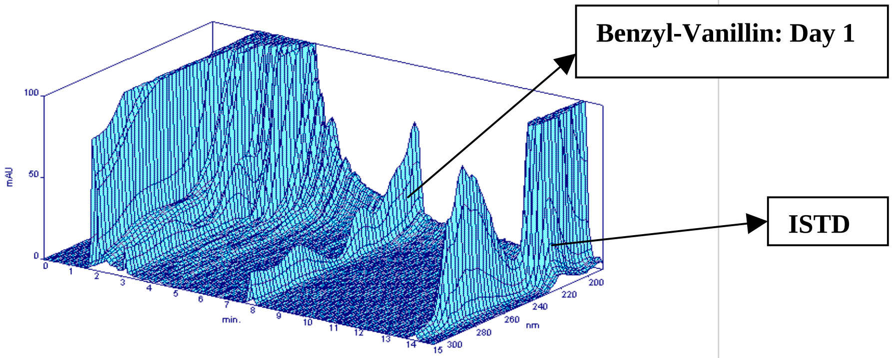
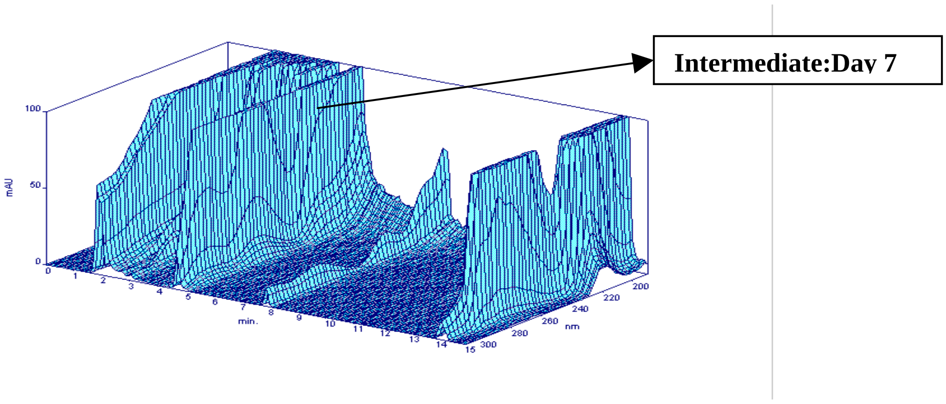


Figure 5.12. Two dimensional HPLC chromatograms showing the biodegradation of benzyl-vanillin and its intermediates.

