ENZYMATIC DEPOLYMERIZATION OF LIGNIN BY LACCASES

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In the name of Allah, the most gracious and most merciful

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DECLARATIONS

No portion of work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

Nor Hanimah Hamidi

I lovingly dedicate this thesis to my parents, ***Hamidi Abd Rashid*** *and* ***Fatimah Hj Ismail*** *who supported me each step of the way*

ABSTRACT

More than half of platform petrochemicals are aromatic, whereas the only large-scale, naturally-occurring, renewable source of aromatics is lignin. Chemical depolymerization of lignin requires extreme conditions, and results in extensive destruction of the aromatic rings and/or char formation. By contrast, enzymatic lignin depolymerization occurs under mild conditions with retention of the aromatic nuclei. Therefore, laccase from *Agaricus bisporus* (LAB) and from *Trametes versicolor* (LTV) with the mediator, ABTS (2,2'-azino-bis(3 ethyl benzthiazoline-6-sulphonic acid)) were used to depolymerize lignin (sodium lignosulphonate) under mild reaction conditions with the aim to obtain high concentrations of value-added chemicals. The depolymerization in the presence of LTV was higher than LAB, which resulted from the high catalytic activity of LTV. Lignin degradation resulted in formation of complex product mixtures. Therefore the products were fractionated and analyzed by different analytical techniques including GPC (for preliminary screening), HPLC and GCMS (for product characterization and quantification), and NMR (for fingerprint analysis). Products included guaiacol, vanillin, acetovanillone, vanillic acid, homovanillyl alcohol, phenol, 4- methylbenzaldehyde, catechol, *p*-toluic acid, 4-hydroxybenzaldehyde, tyrosol, isovanillin, and 3-hydroxy-1-(4-hydroxy-3-methoxyphenyl) propan-1-one, and the total yield of monomers from lignin was 9.8 % in the presence of LTV. The parameters involved in the depolymerization process were optimized to increase the yield of monomers. The efficiency of laccase mediators was also explored by the use of 2,2,6,6-tetramethylpiperidin-1-yloxy (TEMPO), 1-hydroxybenzotriazole (HBT), *N*-hydroxyphthalimide (HPI) and violuric acid (VLA) in the depolymerization of sodium lignosulphonate. However, the catalytic depolymerization in the presence of these mediators was lower than ABTS. In order to improve the solubility of the substrate for the depolymerization process, screening of ionic liquids that are compatible with LAB was deployed in order to find laccase-friendly ionic liquids for further use in lignin depolymerization. The study has found [C_4mim] [L-tartrate] as the best ionic liquid tested, that increased the activity of LAB by 90 %. In conclusion, enzymatic depolymerization of lignin offers a greener process than the chemical methods, and also provides a more efficient method to obtain monomers of valuable specialty chemicals under mild reaction conditions.

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LIST OF ABBREVIATIONS

Time, length, weight, volume and concentration:

h	hours
min	minutes
s	seconds
n	nanometres
μm	micrometres
g	gram
mg	milligram
μl	microliters
ml	milliliters
L	litres
mM	milimolar
M	molar

General abbreviations:

A	Absorbance
ACN	Acetonitrile
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
BSTFA	bistrimethylsilyltrifluroacetamide
°C	degree celcius
DCM	dichloromethane
DMSO-d_6	deutrated dimethyl sulfoxide
EA	elementary analysis
EI	electron ionization
ET	electron transfer
EPR	electron paramagnetic resonance
GC	gas chromatography
GCMS	gas chromatography mass spectroscopy
GPC	gel permeation chromatograpgy

HAT	hydrogen atom transfer
HBT	hydroxybenzotriazole
HPI	*N*- hydroxyphthalimide
HPLC	high performance liquid chromatography
K_m	value of substrate concentration at 1/2 V_{max}
L	light path length
LiP	lignin peroxidase
LAB	laccase from *Agaricus bisporus*
LTV	laccase from *Trametes versicolor*
LMS	laccase mediator system
mV	millivolts
MnP	manganese peroxidase
MeOH	methanol
mol	moles
NMR	nuclear magnetic resonance
OD	optical density
ppm	part per million
[P]	product concentration
rpm	round per minute
RI	refractive index
SD	standard deviation
SEC	size exclusion chromatography
SEM	screening electron microscope
[S]	substrate concentration
$[S_o]$	initial substrate concentration
t	time
THF	tetrahydrofuran
TMCS	trimethylchlorosilane
TEMPO	2,2,6,6-tetramethylpiperidin-1-yloxy
uv	ultra violet
v_o	initial rate of reaction

V	rate of reation
VOC	volatile organic compound
VLA	violuric acid
V_{max}	maximum velocity
v/v	volume per volume
w/w	weight per weight
w/v	weight per volume
λ	wavelength
ε	extinction coefficient

Chapter 1

AIM AND SCOPE OF THE THESIS

The aim and scope of the thesis is to explore the depolymerization of sodium lignosulphonate to value-added chemicals by an enzymatic process. In this study, commercial laccase was used since the isolation of laccase from lignin-degrading microorganisms such as white rot fungi are generally slow growing and may be difficult to cultivate at scale. In addition, laccase is produced on a large scale due to the widespread applications in biotechnology including in paper manufacturing, detergent formulations, bioremediation, biotransformation, lignocellulose processing, *etc.* (Yaver *et al.*, 2001). Therefore, a commercially available laccase was preferred. There are several factors that contribute towards the efficiency of the enzymatic conversion of lignin by laccase, which is complex from the chemical and biological points of view. A process was developed to study the effect of laccase from *Agaricus bisporus* (LAB) on the degradation of sodium lignosulphonate in the presence of 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) as a mediator. After the enzymatic depolymerization by laccase, a complex mixture of the products was formed, that is extremely difficult to analyze. Therefore, a fractionation method was implemented to simplify the analysis process and a combination of analytical methods was deployed to identify the products.

Several studies have implemented size exclusion chromatography (SEC) to study the molecular weight distribution of the products (Majcherczyk and Huttermann, 1997; Nugroho *et al.*, 2010; Shleev *et al.*, 2006). Gel permeation chromatography (GPC) is a type of SEC that separates on the basis of size. Bourbonnais *et al.* (1995) have

demonstrated the use of GPC to analyze the oxidation of Kraft lignin by laccase from *Trametes versicolor*. From their observation, the process produced molecular weight averages between 7800 to 10500 $gmol^{-1}$ after several days of treatment. In this project, the aim would be to produce compounds that have a molecular weight below 1000 $gmol^{-1}$. Therefore, GPC was adopted as a part of the preliminary screening of the product distribution after fractionation.

Other than GPC, proton nuclear magnetic resonance (^{1}H-NMR) was implemented to provide chemical information about the products. In ^{1}H-NMR, a chemical shift is associated with the occurrence of various types of chemical resonance present in the sample. Therefore, this technique was used as a fingerprint analysis of the products. NMR analysis has become one of the important milestones for lignin chemistry. However, it has to be noted that the characterization of lignin depolymerization products is difficult due to the complex mixture of products and overlapping signals.

Therefore, gas chromatography mass spectroscopy (GCMS) was also employed to characterize the products. Pecina *et al.* (1986) demonstrated the use of GCMS for the analysis of lignin degradation products. In their work, a method of derivatization was implemented to increase the volatility as well as the detectability of the products. However, derivatization is not always necessary for GCMS unless the compound of interest cannot be detected. In addition, quantification by GCMS was carried out by measuring the peak area of individual components and comparing with authentic standards. Besides GCMS, high performance liquid chromatography (HPLC) has been used for quantification in several studies (Pecina *et al.*, 1986; Bourbonnais and Paice, 1990; Bourbonnais *et al.*, 1997; Vigneault *et al.*, 2007). Thus, an attempt was made to develop an analytical method by using reversed-phase high performance liquid chromatography (RP-HPLC) for the quantification of lignin depolymerization products in conjunction with GCMS analysis. In this study, the identification of the products by GCMS revealed five compounds formed after the enzymatic depolymerization by LAB. However, the yield was only 7.8 % of the total lignin used.

Therefore, the next aim was to further increase the product yield by using laccase from a different source, to influence the efficiency of product formation from the breakdown of sodium lignosulphonate. Therefore, laccase from *Trametes versicolor* (LTV) was studied. The optimum reaction condition in the presence of LTV was explored, with respect to the reaction time, temperature and also the stability of LTV during the course of the reaction.

Even though ABTS is known as the best mediator for laccase (Morozova *et al.*, 2007; Bourbonnais and Paice, 1992), there are more than 100 possible mediators which have been classified into two types, namely natural and synthetic mediators (Canas and Camarero, 2010). Since the synthetic mediators have been proven to be the most effective mediators by several authors (d'Acunzo *et al.*, 2002; Fabbrini *et al.*, 2002), a study on the effect of five synthetic mediators on lignin depolymerization was implemented. Despite the addition of mediators into the reaction however, there is a major drawback since they are expensive (Li *et al.*, 1999; Couto *et al.*, 2005). Therefore, the process for lignin depolymerization by LTV was designed to use the least amount of mediator as possible.

Laccase has a variety of applications. In some cases however, the processes are inefficient because the substrate is insoluble in water. Therefore, it would be desirable to identify enzyme-friendly solvents that can be used to solubilize the substrates. Ionic liquids are a relatively new type of non-aqueous solvent which often perform better in biocatalytic processes than conventional solvents (Cull *et al.*, 2000). Most importantly, there are millions of ionic liquids, offering a variety of chemical and physical properties. This allows the structure of the ionic liquids to be fine tuned to match the specific requirements of the desired process. Therefore, 106 ionic liquids were tested for their effect on laccase from *Agaricus bisporus* (LAB) using a new high throughput screening method (Rehmann *et al.*, 2012). 2,2'-Azino-*bis*(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) was used as the substrate, and the Michaelis-Menten kinetic parameters were determined.

Chapter 2

LITERATURE REVIEW

2.1 The Need for Lignin Utilization

Petroleum feedstocks are used in industry to produce a variety of products including fine chemicals, *etc.* (Bender, 2000; Demirbas, 2005). As reported by the Association for the Study of Peak Oil and gas (ASPO), the production of petroleum will decline gradually every year starting from 2010 (Fig. 2.1). The decreasing supply of this feedstock has forced the need to find new alternatives to meet the high demands of value-added chemicals in various applications.

In a sense, the fossil fuels are a one-time gift that lifted us up from subsistence agriculture and eventually should lead us to a future based on renewable resources - Kenneth Deffeyes (2001).

Therefore, an alternative approach was explored based on the potential of lignin as a renewable feedstock for the production of valuable aromatic chemicals that are usually derived from petroleum. According to Gargulak and Lebo (2000), there is an estimated 50 million tonnes of lignin available per year from pulping processes worldwide and only 2 % is in use for commercial applications (Gargulak and Lebo, 2000).

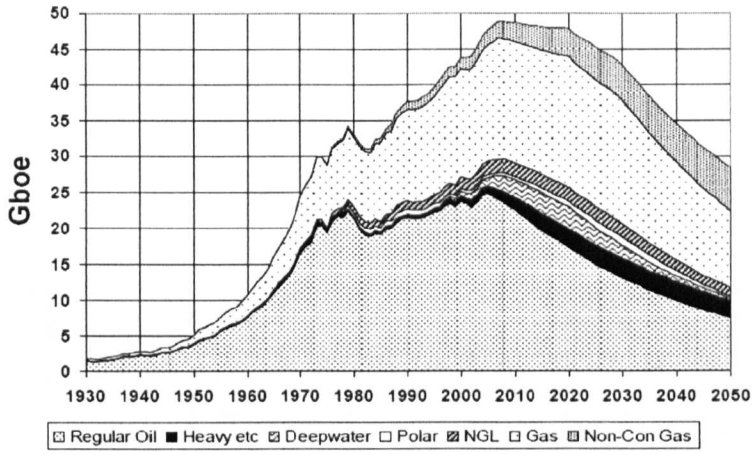


Figure 2.1 Oil and gas production profiles around the globe in 2008 taken from ASPO newsletter No. 100, April 2009. Gboe represents a giga barrel oil equivalent (ASPO, 2009).

2.2 Lignocellulose and lignin

The past decade has seen the rapid development of lignocellulosic biomass as a sustainable source of sugars for biotransformation into biofuels and valuable chemicals (Li *et al.*, 2008; Himmel *et al.*, 2007) especially in the fibre, paper, membrane, polymer and paint industries (Swatloski *et al.*, 2002). Lignocellulosic materials consist mainly of complex structures of the carbohydrates, cellulose (35-50%) and hemicelluloses (20 – 35 %), and lignin (5 - 30 %), a polyphenolic structure (Lnyd *et al.*, 2002; Zavrel *et al.*, 2009; Fig. 2.2).

Cellulose and hemicelluloses are easy to hydrolyze to their subunits (*e.g.* glucose, fructose, galactose, mannose, xylose). The transformations of celluloses and hemicelluloses to the monomer units are a relatively simple process. Numerous studies have attempted to obtain the conversion of cellulose to other products such as bioethanol as a promising alternative energy source to replace crude oil that is likely to suffer limited availability (Demirbas, 2005; Sun and Cheng, 2002). In 2009, Buckeye Technologies, Inc in association with Myriant and University of Florida have announced the development of a new generation bioethanol plant from cellulose which was believed

to be a step forward towards new source of fuel from renewable feedstock (Buckeye Technologies Inc., 2009).

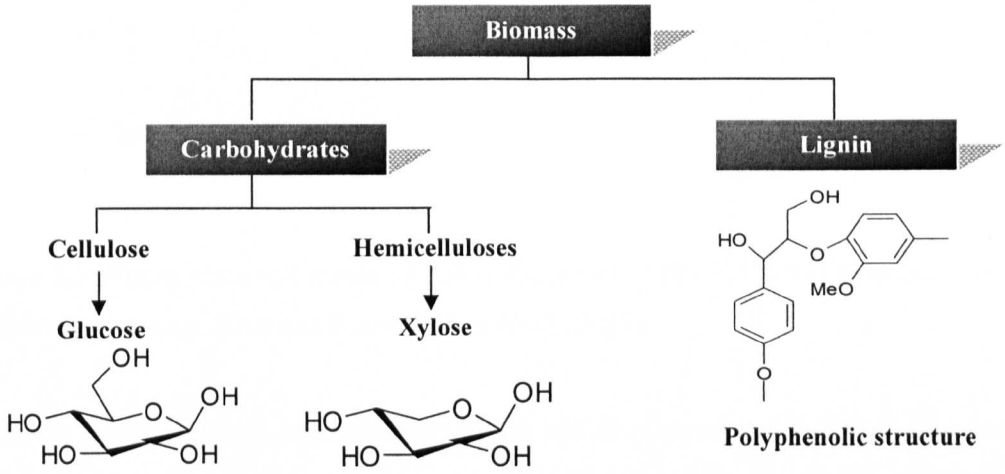


Figure 2.2 Components of lignocellulosic biomass taken from Rogers *et al.* (2002).

On the other hand, lignin is a polyphenolic material composed of phenylpropane units (Rogers *et al.*, 2002). Lignin is practically impossible to dissolve in water in its native form due to the irregular three dimensional cross-linked networks that bind the whole wood structure together to make a strong and resistant plant wood (Kilpelainen *et al.*, 2007). It may also play an important role in defence against pathogen attack and mechanical wounding (Hawkins *et al.*, 1997). The toughness of a plant depends on the percentage of lignin in the cell wall structure. For example, hardwood plants (Fig. 2.3a) contain more lignin compared to softwood plants (Fig. 2.3b) (Antai and Crawford, 1981).

The first serious discussion and analyses of lignin emerged in 1838 in a study by Anselme Payen (Frenh, 2000). He treated wood with nitric acid to remove part of the wood substances and left behind fibrous materials which he called 'cellulose'. He realized that the part that had been removed from the wood materials was rich in carbon content compared to the cellulose. He called the carbon-rich substance as an 'encrusting material' (French, 2000).

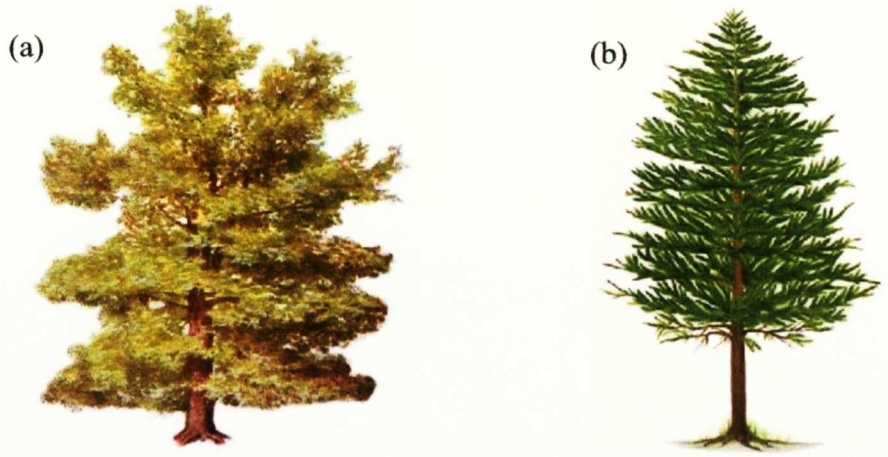


Figure 2.3 Woody plant (a): Hardwood plant (beech tree); (b): Softwood plant (pine tree) (taken from Karen whimsy, 2013 and Peacock river ranch, 2012)

Over the past 100 years, research into lignin has developed and enlarged beginning with work by Schulze in 1865 who first introduced the term 'lignin' (Lu and Ralph, 2010). Three years later, Erdmann in 1868 concluded that the non-cellulosic constituent in wood substances was aromatic. Further investigation of lignin was then demonstrated by Benedikt and Bamberger in 1890 in which they found that methoxyl groups were present in wood tissue but such tissues were lacking in cellulose materials (Brunow, 2001). Further research was done by Klason who came up with the idea in 1897 that lignin was chemically related to coniferyl alcohol (Sjöström, 1993).

Lignin is the second most abundant polymer in nature after cellulose (Annele, 1994; Leonowicz *et al.*, 1999; Li *et al.*, 2008; Zavrel *et al.*, 2009; Kilpelainen *et al.*, 2007; Adler, 1977). It is classified into three major groups which are softwood lignin, hardwood lignin and grass lignin based on the chemical structure of the monomer units (Adler, 1977) which build to form an aromatic, 3-dimensional and amorphous structure (Brown, 1985). Lignin is built from three precursors which are *p*-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol as shown in Figure 2.4 and these precursors are incorporated in lignin as *p*-hydroxyphenyl (H), guaiacyl (G) and syringyl (S), respectively (Grabber *et al.*, 1997).

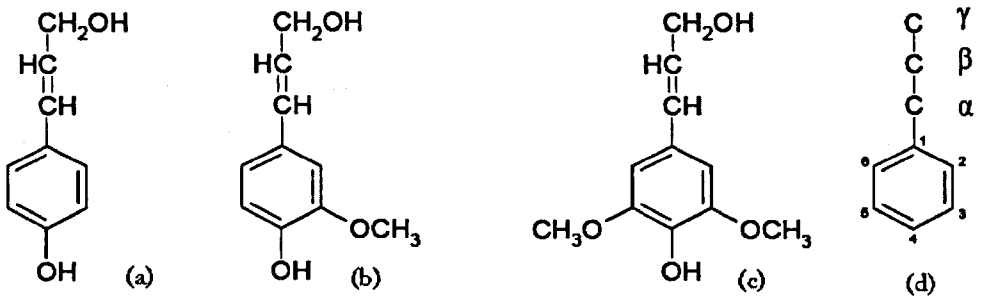


Figure 2.4 Lignin precursors (a) *p*-coumaryl alcohol **(H)**; (b) coniferyl alcohol **(G)**; (c) sinapyl alcohol **(S)**; (d) model for numeration of a carbon skeleton which consist of the aromatic nucleus and 3-carbon side chain represented by γ, β and α (taken from Buswell and Odier, 1987).

These precursors then form different types of subunits of lignin macromolecules where the most abundant subunit is the guaiacylglycerol-β-aryl ether (β-O-4) substructure (40-60 %) followed by biphenyl and dibenzodioxocin, 5-5 (18 – 25 %), phenylcoumaran, β-5 (9 – 12 %), 1,2-diaryl propane, β-1 (7 – 10 %), phenylpropane α-aryl ether, α-O-4 (6 – 8 %), diaryl ether, 4-O-5 (4 -8 %) and β-β linked structures (Adler, 1977; Higuchi, 1990; Sakakibara, 1983; Fig. 2.5) *etc.* A large and growing body of literature has shown that there are no single repeating bonds between the subunits, but a random distribution of at least ten types of bonds (Argyropoulos and Menachen, 1997). The β-aryl ether (β-O-4) bond was the most common bond found in lignin molecule as shown in Fig. 2.5 (Buswell and Odier, 1987). The bonds in lignin are complicated and non hydrolysable, and are much more difficult to break down compared to cellulose and hemicelluloses that are just made from a simple structures and linked with β-1, 4-glucosidic bonds (Kuhad *et al.*, 1997).

Lignin has a high molecular weight which makes it a tough structure and prevents its uptake into the microbial cells (Eriksson *et al.*, 1990). Due to this fact, biological degradation of native lignin must occur through the activity of extracellular enzymes (Adler, 1977; Argyropoulos and Menachen, 1997; Kuhad *et al.*, 1997; Eriksson *et al.*, 1990) from lignin degrading microorganisms such as white rot fungi (Hatakka, 1994; Leonowicz *et al.*, 1999). White rot fungi have a unique ability to produce ligninolytic enzymes to degrade lignin. Wood-rotting fungi are divided into three groups which are

white-rot, brown-rot and soft-rot fungi depending on the types of rot they cause in wood. In nature, a white-rot fungus attaches to wood and slowly degrades the lignin, leaving the cellulose and hemicelluloses untouched. Due to this fact, they are called selective degraders (Hofrichter, 2002; Hatakka, 2001).

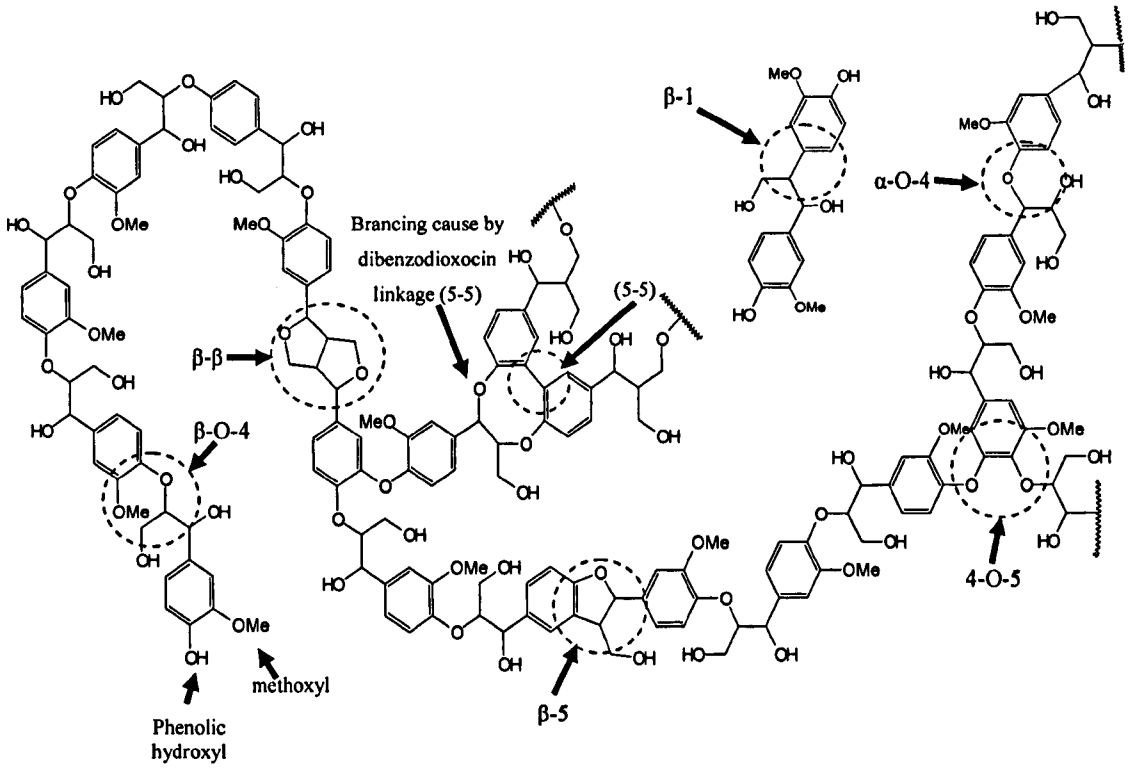


Figure 2.5 Representation of softwood lignin polymer (adapted from Zakzeski *et al.*, 2010).

In the pulp and paper industry, these fungi can be used to degrade lignin from wood, leaving the cellulose unaffected for paper making. Lignin is discharged as a byproduct and can be used as an energy source (Huttermann *et al.*, 2001; Himmel *et al.*, 2007; Kilpelainen *et al.*, 2007; Hofrichter, 2002). According to the literature to date, lignin-degrading enzymes are extracellular as they are secreted from the cell and exist in solution in a free form, and have nonspecific activity, in which these enzymes participate in different oxidative reactions where the lignin aromatic structure and bonds between the subunits are broken (Kuhad *et al.*, 1997; Eriksson *et al.*, 1990; Orth and Tien, 1995).

2.2.1 Lignin Preparation

Various pretreatment technologies are employed to separate lignocellulosic materials to each individual component. Pulping is the major process to remove lignin from cellulose and hemicelluloses for producing pulp that is suitable to be used for pulp manufacturing (Pulp and Paper Manufacture, 1987). Other than the pulp and paper industry, the separation of these materials is important for further conversion of cellulose and hemicelluloses into fuels and fine chemicals (Pan *et al.*, 2005). The techniques used to isolate lignin are varied which results in various types of lignin. This lignin is also referred to as 'isolated lignin' or 'modified lignin'. Isolated lignin is currently used in major markets including construction, mining, animal feed and agriculture. There are diverse isolation techniques including the lignosulphonate process, the Kraft process and the organosolv process. Most of the lignin from pulping processes is burned to provide steam for heat and power production. Thus, the whole process of lignin conversion needs to be competitive with the use of lignin as an energy source.

2.2.1.1 Lignosulphonate Lignin

The sulphite process is a common process in the pulp and paper industry which produces lignosulphonate as a byproduct. The process involves the production of cooking liquor by the oxidation of sulphur to produce sulphur dioxide (SO_2) which is then hydrolyzed to become sulphurous acid (H_2SO_3). The addition of a base, for example sodium hydroxide (NaOH) to H_2SO_3 produces cooking liquor (Reknes, 2004) which is then contacted with the pulp for 3 to 4 hours with a temperature of between 105 to 110 °C (Juan and Huaiyu, 2008) to produce sodium lignosulphonate. The chemical reactions involved in this process are as follows:

$$S + O_2 \rightarrow SO_2$$

$$SO_2 + H_2O \rightarrow H_2SO_3$$

$$H_2SO_3 \rightarrow H^+ + HSO_3^-$$

$$NaOH \rightarrow Na^+ + OH^-$$

The net reaction: $NaOH + H_2SO_3 \rightarrow Na^+ + \mathbf{HSO_3^-} + H^+ + OH^-$

The addition of cooking liquor to the pulp chemically modifies the structure of the lignin by incorporating the sulphonate groups (HSO_3^-) (Fig. 2.6), thus increasing the solubility in water and also increasing the molecular weight (Holladay *et al.*, 2007).

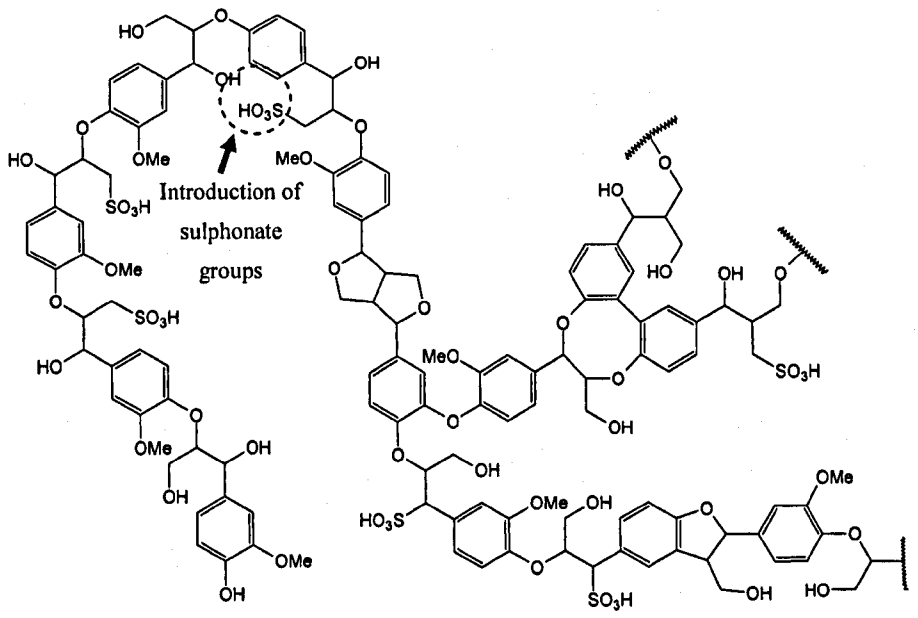


Figure 2.6 Representation of isolated lignosulphonate polymer (adapted from Zakzeski *et al.*, 2010) with some modifications of the side chain with SO_3H-groups at the α- and β-positions (Zakis, 1994).

Lignosulphonates are used in a wide range of applications such as cement additives, the adhesive industry, detergents, surfactants, dispersing agents, stabilizer in colloidal suspension, cements additives, *etc.* (Vishtal and Kraslawski, 2011). The variety of functional groups in the structure of lignosulphonate including phenolic hydroxyl groups, carboxylic groups and sulphonate groups have made this isolated lignin the first choice in a broad range of applications (Juan and Huaiyu, 2008). Lignosulphonate has been produced chemically by several companies and the major producer is Borregaard LignoTech, with a capacity of about 500 000 tonnes of lignosulphonate per year (Belgacem and Gandini, 2008; Ek, 2005). Thus, this company was selected to supply sodium lignosulphonate as the main material used in this study.

2.2.1.2 Kraft Lignin

The Kraft process has been employed since 1879 and since then has become the main pulping technique since then (Gierer, 1980; Chakar and Ragauskas, 2004). The process involves an operation at a high pH (12 – 14) in the presence of aqueous sodium hydroxide (NaOH) and sodium sulphide (Na_2S), also known as white liquor. The chemical reactions involved in this process are as follows:

$$NaOH \rightarrow Na^+ + OH^-$$

$$Na_2S \rightarrow 2\,Na^+ + S^{2-}$$

$$S^{2-} + H_2O \rightarrow SH^- + OH^-$$

The net reaction: $NaOH + Na_2S + H_2O \rightarrow 3\,Na^+ + \mathbf{2OH^-} + \mathbf{SH^-}$

This liquor is reacted with the pulp in a large vessel at temperatures between 70 to 170 °C for 1 – 2 hours (Gierer, 1980; Smook, 1992). The hydroxide (OH^-) and hydrosulphide (SH^-) anions reacted with the lignin causing the breakdown of this polymer into smaller fragments that are soluble in alkali (Chakar and Ragauskas, 2004). This process would then separate the lignin from the cellulose fibres. The isolated lignin is disposed of as a black liquor and chemically modified by altering the β-aryl-ether bonds by hydrosulphide anions (SH^-) as illustrated in Fig. 2.7 (Zakzeski *et al.*, 2010).

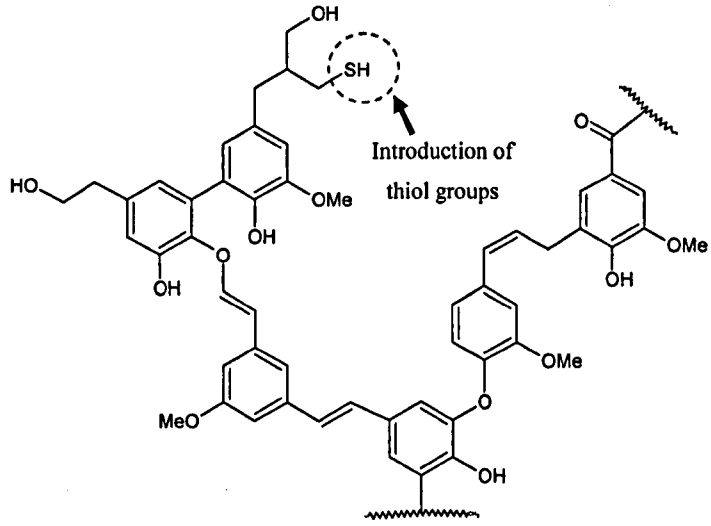


Figure 2.7 Representation of an isolated Kraft lignin with the introduction of thiol-groups at the β-position (adapted from Zakzeski *et al.*, 2010).

For industrial applications, Kraft lignin is use as a dispersant for dyes, pesticides, carbon fibres, blends with thermoplastics, polymers binders and resin, activated carbon, *etc.* (Vishtal and Kraslawski, 2011). The use of Kraft lignin is limited compared to lignosulphonate due to the fact that Kraft lignin is not soluble in water, but only in alkaline solution, which also restricted its use in this study.

2.2.1.3 Organosolv Lignin

In contrast to the lignosulphonate and Kraft process, the organosolv process typically has no sulphur content, has higher purity and a lower molecular weight. Thus, it adds a higher value to the chemical products formed after the depolymerization of organosolv lignin. In addition, this process is considered environmentally friendly since the process does not employ extreme conditions and avoids the use of sulphide (Zakzeski *et al.*, 2010). Lignin is separated from the pulp fibres by solubilizing the pulp in aqueous organic solvents at temperatures between 135 to 165 °C for 1 to 6 hours (Sarkanen *et al.*, 1981). Various organic solvents are employed such as acetone, methanol, ethanol, butanol, ethylene glycol, formic acid and acetic acid (Sarkanen, 1990; Huijgen *et al.*, 2010; Pan *et al.*, 2005; Mabee *et al.*, 2006; Pye and Lora, 1991). In the process that involves ethanol as a solvent, approximately 50 % (w/w) of the mixture of ethanol in water was used (Pye and Lora, 1991). The contact between pulp and the solvent/water mixture causes the breakdown of the lignin (Hergert and Pye, 1992) and produces organosolv lignin without chemically modifying the structure.

Organosolv lignin is usually used for varnishes and paints (Belgacem *et al.*, 2003). Contrary to lignosulphonate and Kraft lignin, the applications of organosolv lignin are limited due to its low molecular weight, which hinders its use as adhesives and binders as offered by other technical lignins. However, when considering the use of organosolv lignin as a feedstock for the production of value-added chemicals, this type of lignin has a bright future. However, this lignin is not yet commercially available at large scale (Vishtal and Kraslawski, 2011), limiting its use in this study.

In contrast, lignosulphonate and Kraft lignin are produced commercially. Lignosulphonate leads by the production of 1 million tonnes of lignosulphonate per year, and the remaining 100 000 tonnes is derived from the Kraft pulping process (Gosselink *et al.*, 2004). Therefore, the study on the enzymatic conversion of lignin was motivated by the high production of sodium lignosulphonate by industry and also because of its high solubility in water which is important for the preliminary study of the enzymatic conversion of lignin.

2.3 Lignin Depolymerization

Various methods have been developed in attempts to convert lignin to value-added chemicals including chemical and biological methods. The latter methods offer more benefits in term of selectivity and mild reaction conditions, and thus require a lower energy demand (Chen *et al.*, 2012). The depolymerization of lignin with selective cleavage is a major challenge for converting this complex polymer into valuable chemicals, thus by using the enzymatic process, selectivity can be achieved.

Enzymatic depolymerization of lignin is unlikely to compete with bulk chemical depolymerization methods unless they can produce the desired product more economically. The enzymatic catalysis process has recently been challenged by chemical catalysis subjected to the depolymerization of lignin under thermal and ultrasonic activation (Finch *et al.*, 2012). Furthermore, Lavoie *et al.* (2011) have also demonstrated the depolymerization of pre-treated lignin for the production of chemicals. In this work, they have reported the production of 10 % monomers from the total of pre-treated lignin used. Serious discussion of the mechanism of lignin depolymerization for specific bond types on lignin has been implemented by Roberts *et al.* (2011). They have demonstrated the production of monomers by the addition of appropriate concentrations of sodium hydroxide (NaOH).

However, it has to be noted that these studies were conducted at extremely high temperature and using chemical catalysts that have inherent drawbacks from a

commercial and environmental point of view. In addition, chemical methods such as pyrolysis, gasification, hydrogenolysis, chemical oxidation and hydrolysis under supercritical conditions are the major methods which be applied to obtain the small fragments of lignin (Pandey and Kim, 2011; Lavoie *et al.*, 2011) and also employ harsh conditions. The processes are conducted at a temperature range of between 300 – 500 °C (Pandey and Kim, 2011) at a high elevated pressure (Zakzeski *et al.*, 2010) that leads to high energy costs and may also contribute to the 'non-green' process. Many of the processes use hazardous catalysts that are often expensive and toxic. In contrast, biocatalytic reactions take place under mild conditions and are often conducted at room temperature. In addition, the reactions catalyzed by ligninolytic enzymes are very selective that are hardly accessible by chemical conversion methods. Therefore, exploitation of enzymatic depolymerization of lignosulphonate under mild reaction conditions was conducted with the aim of obtaining a better understanding of the factors that influence the behaviour of the enzyme to lignin breakdown process.

Waste treatment might well become the first directed use of a bio-ligninolytic system - Kirk (1983)

2.4 Enzymatic Depolymerization of Lignin

The enzymatic conversion of lignosulphonate could be performed in the presence of ligninolytic enzymes, which are known as the main enzymes for lignin degradation (Hatakka, 1994; Leonowicz *et al.*, 1999). There has been an increasing amount of literature highlighting ligninolytic enzymes after the discovery of these enzymes from white rot fungi (Tien and Kirk, 1983; Glenn and Gold, 1985). White-rot fungi produce the main enzymes involved in lignin degradation including heme-containing lignin peroxidase (LiP), manganese peroxidase (MnP), versatile peroxidase (VP) and Cu-containing laccase (benzenediol: oxidoreductase) (Hatakka, 1994) as in Fig. 2.8.

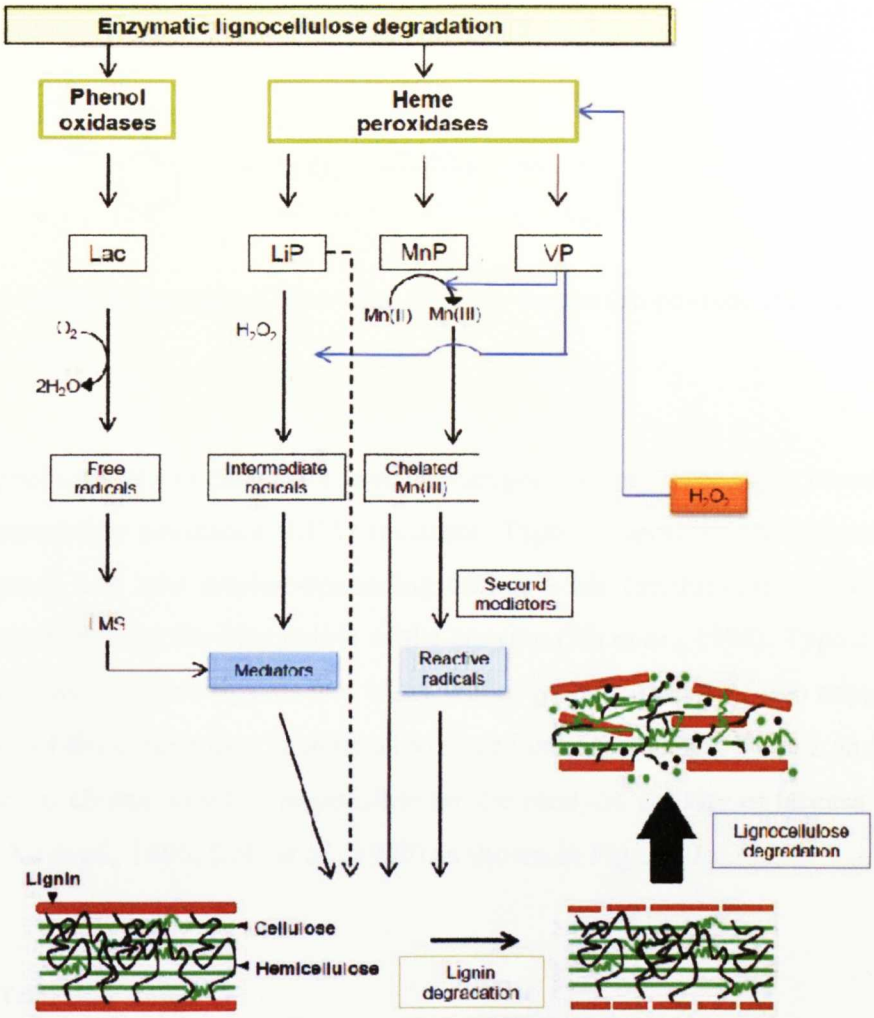


Figure 2.8 Schematic diagram of the lignin degradation steps and enzymes involved (taken from Dashtban *et al.*, 2010).

2.4.1 Laccase

Among the ligninolytic enzymes, laccase offer more stability than the others, especially when compared to peroxidases (Kunamneni *et al.*, 2007) due to the fact that laccase do not use hydrogen peroxide (H_2O_2) as a cofactor. This has also led to the possibility of laccase to be utilized in an immobilized form (Mansur *et al.*, 1997). Laccase belong to the blue copper-containing oxidase group that are able to reduce both atoms of molecular oxygen to water (Baldrian and Gabriel, 2002) in the presence of a substrate as shown in Fig. 2.9 (Eggert *et al.*, 1996).

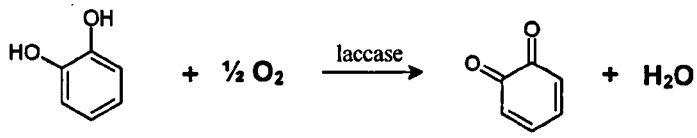


Figure 2.9 Reduction of dioxygen (O_2) to water (H_2O) by laccase (Shipovskov *et al.*, 2008; Octavia *et al.*, 2006).

The four copper ions in a laccase active site are categorized into three types based on the electron paramagnetic resonance (EPR) spectrum. Type 1 copper is attached with two histidine ligands and two sulphur-containing amino acids (methionine and cysteine) which are responsible for the blue colour of the enzyme (Xu *et al.*, 1996). Type 2 copper is attached via two histidine ligands and water whilst type 3 consists of two copper ions of which each of the copper ions is attached to three histidine ligands. Type 2 and type 3 form a trinuclear cluster which is responsible for the catalytic activity of laccase (Duran *et al.*, 2002; Xu *et al.*, 1996; Cole *et al.*, 1990) as shown in Fig. 2.10.

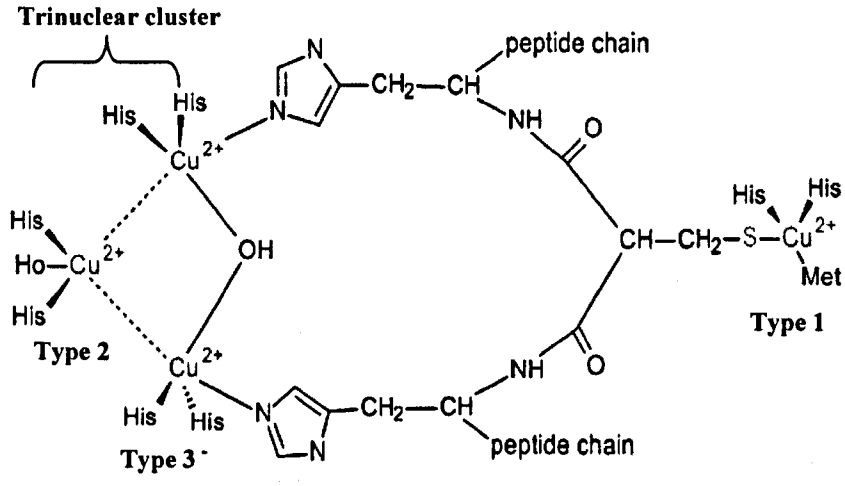


Figure 2.10 Laccase active site containing four copper which belong to type 1 or blue, type 2 or normal and type 3 or a coupled binuclear copper site based on their electron paramagnetic resonance (EPR) (adapted from Duran *et al.*, 2002).

The catalytic activity of laccase essentially depends upon these four types of copper, with three binding sites. Type 1 copper acts as a primary electron acceptor. The electron is then transferred to trinuclear cluster consisting of type 2 and type 3 copper. The reduction of O_2 to H_2O also occurs in these binding sites. Laccase oxidizes its substrate by removing only one electron, and the total reduced state of laccase contains a total of four electrons, thus the electrons are transferred to O_2 to form H_2O (Gianfreda *et al.*, 1999; Octavio *et al.*, 2006) which is illustrated in Fig. 2.11. The removal of protons from the substrate can spontaneously rearrange its structure to form a new compound or form a free radical (Kunamneni *et al.*, 2007).

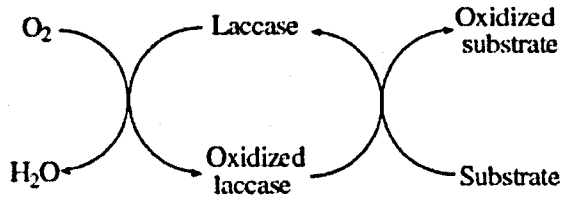


Figure 2.11 Schematic representation of laccase catalyzed redox cycles for oxidation of substrates (taken from Kunamneni *et al.*, 2007)

A large and growing body of literature has investigated the sources of laccase from plants and fungi. Laccase activity is also found in bacteria such as *Azospirillum lipoferum*, *Marinomonas mediterranea*, *Streptomyces griseus*, and *Bacillus subtilis* but the role is as yet not clear (Alexandre and Bally, 1999; Endo *et al.*, 2002; Givaudan *et al.*, 1993; Hullo *et al.*, 2001; Sanchez-Amat *et al.*, 2001; Solano *et al.*, 2001). There are numerous fungi that can produce laccase such as *Polyporus versicolor* A, B, *Pleurotus*, *Pholiata*, *Podospora anserine*, *Neurospora crassa*, *Aspergillus nidulans* and *Pyricularia bryzae* (Gardiol *et al.*, 1998). However, research interest has significantly increased in white rot fungi or basidiomycetes such as *Trametes versicolor*, *Lentimus edodes*, *Pleurotus ostreatus* and *Agaricus bisporus* due to the fact that these fungi have the ability to produce laccase that are involved in lignin depolymerization (Goodell *et al.*, 1998; Crestini and Argyropoulos, 1998; Ardon *et al.*, 1998). Among these are basidiomycetes, and laccase from *Agaricus bisporus* (LAB) and *Trametes versicolor* (LTV) as shown in Fig. 2.12, which are commercially available on a large scale for

various applications including the pulp and paper industry, textiles, organic synthesis, environmental aspects, the food industry, pharmaceutical and nanobiotechnology (Kunamneni *et al.*, 2007).

Figure 2.12 Two types of mushrooms (a) *Trametes versicolor* (LTV) and (b) *Agaricus bisporus* (LAB) (Wilson, 2002 and Cervini, 2005)

In addition, LAB and LTV are the most-studied laccase producing fungus based on the voluminous literature available concerning the production and reactions of LTV (Bourbonnais *et al.*, 1995; Schlosser *et al.*, 1997; Khan and Overend, 1990; Hossain and Anantharaman, 2006). Kawai *et al.* (1998) suggested that fungal laccase, especially produced by white-rot fungi including LTV and LAB have an ability to degrade the lignin due to the capability of these enzymes to cause further rearrangement of the phenoxyl radical, by $C_\alpha - C_\beta$ cleavage on the side chain of the lignin model compounds (Kawai *et al.*, 1988) and the oxidation of the benzyl hydroxyls (Kawai *et al.*, 1999). However, lignin could not be oxidized directly by laccase, due to the fact that this polymer is too large to penetrate into the laccase active sites. To overcome this limitation, the addition of a compound called a mediator is required.

2.4.1.1 Laccase Mediator System (LMS)

Laccase catalyzed depolymerization of lignin requires the presence of a mediator (Elegir *et al.*, 2005). A mediator is also known as an intermediary substance that acts as a mediating agent in chemical or biological processes. Most of the laccase mediators are aromatic compounds which are known to be phenolic fragments of lignin. Due to this fact, they can be lignin model compounds (Morozova *et al.*, 2007). The structures of some laccase mediators are shown in Fig. 2.13. Over the past 20 years, the range of compounds for a laccase mediator system was discovered and dramatically increased after 2,2'-azino-bis(3-ethylbenthiazoline-6-sulphonic acid) (ABTS) was found to be the best mediator for laccase (Morozova *et al.*, 2007; Bourbonnais and Paice, 1992).

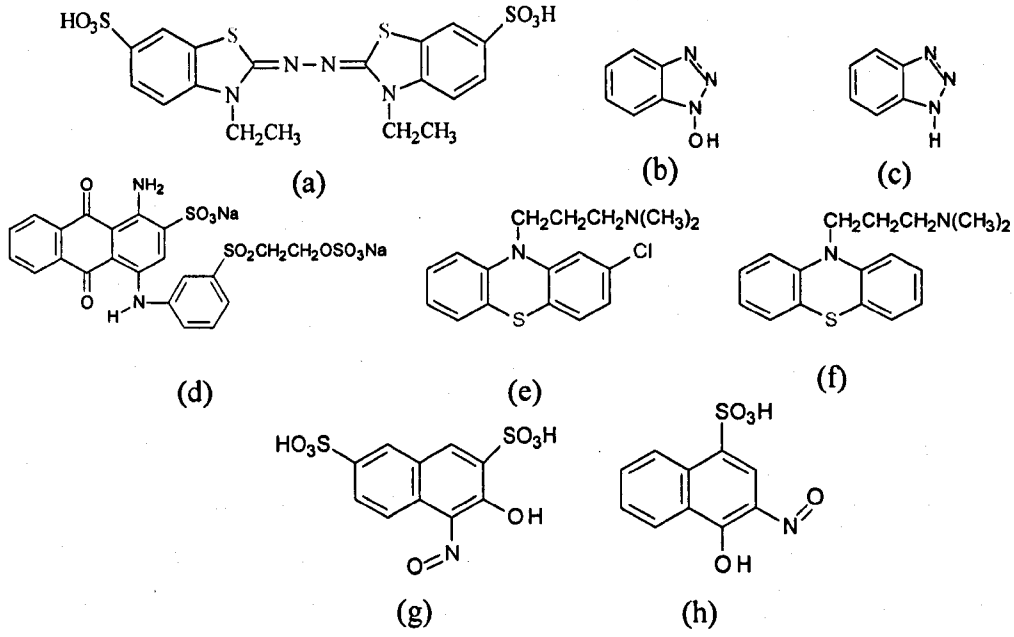


Figure 2.13 Structures of some laccase mediators; (a) 2,2'-azino-bis(3-ethylbenthiazoline-6-sulphonic acid) (ABTS); (b) 1-hydroxybenzotriazole (HBT); (c) benzotriazole; (d) remazol brilliant blue; (e) chlorpromazine; (f) promazine; (g) 1-nitroso-2-naphthol-3,6-disulphonic acid; (h) 2-nitroso-1-naphthol-4-sulphonic acid (Bourbonnais *et al.*, 1997).

The oxidation of the more complex compounds such as lignin does not occur with just laccase alone (Bourbonnais and Paice, 1992) in the system. The oxidized laccase

promotes the oxidation of the mediator and is returned to its original form. The oxidized mediator is reduced to its original form by the substrate to be oxidized which is lignin (Bourbonnais *et al.*, 1998; Fabbrini *et al.*, 2002) (Fig. 2.14).

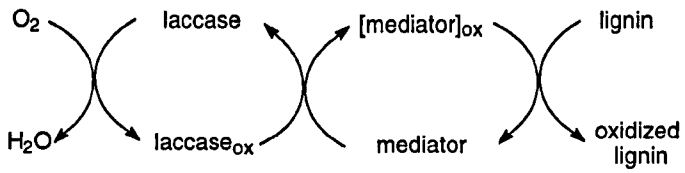


Figure 2.14 Schematic representation of laccase catalyzed redox cycles for lignin oxidation in the presence of a mediator (taken from Bourbonnais *et al.*, 1998)

As mentioned before, ABTS has been found to be the best substrate mediator for laccase (Bourbonnais and Paice, 1992). ABTS is the organic compound best fitting the term "redox mediator" in which ABTS speeds up the reaction rate by shuttling electrons from the substrate (compounds to be oxidized) of primary electron donors to the electron accepting compounds (Bourbonnais and Paice, 1990). The oxidation of ABTS involves two stages. In the first stage, the $ABTS^{+\bullet}$ cation radical is formed by fast oxidation followed by the formation of the $ABTS^{2+}$ dication in the slow oxidation mode of the cation radical (Bourbonnais and Paice, 1990) as shown in Fig. 2.15.

Numerous studies have attempted to explain the use of laccase enzyme in lignin degradation in the presence of ABTS (Bourbonnais *et al.*, 1995; Bourbonnais and Paice, 1992). In order to oxidize the subunits of lignin, the inclusion of mediators such as ABTS was found to be important. Bourbonnais *et al.* (1995) completed a study on the oxidation of Kraft lignin by laccase from *Trametes versicolor* (LTV), which showed that the laccase catalytic activity increased in the presence of ABTS as a mediator, and was able to produce small fragments of lignin in the average molecular weight of 5300 g/mol (Bourbonnais *et al.*, 1995). Three years later, Bourbonnais *et al.* (1998) explained the mechanism of ABTS oxidation by electrochemical analysis. In their study, they determined that the cation radical ($ABTS^{+\bullet}$) reacted only with phenolic structures of lignin, whereas the dication ($ABTS^{2+}$) was shown to be responsible as the intermediate

for the oxidation of non-phenolic structures. Therefore, ABTS was used as an electron carrier for the oxidation of lignin as a substrate in two different mechanisms according to the intermediates produced from the oxidation of ABTS by laccase (Bourbonnais *et al.*, 1998).

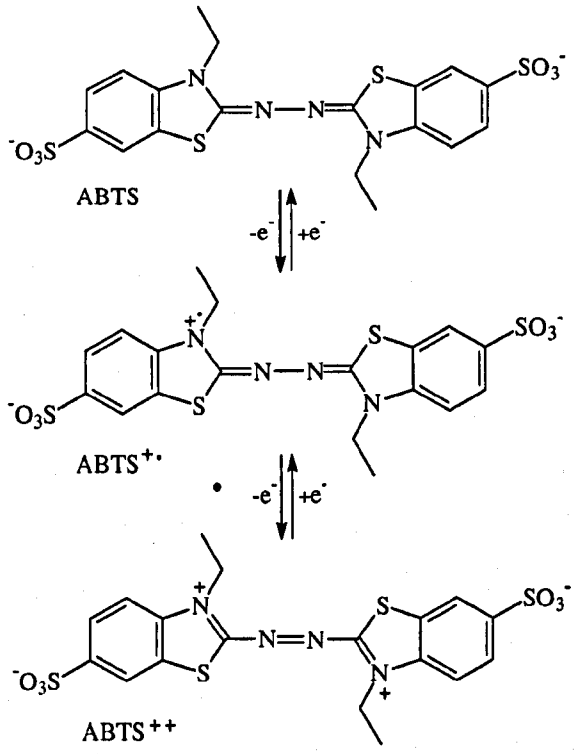


Figure 2.15 Oxidation of ABTS in the presence of laccase taken from Fabbrini *et al.*, 2002.