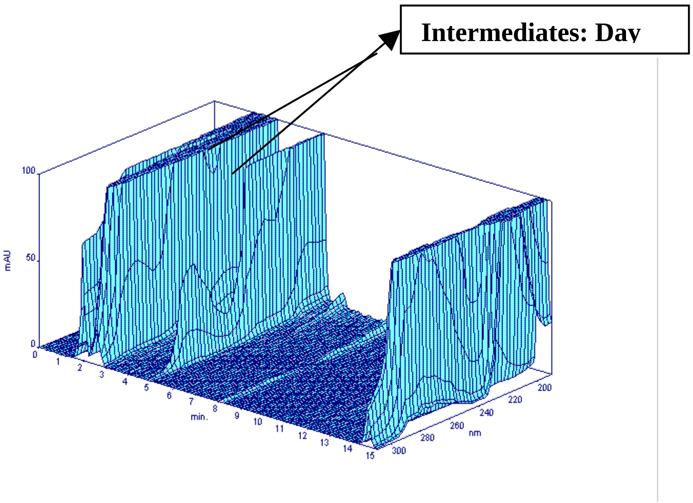
F



G

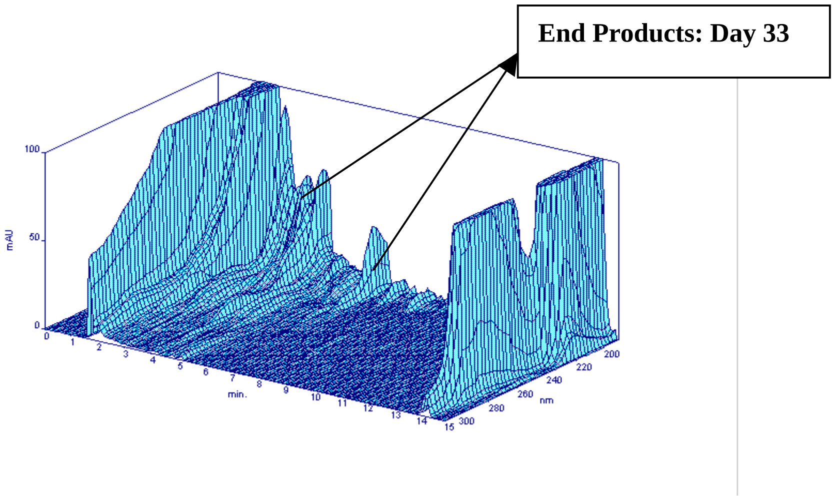


H



Intermediates: Day

I



End Products: Day 33

Figure 5.13. PDA chromatograms showing the biodegradation of benzyl-vanillin and its intermediates.

The intermediate product for benzyl-vanillin was analysed by GC-MS and the results are reported in figure 5.14. Benzyl-Vanillyl alcohol was shown to be one of the intermediate products in the degradation of benzyl-vanillin by SRB consortia. Acetate was also produced as intermediate product.

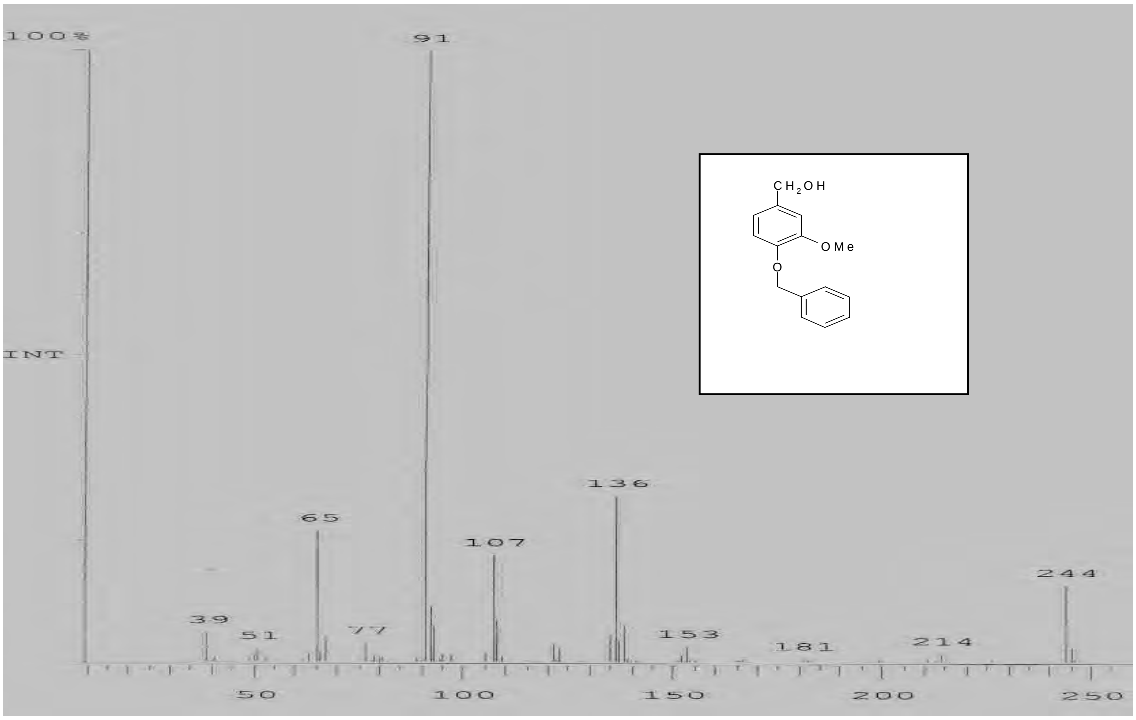


Figure 5.14. Benzyl-vanillyl alcohol as one of the intermediates of benzyl vanillin biodegradation by sulphate reducing consortia.

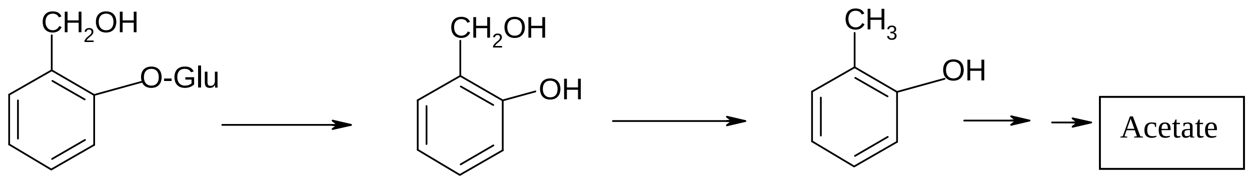
5.8. Discussion

Interestingly, salicin underwent hydrolysis across the glycosidic bond rapidly (within 3 days) resulting in the formation of salicyl alcohol (figure 5.1). This was evidenced by both HPLC and GC-MS. This product of initial degradation was further converted to o-cresol with the alcoholic functional group of salicyl alcohol being reduced to a methyl group. Further cleavage of o-cresol did not happen in more than 30days. PDA analysis showed the loss of aromaticity and the production of aliphatic degradation products. Salicin is a model compound simulating the lignin carbohydrate complex. Biodegradation of salicin implies that the lignin carbohydrate complex is biodegradable under sulphidogenic conditions. Also, the ether bond in salicin was rapidly cleaved which indicates the ability of the SRB consortia to cleave these types of bonds which are predominant in lignin as indicated in the case of mono-aromatic model compounds in chapter 4.

Ferulic acid dimer is composed of carbon-carbon bonds and is also representative of lignin bonds. Its biodegradation to cyclohexane carboxylic acid and adipic acid (figure 5.10.) has also shown that the SRB consortia can cleave these aromatic lignin structures by reduction of the aromatic ring structure to produce cyclohexane carboxylic acid. This is followed by ring fission to produce adipic acid and then the aliphatic compounds. This mechanism has also been observed in chapter 4.

Vanillin benzyl ether was considered to test the difference in degradation of different ether linkages. It is known that ether bonds undergo cleavage under extreme conditions as mentioned for the Kraft process. Benzylic ether was expected to undergo rapid cleavage and in this case vanillin should form as one of the products with toluene being the other and that should inhibit SRB to a certain extent (depending on the concentration of toluene produced). This should enable accumulation of vanillin and this could be easily analysed. However, under the set conditions, vanillin part of the molecule underwent reduction resulting in the formation of vanillyl alcohol. This was evidenced by the lowering in the retention time for the molecule on HPLC (as alcohol is more polar than the corresponding aldehyde) and the GC-MS gave a molecular ion peak at 244(M^+) with a splitting pattern identical to that of standard benzyl vanillin alcohol (figure 5.14.). However, the results in figure 5.12 indicate the production a second aromatic intermediate other than the benzyl vanillin alcohol and then the end products. Figure 5.15 below summarises the proposed pathway for biodegradation of lignin dimeric model compounds; **A**=Salicin, **B**=Ferulic acid dimmer and **C**=Benzyl Vanillin.