Much of the research into the catalytic reaction of laccase has concentrated on the oxidation of alcohols (Fabbrini *et al.*, 2001; Arends *et al.*, 2006), ethers (d'Acunzo *et al.*, 2002) and lignin model compounds (Bourbonnais *et al.*, 1997; Li *et al.*, 1999; Fabbrini *et al.*, 2001). So far, however, there has been little discussion about the catalytic reaction of laccase on lignin. Bourbonnais *et al.* (1995) and Shleev *et al.* (2006) have reported the interaction of Kraft lignin with laccase. However, no attempt was made to discover the products of the reaction and the factors that may influence the process. The reason behind the considerable amount of research describing the effect of laccase and mediators on lignin model compounds is very clear. Due to the complex structure of the lignin polymer, lignin model compounds are used to understand the laccase reaction.

Baiocco *et al.*, (2003) demonstrated the mechanism of the laccase mediator towards non-phenolic substrates by following either an electron transfer (ET) or a radical hydrogen atom transfer (HAT). Therefore, attempts have been made to discover the efficiency of using laccase for the depolymerization of isolated lignin from industry. The method was developed to optimize the yield of depolymerization products under mild reaction conditions.

2.4.1.2 Lignin Model Compounds

There is a large volume of published studies describing the oxidation of lignin model compounds by either chemical (Jia *et al.*, 2011; Train and Klein, 1991) or biological methods (Baiocco *et al.*, 2003; Li *et al.*, 1999; d'Acunzo *et al.*, 2002). The idea of using the lignin model compound is governed by several factors; (1) to understand the interaction between enzymes and the lignin by representing the lignin through the model compounds which is much simpler than the lignin polymer; (2) most of the lignin model compounds consist of lignin related linkages such as β-O-4, α-O-4, β-5, 4-O-5 *etc* which represent those linkages found in lignin and the reaction between the lignin model compound and laccase provides knowledge that may lead to the idea to lignin degradation; (3) lignin model compound often contain only one type of linkage, therefore the analysis of these compounds and the products is less complicated compared to lignin polymers (Zakzeski *et al.*, 2010). Thus, the vast amount of publications on lignin model compounds have provided additional knowledge regarding the chemistry of the interaction with the laccase, however, the mechanism involved in lignin depolymerization is more complex, and yet still unknown.

2.4.2 Lignin Peroxidase

Lignin peroxidases (LiP) are a heme-containing peroxidases in which the heme groups act independently and reduce its substrate (*e.g.* veratryl alcohol) in the presence of H_2O_2 (Bloois *et al.*, 2010). LiPs were first isolated from the lignin-degrading fungus *Phanerochaete chrysosporium.* This enzyme catalyzes a wide range of lignin

depolymerization reactions with soluble lignin models compounds and has been fully characterized (Tien, 1987). The catalytic mechanism of LiPs in oxidizing substrates was reported in the studies by Dunford and Stillman (1976) and Tien *et al.* (1986) and followed the same mechanism as other peroxidases. As illustrated in Fig. 2.16, the enzyme is oxidized by H_2O_2 to form LiPI (two electron oxidized intermediate of LiP) and water. LiPI then oxidizes the first molecule of veratryl alcohol (VA) by one electron reduction producing LiPII (oxidized intermediate of LiP) and a substrate radical ($VA^{+\bullet}$). LiPII then uses another veratryl alcohol molecule (VA) by reducing one electron of the substrate and returning to the original form of the enzyme (Dunford and Stillman, 1976; Tien *et al.*, 1986). The substrate cation radical ($VA^{+\bullet}$) is then combined with other radical product to form a new chemical/product (Hiner *et al.*, 2001) or to spontaneously rearrange its structure.

$$\text{Enzyme (LiP)} + H_2O_2 \rightarrow \text{LiPI} + H_2O$$

$$\text{LiP I} + \text{VA} \rightarrow \text{LiPII} + VA^{+\bullet}$$

$$\text{LiP II} + \text{VA} \rightarrow \text{Enzyme (LiP)} + VA^{+\bullet}$$

Figure 2.16 Schematic representation of lignin peroxidase (LiP) catalyzed redox cycles for veratryl alcohol (VA) oxidation (adapted from Schoemaker and Piontek, 1996)

Since 1986, veratryl alcohol has been proposed to be a natural redox mediator for LiP (Palmer *et al.*, 1986). From the study done by Hammel and Moen (1991), lignin did not react with LiP unless veratryl alcohol was added. In the presence of veratryl alcohol, the depolymerization of lignin by LiP occurred (Hammel and Moen, 1991).

2.4.3 Manganese Peroxidase

Over the past 25 years, there has been an increasing amount of literature concerning the production of heme-peroxidases produced from *Phanerochaete chrysosporium* which includes lignin peroxidase (LiP) as discussed earlier, and also manganese peroxidase (MnP) (Gold *et al.*, 1984; Wariishi *et al.*, 1989; Kuwahara *et al.*, 1984; Tien and Kirk, 1984; Glenn and Gold, 1985; Renganathan *et al.*, 1985; Paszczynskia *et al.*, 1986;

Buswell and Odier, 1987). Like LiP, MnP also uses H_2O_2 as a co-substrate in the oxidation of substrates (Dunford and Stillman, 1976). The catalytic cycle of MnP is similar to lignin peroxidase, and involves the production of oxidized intermediates (MnP-compound I and MnP-compound II) (Dunford and Stillman, 1976; Renganathan and Gold, 1986) as seen in Fig. 2.17. However, MnP uses Mn^{2+} as a substrate that is available naturally in all lignocellulosic and in soil (Hofrichter, 2002).

The H_2O_2 is bound to the native ferric MnP to forms an iron-peroxide complex. The transfer of two electrons from MnP resulting in the formation of a MnP-compound I (Fe^{4+}-oxo-porphyrin-radical complex) and produces one molecule of water. MnP-compound I is converted to MnP-compound II (Fe^{4+}-oxo-porphyrin complex), during this process, the Mn^{2+} is oxidized to Mn^{3+} (Glenn and Gold, 1985; Paszczynskia *et al.*, 1986; Glenn *et al.*, 1986; Wariishi *et al.*, 1988) and donate one electron for the porphyrin intermediate. The reduction of MnP-compound II proceeds in a similar extent, thereby the native MnP is re-generated and a second molecule of water is released as shown in Fig. 2.17. The chelates of Mn^{3+} with organic acid such as lactate, malate, *etc.*, facilitate the detachment of Mn^{3+} from the MnP active site and stimulate the MnP activity by increasing the rate of oxidation (Wariishi *et al.*, 1989). The chelates of Mn^{3+} also cause one electron oxidations of various substrates (*e.g.* phenol, amine, *etc.*) that leads to substrate modification or the production of free radicals (Hofrichter, 2002).

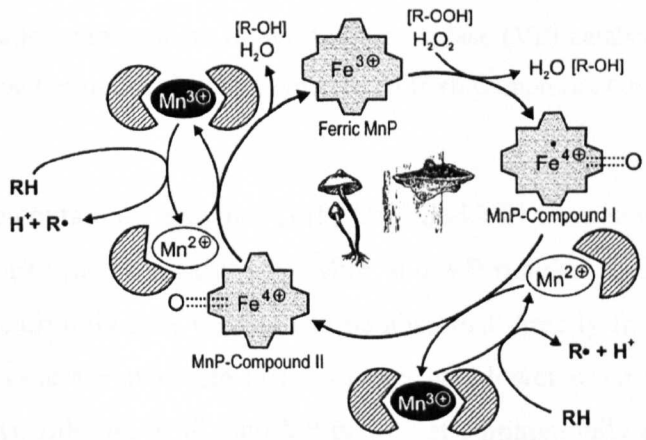


Figure 2.17 Schematic representation of manganese peroxidase (MnP) catalyzed redox cycles for Mn^{2+} (taken from Hofrichter, 2002)

2.4.4 Versatile Peroxidase

In 1999, Camarero *et al.* found a new peroxidase enzyme combining two major peroxidase properties from LiP and MnP which is secreted by the fungus, *Pleurotus eryngii* in lignocellulosic media. This enzyme is called versatile peroxidase (VP) (Camarero *et al.*, 1999). Referring to the fact that VP has both MnP and LiP properties (Fig. 2.18), VP are able to oxidize Mn^{2+} and phenolic compounds as well as non-phenolic aromatic compounds such as veratryl alcohol (Camarero *et al.*, 1999). VP can also be isolated from other types of white rot fungi such as *Pleurotus ostreatus* (Cohen *et al.*, 2001), *Bjerkandera adusta* (Heinfling *et al.*, 1998; Wang *et al.*, 2003), and *Bjerkandera* sp. strain BOS55 (Mester and Field, 1997; Palma *et al.*, 2000).

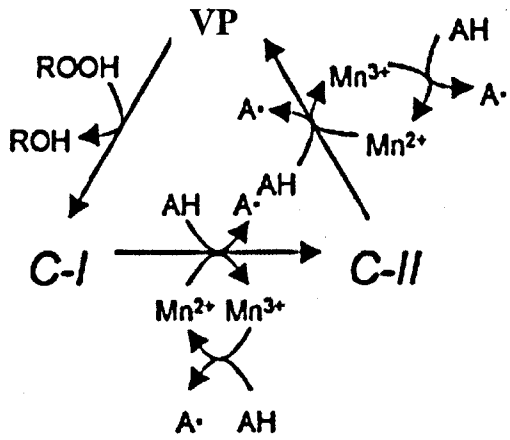


Figure 2.18 Schematic representation of versatile peroxidase (VP) catalyzed redox cycles for Mn^{2+} having the properties of both LiP and MnP (taken from Camarero *et al.*, 1999)

In summary, heme-containing enzymes (LiP, MnP and VP) have several disadvantages that hold back its use in this study. LiP, MnP and VP require H_2O_2 for the catalytic cycle, whereas laccase only uses O_2 that can be absorbed directly from the atmosphere. In comparison, laccase are available in the market at a lower price if compared to the extremely expensive LiP and MnP, and VP is not yet commercially available as shown in Table 2.1. In contrast to heme-containing peroxidase, laccase offers a variety of mediators/substrates that can be chosen according to a particular design of the process

and also the market price of the mediator. Thus, laccase is a promising enzyme for lignin degradation with a great amount of potential applications that could improve productivity and efficiency without high investment cost.

Table 2.1 Comparison of reactions and prices of linginolytic enzymes.

Enzyme and abbreviation	Co-factor	Price (GBP)/g*	Substrate, Mediator	Reaction
Laccase	O_2	15.1 (LAB); 22.3 (LTV)	Phenols, hydroxybenzotriazole (HBT), ABTS, syringaldehyde, TEMPO, violuric acid, *N*-hydroxyphthalimide (HPI), *etc.*	Phenols are oxidized to phenoxyl radicals; other reactions in the presence of mediators
Lignin peroxidase (LiP)	H_2O_2	5420	Veratryl alcohol	Aromatic rings oxidized to cation radical
Manganese peroxidase (MnP)	H_2O_2	4210	Mn, organic acids as chelators, thiols, unsaturated fatty acids	Mn^{2+} oxidized to Mn^{3+}; chelated Mn^{3+} oxidizes phenolic compounds to phenoxyl radicals; other reactions in the presence of additional compounds
Versatile peroxidase (VP)	H_2O_2	N/A**	Mn, veratryl alcohol, compounds similar to LiP and MnP	Mn^{2+} oxidized to Mn^{3+}, oxidation of phenolic and non-phenolic compounds, and dyes

*Prices of commercially available lignolytic enzymes from Sigma-Aldrich (www.sigmaaldrich.com)
**Versatile peroxidase (VP) is not commercially available.

2.5 Ionic Liquids

As discussed earlier, laccases have a variety of applications especially in lignocellulose processing. In some cases, the processes are inefficient because the substrate is insoluble in water. Therefore, it would be desirable to identify enzyme-friendly solvents that can be used to solubilise the substrates. Ionic liquids offers better performance in biocatalytic processes than conventional solvents (Park and Kazlauskas, 2001; 2003; Chin *et al.*, 1994). This solvent is considered as 'green' since it does not have a vapour pressure, which provides environmental advantages. Green technology is concerned with generating less waste from an industrial chemical process. By reducing waste, the opportunity arises to develop more cost effective processes and products (Lancaster,

2000). Ionic liquids are recently developed solvents that offer much greener solvent properties that can replace existing solvents that generate 'dirty' waste in the speciality chemical industry, oil refining and bulk chemicals industry (Seddon, 1997; Huddleston *et al.*, 1998).

An ionic liquid typically contains organic cations and anions, and has a low melting point at less than 150 °C and is liquid at room temperature (Rogers and Seddon, 2002). Other than that, ionic liquids are non-volatile and do not easily evaporate in the environment. Ionic liquids have thermal stability to over 350 °C (Othmer, 2009; Rogers and Seddon, 2002), making them initially useful as replacements for volatile organic compounds (VOCs) (Wasserscheid, 2006; Deetlefs *et al.*, 2006).

According to Holbrey and Seddon (1999), ionic liquids can be divided into three categories; first generation; second generation; and third generation ionic liquids (Holbrey and Seddon, 1999). The first generation ionic liquids are not stable in the presence of air and moisture and consist of cations such as *N,N'*-dialkyl-imidazolium or *N*-alkyl-pyridinium. First generation ionic liquid anions are principally based on haloaluminate (III) (*e.g* chloroaluminate (III)) (Wilkes *et al.*, 1982; Abdul-Saka *et al.*, 1993). This is because the first generation ionic liquids are more sensitive, in which the hydroxoaluminate (III) species with aluminium (III) chloride are formed when reacted with water. Therefore, the ionic liquids tend to decompose (Zawodzinski and Osteryoung, 1987). The second generation ionic liquids are usually stable in air, water and most organic chemicals, except for ionic liquids based on hexafluorophosphate (PF_6^-) and tetrafluoroborate (BF_4^-) anions. The latter anions generate HF when reacted with water, which is extremely toxic and corrosive as shown below:

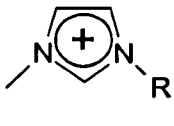
$$[PF_6]^- + 4H_2O \rightarrow 2H^+ + 6HF + [PO_4]^{3-} \quad \text{(Zawodzinski and Osteryoung, 1987)}$$

$$[BF_4]^- + 3H_2O \rightarrow 2H^+ + 4HF + [BO_3]^- \quad \text{(Koch } \textit{et al.}\text{, 1976)}$$

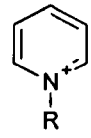
The third generation ionic liquids are also known as 'task specific' ionic liquids and are designed for specific applications by knowing the properties of the anion and cation (Rogers and Seddon, 2002) which means that their properties can be adjusted to suit the

requirements of a particular process. Up to now, very little is known about third generation ionic liquid physical properties, or the synthesis method *etc.* (Rogers and Seddon, 2002). Fig. 2.19 shows some of the commonly used anions and cations of ionic liquids. Wilkes and Zaworotko (1992) proved the concept of altering the ions to vary the properties of ionic liquids, such as the melting point, viscosity, density and hydrophobicity (Wilkes and Zaworotko, 1992; Abdul-Sada *et al.*, 1995).

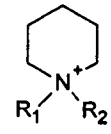
Most commonly used cations:



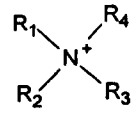
1-alkyl-3-methyl-imidazolium



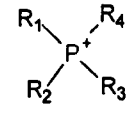
N-alkyl-pyridinium



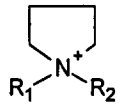
N,N-Dialkyl-piperidinium



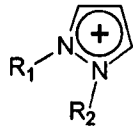
Tetraalkyl-ammonium



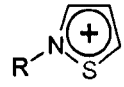
Tetraalkyl - phosphonium



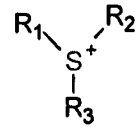
N,N-Dialkyl-pyrrolidinium



1,2-Dialkyl-pyrazolium



N-alkyl-thiazolium



Trialkyl-sulfonium

$R_{1,2,3,4} = CH_3 (CH_2)_n$, (n = 0, 1, 3, 5, 7, 9); aryl; *etc.*

Some possible anions:

Water immiscible ⟶ Water miscible

$[PF_6]^-$	$[BF_4]^-$	$[CH_3CO_2]^-$
$[NTf_2]^-$	$[OTf]^-$	$[CF_3CO_2]^-$, $[NO_3]^-$
$[BR_1R_2R_3R_4]^-$	$[N(CN)_2]^-$	Br^-, Cl^-, I^-

Figure 2.19 Some commonly used ionic liquid systems (taken from Plechkova and Seddon, 2007).

For instance, melting points of the ionic liquids can vary with the length of functional groups such as the 1-alkyl group, with liquid crystalline phases that form for alkyl chains that contain more than 12 carbon atoms. Other than that, the miscibility of ionic liquids in water can be adjusted with changes of the ion structures. For example, 1-alkyl-3-methylimidazolium tetrafluoroborate salts are miscible in water when the alkyl chain contains less than six carbon atoms. Above six carbon atoms, the miscibility of this ionic liquid in water decreases, and forms a biphasic system as a result (Holbrey and Seddon, 1999; Gordon *et al.*, 1998).

Ionic liquids are excellent solvents in many processes. In particular, numerous ionic liquids are hydrophobic and dissolve both organic and inorganic molecules, except alkanes and alkylated aromatics (Huddleston *et al.*, 1998). The ability to dissolve hydrophobic molecules in ionic liquids gives an advantage for clean synthesis. For example, the use of transition-metal catalysts which can be dissolved in ionic liquids allows the separation of the products and by-products from ionic liquids by solvent extraction (Blanchard *et al.*, 1999). Thus, ionic liquids and expensive catalysts can be re-used and recycled. It is worth noting that ionic liquids have effectively no vapour pressure and therefore cannot be lost to the atmosphere. This allows some volatile products to be separated from an ionic liquid and catalyst by distillation. Alternatively, supercritical carbon dioxide (CO_2) can also be used to separate products and by-products from an ionic liquid and catalyst (Blanchard *et al.*, 1999).

2.5.1 Ionic Liquids as Solvents for Lignin

In recent years, there has been an increasing amount of literature available on the use of ionic liquids as solvents for lignin dissolution (Moniruzzaman and Ono, 2012; Cheng *et al.*, 2012; Polaskova *et al.*, 2013; Tan *et al.*, 2009; Fort *et al.*, 2007; Kilpelainen *et al.*, 2007; Zavrel *et al.*, 2009). Recently, Moniruzzaman and Ono (2012) demonstrated the use of ionic liquids (1-ethyl-3-methylimidazolium acetate; [C_2mim][OAc]) in the delignification of wood chips from *Chamaecyparis obtusa*. In their study, the wood chips were treated with [C_2mim][OAc] for 1 hour then treated with laccase from

Trametes sp., to remove lignin. The isolated α-cellulose was increased from 46.3 to 73.1 % compared with the untreated wood chips, thus the delignification was improved. A scanning electron microscope (SEM) has shown the difference between untreated, seen in Fig. 2.20a and treated wood chips, seen in Fig. 2.20b, with $[C_2mim][OAc]$ and laccase from *Trametes sp.* The surfaces of the untreated wood chips are very rough due to the coating of lignin, whereas, those surfaces of treated wood chips are plane, indicates that the lignin has been successfully removed from the cellulose fibres.

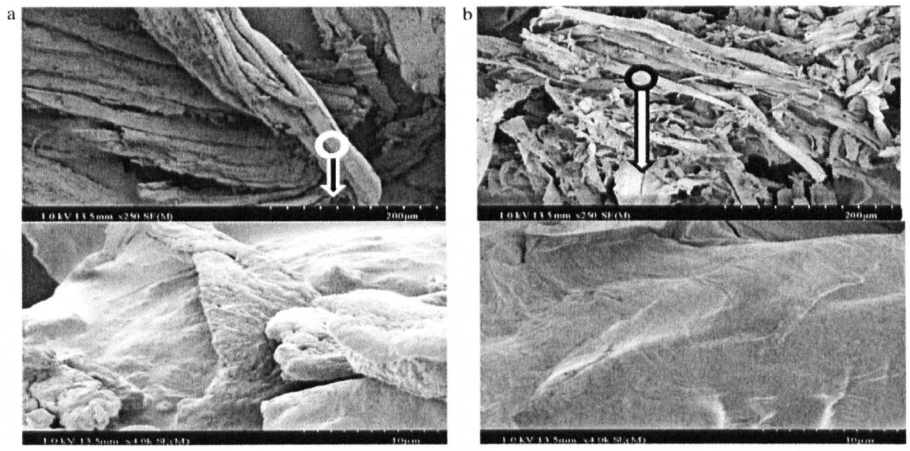


Figure 2.20 SEM images of (a) untreated wood chips and (b) after treatment of wood chips with $[C_2mim][OAc]$ and laccase from *Trametes sp.* (taken from Moniruzzaman and Ono, 2012)

The use of lignin as renewable feedstock has become increasingly important due to the fact that lignin is cheap and can be recycled from agricultural waste such as baggase, the residue from sugarcane processing (Tan *et al.*, 2009). Following this, Cheng *et al.* (2012) took an approach to study the shape of lignin subunits as an elongated shape, described well by ellipsoidal and cylindrical models, released by the treatment of three types of lignin, namely organosolv, Kraft and low sulphonate lignin, with ionic liquid, $[C_2mim][OAc]$ as illustrated in Fig. 2.21. One important finding that emerged from this study is that the sulphur content in sulphonate lignin can be reduced in the presence of this ionic liquid (Cheng *et al.*, 2012). This is an important strategy that can be used to increase the purity of the chemical products formed from the depolymerization of lignosulphonate. In addition, ionic liquid offers several advantages in the process

including the operation at atmosphere pressure, no hazardous waste is generated and the ionic liquid has the ability to be recycled (Tan *et al.*, 2009; Fort *et al.*, 2007). However, it has to be noted that the study described above is based on chemical processes that have inherent drawbacks from a commercial and environmental point of view, as discussed earlier. Table 2.2 shows a summary of publications on the dissolution of lignin by ionic liquids and the operating conditions.

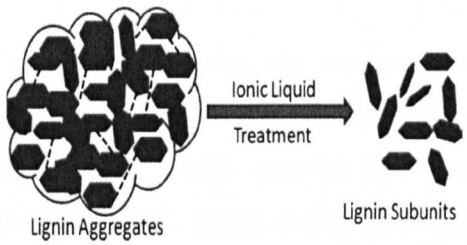


Figure 2.21 The depolymerization of lignin to smaller lignin subunits prior to the treatment with ionic liquid (taken from Cheng *et al.*, 2012).

Table 2.2 Summary of publications of ionic liquids as solvent for lignin dissolution

Research paper	Ionic liquid used	Condition (temperature)	Lignin/wood dissolved	Reaction with water
Pu *et al.* (2007)	$[C_1mim][C_1SO_4]$ $[C_4mim][C_1SO_4]$	50 °C	20 wt%	Not mentioned
Zavrel *et al.* (2009)	[amim][Cl] $[C_2mim][Ac]$	90 °C	5 wt%	Ionic liquids are unstable in water
Kilpelainen *et al.* (2007)	$[C_4mim]$ [Cl]	130 °C	8 wt%	Water was found to significantly reduce the solubility of wood in ionic liquids
Fort *et al.* (2006)	$[C_4mim]$ [Cl]	100 °C	2 wt%	Not mentioned
Cheng et al. (2012)	$[C_2mim][OAc]$	120 °C	45 - 70 wt%	Ionic liquid is hydrophilic, however lignin/ionic liquid mixture are less soluble
Moniruzzaman and Ono (2012)	$[C_2mim][OAc]$	80 °C	7 wt % Improved to 50.1 % after treatment with laccase from *Trametes sp.*	Ionic liquid is hydrophilic

* Note: 1-allyl-3-methylimidazolium [amim]

2.5.2 Ionic Liquids as Solvents for Laccase

The amount of publications regarding ionic liquid as a solvent for the process involving laccase as a bio-catalyst have significantly increased due to the fact that both the ionic liquid and laccase have their own unique abilities and are claimed to be "green" to the environment (Seddon, 1997; Lancaster, 2000; Huddleston *et al.*, 1998; Blanchard *et al.*, 1999). Several studies have attempted to explain the activity and stability of proteins in ionic liquids (Diego *et al.*, 2005; Fujita *et al.*, 2006; Lau *et al.*, 2004; Lozano *et al.*, 2001; Park and Kazlauskas, 2003) and also the performance of ionic liquids as co-solvents for the catalytic activity of an enzyme (Baumann *et al.*, 2005; Kragl *et al.*, 2002) such as lipase (Barahona *et al.*, 2006), epoxide hydrolase (Chiapple *et al.*, 2007), D-amino acid oxidase (Lutz-Wahl *et al.*, 2006) and horseradish peroxidase (Sgalla *et al.*, 2007).

In 2008, Tavares *et al.* and Shipovskov *et al.* focused on the use of ionic liquids as a solvent for laccase-catalyzed reactions (Shipovskov *et al.*, 2008; Tavares *et al.*, 2008). Tavares *et al.* (2008) used three different water soluble ionic liquids (1-ethyl-3-methylimidazolium 2-(2-methoxyethoxy) ethylsulphate; [C_2mim] [$MDEGSO_4$], 1-ethyl-3-methylimidazolium ethylsulphate; [C_2mim] [C_2SO_4], and 1-ethyl-3-methylimidazolium methanesulphonate; [C_2mim] [C_1SO_4]) for the oxidation of ABTS in the presence of commercial laccase as a catalyst (Tavares *et al.*, 2008). Among these ionic liquids, [C_2mim] [$MDEGSO_4$] was the most promising ionic liquid to support laccase activity. The most important finding was that the activity of laccase was decreased when the concentration of ionic liquid increased. The highest laccase activity was obtained at 10 % of the ionic liquid in the assay (Tavares *et al.*, 2008). On the other hand, Shipovskov *et al.* (2008) used different types of laccase from *Agaricus bisporus* (LAB) and *Trametes versicolor* (LTV) for the oxidation of catechol in the presence of three different ionic liquids, 1-butyl-3-methylimidazolium bromide ([C_4mim] [Br]); 1-butyl-3-methylimidazolium dicyanamide ([C_4mim] [$N(CN)_2$]); and 1-butyl-3-methylimidazolium tetrafluroborate ([C_4mim] [BF_4]). From this study, [C_4mim] [Br] and [C_4mim] [$N(CN)_2$] stimulated the activity of both LAB and LTV. However the activity

of these enzymes was inhibited at higher and lower concentrations of ionic liquid. The activity increased with the concentration of ionic liquid between 10 - 20 % and 50 - 60 % (v/v) in water (Shipovskov *et al.*, 2008).

Following this, however, the limitation lies in the fact that many ionic liquids cause the deactivation of enzymes. Most of the research on the activity of laccase in ionic liquids does not cover all of the ionic liquids available because there are millions of ionic liquids known. Therefore, the screening of enzyme-friendly ionic liquids is necessary to obtain a wide selection of this 'green' solvent. Recently, Rehmann *et al.* (2012) screened 63 ionic liquids for their compatibility with LTV. By taking the study by Rehmann *et al.* (2012) as a benchmark, further screening was conducted in this current project. 106 ionic liquids were screened to determine the best ionic liquids that can stimulate the activity of LAB for the oxidation of ABTS as a substrate. Future attempts should then focus on the discovery of the effect of laccase in the presence of ionic liquids for the depolymerization of lignin, which can offer great opportunities due to the fact that both laccase and ionic liquids are claimed to be good stimulators of lignin depolymerization.

Chapter 3

MATERIALS & METHODS

3.1 Materials

Laccase from *Trametes versicolor* (LTV), laccase from *Agaricus bisporus* (LAB) and all chemicals and solvents used in this study were obtained from Sigma Aldrich (UK) and Fisher Scientific (UK) Ltd. Ionic liquids were synthesized and supplied by QUILL (Queen's University Ionic Liquid Laboratories).

3.1.1 Buffer Preparation

For the preparation of the 0.1 M ammonium acetate buffer solution, 3.85 g ammonium acetate was dissolved in 500 ml of distilled water. The solution in beaker was then stirred using a magnetic stirrer and the pH was adjusted to 4.5 by added droplets of concentrated acetic acid as necessary. The pH of this mixture was measured using a pH meter.

The routine preparation of 1M stock of sodium citrate buffer solution pH 6.0, dipotassium hydrogen orthophosphate anhydrous (174.18 g) and tri-sodium citrate (294.10 g) were dissolved in water (500 ml). Citric acid (0.5 M) was then added to the mixture to give pH 6. The mixture was then poured into 1 L volumetric flask and distilled water was then added to the mixtures to give 1 L (1 M) sodium phosphate citrate buffer solution. In order to get 25 mM (500 ml) buffer solution, 12.5 ml of 1 M sodium phosphate citrate buffer solution was used and the pH was rechecked. The solution was then stored in a glass bottle at room temperature for future use.

3.1.2 Laccase

Laccase (0.01 g) was added to water (10 ml) to make 1 mg/ml LTV solution. This solution was then pipetted into tubes such that each tube contained 500 µl LTV to produced 20 tubes of stock. These tubes were stored in a freezer below -18 °C for future use. Each tube was diluted four times to 0.25 mg/ml for the routine experiments, used only once and then discarded after use. The same procedure was applied for the preparation of 1 mg/ml LAB stock solution.

3.1.3 Lignin

Sodium lignosulphonate was supplied by Borregard LignoTech and was soluble in water that can be easily dissolved for further reaction.

3.2 Laccase Activity

3.2.1 Laccase from *Trametes versicolor* (LTV)

LTV activity was determined spectrophotometrically by the oxidation of 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) at 420 nm in buffered solution (pH 4.5; 0.1 M in ammonium acetate buffer). The concentration of the reagents was: [ABTS], 4 mM; with [LTV], 0.25 mg/ml. The oxidation was measured for 30 min at room temperature. For the effect of ABTS concentration, the activity was determined over a range of substrate concentrations varying from 2 to 10 mM at 2mM intervals. The same procedure was applied for the temperature effect on LTV activity with a constant amount of reagents, [ABTS], 4mM. The temperature was maintained by using a temperature controlled water-bath (Grant Instruments (Cambridge) Ltd.) with the rotary tube (Gilson Minipuls 3) attached to spectrophotometer. The absorbance at different reaction temperature varying from 30 to 80 °C at 10 °C intervals was recorded for further analysis.

For the effect of temperature on LTV stability, the activity was determined by incubating the LTV solution (0.25 mg/ml) in an ammonium acetate buffer (pH 4.5;

0.1 M) at 30, 40, 50 and 60 °C by shaking the mixture at 200 rpm (Sartorius Certomat BS-1). For each reaction temperature, the LTV solution was incubated for 1 to 8 h at 1 h intervals for 24 h. After each hour, 900 µl of LTV/buffer solution was poured into a 1 ml cuvette, the reaction started by the addition of ABTS (4 mM, 100 µl) and the absorbance was determined spectrophotometrically at 420 nm. The absorbance changes were monitored for 30 min for each sample.

3.2.2 Laccase from *Agaricus bisporus* (LAB)

The experiment was performed spectrophotometrically by measuring the LAB activity during the course of the reaction at 420 nm. LAB was assayed with a sodium citrate buffer (pH 6, 25 mM, 900 µl) and LAB (5 µl, 0.25 mg/ml). The reaction was started with the addition of 4 mM ABTS solution (100 µl). For the optimization of the ABTS concentration, LAB was assayed at different ABTS concentrations varying from 2 to 10 mM with a fixed amount of sodium citrate buffer (pH 6, 25 mM, 900 µl) and LAB (5 µl, 0.25 mg/ml). The reaction without ABTS was run as a control.

The activity was determined over a range of substrate concentrations (2 to 10 mM) as the rate of reaction (V) will tend towards a maximum value as the substrate concentration ([S]) increases as in Eq. 1. The kinetic parameters of the ABTS oxidation were directly determined from the reaction progress curves using the Michaelis-Menten equation (Blanch and Clark, 1995):

$$V = \frac{V_{max}[S]}{K_m + [S]} \qquad \text{Eq. 1}$$

For routine determinations of the LAB activity, three replicate data sets were used and errors were calculated as a standard deviation.

3.3 Mediated Oxidation with Laccase

A routine experiment was carried out using sodium lignosulphonate depolymerized by LTV and mediated by ABTS. 1.7 g sodium lignosulphonate was dissolved in 23 ml ammonium acetate buffer (0.1 M, pH 4.5). The ABTS solution was prepared by

dissolving 0.02 g ABTS to 11 ml of ammonium acetate buffer (0.1 M, pH 4.5). The ABTS solution was then added to the lignin solution (23 ml) to give a final lignin concentration of 50 g/L and ABTS concentration of 5 mM. The reaction was started by the addition of LTV (0.25 mg/ml, 250 µl) and was shaken at 200 rpm by using Sartorrius Certomat BS-1 for 6 h at 60 °C in a 250 ml Erlenmeyer flask. The effect of lower temperature on the lignin depolymerization by LTV was determined by incubating the assay at 30 °C for 6 h. The reaction mixture was incubated in triplicate.

After 6 h, the reaction mixture was left to cool to room temperature and the fractionation method was applied. Various fractionation methods have been used to assess complex phenolic substances such as tea (Roberts *et al.*, 1957, Roberts and Williams, 1958), highbush blueberries (Kader *et al.*, 1996) and grapes (Jaworski and Lee, 1987). Among these methods, Roberts *et al.* (1957) established a method for mass balance determination. According to that particular study, the application of a fractionation process to the complex mixture of phenolic substances is to separate mixtures of chemical compounds based on the relative solubility of these substances in ethyl acetate and water (Roberts *et al.*, 1957, Roberts and Williams, 1958). Based on the study by Vigneault *et al.* (2007), ethyl acetate and diethyl ether show the best performance for monomer extraction among the five solvents studied in the acidified aqueous phase. However, diethyl ether generates unstable peroxides (Vigneault *et al.*, 2007). In view of these two earlier studies, ethyl acetate was used as the extraction solvent in this current study.

Following this, the sample was acidified with concentrated H_2SO_4 (100 µl). The mixture was then centrifuged by using an Eppendorf centrifuge 5810R at 10 000 rpm for 15 min. After separation, the aqueous fraction was poured into a 100 ml separating funnel and then extracted with 40 ml of ethyl acetate. The extraction process was repeated three times producing 120 ml aqueous ethyl acetate extract. Both fractions were evaporated to dryness by a Buchi Switzerland Rotavapor R-210 rotary evaporator at 60 °C leaving a light brown residue for the ethyl acetate fraction and a dark brown residue for the aqueous fraction. The remaining solid fraction was washed with ethyl acetate (40 ml) and then filtered to give a colourless solid ethyl acetate extract and a black solid residue. Both fractions were evaporated to dryness in

a fume hood for 30 min. Once dried, the dry weight of each fraction was accurately weighed to four decimal places for mass balance and quantification analysis. The scheme for the fractionation method applied for lignin depolymerization products is represented by Fig. 3.1. Dry samples were then kept for analysis by gas chromatography mass spectroscopy (GCMS), high performance liquid chromatography (HPLC), nuclear magnetic resonance (NMR), gel permeation chromatography (GPC) and elementary analysis (EA).

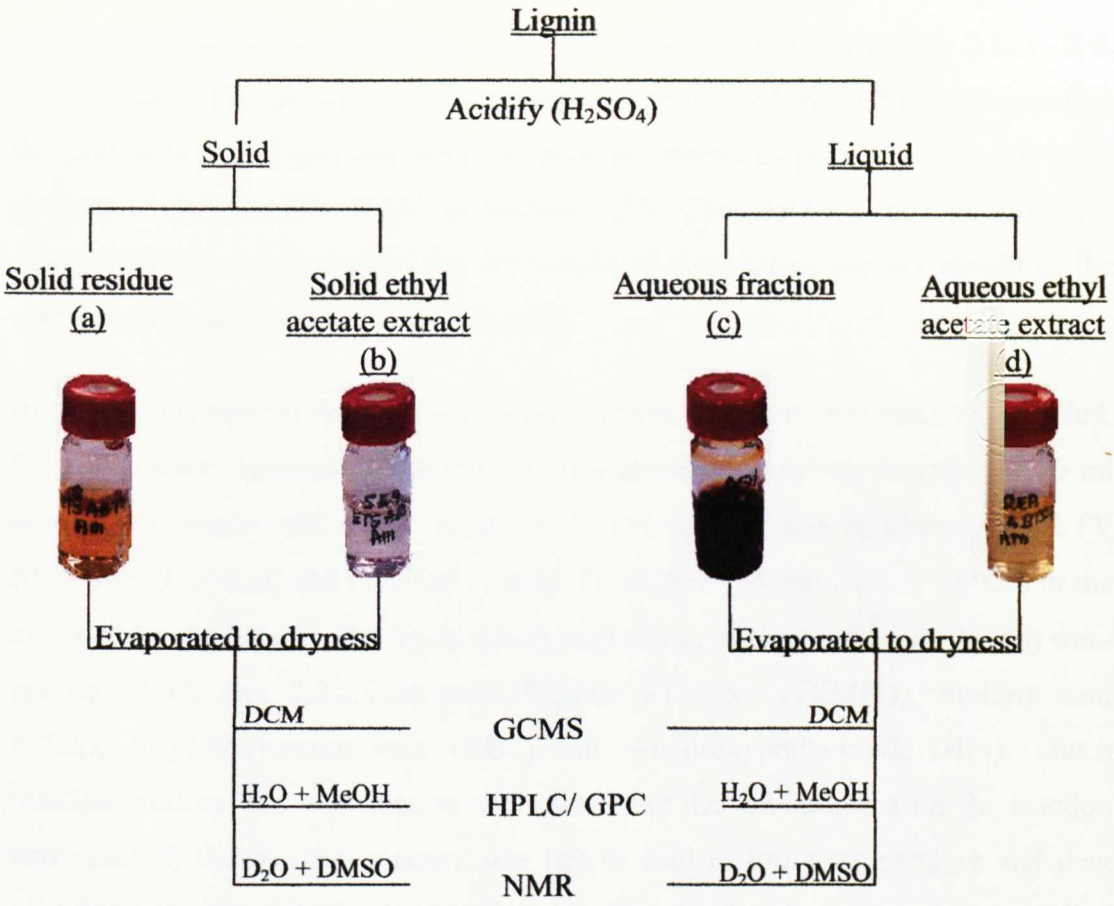


Figure 3.1 Scheme for the fractionation method of lignin depolymerization products for analytical investigation: Figure shows the colour intensity of the lignin depolymerization products after fractionation. (a) Solid residue; (b) solid ethyl acetate extract; (c) aqueous fraction; and (d) aqueous ethyl acetate extract. DCM (dichloromethane); H_2O (water); MeOH (methanol); DMSO (dimethyl sulphoxide); D_2O (deuterium oxide).

The effect of LTV and mediators on sodium lignosulphonate was compared with the control experiments by: (1) reacted sodium lignosulphonate (50 g/L final concentration) in an ammonium acetate buffer (0.1 M, pH 4.5) with LTV (0.25 mg/ml) and without ABTS, (2) sodium lignosulphonate in a buffer without LTV and ABTS, (3) reacted sodium lignosulphonate in a buffer with ABTS without LTV. Fractionation was applied for each of the control experiments and the reaction was performed in triplicate for data accuracy.

Product optimization at 30 °C was achieved by incubating the assay for 0.5, 1, 2, 4, 6, 8 and 24 h. The product concentrations were quantified by GCMS by comparing the peak area of the products with the authentic standards peak. The quantification analysis is discussed in detail in Section 3.5.5. The yield of each fraction was represented as a percentage of the dry weight of the fraction per dry weight of the starting materials (sodium lignosulphonate).

In order to understand the laccase mediator system, the same procedure was applied. 1.7 g of sodium lignosulphonate (50 g/L final concentration) was dissolved in 23 ml ammonium acetate buffer (0.1 M, pH 4.5). The mixture was incubated with LTV (0.25 mg/ml, 250 µl) and mediator (5 mM, 11 ml) for 2, 6 and 24 h at 30 °C. For the effect of the mediator on the lignin depolymerization, mediators (beside ABTS) were choose which are; 2,2,6,6-tetramethylpiperidin-1-yloxy (TEMPO), violuric acid (VLA), 1-hydroxybenzotriazole (HBT) and *N*-hydroxyphthalimide (HPI). Each reaction mixture was incubated in triplicate. After the time allotted for the reaction was reached, the reaction mixture was left to cool to room temperature and then acidified with concentrated H_2SO_4 (100 µl). The fractionation method was applied for each sample as described previously.

The effect of laccase from *Agaricus bisporus* (LAB) on lignin depolymerization was investigated. This experiment was performed by incubating 1.7 g sodium lignosulphonate (50 g/L final concentration) in a sodium citrate buffer (pH 6, 25 mM, 23 ml) with LAB (0.25 mg/ml, 250µl) and ABTS (5 mM, 11 ml) for 1, 3, 4, 6, 8 and 24 h at 30 °C. The reaction mixture was shaken at 200 rpm and was incubated in triplicate. The fractionation method was also applied to each sample.

3.4 Lignin Derived Compounds as a Substrate

In an attempt to understand the LTV behaviour towards lignin depolymerization products, five compounds were incubated in separate Erlenmeyer flask with LTV (0.25 mg/ml, 250 µl) and ABTS (5 mM, 11 ml) as listed: vanillin, acetovanillone, guaiacol, vanillic acid and homovanillyl alcohol. An amount of 800 mg of compound was dissolved in 23 ml ammonium acetate buffer (0.1 M, pH 4.5, 15 ml). The mixture was then poured into a 250 ml Erlenmeyer flask and shaken at 200 rpm for 2, 6 and 24 h at 30 °C. After each period of time was reached, the products mixture was acidified with concentrated H_2SO_4 (44 ml). In order to precipitate the solid residue, the mixture was centrifuged at 10 000 rpm for 15 min. After separation, the mixture was extracted with ethyl acetate and the fractionation method was applied for each sample as described previously. Each sample was analyzed further by GCMS and NMR for identification of the products and GCMS for quantitative analysis.

3.5 Analysis Strategy

In order to assess the products formed after the enzymatic treatment of sodium lignosulphonate by laccase, an analytical strategy was developed as summarized in Fig. 3.3. GPC was chosen as the first analytical approach to screen the distribution of product molecular weight. Following the screening by GPC, a fingerprint analysis was employed by using ^{1}H-NMR to identify the modification in the chemical composition before and after the enzymatic treatment. In an attempt to study the effect of different lignin, LAB and ABTS concentrations, ^{1}H-NMR can provide the occurrence and distribution of various types of functional groups in the lignin depolymerization samples. In addition, the distribution of functional groups of interest could be identified by using this technique. Next, the indication of element changes in the sample before and after the enzymatic treatment was confirmed by using EA especially with an interest to identify the occurrence of sulphur in the sample. The results from the screening by GPC and fingerprinting analysis by ^{1}H-NMR contributed to the identification and quantification of the low molecular weight products by HPLC and GCMS. GCMS has played an important role in the

identification and quantification of the products as this technique was found to be more suitable than HPLC as discussed later in Chapter 5 (Section 5.5).

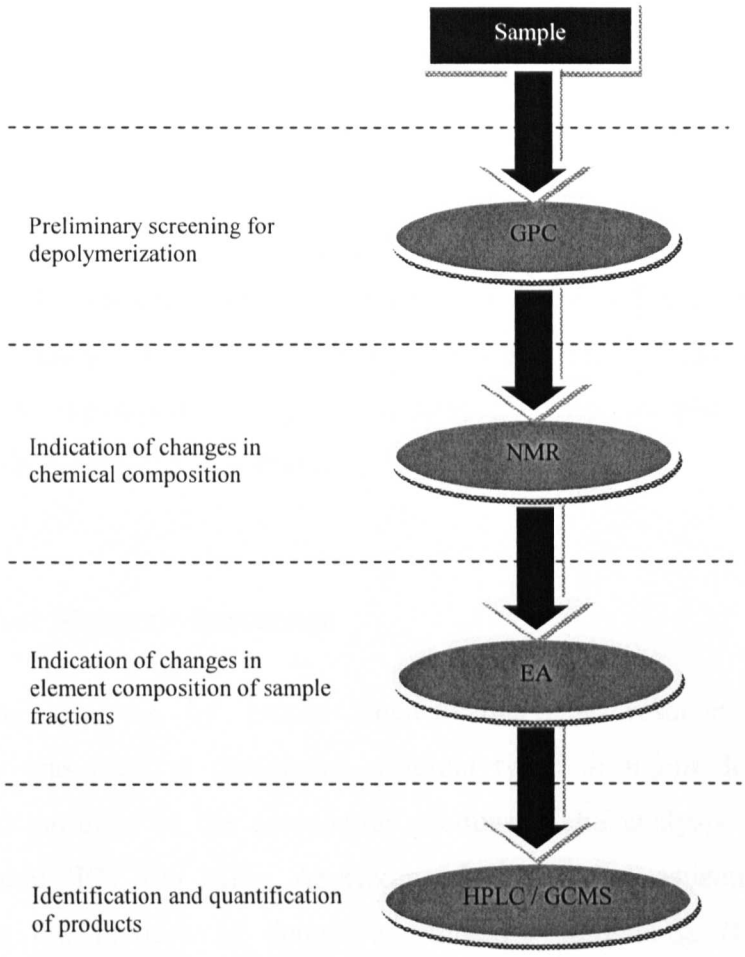


Figure 3.2 Summary of the analysis strategy. **GPC:** gel permeation chromatography; **NMR:** nuclear magnetic resonance; **EA:** elemental analysis; **HPLC:** high performance liquid chromatography; **GCMS:** gas chromatography mass spectrometry

3.5.1 Gel Permeation Chromatography

The gel permeation chromatography (GPC) system is well suited for the reliable determination of the molecular weight of various chemical mixtures produced after lignin depolymerization. Additionally, the molecular weight distribution of products was determined in order to verify the effect of mediators on lignin depolymerization and as a preliminary screening of products formation.

The analysis was performed using an Agilent 1200 series GPC-SEC system consisting of a vacuum degasser, autosampler, pump, column oven, UV-detector, refractive index (RI) detector and Agilent ChemStation software. The system was equipped with a Jordi gel sulphonated DVB 10^Å 250 x 10 mm column. Methanol: water (1:9) with the addition of sodium hydroxide (NaOH) to pH 12 was used as an eluent with a GPC column set base with a flow rate of 1 ml/min at 37 °C. The detection was performed at 280 nm with a refractive index (RI) detector. The same eluent was used to dissolve the dry samples after the reaction with a 1 mg sample per 1 ml eluent. The samples were filtered with a 0.2 µm cellulose membrane filter before further analysis by GPC. An amount of 5µl of sample was injected into the column and the separation took 20 min to complete. Once completed, the data was analyzed by the ChemStation software.

3.5.2 Nuclear Magnetic Resonance

Fingerprinting analysis by proton nuclear magnetic resonance (^{1}H NMR) spectroscopy was used to distinguish different types of lignin depolymerization products and varieties of the compounds produced. The analysis was performed using a Bruker (III) 400 MHz. Approximately 10 mg of aqueous fraction was dissolved in the mixture of deuterated dimethyl sulphoxide (DMSO-d_6) and deuterium oxide (D_2O) in the ratio of 8:2 (800µl). The sample was then poured into a NMR tube (Wilmad 507PP) for further analysis.

3.5.3 Elemental Analysis

Carbon, hydrogen, sulphur and nitrogen contents were determined using a Thermo Flash EA1112 Elemental Analyzer coupled with a MAS 200R autosampler and controlled by Eager Xperience software. The sample was prepared by using sulphanilamide as a standard. In order to determine the sulphur content, approximately 5 mg vanadium pentoxide was used for each sample preparation. Vanadium pentoxide was weighed in a tin capsule followed by the addition of 2.5 mg sulphanilamide for the bypass sample. The blank sample just contained vanadium pentoxide. The tin capsule was removed from the balance and was placed gently in

the capsule holder. Because of the small size of the tin capsule, it was held with tweezers. The tin capsule was then folded gently using the tweezers expelling as much air as possible and to ensure that no sample was lost. The samples were prepared as summarized in Table 3.1.

Table 3.1 Sample preparation for elementary analysis and the purpose of each sample.

Name	Purpose	Capsule preparation
Bypass	Condition the instrument ready for analysis	Vanadium pentoxide and sulphanilamide
Blank	Provides baseline signal to be subtracted from sample signals	Vanidium pentoxide only
Standard	Provides calibration data for sample calculation	Vanadium pentoxide and sulphanilamide
Sample	Sample for elemental determination	Vanadium pentoxide and sample
Standard check	Quality control measure	Vanadium pentoxide and sulphanilamide

It is important to note that the weight of standards and samples have to be measured accurately for the precise elements calculation except for the bypass. The sample was then transferred to the autosampler. Before starting the analysis, the flow of oxygen was checked and the inlet pressure was approximately 4 bars.

3.5.4 High Performance Liquid Chromatography with UV detector

A high performance liquid chromatography (Agilent 1220 Infinity LC system) with an integrated data system, a column oven compartment and an autosampler was applied for the preliminary screening of fractions and for the purpose of quantification analysis. The detection of the compounds was carried out with a variable-wavelength UV-detector at 280 nm with a Waters XTerra RP18 (5.0 μm 3.0 mm x 250 mm) column. The separation was performed using isocratic elution with water (pH 4): acetonitrile (9:1). The column temperature was maintained at 37 °C and the flow rate at 0.7 ml/min. The sample was prepared by using the same eluant as for the GPC sample preparation with methanol: water (1:9) at pH 12 dissolving the dry lignin depolymerization product sample. Each sample took 30 min for a maximum elution time.

Quantification of the lignin depolymerization products was carried out by using authentic standards. The full standard calibration curve was applied with a five point concentration, ranging between 2 to 10 mM with 2 mM intervals, and each in triplicate. Thirteen standards were calibrated as listed: vanillin, vanillic acid, acetovanillone, isovanillic acid, tyrosol, homovanillyl alcohol, guaiacol, syringaldehyde, 4-hydroxybenzaldehyde, catechol, isovanillin and syringic acid. Points were plotted based on the peak area of each standard resulting in a peak area versus concentration (mM). The standard calibration curves passed through the origin, and showed good agreement among the triplicates and linearity in the concentration ranges studied. The products were then identified based on the retention time of the standards and the concentration of the products was determined by comparing the peak area with the standard calibration curve.

3.5.5 Gas Chromatography Mass Spectroscopy

Product identification was analyzed by an Agilent Technologies 7890A gas chromatography (GC) system with MS-5975C triple-axis detector mass spectroscopy (MS) and an integrated autosampler (model number 7693). The column used was a HP-5MS (Agilent technologies) with 30 m x 0.25 mm (internal diameter) non polar bonded phase capillary column with a phase thickness of 0.25 μm. The carrier gas was high purity helium at 0.55 ml/min (30 cm/s) with a split ratio of 65:1. The temperature program started at 70 °C for 2 min and then increased to 230 °C at a rate of 7 °C/min. The column was held at this temperature for 5 min. The injector and the GCMS detector were kept at 250 and 280 °C, respectively. The solution containing the sample was injected (1μl) from the autosampler. Fragmentation was achieved by electron ionization (EI) (positive ion ionization) at 70 eV, the source temperature was 180 °C, the interface temperature was 240 °C and the mass resolution was 300 units.

For the sample preparation, after the reaction, the sample was further fractionated following the method described previously. In order to concentrate the sample, the ethyl acetate extract for both the aqueous and solid phase were evaporated to dryness and dissolved in dichloromethane (DCM). Before being injected, the sample was filtered with a special filter design for organic solvents (Sartorius filter Minisart SRP, Scientific Laboratory Supplies) with a 0.2 μm pore size. On the other hand, the

aqueous fraction and solid residue were screened for different solvents since these fractions were not fully dissolved in the DCM. This experiment was conducted by dissolving 10 mg of each fraction in 1 ml of organic solvent as listed: ethanol, acetone, tetrahydrofuran (THF) and water.

In the work described above, the sample was also derivatized to increase the volatility of the compounds that could otherwise not be detected. After the reaction, the sample was fractionated and each fraction was evaporated to dryness by a rotary evaporator. Once dried, 5 mg of sample was dissolved in 1 ml acetonitrile (ACN), 10 µl trimethylchlorosilane (TMCS) and 600 µl bistrimethylsilyltrifluroacetamide (BSTFA). The reaction was performed in a fume hood since the solvents used were highly volatile and flammable. The reaction vessel was securely closed and heated at 70 °C in a water-bath (Grant Instruments (Cambridge) Ltd.) for 1 h. After this time, 1 ml of sample was filtered (Sartorius filter Minisart SRP, Scientific Laboratory Supplies; 0.2 µm pore size) for GCMS identification.

The product concentrations were quantified by GCMS by comparing the peak area of the products with the authentic standards peak. The identification of the products was confirmed *via* the retention times of the authentic standards and the NIST library. The sample peak areas are proportional to the amount of the compound in the sample. Therefore, the peak areas were used with the calibration curves (Appendix A.3.2) (Page 223 – 224) to quantify the amount of the compound in the sample. Calibration curves were generated by analyze the standard with the concentration varies from 2 to 10 mM.

3.6 Ionic Liquids as Potential Solvents for Lignin Depolymerization

3.6.1 Ionic Liquids

The ionic liquids used in this study are listed according to structural groups and are categorized into six groups which are:

1) Imidazolium based ionic liquids
2) Pyridinium based ionic liquids
3) Quaternary ammonium based ionic liquids

4) Phosphonium based ionic liquids
5) Piperidinium based ionic liquids and
6) Pyrrolidinium based ionic liquids.

In all, 106 ionic liquids were tested in this study. Each ionic liquid had different chemical and physical properties based on their structural groups, anions and cations as explained in Chapter 2.

3.6.2 Assays for Laccase from Agaricus Bisporus Activity in Ionic Liquids

Oxidation of ABTS by laccase from *Agaricus Bisporus* (LAB) was measured in 96 well quartz plates using a FLUOstar Optima Microplate Reader (BMG Labtech Ltd., UK). A quartz plate was used in this study because some ionic liquids can dissolve in disposable polyisoprene or polypropylene plates. The assay was then prepared with 300 µl total volume in each well. Each well contained 2.3 µl (0.25 mg/ml) LAB in a 260 µl sodium citrate buffer (25 mM, pH 6.0) and 3 % v/v ionic liquid (about 8.7 µl). Each assay was done in triplicate to ensure the accuracy of the data. An assay without ionic liquid was also prepared as the control for the system.

The assay was incubated for 22 min to equilibrate the mixtures before adding ABTS. Meanwhile, the mixture was shaken for 1 min before the ABTS was added in order to make sure the solution was mixed well. The reaction was started by the addition of ABTS solution (5 mM final concentration, 28 µl for each well). The oxidation of ABTS was measured at 420 nm since this was the maximum absorbance measured using a UV-visible spectrophotometer. This value has good agreement with the studies by Marjasvaara *et al.* (2008) and Branchi *et al.* (2005). Each run was completed in 6 hours 40 minutes. The data generated from the experiment was then processed to produce initial rates of reaction and Michaelis-Menten parameters, K_m and V_{max}.