A



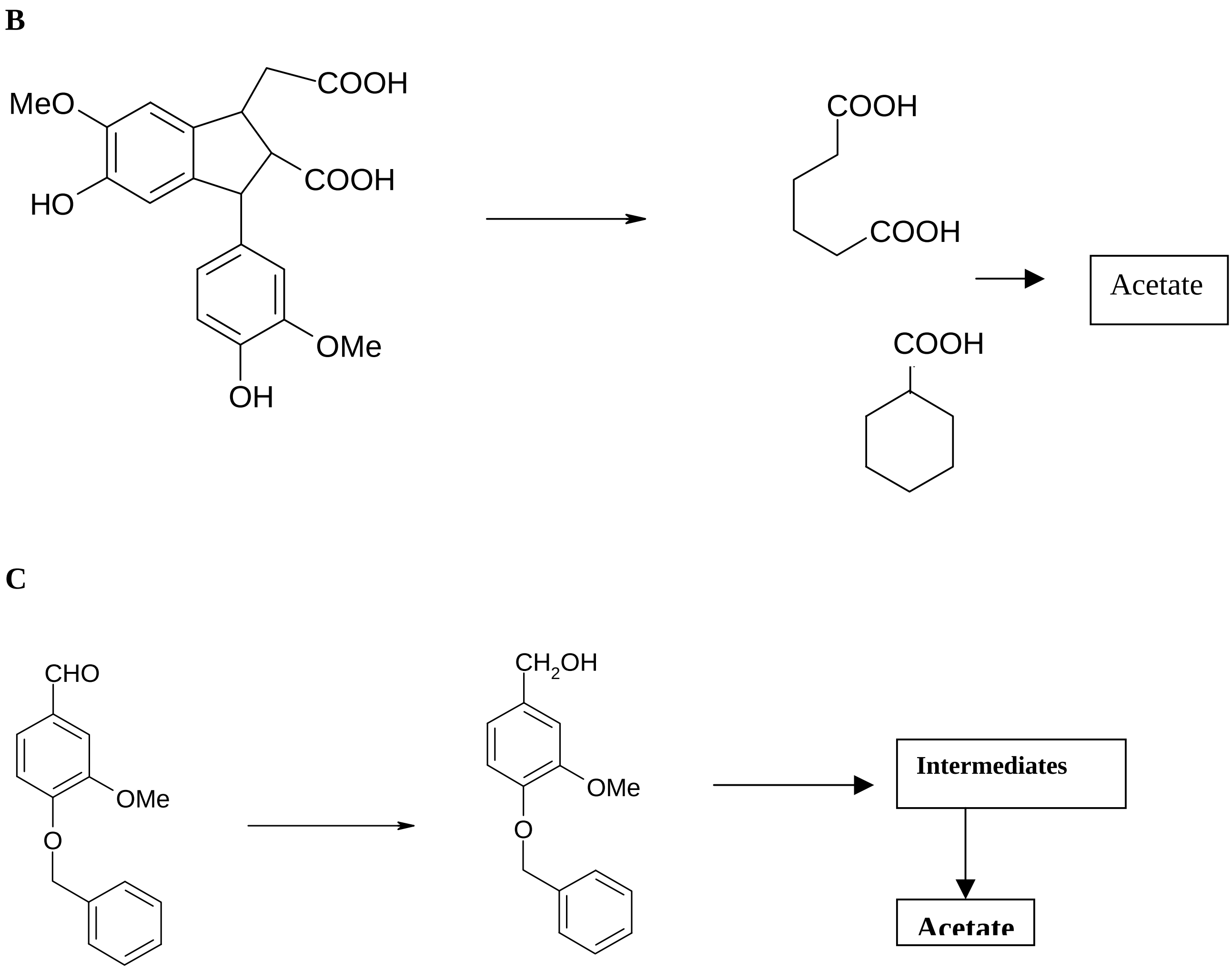


Figure 5.15. Summary of the proposed biodegradation pathway for: **A**=salicin, **B**=Ferulic acid dimer and **C**=Benzyl-Vanillin.

5.9. Conclusion

The use of dimeric lignin model compounds in this investigation has enabled us to have a better understanding of lignin biodegradation under sulphate reducing conditions.

- Our results support the fact that molecular oxygen is not a major factor required for lignin biodegradation as indicated in previous studies.

- These model compounds are degraded by reductive ring cleavage, which results in the reduction of the aromatic ring and then fission to produce aliphatic acids as shown by PDA analysis.
- Cleavage of the ether bonds in lignin is not a limiting factor in biosulphidogenic cleavage of lignin as shown in chapter 3 and in the salicin biodegradation result. Biodegradation of benzyl vanillin into acetate is a further indication of ether bond cleavage.

Chapter 6

General Conclusion

Lignin is characterised by several different carbon-carbon and ether linkages between monomeric phenylpropane units, most of which are not readily hydrolysable (Higutchi, 1990). Progress has been made in understanding aerobic mechanisms of lignin catabolism, the white rot basidiomycetes such as *Phanerochaete chrysosporium, Phlebia radiata* and *Coriolus versicolor* have been shown to be typical lignin degrading microorganisms (Buswell and Odier, 1987).

Lignin degradation under methanogenic conditions is well known and has been studied thoroughly using the lignin model compounds (Colberg and Young, 1985). Little is known about the specific chemical and biochemical transformation reactions under biosulphidogenic conditions. In Kraft pulping process, lignin is chemically degraded at ±170°C under very high sulphide and pH conditions (Clayton, 1969). Sulphide is known to play a major role in preventing re-polymerisation of monomeric lignin aromatic compounds during this reaction (figure 1. 6b).

The SRB consortium is known to generate moderately high levels of both sulphide and alkalinity and they require organic compounds as electron donors during this process. The chemical conditions generated by SRB are similar to those described for Kraft pulping, although occurring at room temperatures, little is known about lignin degradation under these biosulphidogenic conditions.Based on the investigation of the abiotic chemical effects of both sulphide and alkalinity on wood powder, it has been demonstrated that there is an increased release of wood aromatic compounds and extractives in the presence of sulphide and alkalinity at room temperature and pressure. The release of these aromatic compounds was also observed in depectinated wood, which is extractive free. These observations imply that wood can be hydrolysed under biosulphidogenic conditions with the release of lignin derived aromatic compounds at least partly as a result of sulphide and alkalinity producing reactions.

These results are in support of the observation made by Whittington-Jones (2000) that sulphide and alkalinity enhance solubilisation of lignocellulose at room temperatures. Bioreactor studies with mixed SRB consortia indicated that the sulphide and alkalinity levels generated were comparable to those used in the abiotic chemical degradation studies. The amount of aromatic compounds released in both untreated and depectinated wood bioreactor was 20 to 50% of that in the chemical investigation experiments. There was also an observable fluctuation in the release of these aromatic compounds, and this was taken to indicate their utilization as an electron donor sources by the SRB consortia.

Studies with lignin as the sole carbon and electron sources indicated high levels of aromatic compound release with concomitant sulphate reduction and utilisation of these aromatic compounds. Sulphate reduction analysis in wood bioreactors showed an increased sulphate reduction rate per day in untreated wood (94.78l/day) compared to depectinated wood (47.67l/day). This is probably due to the fact that untreated wood has readily available carbon sources such as pectin and simple sugars compared to depectinated wood. It also indicates that lignin on its own is nevertheless still mobilized by the SRB consortia. Surface modification due to sulphide and alkalinity on wood surface was observed by SEM.

These observations indicate that both the chemical and biological effect are important in biosulphidogenic hydrolysis of lignin. Biosulphodogenic hydrolysis of lignocellulose has similarities to the Kraft pulping process, although occurring at room temperature. There is a generation of both sulphide and alkalinity, which are among the conditions, required for lignin degradation. In the biosulphogenic hydrolysis both sulphide and alkalinities play a role in the release of wood aromatics, and these can be used as electron and carbon sources to drive the biological sulphate reduction process.

The hydrolysis of lignin results in the production of oligolignols, dimeric and monomeric lignin-derived aromatic compounds (Kawai *et al.,* 1995). Understanding the degradation of these lignin-derived aromatics is a key factor in elucidating the mechanism of lignin biodegradation under biosulphidogenic conditions. Based on the results from utilisation of lignin model compounds (monomeric and dimeric) by the

SRB consortia, it has been shown that the SRB consortia can use these compounds as electron and carbon sources for sulphate reduction processes. These model compounds are degraded readily within a 30-day period with the production of aromatic and aliphatic intermediates such as acetate as end products were demonstrated.

The major hydrolysis intermediates identified by GC-MS include catechols and aliphatic products such cyclohexane carboxylic acid and adipic acid, which are derived from cleavage of the aromatic ring. The major conclusion based on the identification of these intermediate is that, lignin is hydrolysed under biosulphidogenic conditions to produce monomeric and dimeric lignin derivatives, and they are further broken down by reductive aromatic ring cleavage resulting in the production of aliphatic compounds.

In summary, the results of this investigation indicate that:

- Both chemical and biological effects play a role in lignin hydrolysis;
- The presence of inter-unit ether bonds does not limit anaerobic biodegradation of lignin;
- The presence of molecular oxygen is not a major factor necessary for lignin biodegradation;
- Lignocellulose can be used as an electron donor and carbon source to drive biotechnological processes such as AMD treatment by the SRB consortia.

While the above results provide a preliminary indicator of the mechanisms involved in biosulphidogenic hydrolysis of lignin, further work will be needed to clarify a number of outstanding issues including:

- The use of ^{14}C-labeled model compounds would indicate total mineralisation of the lignin model compound.
- Pure culture studies of the SRB consortia would indicate the particular species responsible for biodegradation of lignin.

- More work is needed to establish the relationship between sulphide and enzymatic system involved in lignin biodegradation.

- Identification of the enzymes involved in lignin degradation under sulphidogenic conditions will also give a better understanding of the system.