3.6.3 Ionic Liquid Miscibility in Water

The water miscibility of ionic liquids used in this study is listed in Appendix A.7 (Page 247). Due to the different physical properties (viscosity and phase) of each ionic liquid, highly viscous or solid ionic liquids were weighed and dissolved in water to produce 20 % (w/v) solution. This made it easier for these ionic liquids to be pipetted accurately into the assay mixture. The water miscible ionic liquids will produce a single phase system while a biphasic system will be produced when water immiscible ionic liquids are applied. The schematic diagram of this dilution and phase system is shown in Fig. 3.4. In this study, not all of the ionic liquids were liquid at room temperature, and in these cases the solids ionic liquids were mixed with water (20 % w/v) and added to the reaction mixtures as a suspension. The room temperature ionic liquids were also mixed with water (20 % w/v) until the ionic liquid phase was saturated with water, and then the ionic liquid phase (approximately 8.7 µl) was added directly to the reaction mixture.

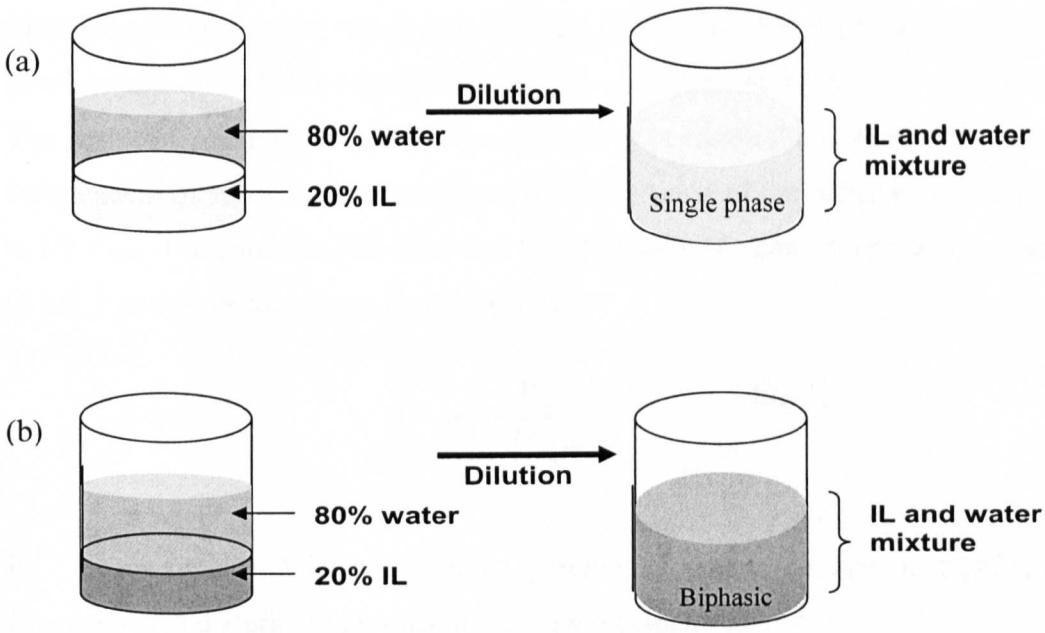


Figure 3.3 Single phase and biphasic system of ionic liquids and water mixture (a) production of single phase system by water miscible ionic liquid; (b) production of biphasic system by water immiscible ionic liquid.

3.7 Determination of Michaelis-Menten Parameters

In enzyme kinetics, the reaction rate is measured and the effect of varying the conditions of the reaction is investigated. The enzyme kinetics is represented by the Michaelis-Menten kinetic parameter (K_m and V_{max}) as well as the initial rate of reaction (v_o). These values were calculated to understand the various reaction conditions that affect the activity of laccase in the oxidation of ABTS or other substrates. In this section, step by step calculation of Michaelis-Menten parameters will be shown in detail using two different approaches.

3.7.1 Enzyme kinetics by Michaelis-Menten and Lineweaver-Burke Plot

In order to determine the Michaelis-Menten kinetic parameters (K_m and V_{max}), the initial rate of reaction (v_o) values were determined over a range of various concentration of substrates as presented in Fig. 3.4a. This figure presents a Michaelis-Menten curve which describes the relationship between the initial rates of reaction and the substrate concentration, [S]. v_o is present in the unit of mM per s. The term V_{max} is defined as the velocity (mM/s) at which the v_o eventually becomes independent of substrate concentration. K_m is the value of the substrate concentration at 1/2 V_{max}. The equation that describes the Michaelis-Menten curve was given earlier as Eq. 1 and is repeated here for convenience:

$$v_o = \frac{V_{max}.[S]}{[S]+K_m} \qquad \text{(Eq. 1)}$$

Eq. 1 presents the Michaelis-Menten parameters that were then manipulated by Lineweaver and Burke to produce the following equation:

$$\frac{1}{v_o} = \frac{1}{V_{max}} + \frac{K_m}{V_{max}.[S]} \qquad \text{(Eq. 2)}$$

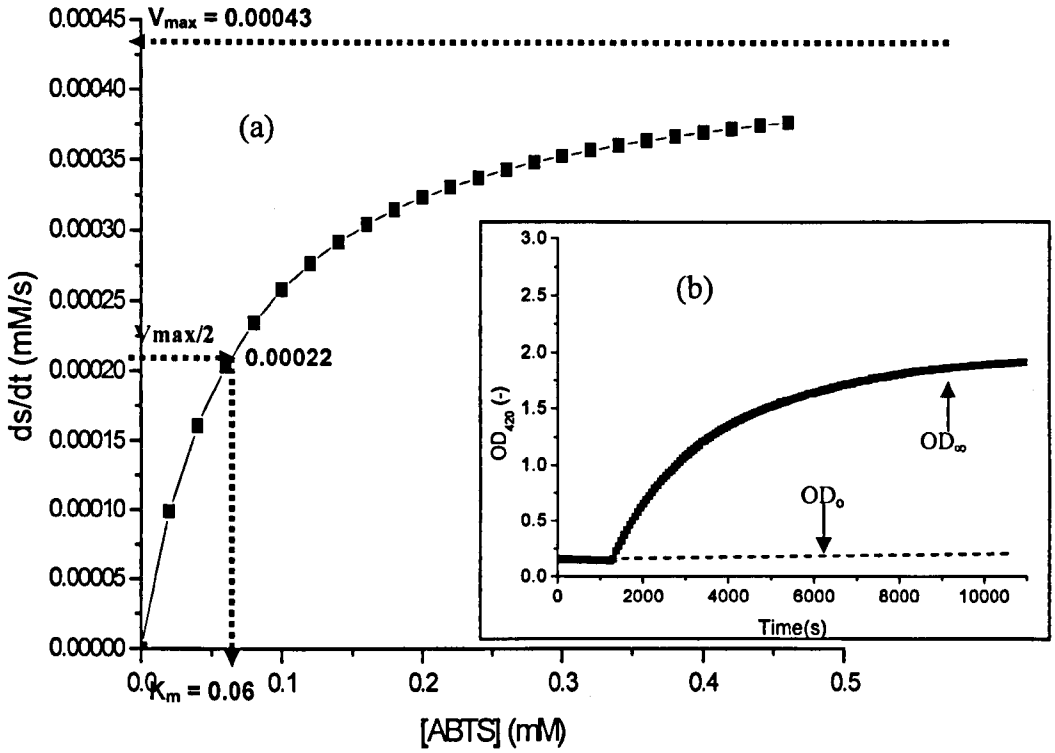


Figure 3.4 Determination of Michaelis-Menten parameters. (a) Estimation of kinetics parameters by Lineweaver plot and (b) the absorbance versus time graph for estimation of initial rate of reaction (v_o)

By plotting $1/v_o$ versus 1/[S], a Lineweaver-Burke plot is obtained as the y-intercept is $1/V_{max}$ and the x-intercept is $-1/K_m$. The slope of the linear line is the value of K_m/V_{max}. However, before the values of K_m and V_{max} can be obtained, it is necessary to calculate the value of v_o. After the analysis, the OD (optical density) data was generated spectrophotometrically and for later experiments from the microplate reader are dimensionless and present the absorbance (A) of the reaction in which is defined as:

$$A_\lambda = -\log_{10}(I/I_o) \qquad \text{(Eq. 3)}$$

Where I is the intensity of light at a specific wavelength (λ) that has passed through a sample (transmitted light intensity) and I_0 is the intensity of the light before it enters the sample or incident light intensity. All of these parameters were calculated automatically by an Agilent 8453 UV-visible spectrophotometer (Agilent

Technologies Ltd., UK) or FLUOstar Optima Microplate Reader (BMG Labtech Ltd., UK) and generated the graph of absorbance data at a specified wavelength (420 nm) versus time graph as shown in Fig. 3.4b. The term v_o was calculated by the changes of absorbance per unit time which corresponds to the slope of this graph. For most of the experiments performed, this would be ΔA_{420}/s. The absorbance value at 420 nm was converted to an actual concentration by the Beer-Lambert law:

$$A_\lambda = \varepsilon c L \qquad \text{(Eq. 4)}$$

Under these conditions, the transmittance and absorbance (A) at a certain wavelength (λ) of the sample depends on the molar concentration (c), light path length in centimetres (L) and extinction coefficient (ε) for the dissolved substance (Grünewald, 1976; Dean, 1992). The standard laboratory spectrophotometers are fitted for use with a 1 cm width sample cuvette, hence the path length is generally assumed to be equal to one (Dean, 1992) using the value of 36 mM^{-1} cm^{-1} as the molar extinction coefficient (ε) (Laufer *et al.*, 2006). Thus Eq.4 is rearranged to:

$$c = \frac{A_\lambda}{\varepsilon L} \qquad \text{(Eq. 5)}$$

The initial rate of reaction (v_o) is the concentration of the products formed (mM) per unit time, t (s), therefore:

$$v_o = \frac{c}{t} \qquad \text{(Eq. 6)}$$

$$v_o = \frac{A_\lambda}{\varepsilon L t} \qquad \text{(Eq. 7)}$$

3.7.2 Enzyme kinetics via Non-linear Regression Analysis

The analysis of Michaelis-Menten parameters by non-linear regression analysis was adopted from a recent published study by Rehmann *et al.* (2012). This method was used to minimize the number of experiments since the Lineweaver-Burke method requires numerous assays at different substrate concentrations. Therefore, the method introduced by Rehmann *et al.* (2012) is convenient in terms of minimizing the assays and experimental time. Furthermore, it can also increase the experimental

throughput. To ensure the accuracy of this method, the Michaelis-Menten parameters were compared with the Lineweaver-Burke method. The Michaelis-Menten equation (Eq. 1) was integrated and rearranged as follows (Bisswanger, 2008):

$$v = -\frac{d[S]}{dt} = \frac{V_{max}.[S]}{K_m + [S]} \quad \text{(Eq. 8)}$$

The equation was then modified to become:

$$-\frac{K_m}{[S]}.d[S] - d[A] = V.dt \quad \text{(Eq. 9)}$$

Integrating from the initial substrate concentration, $[S]_o$ to time t=0 until [S] to time t gives:

$$-K_m \int_{[S]_o}^{[S]} \frac{d[S]}{[S]} - \int_{[S]_o}^{[S]} d[S] = V_{max} \int_0^t dt \quad \text{(Eq. 10)}$$

Thus, the integrated Michaelis-Menten equation is:

$$-K_m.\ln\frac{[S]_o}{[S]} + [S]_o - [S] = V_{max}.t \quad \text{(Eq. 11)}$$

and

$$t = \frac{[S]_o - [S] - K_m.\ln\frac{[S]_o}{[S]}}{V_{max}} \quad \text{(Eq. 12)}$$

The absorbance measurement of the product (OD) data was processed to estimate the substrate concentration by using the following formula (Rehmann *et al.*, 2012):

$$[S] = [S]_o - \left[\frac{OD(t) - OD_o}{OD_\infty - OD_o}\right][S]_o \quad \text{(Eq.13)}$$

Where [*S*] is the substrate concentration, $[S]_0$ is initial substrate concentration, *OD (t)* is the OD at the given time and OD_o is the initial OD at the beginning of the reaction and OD_∞ is the OD at the end of the reaction as illustrated in Fig. 3.4b. This assumes that the substrate is converted stoichiometrically to the product absorbing at 420 nm. The term v_o was estimated using a linear regression of product concentration, [P] versus time, t in which [P] was determined by the following equation:

$$[P] = \frac{OD(t).[S]_o}{OD_\infty - OD_o} \qquad \text{(Eq. 14)}$$

In order to estimate the values of K_m and V_{max}, the graph of t versus [S] was plotted. A non-linear regression analysis was employed based on the Levenberg-Marquardt algorithm.

Chapter 4

DEVELOPMENT OF ANALYTICAL METHODS AND THEIR USE IN PRELIMINARY TESTS OF LIGNIN DEPOLYMERIZATION USING LACCASE FROM *AGARICUS BISPORUS*

4.1 Introduction

The first objective of this project is to investigate the depolymerization of lignin by using laccase from *Agaricus bisporus* (LAB) in the presence of 2,2'-azino-bis(3-ethylbenzthiazole-6-sulphonic acid) or ABTS as a laccase mediator. This task is challenging due to the complexity of the lignin polymer and the fact that laccase prefers to catalyze the polymerization of lignin-related substrates to form lignin polymers instead of depolymerization (Rogalski *et al.*, 1990; Bourbonnais *et al.*, 1995). However, depolymerization of lignin may occur in the presence of a mediator. It is preferable to use ABTS as the laccase mediator as it is known as the best mediator for the oxidation of non-phenolic lignin structures (Morozova *et al.*, 2007).

4.2 Activity of Laccase from *Agaricus bisporus* (LAB)

This study sets out to determine the rate of ABTS oxidation by LAB and to determine the Michaelis-Menten parameters. The rate of ABTS oxidation by LAB is influenced by the ABTS concentration, the operating temperature and also the pH of the buffer. To this end, a spectrophotometric assay of ABTS oxidation by LAB was performed under various reaction conditions. The reaction mixture contained sodium citrate buffer, ABTS and LAB.

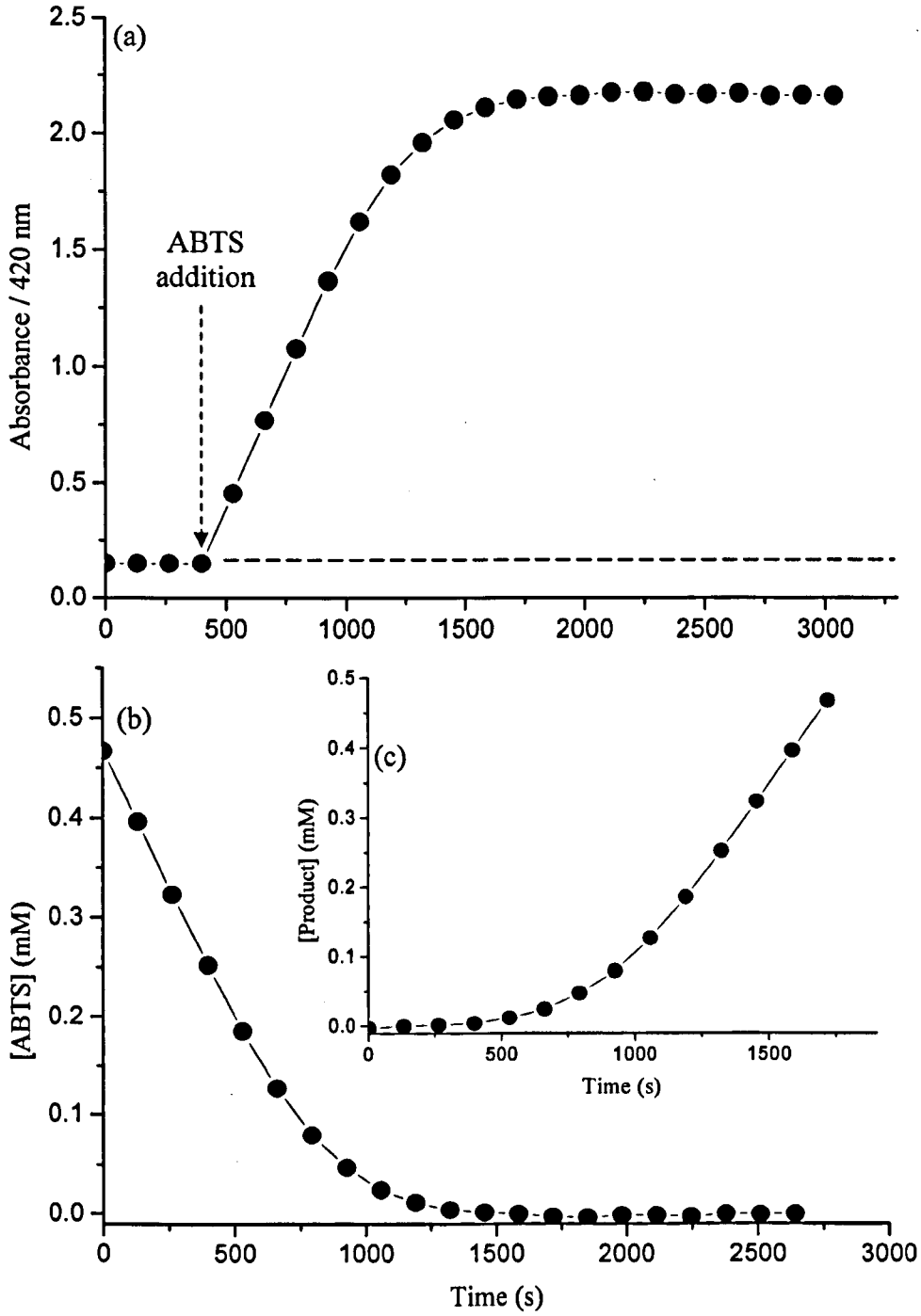


Figure 4.1 The oxidation of ABTS by LAB. The absorbance changes of the oxidized substrate at 420 nm were recorded for 1 h. The data represent the mean of three replicates with an error of less than 1%. The laccase activity was determined at 22 °C using ABTS as a substrate (0.5 mM final concentration). ABTS was added to a sodium citrate buffer (25 mM, pH 6) containing LAB (0.25 mg/ml). (a) Time course of laccase-catalyzed ABTS oxidation; (b) Non-linear regression analysis of LAB-catalyzed ABTS oxidation; (c) Product formation for the first 1500 s of the same experiment which was used to estimate the initial rate of reaction (v_o).

Absorbance values were plotted against time as shown in Fig. 4.1a. In order to minimize the number of experiments, the kinetic parameters (K_m and V_{max}) of the ABTS oxidation by LAB were estimated from a non-linear regression analysis from three replicate experiments as presented in Fig. 4.1b (Rehmann *et al.*, 2012). The reaction started immediately at a linear rate after the ABTS was added after equilibration for 400 s. As the concentration of the substrate decreased (Fig. 4.1b), it was assumed that product formation increased stoichiometrically (Fig. 4.1c). The change of product concentration during this experiment was used to estimate the initial rate of reaction (v_o) and was found to be $5.76 \times 10^{-4} \pm 6.7 \times 10^{-5}$ mMs^{-1}. A dark green precipitate at the bottom of the cuvette was observed as a result of the formation of ABTS cation radical ($ABTS^{\bullet+}$) and ABTS dication ($ABTS^{2+}$) as the products. An enzyme blank reference cuvette was used without the ABTS as a control. The value of K_m was estimated to be 0.48 ± 0.04 mM and V_{max} was found to be $7.8 \times 10^{-4} \pm 1.0 \times 10^{-4}$ mMs^{-1}.

In order to check the efficiency of the non-linear regression analysis method, the values of K_m and V_{max} were calculated by a conventional experiment, with multiple assays at different substrate concentrations varying from 0.1 to 1.1 mM (final concentration). As Fig. 4.2 shows, there was a significant increase in the rate of the reaction when the concentration of ABTS was increased due to the fact that, in an enzymatic reaction, the rate of product formation varies with the substrate concentration (Maragoni, 2003). There was no activity in the absence of the ABTS. The rate of catalysis rose rapidly as the substrate concentration increased but then it began to level off and approached a maximum rate at high substrate concentrations. This is because the active sites of the LAB molecules at a given time were virtually saturated with substrate (Cornish-Bowden, 2004) and the LAB/ABTS (enzyme/substrate) complex had to dissociate before the active sites could become free to accommodate more substrate. Provided that the substrate concentration is high and the temperature and pH are kept constant (22 °C, pH 6), the rate of reaction should be proportional to the enzyme concentration. Further analysis showed that there was no significant increase in the rate of reaction when the concentration of the substrate was increased to 1.0 mM. The initial rate of reaction at 0.5 mM ABTS (final concentration) was found to be approximately $5.81 \times 10^{-4} \pm 0.2 \times 10^{-4}$ mMs^{-1}. The kinetic parameters as determined using a Lineweaver-Burke plot were found to

be K_m= 0.41 ± 0.05 mM and V_{max} = 7.1 x 10^{-4} ± 4.0 x 10^{-5} mMs^{-1}. These values were in good agreement with the values determined by non-linear regression analysis above. Therefore, the method of Rehmann *et al.* (2012) was adopted for the calculation of kinetic parameters. However, measurements at different substrate concentrations may sometimes be needed to confirm the accuracy of the data.

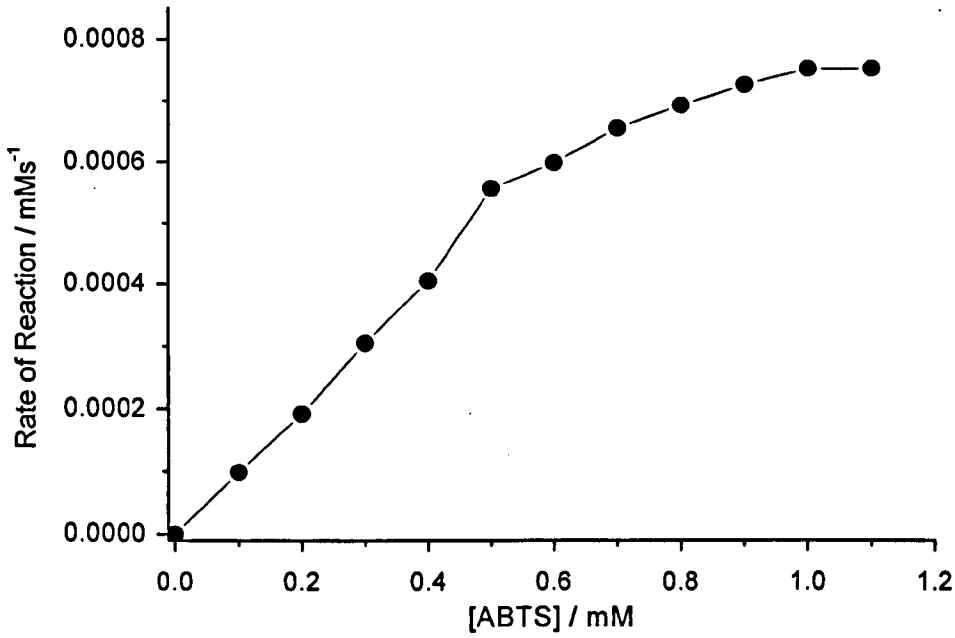


Figure 4.2 Effect of ABTS concentration on the oxidation by LAB. The laccase activity was determined at 22 °C. The ABTS concentration varied from 0 to 1.1 mM (final concentration) in a sodium citrate buffer (25 mM, pH 6) and LAB (0.25 mg/ml). The absorbance changes of the oxidized substrate at 420 nm were recorded for 1 h. The data represents the mean of three replicates with an error less than 1 %.

4.2.1 The Effect of Temperature on the Activity of LAB

Most laccases are very thermostable. Reiss *et al.*, (2011) found that the optimum temperature of laccase from *Bacillus pumilus* is between 55 – 75 °C. This study is in line with the results found for laccase from *B. subtilis* and *B. licheniformis* (Reiss *et al.*, 2011, Koschorreck *et al.*, 2008, Durão *et al.*, 2008). However, different laccases possess different optimum temperatures. For instance, the activity of laccase from *Funalia trogii* was found to be optimum at 50 °C (Patrick *et al.*, 2009). This optimum

temperature was also observed for laccase from *Polyporus sp.* (Gonçalves and Steiner, 1996), *Daedalea quercina* (Baldrian, 2004) and *Trametes hirsute* (Castillo *et al.*, 2012). In reviewing the literature, the optimum temperature for LAB has not yet been studied. Therefore, in order to assess the optimum temperature for ABTS oxidation by LAB, the activity was determined at a range of temperature (Fig. 4.3). Absorbance changes could not be detected above 90 °C because of the limitation of the temperature control on the spectrophotometer.

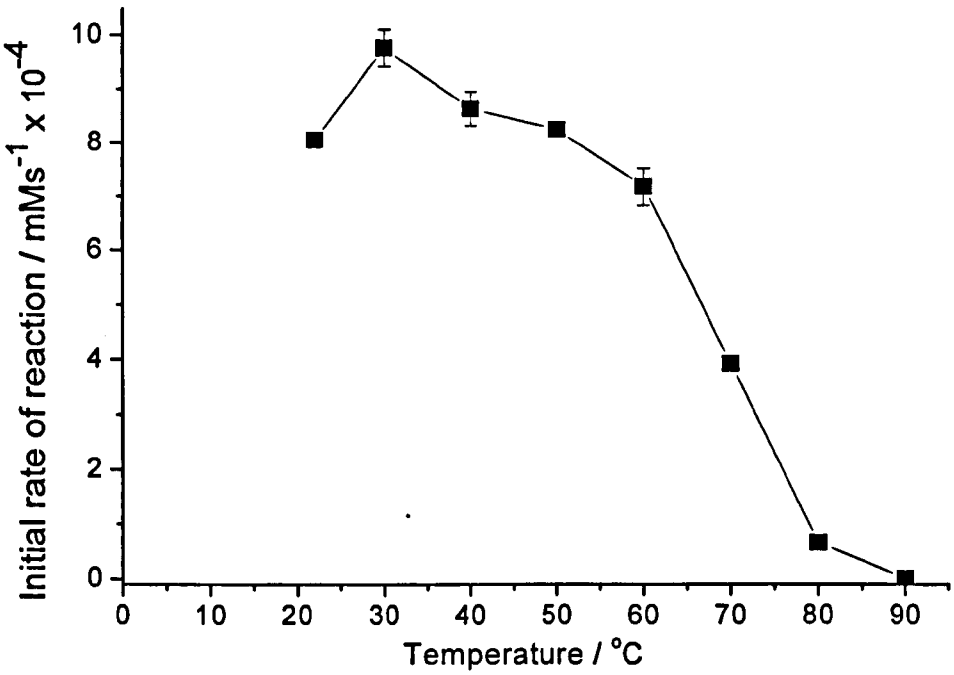


Figure 4.3 Effect of temperature on LAB activity. ABTS (5 mM) was added to start the reaction containing sodium citrate buffer (25 mM, pH 6) and LAB (0.25 mg/ml). The absorbance change at 420 nm for each temperature was recorded for 30 min. The data represent the mean of three replicates with an error of less than 1%.

LAB activity was maximal at 30 °C. However, when the temperature increased beyond 30 °C, the activity gradually decreased up to 60 °C and dramatically dropped thereafter when the temperature was further increased up to 80 °C. The LAB was completely deactivated at 90 °C. Therefore LAB was tested for depolymerization of sodium lignosulphonate at 30 °C.

4.3 LAB catalyses the Oxidation of Sodium Lignosulphonate

The three dimensional structure of lignin does not allow this polymer to be attached to the active sites of laccase. Due to this fact, a mediator is needed as an 'accelerator' to oxidize this complex polymer (Hüttermann *et al.*, 1980). Therefore, the goal of this present study was to investigate the feasibility of using ABTS as mediator for lignin depolymerization. Most of the lignins available are practically water insoluble. However, sodium lignosulphonate contains hydrophilic functional groups which make this lignin water soluble. Therefore, sodium lignosulphonate (Borregard LignoTech, mol. wt. 10,000 $gmol^{-1}$) was used to gain an understanding of the interaction between laccase and lignin. The reaction mixtures containing sodium citrate buffer, sodium lignosulphonate, ABTS and LAB were shaken for 6 h at 30 °C. To verify the effect of the enzymatic depolymerization process on the sodium lignosulphonate, the original lignin was treated using the same conditions, but without ABTS and LAB (Table 4.1d), without ABTS (Table 4.1e) and without LAB (Table 4.1f).

After the reaction, a complex mixture of depolymerization products was formed. Therefore, the products were fractionated to simplify the analysis. The reaction mixture was acidified with concentrated sulphuric acid (H_2SO_4). The mixture was then centrifuged to precipitate the solid residue, separating the soluble oxidation products (Roberts *et al.*, 1957) from the unreacted lignin and repolymerized products. After separation of the solid and liquid fractions, the solid residue was washed with ethyl acetate to extract the chemical compounds which were soluble in this solvent. The liquid fraction was extracted with ethyl acetate to produce aqueous and ethyl acetate extract fractions which were evaporated to dryness using a rotary evaporator and the products were kept for further analysis. It was found that depolymerization produced four fractions: solid residue, ethyl acetate extract of the solid residue (hereinafter referred to as solid ethyl acetate extract), aqueous fraction and ethyl acetate extract of the liquid fraction (hereinafter referred to as aqueous ethyl acetate extract). The colour intensity of each fraction is shown in Fig. 4.4.

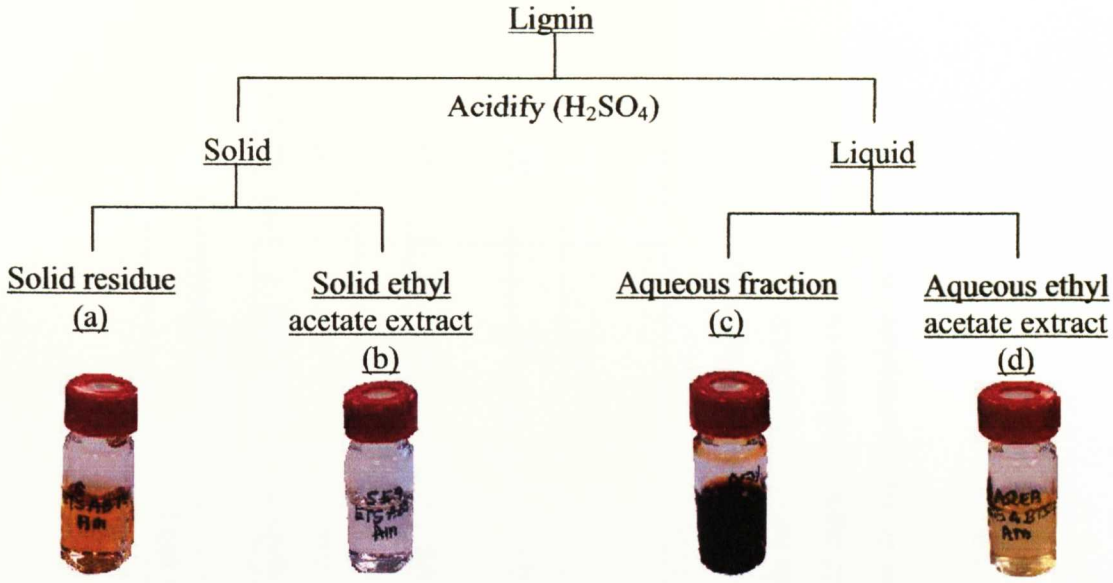


Figure 4.4 The colour intensity of lignin product fractions. (a) Solid residue (dark brown), (b) solid ethyl acetate extract (colourless), (c) aqueous fraction (black) and (d) aqueous ethyl acetate extract (light brown).

The dry weight of each fraction was accurately weighed for mass balance analysis. The yield of each fraction was represented as percentage of dry weight of the fraction (g) per dry weight of the starting material (g). Table 4.1 presents the yield (%) of the dried material in each fraction after the enzymatic depolymerization using three different concentrations of lignin. Most of the material in the controls was found in

Table 4.1 The mass balance of product fractions after conversion of sodium lignosulphonate using LAB.

Fraction	Yield (%) after drying [g product / g lignin x 100]					
	(a) 10 g/L Lignin + LAB + buffer + ABTS	(b) 30 g/L Lignin + LAB + buffer + ABTS	(c) 50 g/L Lignin + LAB + buffer + ABTS	Controls		
				(d) Lignin + buffer	(e) Lignin + LAB	(f) Lignin + ABTS
Solid residue	0	0.1	0.5	0.2	0.3	0.3
Ethyl acetate extract of solid residue	0	0.8	1.4	0.3	0.2	0.4
Aqueous fraction	96	94	90	97	98	98
Ethyl acetate extract of the aqueous fraction	2.5	3.4	7.6	1.1	1.5	1.2

The concentration of lignin was varied from (a) 10 g/L, (b) 30 g/L and (c) 50 g/L of sodium lignosulphonate (final concentration) and compared with controls containing 50 g/L of lignin (d) lignin without LAB and ABTS, (e) lignin and LAB without ABTS, (f) lignin with ABTS without LAB. The reaction mixtures included sodium lignosulphonate dissolved in sodium citrate buffer (25 mM; pH 6), ABTS (5 mM) and LAB (0.25 mg/ml) and were shaken at 200 rpm for 6 h at 30 °C. Fractionation was performed and each fraction was evaporated to dryness and the yield % of mass after drying was calculated based on the mass of the starting materials. Mass of starting materials; 2210 mg. The standard error was less than 1%.

the aqueous fraction and the amount was less in the ethyl acetate extract and solid fraction. It is apparent from this table that only 0.1 and 0.5 % of solid residue were obtained for both 30 and 50 g/L of lignin, and no solid residue was produced at the lower concentration of lignin (10 g/L).

If compared to the controls, there was no significant difference observed. On the other hand, the yield (%) of material in solid ethyl acetate extract fraction increased by 0.5 and 1.1 % for the reaction using 30 and 50 g/L of lignin, respectively compared to the control (Table 4.1d).

The bulk of the product material was found in the aqueous fraction and the ethyl acetate extract. The materials found in the aqueous fraction varied from 96, 94 and 90 % of the total 10, 30 and 50 g/L of lignin respectively, but these yields were lower than the control experiments without ABTS and LAB. As the dry mass of the aqueous fraction decreased, the dry mass of the aqueous ethyl acetate extract increased from 2.5 and 3.4 to 7.6 % as the concentration of lignin increased. This indicated that extractable compounds in ethyl acetate were increased as the concentration of lignin increased from 10 to 50 g/L. It has to be noted that the sodium lignosulphonate sample was not soluble beyond 60 g/L.

The highest amount of products in the ethyl acetate fractions was produced from 50 g/L of lignin and was 6.5 % higher than the control. The mass balance showed that without LAB and ABTS (Table 4.1d), only 1.1 % of materials can be extracted into ethyl acetate compared with 7.6 % when the LAB and ABTS were present. The mass was increased by only 0.4 % with the addition of LAB excluding ABTS (Table 4.1e). This finding further supports the idea that enzymatic depolymerization of lignin may not occur without the occurrence of a mediator (Cañas and Camarero, 2010, Bourbonnais and Paice, 1990, Bourbonnais *et al.*, 1997, Bourbonnais *et al.*, 1998).

It can therefore be concluded that LAB catalyzes a change in the distribution of material between the product fractions. However, this study has found that generally the percentage change was low. Further investigation and experimentation should

therefore concentrate on the factors that influence the reaction and identifying the products formed.

4.4 Preliminary Screening of Fractions by GPC

The distribution of the molecular weight of products can be studied using gel permeation chromatography (GPC). GPC has been implemented in various lignin-related studies since the technique is particularly well suited to study the distribution of different size compounds in a mixture (Pellinen and Salkinoja-Salonen., 1985; Cathala *et al.*, 2003; Majcherczyk *et al.*, 1998). GPC was used as part of the preliminary screening of the product fractions. The sodium lignosulphonate was incubated with LAB and mediated with ABTS. The sample was subsequently fractionated and each fraction was evaporated to dryness and then dissolved in methanol and water in the ratio of 1:9. The enzyme catalyzed extensive depolymerization compared to a control without the enzyme and LAB (Fig. 4.5).

The ethyl acetate extract of the aqueous fraction contained low molecular weight compounds with retention times of between 11 and 13 min and small quantities of high molecular weight compounds were observed between 9 – 11 min (Fig. 4.5, blue line) as compared to the original lignin (Fig. 4.5, green line). This finding gave further information together with the spectroscopic fingerprint of aqueous ethyl acetate extract fraction by ^{1}H-NMR that low molecular weight compounds were produced and more likely to be monomers.

It has been suggested that laccase catalyses both the polymerization and depolymerization of lignin (Leonowicz *et al.*, 1985). Some evidence for polymerization was obtained, since the peak between 9.5 to 10.5 min in the aqueous fraction was slightly increased as compared to the original lignin (Fig. 4.5, red line). These results are in line with other studies by Bourbonnais *et al.* (1995) and Hernandez Fernaud *et al.* (2006) in which lignin seemed to remain polymeric after a longer incubation time with ABTS as a mediator (Bourbonnais *et al.*, 1995, Hernández Fernaud *et al.*, 2006). Furthermore, the aqueous fraction contained

polymeric materials, with a similar composition to the original lignin, suggesting that it may contain unconverted lignin.

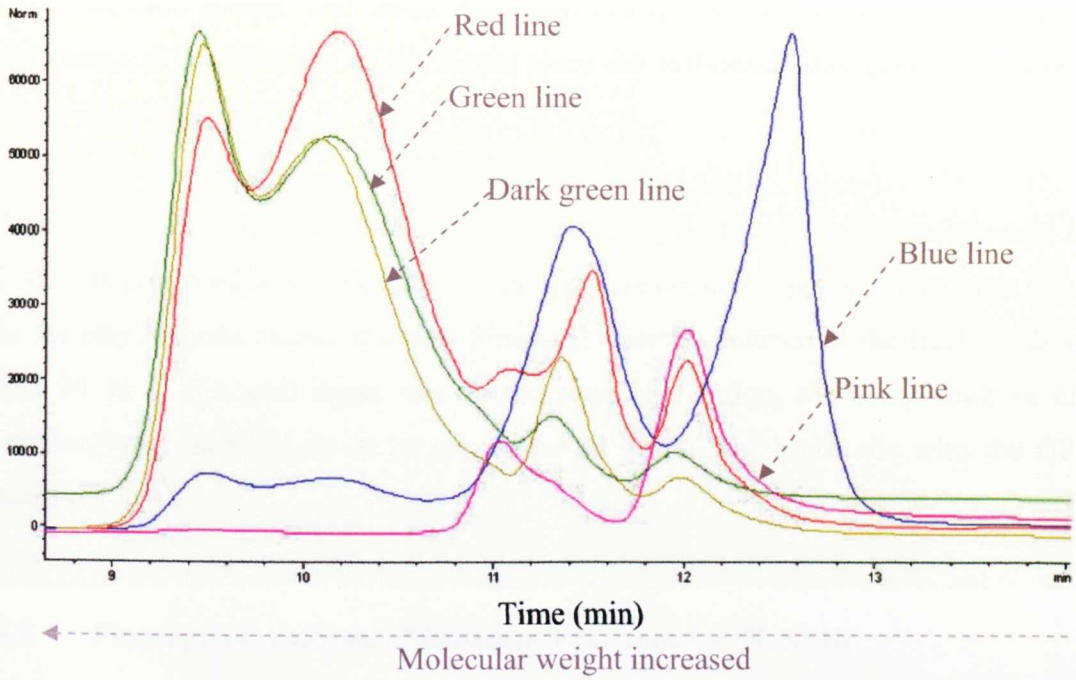


Figure 4.5 Depolymerization of sodium lignosulphonate by LAB as analyzed by GPC. The dried sample was dissolved in a mixture of 10 % methanol and 90 % water at pH 12. The chromatogram presented the refractive index (RI) of fractions and original lignin as monitored at 280 nm. Analysis was done in duplicate with the same representative GPC traces. Green: original sodium lignosulphonate; dark green: solid residue; pink: ethyl acetate extract of solid residue; red: aqueous fraction; blue: ethyl acetate extract of the aqueous fraction.

There was a small apparent increase in materials with intermediate molecular weights (retention time between 9.5 to 10.5 min). This may be significant but it could also equally be due to inadvertent increases in sample concentration during drying and reconstitution of the sample for GPC analysis. Low molecular weight peaks were also observed between 11.2 to 12.2 min.

As expected, high molecular weight compounds were not observed in the material extracted from the solid residue using ethyl acetate (leaving behind the solid) and there was a low intensity of low molecular weight compounds with a retention time

of between 10.8 to 12.4 min (Fig. 4.5, pink line). There was a slight qualitative difference between the chromatogram of the original lignin and the solid residue (Fig. 4.5, dark green). This result suggested that the enzyme catalyzed a change in the chemical composition of the lignin since the difference was observed between 11.2 – 11.4 min.

Thus, it can be concluded that the depolymerization of sodium lignosulphonate by LAB has produced low molecular weight and less polar compounds than were found in the ethyl acetate extract fraction. Since the material balance of the fraction shows that 90 % of the total lignin was in the aqueous fraction, the compounds in this fraction were more likely to be the unreacted lignin which accords with the GPC analysis.

4.5 Fingerprint Analysis of Different Fractions by ^{1}H-NMR

NMR fingerprinting was performed by using a proton nuclear magnetic resonance (^{1}H-NMR) to study the effect of LAB on lignin. The analysis of the NMR spectra was assisted by Dr Adrienne Davis. In ^{1}H-NMR, chemical shifts are associated with the occurrence of the various types of chemical resonance present in the sample. The identification of the depolymerization products by ^{1}H-NMR could not be performed since the products formed were complex mixtures of numerous compounds. Therefore, fingerprinting analysis was used to obtain indications of changes in chemical composition catalyzed by the enzyme.

First, a set of experiments was conducted to investigate the ^{1}H-NMR fingerprints after enzymatic treatment of lignin at different concentrations. Three concentrations of lignin, varying from 10 – 50 g/L were incubated with LAB and ABTS. Initially, this study was conducted using the aqueous ethyl acetate extract fraction because it can be easily evaporated to dryness and redissolved in the mixture of deuterated dimethyl sulphoxide (DMSO-d_6) and deuterium oxide (D_2O). Fig. 4.6 show the different ^{1}H-NMR spectra of the three lignin concentrations studied and the spectrum of the control experiment (lignin without ABTS and LAB). It is apparent from this figure that there is no trace of aldehyde peak observed in the enzyme-treated sample

containing 10 g/L of lignin and the control (Fig. 4.6a and Fig. 4.6b). On the other hand, the occurrence of the aldehyde group was observed between 9 – 10 ppm at 30 g/L of lignin (Fig. 4.6c).

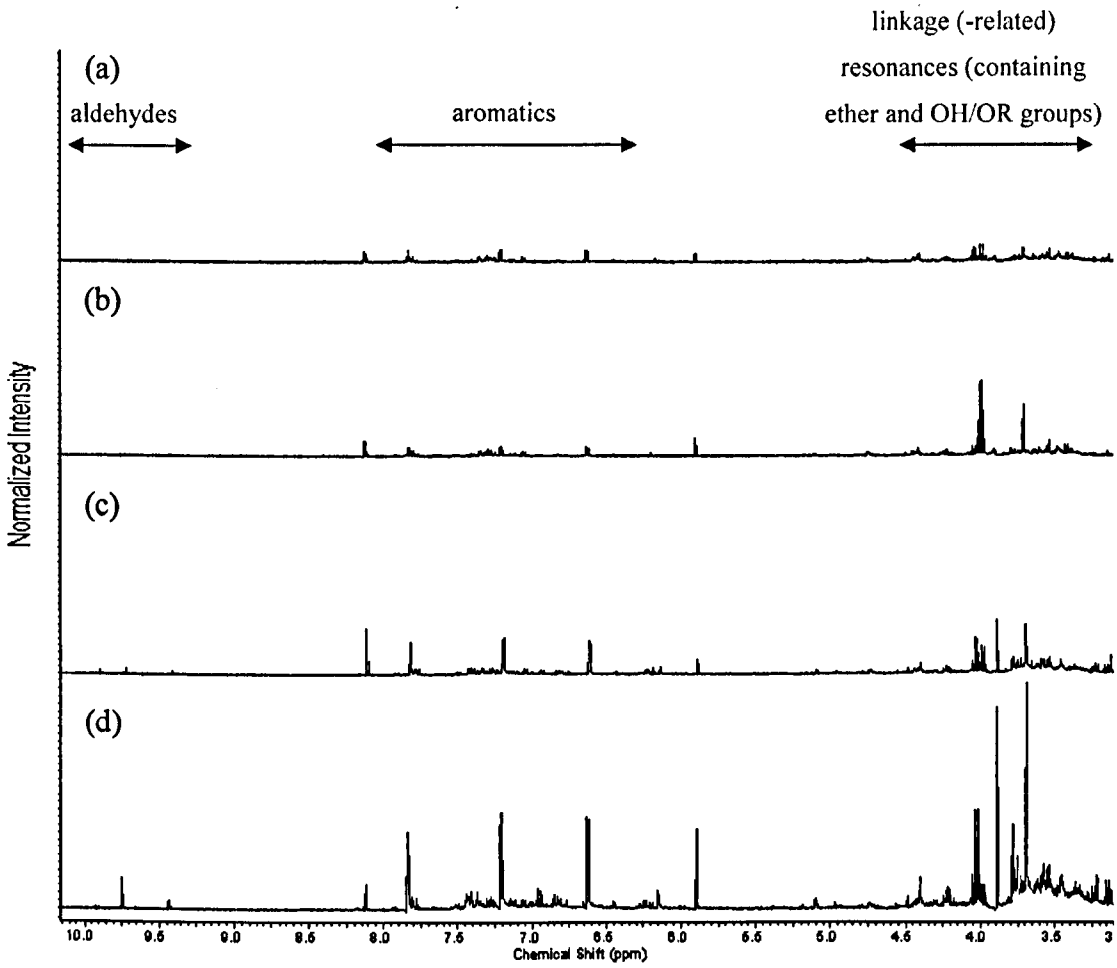


Figure 4.6 The ^{1}H-NMR spectrum of aqueous ethyl acetate extracts of different lignin concentrations. The reaction mixtures contained sodium lignosulphonate (10, 30, 50 g/L final concentration) dissolved in a sodium citrate buffer (25 mM; pH 6), ABTS (5 mM) and LAB (0.25 mg/ml) and shaken at 200 rpm for 6 h at 30 °C. Fractionation was applied and the aqueous ethyl acetate extract fraction was evaporated to dryness and dissolved in DMSO-d_6 and D_2O in the ratio of 8:2. All spectra were scaled on the same scale of intensity. (a) Lignin (50 g/L) without ABTS and LAB, (b) 10 g/L, (c) 30 g/L and (d) 50 g/L.

The intensity of the aldehyde peaks was found to increase when 50 g/L of lignin was used. Furthermore, higher aromatic peak intensities were observed between 6.5 – 8 ppm as the concentration of lignin increased compared with the control. The intensity of hydrogen adjacent to ether and OH/OR groups (between 3 – 4 ppm) was

also increased. Therefore, LAB catalyses a change in the chemical composition of material extracted from lignin. It can also be suggested that 50 g/L of lignin is the best concentration to be used for further study.

Next, ^{1}H-NMR fingerprinting was conducted for each fraction in order to obtain the chemical information of the products. The analysis was conducted by drying a sample of each fraction and dissolving it in DMSO-d_6 and D_2O. The sodium lignosulphonate was used without LAB and ABTS as a control (Fig. 4.7a-d) and was incubated and fractionated under the same conditions as the enzymatic assay. The ^{1}H-NMR analysis of the product fractions provided spectroscopic fingerprints that were visually similar for replicate samples of each fraction. Fig. 4.7 (a1-d1) presents the ^{1}H-NMR spectra of different lignin depolymerization product fractions. The occurrence of the 'hump' between 6.5 – 7.5 ppm in the solid fraction represents the polymeric materials in the fraction (Fig. 4.7a1). Since the 'hump' was relatively small in the solid residue of the control reaction (Fig. 4.7a), it was then assumed that polymerization had occurred in the enzymatic assay containing LAB and ABTS. There are no interpretable differences in the spectroscopic fingerprints of the aqueous fraction (Fig. 4.7b1) and its control (Fig. 4.7b), except that the spectroscopic fingerprint of the aqueous fraction was distinctly different in the aromatic regions between 7 - 8 ppm (Fig. 4.7b1), suggesting that lower molecular weight aromatic compounds had been produced in the aqueous fraction. As observed in solid fraction, polymeric 'hump' was also observed in aqueous fraction. This also accorded with earlier observation by GPC (Fig. 4.5; red line) which showed that polymeric materials were present in the aqueous fraction as well as the low molecular weight aromatic compounds.

Aromatic peaks were also observed in the solid ethyl acetate extract fraction (Fig. 4.7c1). The aqueous ethyl acetate extract of the control reaction exhibited relatively small aromatic peaks (Fig. 4.7d). The aromatic peaks were more intense in the aqueous ethyl acetate extract fraction after enzymatic treatment (Fig. 4.7d1) compared to the other fractions and the control reaction (Fig. 4.7d).

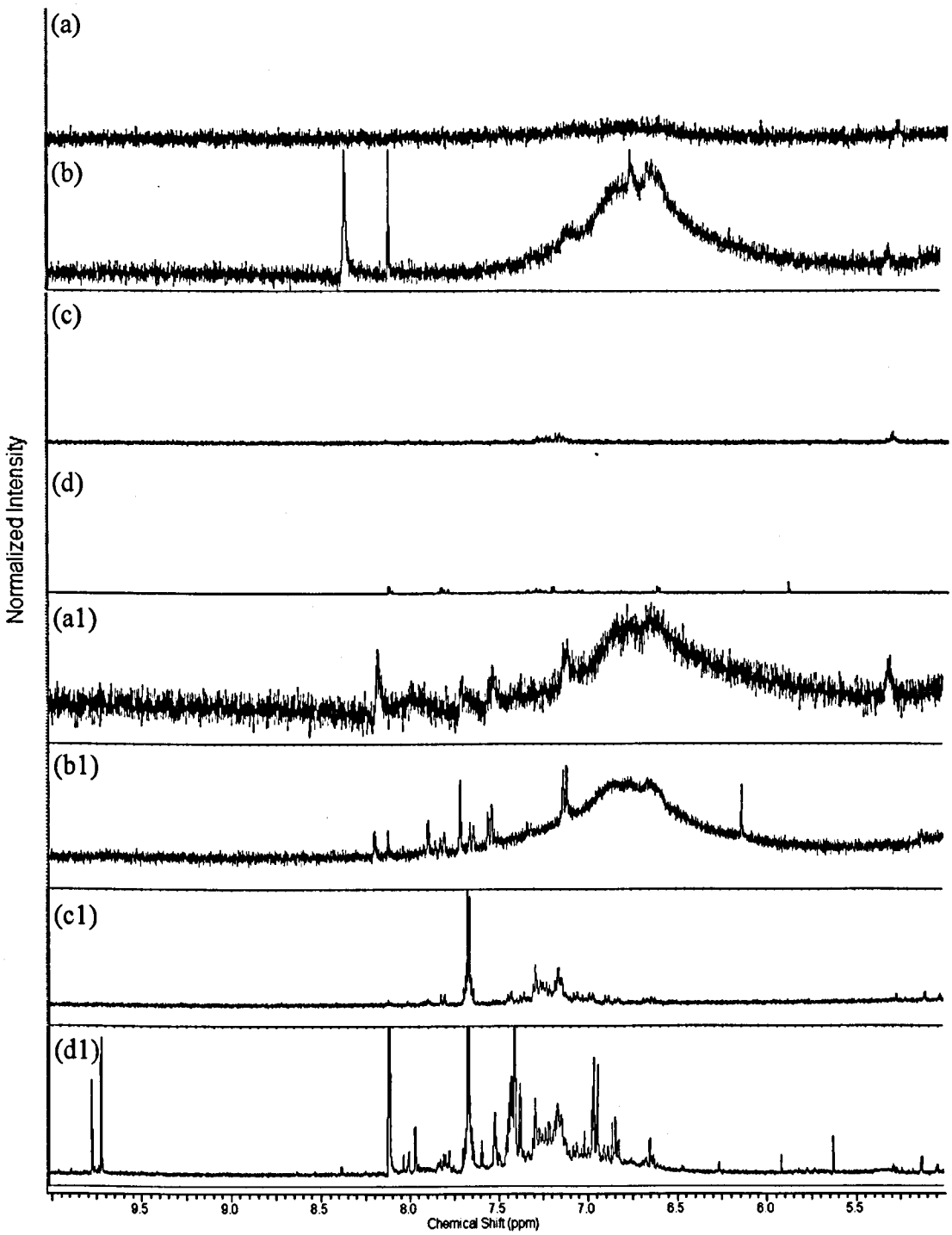


Figure 4.7 ^{1}H-NMR spectra for comparison of functional groups between control fractions [(a) solid residue (b) aqueous fraction, (c) solid ethyl acetate fraction, (d) aqueous ethyl acetate extract fraction] and the fractions after enzymatic reaction [(a1) solid residue, (b1) aqueous fraction, (c1) solid ethyl acetate fraction, (d1) aqueous ethyl acetate fraction]. The reaction mixtures included sodium lignosulphonate (50 g/L final concentration) which was dissolved in a sodium citrate buffer (25 mM; pH 6), ABTS (5 mM) and LAB (0.25 mg/ml) and shaken at 200 rpm for 6 h at 30 °C. Fractionation was applied and the fractions were evaporated to dryness and dissolved in DMSO-d_6 and D_2O in the ratio of 8:2. All spectra were on the same scale of intensity and two replicates representative NMR.

The aromatic compounds tended to be extracted in ethyl acetate which indicates that the enzymatic reaction of sodium lignosulphonate by LAB produced less polar products. Furthermore, aldehyde peaks were also observed between 9.5 – 10 ppm in the aqueous ethyl acetate extract fraction. However this was not observed in the other fractions.

This result suggests that the aldehydes were produced from the depolymerization of sodium lignosulphonate by LAB since these peaks were not observed in the aqueous ethyl acetate extract of the control reaction (Fig. 4.7d). Taken together, these results suggest that the depolymerization of sodium lignosulphonate by LAB and mediated by ABTS was successful, which supports the previous result for the mass balance of the materials produced. This study has also delivered a better understanding of the chemistry of the products formed. Even though the identification of the complex product mixtures could not be performed by ^{1}H-NMR, it did demonstrate the role of LAB and ABTS in the breakdown of sodium lignosulphonate.

4.5.1 The Effect of LAB Concentration

Fingerprint analysis by ^{1}H-NMR was also used to investigate the effect of LAB concentration on product formation. A sample was prepared and fractionated as described previously. In order to study the effect, four different concentrations of LAB were used (Fig. 4.8). The spectroscopic fingerprints of the product fractions described above suggested that the compounds of interest were in the aqueous ethyl acetate extract fraction. Therefore, only the aqueous ethyl acetate extract fraction was studied. The concentration of LAB did not have any significant effect on product formation, except that the intensity of the aldehyde peaks (9 – 10 ppm) was found to be much lower when the LAB concentration was decreased to 0.05 mg/ml (Fig. 4.8). The intensity of the aromatic protons (between 6.5 – 8 ppm) was 33 % higher in the presence of 0.25 mg/ml of LAB compared to the other enzyme concentrations. This finding suggests that the concentration of LAB should be greater than or equal to 0.25 mg/ml. The difference was calculated based on the height of the integration peak at 8 ppm. The same result was observed for the intensity of the chemical shift of

hydrogen adjacent to ether and OH/OR groups (3 – 4 ppm). Thus, it can be suggested that 0.25 mg/ml of LAB concentration is the best concentration to be used for further study.

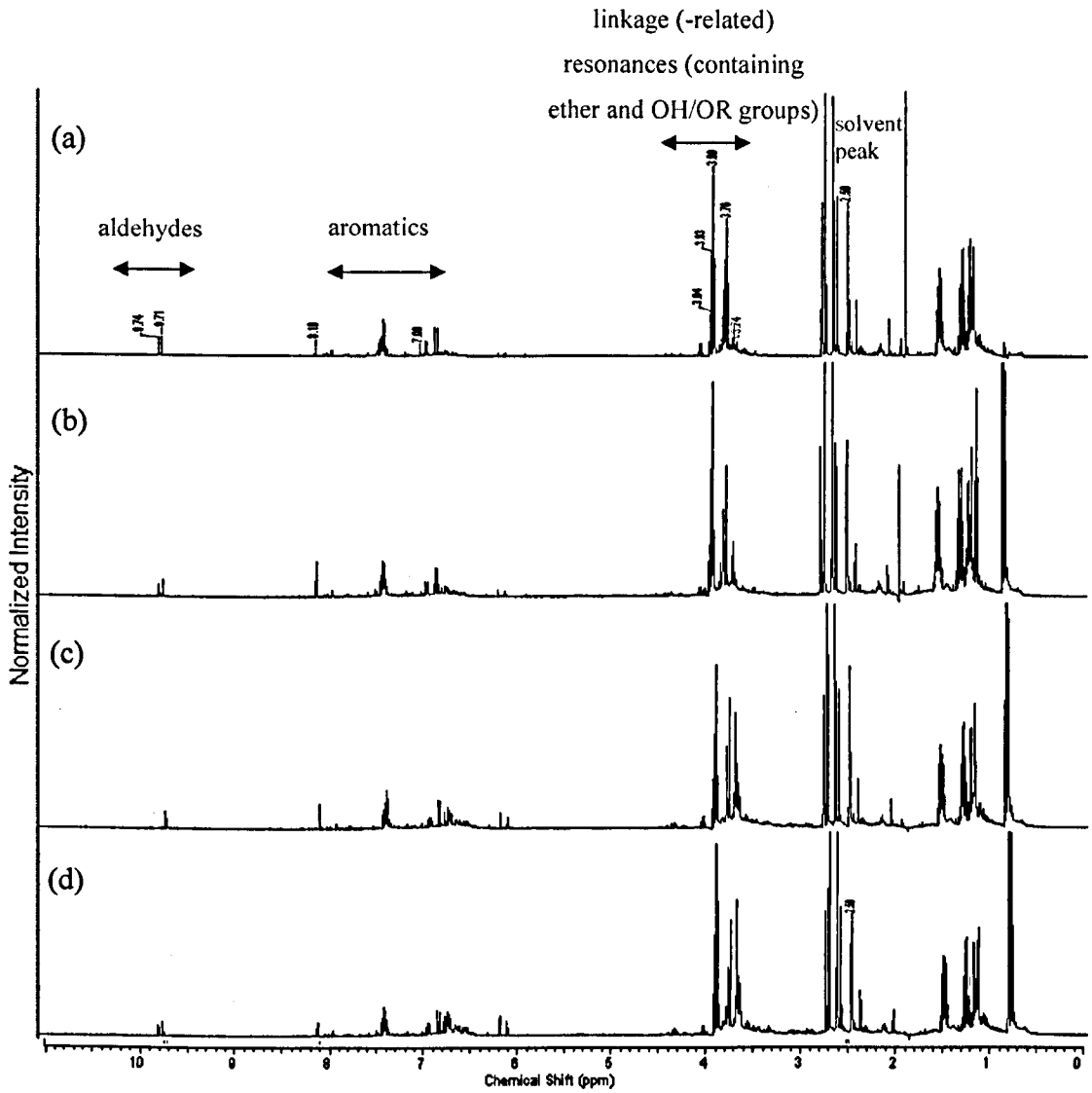


Figure 4.8 The ^{1}H-NMR spectra after treatment of sodium lignosulphonate with different LAB concentrations. The reaction mixtures contained sodium lignosulphonate (50 g/L) dissolved in sodium citrate buffer (25 mM; pH 6), ABTS (5 mM) and LAB, and were shaken at 200 rpm for 6 h at 30 °C. Fractionation was applied and the aqueous ethyl acetate extract fraction was evaporated to dryness and dissolved in DMSO-d_6 and D_2O in the ratio of 8:2. All spectra were on the same scale of intensity and represent two replicates. (a) 0.05 mg/ml, (b) 0.25 mg/ml, (c) 0.45 mg/ml and (d) 0.65 mg/ml.

4.5.2 The Effect of ABTS Concentration on the Formation of Products

Another important factor that needs to be taken into account is the effect of ABTS concentration on the depolymerization process. Fingerprint analysis by ^{1}H-NMR was employed to study this effect. Fig. 4.9a presents the ^{1}H-NMR spectrum of ABTS at 5 mM concentration as a standard. As shown in this figure, the protons that are attached to the aromatic carbons appeared between 7.11 – 7.72 ppm. The second group of protons lies between 3.94 – 4.07 ppm which represents the protons attached to the side chains of ABTS. The third group is the protons of the methyl group between 1.23 – 1.27 ppm. It should be noted that the standard ABTS has not been fractionated as the enzymatic reaction sample.

The next step was to study the partitioning of ABTS between different sample fractions. From a simple experiment that had been conducted on the oxidation of ABTS by LAB, the assay changed colour from light green to dark green. After prolonged incubation period, the product of ABTS (ABTS cation radicals and ABTS dications) (Marjasvaara *et al.*, 2008; Bourbonnais and Paice, 1990; Bourbonnais *et al.*, 1998) formed a dark green precipitate. It was therefore envisaged that the ABTS cation radicals and the dications may be fractionated into the solid residue. Surprisingly, the ABTS proton peaks were not observed in any of the fractions (Fig. 4.9 b-e). It is unclear why the ABTS radical cations could not be detected by the ^{1}H-NMR. Since ABTS was not observed in any of the fractions, thus, the effect of ABTS concentration on the product formation was able to be conducted since there is no interference of ABTS proton peaks.

Fig. 4.10 shows the result obtained from the fingerprint analysis of the aqueous ethyl acetate extract fractions that were produced from the reaction mediated by different concentration of ABTS. The intensity of the peaks increased when the concentration of ABTS increased from 1 to 5 mM (Fig. 4.10b - c). The intensity was higher than the control (Fig. 4.10a). Moreover, the amount of aldehdyde protons was higher in the 5 mM ABTS spectrum (9 -10 ppm) (Fig. 4.10c) than the other ABTS concentrations, and they were absent in the control. Surprisingly, the intensity of all the peaks decreased when the concentration of ABTS increased from 5 to 10 and 20 mM.

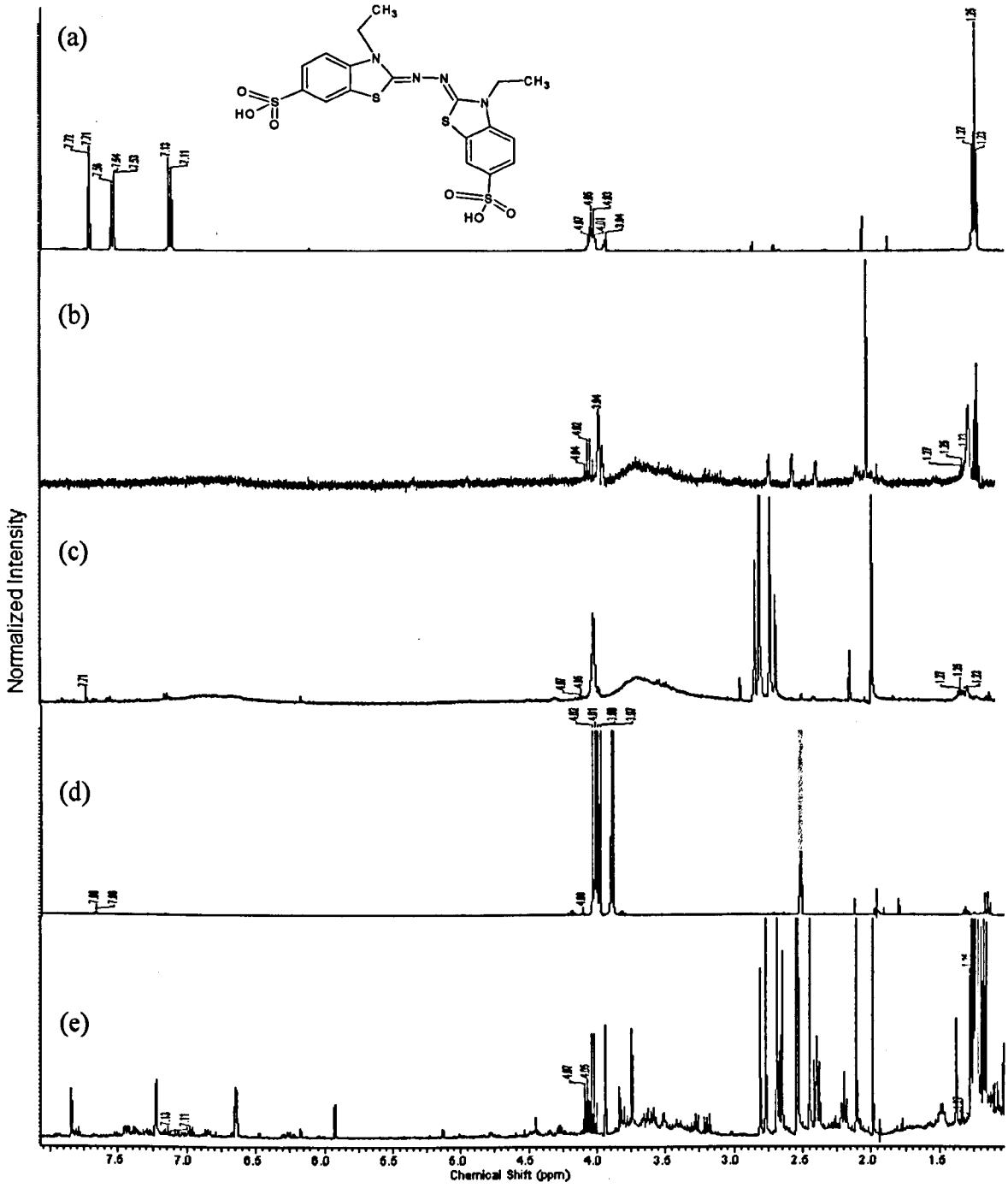


Figure 4.9 Comparison between ABTS intensity before and after the treatment with LAB. The reaction mixtures of (b) - (e) contained sodium lignosulphonate (50 g/L final concentration) which dissolved in a sodium citrate buffer (25 mM; pH 6), ABTS (5 mM) and LAB, and were shaken at 200 rpm for 6 h at 30 °C. Fractionation was applied and fractions were evaporated to dryness and dissolved in DMSO-d_6 and D_2O in the ratio of 8:2. Fractionation was not applied to the ABTS sample. All spectra were scaled to the same scale of intensity except for ABTS which was four times higher than the product fractions. Each spectrum represents two replicates. (a) ABTS without lignin and LAB (control), (b) solid residue, (c) aqueous fraction, (d) solid ethyl acetate extract fraction and (e) aqueous ethyl acetate extract fraction.

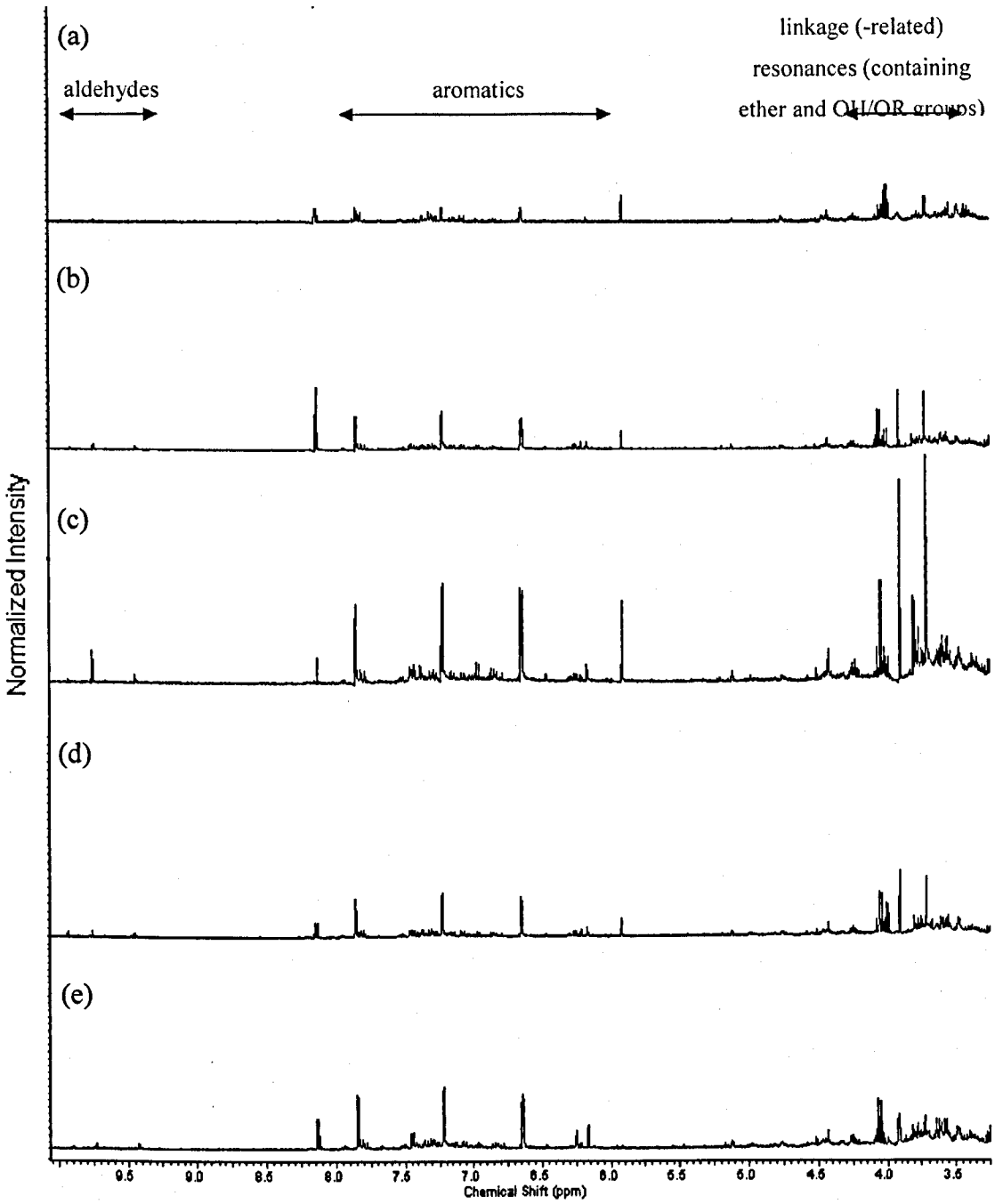


Figure 4.10 The ^{1}H-NMR spectra of aqueous ethyl acetate extract fraction produced from different ABTS concentrations. (a) lignin (control), (b) 1 mM, (c) 5 mM, (d) 10 mM, (e) 20 mM. The reaction mixtures include sodium lignosulphonate (50 g/L final concentration) dissolved in a sodium citrate buffer (25 mM; pH 6). The ABTS concentration varied from 1 mM to 20 mM and LAB (0.25 mg/ml) and was shaken at 200 rpm for 6 h at 30 °C. Fractionation was applied and the aqueous ethyl acetate extract fraction was evaporated to dryness and dissolved in DMSO-d_6 and D_2O in the ratio of 8:2. All spectra were on the same scale of intensity and each spectrum represents two replicates.

The result is in line with the study by Bourbonnais and Paice (1990) who found that the production of veratraldehyde from veratryl alcohol by laccase from *Trametes versicolor* was reduced when the concentration of ABTS increased. According to their study, a higher amount of ABTS contributes to enzyme inhibition. The reason for this was not clear but it may due to the high amount of ABTS dication ($ABTS^{2+}$) that was produced over a long incubation period. The dication of ABTS may undergo a comproportionation reaction with ABTS to produce ABTS cation radicals ($ABTS^{\bullet+}$) (Bourbonnais *et al.*, 1998; Bourbonnais and Paice, 1990; Bourbonnais *et al.*, 1995). Since the enzyme is a polymer as a lignin, $ABTS^{\bullet+}$ may also react with the laccase polypeptide and inactivate the enzyme. On the other hand, the main purpose of the mediated oxidation of lignin is to use ABTS as a mediator and not as a main material. Thus, the amount of ABTS was kept less than or equal to 5% (≈ 2.5 g/L of 50 g/L) of the total lignin used. In view of the fact that ABTS is expensive (Potthast *et al.*, 1996), the experiment was designed to use a lower concentration of ABTS to reduce the cost of the reaction. Since 5 mM of ABTS concentration has been proven to give better product formation, this amount is used in further experimental work.

4.6 Elemental Analysis (EA)

Elemental analysis was performed on the aqueous fractions and solid residues. Both fractions were dried and analyzed using an element analyzer to determine their carbon (C), hydrogen (H), nitrogen (N) and sulphur (S) content, as shown in Table 4.3. To verify the effect of enzymatic treatment by LAB and ABTS, sodium lignosulphonate (as a control) was analyzed following the same experimental procedure. The elemental analysis of aqueous ethyl acetate extract fraction and solid ethyl acetate extract fraction could not be performed because there was insufficient solid material after drying.

After the treatment of sodium lignosulphonate by LAB and ABTS, the C content of the aqueous fraction and solid residue was reduced by 21 and 18 %, respectively compared to the standard sodium lignosulphonate (Table 4.2). Moreover, the H content was also reduced. This result indicated that the breakdown of sodium lignosulphonate was successfully performed since the C and H content were

decreased, suggesting that new compounds had been produced and extracted into the ethyl acetate fractions.

Table 4.2 Elemental composition of (a) standard sodium lignosulphonate (control), (b) aqueous fraction and (c) solid residue.

Element	C	H	N	S
(a) Sodium lignosulphonate	45.74	5.86	0.03	6.05
(b) Aqueous fraction	36.25	3.86	0.19	6.57
(c) Solid residue	37.47	3.94	1.53	5.19

The N content of the aqueous fraction and solid residue was increased by 84 and 98 %, respectively after the reaction. The S content in the aqueous fraction was increased by 8 % and decreased by 14 % in the solid residue. It would appear that this result is due to the use of H_2SO_4 to acidify the sample after the reaction was complete. The S content in H_2SO_4 seems to remain in the aqueous fraction rather than being precipitated into the solid residue. Since ABTS was not observed in either the solid residue or the aqueous fraction as studied previously by ^{1}H-NMR, it is therefore unlikely that the increase of both N and S was caused by ABTS products.

4.7 GCMS Analysis

Gas chromatography mass spectroscopy (GCMS) has been widely used as an analytical tool for characterizing products from thermochemical or chemical lignin degradation (Pecina *et al.*, 1986, Lavoie *et al.*, 2011). This technique allows the identification of the chemical compounds corresponding to the individual component boiling points. However, higher boiling point compounds above the limit of the GC-column could not be identified. Therefore, the sample was also derivatized to increase the volatility of the components. In this study, as a preliminary step, the analysis by GCMS without derivatization was employed to characterize the monomeric composition of the lignin depolymerization products.

Samples were prepared and fractionated following the method and the control reactions described previously. However, the fractions were evaporated to dryness and then redissolved in dichloromethane (DCM) to concentrate the samples to enable

detection by GCMS. In initial tests, samples (120 ml) in ethyl acetate were analyzed directly but products could not be detected. Therefore, the sample was evaporated. Attempts were made to dissolve the residue in a smaller volume of ethyl acetate (2 ml) but the products could not be redissolved. By contrast, the products could be dissolved in 2 ml of DCM. Therefore, this sample procedure was used to concentrate the sample to 60-fold.

Most of the monomers were identified in the aqueous ethyl acetate extract (up to 7.6 % of the mass of the initial lignin). Most of the lignin depolymerization products detected were guaiacyl (G) derivatives as expected because sodium lignosulphonate from softwood was used as the substrate (Matsushita and Yasuda, 2005). Vanillic acid **(5)** was found to be the major products and the other minor peaks were identified as guaiacol **(1)**, vanillin **(2)**, acetovanillone **(3)** and homovanillyl alcohol **(4)** (Fig. 4.11) (Table 4.3). The retention time and mass spectra were in a good agreement with authentic standards, and the NIST library as listed in Table 4.3 and Appendix A.1 (page 207). Only vanillin was observed in the control reaction (Fig. 4.12a and Fig. 4.12b) with a peak area of about 99 % less than the vanillin observed after the enzymatic treatment by LAB and ABTS (Fig. 4.12c). This seems surprising because aldehydes were not detected in the control samples using ^{1}H-NMR. This rather contradictory result may be due to the lower detection efficiency of NMR compared to GCMS.

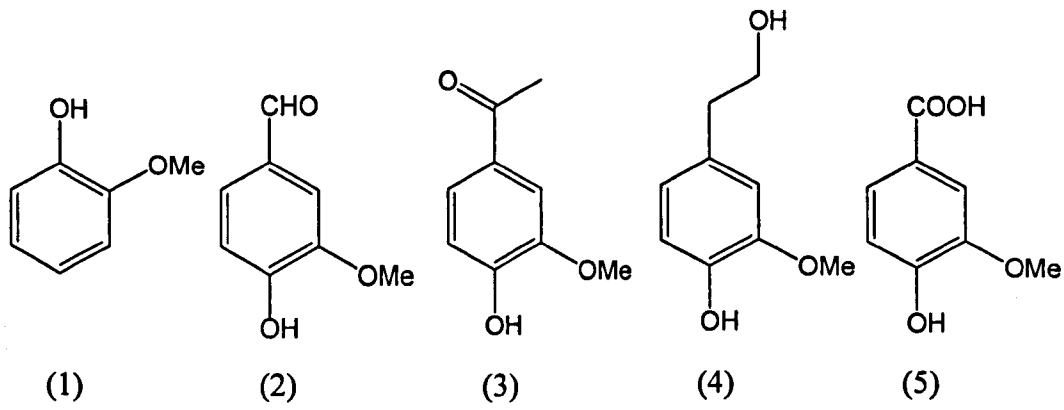


Figure 4.11 Chemical structures of lignin depolymerization products produced from the oxidation of sodium lignosulphonate by LAB and mediated by ABTS. The products were identified by GCMS in aqueous ethyl acetate extract. The identities of the released compounds are listed in Table 4.3.

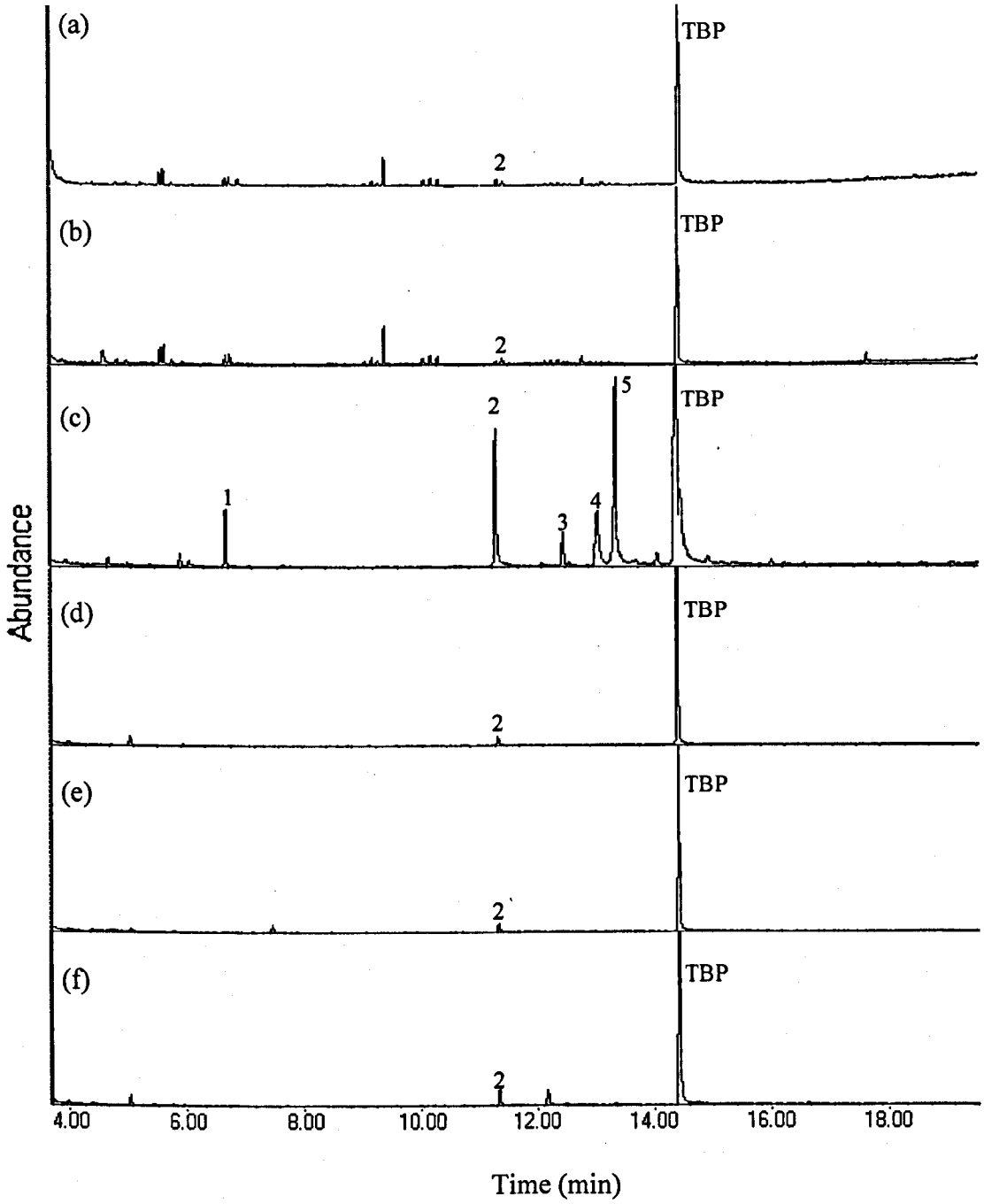


Figure 4.12 The GCMS chromatograms of products that have been extracted in ethyl acetate. The identities of the released compounds are listed in Table 4.3. The samples were incubated under identical conditions (at 30 °C for 6 h, shaken at 200 rpm). Fractionation was applied and the dried samples were redissolved in DCM. (a) Aqueous ethyl acetate extract fraction of the control-sodium lignosulphonate only, (b) aqueous ethyl acetate extract fraction of the control- sodium lignosulphonate plus LAB, (c) aqueous ethyl acetate extract fraction after treatment with LAB and ABTS, (d) solid ethyl acetate extract fraction of the control- sodium lignosulphonate only, (e) solid ethyl acetate extract fraction of the control - sodium lignosulphonate plus LAB, (f) solid ethyl acetate extract fraction after treatment with LAB and ABTS. All chromatograms were on the same scale of intensity. TBP: Tributyl phosphate

Table 4.3 Identification of products formed after liquid-liquid extraction with ethyl acetate. Identification of the products was based on comparison of mass spectra of the NIST library data and authentic standards (Appendix A.1 – page 207). The data represent the mean of three replicates and standard deviation (the variation from the average value). The chemical structure of the compounds is shown in Fig. 4.11. Control samples (sodium lignosulphonate and LTV without ABTS) contained the same amount of lignin (50 g/L) as the reaction mixtures with LTV and ABTS. The concentration of the release compound was calculated based on the peak area of the product as compared to the peak area of the authentic standard (5 mM).

Label	Compounds	Retention time (min)		(a) Aqueous ethyl acetate extract fraction			(b) Solid ethyl acetate extract fraction		
		Products	External Standard	Control (mM)	After LTV treatment (mM)	*Yield (%)	Control (mM)	After LTV treatment (mM)	*Yield (%)
1	Guaiacol	6.80	7.01	-	0.11 ± 0.01	0.02	-	-	
2	Vanillin	11.23	11.52	0.02 ± 0.00	0.28 ± 0.02	0.05	0.02 ± 0.00	0.03 ± 0.00	0.004
3	Acetovanillone	12.37	12.64	-	0.05 ± 0.01	0.01	-	-	
4	Homovanillyl alcohol	12.94	13.20	-	0.37 ± 0.01	0.08	-	-	
5	Vanillic acid	13.24	13.54	-	2.8 ± 0.02	0.60	-	-	
Total yield (%)						0.76			0.004

*Yield of the product was calculated based on the concentration (mol/ L) and the molecular weight (g/ mol) of each compound, and the resulted mass was then compared with the mass of starting material = 2210 mg. The standard error was less than 1 %.

The other peaks without a label were not structurally related to sodium lignosulphonate, and were assumed to be contamination peaks, for example, the major peak was identified as the plasticizer tributyl phosphate (TBP). Surprisingly, no differences were found between the solid ethyl acetate extract fraction and the controls as apparent in Fig. 4.12d-f.

The yield of each product was represented as percentage of mass of the product (g) per mass of starting material (g). As depicted in Table 4.3, the total yield of product in aqueous ethyl acetate extract fraction was only 0.76 %, whereas the total yield of product in this fraction should be 7.6 % as shown previously in Table 4.1 (Page 61). There was approximately 6.84 % product loss that may be occurred during quantification analysis by GCMS including the process of dissolving the sample in DCM and filtration. In addition, there might be compounds in aqueous ethyl acetate extract fraction that could not be characterized by GCMS. It can thus be suggested to employ a large column and high flow rates for product separation by using preparative HPLC technique, thus the isolation and purification of the compounds could be performed. The identity of the purified product can then be characterized by GCMS and NMR.

It was more difficult to analyze the aqueous fraction (the remaining liquid after ethyl acetate extraction) and the solid residue (from the reaction mixture) using GCMS since neither of these fractions was soluble in DCM. It should be noted that both fractions were in solid form after the evaporation of water from the reaction mixture. Therefore, a range of different solvents was screened for dissolution of the solid material. These fractions could be redissolved in water, partially dissolved in ethanol and acetone and were insoluble in DCM and tetrahydrofuran (THF) (Fig. 4.13).

Therefore, water was found to be the only suitable solvent. In both cases, the separation efficiency of the GCMS column was affected by the water and it was difficult to obtain signals for product identification. Another possibility may be that dimers, trimers or polymers maybe produced in both fractions which would not be detectable by mass spectrometry (MS). Therefore, these fractions were analyzed by HPLC, GPC, elemental analysis (EA), and ^{1}H-NMR.

Figure 4.13 Solubility of dried aqueous fraction in different solvent: (1) Dichloromethane (DCM); (2) ethanol; (3) acetone; (4) tetrahydrofuran (THF) and (5) water

4.8 HPLC Analysis with UV detector

An attempt was made to develop an analytical method by using reversed-phase high performance liquid chromatography (RP-HPLC) on the quantification of lignin depolymerization products. This method has been used by several authors (Pecina *et al.*, 1986; Vigneault *et al.*, 2007) since it allows rapid quantification (Pecina *et al.*, 1986). However, the accurate identification of the products is difficult since retention times vary in HPLC (Pecina *et al.*, 1986). For instance, the average retention time of vanillic acid was 9.69 ± 0.04 min (Appendix A.4.1 – page 225). Thus, the separation of peaks was used in the conjunction with GCMS in order to confirm the identity of the compounds.

First, HPLC was used to provide information concerning the compounds that might be produced from lignin depolymerization by LAB. Samples were prepared and fractionated as described previously. To verify the effect of the depolymerization, a control reaction was performed by incubating sodium lignosulphonate under the same reaction conditions as the enzymatic reaction. Fig. 4.14a1-d1 shows the HPLC chromatograms of the four fractions that were compared with the control samples (Fig. 4.14a-d). The aqueous ethyl acetate extract fraction contained more chemicals that can be separated and detected by HPLC than any other fraction (Fig. 4.14d1). Some of the peaks in the aqueous ethyl acetate extract fraction could be identified as guaiacol **(1)**, vanillin **(2)**, acetovanillone **(3)**, homovanillyl alcohol **(4)** and vanillic acid **(5)** by running the authentic standards (Appendix A.4 – page 225) (Table 4.4).

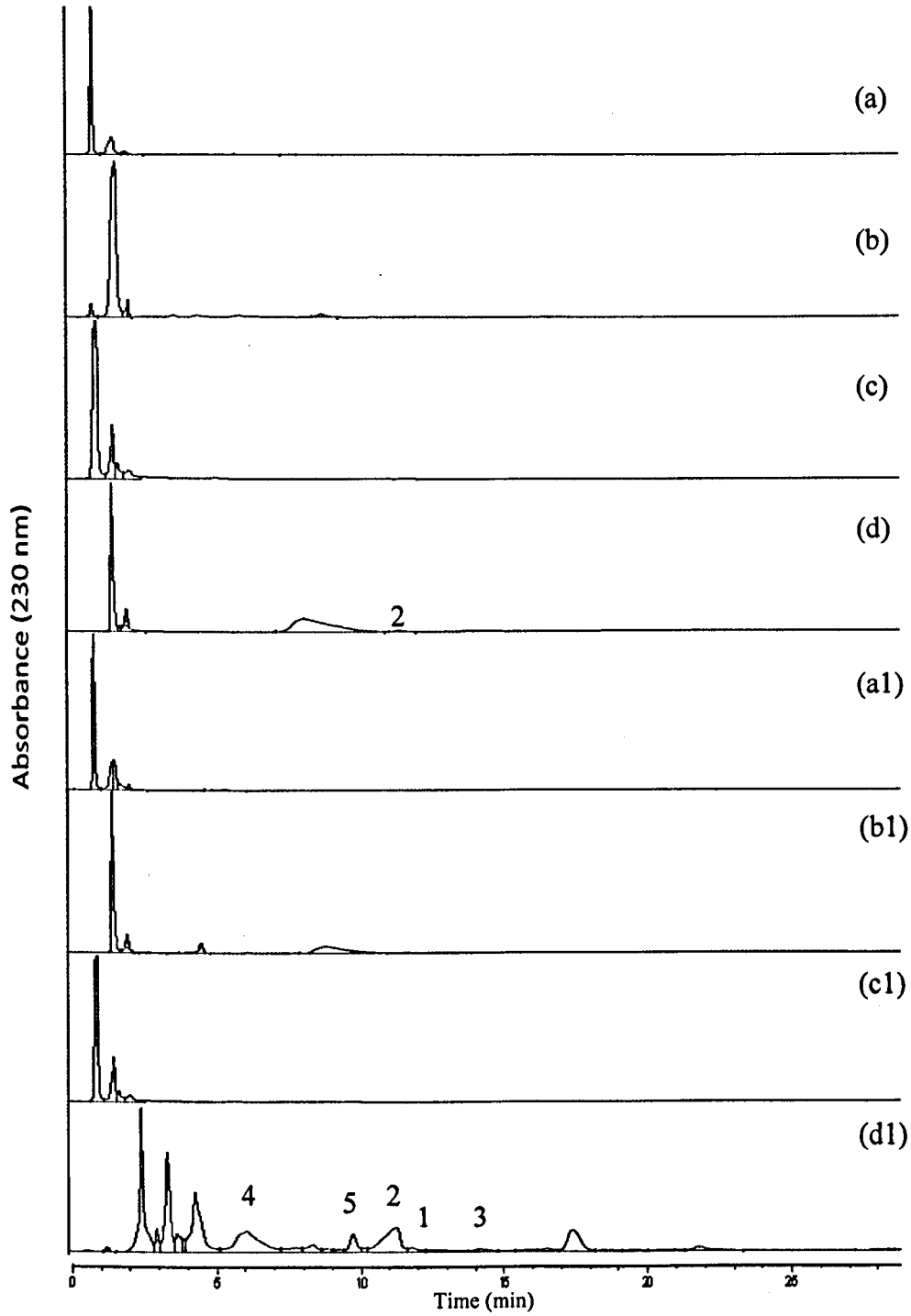


Figure 4.14 HPLC chromatograms of four fractions after LAB treatment of sodium lignosulphonate compared with the control sample. [(a) solid residue (b) aqueous fraction, (c) solid ethyl acetate fraction, (d) aqueous ethyl acetate extract fraction] and the fractions after enzymatic reaction [(a1) solid residue, (b1) aqueous fraction, (c1) solid ethyl acetate fraction, (d1) aqueous ethyl acetate fraction]. The reaction mixtures included sodium lignosulphonate (50 g/L final concentration) which were dissolved in sodium citrate buffer (25 mM; pH 6), ABTS (5 mM) and LAB (0.25 mg/ml) and shaken at 200 rpm for 6 h at 30 °C. Fractionation was applied and fractions were evaporated to dryness and dissolved in methanol and water in the ratio of 1:9. All spectra were scaled to the highest peak in the region. The identity of the compounds is listed in Table 4.4.

These products were also detected in the same sample by GCMS (prepared in DCM). Only vanillin was observed in the control reaction (Fig. 4.14d) which was corroborated by the GCMS analysis on the same sample.

On the other hand, only a few peaks were observed at the beginning of the separation for the other fractions. These peaks could not be identified and did not seem to correspond to any peaks identified by GCMS. Their identification would require screening of many authentic standards following by mass spectroscopic confirmation. In any case, there was no significant difference between the solid residue and solid ethyl acetate extract fraction compared to the control reaction. A new product peak was observed at 4.2 min in the enzyme treated aqueous fraction compared to the control. However, this compound could not be identified.

Table 4.4 The identities of the compounds in the aqueous ethyl acetate extract fraction.

No.	Compound	Retention time (min) ± S.D
1	Guaiacol	12.89 ± 0.02
2	Vanillin	11.29 ± 0.07
3	Acetovanillone	14.35 ± 0.05
4	Homovanillyl alcohol	6.21 ± 0.01
5	Vanillic acid	9.69 ± 0.04

In general, therefore, further investigation would be needed to identify the compounds in each fraction, using preparative HPLC. By using a large column and high flow rates, the isolation and purification of the compounds could possibly be performed. The identity of the purified product can then be characterized by GCMS and NMR. However, an inadequate supply of sodium lignosulphonate limited the possibility of scaling up the sample.

4.9 Effect of incubation time on Product Formation

Next, a quantitative analysis of products was performed to investigate the maximum yield of product. For quantification, the peak area of the GCMS chromatogram was found to be proportional to the amount of substance that was produced (Appendix

A.3.2) (Page 223). A series of experiments was conducted by investigating the effect of incubation time on the depolymerization of sodium lignosulphonate by LAB. In order to achieve this goal, the reaction mixtures containing sodium lignosulphonate, ABTS and LAB were incubated at 30 °C with a time range of between 0 to 24 h. The product concentrations were calculated based on the comparison of the product peaks and authentic standards using the standard calibration curves (Appendix A.4.2) (Page 227). Fig. 4.15 illustrates the pattern of the product formation. The production of vanillic acid was higher than the others with the maximum of 3.70 mM, and increased gradually over 24 h of reaction time (Fig. 4.15a). The maximum production of vanillic acid might be achieved if the reaction time increases over 24 h. On the other hand, homovanillyl alcohol achieved the optimum production after 6 h (0.37 mM), and decreased slightly thereafter. Highest production of vanillin and guaiacol were obtained after 8 h by 0.30 and 0.12 mM, respectively. The production of acetovanillone remains constant during 1 to 6 h (0.05 mM) and increased slightly after 8 h (0.06 mM) (Fig. 4.15b).

In summary, the reaction did not really achieve a high yield and product concentration decreased slightly over a prolonged period of time especially in the production of guaiacol, vanillin, and homovanillyl alcohol. It is unclear whether the reduction was caused by the repolymerization of the product or its stability during the period of analysis. In order to achieve the optimum yield, it can thus be suggested that the reaction for guaiacol and vanillin should be run for 8 h, and 6 h for homovanillyl alcohol.

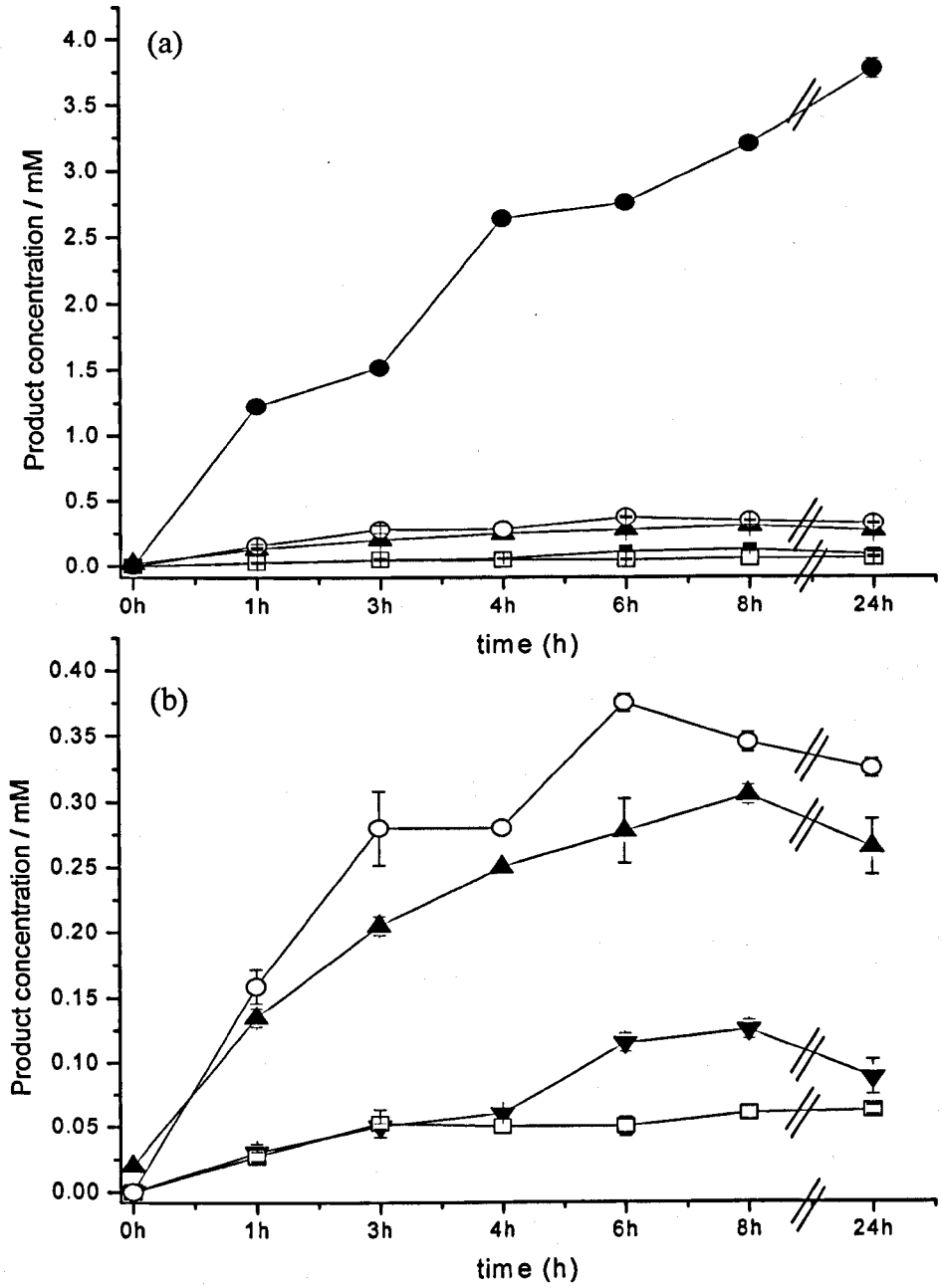


Figure 4.15 Effect of incubation time on the production of chemicals from lignin by the LAB-ABTS system, homovanillyl alcohol (○), vanillic acid (●), vanillin (▲), guaiacol (▼) and acetovanillone (□). The reaction mixtures contained sodium lignosulphonate (50 g/L final concentration) dissolved in a sodium citrate buffer (25 mM; pH 6), ABTS (5mM) and LAB (0.25 mg/ml) and were shaken at 200 rpm for a time varying from 0 to 24 h at 30 °C. Fractionation was applied and the aqueous ethyl acetate extract fraction was evaporated to dryness and dissolved in DCM and analyzed by GCMS. (a) shows all product concentration on the scale; (b) shows on homovanillyl alcohol, vanillin, acetovanillone and guaiacol on a larger diagram (smaller scale), to demonstrate differences in production.

4.10 Discussion

Taken together, the preliminary study of lignin depolymerization by LAB confirms a role for a mediator (ABTS) in promoting the reaction. The evidence from this study in comparison with the control reaction suggests the use of ABTS in the depolymerization of sodium lignosulphonate by LAB has contributed towards the breakdown of this complex polymer. The incubation time and temperature contributed towards the production of compounds from the reaction. A temperature of 30 °C was found to be the optimal temperature for LAB activity. Thus, this temperature was used for the depolymerization of sodium lignosulphonate. If compared to major thermochemical lignin conversion processes such as pyrolysis, thermolysis, hydrogenolysis and supercritical solvents (Pandey and Kim, 2011), the enzymatic conversion of lignin is by far more environmentally friendly and energy saving (Leonowicz *et al.*, 1999), especially since the reaction temperature of the enzymatic reaction is low compared to the chemical reaction which is conducted at temperatures of between 300 – 500 °C (Pandey and Kim, 2011; Lavoie *et al.*, 2011).

Clearly, the complex puzzle regarding the breakdown of lignin has yet to be resolved. The method of fractionation has provided a major contribution to this study especially from the analytical point of view. Summarizing the applied analytical methods to determine the effects of laccase on the depolymerization of sodium lignosulphonate is a little challenging. For the analysis of the complex mixture of products formed after the reaction, this requires different analytical methods to be taken into consideration. It was necessary to gather the information from different analytical instruments. For the preliminary screening of the product mixture, GPC and ^{1}H-NMR were mostly preferred. The effect of laccase on the breakdown of sodium lignosulphonate was studied by the distribution of the molecular mass of products using GPC and fingerprinting analysis by ^{1}H-NMR. However, identification of the products could not be performed by either HPLC or ^{1}H-NMR since the product mixture was complex. Taking this into account, GCMS was used to identify the individual monomers in the product mixture. Quantification analysis was also performed by GCMS. GCMS procedure was convenient and provides a rapid quantification of the product (Jham *et al.*, 2002; Pecina *et al.*, 1986). The combination of different analytical methods has enabled a better understanding of the

effect of LAB on lignin. One interesting fact to point out is that the use of a mediator has accelerated the reaction. A further trial assessed the possibility of using elemental analysis (EA) to investigate the individual components in the aqueous fraction and solid residue. The reduction of carbon and hydrogen content in these fractions after the reaction further proved that the depolymerization method used in this study was successful.

On the other hand, polymerization was also observed by GPC since the intensity of the high molecular weight products was increased after the enzymatic reaction. This result confirmed recent observations that the polymerization of lignin may occur in the present of laccase from *Trametes versicolor* (Kolb *et al., 2012).* Kolb *et al.*, (2012) identified a few chemicals from the pretreatment of wheat straw by a high pressure autoclave before further treatment by laccase. However, the amount of the products decreased very quickly towards 2 h of reaction time in the presence of laccase. This quick consumption of monomers was attributed to the polymerization of the compounds.

The challenge is to develop a methodology for lignin breakdown by laccase with the aim of maximizing the yield and selectivity of the enzyme towards the breakdown under mild conditions. Taking this into consideration, a number of possible future studies using the same experimental set up are apparent. It would be interesting to compare the effect of LAB with different types of laccase, such as from *Trametes versicolor* (LTV) which seems to be the answer to achieve this goal. Further study was therefore concentrated on the investigation of LTV towards the breakdown of sodium lignosulphonate.

Chapter 5

LACCASE FROM *TRAMETES VERSICOLOR* AS A POTENTIAL ENZYME FOR DEPOLYMERIZATION OF SODIUM LIGNOSULPHONATE

5.1 Introduction

There is growing interest in developing a process using laccase from *Trametes versicolor* due to the fact that this basidiomycete is a good source of laccase (Tanaka *et al.*, 1999, Minussi *et al.*, 2007). Reports suggest that laccase from different fungi have catalyzed many reactions and possess different behaviour towards the reaction (Tanaka *et al.*, 1999, Bollag and Leonowicz, 1984). Therefore, attempts have been made to investigate the effect of laccase from *T. versicolor* (LTV) for the oxidation of sodium lignosulphonate. An experiment was set up to study the effect of temperature towards the oxidation of ABTS by LTV. Since these factors affect the activity, the previous method for the depolymerization of sodium lignosulphonate by LAB was modified. Thus, the overall aim of the study was to determine the feasibility of using LTV to maximize the yield and selectivity towards the breakdown of sodium lignosulphonate under mild reaction conditions.

5.2 Laccase from *Trametes versicolor* (LTV) as a Potential Enzyme

In the previous chapter, LAB was demonstrated to catalyze the depolymerization of sodium lignosulphonate with the cooperation of ABTS. However, the yield of products was relatively low. Therefore, LTV was selected as an alternative enzyme to catalyze the reaction. In order to assess the potential of LTV, the activity was measured spectrophotometrically by the oxidation of ABTS as a substrate. As Fig.

5.1 shows, there is a significant increase in the LTV reaction rate compared to LAB. Provided that the substrate concentration and temperature are kept constant, the rate of reaction is determined by the ABTS concentration.

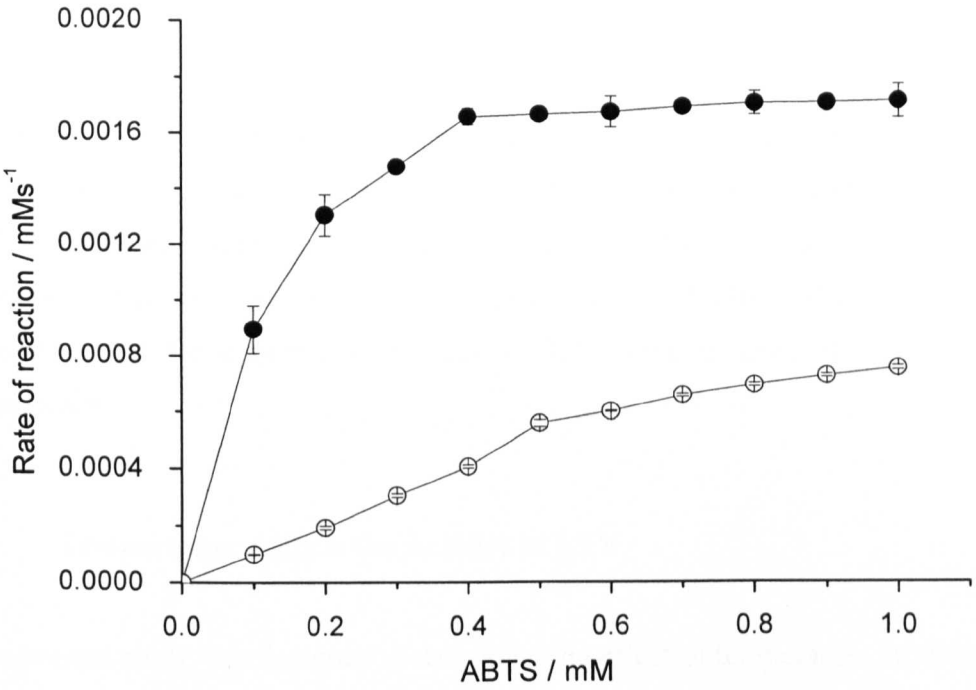


Figure 5.1 Effect of ABTS concentration on reaction rate of different laccase LTV (•) and LAB (○). ABTS was added to start the reaction containing ammonium acetate buffer (100 mM, pH 4.5) and LTV (0.25 mg/ml). The absorbance change at 420 nm was recorded for 30 min. The data represent the mean of three replicates and the error bars show that the errors were less than 1%. LAB data was taken from previous experiment in Chapter 4 (Fig. 4.2).

There was no further increase in the rate of reaction when the concentration of the substrate was increased beyond 0.4 mM. The initial rate of reaction (v_o) of LTV at 0.5 mM was determined to be $9.8 \times 10^{-4} \pm 1.0 \times 10^{-4}$ mMs^{-1} which was an increase of 41 % compared to the oxidation of ABTS by LAB. The Michaelis-Menten parameters of the ABTS oxidation by LTV were found to be approximately $K_m = 0.1 \pm 0.09$ mM; $V_{max} = 17 \times 10^{-4} \pm 0.4 \times 10^{-4}$ mMs^{-1}). The Michaelis-Menten parameters of both laccases are summarized in Table 5.1.

Table 5.1 Kinetic parameters for the oxidation of ABTS by different laccases. Parameters are kept constant (22 °C, ABTS concentration varied between 0.1 and 1 mM final concentration). The kinetic parameters were calculated based on Lineweaver-Burke method.

Laccase	v_o (mMs^{-1}) x 10^{-4}	K_m (mM)	V_{max} (mMs^{-1}) x 10^{-4}
LAB	5.8 ± 0.2	0.4 ± 0.04	7.1 ± 0.4
LTV	9.8 ± 1.0	0.1 ± 0.01	17 ± 0.4

It can therefore be concluded that the activity of LTV is higher than for LAB. The lower value of K_m indicates that LTV has a higher affinity for the substrate than LAB which results in a higher reaction rate. In addition, LTV can convert more substrate to product per unit time which is represented by the higher value of V_{max}. It was predicted that the higher reaction rate of LTV may increase the rate of lignin breakdown.

5.3 Temperature Affects the Activity of LTV

The present study was designed to determine the effect of temperature on the activity of LTV with regard to the oxidation of ABTS. Spectrophotometric assays were conducted in a 1ml cuvette at different temperatures varying from 30 to 80 °C and compared with the activity at room temperature. LTV activity increased as the temperature increased to a maximum at 60 °C, and decreased slightly thereafter (Fig. 5.2). Surprisingly, LTV was still active at 80 °C which indicates that this enzyme is highly thermostable. The finding of this maximal temperature of LTV is consistent with those of Rancaño *et al.* (2003) who found the same result for the activity of this enzyme although they used purified enzyme instead of the commercial enzyme used in this study.

According to Baldrian (2004) the optimum temperature for laccase activity varies between 50 to 70 °C depending on the type of enzyme, the pH and the buffer used. The finding of the current study suggests that 60 °C is the optimum temperature for LTV activity. Therefore, the depolymerization of sodium lignosulphonate was conducted initially at this temperature.