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Appendices

Appendix 1. Sulphate and sulphide standard curves

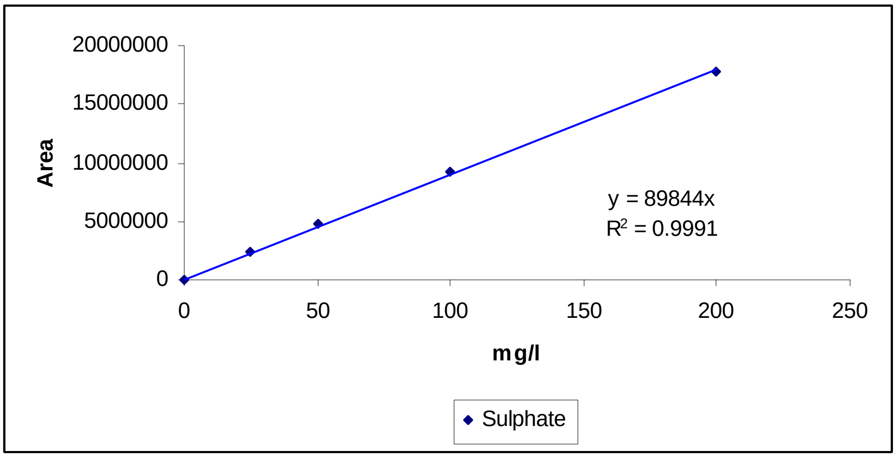

Figure A.1.1. Sulphate standard curve.

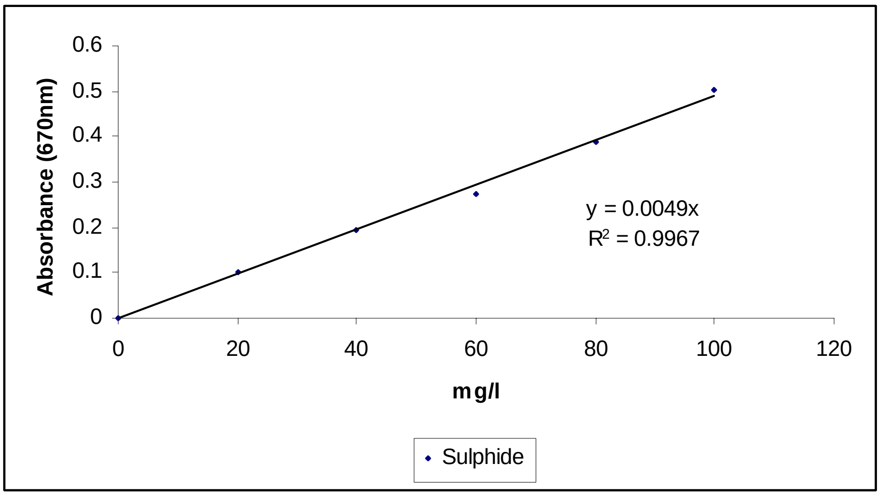

Figure A.1.2. Sulphide standard curve.

Appendix 2. Sulphide analysis

A2.1. Reagents

1. N, N-dimethyl-p-phenylene diamine dihydrochloride (2g) in 500ml 6mol.dm^{-3} HCl
2. $FeCl_3$ (8g) in 500ml 6mol.dm^{-3} HCl
3. Zinc Acetate (10.44g) in 1000ml dH_2O
4. $Na_2S.9H_2O$ (0.750g) in 1000ml degassed dH_2O

A2.2. Method

1. Pippette a suitable sample volume of Zinc acetate (100µl) into a test tube and use dH2O as a control.
2. Add a 1000µl of sample
3. Add 0.5ml of the N, N-dimethyl-p-phenylene damine dihydrochloride solution
4. Add 0.5ml $FeCl_3$ and mix.
5. Leave the sample at room temperature for 60 min.
6. Read absorbance at 670nm.

Appendix 3. Sulphate sample preparation

A3.1. Method

1. Dilute 1ml of sample with 1ml of 0.05M Zinc Acetate solution and leave the sample at room temperature for 15 min.
2. Filter the dilute sample through 0.22µm nylon filter.
3. Pass the sample solution through a C_{18} column filter to remove organic compounds.
4. Dilute the sample to 10ml in a volumetric flask
5. Analyse the 1:10 diluted sample for sulphate

Appendix 4. GC and HPLC sample preparation

A.4.1. GC-sample preparation

1. Filter 1ml of sample through 0.22μm nylon filter.
2. Extract with 1ml of dichloromethane
3. Separate the organic layer from the aqueous layer
4. Add 10mg of Na_2SO_4 to the organic layer and the clear organic layer and store in sample tubes
5. Inject 1μl in GC for analysis

A4.2. HPLC sample preparation

1. Take 1ml of sample and mix it with 1ml of 1mg/ml Naphthalene an internal standard.
2. Filter the mixture through a 0.22μm nylon filter.
3. Inject the sample and the standard 0.5mg/ml Naphthalene
4. Calculate the correction factor using a Naphthalene standard curve to compensate for sample loss.