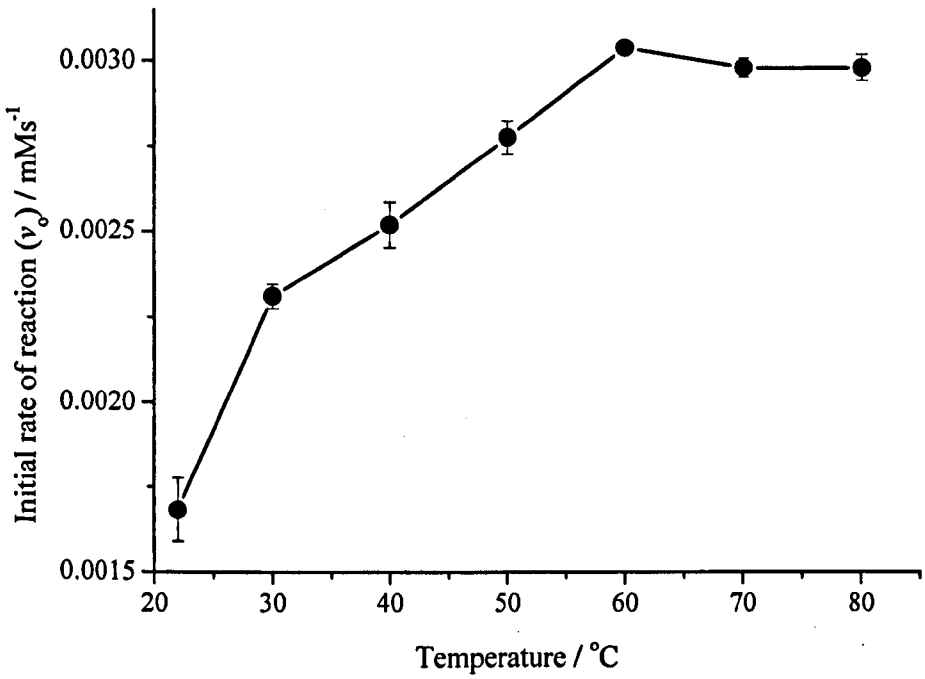


Figure 5.2 The effect of temperature on LTV activity. ABTS was added to start the reaction containing ammonium acetate buffer (100 mM) and LTV (0.25 mg/ml). An absorbance change at 420 nm for each temperature was recorded for 30 min. The data represent the mean of three replicates with an error of less than 1 %.

5.4 Mediated oxidation of Sodium Lignosulphonate by LTV

Several studies have attempted to use laccase from *T. versicolor* as a catalyst for lignin oxidation (Bourbonnais *et al.*, 1995, Bourbonnais *et al.*, 1997). For instance, Bourbonnais *et al.* (1995) isolated two laccases from *T. versicolor* and found out that this enzyme catalyzed both polymerization and depolymerization of Kraft lignin. Depolymerization of lignin was observed with the addition of ABTS to an average molecular weight of 5300 gmol^{-1} but no attempts were made to measure formation of monomers (Bourbonnais *et al.*, 1995). By taking the study by Bourbonnais *et al.* (1995) as a bench mark, an attempt was made to produce compounds with a much lower molecular weight from the breakdown of sodium lignosulphonate by LTV and mediated by ABTS. The reaction mixtures containing sodium lignosulphonate (50 g/L, as for LAB Section 4.3), LTV and ABTS were incubated at 60 °C for 6 h following the optimized reaction time of LAB. To verify the effect of enzymatic

depolymerization, sodium lignosulphonate was treated under the same reaction conditions but without ABTS and LTV, without ABTS and without LTV.

With previous LAB-catalyzed reactions, the product concentration fell over 24 h of reaction time except for vanillic acid. The reaction had changed colour, to black, suggesting that repolymerization might have occurred. For this reason, the reaction time with LAB & LTV was reduced to 6 h, in attempt to observe the monomers before repolymerization could occur. Table 5.2 presents the yield (%) of the dried material in each fraction after the enzymatic treatment by LTV compared to the reaction catalyzed by LAB and the control samples. The mass of the aqueous ethyl acetate extract fraction was increased by approximately 2.2 % compared to the reaction catalyzed by LAB (Table 5.2a and Table 5.2b) which is consistent with the higher activity of LTV for ABTS oxidation. The yield was higher by far than the control sample by 8.9-fold (Table 5.2c). The bulk of the product material was still found in the aqueous fraction and the yield (%) after drying was slightly decreased by 7 and 14 %, compared to the reaction catalyzed by LAB and the control sample respectively. This suggests that LTV had converted a greater proportion of the water-soluble substrate than LAB. The solid ethyl acetate extract fraction was slightly increased by 1 % compared to the control. There is no significant difference observed in the yield of solid residue since the yield of this fraction after the reaction with LTV increased by only 0.2 %.

The mass balance showed that without LTV and ABTS (Table 5.2c), only 1.1 % of materials can be extracted into the ethyl acetate compared with 9.8 % when the LTV and ABTS were used. The mass was increased by only 0.4 % with the addition of LTV excluding ABTS (Table 5.2d). This finding further supports the idea that enzymatic depolymerization of sodium lignosulphonate may not occur without ABTS as observed in the reaction catalyzed by LAB. The finding of this study has demonstrated an increase in the low molecular weight products extracted into the ethyl acetate. It can therefore be concluded that the use of LTV has enhanced the breakdown of the sodium lignosulphonate.

Table 5.2 The mass balance of product fractions after conversion of sodium lignosulphonate using LTV. The yield (%) was compared with (a) the reaction catalyzed by LAB and with controls containing 50 g/L of lignin (c) lignin without LTV and ABTS, (d) lignin and LTV without ABTS, (e) lignin with ABTS without LTV.

Fraction	Yield (%) after drying [g product / g lignin x 100]				
	(a) Lignin + LAB + buffer + ABTS	(b) Lignin + LTV + buffer + ABTS	Controls		
			(c) Lignin + buffer	(d) Lignin + LTV	(e) Lignin + ABTS
Solid residue	0.5	0.4	0.2	0.3	0.3
Ethyl acetate extract of solid residue	1.4	1.3	0.3	0.2	0.4
Aqueous fraction	90	83	97	98	98
Ethyl acetate extract of the aqueous fraction	7.6	9.8	1.1	1.5	1.2

The reaction mixtures catalyzed by LTV included sodium lignosulphonate (50 g/L) dissolved in ammonium acetate buffer (100 mM; pH 4.5), ABTS (5 mM) and LTV (0.25 mg/ml) and were shaken at 200 rpm for 6 h at 60 °C. The reaction mixtures catalyzed by LAB contained sodium lignosulphonate (50 g/L) dissolved in sodium citrate buffer (25 mM; pH 6),, ABTS (5 mM) and LAB (0.25 mg/ml). Fractionation was performed, each fraction was evaporated to dryness and the yield % of mass after drying was calculated based on the mass of the starting materials. Mass of starting materials; 2210 mg. Standard error was less than 1%.

Next, the products from both ethyl acetate fractions were characterized. The aqueous ethyl acetate extract fraction (9.8 % yield of the mass of the initial lignin) and solid ethyl acetate extract fraction (1.3 % yield) were analyzed by GCMS. Samples were prepared and fractionated following the method described in the previous chapter. It has to be noted that the quantification was carried out by measurement of the relative areas under each peak which was proportional to the amount of substance that was produced. Thus, the product concentrations were calculated using the standard calibration curves (Appendix A.3.2) (Page 223 – 224). The control reaction was treated under the same conditions as the enzymatic reaction. It was expected that the products resulting from the breakdown of sodium lignosulphonate would be guaiacyl (G) derivatives (Matsushita and Yasuda, 2005) as observed in the breakdown by LAB.

The actual products were a complex mixture of components and thirteen compounds were identified by GCMS as a result of the breakdown of sodium lignosulphonate by LTV. Vanillic acid **(5)** (12.9 mM) and vanillin **(2)** (3.13 mM) were identified as the major products. The concentration of vanillic acid and vanillin were significantly increased compared to the control (Table 5.3). In comparison with the reaction catalyzed by LAB, this is encouraging since these compounds have a wide range of industrial uses. Other minor peaks were identified including guaiacol **(1)**, acetovanillone **(3)**, homovanillyl alcohol **(4)**, phenol **(6)**, 4- methylbenzaldehyde **(7)**, catechol **(8)**, *p*-toluic acid **(9)**, 4-hydroxybenzaldehyde **(10)**, tyrosol **(11)**, isovanillin **(12)**, and 3-hydroxy-1-(4-hydroxy-3-methoxyphenyl) propan-1-one **(13)** were also observed in aqueous ethyl acetate extract fraction (Table 5.3). The identification of these compounds shows a good agreement with the authentic standard and the NIST library (Appendix A.2, page 210). The comparison of 4-methylbenzaldehyde **(7)** and 3-hydroxy-1-(4-hydroxy-3-methoxyphenyl) propan-1-one **(13)** with the authentic standards could not be performed since the standards are not available. Therefore, the identification of these compounds was based on the NIST library (Appendix A.2, page 210).

The yield of each product was represented as percentage of mass of the product (g) per mass of starting material (g). As depicted in Table 5.3, the total yield of product in aqueous ethyl acetate extract fraction was only 4.5 %, whereas the total yield of

Table 5.3 Identification of products formed after liquid-liquid extraction with ethyl acetate. Identification of the products was based on a comparison of mass spectra of authentic standards and the NIST library data (Appendix A.2, page 210). The data represent the mean of three replicates and standard deviation. The chemical structure of the compounds is shown in Fig. 5.3. Control samples (sodium lignosulphonate and LTV without ABTS) contained the same amount of lignin (50 g/L) as the reaction mixtures with LTV and ABTS. The concentration of the released compound was calculated based on the peak area of the product compared to the peak area of the authentic standard (5 mM). N/A represents a compound without authentic standard and the molar concentration could not be calculated.

Label	Compounds	Retention time (min)		(c) Concentration of the product in aqueous ethyl acetate extract fraction			(d) Concentration of the product in solid ethyl acetate extract fraction		
		Products	External Standard	Control (mM)	After LTV treatment (mM)	*Yield (%)	Control (mM)	After LTV treatment (mM)	*Yield (%)
1	Guaiacol	6.99	7.01	-	0.87 ± 0.14	0.14	-	-	-
2	Vanillin	11.52	11.52	0.17 ± 0.01	3.13 ± 0.19	0.60	0.03 ± 0.00	0.05 ± 0.00	0.01
3	Acetovanillone	12.63	12.65	-	1.14 ± 0.09	0.24	-	-	-
4	Homovanillyl alcohol	13.19	13.20	-	1.04 ± 0.12	0.22	-	-	-
5	Vanillic acid	13.53	13.54	0.80 ± 0.01	12.9 ± 1.20	2.76	0.31 ± 0.02	1.35 ± 0.01	0.29
6	Phenol	5.21	5.21	-	0.09 ± 0.01	0.01	-	-	-
7	4-Methylbenzaldehyde	6.92	N/A	-	N/A	N/A	-	-	-
8	Catechol	8.54	8.55	-	0.60 ± 0.04	0.09	-	-	-
9	*p*-Toluic acid	9.56	9.73	-	0.50 ± 0.07	0.08	-	-	-
10	4-Hydroxybenzaldehyde	11.03	10.90	-	0.85 ± 0.03	0.13	-	-	-
11	Tyrosol	11.79	11.77	-	0.28 ± 0.03	0.04	-	-	-
12	Isovanillin	12.24	12.25	-	0.71 ± 0.09	0.11	-	-	-
13	3-hydroxy-1-(4-hydroxy-3-methoxyphenyl)propan-1-one	14.86	N/A	N/A	N/A	N/A	-	N/A	N/A

*Yield of the product was calculated based on the concentration (mol/ L) and the molecular weight (g/ mol) of each compound, and the resulted mass was then compared with the mass of starting material = 2210 mg. The standard error was less than 1 %.

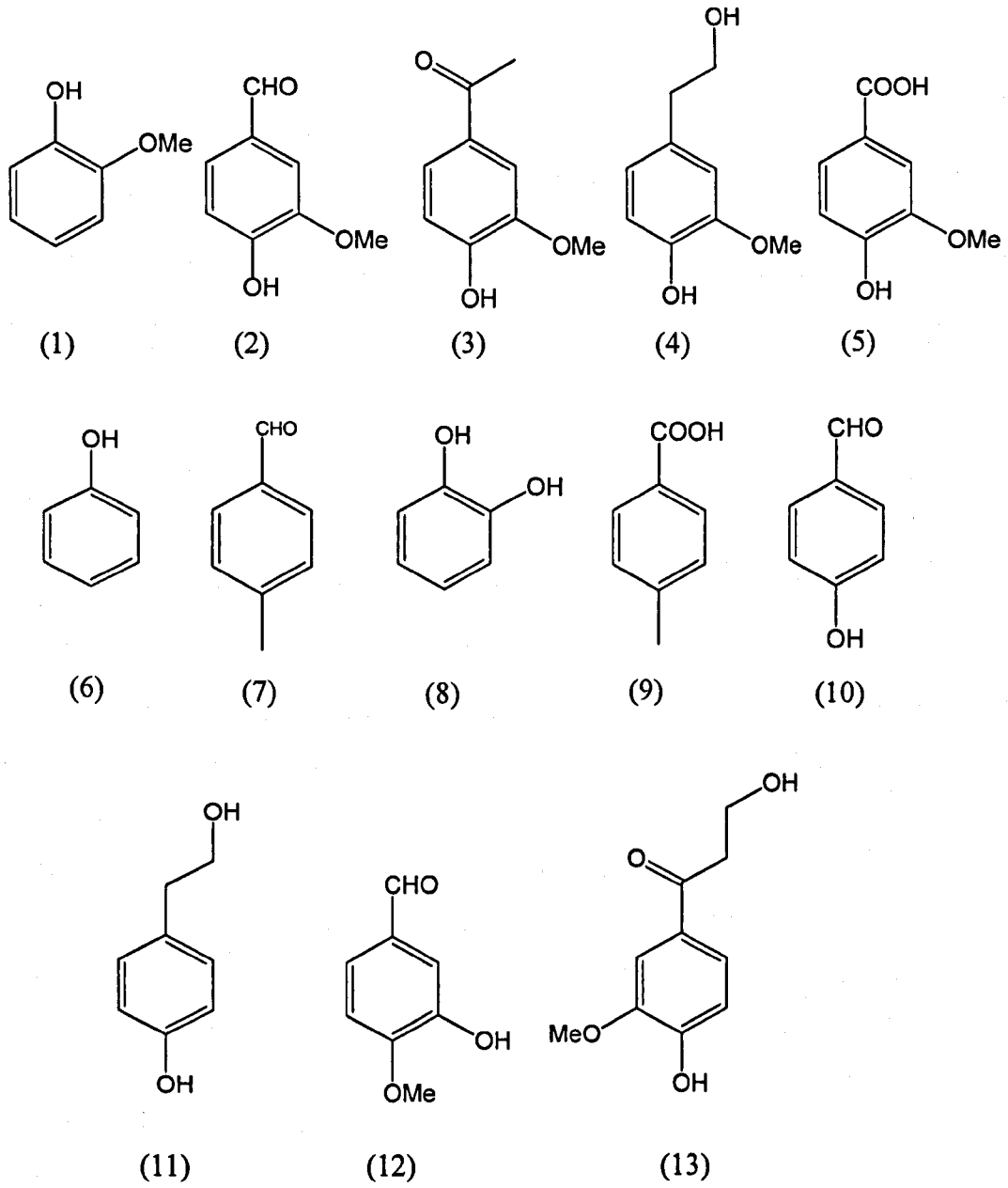


Figure 5.3 Chemical structures of lignin depolymerization products produced from the oxidation of sodium lignosulphonate by LTV and mediated by ABTS. The products were identified by GCMS in the aqueous ethyl acetate extract. The identities of the released compounds are listed in Table 5.3.

product in this fraction should be equal to 9.8 % as shown previously in Table 5.2 (Page 92). There was approximately 5.3 % product loss that may be occurred during quantification analysis by GCMS including the process of dissolving the sample in DCM and filtration. In addition, there might be compounds in aqueous ethyl acetate extract fraction that could not be characterized by GCMS. As discuss earlier in Chapter 4, it can thus be suggested to employ a large column and high flow rates for

product separation by using preparative HPLC technique, thus the isolation and purification of the compounds could be performed. The identity of the purified product can then be characterized by GCMS and NMR.

Fig. 5.4 shows the GCMS chromatograms of both ethyl acetate extract fractions (aqueous and solid). Only vanillin **(2)** was identified in the control sample (Fig. 5.4a and Fig. 5.4d). In the presence of LTV without ABTS, three compounds were observed for the aqueous ethyl acetate extract fraction (Fig. 5.4b) and two compounds in the solid ethyl acetate extract fraction (Fig. 5.4e). These compounds were identified as vanillin **(2)**, vanillic acid **(5)** and 3-hydroxy-1-(4-hydroxy-3-methoxyphenyl) propan-1-one **(13)**. The intensity of the peaks was significantly increased in the presence of ABTS and LTV (Fig. 5.4c).

Three compounds were observed in the solid ethyl acetate extract fraction after the enzymatic treatment with LTV and ABTS which were vanillic acid **(5)**, vanillin **(2)** and 3-hydroxy-1-(4-hydroxy-3-methoxyphenyl) propan-1-one **(13)** (Table 5.3). The concentration of vanillin **(2)** and vanillic acid **(5)** in the solid ethyl acetate fraction were increased by only 0.02 and 1.04 mM respectively after treatment with LTV compared to the control, whereas the concentrations in the aqueous ethyl acetate extract fraction had significantly increased by 1.77 and 12.9 mM respectively.

The above results confirm that LTV has successfully catalyzed the depolymerization of sodium lignosulphonate and that LTV is more active than LAB. The production of guaiacol **(1)**, vanillin **(2)**, acetovanillone **(3)**, homovanillyl alcohol **(4)** and vanillic acid **(5)** were significantly increased compared to the reaction catalyzed by LAB. The comparison of the product formation is shown in Table 5.4.

Table 5.4 The comparison of products formed after enzymatic treatment of sodium lignosulphonate by LAB and LTV.

Compounds	LAB (mM)	LTV (mM)
Guaiacol	0.11 ± 0.01	0.87 ± 0.02
Vanillin	0.28 ± 0.02	3.13 ± 0.10
Acetovanillone	0.05 ± 0.01	1.14 ± 0.02
Homovanillyl alcohol	0.37 ± 0.01	1.04 ± 0.08
Vanillic acid	2.80 ± 0.02	12.9 ± 5.20

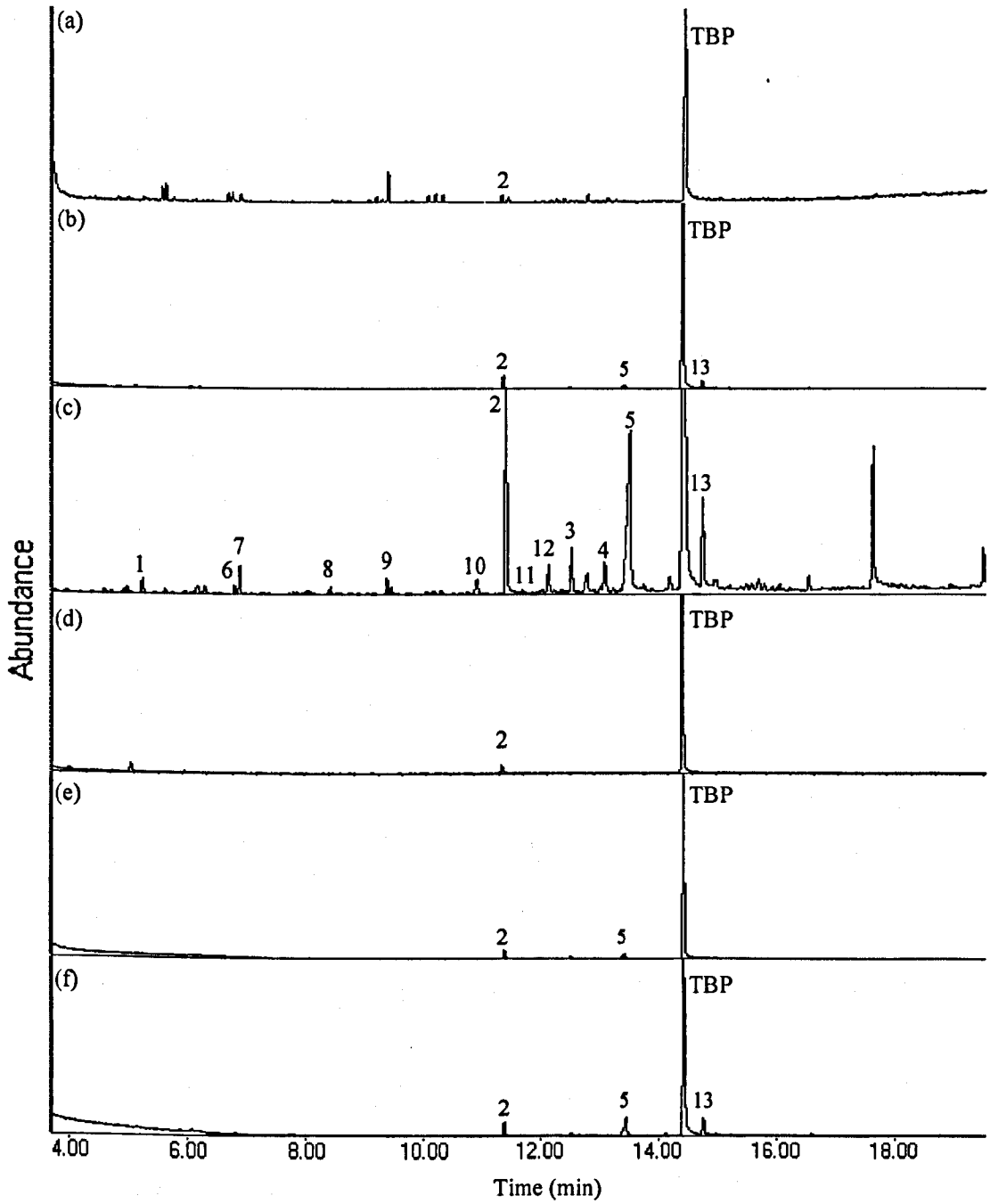


Figure 5.4 The GCMS chromatograms of products that were extracted in ethyl acetate. The identities of the released compounds are listed in Table 5.3. Samples were incubated under identical conditions (at 60 °C for 6 h, shaken at 200 rpm). Fractionation was applied and the dried samples were redissolved in DCM. (a) Aqueous ethyl acetate extract fraction of the control (i), (b) aqueous ethyl acetate extract fraction of the control (ii), (c) aqueous ethyl acetate extract fraction after treatment with LTV and ABTS, (d) solid ethyl acetate extract fraction of the control (i), (e) solid ethyl acetate extract fraction of the control (ii), (f) solid ethyl acetate extract fraction after treatment with LTV and ABTS. All chromatograms were on the same scale of intensity.

5.4.1 Elemental Analysis of the Aqueous Fraction and the Solid Residue

The bulk of the product material was found in the aqueous fraction. However, the characterization of the products in the aqueous fraction and solid residue could not be performed by GCMS (Chapter 4, Section 4.7). Therefore, elemental analysis was implemented in order to obtain the differences between the elements before and after treatment by LTV. Elemental analysis was conducted to obtain the carbon (C), hydrogen (H), nitrogen (N) and sulphur (S) content, as shown in Table 5.5.

Table 5.5 Elemental composition of (a) blank sodium lignosulphonate (control), (b)(i) aqueous fraction catalyzed by LAB, (b)(ii) aqueous fraction catalyzed by LTV, (c)(i) solid residue catalyzed by LAB and (c)(ii) solid residue catalyzed by LTV.

	Element	C	H	N	S
(a)	Sodium lignosulphonate	45.74	5.86	0.03	6.05
(b)	(i) Aqueous fraction (LAB)	36.25	3.86	0.19	6.57
	(ii) Aqueous fraction (LTV)	34.23	3.48	1.19	6.70
(c)	(i) Solid residue (LAB)	37.47	3.94	1.53	5.19
	(ii) Solid residue (LTV)	37.51	3.89	1.48	5.11

After the treatment of sodium lignosulphonate by LTV, the C content of both the aqueous fraction and the solid residue was reduced by 25 and 18 %, respectively. The C content for the LTV-catalyzed reaction was 2 % less than the reaction catalyzed by LAB in the aqueous fraction. Moreover, the H content was also reduced by 41 and 34 % respectively for the aqueous fraction and the solid residue which is slightly lower than for the LAB-catalyzed reaction. This result indicates that the rate of sodium lignosulphonate breakdown by LTV was higher than for the LAB. It is apparent from Table 5.5 that the N and S content in the aqueous fraction were increased by 97 and 9.7 %, respectively. This result may be due to the use of H_2SO_4 to acidify the sample after the reaction was completed and useful information about S content could not be obtained. The S content in H_2SO_4 seems to remains in the aqueous fraction rather than being precipitated into the solid residue. On the other hand the S content in the solid residue decreased by 15 % and the N content

increased by 98 % from the total amount of this element in the standard sodium lignosulphonate.

As discussed earlier in Chapter 4 (Section 4.7), with a small sample size the elemental analysis of the aqueous ethyl acetate extract fraction and the solid ethyl acetate extract fraction could not be performed since there was insufficient solid material after drying. It appears favourable to use either elemental analysis or ^{1}H-NMR to understand the interaction between sodium lignosulphonate and LTV.

5.4.2 GCMS analysis after Derivatization

It was possible that some products of lignin degradation are not volatile and cannot be detected by GCMS without derivatization (Pecina *et al.*, 1986, Takada *et al.*, 2004). Therefore, samples were prepared and fractionated as described previously, evaporated to dryness using a rotary evaporator and then derivatized by adding acetonitrile, trimethylchlorosilane (TMCS) and N,O(bistrimethylsilyl)trifluoroacetamide (BSTFA).

Fig. 5.5 shows the representative chromatograms for the aqueous ethyl acetate extract after derivatization. The peaks present in the chromatogram were identified by comparison with mass spectra in the NIST library and these are attached in Appendix A.3.1 (Page 216). It has to be noted that the quantification was carried out by measurement of the relative areas under each peak which was proportional to the amount of substance that was produced. In order to verify the effect of lignin depolymerization by LTV and mediated by ABTS, the fractionation and derivatization procedure was applied to both controls under the same reaction conditions (Fig. 5.5a and Fig. 5.5b). Propane-1,2-diol **(14)**, 2-hydroxypropanoic acid **(15)** and succinic acid **(22)** were found in the control sample of sodium lignosulphonate without LTV and ABTS (Fig. 5.5a). These compounds were also observed in the control sample of sodium lignosulphonate and LTV without ABTS (Fig. 5.5b).

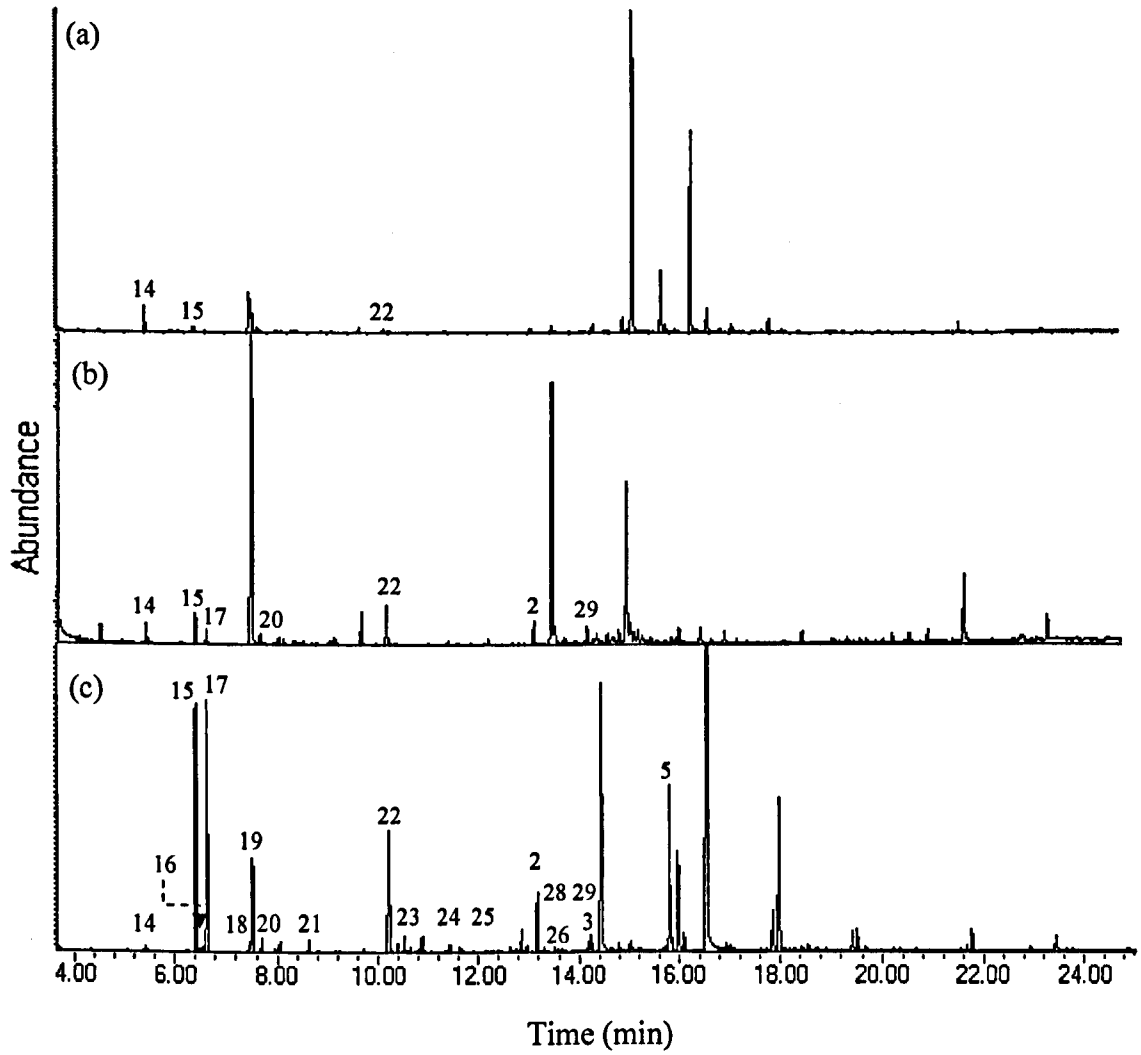


Figure 5.5 The GCMS chromatograms of products that have been extracted in ethyl acetate after derivatization. The identities of the released compounds are listed in Table 5.6. Samples were incubated under identical conditions (at 60 °C for 6 h, shaken at 200 rpm). Fractionation was applied and the dried samples were derivatized by adding acetonitrile (1 ml), trimethylchlorosilane (TMCS) (10 µl) and bistrimethylsilyltrifluroacetamide (BSTFA) (600 µl). The reaction vessel was closed and heated at 70 °C for 1 h. (a) Aqueous ethyl acetate extract fraction of the control (sodium lignosulphonate without LTV and ABTS); (b) aqueous ethyl acetate extract fraction of the control (sodium lignosulphonate and LTV without ABTS); (c) aqueous ethyl acetate extract fraction after treatment with LTV and ABTS. All chromatograms were on the same scale of intensity.

Table 5.6 Identification of products formed after liquid-liquid extraction and derivatization by GCMS. Identification of the products was based on comparison of mass spectra of the NIST library data (Appendix A.3.1, page 216). The data represent the mean of three replicates. The retention times had good reproducibility, with a standard deviation of as shown in the table. The chemical structure of each compound is shown in Fig. 5.6.

Label	Compound	NIST library match	Retention time (min)	Peak area of products $x10^7 \pm$ SD (Lignin + LTV + ABTS)	Peak area of control $x10^7 \pm$ SD	
					Lignin	Lignin +LTV
2	Vanillin	√	13.24	39.8 ± 7.0	-	2.0 ± 0.1
3	Acetovanillone	√	14.25	7.1 ± 1.0	-	-
5	Vanillic acid	√	15.84	7.3 ± 0.4	-	-
14	Propane-1,2-diol	√	5.60	2.8 ± 0.5	2.3 ± 1.2	1.4 ± 0.1
15	2-hydroxypropanoic acid	√	6.54	165.2 ± 23	4.4 ± 0.3	2.3 ± 0.3
16	Hexanoic acid	○	6.68	3.6 ± 0.3	-	-
17	2-hydroxyacetic acid ?	•	6.79	175.3 ± 26	-	1.0 ± 0.1
18	2-hydroxybutanoic acid ?	•	7.61	5.6 ± 0.1	-	-
19	Oxalic acid ?	•	7.68	73.5 ± 12	-	-
20	3-hydroxypropanoic acid	√	7.85	8.0 ± 0.7	-	1.5 ± 0.2
21	Malonic acid ?	•	8.77	7.3 ± 0.5	-	-
22	Succinic acid	○	10.32	80.7 ± 11	3.1 ± 0.2	3.3 ± 0.3
23	2,3-dihydroxypropanoic acid	√	10.63	6.2 ± 0.9	-	-
24	Glutaric acid ?	•	11.53	6.0 ± 0.7	-	-
25	2,4-dihydroxybutanoic acid	○	11.72	3.3 ± 0.3	-	-
26	5-(hydroxymethyl)furan-2-carboxylic acid	√	13.4	3.2 ± 0.3	-	-
27	3-hydroxybenzoic acid	√	13.58	3.4 ± 0.2	-	-
28	2-hydroxypentanedioic acid	√	13.73	2.3 ± 0.1	-	-
29	4-hydroxybenzoic acid	√	14.30	11.3 ± 0.8	-	1.7 ± 0.3

(√: a good match with the NIST library, ○: tentatively identified and •: poor match with NIST library)

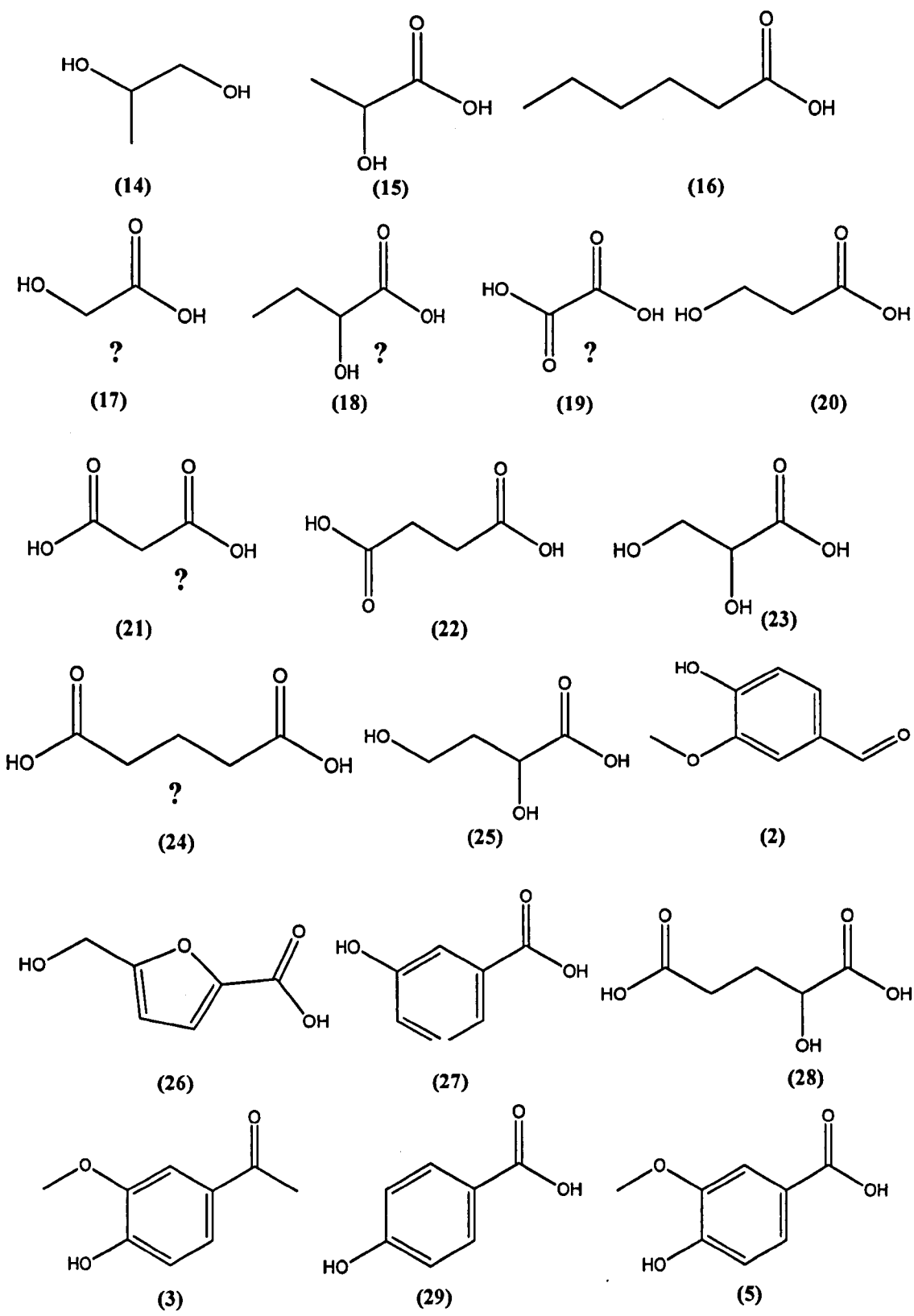


Figure 5.6 Chemical structures of the lignin depolymerization products identified by GCMS in the aqueous ethyl acetate extract after derivatization. The identities of the compounds released are listed in Table 5.6.

In addition, 3-hydroxypropanoic acid **(20)**, vanillin **(2)** and 4-hydroxybenzoic acid **(29)** were also apparent together with an unknown compound labelled as **(17)**. The intensity of the peaks for these compounds was increased by the addition of ABTS to the reaction mixtures. This finding further supports the idea that depolymerization of lignin may not occur if ABTS is not present. The other peaks without a label were contamination peaks. It is apparent from Table 5.6 that the amount of 2-hydroxypropanoic acid **(15)** was increased by 98 % if compared to the control sample of sodium lignosulphonate and LTV without ABTS. 3-hydroxypropanoic acid **(20)**, succinic acid **(22)**, vanillin **(2)**, 4-hydroxybenzoic acid **(29)** and the unknown compound **(17)** were also increased by 81, 96, 95, 85 and 99 % respectively. The other compounds that have been identified are listed in Table 5.6; of these, a close match of the unknown compound with the NIST library was labelled as compound number **(17)**, **(18)**, **(19)**, **(21)** and **(24)** were listed. The production of 2,3-Dihydroxypropanoic acid **(23)** may be resulted from the oxidation of α-carbon and the reduction of β-carbon as illustrated in Fig. 5.7. 2,3-Dihydroxypropanic acid **(23)** was further oxidized to produce 2-hydroxypropanoic acid **(15)** and 3-hydroxypropanic acid **(20)**. Propane-1,2-diol **(14)** was also one of the products resulted from the cleavage of β-O-4 linkage (Fig. 5.7).

One interesting finding that needs to be pointed out is that the majority of the compounds that have been identified were aliphatic. If compared to the method of GCMS analysis without derivatization, the aliphatic compounds could not be detected. Surprisingly, the aromatic compounds that were identified without using the derivatization method were not apparent except for vanillin **(2)**, acetovanillone **(3)** and vanillic acid **(5)**. Since the production of these compounds was higher than for the other compounds (Table 5.3), it can thus be suggested that the detection after derivatization by GCMS was limited by the concentration of the compounds. Therefore, only a few compounds with a higher concentration were able to be silylated using the derivatization method applied in this project and further optimization is needed.

The results of this study suggest that the derivatization method for GCMS analysis has identified the compounds that could not be detected without silylating the samples. Therefore the method was successfully adapted for the identification of 11

new compounds from the depolymerization of sodium lignosulphonate by LTV and mediated by ABTS. Further work is needed to verify the identity of the products and determine the product concentrations using authentic standards.

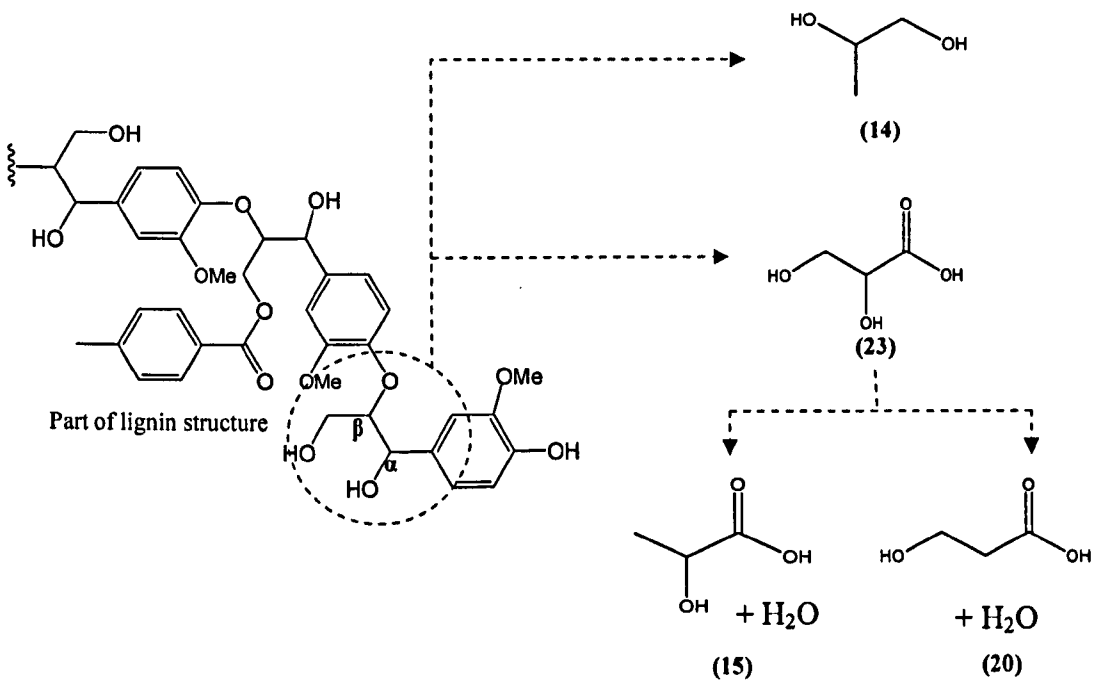


Figure 5.7 The production of aliphatic compounds from lignin. 2,3-dihydroxypropanoic acid **(23)**, 2-hydroxypropanoic acid **(15)**, 3-hyroxypropanoic acid **(20)** and propane-1,2-diol **(14)**.

5.5 Attempts to Quantify Products by HPLC with UV detector

Following the identification of the products by GCMS, an attempt was made to quantify the products by HPLC using the same sample but without fractionation in order to obtain the identity of products that could not be detected by GCMS. After the enzymatic treatment of sodium lignosulphonate by LTV, the aqueous sample was injected directly *via* autosampler for HPLC analysis. There was only one peak observed in the control sample (sodium lignosulphonate). After enzymatic treatment, there were three peaks observed (Fig. 5.8). However, the identity of these compounds could not be confirmed since the products peak did not match the retention time of the authentic standards. Contradictory to expectation, the HPLC analysis without fractionation had failed to separate each individual compounds from the reaction.

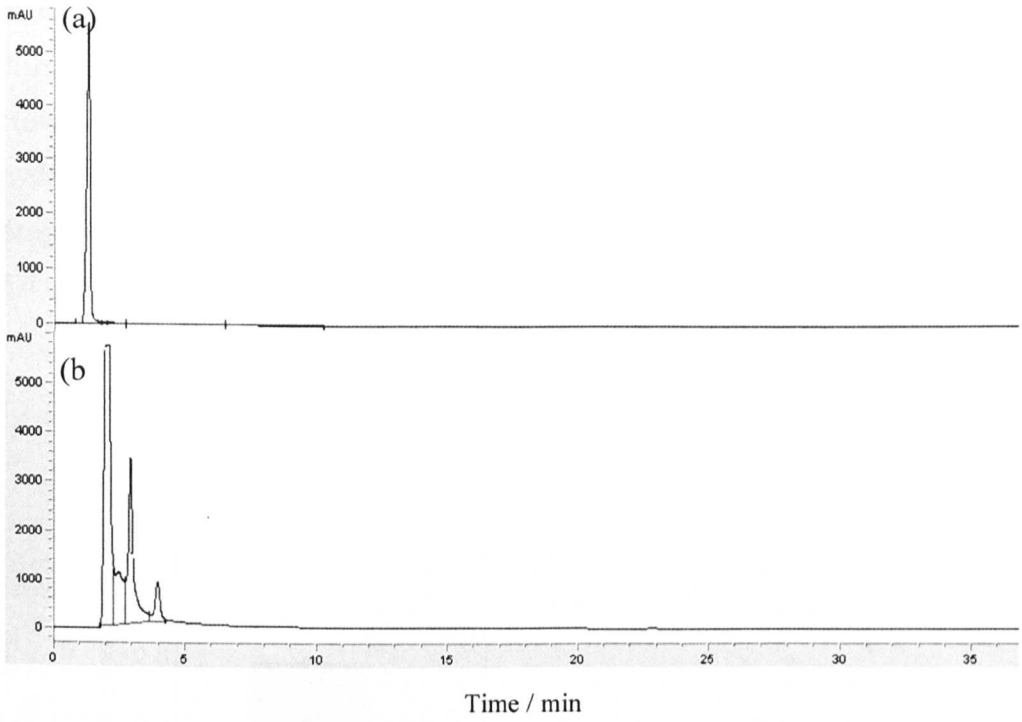


Figure 5.8 HPLC chromatograms of the products formed after enzymatic treatment of sodium lignosulphonate by LTV and mediated by ABTS. Samples were incubated under identical conditions (at 60 °C for 6 h, shaken at 200 rpm). Fractionation was not applied. (a) the chromatogram of sodium lignosulphonate (control) and (b) the chromatogram after enzymatic treatment.

Therefore, fractionation was employed and the samples were prepared by extracting 32 ml of reaction mixture with 120 ml of ethyl acetate. The extract was divided, dried and the residues were dissolved in dichloromethane (DCM) (1 ml) for GCMS analysis and methanol/water (1 ml) for HPLC analysis. The product concentrations were calculated using the standard calibration curves (Appendix A.4.2 – page 227). The identification of the products was confirmed *via* the retention times of the authentic standards. Although numerous peaks were observed by HPLC, only five peaks were large enough to obtain firm identification and quantification of the chemicals. Fig. 5.9 presents the concentration of products formed after enzymatic treatment at 60 °C for 6 h.

In contrast to the quantification by GCMS, only 1.32 mM of vanillic acid and 0.56 mM of vanillin were observed after 6 h. GCMS analysis indicated that vanillic acid and vanillin could be detected by up to 12.9 and 3.13 mM respectively, after 6 h

of incubation. In addition, homovanillyl alcohol, acetovanillone and guaiacol were also observed in concentrations of 0.78 mM, 0.09 mM and 0.46 mM, respectively. However, phenol, 4-methylbenzaldehyde, catechol, *p*-toluic acid, 4-hydroxybenzaldehyde, tyrosol and 3-hydroxy-1-(4-hydroxy-3-methoxyphenyl) propane-1-one were detected in GCMS but could not be detected by HPLC (Table 5.7).

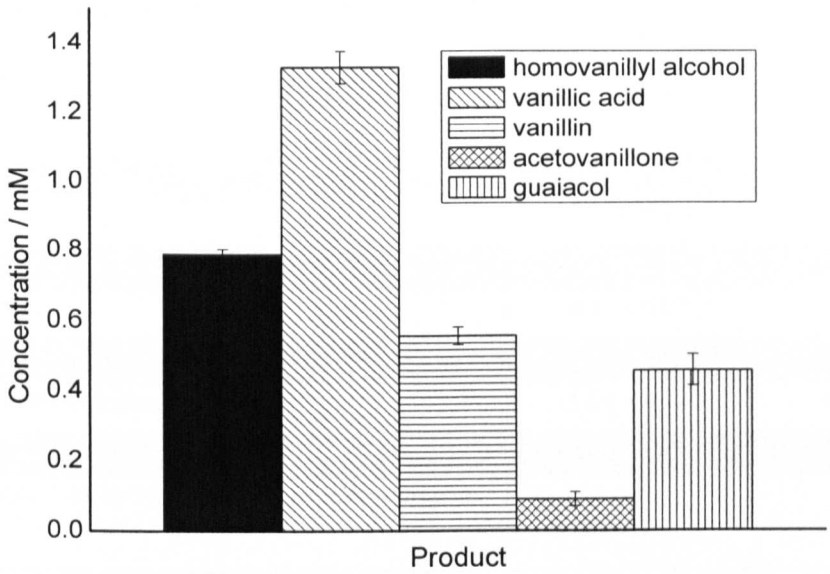


Figure 5.9 Concentration of lignin depolymerization products formed at 60 °C. The reaction mixtures included ammonium acetate buffer (pH 4.5, 100 mM), lignin (50 g/L), ABTS (5 mM) and LTV (0.25 mg/ml) and were shaken at 200 rpm for 6 h. Fractionation was applied and the aqueous ethyl acetate extract was evaporated to dryness and redissolved in methanol and water in the ratio of 1:9. Product was quantified by HPLC based on standards calibration curve. The data represent the mean of three replicates.

It has to be noted that the quantification analysis by GCMS and HPLC was different. This may be due to the use of different solvents in both analyses (DCM for GCMS analysis and methanol/water (1:9) for HPLC analysis). For instance, DCM is a less polar solvent than water. Since the products were soluble in ethyl acetate (polarity of ethyl acetate is less than the water/methanol), it is possible that the products mixture used for HPLC were less soluble in water/methanol than ethyl acetate. As a result, a smaller amount of product was observed by HPLC analysis. It can thus be suggested

that HPLC analysis is not a suitable technique to quantify the lignin depolymerization products.

Table 5.7 Comparison of products concentration between GCMS and HPLC.

Compounds	Product concentration (mM)	
	GCMS	HPLC
Phenol	0.09 ± 0.01	n.d
4-Methylbenzaldehyde	n.a	n.d
Guaiacol	0.87 ± 0.14	0.46 ± 0.05
Catechol	0.60 ± 0.04	n.d
p-Toluic acid	0.50 ± 0.07	n.d
4-Hydroxybenzaldehyde	0.85 ± 0.03	n.d
Vanillin	3.13 ± 0.19	0.56 ± 0.01
Tyrosol	0.28 ± 0.03	n.d
Isovanillin	0.71 ± 0.09	n.d
Acetovanillone	1.14 ± 0.09	0.09 ± 0.01
Homovanillyl alcohol	1.04 ± 0.12	0.78 ± 0.01
Vanillic acid	12.9 ± 1.20	1.32 ± 0.04
3-hydroxy-1-(4-hydroxy-3-methoxyphenyl)propan-1-one	n.a	n.d

n.a: not applicable (authentic standard is not available, therefore molar concentration could not be calculated; n.d: not detected (compound not detected by HPLC)

5.5.1 Effect of Incubation Time on Product Formation at 60 °C

In previous experiments with LAB, the optimum incubation time for most of the products was between 6 – 8 h. However, the optimum incubation time for LTV was unknown. Therefore, a study into the effect of incubation time on the distribution of lignin depolymerization products was performed at 60 °C by incubating lignin, ABTS and LTV over 24 h. Samples were taken, fractionated and analyzed by GCMS. The aqueous ethyl acetate extract was evaporated to dryness and redissolved in DCM. Vanillic acid **(5)** was observed as the major product of the enzymatic treatment of sodium lignosulphonate by LTV. The production of this compound was dramatically increased in the first hour and then slightly increased until 4 h of incubation time. The concentration then dropped until 24 h (Fig. 5.10). The same

pattern was also observed in the production of acetovanillone (3) and guaiacol (1). It can therefore be concluded that the optimum production of vanillic acid, acetovanillone and guaiacol can be achieved during 4 h of incubation time. It is interesting to note that amongst the five compounds detected, only the production of vanillin increased over time, and appeared to be continuing even after 24 h. In contrast to vanillin, the optimum production of homovanillyl alcohol was achieved during 1 h and then dropped over time until it disappeared after 8 h (Fig. 5.10). Most of the products were reduced over longer incubation period which suggests that LTV was losing activity at 60 °C, and that the products were being transformed to other substances. Therefore, further experiments were designed to test these hypotheses.

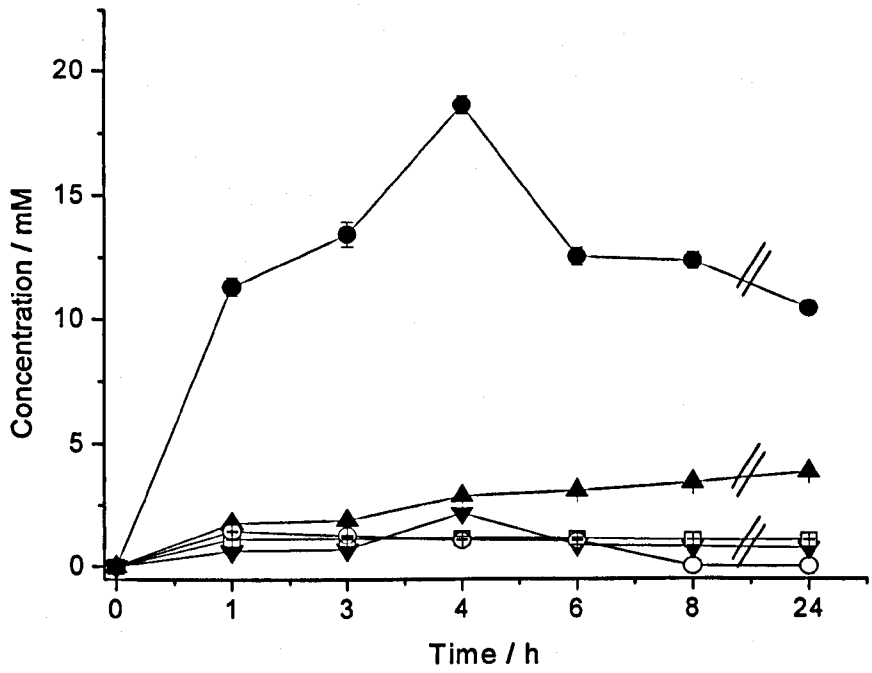


Figure 5.10 Effect of incubation time on products formation between 1 to 24 h, vanillic acid (•), homovanillyl alcohol (○), vanillin (▲), acetovanillone (□) and guaiacol (▼).The reaction mixtures included ammonium acetate buffer (pH 4.5, 100 mM), lignin (50 g/L), ABTS (5 mM) and LTV (0.25 mg/ml) and were shaken at 200 rpm at 60 °C for 6 h. Fractionation was applied and the aqueous ethyl acetate extract was evaporated to dryness and redissolved in DCM. The samples were sacrificed at intervals to measure the activity. The data represent the mean of three replicates.

5.5.1.1 Thermal Stability of LTV

The effect of temperature on LTV stability was determined spectrophotometrically following the LTV catalyzed oxidation of ABTS at temperatures ranging from 30 to 60 °C at 10 °C intervals. The thermal stability of LTV at each set temperature was investigated by performing assays at time intervals (Fig. 5.11). LTV lost 85 % of the activity after only 1 h of incubation at 60 °C. The activity decreased steadily until LTV was completely deactivated at 7 h. This result explains why product formation had stopped when LTV was used for lignin oxidation at 60 °C. Although LTV was most active at 60 °C (Fig. 5.2), it is not stable at this temperature over the long incubation period needed to obtain lignin depolymerization. Furthermore, LTV lost approximately 70, 60 and 40 % of its activity when it was incubated at 50, 40 and 30 °C after 4 h respectively (Fig. 5.11). This confirms that LTV is not stable at 60 °C. It is therefore envisaged that the products yield will be increased by reducing the incubation temperature. Therefore, the incubation temperature was reduced to 30 °C for further experimental work.