Appendix 5. Standard curves for lignin model compounds

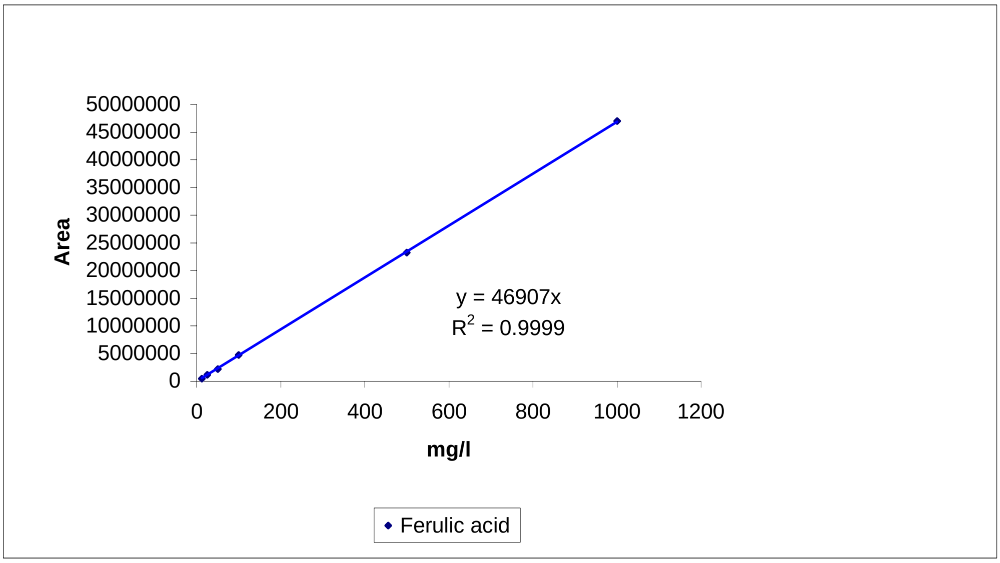

Figure A 5.1. Ferulic acid standard curve.

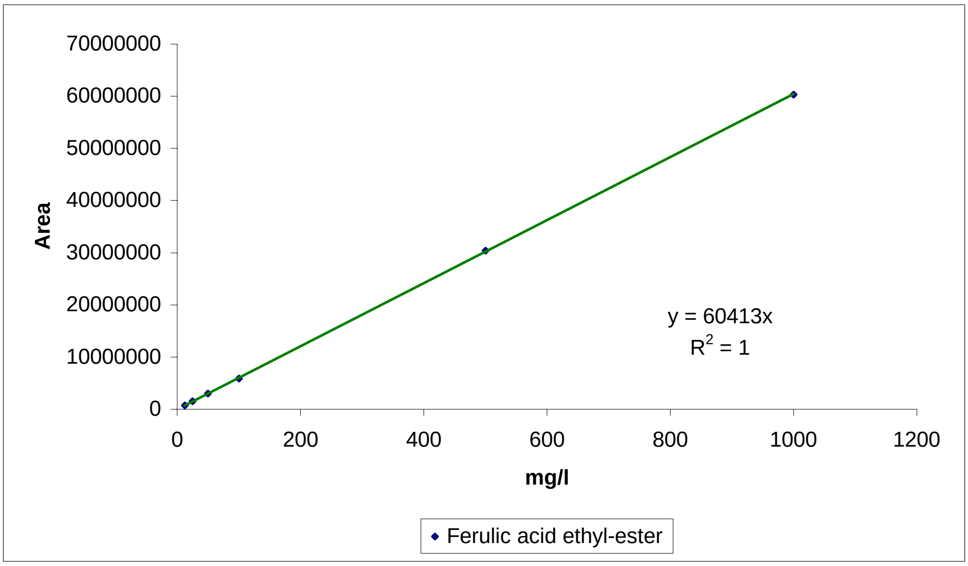

Figure A 5.2. Ferulic acid ethyl ester standard curve.