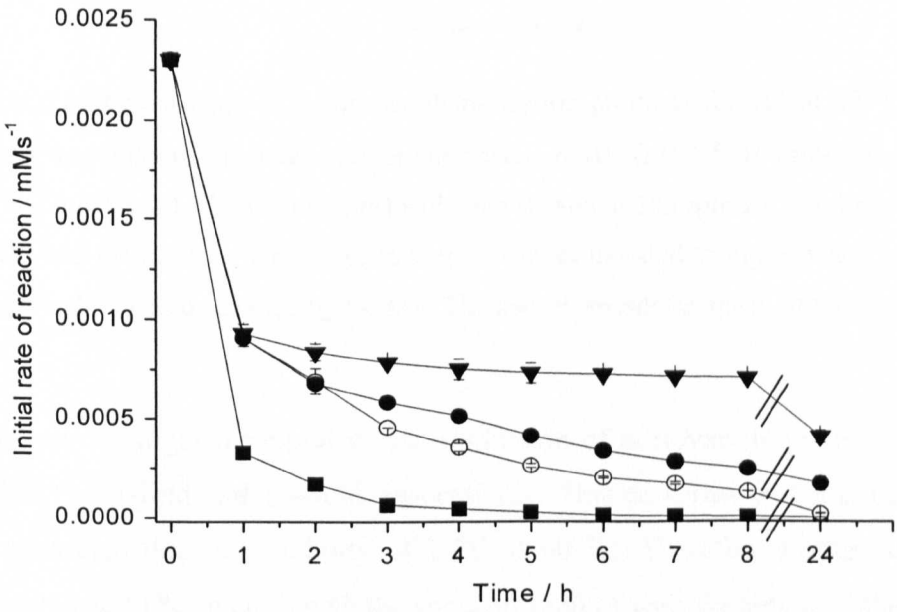


Figure 5.11 Thermal stability of LTV at 30 to 60°C from 1 to 24 h of incubation period. The stability of LTV was determined spectrophotometrically by incubating LTV solution in ammonium acetate buffer (pH 4.5, 100 mM) in cuvettes at 30 (▼), 40 (●), 50 (○) and 60°C (■). The samples were sacrificed at intervals to measure the activity. The activity was started by the addition of ABTS (5 mM). The absorbance changes were determined at 420nm. The data represent the mean of three replicates.

5.5.2 Effect of Temperature on Lignin Depolymerization

The effects of incubation temperature on the production of chemicals from lignin by LTV were investigated (Fig. 5.12). The quantification was conducted by GCMS. The product concentrations were higher at 30 °C than at 60 °C except for vanillin. The production of vanillic acid and guaiacol were increased dramatically to 3-fold and 2.4-fold, respectively.

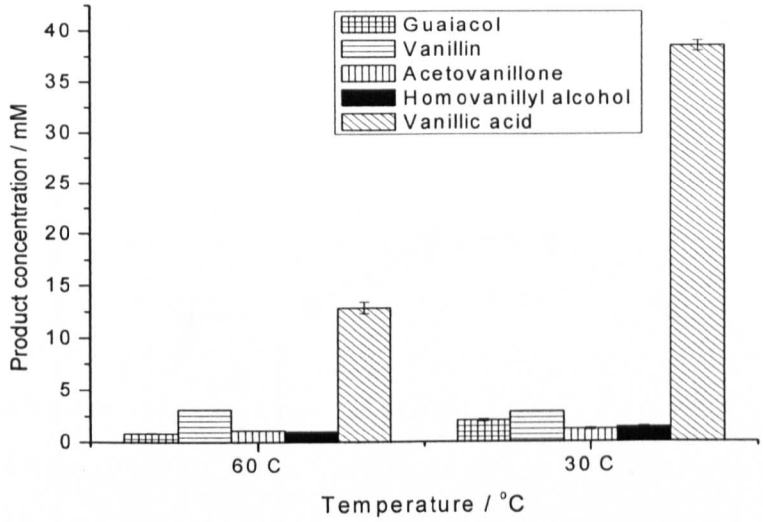


Figure 5.12 Comparison of lignin depolymerization products formed at 60 °C and 30 °C. The reaction mixtures included ammonium acetate buffer (pH 4.5, 100 mM), lignin (50 g/L), ABTS (5mM) and LTV (0.25 mg/ml) and were shaken at 200 rpm for 6 h. Fractionation was applied and the aqueous ethyl acetate extract was evaporated to dryness and redissolved in DCM. Product was quantified by GCMS. The data represent the mean of three replicates.

There were a slight increased in the production of acetovanillone and homovanillyl alcohol to 1.1-fold and 1.4-fold, respectively. This confirms that product formation was restricted due to instability of LTV at 60 °C. Therefore further studies were conducted at 30 °C even though the concentration of vanillin remained the same.

5.6 Effect of Incubation Time on Product Formation at 30 °C

In this work, the effect of incubation time on lignin depolymerization by LTV at 30 °C was explored. Samples were prepared by incubating sodium lignosulphonate,

LTV and ABTS for different reaction times, and fractionating the samples and analyzing by GCMS. All of the products were produced rapidly, within the first hour of the incubation period. The control (sodium lignosulphonate without LTV and ABTS) contained very small quantities of vanillin and vanillic acid but the quantities increased by 97 and 98 % respectively during the first hour of incubation with LTV and ABTS. Vanillic acid was observed as the major product (Fig. 5.13), which is consistent with the finding at 60 °C. The concentration of vanillic acid and guaiacol were increased slightly by 2.4 and 1.8 %, respectively from 1 to 3 h and these compounds reached optimum production at 4 h with 39.4 and 2.88 mM, respectively.

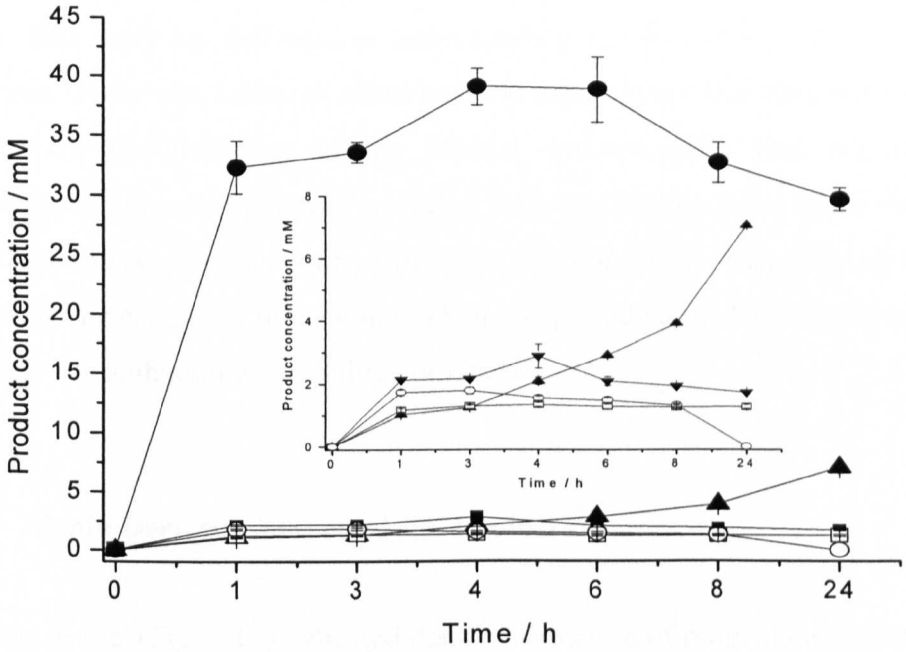


Figure 5.13 The effect of incubation time on the product yield formed from the depolymerization of sodium lignosulphonate by LTV at 30 °C. Vanillic acid (●), homovanillyl alcohol (○), vanillin (▲), acetovanillone (□) and guaiacol (▼). The reaction mixtures include ammonium acetate buffer (pH 4.5, 100 mM), lignin (50 g/l), ABTS (5mM) and LTV (0.25 mg/ml). The reaction mixture was shaken at 200 rpm over different incubation times. Fractionation was applied and the aqueous ethyl acetate extract was redissolved in DCM. Each product was quantified based on the authentic standard. The data represent the mean of three replicates. (a) shows all product concentration on the scale; (b) shows on homovanillyl alcohol, vanillin, acetovanillone and guaiacol on a smaller scale, to demonstrate differences in production kinetics.

After 6 h, a reduction in vanillic acid and guaiacol concentration were observed and the concentrations steadily declined thereafter until 24 h. The optimum production of acetovanillone was also achieved at 4 h (1.33 mM). However, the production of this compound remained constant between 8 to 24 h. On the other hand, the concentration of vanillin was increased over time until the maximum concentration was achieved (7.05 mM) at 24 h. It can therefore be assumed that the production of vanillin may be increased over a longer incubation period. In contrast to vanillin, homovanillyl alcohol reached maximum production at 1 h and reduced slightly over 8 h of incubation time. No trace of homovanillyl alcohol was observed after 24 h.

Contrary to expectations, the product yield decreased over a longer incubation period. This study has delivered an understanding that the stability of LTV is not the only issue. There are, however, other possible explanations that may possibly be put forward for the reduction of the product concentrations that require further investigation. Some of the issues emerging from this finding relate specifically to the individual compounds that formed from the reaction. It can therefore be suggested that further investigation of each individual compound has to be undertaken to study the effect of incubation time on these products.

5.7 Lignin Derived Compounds as a Substrate

Previous results (Fig. 5.13) indicated that the formation of products was reduced over a longer incubation period except for vanillin, suggesting that they were further converted to other products. This hypothesis was tested by using five lignin derived compounds (guaiacyl derivative units) as the substrate. The method was adapted following the method described by Fabbrini *et al.* (2001) on the oxidation of non-phenolic substrates by laccase from *Trametes villosa* in the presence of various mediators. However, a slight change was employed by shaking the mixtures at the optimized temperature for LTV at 30 °C. The enzymatic conversions of vanillin, acetovanillone, guaiacol, vanillic acid and homovanillyl alcohol were catalyzed by LTV in the presence of ABTS. All five substrates were chosen because they were formed during depolymerization of sodium lignosulphonate.

5.7.1 The Oxidation of Vanillin

The transformation of vanillin by LTV produced a compound which was identified as 2-methoxyhydroquinone (**30**) after 2 h of reaction by comparison of the mass spectrum of this compound with the NIST library (Fig. 5.14). The production of 2-methoxyhydroquinone is perhaps significant in this process as hydroquinone is well known as an intermediate in the lignin degradation process (Szklarz and Leonowicz, 1986) and 2-methoxyhydroquinone is a known product of vanillic acid degradation (Ander *et al.*, 1983). However, the production of 2-methoxyhydroquinone was relatively low with a 0.01 % yield (Table 5.8). The disappearance of 2-methoxyhydroquinone after 6 h confirmed that this compound further reacted with the LTV. It would appear that the LTV oxidized vanillin to become vanillic acid identified by comparison of the retention time and mass spectrum of an authentic standard as attached in Appendix A.5.1 (page 228) after 6 h of incubation time. This compound was increased from 0.24 to 0.46 % yield (Table 5.8) until 24 h. Furthermore, acetovanillone (identified by comparison with the authentic standard) was also observed at 6 h but had disappeared after 24 h.

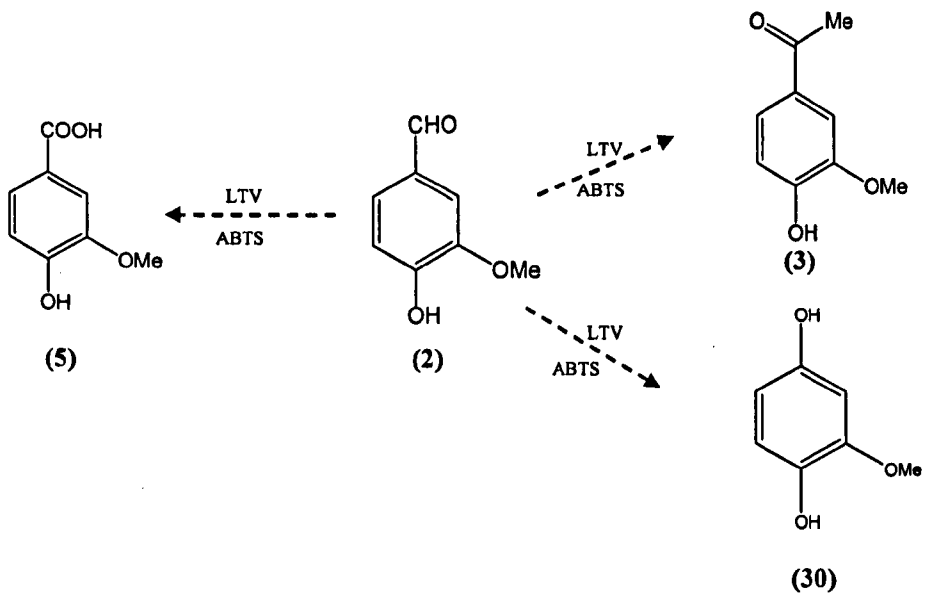


Figure 5.14 Oxidation of vanillin (**2**) to 2-methoxyhydroquinone (**30**), acetovanillone (**3**) and vanillic acid (**5**). The reaction mixtures included ammonium acetate buffer (pH 4.5, 100 mM), vanillin (20 mM), ABTS (5 mM) and LTV (0.25 mg/ml) and were shaken at 200 rpm for 2, 6 and 24 h. Fractionation was applied and the ethyl acetate extract was evaporated to dryness and redissolved in DCM for GCMS analysis. The result shows a good agreement with the NIST library and the authentic standards which were available for (**3**) and (**5**) but not (**30**) as attached in Appendix A.5.1, page 228.

Table 5.8 Products formed in the conversion of compounds representative of lignin catalyzed by LTV in the presence of ABTS.

Reactant (20 mM)	Converted products		NIST library match	Availability of standard	RT (min)	Yield (%)		
	Label	Product				2 h	6 h	24 h
Vanillin	30	2-methoxyhydroquinone	√	X	11.31	0.01 ± 0.00	n.d	n.d
	3	Acetovanillone	√	√	12.56	n.d	0.44 ± 0.01	n.d
	5	Vanillic acid	√	√	13.37	n.d	0.24 ± 0.00	0.46 ± 0.01
Acetovanillone	31	2-methoxyphenyl acetate	○	X	9.48	0.06 ± 0.00	0.07 ± 0.00	0.07 ± 0.00
	32	4-acetyl-2-methoxyphenyl acetate	○	X	14.20	0.17 ± 0.01	0.05 ± 0.00	0.06 ± 0.00
	33	1-(2,6-dihydroxy-4-methoxyphenyl)-ethanone	○	X	16.53	0.12 ± 0.00	0.07 ± 0.00	n.d
	34	Benzoic acid, 4-methoxy-3-(4-methoxycarbonylphenoxy)-, methyl ester	•	X	22.8	n.d	0.01 ± 0.00	0.01 ± 0.00
Guaiacol	35	1-hydroxy-3,5,6-trimethoxyxanthone	•	X	19.17	n.d	0.04 ± 0.00	0.05 ± 0.00
	36	4-4'-biguaiacol	√	X	20.42	n.d	0.09 ± 0.00	0.14 ± 0.01
Vanillic acid	1	Guaiacol	√	√	6.98	4.63 ± 0.06	4.18 ± 0.05	n.d
	30	2-methoxyhydroquinone	√	X	11.31	1.71 ± 0.04	2.10 ± 0.01	1.03 ± 0.02
	2	Vanillin	√	√	11.26	8.08 ± 0.10	14.18 ± 0.90	26.0 ± 3.28
	37	Methyl vanillate	•	X	12.74	0.40 ± 0.03	0.74 ± 0.08	1.47 ± 0.07
Homovanillyl alcohol	2	Vanillin	√	√	11.26	n.d	0.16 ± 0.01	0.24 ± 0.02
	38	2-methoxy-4-propyl phenol	√	X	12.02	0.33 ± 0.05	1.17 ± 0.06	4.90 ± 0.43
	39	Homovanillic acid	○	X	12.40	0.06 ± 0.00	0.14 ± 0.08	0.21 ± 0.04
	40	4-hydroxy-3-methoxyphenylglycol	•	X	15.12	n.d	5.68 ± 0.87	24.9 ± 2.49

(√: a good match with the NIST library, ○: tentatively identified and •: poor match with NIST library). n.d, not detected.

The conversion of vanillin was increased over time. The remaining vanillin in the reaction mixture was decreased from 59.6 after 2 h and 35.3 after 6 h to 22 % after 24 h (Table 5.9) of the starting vanillin. However, the yield of detectable products did not increase. This result suggested that the longer incubation period may have produced compounds that could not be extracted in ethyl acetate or with higher boiling points that could not be detected by GCMS.

Table 5.9 The percentage of unconverted reactant and undetected product. The percentage was calculated based on the peak area of the authentic standard (5 mM).

Reactant	Reactant unconverted (%)			Product undetected (%)		
	2 h	6 h	24 h	2 h	6 h	24 h
Vanillin	59.6 ± 1.4	35.3 ± 2.1	22.0 ± 3.5	40.4 ± 2.4	64.0 ± 4.3	77.5 ± 5.8
Acetovanillone	29.6 ± 1.7	16.6 ± 0.9	11.0 ± 0.9	70.1 ± 4.8	83.2 ± 5.9	88.9 ± 8.2
Guaiacol	-	1.1 ± 0.01	1.46 ± 0.02	-	98.8 ± 9.6	98.4 ± 8.7
Vanillic acid	20.5 ± 1.2	39.1 ± 1.5	45.0 ± 3.5	64.6 ± 4.5	40.0 ± 3.7	11.5 ± 2.5
Homovanillyl alcohol	50.5 ± 3.4	32.5 ± 4.1	31.2 ± 3.2	49.1 ± 3.5	60.4 ± 5.9	38.6 ± 4.7

Therefore, fingerprint analysis by ^{1}H-NMR was used to obtain indications of changes in chemical composition catalyzed by the enzyme (Fig. 5.15). The spectrum of vanillin shows the chemical shift of the aldehyde proton (9.66 ppm), aromatics quartet, duplet and singlet at 7.28, 7.24 and 6.91 ppm respectively. The methoxy group was observed at 3.95 ppm (Fig. 5.15a).

The NMR spectrum shows the remaining vanillin after the enzymatic treatment by the observation of aromatic, methoxy and aldehyde proton peaks of this compound (Fig. 5.15b). The solvent (DMSO) and buffer (acetate) proton peaks were observed at 2.50 and 1.80 ppm, respectively. There was a major compound with the quartet aromatic proton peaks observed at 6.70 and 7.09 ppm, and singlet peak of proton attached to the H-C-OR group at 4.34 ppm. There are other minor products observed with the aromatic proton between 7.51 to 8.13 ppm and H-C-OR groups at 4.25 to 4.39 ppm. The finding of the new proton peaks indicates the production of new chemicals from vanillin. However, the identification of the products formed could not be confirmed.

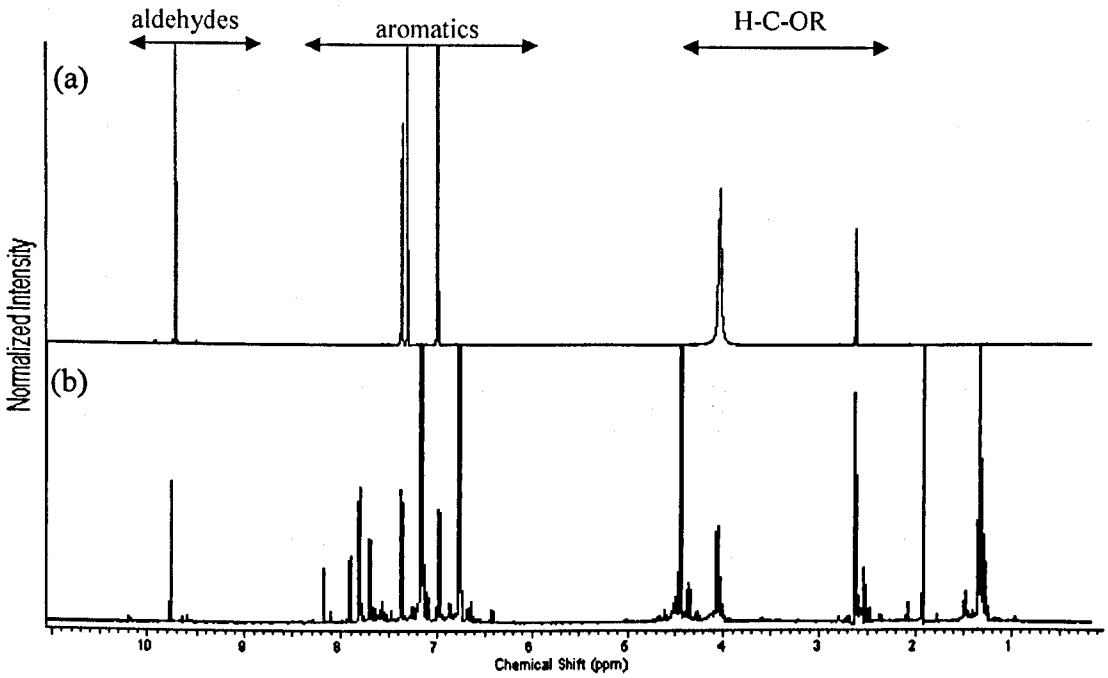


Figure 5.15 The ^{1}H-NMR spectrum of the products formed after enzymatic treatment of vanillin by LTV. The reaction mixtures contained vanillin (20 mM) dissolved in ammonium acetate buffer (100 mM; pH 4.5), ABTS (5 mM) and LTV (0.25 mg/ml) and shaken at 200 rpm for 6 h at 30 °C. The product mixtures were evaporated to dryness and dissolved in DMSO-d_6 and D_2O in the ratio of 8:2. Spectra were on different scales (the spectrum of authentic standard was 4.5-fold higher than the spectrum of the reaction mixtures and double solvent presaturation at 4.10 and 2.44 ppm was applied on the analysis after enzymatic treatment) (a) Authentic standard of 5 mM vanillin (b) The product mixture after enzymatic treatment of 20 mM vanillin by LTV and ABTS.

5.7.2 The Oxidation of Acetovanillone

LTV catalyzed the oxidation of acetovanillone and produced four different compounds in which two of the compounds were very tentatively identified as acetate esters (Fig. 5.16) by comparing the mass spectra of the released compound with the NIST library. The possibility of obtaining acetate esters may have been caused by the use of ammonium acetate as the reaction buffer. It can therefore be assumed that the acetate buffer reacted with the products that resulted in the production of acetate based compounds. Possible dimerization was observed

although the identification as 4-methoxy-3-(4-methoxycarbonylphenoxy) benzoic acid (**34**) cannot be confirmed. There was 29.6 % unreacted acetovanillone during 2 h of incubation period and this amount was reduced to 16.6 and 11 % after 6 and 24 h, respectively (Table 5.9). There were more than 88.9 % of products that could not be detected by GCMS (Table 5.9).

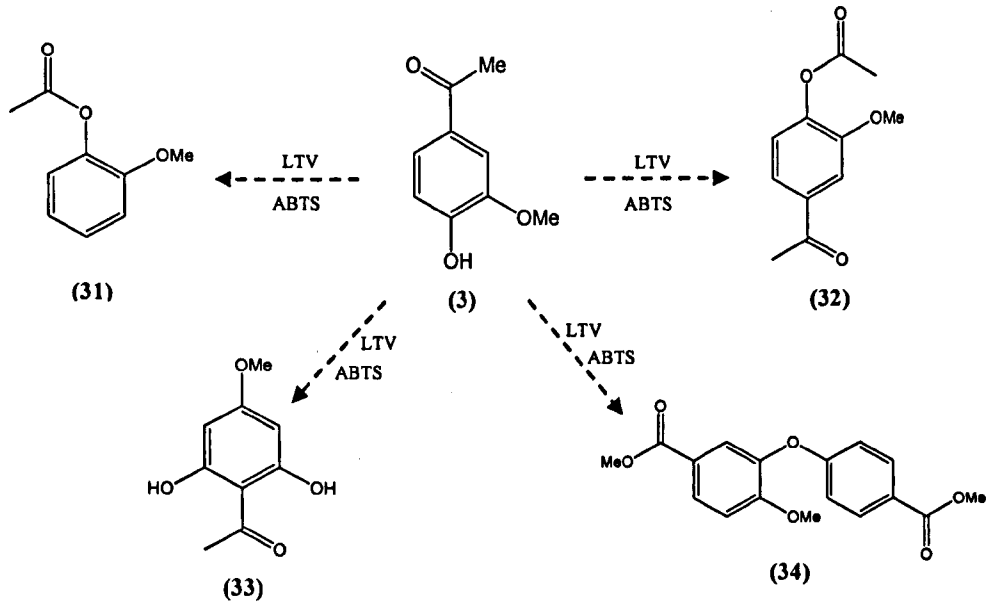


Figure 5.16 Oxidation of acetovanillone (**3**) to 2-methoxyphenyl acetate (**31**), 4-acetyl-2-methoxyphenyl acetate (**32**), 1-(2,6-dihydroxy-4-methoxyphenyl)-ethanone (**33**) and 4-methoxy-3-(4-methoxycarbonylphenoxy)benzoic acid (**34**). The reaction mixtures included ammonium acetate buffer (pH 4.5, 100 mM), acetovanillone (20 mM), ABTS (5 mM) and LTV (0.25 mg/ml) and were shaken at 200 rpm for 2, 6 and 24 h. The sample was evaporated to dryness and redissolved in DCM for GCMS analysis. The compounds were identified by comparison of the mass spectrum with the NIST library as attached in Appendix A.5.2, page 230.

The ^{1}H-NMR spectrum of acetovanillone shows the chemical shift of the aromatics quartet, duplet and singlet at 6.53, 6.71 and 6.63 ppm respectively. The methoxy group was observed at 3.94 ppm and the methyl at 2.17 ppm (Fig. 5.17a). The solvent (DMSO) and buffer (acetate) proton peaks were observed at 2.50 and 1.90 ppm, respectively. In contrast to the result by GCMS, acetovanillone protons were not detected in the reaction media after enzymatic treatment indicates that acetovanillone was fully converted (Fig. 5.17b). ^{1}H-NMR spectrum shows that there are five methoxy groups observed as a single peak at 3.70, 3.71, 3.79, 3.81 and a

quartet at 3.93 ppm. There was also a single peak of hydrogen adjacent to H-C-OR proton at 4.39 ppm. From the observation, the aromatic protons between 6.80 to 8.12 ppm were increased, which indicates the production of new compounds from the reaction. The aldehyde proton was also observed at 9.79 ppm which indicates the production of aldehyde compound from acetovanillone. However, this compound could not be detected by GCMS. Since the product mixture was complex, the identification of each individual compound could not be performed.

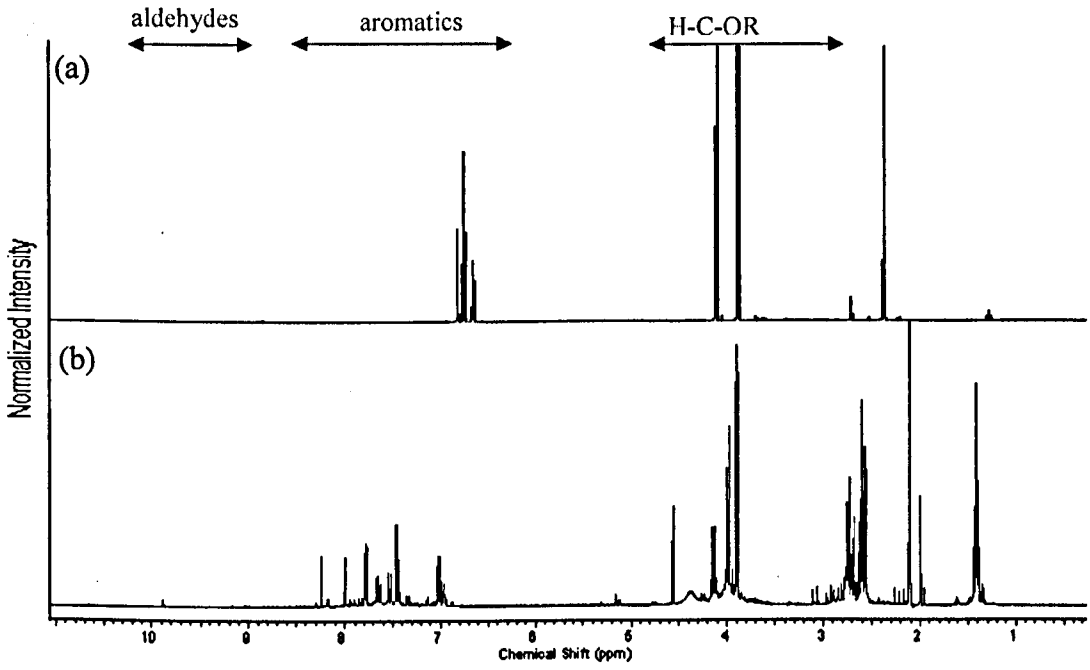
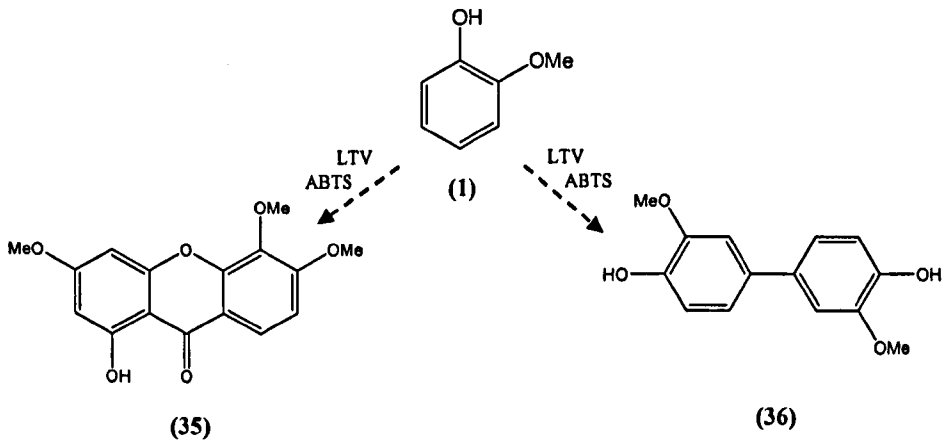


Figure 5.17 The ^{1}H-NMR spectrum of the products formed after enzymatic treatment of acetovanillone by LTV. The reaction mixtures contained acetovanillone (20 mM) dissolved in ammonium acetate buffer (100 mM; pH 4.5), ABTS (5 mM) and LTV (0.25 mg/ml) and shaken at 200 rpm for 6 h at 30 °C. The product mixtures were evaporated to dryness and dissolved in DMSO-d_6 and D_2O in the ratio of 8:2. Spectra were on different scales (the spectrum of authentic standard was 4.5-fold higher than the spectrum of the reaction mixtures and double solvent presaturation at 4.20 and 2.53 ppm was applied on the analysis after enzymatic treatment) (a) Authentic standard of 5 mM acetovanillone (b) The product mixture after enzymatic treatment of 20 mM acetovanillone by LTV and ABTS.

5.7.3 The Oxidation of Guaiacol

It was envisaged that LTV would tend to polymerize the guaiacol with the production of 1-hydroxy-3,5,6-trimethoxyxanthane **(35)** and 4-4'-biguaiacol **(36)** after 6 h (Fig. 5.18). These compounds were not observed during 2 h of reaction time (Table 5.8). After 6 h, 1-hydroxy-3,5,6-trimethoxyxanthane **(35)** was slightly increased from 0.04 to 0.05 % yield. The production of 4-4'-biguaiacol **(36)** was also increased (Table 5.8). Surprisingly, there are only 1.1 – 1.46 % of guaiacol remained after the reaction which indicates that more than 98.5 % of guaicol have been converted to new compounds. However, these compounds could not be detected by GCMS (Table 5.9).



(35) (36)

Figure 5.18 Oxidation of guaiacol **(1)** to 1-hydroxy-3,5,6-trimethoxyxanthone **(35)** and 4-4'-guaiacol **(36)**. The reaction mixtures included ammonium acetate buffer (pH 4.5, 100 mM), acetovanillone (20 mM), ABTS (5 mM) and LTV (0.25 mg/ml) and were shaken at 200 rpm for 2, 6 and 24 h. Fractionation was not applied and the sample was evaporated to dryness and redissolved in DCM for GCMS analysis. The compounds were identified by comparison of the mass spectrum with the NIST library as attached in Appendix A.5.3, page 232.

This finding supports the previous results in which guaiacol was produced from lignin and then was consumed over 24 h (Fig. 5.13). Furthermore, increasing quantities of a solid product was formed over time is apparent during the reaction (Fig. 5.19). This suggests that LTV catalyzed the polymerization of guaiacol in the presence of ABTS. The putative dimers may be intermediates in this process, whereas higher molecular weight oligomers and polymers may not be detectable by GCMS (Potthast *et al.*, 1999). The present findings seem to be consistent with other

research which found that laccase are able to polymerize guaiacol in the presence of ABTS, even though the laccase used was from *Trametes hirsuta* (Rittstieg *et al.*, 2003).

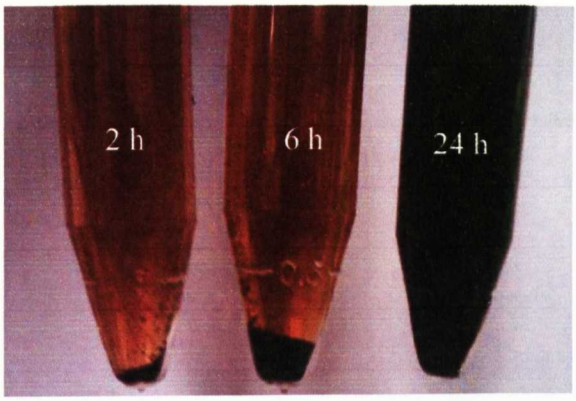


Figure 5.19 Conversion of guaiacol by LTV in the presence of ABTS over 2, 6 and 24 h.

The ^{1}H-NMR spectrum of guaiacol shows the chemical shift of the aromatics protons were observed between 6.77 to 6.88 ppm and the methoxy group proton at 3.83 ppm (Fig. 5.20a). The fingerprint of the products formed after enzymatic treatment of guaiacol by LTV shows a complex mixture of compounds (Fig. 5.20b). The solvent (DMSO) and buffer (acetate) proton peaks were observed at 2.50 and 1.83 ppm, respectively.

Low intensity of guaiacol proton peaks were detected after enzymatic treatment with LTV which indicates that guaiacol was almost fully converted. This result is consistent with GCMS analysis in which only 1.1 to 1.46 % of guaicol remained after enzymatic treatment. There are increasing numbers of aromatic proton between 6.24 to 8.12 ppm and hydrogen adjacent to H-C-OR proton between 3.45 to 4.42 ppm. The fingerprint of aromatics proton has indicates the complex mixtures of new aromatic chemicals produced from the enzymatic treatment of guaiacol by LTV. This result suggests that there are large numbers of possible chemical modification and substitution that may occur between guaiacol, LTV and ABTS that need further investigation for better understanding of the mechanism.

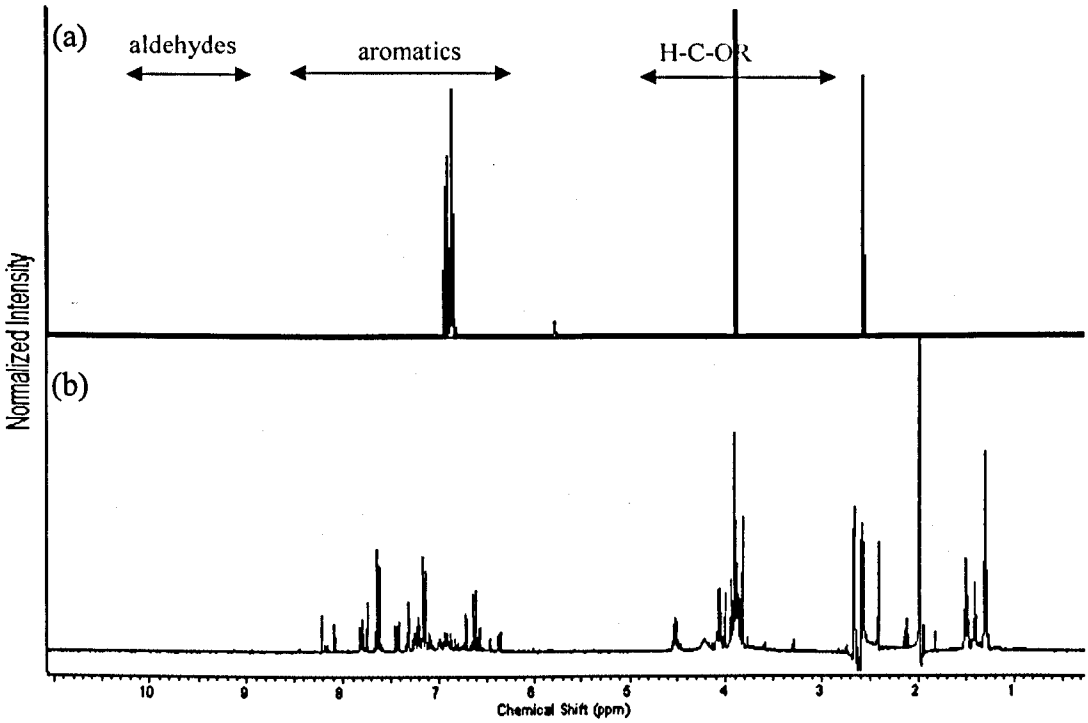


Figure 5.20 The ^{1}H-NMR spectrum of the products formed after enzymatic treatment of guaiacol by LTV. The reaction mixtures contained guaiacol (20 mM) dissolved in ammonium acetate buffer (100 mM; pH 4.5), ABTS (5 mM) and LTV (0.25 mg/ml) and shaken at 200 rpm for 6 h at 30 °C. The product mixtures were evaporated to dryness and dissolved in DMSO-d_6 and D_2O in the ratio of 8:2. Spectra were on different scales (the spectrum of authentic standard was 4.5-fold higher than the spectrum of the reaction mixtures and double solvent presaturation at 4.09 and 2.43 ppm was applied on the analysis after enzymatic treatment) (a) authentic standard of 5 mM guaiacol (b) The product mixture after enzymatic treatment of 20 mM guaiacol by LTV and ABTS.

5.7.4 The Oxidation of Vanillic Acid

Most of the compounds produced from the reaction of vanillic acid with LTV in the presence of ABTS were monomers (Fig. 5.21). These compounds were identified by GCMS with a good agreement with the NIST library and the identification of guaiacol and vanillin were confirmed by authentic standards. In contrast to the reaction of LTV towards guaiacol, LTV tended to break or modify the substituent and produce new chemicals as illustrated in Fig. 5.21. Four compounds were observed after enzymatic treatment of vanillic acid with LTV. Esterification is involved in the modification of vanillic acid to become methyl vanillate **(37)**

(Fig. 5.21a). The production of this compound was increased from 0.4 to 1.47 % yields over 24 h of reaction time (Table 5.8). LTV was also able to decarboxylate vanillic acid to guaiacol **(1)** (Figure 5.21b) which is consistent with the result found by Huang *et al.* (1993) using *Rhodotorula rubra* and 2-methoxyhydroquinone **(30)** (Fig. 5.21c) as observed by Ander *et al.* (1983) using *Sporotrichum pulverulentum.* According to Huang *et al.* (1993), the conversion of vanillic acid to guaiacol was achieved after 42 h of incubation. However, it only takes 2 h in the presence of LTV and ABTS.

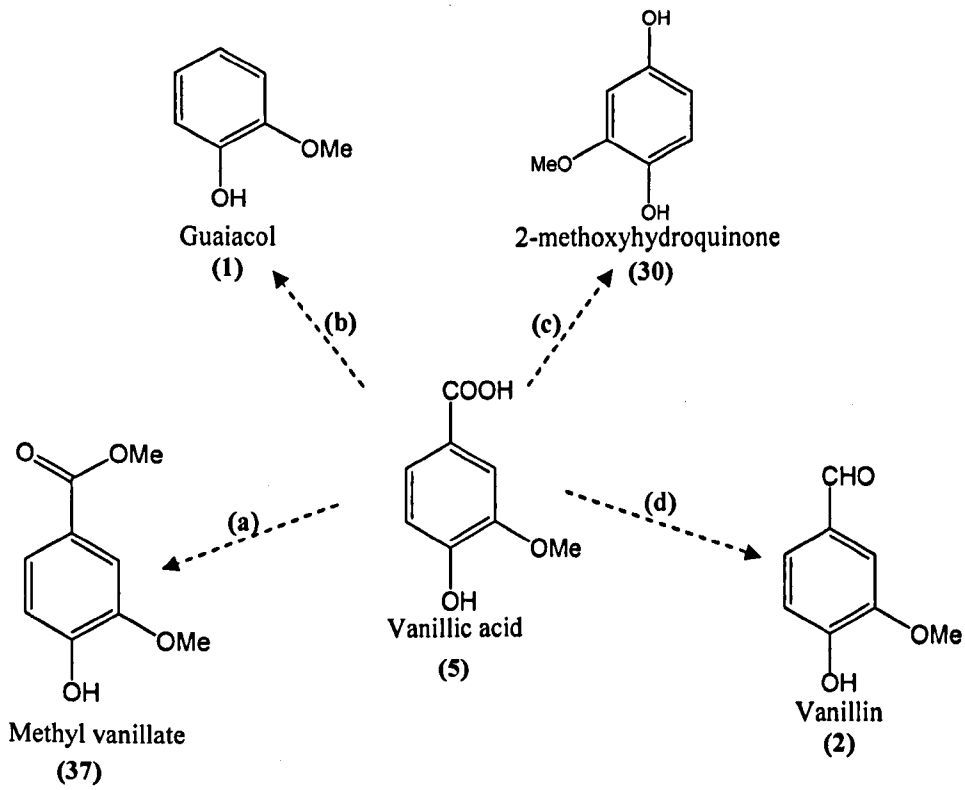


Figure 5.21 The conversion of vanillic acid **(5)** to methyl vanillate **(37)**, guaiacol **(1)**, 2-methoxyhydroquinone **(30)** and vanillin **(2)** by (a) esterification, (b) and (c) decarboxylation, (d) reduction and (e) isomerisation. The reaction mixtures included ammonium acetate buffer (pH 4.5, 100 mM), vanillic acid (20 mM), ABTS (5 mM) and LTV (0.25 mg/ml) and were shaken at 200 rpm for 2, 6 and 24 h. Fractionation was not applied and the sample was evaporated to dryness and redissolved in DCM. Compounds were identified by GCMS with a good agreement with the NIST library. The identification of **(1)** and **(2)** were confirmed by authentic standards and **(30)** and **(37)** by the comparison with mass spectrum of the NIST library as attached in Appendix A.5.4, page 233.

This is due to the fact the whole cell of *R. rubra* was employed for the conversion (Huang *et al.*, 1993), which is much slower than using the isolated enzyme. A reduction takes place in the modification of vanillic acid to vanillin (**2**) (Fig. 5.21d). High amount of vanillin was produced over time in which increased from 8.08 and 14.18 to 26 % yield at 2, 6 and 24 h, respectively (Table 5.8). The unconverted vanillic acid was increased from 20.5 to 45 % over 24 h of reaction time (Table 5.9).

The ^{1}H-NMR spectrum of vanillic acid shows the chemical shift of the aromatics doublet duplet, and duplet at 6.91, 7.28 and 7.24 ppm respectively. The methoxy group was observed at 3.95 ppm and the hydroxyl proton at 3.99 ppm (Fig. 5.22a).

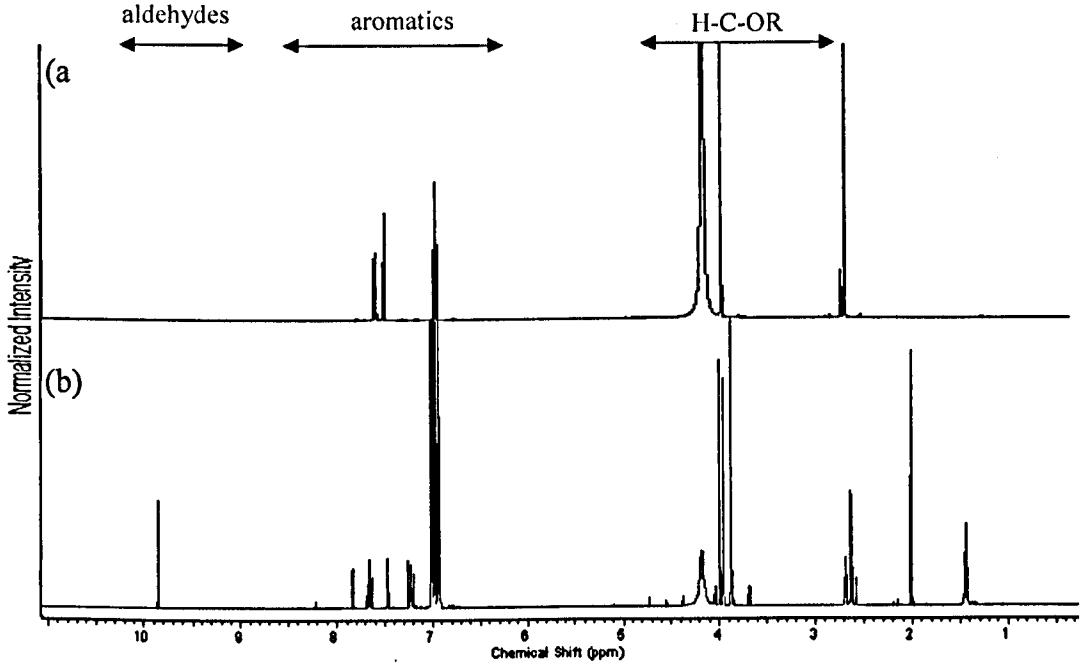


Figure 5.22 The ^{1}H-NMR spectrum of the products formed after enzymatic treatment of vanillic acid by LTV. The reaction mixtures contained vanillic acid (20 mM) dissolved in ammonium acetate buffer (100 mM; pH 4.5), ABTS (5 mM) and LTV (0.25 mg/ml) and shaken at 200 rpm for 6 h at 30 °C. The product mixtures were evaporated to dryness and dissolved in DMSO-d_6 and D_2O in the ratio of 8:2. Spectra were on different scales (the spectrum of authentic standard was 4.5-fold higher than the spectrum of the reaction mixtures and double solvent presaturation at 3.70 and 4.40 ppm was applied on the analysis after enzymatic treatment) (a) Authentic standard of 5 mM vanillic acid (b) The product mixture after enzymatic treatment of 20 mM vanillic acid by LTV and ABTS.

The solvent (DMSO) and buffer (acetate) proton peaks were observed at 2.50 and 1.83 ppm, respectively. Vanillic acid proton peaks were detected after enzymatic treatment with LTV which indicates that some of the vanillic acid was still remains unconverted. This result is consistent with GCMS analysis in which around 39.1 % of vanillic acid was observed after enzymatic treatment. (Fig. 5.22b).The indications of changes in chemical composition of vanillic acid after the enzymatic treatment with LTV were shown in Fig. 5.22b. The production of an aldehyde compound was confirmed by the observation of aldehyde proton peak at 9.78 ppm. However, this compound could not be vanillin since the ^{1}H-NMR spectrum of this unknown compound did not match the authentic standard of vanillin.

The numbers of aromatic proton between 6.60 to 8.12 ppm were increased as well as the hydrogen adjacent to H-C-OR proton between 3.52 to 4.58 ppm indicating the production of new chemicals from the reaction. The observation of methoxy singlet proton peak at 3.52, 3.53, 3.70, 3.72, 3.80, 3.84, 3.88 and 3.90 indicating the production of new chemicals by at least 5 new compounds with the methoxy group attached to the side chain. However, GCMS can only identified four compounds with five methoxy groups (Fig. 5.21).

5.7.5 The Oxidation of Homovanillyl Alcohol

The effect of incubation time on homovanillyl alcohol reacted with LTV and mediated by ABTS is shown in Fig. 5.23. The colourless homovanillyl alcohol turned brown after 2 h and the colour intensity increased until 24 h. The coloured material remained in solution even after centrifugation. The production of new chemicals by LTV may likely contribute to the colour changes with time.

Fig. 5.24 shows four compounds produced from the enzymatic digestion of homovanillyl alcohol by LTV as identified by GCMS. 2-methoxy-4-propyl phenol **(38)** and homovanillic acid **(39)** were produced after 2 h of reaction. The production of these compounds was increased by 4.57 and 0.15 % yield respectively (Table 5.8). After 6 h, 4-hydroxy-3-methoxyphenylglycol **(40)** and vanillin **(2)** were observed.

The production of 4-hydroxy-3-methoxyphenylglycol **(40)** was dramatically increased from 5.68 to 24.9 % yield after 24 h (Table 5.8).

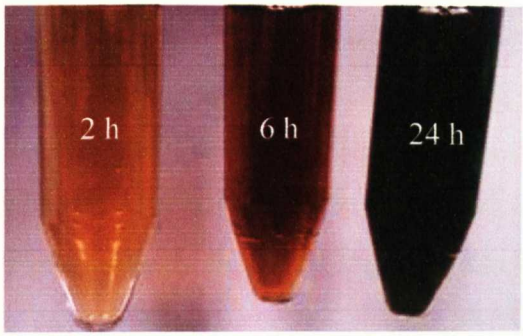


Figure 5.23 Conversion of homovanillyl alcohol by LTV in the presence of ABTS over 2, 6 and 24 h.

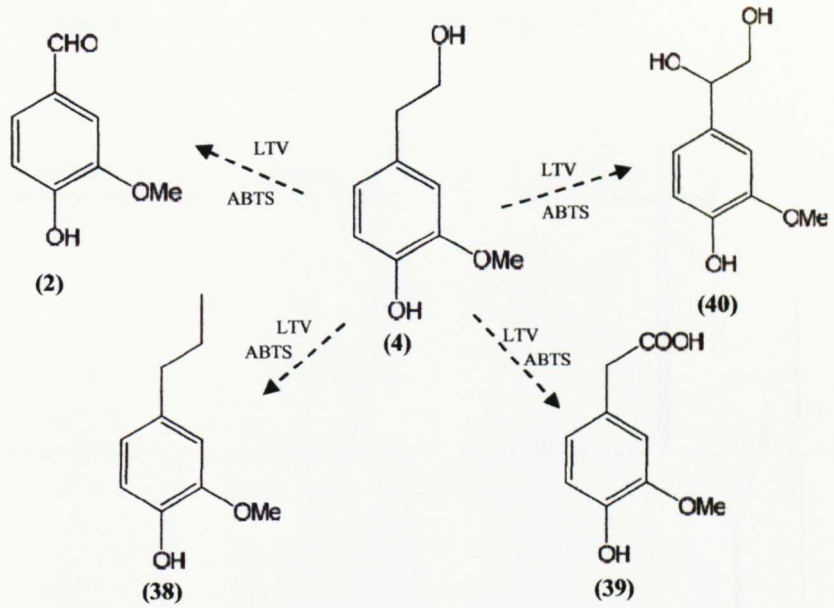


Figure 5.24 The conversion of homovanillyl alcohol **(4)** to vanillin **(11)**, 2-methoxy-4-propyl phenol **(13),** homovanillic acid **(14)** and 4-hydroxy-3-methoxyphenylglycol **(15).** The reaction mixtures included ammonium acetate buffer (pH 4.5, 100 mM), homovanillyl alcohol (20 mM), ABTS (5 mM) and LTV (0.25 mg/ml) and were shaken at 200 rpm for 2, 6 and 24 h. Fractionation was not applied and the sample was evaporated to dryness and redissolved in DCM. Compounds were identified by GCMS with a good agreement with the NIST library. The identification of **(2)** was confirmed by authentic standard and **(38), (39)** and **(40)** by the comparison of the mass spectrum with the NIST library as attached in Appendix A.5.5, page 236.

However, the production of vanillin **(2)** was remained constant up to 24 h of reaction. This study produced results which corroborate the findings of the previous experiment for optimization of the products by time courses (Section 5.6). The production of homovanillyl alcohol was optimum at 4 h, however, it declined thereafter. This result may be explained by the fact that LTV further reacts with homovanillyl alcohol and converts it to new chemicals as observed in the current study. The unconverted homovanillyl alcohol was reduced over time from 50.5 to 31.2 % (Table 5.9) which indicates that more reactant has been converted to new compounds.

The ^{1}H-NMR spectrum of homovanillyl alcohol shows the chemical shift of the aromatics proton between 6.80 to 6.87 ppm. The methoxy group was observed at 3.82 ppm and the hydroxyl proton at 4.95 ppm (Fig. 5.25a).

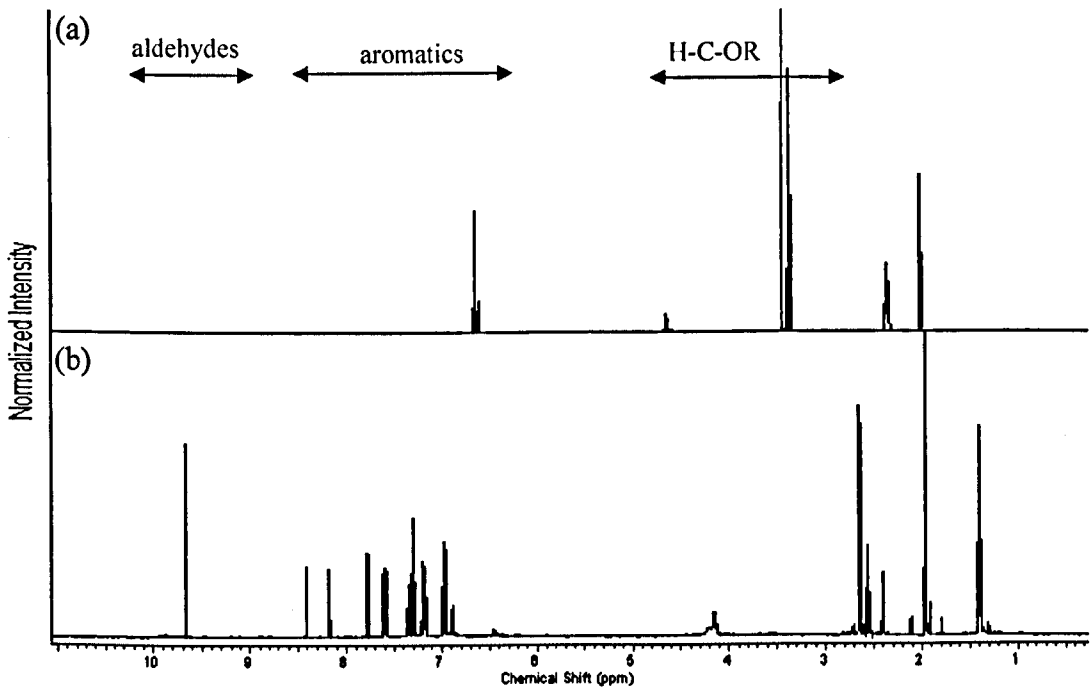


Figure 5.25 The ^{1}H-NMR spectrum of the products formed after enzymatic treatment of homovanillyl alcohol by LTV. The reaction mixtures contained homovanillyl alcohol (20 mM) dissolved in ammonium acetate buffer (100 mM; pH 4.5), ABTS (5 mM) and LTV (0.25 mg/ml) and shaken at 200 rpm for 6 h at 30 °C. The product mixtures were evaporated to dryness and dissolved in DMSO-d_6 and D_2O in the ratio of 8:2. Spectra were on different scales (the spectrum of authentic standard was 4.5-fold higher than the spectrum of the reaction mixtures) (a) Authentic standard of 5 mM homovanillyl alcohol (b) The product mixtures after enzymatic treatment of 20 mM homovanillyl alcohol by LTV and ABTS.