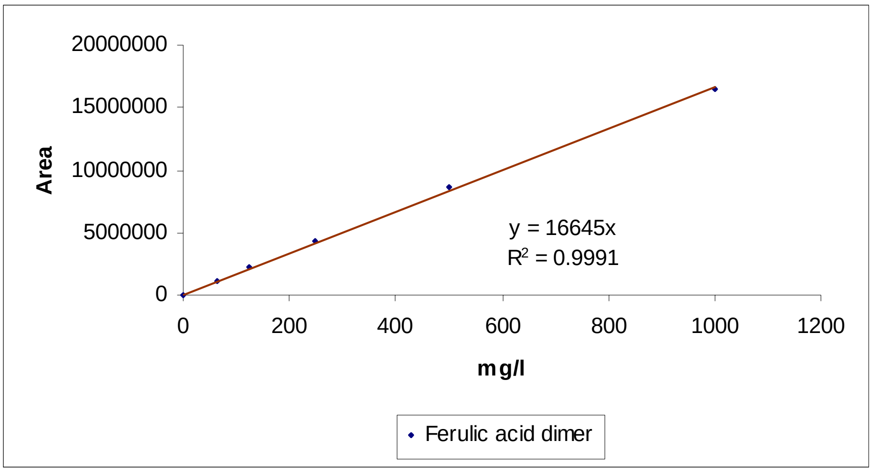

Figure A 5.3. Ferulic acid dimer standard curve.

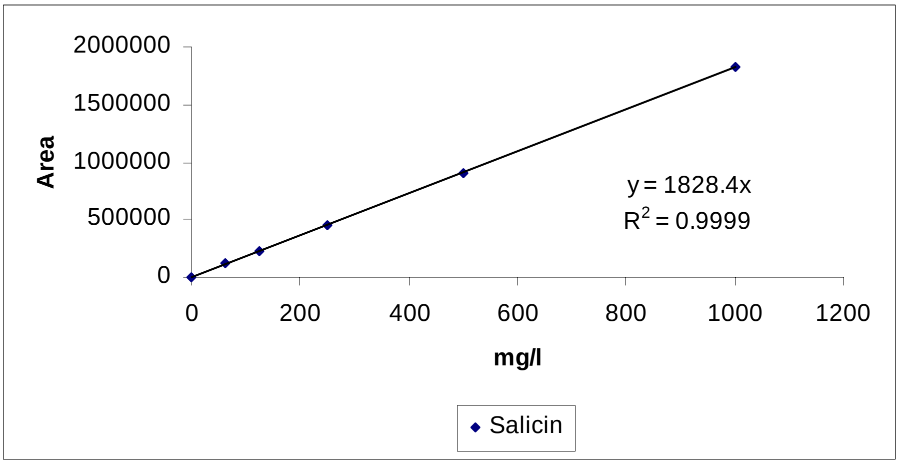

Figure A 5.4. Salicin standard curve

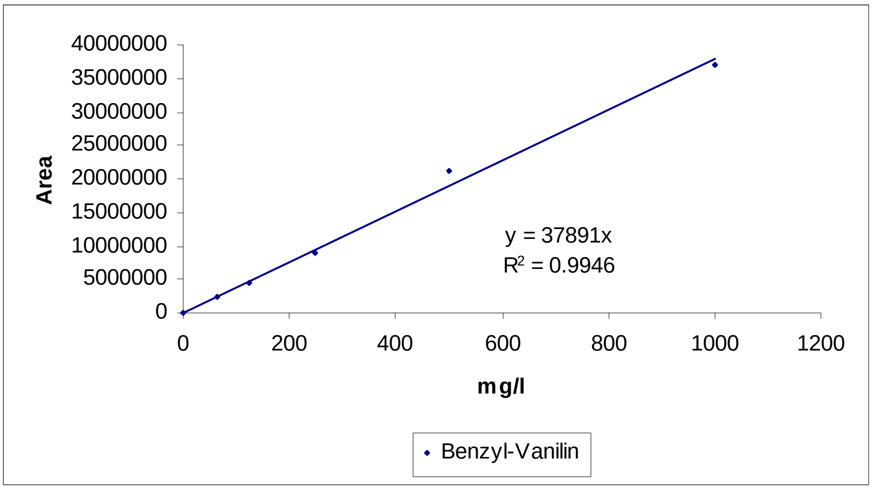

Figure A 5.5. Benzyl-Vanillin standard curve

Appendix 6. Mineral media

2g $MgSO_4$

1g NH_4Cl

1g Na_2SO_4

0.5g K_2HPO_4

0.1g $CaCl_{2}.2H_2O$

Add 950ml of distilled H_2O, adjust the pH to about 7.5 and then fill up to 1l and the autoclave.