The solvent (DMSO) and buffer (acetate) proton peaks were observed at 2.50 and 1.80 ppm, respectively. In contrast to the result by GCMS, homovanillyl alcohol protons were not detected in the reaction media after enzymatic treatment indicates that homovanillyl alcohol was fully converted (Fig. 5.25b). The numbers of aromatic proton were increased and observed between 6.83 to 8.36 ppm. The fingerprint of aromatics proton has indicates the complex mixtures of new aromatic chemicals produced from the enzymatic treatment of homovanillyl alcohol by LTV. However, the identification of each individual chemical could not be performed. The production of aldehyde compound was confirmed by the observation of aldehyde proton peak at 9.66 ppm which corresponds to the authentic standard of vanillin. The aromatic double duplet and duplet peaks of vanillin was also observed at 6.85, 7.49 and 7.41 ppm, respectively. The methoxy group of vanillin **(2)** was also observed at 3.95 ppm. Therefore, the production of vanillin from the enzymatic treatment of homovanillyl alcohol by LTV was confirmed by both GCMS and ^{1}H-NMR analysis.

5.8 Discussion

The combination of findings demonstrates that LTV can breakdown lignin in the presence of ABTS. In addition, this study shows that LTV is a more efficient biocatalyst for the breakdown of sodium lignosulphonate than LAB. Modification of the previous method was performed since the activity of LTV was affected by the pH and the reaction temperature. A temperature of 60 °C was observed as the optimum for LTV and the assay for lignin depolymerization was conducted at pH 4.5. However, the enzyme was not stable at 60 °C throughout the 24 h of the reaction time which resulted in a decrease of product yield after 4 h of incubation. By modifying the temperature to 30 °C, the product concentration was slightly increased. The modification of the incubation temperature contributed towards a higher concentration of product formation. Even though the concentration was increased, a reduction of the concentration over 24 h of the incubation period was observed. Taking this positively, the finding has important implications for producing chemicals from lignin in a specific time. Most of the chemicals were produced after a period of 1 h of incubation.

Both types of laccases produced different effects on the breakdown of the lignin. The most striking observation to emerge from the data comparison of LTV and LAB was the production of vanillic acid. A significantly higher proportion of vanillic acid was observed in the breakdown by LTV compared to LAB. The production of acetovanillone and vanillin were also higher than LAB especially for an incubation time of between 1 to 4 h. Furthermore, LTV presented a different trend for product formation over time in which the optimum product formation was achieved between 4 to 6 h of the incubation period. LTV might possibly possess a repolymerization of the products that results in a decrease of the yield of products over a longer incubation period. Since LTV shows a high potential for an optimum product yield, a set of experiments was set up to investigate this hypothesis. In addition, this study can also deliver a better understanding of the reduction of product yield by time as discussed earlier. A comparison between the breakdown of sodium lignosulphonate by LAB and LTV has gone some way towards enhancing the understanding of the different laccase behaviours in this reaction. Since the product concentrations were low in the presence of LAB, it can thus be suggested that the concentration of LAB could be increased in future study in order to obtain the same product pattern as the reaction catalyzed by LTV.

In order to understand the behaviour of LTV with regard to the products formed, further investigation was conducted by using five major products observed in the study as a substrate. Vanillin, acetovanillone, guaiacol, vanillic acid and homovanillyl alcohol were assigned. The most striking result to emerge from this study is that the LTV possessed the ability to reduce vanillic acid to vanillin. It was expected that LTV may catalyze the polymerization of guaiacol even in the presence of ABTS. Several studies have claimed the same result for the catalytic reaction of guaiacol by laccase. Polymerization was also observed in the reaction of acetovanillone by LTV. The combination of findings provides some support for the previous results obtained for the breakdown of sodium lignosufonate by LTV. For instance, the decrease of guaiacol concentration after 4 h of incubation time (Fig. 5.13) has enhanced the understanding that this compound tends to be repolymerized over a longer period of time. In addition, the increase of vanillin concentration over time (Fig. 5.13) could be related to the conversion of vanillic acid and homovanillyl alcohol to vanillin as illustrated in Table 5.8. The fingerprint analysis by ^{1}H-NMR

has provided the chemical changes of the substrate after the enzymatic treatment with LTV. The identification of the product can be performed by corroborating the ^{1}H-NMR with carbon NMR (^{13}C-NMR). The production of heteronuclear single quantum coherence (HSQC) data from ^{1}H-NMR and ^{13}C-NMR may provide further information in identifying the products. Furthermore, the diffusion NMR is another approach to separate the compounds in the sample based on the differing translation coefficients. Therefore, the identification of each individual component could be performed. However, since the time is limited, it can thus be suggested that these analyses can be conducted in the future for better understanding of the mechanism involve in the reaction catalyze by LTV.

The most important limitation lies in the fact that the chemical compounds produced from lignin depolymerization by LTV were numerous, complex and have different chemical properties. Thus, the selectivity of the compounds extracted in the different organic solvents may vary following these properties. The empirical findings in this experiment suggest that the screening of different extraction solvents for each product may be necessary to improve the characterization and quantification of the lignin depolymerization products. Furthermore, more research on this topic needs to be undertaken before the association between LTV and sodium lignosulphonate is more clearly understood. In reviewing the literature, no data were found on the association between LTV and sodium lignosulphonate which suggests that the mechanism of oxidation by LTV is still poorly understood.

Chapter 6

TOWARDS UNDERSTANDING OF THE LACCASE-MEDIATOR SYSTEM

6.1 Introduction

The laccase-mediator system is based on the oxidation of a mediator by laccase to form radical cation, and the radical can then oxidize the lignin. Synthetic mediators are the most efficient mediators for oxidation of aromatic compounds such as lignin (Cañas and Camarero, 2010; Bourbonnais *et al.*, 1997; Srebotnik and Hammel, 2000). In order to be a good mediator, the compound needs to have a stable radical of the oxidized intermediate that has the ability to interact with the lignin and not deactivate the laccase. According to Fabbrini *et al.* (2002), the mediator can interact with lignin model compounds *via* an electron transfer (ET) route (Fig. 6.1) which is more feasible with laccase-ABTS (2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid)) system. On the other hand, 1-hydroxybenzotriazole (HBT), *N*-hydroxyphthalimide (HPI) and violuric acid (VLA) were following the hydrogen atom transfer (HAT) route (Fabbrini *et al.*, 2002; Baiocco *et al.*, 2003) (Fig. 6.1 and Fig. 6.2). Empirically, the HAT route provides more efficient degradation of the lignin model compounds than the ET. By contrast, 2,2,6,6-tetramethylpiperidin-1-yloxy (TEMPO) follows a non-radical-ionic mechanism which is more complex (Fabbrini *et al.*, 2002) but results in even better degradation. Laccase is unique amongst lignolytic enzyme due to the wide range of mediators. Since different mediators provide a range of efficiencies for degradation of lignin model compounds, their efficiencies for degradation of sodium lignosulphonate was studied in this project. The four synthetic mediators (TEMPO, VLA, HBT and HPI) used by Fabbrini *et al.* (2002) were employed. The effect of these mediators towards lignin depolymerization was then compared with ABTS.

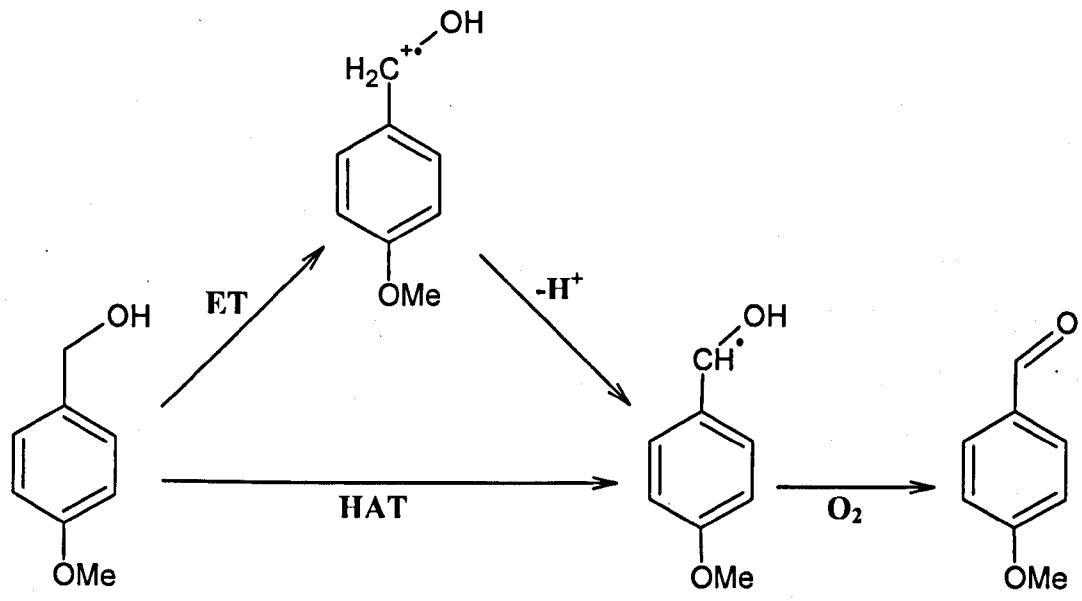


Figure 6.1 The oxidation of *p*-anisilic alcohol by laccase from *Trametes villosa* following two different oxidation mechanisms: ET (electron transfer) and HAT (hydrogen atom transfer) adapted from Fabbrini *et al.* (2002).

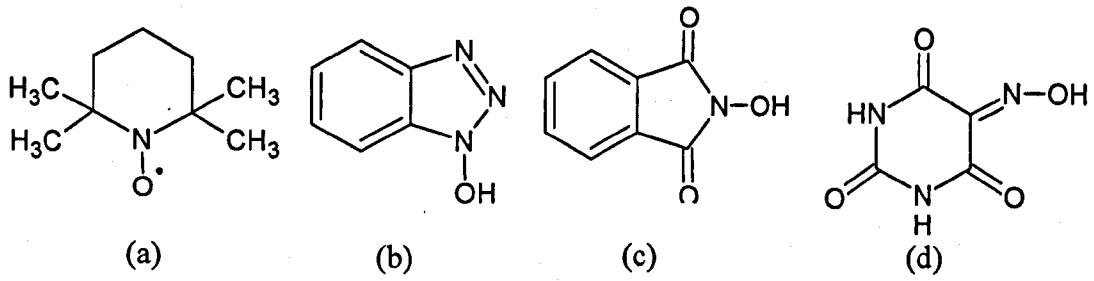


Figure 6.2 Synthetic mediators used in this study (a) 2,2,6,6-tetramethyl-piperidin-1-yl)oxyl **(TEMPO)**, (b) 1-hydroxybenzotriazole **(HBT)**, (c) *N*-hydroxyphthalimide **(HPI)** and (d) violuric acid **(VLA)**.

6.2 Laccase Activity in the Presence of TEMPO and HBT

There is a large volume of published studies describing the role of laccase mediators (Baiocco *et al.*, 2003; Bourbonnais *et al.*, 1998; Cañas and Camarero, 2010; Bourbonnais *et al.*, 1997; Hernández Fernaud *et al.*, 2006), however, a comparison between mediators is limited by the fact that different reaction conditions were implemented and diverse source of laccases were used. From previous chapter, it has

been demonstrated that LTV shows different behaviour under varied reaction conditions. Therefore, the activity of LTV on the oxidation of TEMPO and HBT were investigated. This experiment was performed spectrophotometrically at 30 °C. The concentration of TEMPO and HBT was varied from 0 to 10 mM, at 1 mM interval. The enzyme assay without the substrate was used as a control. Fig. 6.3 compares the result of LTV activity on the oxidation of HBT, TEMPO and ABTS. It is apparent from this figure that the initial rate of reaction at 4 mM of ABTS was higher than HBT and TEMPO. The control assay gave a reaction rate equal to 0 mMs^{-1}.

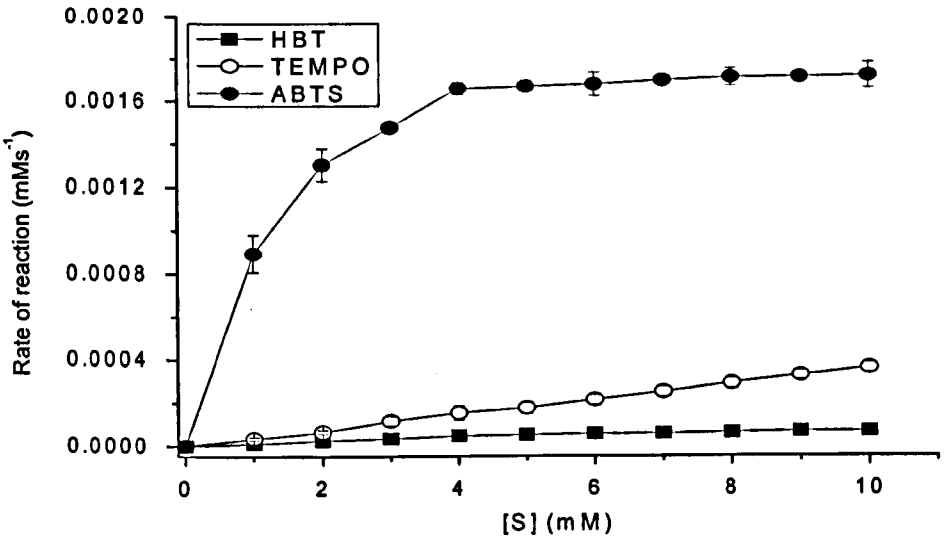


Figure 6.3 Comparison of the rate of oxidation between ABTS, TEMPO and HBT by LTV. The LTV activity was determined at 30 °C. TEMPO and HBT concentration varied from 0 to 10 mM in ammonium acetate buffer (25 mM, pH 4.5) and LTV (0.25 mg/ml). The data represent the mean of three replicates with error less than 1%. Absorbance changes were monitored at 420 nm (ABTS), 408 nm (HBT; Ander and Messner, 1998) and 245 nm (TEMPO; Kulys and Vidziunaite, 2005) for 1 h.

The initial rate of TEMPO at 4 mM was found to be approximately $1.1 \times 10^{-4} \pm 1.0 \times 10^{-5}$ mMs^{-1} which is 88 % less than the rate of oxidation of ABTS. With HBT, the rate of oxidation was decreased by 97 %. The comparison of the Michaelis-Menten parameters on the oxidation of ABTS, TEMPO and HBT are summarized in Table 6.1, and show that both the substrate affinity and maximum reaction rate were lower with TEMPO and HBT than ABTS.

Table 6.1 Effect of different substrate on the oxidation by LTV. Parameters are kept constant (22 ^{0}C, ABTS concentration varied between 0.1 and 1 mM, at 0.1 mM interval, final concentration).

Laccase	v_o, (mMs^{-1}) x 10^{-4}	K_m, (mM)	V_{max}, (mMs^{-1}) x 10^{-4}
ABTS	9.8 ± 1.0	0.1 ± 0.01	17 ± 0.4
TEMPO	1.1 ± 0.1	0.5 ± 0.02	5.3 ± 0.03
HBT	0.2 ± 0.01	0.7 ± 0.01	1.2 ± 0.04

The LTV-catalyzed VLA and HPI oxidation rate could not be measured spectrophotometrically and Michaelis-Menten kinetic parameters have not been determined. It is therefore could be conceivably be hypothesised that the rate of lignin depolymerization mediated by TEMPO, HBT, VLA and HPI may be lower than the reaction mediated by ABTS. A better understanding of what governs lignin depolymerization efficiency by mediators would require further investigation towards the oxidation mechanism of each mediator by LTV.

6.3 Mediation Efficiency towards Lignin Depolymerization

From previous chapter, it has been proved that the breakdown of sodium lignosulphonate by LTV could not be performed without the presence of the mediator (ABTS). Thus, a further study was set up to determine the efficiency of using TEMPO, HBT, VLA and HPI towards the breakdown of sodium lignosulphonate by LTV.

6.3.1 TEMPO

A large and growing body of literature has investigated the role of 2,2,6,6-tetramethylpiperidin-1-yloxy (TEMPO) as a laccase mediator (Arends *et al.*, 2006; Fabbrini *et al.*, 2001; Fabbrini *et al.*, 2002; Baiocco *et al.*, 2003; Bourbonnais *et al.*, 1997; Galli and Gentili, 2004; d'Acunzo *et al.*, 2003). Most studies so far focused on the oxidation of various alcohol induced by the laccase-TEMPO system (Arends *et al.*, 2006; Fabbrini *et al.*, 2001). According to Fabbrini *et al.* (2002), laccase oxidize

TEMPO to oxoammonium ion by non-radical-ionic mechanism. The oxoammonium ion is then been attacked by the substrate (4-methoxybenzyl alcohol) to produced a reduced form of TEMPO. Laccase would then regenerates TEMPO aminoxyl radical from a reduced form of TEMPO as part of a recycle process (Fig. 6.4).

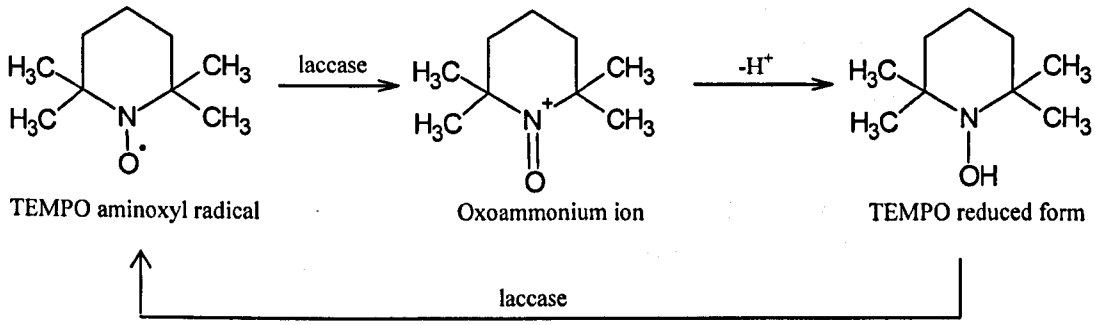


Figure 6.4 Mechanism of TEMPO oxidation by laccase as suggested by Fabbrini *et al.* (2002).

In reviewing the literature, however, there is no published study on the effect of TEMPO mediated oxidation of lignin by laccase. Therefore, this study was conducted to understand the interaction between laccase-TEMPO systems with sodium lignosulphonate. It is predicted from the activity of LTV on the oxidation of TEMPO that the breakdown of lignin may be lower than ABTS. To study the effect, sodium lignosulphonate was incubated in ammonium acetate buffer, LTV and TEMPO by following the previous optimized condition. Control samples of (i) sodium lignosulphonate (without TEMPO and LTV) (ii) TEMPO and LTV were treated under the same reaction conditions.

No product could be detected from the reaction between TEMPO and LTV without sodium lignosulphonate (control) using mass spectrometry (MS). Since the oxidation of TEMPO by laccase produces a nitroxyl radical (Galli and Gentili, 2004; Fabbrini *et al.*, 2002), it is not clear whether this radical could be detected by the MS. Vanillin **(2)**, vanillic acid **(5)** and 3-hydroxy-1-(4-hydroxy-3-methoxyphenyl)propan-1-one **(13)** were observed in the control sample containing sodium lignosulphonate without TEMPO and LTV (Fig. 6.5a and Table 6.2).

When LTV and TEMPO were present, 0.28 mM of vanillin **(2)** was formed compared to 0.17 mM in the control (Table 6.2). This compound was increased by 39 % after 2 h of reaction time and the concentration was decreased to 0.12 mM over 24 h. This suggests that LTV has either repolymerized the vanillin **(2)** or has catalyzed the production of new compounds. On the other hand, guaiacol **(1)**, acetovanillone **(3)** and isovanillic acid **(41)** were not found in the control and were produced after 2 h, indicating that there was depolymerization of sodium lignosulphonate. The intensity of the product peaks decreased over time (Fig. 6.5).

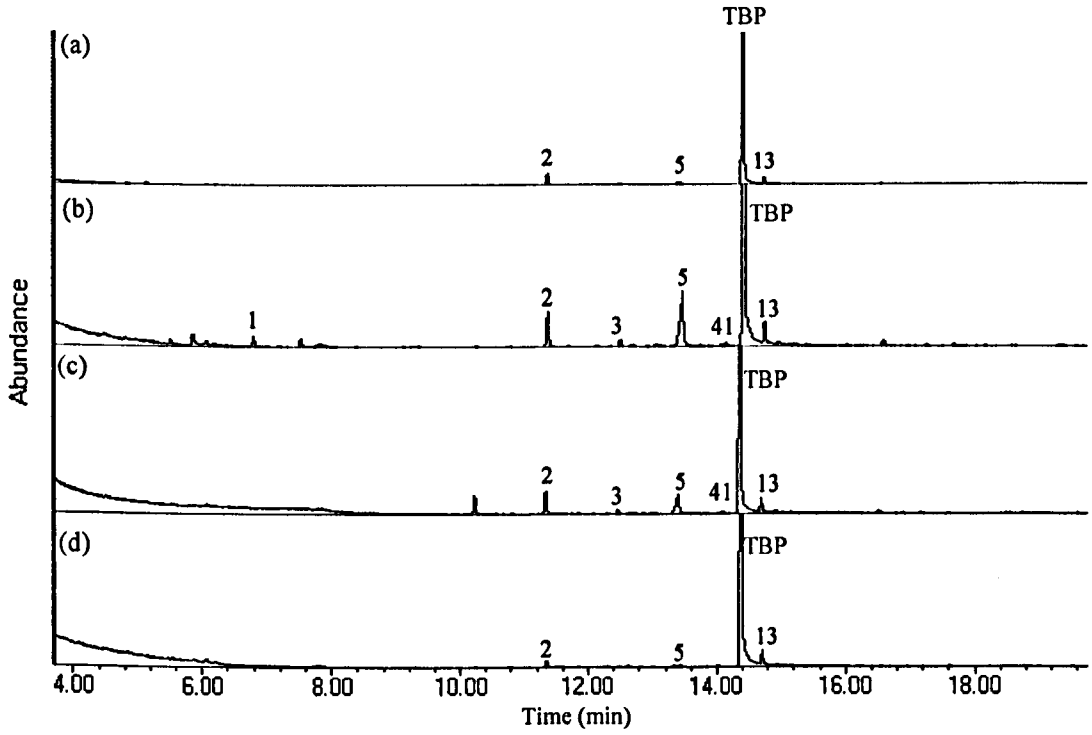


Figure 6.5 The effect of incubation time on the depolymerization of sodium lignosulphonate mediated by TEMPO. The identities of the released compounds are listed in Table 6.2. Samples were incubated under identical condition (at 30 °C, shaken at 200 rpm). Fractionation was applied and the aqueous ethyl acetate extract fraction was evaporated to dryness and redissolved in DCM. TBP (tributylphosphate). (a) The aqueous ethyl acetate extracts of the control (b) 2 h, (c) 6 h, (d) 24 h of incubation time. All chromatograms were on the same scale of intensity.

In contrast to earlier findings with laccase-ABTS system, the products of laccase-TEMPO system were consumed faster. For instance, guaiacol **(1)** disappeared after

2 h of incubation time. In addition, the concentration of vanillic acid **(5)** was decreased by 96 % over 24 h of reaction time (Table 6.2). The maximum production of vanillic acid **(5)** was achieved after 2 h (1.42 mM).

Table 6.2 Identification of products formed after the depolymerization of sodium lignosulphonate by LTV and mediated by TEMPO. Identification of the products was based on comparison of mass spectra of authentic standards (Appendix A.6.1 – page 238) and the retention time of the products was matched to the standards. The data represent the mean of three replicates and standard deviation (SD). A control sample was treated under the same condition. The concentration of the released compounds was calculated based on the peak area of the product compared to the peak area of an authentic standard (5 mM).

Label	Compounds	Concentration of the product in aqueous ethyl acetate extract fraction ± SD			
		Control (mM)	2 h (mM)	6 h (mM)	24 h (mM)
1	Guaiacol	n.d	0.03 ± 0.00	n.d	n.d
2	Vanillin	0.17 ± 0.01	0.28 ± 0.00	0.18 ± 0.00	0.12 ± 0.00
3	Acetovanillone	n.d	0.02 ± 0.00	0.01 ± 0.00	n.d
5	Vanillic acid	0.80 ± 0.01	1.42 ± 0.04	0.93 ± 0.02	0.05 ± 0.00
13	3-hydroxy-1-(4-hydroxy-3-methoxyphenyl)propan-1-one	n.a	n.a	n.a	n.a
41	Isovanillic acid	n.d	0.19 ± 0.01	0.06 ± 0.00	n.d

*n.d: not detected; *n.a: not applicable (the compound without authentic standard for which the molar concentration could not be calculated)

The peak area of 3-hydroxy-1-(4-hydroxy-3-methoxyphenyl)propan-1-one **(13)** also decreased with time (Fig. 6.5), however, the molar concentration of this compound could not be measured. Other than these compounds, actovanillone **(3)** and isovanillic acid **(41)** could also be observed between 2 and 6 h and completely disappeared after 24 h of incubation time. During this period, the concentration was decreased from 0.02 to 0.01 mM for acetovanillone **(3)** and 0.19 to 0.06 mM for isovanillic acid **(41)** (Table 6.2). The finding of this study suggests that the reaction mediated by TEMPO has little effect on the depolymerization of sodium lignosulphonate compared to the reaction mediated by ABTS.

6.3.2 HBT

Other than TEMPO, 1-hydroxybenzotriazole (HBT) is among the most commonly used laccase mediators (Nugroho *et al.*, 2010; Fabbrini *et al.*, 2002; Shleev *et al.*, 2006; Baiocco *et al.*, 2003; Minussi *et al.*, 2007). Beside ABTS, HBT has a high mediation efficiency for the oxidation of non-phenolic compounds (Fabbrini *et al.*, 2002). However, all of the previously published studies reported different behaviour of HBT for different reaction conditions and substrates. For instance, Li *et al.* (1999) and d'Acunzo *et al.* (2002) have observed high oxidation rates for a variety of substrates in the presence of HBT (Li *et al.*, 1999, d'Acunzo *et al.*, 2003). On the other hand, Minussi *et al.* (2005) failed to demonstrate the efficiency of using HBT as a mediator in their system (Minussi *et al.*, 2007). Among these published studies, Shleev *et al.* (2006) have demonstrated the interaction of HBT and lignin catalyzed by laccase from *Trametes hirsuta, T. ochracea and T. pubescens*. They proposed that the interaction of laccase-mediator system is a very complex process and needs further investigation.

According to Fabbrini *et al.* (2002), laccase oxidize HBT to produce radical cations which is then deprotonated to aminoxyl radicals (>N-O$^{\bullet}$) (Fig. 6.6). The oxidation of lignin is governed by this radical. Therefore, an attempt was made to study the efficiency of using HBT as laccase mediator on the depolymerization of sodium lignosulphonate. Since the activity of LTV in the presence of HBT was lower than ABTS, it was predicted that the product yields would be correspondingly low. In order to investigate this hypothesis, the sodium lignosulphonate was incubated in ammonium acetate buffer, LTV and HBT. The control samples were incubated under the same reaction conditions.

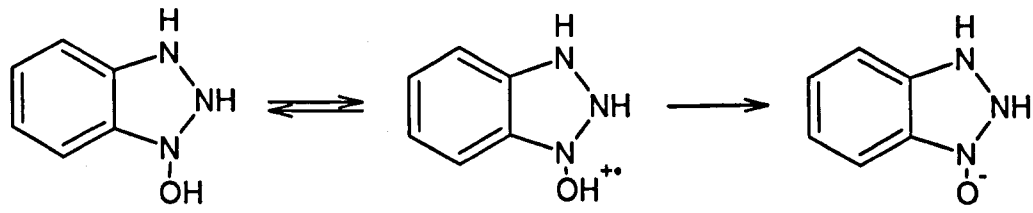


Figure 6.6 Mechanism of HBT oxidation by laccase as suggested by Fabbrini *et al.* (2002).

There was no product detected after the enzymatic reaction of HBT and LTV (control) by GCMS. Unlike the laccase-TEMPO system, guaiacol **(1)** and isovanillic acid **(41)** were not observed in the presence of HBT after the reaction of sodium lignosulphonate by LTV and mediated by HBT. The concentration of vanillin **(2)** was increased from 0.17 (control) to 0.25 mM (Table 6.3) over 2 h and the decreased to 0.12 mM over 24 h indicating that the repolymerization or formation of other compounds might occur. In addition, the production of vanillic acid **(5)** was also increased from 0.8 to 1.38 mM after 2 h. It was then decreased to 0.94 mM after 6 h of incubation time, and this compound totally disappeared after 24 h. There was a low production of acetovanillone **(3)** and the production remained constant until 6 h (0.01 mM) (Table 6.3). As for 3-hydroxy-1-(4-hydroxy-3-methoxyphenyl)propan-1-one **(13)**, the production was maximum after 2 h and was decreased over time. The most striking result to emerge from the data is that HBT was observed after the enzymatic reaction. The comparison of the peak area of HBT in the product mixtures and authentic standard reveals that there was approximately 32 % of unconverted or recycled HBT after 24 h (Fig. 6.7).

Table 6.3 Identification of products formed after the depolymerization of sodium lignosulphonate by LTV and mediated by HBT. Identification of the products was based on comparison of mass spectra of authentic standards (Appendix A.6.2 – page 240) and the retention time of the products was matched to the standards. The data represent the mean of three replicates and standard deviation (SD). A control samples (sodium lignosulphonate without LTV and HBT) contained the same amount of lignin (50 g/L) treated under the same condition. The concentration of the released compound was calculated based on the peak area of the product been compared to the peak area of an authentic standard (5 mM).

Label	Compounds	Concentration of the product in aqueous ethyl acetate extract fraction ± SD			
		Control (mM)	**2 h (mM)**	**6 h (mM)**	**24 h (mM)**
2	Vanillin	0.17 ± 0.01	0.25 ± 0.00	0.26 ± 0.00	0.12 ± 0.00
3	Acetovanillone	n.d	0.01 ± 0.00	0.01 ± 0.00	n.d
5	Vanillic acid	0.80 ± 0.01	1.38 ± 0.04	0.94 ± 0.03	n.d
13	3-hydroxy-1-(4-hydroxy-3-methoxyphenyl)propan-1-one	n.a	n.a	n.a	n.a

*n.d: not detected; *n.a: not applicable (the compound without authentic standard for which the molar concentration could not be calculated)

This finding indicates that LTV could not oxidize more HBT to product per unit time. It is therefore likely that the active site of LTV could not accommodate 5 mM of HBT in the reaction mixture. As a result, low HBT radical was produced to enable the depolymerization of sodium lignosulphonate to occur. It should be possible to optimize the process by investigating the optimum concentration of each reaction material, however, due to time limitations, no further attempts were made to optimize the reaction.

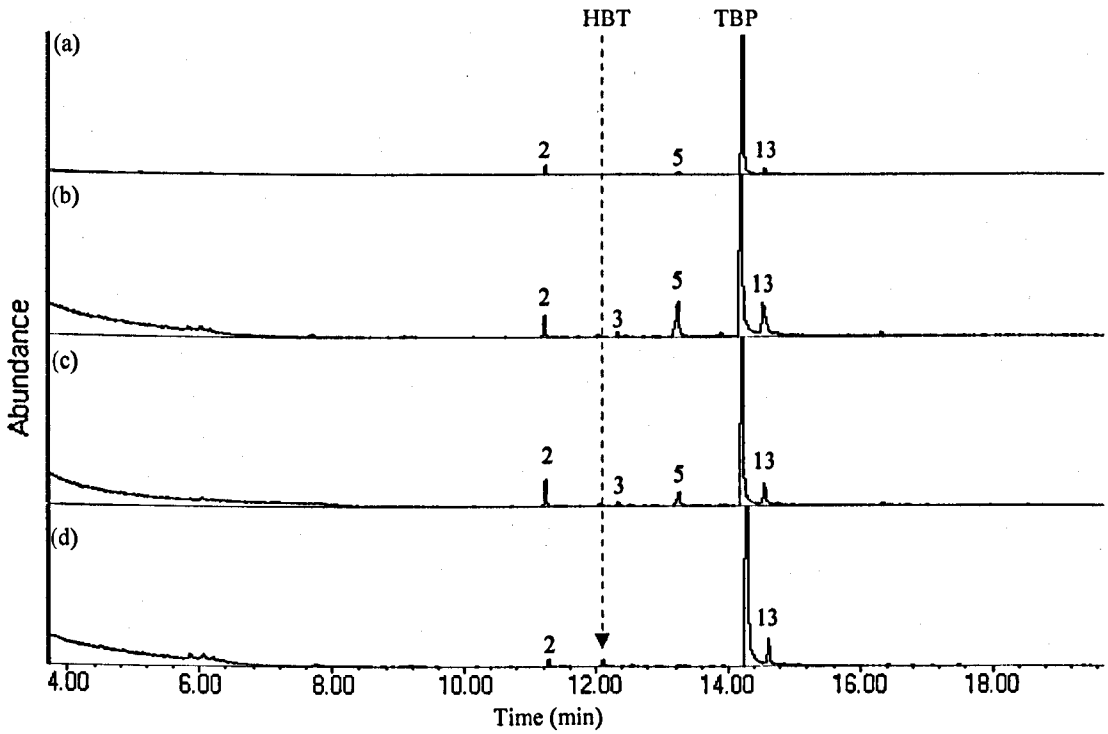


Figure 6.7 The effect of incubation time on the depolymerization of sodium lignosulphonate mediated by HBT. The identities of the released compounds are listed in Table 6.3. Samples were incubated under identical condition (at 30 °C, shaken at 200 rpm). Fractionation was applied and the aqueous ethyl acetate extract fraction was evaporated to dryness and redissolved in DCM. TBP (tributylphosphate). (a) The aqueous ethyl acetate extracts of the control (b) 2 h, (c) 6 h, (d) 24 h of incubation time. All chromatograms were on the same scale of intensity.

In general, therefore, it seems that HBT was not a suitable mediator for LTV-catalyzed sodium lignosulphonate depolymerization. The results confirm that the lower residual activity of LTV in the presence of HBT had contributed towards the

lower product formation. Better results might be achieved if further experimental work could be done to investigate the cause of this result.

6.3.3 HPI

N-hydroxyphthalimide (HPI) shared the structural feature of being an N-OH derivative as with HBT and violuric acid (VLA). As the other N-OH mediators, the efficiency of HPI to oxidize various substrates has been previously studied. For instance, Sealey *et al.* (1999) have applied HPI in the degradation of softwood Kraft pulp by laccase from *Polyporus* fungus. In this study, the mediator efficiency in the presence of HPI was lower than HBT as another mediator used in their study (Sealey *et al.*, 1999). Several studies have reported the phthalimide-*N*-oxyl (PINO) radical is considered to be the active oxidant (Annunziatini *et al.*, 2005; Galli and Gentili, 2004) which is already known to be involved in the radical oxidation procedure by chemicals oxidant (Galli and Gentili, 2004). Laccase catalyze the oxidation of HPI to radical cation, which is then deprotonated to aminoxyl radical (Fig. 6.8) (Fabbrini *et al.*, 2002). However, there is no study to date reporting the use of HPI in the degradation of isolated lignin. It has to be noted that the effect of HPI may vary depending on the types of laccase, the substrate and the optimized reaction condition (Fabbrini *et al.*, 2002). Therefore, an attempt was made to study the effect of HPI in the depolymerization of sodium lignosulphonate by LTV. To study the effect, sodium lignosulphonate was incubated in ammonium acetate buffer in the presence of LTV and HPI. The control samples were incubated under the same reaction conditions.

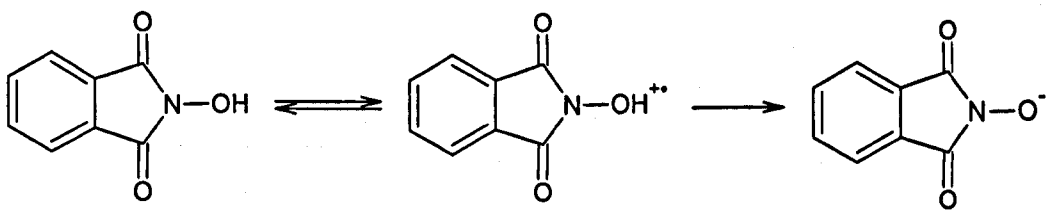


Figure 6.8 Mechanism of HPI oxidation by laccase as suggested by Fabbrini *et al.* (2002).

Only vanillin **(2)**, vanillic acid **(5)** and 3-hydroxy-1-(4-hydroxy-3-methoxyphenyl)propan-1-one **(13)** were observed after the enzymatic treatment of sodium lignosulphonate by LTV and mediated by HPI. These compounds were also observed in the control sample (sodium lignosulphonate without LTV and HPI), and the intensity was increased in the presence of LTV and HPI. This result is rather disappointing since the concentration of these compounds were increased slightly than the control after 2 h of reaction time (Table 6.4). The concentration was further decreased over 24 h which indicates the products have either been converted to other compounds or repolymerization had occurred. It is apparent in Fig. 7.10 that the 3-hydroxy-1-(4-hydroxy-3-methoxyphenyl)propan-1-one **(13)** peak was overlapped with unconverted HPI (14.78 min) and the concentration of HPI was decreased over time.

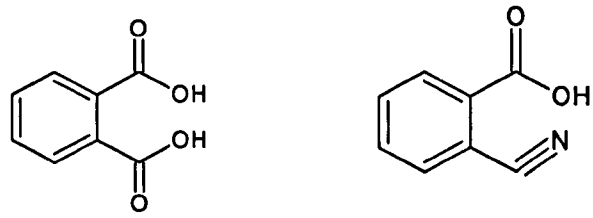
Table 6.4 Identification of products formed after the depolymerization of sodium lignosulphonate by LTV and mediated by HPI. Identification of the products was based on comparison of mass spectra of authentic standards (Appendix A.6.3 – page 242) and the retention time of the products was matched to the standards. The data represent the mean of three replicates and standard deviation (SD). A control samples (sodium lignosulphonate without LTV and HPI) contained the same amount of lignin (50 g/L) treated under the same condition. The concentration of the released compound was calculated based on the peak area of the product been compared to the peak area of an authentic standard (5 mM).

Label	Compounds	Concentration of the product in aqueous ethyl acetate extract fraction ± SD			
		Control (mM)	**2 h (mM)**	**6 h (mM)**	**24 h (mM)**
2	Vanillin	0.17 ± 0.01	0.25 ± 0.00	0.24 ± 0.00	0.01 ± 0.00
5	Vanillic acid	0.80 ± 0.01	1.01 ± 0.03	0.27 ± 0.01	n.d
13	3-hydroxy-1-(4-hydroxy-3-methoxyphenyl)propan-1-one	n.a	n.a	n.a	n.a

*n.d: not detected; *n.a: not applicable (the compound without authentic standard for which the molar concentration could not be calculated)

As proposed by Galli and Gentili (2004), the oxidation of HPI by laccase produces a HPI radical cation and further deprotonates to nitroxyl radicals. This radical then oxidizes lignin. However, in the current study, 1,2-benzenedicarboxylic acid **(HPI-**

P1) and 2-cyanobenzoic acid **(HPI-P2)** were tentatively identified (by the comparison of the mass spectrum of the unknown compound with the NIST library) to be the product of HPI (Fig. 6.9 and Fig. 6.10).



HPI-P1 **HPI-P2**

Figure 6.9 The chemical structure of 1,2-benzedicarboxylic acid **(HPI-P1)** and 2-cyanobenzoic acid **(HPI-P2).**

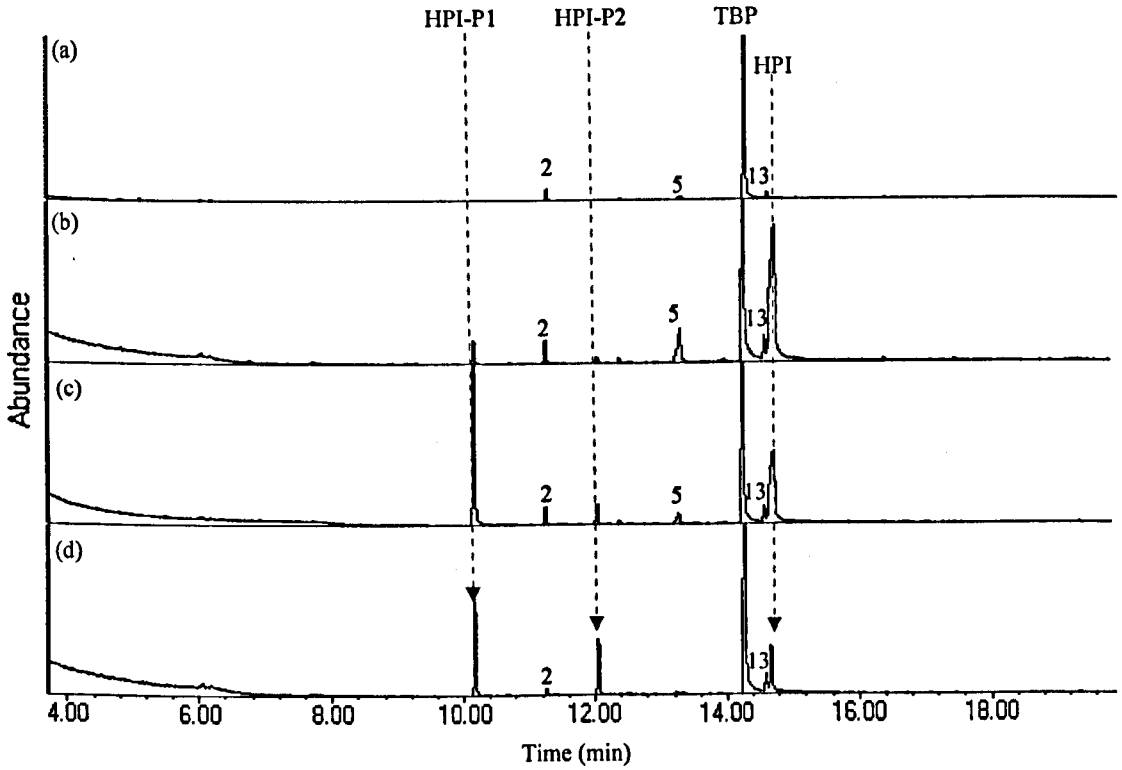


Figure 6.10 The effect of incubation time on the depolymerization of sodium lignosulphonate mediated by HPI. The identities of the released compounds are listed in Table 6.4. The reaction mixtures containing ammonium acetate buffer, sodium lignosulphonate (50 g/L), HPI (5 mM) and LTV (0.25 mg/ml) were incubated under identical condition (at 30 °C, shaken at 200 rpm). Fractionation was applied and the aqueous ethyl acetate extract fraction was evaporated to dryness and redissolved in DCM. TBP (tributylphosphate). (a) The aqueous ethyl acetate extracts of the control (b) 2 h, (c) 6 h, (d) 24 h of incubation time. All chromatograms were on the same scale of intensity.

These compounds were also observed after the enzymatic treatment of HPI by LTV without sodium lignosulphonate (control). The mechanism of this reaction is unclear, since there is no publish study regarding the production of these compounds from HPI. It can therefore be suggested that further works need to be taken into account in order to understand the system. The production of 1,2-benzedicarboxylic acid **(HPI-P1)** was increased by 22-fold after 6 h of reaction and was then reduced to 6.6-fold over 24 h. The production of 2-cyanobenzoic acid **(HPI-P2)** was increased over time (Fig. 6.10).

The production of 1,2-benzenedicarboxylic acid **(HPI-P1)** and 2-cyanobenzoic acid **(HPI-P2)** have reduced the production of aminoxyl radicals (>N-O•). Therefore, the depolymerization of sodium lignosulphonate was also reduced due to the insufficient amount of aminoxyl radical to oxidize this complex polymer. As a result, lower product distribution was observed. The evidence of this study suggests that HPI is not a suitable mediator for LTV. However, a number of possible future studies using the same experimental set up are apparent in order to optimize the reaction.

6.3.4 VLA

Other than TEMPO, HBT and HPI, violuric acid (VLA) is another potential mediator for laccase. Galli and Gentili (2004) have proved that among these mediators, VLA was found to be the most efficient. They have found out that the conversion of benzylic alcohol to products by laccase from *Polyporus pinsitus* was high in the presence of VLA (Galli and Gentili, 2004). As N-OH mediator, laccase oxidize VLA to produce radical cations and be deprotonated to aminoxyl radical (>N-O$^{\bullet}$) (Fig. 6.11) which involve in the depolymerization of lignin. An attempt was made to study the effect of LTV on the depolymerization of sodium lignosulphonate using VLA. In order to obtain the result, sodium lignosulphonate was incubated in ammonium acetate buffer and LTV in the presence of VLA using the same experimental setup as previously described.

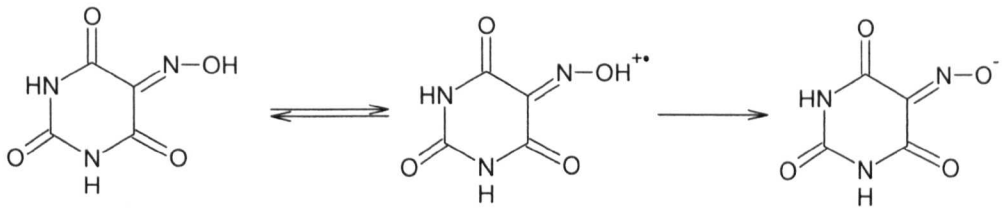


Figure 6.11 Mechanism of VLA oxidation by laccase as suggested by Fabbrini *et al.* (2002).

Six different compounds were produced after 2 h of reaction. This result is by far similar to the product formation with the laccase-TEMPO system. There was only 0.01 mM of guaiacol **(1)** detected after 2 h of reaction; however, this compound disappeared after 6 h (Table 6.5).

Table 6.5 Identification of products formed after the depolymerization of sodium lignosulphonate by LTV and mediated by VLA. Identification of the products was based on comparison of mass spectra of authentic standards (Appendix A.6.4 – page 244) and the retention time of the products was matched to the standards. The data represent the mean of three replicates and standard deviation (SD). A control samples (sodium lignosulphonate without LTV and VLA) contained the same amount of lignin (50 g/L) treated under the same condition. The concentration of the released compound was calculated based on the peak area of the product been compared to the peak area of an authentic standard (5 mM).

Label	Compounds	Concentration of the product in aqueous ethyl acetate extract fraction ± SD			
		Control (mM)	**2 h (mM)**	**6 h (mM)**	**24 h (mM)**
1	Guaiacol	n.d	0.01 ± 0.00	n.d	n.d
2	Vanillin	0.17 ± 0.01	0.27 ± 0.00	0.25 ± 0.00	0.12 ± 0.00
3	Acetovanillone	n.d	0.02 ± 0.00	0.01 ± 0.00	n.d
5	Vanillic acid	0.80 ± 0.01	1.39 ± 0.04	0.89 ± 0.01	-
13	3-hydroxy-1-(4-hydroxy-3-methoxyphenyl)propan-1-one	n.a	n.a	n.a	n.a
41	Isovanillic acid	-	0.41 ± 0.01	0.06 ± 0.00	-

*n.d: not detected; *n.a: not applicable (the compound without authentic standard for which the molar concentration could not be calculated)

As observed in the reaction mediated by TEMPO, HBT and HPI, the concentration of vanillin **(2)** was increased from 0.17 (control) to 0.27 mM over 2 h and then slightly reduce to 0.25 mM after 2 h. This compound was further decreased over 24 h to 0.12 mM of concentration (Table 6.5). The production of acetovanillone **(3)** and isovanillic acid **(41)** was observed after 2 h with concentrations of 0.02 and 0.41 mM, respectively and slightly decreased thereafter. The maximum production of vanillic acid **(5)** was also obtained after 2 h of reaction with the production of 1.39 mM vanillic acid. However, the amount was deceased to 0.89 mM after 6 h (Table 6.5). It is apparent in Fig. 6.12 that the intensity of 3-hydroxy-1-(4-hydroxy-3-methoxyphenyl)propan-1-one **(13)** was increased after 2 h and slightly reduced over 24 h of reaction time.

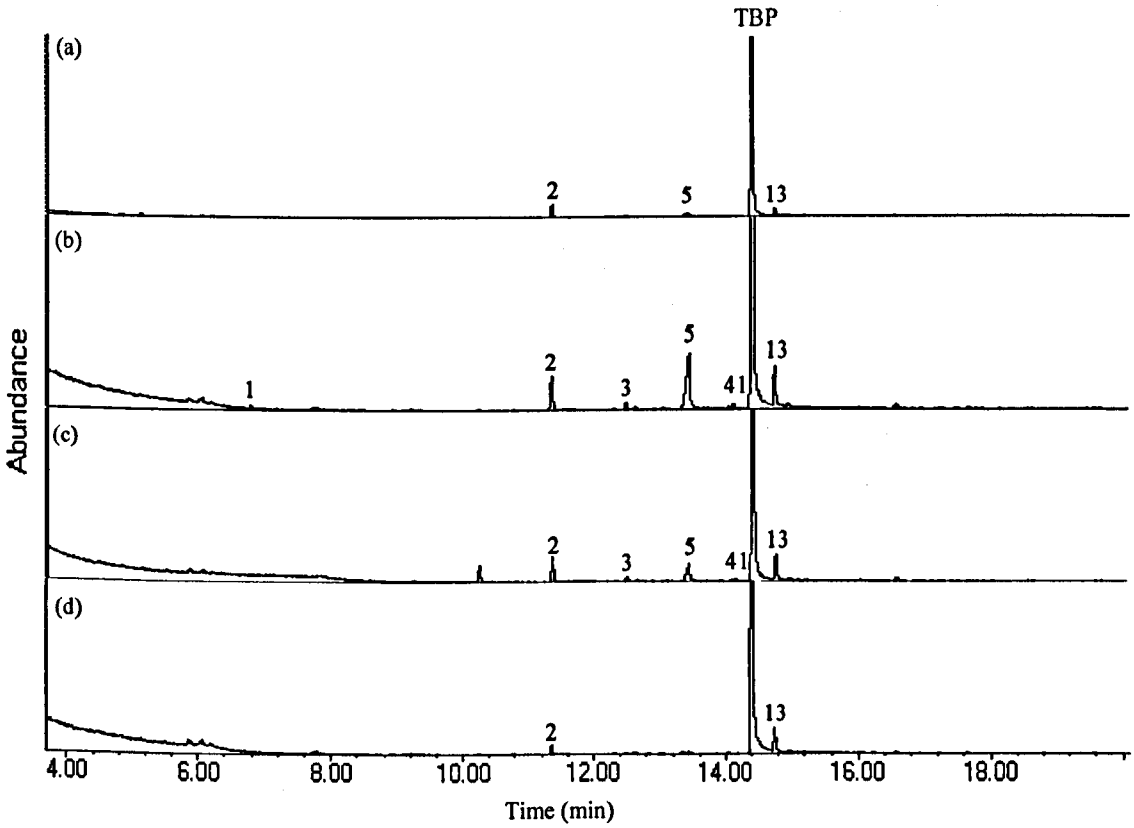


Figure 6.12 The effect of incubation time on the depolymerization of sodium lignosulphonate mediated by VLA. The identities of the released compounds are listed in Table 6.5. Samples were incubated under identical condition (at 30°C, shaken at 200rpm). Fractionation was applied and the aqueous ethyl acetate extract fraction was evaporated to dryness and redissolved in DCM. TBP (tributylphosphate). (a) The aqueous ethyl acetate extracts of the control (b) 2 h, (c) 6 h, (d) 24 h of incubation time. All chromatograms were on the same scale of intensity.

VLA is less effective as a mediator compared to ABTS, since VLA does not improve the yields of product formation. This result is rather disappointing. However, earlier it has been pointed out that there are several factors that may contribute to this result such as the lack of optimization of the process for each of the mediators. It can therefore be suggested that further studies need to be done to investigate each of the variables that contributes towards a better process.

6.4 Discussion

According to the study by Fabbrini *et al.* (2002), TEMPO possessed high mediation efficiency in the conversion of benzyl alcohol and 4-methylbenzyl alcohol to oxidised products using laccase from *Trametes villosa.* The yield was observed to be 99 % conversion in the reaction mediated by TEMPO and if compared to ABTS, only 22 % product yield was observed (Fabbrini *et al.*, 2002; Baiocco *et al.*, 2003). In addition, N-OH mediators such as HBT, HPI and VLA were the most efficient laccase mediator for the oxidation of aromatic compounds such as non-phenolic lignin (Srebotnik and Hammel, 2000; Xu *et al.*, 2000). The oxidation of N-OH type mediators produces highly active aminoxyl radicals (>N-O$^{\cdot}$) which result in a high efficiency of substrate oxidation (Bourbonnais *et al.*, 1997). In contrast to previous studies, the rate of sodium lignosulphonate breakdown by LTV and mediated by ABTS was significantly higher than the reaction mediated by TEMPO, HBT, VLA and HPI. This result might be due to the different mediated reaction condition and the source of the laccase. In addition, it has to be noted that most of the previous studies were focused on the oxidation of lignin model compounds by purified laccases, which might contribute towards different observation. One of the important results observed from this finding is that only five compounds were identified by GCMS on the reaction mediated by TEMPO and VLA, four compounds in the presence of HBT, and three compounds from the reaction with HPI after 6 h of reaction time. In contrast, 13 compounds were produced after the enzymatic treatment of sodium lignosulphonate by LTV-ABTS system (Chapter 5, Section 5.4).

It is quite disappointing that there were no further improvement of the products concentrations in the reaction mediated by TEMPO, HBT, VLA and HPI compared

to ABTS. For instance, the concentration of five major compounds produced from the laccase-ABTS system were significantly higher than the products formed after enzymatic treatment of sodium lignosulphonate mediated by TEMPO, HBT, HPI and VLA (Table 6.6). It is apparent from Table 6.6 that homovanillyl alcohol and guaiacol was not observed in the presence of these mediators after 6 h. Vanillic acid was observed to be the major product from the breakdown of sodium lignosulphonate, however, the production of vanillic acid was significantly lower in the reaction containing TEMPO, HBT, HPI and VLA compared to ABTS. The production of acetovanillone remained constant after the reaction with TEMPO, HBT and VLA. However, this compound was absent in the presence of HPI. There was only 0.26 mM of vanillin produced from the reaction mediated by HBT and this amount was slightly decreased to 0.25 and 0.24 mM for VLA and HPI, respectively and further decreased to 0.18 mM in the presence of TEMPO (Table 6.6).

Table 6.6 Comparison of the products formed after enzymatic treatment of sodium lignosulphonate in the presence of ABTS, TEMPO, HBT, HPI and VLA.

Product\Mediator	Concentration of the product in aqueous ethyl acetate extract fraction (mM) ± SD				
	ABTS	**TEMPO**	**HBT**	**HPI**	**VLA**
Guaiacol	2.08 ± 0.14	0.18 ± 0.00	n.d	n.d	n.d
Vanillin	2.90 ± 0.06	0.18 ± 0.00	0.26 ± 0.00	0.24 ± 0.00	0.25 ± 0.00
Acetovanillone	1.27 ± 0.08	0.01 ± 0.00	0.01 ± 0.00	n.d	0.01 ± 0.00
Homovanillyl alcohol	1.47 ± 0.08	n.d	n.d	n.d	n.d
Vanillic acid	38.8 ± 2.76	0.93 ± 0.02	0.94 ± 0.03	0.27 ± 0.01	0.89 ± 0.01

*The reaction mixtures include ammonium acetate buffer (pH 4.5, 100 mM), lignin (50 g/l), mediator (5mM) and LTV (0.25 mg/ml). The reaction mixture was incubated at 30 °C and shaken at 200 rpm for 6 h. Fractionation was applied and the aqueous ethyl acetate extract was redissolved in DCM. Each product was quantified from GCMS data based on the peak area of authentic standard. The data represent the mean of three replicates. n.d: not detected (compound not detected by GCMS)

Although these results differ from some published studies (Fabbrini *et al.*, 2002; Baiocco *et al.*, 2003; Camarero *et al.*, 2005), they are consistent with those found in a later study for the decolorization of textile dyes by laccase from *Brassica juncea* (Telke *et al.*, 2011) and laccase from *Aspergillus* (Tavares *et al.*, 2010). Those studies have demonstrated the efficiency of ABTS as a laccase mediator compared to other compounds (HBT, acetosyringone, vanillin, syringaldazine *etc.*).

According to Johannes and Majcherczyk (2000), the rate of oxidation of the substrate by laccase will decrease according to the increase of the redox potential of the substrate. The redox potential of laccase is approximately between 0.5 to 0.8 V depending on the type of laccase. In order for the rate of oxidation to increase, the redox potential of the mediator should be less than the redox potential of laccase (Johannes and Majcherczyk, 2000). This factor may explain the relative correlation of redox potential between the laccase and the mediator. Since the redox potential of ABTS is 0.67 V (Fabbrini *et al.*, 2002), which is much lower than the laccase, the oxidation ability of the laccase mediator system using ABTS increases with this correlation. On the other hand, the redox potential of HBT and VLA are higher than the laccase (1.04 and 0.91 V, respectively) (Heitner *et al.*, 2011) which resulted in the lower rate of oxidation. Even though the redox potential of TEMPO was lower than laccase which is found to be 0.2 V (Fabbrini *et al.*, 2001), the rate of oxidation was still decreased. This contradictory result may be due to other factors. It can generally be assumed that under the reaction conditions discussed above, all radicals were formed in low rate on the oxidation of TEMPO, HBT, HPI and VLA by LTV. As a result, the breakdown of sodium lignosulphonate was reduced in the presence of these mediators. On the other hand, the process involve in laccase-ABTS system was successful since the reaction condition were optimized based on the oxidation of ABTS. It can therefore be suggested that further experimental investigation are needed to optimize the conditions for each of the potential mediators. The yield of products formation may be increase if the optimum conditions can be achieved. The desire to find alternative mediator to replace ABTS was driven by the fact that this compound are expensive (Table 6.7) and toxic (Johannes *et al.*, 2006). In order to reduce the capital cost of the process, and to develop a 'greener' process, the more efficient and less expensive mediators should therefore be explored.

Table 6.7 Current market price for selected mediators (source: www.sigmaaldrich.com)

Mediator	Price (£) per g
ABTS	38.30
TEMPO	17.30
HBT	1.20
HPI	1.52
VLA	2.52

In addition, it would be interesting to assess the effects of natural mediators such as vanillin, syringaldehyde, acetosyringone *etc*. Natural mediators are derived naturally from the biodegradation process of lignin that is supposed to act as redox mediators. Therefore, the high rate of oxidation may be achieved in the presence of these mediators. Future studies on this topic are therefore recommended and it would be interesting to compare the effect of 'synthetic mediator' and 'natural mediator' on the depolymerization of lignin by laccase using the same experimental set up.

Chapter 7

IONIC LIQUIDS AS POTENTIAL SOLVENTS FOR LIGNIN DEPOLYMERIZATION

7.1 Introduction

In this chapter the use of laccase from *Agaricus bisporus* (LAB)-ABTS systems in ionic liquids is explored with the aim to apply laccase in ionic liquids for the transformation of lignin to high value chemicals. The vast amount of publications regarding ionic liquids covers many different types of applications (Liu *et al.*, 2005; Kubisa, 2004; Zhao *et al.*, 2002; Plechkova and Seddon, 2007) due to the potential of ionic liquids as 'green solvents' (Earle and Seddon, 2002; Yang and Pan, 2005). According to several studies, ionic liquids can dissolve larger amounts of lignin compared to other solvents (Pu *et al.*, 2007; Stark *et al.*, 2010). Due to the limited solubility of lignin in organic solvents, ionic liquids have provided a way to enhance the dissolution of lignin. However, the limitation lies in the fact that different ionic liquids may have different behaviours for lignin dissolution and also for enzyme activity. Therefore, the compatibility of ionic liquids with LAB was discovered by performing high throughput screening to identify the ionic liquids that are able to support LAB activity. In this study, ABTS was used as a substrate for LAB. Most of the studies on the compatibility of ionic liquids in laccase assays were only focused on small numbers of ionic liquids (Shipovskov *et al.*, 2008; Tavares *et al.*, 2008, 2012; Rodriguez *et al.*, 2011; Domínguez *et al.*, 2011). Therefore, in this study the effect of 106 ionic liquids on the activity of LAB was demonstrated. In order to achieve this goal, six groups of cations were selected and these, together with the anions used, are presented in Fig. 7.1.

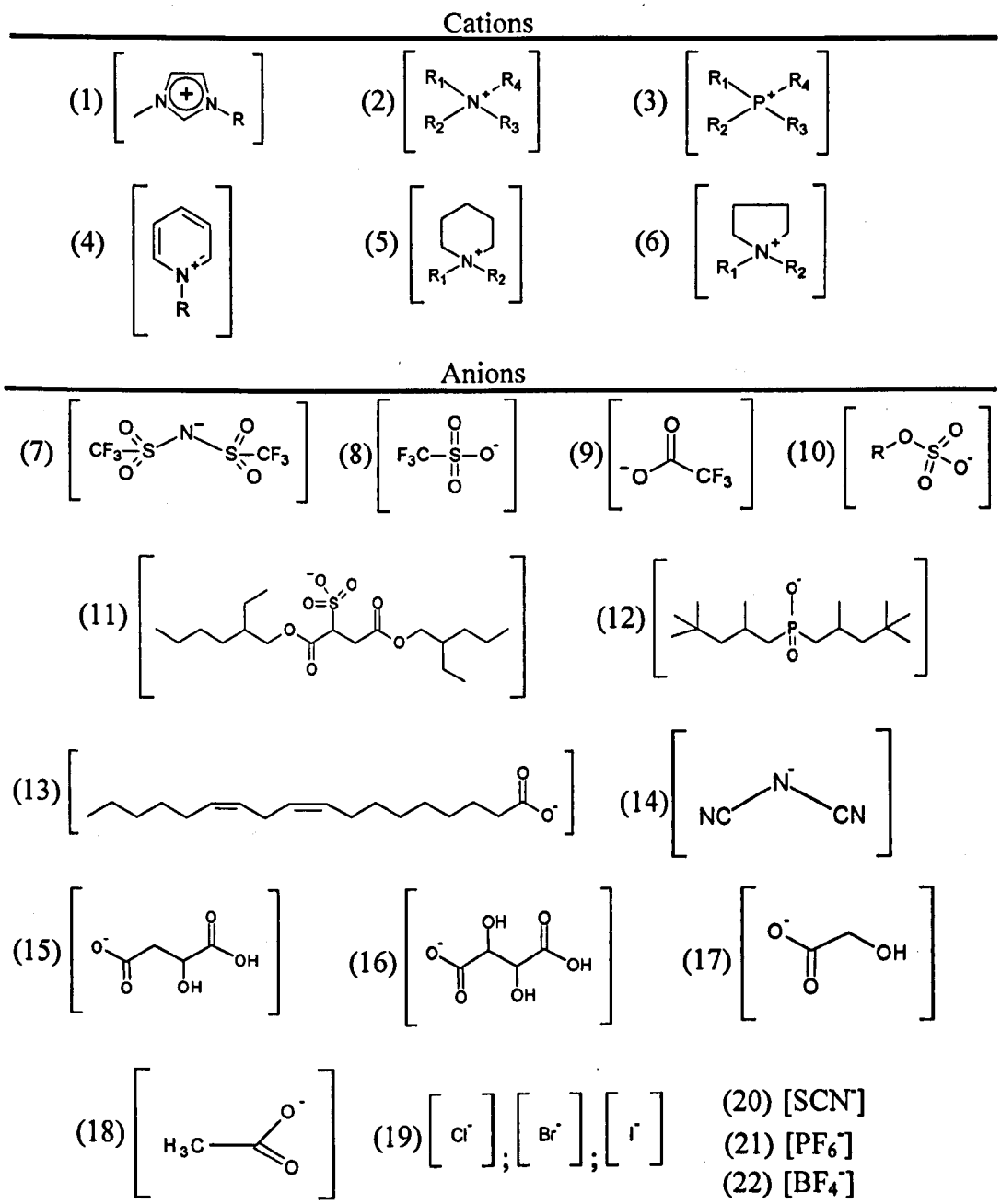


Figure 7.1 Cations and anions used in this study. **(1)** 1-Alkyl-3-methyl-imidazolium (imidazolium based cation);**(2)** tetraalkyl-ammonium (quaternary ammonium based cation); **(3)** tetraalkyl-phosphonium (phosphonium based cation); **(4)** *N*-alkyl pyridinium (pyridinium based cation); **(5)** *N,N*-dialkyl-piperidinium (piperidinium based cation); **(6)** *N,N*-dialkyl-pyrrolidinium (pyrrolidinium based cation); **(7)** bis(trifluoromethylsulphonyl) imide [NTf_2]; **(8)** triflate [OTf]; **(9)** trifluoroacetate [TFA]; **(10)** alkyl sulphate; **(11)** 1,4-*bis* (2-ethylhexyl)-sulfosuccinate [AOT] ; **(12)** *bis*(2,4,4-trimethylpentyl)phosphinate [DIOPN] ; **(13)** [linoleate]; **(14)** dicyanamide [$N(CN)_2$]; **(15)** [DL-malate]; **(16)** [L-tartrate]; **(17)** [lactate]; **(18)** [acetate]; **(19)** halides; **(20)** thiocyanate [SCN]; **(21)** hexafluorophosphate [PF_6]; **(22)** hexafluoroborate [BF_4].

7.2 The Activity of ABTS in the Presence and Absence of [C_4eim] [C_2SO_4]

The effect of ionic liquids on the activity of LAB using ABTS as a substrate was studied by measuring LAB activity in a control reaction without ionic liquid (control) and in the presence of [C_4eim] [C_2SO_4] and measuring the spectra during the course of the reaction as shown in Fig. 7.2. [C_4eim] [C_2SO_4] is a liquid at room temperature, has low viscosity, and therefore is chosen because it can be pipetted accurately into the assay, which makes this ionic liquid a suitable candidate for this experiment. The assay was prepared in a 1 ml cuvette containing sodium citrate buffer, LAB and the [C_4mim] [C_2SO_4]. The aim was therefore to show any interference of the ionic liquid with the absorbance spectrum of ABTS.

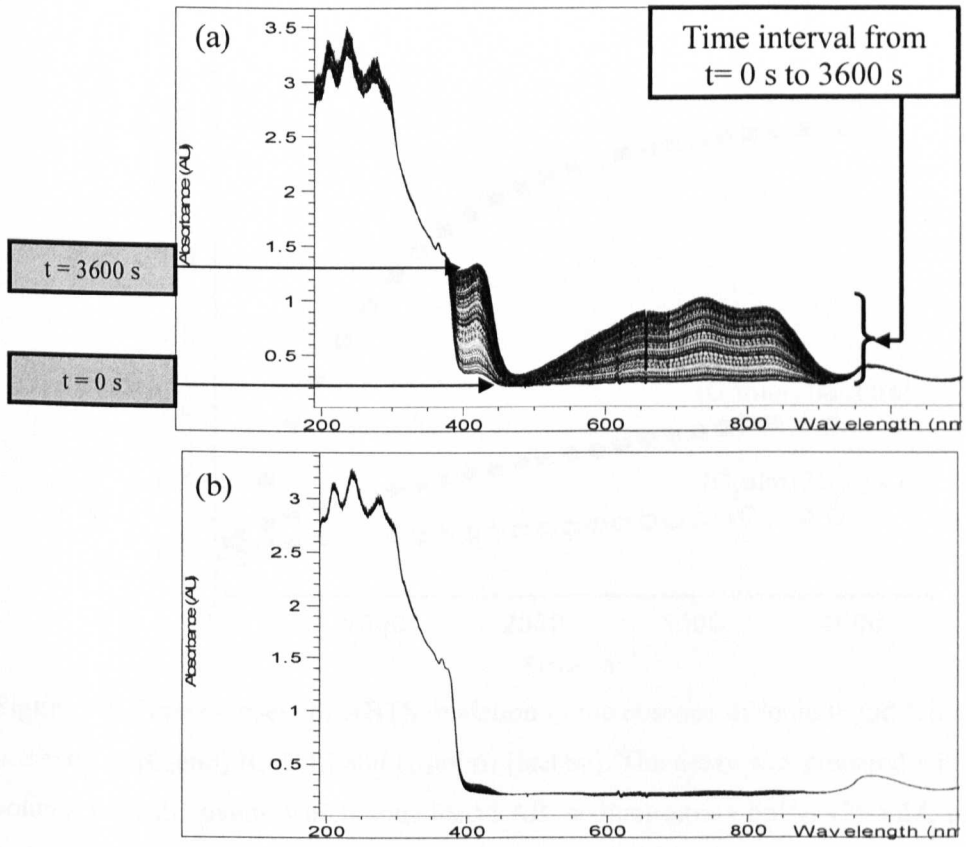


Figure 7.2 The absorbance changes during the oxidation of ABTS by LAB in the (a) absence of [C_4eim] [$EtSO_4$] (control) and (b) in the presence of [C_4eim] [C_2SO_4]. The coloured lines represent the time interval from 0 to 3600 s. The assay was prepared with 1 ml total volume in each cuvette which contained LAB, sodium citrate buffer (25 mM, pH 6.0) and 3 % v/v ionic liquid. The reaction was started by adding ABTS (5 mM) and the absorbance was measured spectrophotometrically at 420 nm at room temperature. The spectra are representative of triplicate experiment.

There was a non-specific absorbance at less than 390 nm in both the control and the reaction mixture with the ionic liquid, but ABTS oxidation resulted in an increased absorbance between 390 nm and 450 nm and from 600 nm to 850 nm. The maximum absorbance was at 420 nm, and was not changed in the presence of the ionic liquid. A good agreement was found between this experimental result and studies by Marjasvaara *et al.* (2008) and Tavares *et al.* (2008). However, [C_4eim] [C_2SO_4] inhibited the activity of LAB since the absorbance was decreased compared to the control. As shown in Fig. 7.2a, a broad absorbance change was observed from time 0 to 3600 s for the control. However, in the presence of [C_4eim] [C_2SO_4], lower absorbance changes were observed as in Fig. 7.2b. The kinetic curves of the sample without the ionic liquid and the sample with 3 % v/v [C_4eim] [C_2SO_4] shows that LAB is inhibited in the presence of this ionic liquid (Fig. 7.3)

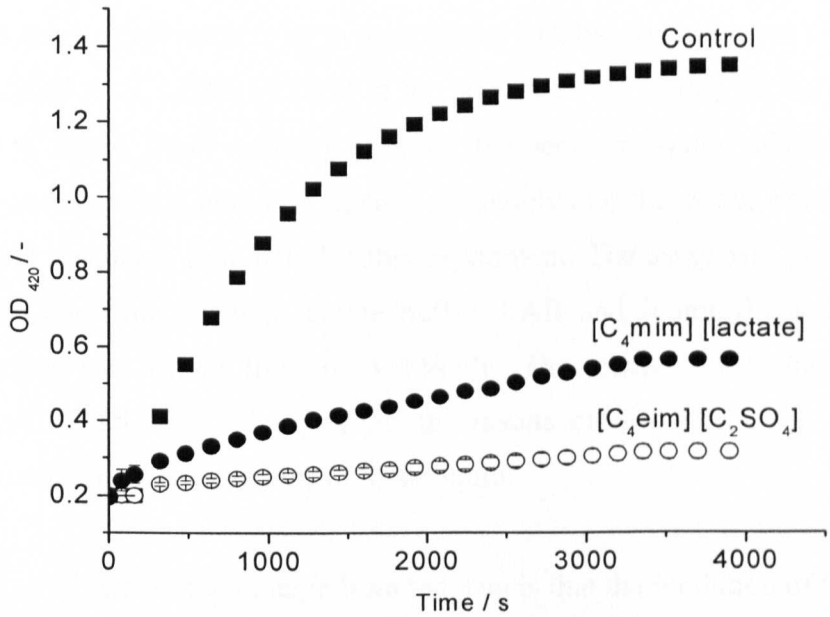


Figure 7.3 Time courses for ABTS oxidation in the absence of ionic liquid (control), in the presence of [C_4eim] [C_2SO_4] and [C_4mim] [lactate]. The assay was prepared with 1 ml total volume in each cuvette which contained LAB, sodium citrate buffer (25 mM, pH 6.0) and 3 % v/v ionic liquid. The reaction was started by adding ABTS (5mM) and the absorbance was measured spectrophotometrically at 420 nm at room temperature. The data represent the mean of three replicates with an error of less than 1 %.

Figure 7.3 illustrates that the initial rate of reaction was calculated from the initial slope of the OD versus time at 5 mM of ABTS and was found to be 2.88 x 10^{-5} mMs^{-}

^{1} in the presence of ionic liquid, which is 96 % lower than the activity of LAB in the absence of [C_4eim] [C_2SO_4] (7.21 x 10^{-4} mMs^{-1}). This study has delivered a basic understanding of the interference of [C_4eim] [C_2SO_4] on the oxidation of ABTS. It has to be noted that different ionic liquids would have different effects on the oxidation of the substrate. Therefore, more work on the effect of ionic liquids on LAB activity was conducted to explore the ionic liquids that are able to support the activity of LAB. Next, the effect of ionic liquid concentrations on LAB activity was studied by using [C_4mim] [lactate], since the activity of LAB in the presence of [C_4mim] [lactate] was higher than [C_4eim] [C_2SO_4] (Fig. 7.3).

7.3 Effect of [C_4mim] [lactate] Concentration on LAB activity

The activity of LAB in the presence of different concentrations of ionic liquids was measured spectrophotometrically to determine a suitable concentration of ionic liquid for the oxidation of ABTS by LAB in the presence of [C_4mim] [lactate]. This ionic liquid is a liquid (light yellow) at room temperature, water miscible, has low viscosity and therefore, could be pipetted accurately into the assay, which makes this ionic liquid a suitable candidate for this experiment. The assay was prepared in a 1 ml cuvette containing sodium citrate buffer, LAB and [C_4mim] [lactate] and the concentration was varied from 0 to 6 % v/v. The reaction was started with the addition of ABTS. Fig. 7.4 represents the results obtained from the oxidation of ABTS in different concentrations of ionic liquid.

The most striking result to emerge from the data is that the inhibition of LAB activity was observed at all concentration tested and the rate of oxidation decreased when the concentration of [C_4mim] [lactate] increased. This result is in line with the findings reported by Ventura *et al.* (2012) by using *Candida antarctica lipase B* (CaLB) in the presence of ten different ionic liquids. There was some residual activity observed in the presence of 4 to 5 % v/v of [C_4mim] [lactate], however LAB was deactivated completely when 6 % v/v of [C_4mim] [lactate] was employed. The lower amount of [C_4mim] [lactate] between 0.6 to 1 % v/v concentration shows a good activity of LAB, however, the concentration of ionic liquid within this range would have little effect on the dissolution of lignin. Thus, it was then proposed to use moderate

amounts of ionic liquid in the range between 2 to 3 % v/v for further experimental work.

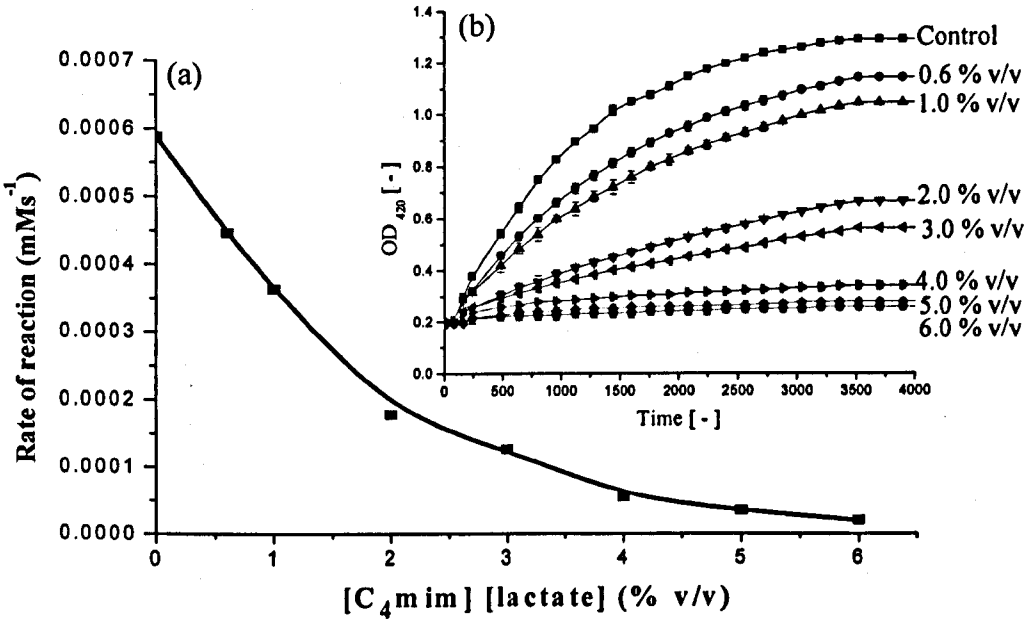


Figure 7.4 Effect of [C_4mim] [lactate] concentration on the oxidation of ABTS by LAB. (a) rate of reaction in the presence of different concentrations of [C_4mim] [lactate] and (b) raw data of ABTS oxidation by LAB. The initial rate of reaction was calculated based on the slope of the OD versus time graph. The assay was prepared with 1 ml total volume in each cuvette which contained LAB, sodium citrate buffer (25 mM, pH 6.0) and various concentrations of ionic liquids. The reaction was started by adding ABTS (5 mM) and the absorbance changes were measured spectrophotometrically at 420 nm at room temperature. The data represents the mean of three replicates with an error of less than 1 %.

7.4 Screening of Ionic Liquids

Oxidation of ABTS by LAB was measured in 96 well quartz plates using a FLUOstar Optima Microplate Reader (BMG Labtech Ltd., UK). A quartz plate was used in this study because some ionic liquids can dissolve disposable polyisoprene or polypropylene plates. The assay was prepared with 300 µl total volume in each well which contained LAB, sodium citrate buffer (25 mM, pH 6.0) and 3 % v/v ionic liquid. The assay was incubated without shaking for 10 min to equilibrate the mixtures, and then shaken for a further 1 min to make sure the solutions were mixed well. The reaction was started by adding ABTS (5 mM) and the absorbance was measured at 420 nm at room temperature. The Michaelis-Menten parameters were calculated based on the method by Rehmann *et al.* (2012) (See Chapter 3; Section

3.7.2). The ionic liquids were grouped into six categories based on the structure of the cation.

7.4.1 Effect of Imidazolium Based Ionic Liquids on LAB Activity

Screening was used to study the effect of 36 imidazolium based ionic liquids on LAB. The basic structure of the imidazolium cation is shown below where R is an alkyl chain and x is the number of carbon atoms in the alkyl chain:

1-alkyl-3-methyl-imidazolium ($C_x$mim)

1-alkyl-3-ethyl-imidazolium ($C_x$eim)

There were ten types of anion groups tested and the list of imidazolium ionic liquids structures are shown in Appendix A.7.1 (page 247). According to Zhao (2007), halide anions with imidazolium cations that contain more than four carbon atoms in the side chain will inhibit the activity of the enzyme completely. This also accords with the current observation, which showed complete inhibition of LAB activity in the presence of halide anions except for [C_1mim] [Cl] (Table 7.1).

Table 7.1 Activity of LAB in the presence of imidazolium based ionic liquids and halides anion.

IMIDAZOLIUM BASED IONIC LIQUID (Halide anions)					
Cation	**Anion**	**Miscibility**	V_o [mMs^{-1}] x 10^{-4}	V_{max} [mMs^{-1}] x 10^{-4}	K_m [mM] x 10^{-2}
Control			3.70 ± 0.09	1.59 ± 0.17	24.6 ± 11.0
[C_1mim]	[Cl][3]	√	1.19 ± 0.07	0.66 ± 0.03	7.29 ± 1.05
[C_8mim]	[Cl][3]	√	n.d	-	-
[C_{13}mim]	[Cl][3]	√	n.d	-	-
[C_{16}mim]	[Cl][3]	√	n.d	-	-
[C_{18}mim]	[Cl][4]	•	n.d	-	-
[C_4mim]	[Br][4]	•	n.d	-	-
[C_{10}mim]	[Br][4]	•	n.d	-	-
[C_4mim]	[I][3]	√	n.d	-	-
[C_6mim]	[I][3]	√	n.d	-	-

(V_{max} is the maximum reaction velocity (mMs^{-1}), K_m is the half saturation constant (mM), V_o is the initial reaction velocity (mMs^{-1}), n.d, indicates complete inhibitory and the parameters cannot be measured; -, indicates the parameters cannot be calculated; [3] indicates single phase; and [4] indicates biphasic system; √ indicates a water miscible ionic liquid; • indicates a water immiscible ionic liquid).

Other than halides, dicyanamides and thiocyanates are also known to inhibit the activity of LTV (Rehmann *et al.*, 2012). Complete inhibition in the presence of these anions was observed in the current study which corroborates the earlier findings by Rehmann *et al.* (2012) (Table 7.2). In addition, the miscibility of ionic liquids in water is also a factor that contributes towards enzyme inhibition (Rehmann *et al.*, 2012). For instance, all of the dicyanamides and thiocyanates anions in this study were water miscible and complete inhibition was observed in the presence of these ionic liquids.

Table 7.2 Activity of LAB in the presence of imidazolium based ionic liquids and thiocyanates and dicyanamides.

IMIDAZOLIUM BASED IONIC LIQUIDS (Thiocyanates and dicyanamides anions)				
Cation	**Anion**	V_o [mMs^{-1}] x 10^{-4}	V_{max} [mMs^{-1}] x 10^{-4}	K_m [mM] x 10^{-2}
Control		3.70 ± 0.09	1.59 ± 0.17	24.6 ± 11.0
[C_2mim]	[SCN]^3	n.d	-	-
[C_{10}mim]	[SCN]^3	n.d	-	-
[C_4mim]	[N(CN)$_2$]^3	n.d	-	-
[C_{10}mim]	[N(CN)$_2$]^3	n.d	-	-

(V_{max} is the maximum reaction velocity (mMs^{-1}), K_m is the half saturation constant (mM), V_o is the initial reaction velocity (mMs^{-1}), n.d, indicates complete inhibitory and the parameters cannot be measured; -, indicates the parameters cannot be calculated; ^{3} indicates single phase.

The majority of imidazolium ionic liquids containing alkyl sulphate anions were water miscible. Contrary to earlier findings with water miscible ionic liquids, the alkyl sulphate anions supported the activity of LAB and the majority of these ionic liquids did not inhibit the activity of LAB completely (Table 7.3). Complete inhibition was observed in the presence of *N*-butyl-*N*-ethyl-imidazolium containing ethyl sulphate anion ([C_4eim] [C_2OSO_3]). For the alkyl sulphate anions, the inhibition of LAB was influenced by the alkyl chain length of the imidazolium cation and by the alkyl substituent [C_nOSO_3] or isoalkyl substituent [$C_n(C_1)OSO_3$] of the anion. A low residual activity of LAB was observed in the presence of [C_2mim] [C_8OSO_3] and [C_4mim] [C_1OSO_3]. On the other hand, [C_4mim] [C_2OSO_3], [C_4mim] [C_3OSO_3], [C_4mim] [$C_1OC_2OSO_3$], [C_4mim] [$C_2OC_2OSO_3$] and [C_4mim] [$C_2(C_1)OSO_3$] caused a very low residual activity of LAB and the rate could not be measured accurately.

Table 7.3 Activity of LAB in the presence of imidazolium based ionic liquids and alkyl sulphate anion.

IMIDAZOLIUM BASED IONIC LIQUID (alkyl sulphate anions)					
Cation	**Anion**	**Miscibility**	V_o [mMs^{-1}] x 10^{-4}	V_{max} [mMs^{-1}] x 10^{-4}	K_m [mM] x 10^{-2}
Control			3.70 ± 0.09	1.59 ± 0.17	24.6 ± 11.0
[C_2mim]	[C_8OSO_3]^3	√	1.49 ± 0.04	0.83 ± 0.04	10.7 ± 1.34
[C_4mim]	[C_1OSO_3]^3	√	0.24 ± 0.02	0.65 ± 0.08	5.04 ± 0.40
[C_4mim]	[C_2OSO_3]^3	√	+	+	+
[C_4mim]	[C_3OSO_3]^3	√	+	+	+
[C_4eim]	[C_2OSO_3]^3	√	+	+	+
[C_4mim]	[$C_1OC_2OSO_3$]^3	√	+	+	+
[C_4mim]	[$C_2OC_2OSO_3$]^3	√	+	+	+
[C_4mim]	[$C_2(C_1)OSO_3$]^3	√	+	+	+
[C_4mim]	[$C_3(C_1)OSO_3$]^4	•	2.45 ± 0.18	0.91 ± 0.05	12.3 ± 1.47

(V_{max} is the maximum reaction velocity (mMs^{-1}), K_m is the half saturation constant (mM), V_o is the initial reaction velocity (mMs^{-1}), n.d, indicates complete inhibitory and the parameters cannot be measured; -, indicates the parameters cannot be calculated; +, indicates low activity which could not be quantified accurately; ^{3} indicates single phase; and ^{4} indicates biphasic system; √ indicates a water miscible ionic liquid; • indicates a water immiscible ionic liquid).

In general, the water immiscible ionic liquids were less inhibitory than the water soluble ionic liquids. For instance, all of the imidazolium based ionic liquids containing either [AOT] or [NTf_2] which formed a biphasic system appeared to increase the activity of LAB (*e.g.* [C_2mim] [AOT], [C_6mim] [AOT] and [C_4mim] [NTf_2]) except for [C_2mim] [NTf_2] (Table 7.4).

Table 7.4 Activity of LAB in the presence of imidazolium based ionic liquids and [AOT], [NTf_2] and [OTf] anions.

IMIDAZOLIUM BASED IONIC LIQUID ([AOT], [NTf_2] and [OTf] anions)					
Cation	**Anion**	**Miscibility**	V_o [mMs^{-1}] x 10^{-4}	V_{max} [mMs^{-1}] x 10^{-4}	K_m [mM] x 10^{-2}
Control			3.70 ± 0.09	1.59 ± 0.17	24.6 ± 11.0
[C_2mim]	[AOT]^4	•	4.72 ± 0.09	2.48 ± 0.14	20.6 ± 3.07
[C_6mim]	[AOT]^4	•	4.22 ± 0.01	2.32 ± 0.12	20.45 ± 1.06
[C_2mim]	[NTf_2]^4	•	2.79 ± 0.38	1.39 ± 0.16	27.3 ± 3.91
[C_4mim]	[NTf_2]^4	•	4.50 ± 0.12	2.29 ± 0.30	23.03 ± 7.5
[C_4mim]	[OTf]^3	√	n.d	-	-

(V_{max} is the maximum reaction velocity (mMs^{-1}), K_m is the half saturation constant (mM), V_o is the initial reaction velocity (mMs^{-1}), n.d, indicates complete inhibitory and the parameters cannot be measured; -, indicates the parameters cannot be calculated; ^{3} indicates single phase; and ^{4} indicates biphasic system; √ indicates a water miscible ionic liquid; • indicates a water immiscible ionic liquid).

[C_2mim] [NTf_2] and [C_4mim] [NTf_2] had a different effect on the activity of LAB. [C_4mim] [NTf_2] increased the activity of LAB by 22 % compared to the control, whereas, [C_2mim] [NTf_2] decreased the activity by 24 %. The absorbance changes of the assays containing [C_2mim] [AOT], [C_6mim] [AOT] and [C_4mim] [NTf_2] were higher than the control. In addition, the K_m value in the presence of these ionic liquids was lower than the control, indicating that the affinity of LAB for the substrate was higher, and thus explaining the higher reaction rate. On the other hand, complete inhibition was observed in the presence of the water miscible ionic liquid, [C_6mim] [OTf] (Table 7.4).

Complete inhibition was also observed in the assay containing [C_6-C_8mim] [BF_4] (Table 7.5) which was also soluble in water. In contrast, a high activity of LAB was observed in the presence of water immiscible ionic liquids containing hexafluorophosphate ([PF_6]) anions, [C_2-C_4mim] [PF_6]. However, the initial rate of reaction of LAB in the presence of [C_2mim] [PF_6] and [C_4mim] [PF_6] was still lower than the control in which the activity was reduced by 19 and 52 % respectively (Table 7.5).

Table 7.5 Activity of LAB in the presence of imidazolium based ionic liquids and [PF_6] and [BF_4] anions.

IMIDAZOLIUM BASED IONIC LIQUID ([PF_6] and [BF_4] anions)					
Cation	**Anion**	**Miscibility**	V_o [mMs^{-1}] x 10^{-4}	V_{max} [mMs^{-1}] x 10^{-4}	K_m [mM] x 10^{-2}
Control			3.70 ± 0.09	1.59 ± 0.17	24.6 ± 11.0
[C_2mim]	[PF_6][4]	•	2.97 ± 0.45	2.08 ± 0.25	37.2 ± 6.01
[C_4mim]	[PF_6][4]	•	1.76 ± 0.11	1.21 ± 0.16	16.7 ± 1.02
[C_6mim]	[BF_4][3]	√	n.d	-	-
[C_8mim]	[BF_4][3]	√	n.d	-	-

(V_{max} is the maximum reaction velocity (mMs^{-1}), K_m is the half saturation constant (mM), V_o is the initial reaction velocity (mMs^{-1}), n.d, indicates complete inhibitory and the parameters cannot be measured; -, indicates the parameters cannot be calculated; [3] indicates single phase; and [4] indicates biphasic system; √ indicates a water miscible ionic liquid; • indicates a water immiscible ionic liquid).

From 11 ionic liquids that supported LAB activity, [C_4mim] [L-tartrate] showed the best reaction rate by increasing the activity of this enzyme by more than 90 %, twice the activity of the control (Table 7.6). Low residual activity was observed in the presence of [C_4mim] [lactate] and [C_4mim] [DL-malate], however, the reaction rate

was too low to be determined accurately. On the other hand, [C_4mim] [acetate] caused complete inhibition of LAB activity (Table 7.6).

Table 7.6 Activity of LAB in the presence of imidazolium based ionic liquids and carboxylate anion.

IMIDAZOLIUM BASED IONIC LIQUID (carboxylates anion)				
Cation	**Anion**	V_o [mMs^{-1}] x 10^{-4}	V_{max} [mMs^{-1}] x 10^{-4}	K_m [mM] x 10^{-2}
Control		3.70 ± 0.09	1.59 ± 0.17	24.6 ± 11.0
[C_4mim]	[acetate][3]	n.d	-	-
[C_4mim]	[lactate][3]	+	+	+
[C_4mim]	[DL-malate][3]	+	+	+
[C_4mim]	[L-tartrate][3]	7.86 ± 0.35	1.76 ± 0.77	22.9 ± 5.6

(V_{max} is the maximum reaction velocity (mMs^{-1}), K_m is the half saturation constant (mM), V_o is the initial reaction velocity (mMs^{-1}), n.d, indicates complete inhibitory and the parameters cannot be measured; -, indicates the parameters cannot be calculated; +, indicates low activity which could not be quantified accurately; [3] indicates single phase.

In general, the water miscible ionic liquids tended to reduce or inhibit the activity of LAB completely (Rehmann *et al.*, 2012), however, a higher activity of LAB was observed in the presence of [C_4mim] [L-tartrate] which is water soluble (Table 7.6). This result indicates that the solubility of ionic liquids in water is not the only issue and the types of anion have a strong influence on LAB activity. For instance, [NTf_2] and [AOT] anions were compatible to be use with LAB. The activity of LAB was increased in the presence of these ionic liquids (Table 7.4). In addition, [PF_6] can also be a potential anion even though the activity of LAB was decreased in the presence these ionic liquids (Table 7.5). However, the hydrolysis of [PF_6] anion in water to produce hydrogen fluoride (HF) needs to be taken into consideration since HF is extremely toxic and corrosive when it reacts with water (Othmer, 2009).

7.4.2 Effect of Quaternary Ammonium Based Ionic Liquid on LAB Activity

A total of 35 quaternary ammonium based ionic liquids were tested and the list of quaternary ammonium ionic liquids structures is shown in Appendix A.7.2 (page 250). As with imidazolium ionic liquids, complete inhibition was observed in the presence of halide anions especially the ionic liquid containing [Cl] and [I] anions, which were [N_{1148}] [Cl], [N_{1888}] [Cl] and [N_{1148}] [I] (Table 7.7). A very low residual

activity of LAB was observed in the presence of $[N_{1124}]$ [Br] and $[N_{112}C_2OH]$ [Br] and the Michaelis-Menten parameters could not be determined.

Table 7.7 Activity of LAB in the presence of quarternary ammonium based ionic liquids and halide anions.

QUATERNARY AMMONIUM BASED IONIC LIQUID (Halide anions)					
Cation	**Anion**	**Miscibility**	V_o [mMs^{-1}] x 10^{-4}	V_{max} [mMs^{-1}] x 10^{-4}	K_m [mM] x 10^{-2}
Control			3.70 ± 0.09	1.59 ± 0.17	24.6 ± 11.0
$[N_{1148}]$	$[Cl]^3$	√	n.d	-	-
$[N_{1888}]$	$[Cl]^4$	•	n.d	-	-
$[N_{1124}]$	$[Br]^3$	√	+	+	+
$[N_{112}C_2OH]$	$[Br]^3$	√	+	+	+
$[N_{1148}]$	$[I]^3$	√	n.d	-	-

(V_{max} is the maximum reaction velocity (mMs^{-1}), K_m is the half saturation constant (mM), V_o is the initial reaction velocity (mMs^{-1}), n.d, indicates complete inhibitory and the parameters cannot be measured; -, indicates the parameters cannot be calculated; +, indicates low activity which could not be quantified accurately; [3] indicates single phase; and [4] indicates biphasic system; √ indicates a water miscible ionic liquid; • indicates a water immiscible ionic liquid).

As expected, there was no activity in the presence of the dicyanamide anion, for $[N_{114}C_2OH]$ $[N(CN)_2]$ and $[N_{24}(C_2OH)_2]$ $[N(CN)_2]$ (Table 7.8). Complete inhibition was also observed in the presence of $[N_{1148}]$ $[NO_3]$. On the other hand, the assays containing [DIOPN] anions supported the activity of LAB. $[N_{1148}]$ [DIOPN] caused low residual activity of LAB but the Michaelis-Menten parameters could not be determined accurately.

Table 7.8 Activity of LAB in the presence of quarternary ammonium based ionic liquids and dicyanamides, nitrate and [DIOPN] anions.

QUATERNARY AMMONIUM BASED IONIC LIQUID (Dicyanamides, nitrate, DIOPN anions)					
Cation	**Anion**	**Miscibility**	V_o [mMs^{-1}] x 10^{-4}	V_{max} [mMs^{-1}] x 10^{-4}	K_m [mM] x 10^{-2}
Control			3.70 ± 0.09	1.59 ± 0.17	24.6 ± 11.0
$[N_{114}C_2OH]$	$[N(CN)_2]^3$	√	n.d	-	-
$[N_{24}(C_2OH)_2]$	$[N(CN)_2]^3$	√	n.d	-	-
$[N_{1148}]$	$[NO_3]^3$	√	n.d	-	-
$[N_{1148}]$	$[DIOPN]^4$	•	+	+	+
$[N_{1888}]$	$[DIOPN]^4$	•	4.86 ± 0.08	1.95 ± 0.22	34.2 ± 5.37

(V_{max} is the maximum reaction velocity (mMs^{-1}), K_m is the half saturation constant (mM), V_o is the initial reaction velocity (mMs^{-1}), n.d, indicates complete inhibitory and the parameters cannot be measured; -, indicates the parameters cannot be calculated; +, indicates low activity which could not be quantified accurately; [3] indicates single phase; and [4] indicates biphasic system; √ indicates a water miscible ionic liquid; • indicates a water immiscible ionic liquid).

In addition, the activity of LAB was increased by 24 % in the presence of [N_{1888}] [DIOPN] compared to the control (Table 7.8).

The effect of alkyl sulphate salts was strongly dependent on the structure of the cation. [$N_{112}C_2OH$] [C_2OSO_3], [$N_{112}C_3OH$] [C_2OSO_3], [$N_1(C_2OH)_3$] [C_1OSO_3] and [N_{1288}] [C_6OSO_3] inhibited the activity of LAB completely (Table 7.9). It has to be noted that all of the quaternary ammonium ionic liquids containing alkyl sulphate anions were water miscible. A very low residual activity was observed in the presence of [N_{1124}] [C_4OSO_3] and [N_{1128}] [C_2OSO_3], however the rate could not be determined accurately. In addition, [N_{1124}] [C_2OSO_3] decreased LAB activity by 20 %. On the other hand, the activity of LAB in the presence of [$N_2(C_1OC_2OC_2)_3$] [$CH_3CH_2OSO_3$] was increased by 44 % when compared to control (Table 7.9).

Table 7.9 Activity of LAB in the presence of quarternary ammonium based ionic liquids and alkyl sulphate anions.

QUATERNARY AMMONIUM BASED IONIC LIQUID (alkyl sulphate anions)				
Cation	**Anion**	V_o [mMs^{-1}] x 10^{-4}	V_{max} [mMs^{-1}] x 10^{-4}	K_m [mM] x 10^{-2}
Control		3.70 ± 0.09	1.59 ± 0.17	24.6 ± 11.0
[N_{1124}]	[C_2OSO_3]^3	2.94 ± 0.13	8.06 ± 0.83	84.3 ± 3.34
[N_{1124}]	[C_4OSO_3]^3	+	+	+
[N_{1128}]	[C_2OSO_3]^3	+	+	+
[$N_{112}C_2OH$]	[C_2OSO_3]^3	n.d	-	-
[$N_{112}C_3OH$]	[C_2OSO_3]^3	n.d	-	-
[$N_1(C_2OH)_3$]	[C_1OSO_3]^3	n.d	-	-
[N_{1288}]	[C_6OSO_3]^3	n.d	-	-
[$N_2(C_1OC_2OC_2)_3$]	[$CH_3CH_2OSO_3$]^3	5.30 ± 0.11	2.37± 2.29	20.1 ± 3.12

(V_{max} is the maximum reaction velocity (mMs^{-1}), K_m is the half saturation constant (mM), V_o is the initial reaction velocity (mMs^{-1}), n.d, indicates complete inhibitory and the parameters cannot be measured; -, indicates the parameters cannot be calculated; +, indicates low activity which could not be quantified accurately; ^{3} indicates single phase.

Most of the quaternary ammonium ionic liquids containing the [AOT] anion were water immiscible except for [N_{14}(propylacetate)$_2$] [AOT] and complete inhibition was observed in the presence of this ionic liquid. Other than that, assays containing [$N_{112}C_2OH$] [AOT], [$N_{112}C_3OH$] [AOT], [N_{1148}] [AOT] and [N_{1888}] [AOT] decreased the activity of LAB and the rate could not be calculated. In contrast, [$N_{114}C_2OH$] [AOT] supported the activity, and activity was increased by 15 % (Table 7.10). No

activity was detected in the presence of water immiscible ionic liquids containing $[NTf_2]$ anions ($[N_{112}(C_2OH)_2]$ $[NTf_2]$ and $[N_{1888}]$ $[NTf_2]$). The activity was slightly decreased in the presence of $[N_{1148}]$ $[NTf_2]$ by only 12 % compared to the control.

Table 7.10 Activity of LAB in the presence of quarternary ammonium based ionic liquids and [AOT], $[NTf_2]$ and [OTs] anions.

QUATERNARY AMMONIUM BASED IONIC LIQUID ([AOT], $[NTf_2]$ and [OTs] anions)					
Cation	**Anion**	**Miscibility**	**V_o [mMs^{-1}] x 10^{-4}**	**V_{max} [mMs^{-1}] x 10^{-4}**	**K_m [mM] x 10^{-2}**
Control			3.70 ± 0.09	1.59 ± 0.17	24.6 ± 11.0
$[N_{112}C_2OH]$	[AOT][4]	•	+	+	+
$[N_{112}C_3OH]$	[AOT][4]	•	+	+	+
$[N_{1148}]$	[AOT][4]	•	+	+	+
$[N_{114}C_2OH]$	[AOT][4]	•	4.37 ± 0.73	2.41 ± 0.51	43.1 ± 11.76
$[N_{1888}]$	[AOT][4]	•	+	+	+
$[N_{14}(propylacetate)_2]$	[AOT][3]	√	n.d	-	-
$[N_{112}C_1CN]$	$[NTf_2]$[3]	√	5.76 ± 0.16	1.41 ± 0.13	26.5 ± 3.86
$[N_{1112}(C_2OH)_2]$	$[NTf_2]$[4]	•	n.d	-	-
$[N_{1148}]$	$[NTf_2]$[4]	•	3.24 ± 0.13	1.15 ± 0.08	17.1 ± 2.20
$[N_{1888}]$	$[NTf_2]$[4]	•	n.d	-	-
$[N_{1288}]$	[OTs][4]	•	n.d	-	-

(V_{max} is the maximum reaction velocity (mMs^{-1}), K_m is the half saturation constant (mM), V_o is the initial reaction velocity (mMs^{-1}), n.d, indicates complete inhibitory and the parameters cannot be measured; -, indicates the parameters cannot be calculated; +, indicates low activity which could not be quantified accurately; [3] indicates single phase; and [4] indicates biphasic system; √ indicates a water miscible ionic liquid; • indicates a water immiscible ionic liquid).

On the other hand, the $[N_{112}C_1CN]$ $[NTf_2]$ had supported good enzymatic activity by increasing the activity by approximately 36 %. Thus, $[N_{112}C_1CN]$ $[NTf_2]$ was the best ionic liquid among the quaternary ammonium based ionic liquids tested. However the use of the $[NTf_2]$ anion does not always produce a laccase-friendly ionic liquid. There was no activity observed in the presence of $[N_{1288}]$ [OTs] (Table 7.10) and $[N_1(C_1OC_2OC_2)_3]$ [linoleate] (Table 7.11). The activity was slightly increased in the assay containing $[N_{1114}]$ $[C_2H_6PO_4]$ by only 2 % compared to the control, whereas, $[N_{1888}]$ [TFA] supported the enzymatic activity of LAB by increasing the activity by 28 % (Table 7.11).

Table 7.11 Activity of LAB in the presence of quarternary ammonium based ionic liquids and phosphate, [TFA] and [linoleate] anions.

QUATERNARY AMMONIUM BASED IONIC LIQUID (phosphate, TFA and linoleate anions)					
Cation	**Anion**	**Miscibility**	V_o [mMs^{-1}] x 10^{-4}	V_{max} [mMs^{-1}] x 10^{-4}	K_m [mM] x 10^{-2}
Control			3.70 ± 0.09	1.59 ± 0.17	24.6 ± 11.0
$[N_{1114}]$	$[C_2H_6PO_4]^3$	√	3.78 ± 0.14	1.69 ± 0.31	21.2 ± 6.03
$[N_{1888}]$	$[TFA]^4$	•	4.73 ± 0.19	2.01 ± 0.37	35.3 ± 8.95
$[N_1(C_1OC_2OC_2)_3]$	$[linoleate]^3$	√	n.d	-	-

(V_{max} is the maximum reaction velocity (mMs^{-1}), K_m is the half saturation constant (mM), V_o is the initial reaction velocity (mMs^{-1}), n.d, indicates complete inhibitory and the parameters cannot be measured; -, indicates the parameters cannot be calculated; +, indicates low activity which could not be quantified accurately; ^{3} indicates single phase; and ^{4} indicates biphasic system; √ indicate a water miscible ionic liquid; • indicate a water immiscible ionic liquid).

7.4.3 Effect of Phosphonium Ionic Liquids on LAB Activity

The third group was phosphonium based cations which consists of 14 ionic liquids and the list of phosphonium ionic liquids structures which are shown in Appendix A.7.3 (page 253). Most of the phosphonium based ionic liquids used in this study were water immiscible and formed biphasic systems except for $[P_{1888}]$ $[C_1OSO_3]$. From 14 ionic liquids studied, only three ionic liquids supported the activity of LAB and produced high absorbance at 420 nm that enabled the initial rate of reaction, Michaelis-Menten parameters (K_m and V_{max}) and the extinction coefficient to be calculated. $[P_{88814}]$ [Br] increased the V_{max}, reduced the value of K_m and the activity of LAB was increased by 9.7 % compared to the control. On the other hand, $[P_{66614}]$ [Br] caused complete inhibition of LAB (Table 7.12).

The ionic liquids containing other anions $[P_{66614}]$ [SCN], $[P_{66614}]$ [TFA] and $[P_{66614}]$ [DIOPN] and $[P_{66614}]$ [decanoate] were also observed to inhibit the activity to a similar extent. As observed for quaternary ammonium salts containing alkyl sulphate anions, $[P_{1888}]$ $[C_1OSO_3]$ supported LAB activity and increased the activity by 45 % (Table 7.12). In contrast, imidazolium and quaternary ammonium ionic liquids containing $[C_1OSO_3]$ anions reduced the activity by 93 % for imidazolium (Table 7.3) and complete inhibition was observed in the presence of the quaternary ammonium cation (Table 7.9). Thus, it can be concluded that this salt was enzyme

friendly and stimulated the activity of LAB in the presence of phosphonium cations, but not in the presence of imidazolium and quaternary ammonium cations. In addition, $[P_{66614}]$ $[NTf_2]$ supported the activity of LAB and this anion was observed to be the most enzyme friendly anion among the phosphonium ionic liquids tested. This is due to the increase in the initial reaction rate (v_o) from 3.7 x 10^{-4} mMs^{-1} (control) to 6.96 x 10^{-4} mMs^{-1} (Table 7.12) which gives an increase in the activity of LAB by 89 % compared to the control. $[P_{66614}]$ $[BF_4]$ increased the activity by only 8 %.

Table 7.12 Activity of LAB in the presence of phosphonium ionic liquids

	PHOSPHONIUM				
Cation	**Anion**	**Miscibility**	**V_o [mMs^{-1}] x 10^{-4}**	**V_{max} [mMs^{-1}] x 10^{-4}**	**K_m [Mm] x 10^{-2}**
Control			3.70 ± 0.09	1.59 ± 0.47	24.6 ± 11.0
$[P_{88814}]$	$[Br]^4$	•	4.04 ± 0.11	1.86 ± 0.16	19.38 ± 3.74
$[P_{66614}]$	$[Br]^4$	•	n.d	-	-
$[P_{66614}]$	$[SCN]^4$	•	n.d	-	-
$[P_{66614}]$	$[TFA]^4$	•	n.d	-	-
$[P_{66614}]$	$[DIOPN]^4$	•	n.d	-	-
$[P_{66614}]$	$[decanoate]^4$	•	n.d	-	-
$[P_{1888}]$	$[C_1OSO_3]^3$	√	5.36 ± 0.02	1.63 ± 0.16	27.9 ± 4.07
$[P_{66614}]$	$[NTf_2]^4$	•	6.96 ± 0.04	1.59 ± 0.25	24.9 ± 6.04
$[P_{66614}]$	$[BF_4]^4$	•	3.39 ± 0.08	1.52 ± 0.14	24.2 ± 3.40
$[P_{66614}]$	$[N(CN)_2]^4$	•	+	+	+
$[P_{66614}]$	$[PF_6]^4$	•	+	+	+
$[P_{66614}]$	$[AOT]^4$	•	n.d	-	-
$[P_{88814}]$	$[AOT]^4$	•	n.d	-	-

(V_{max} is the maximum reaction velocity (mMs^{-1}), K_m is the half saturation constant (mM), V_o is the initial reaction velocity (mMs^{-1}), n.d, indicates complete inhibitory and the parameters cannot be measured; -, indicates the parameters cannot be calculated; [3] indicates single phase; and [4] indicates biphasic system; √ indicates a water miscible ionic liquid; • indicates a water immiscible ionic liquid).

Contrary to the result obtained for imidazolium and quaternary ammonium based ionic liquids, phosphonium ionic liquids containing dicyanamide $[N(CN)_2]$ and hexafluorophosphate $[PF_6]$ anions supported the activity of LAB (Table 7.12). However, the initial rates of reaction and kinetic parameters of LAB in the presence of this ionic liquid could not be determined accurately due to very low residual activity. In contrast to the results obtained from imidazolium and quaternary ammonium ionic liquids, the docusate [AOT] anion did not support the activity of

LAB in the presence of phosphonium cations. Both [P_{66614}] [AOT] and [P_{88814}] [AOT] completely inhibited the activity of LAB. Both of these ionic liquids formed an emulsion and foamed once it was added to the buffer solution.

7.4.4 Effect of Pyridinium Ionic Liquids on LAB Activity

The fourth group consists of 16 ionic liquids based on pyridinium cations and the list of pyridinium ionic liquids structures is shown in Appendix A.7.4 (page 255). None of the pyridinium ionic liquids tested supported the activity of LAB (Table 7.13). As expected, no activity was observed in the presence of [C_6py] [Br] and [C_6py] [Cl], however, some residual activity was observed in the presence of [C_4(3pic)] [Cl] although the activity was decreased by 56 %. For the iodide anion ([C_6py] [I]), a very low activity of LAB was detected, but the rate could not be measured accurately.

Table 7.13 Activity of LAB in the presence of pyridinium ionic liquids.

PYRIDINIUM					
Cation	**Anion**	**Misciblity**	**V_o [mMs^{-1}] x 10^{-4}**	**V_{max} [mMs^{-1}] x 10^{-4}**	**K_m [Mm] x 10^{-2}**
Control			3.70 ± 0.095	1.59 ± 0.47	24.6 ± 11.0
[C_4(3pic)]	[Cl][3]	√	1.63 ± 0.10	1.27 ± 0.09	21.5 ± 2.77
[C_6 py]	[Cl][3]	√	n.d	-	-
[C_6 py]	[Br][3]	√	n.d	-	-
[C_6 py]	[I][3]	√	+	+	+
[C_4(3pic)]	[$N(CN)_2$][3]	√	n.d	-	-
[C_6(3pic)]	[DIOPN][3]	√	n.d	-	-
[C_8(3pic)]	[DIOPN][4]	•	n.d	-	-
[C_6 py]	[TFA][3]	√	n.d	-	-
[C_8(3pic)]	[linoleate][3]	√	n.d	-	-
[C_4(3pic)]	[AOT][3]	√	+	+	+
[C_6(3pic)]	[AOT][4]	•	2.34 ± 0.07	1.49 ± 0.19	21.8 ± 4.35
[C_8(3pic)]	[AOT][4]	•	+	+	+
[C_4 py]	[NTf_2][4]	•	3.27 ± 0.19	1.76 ± 0.27	28.9 ± 6.47
[C_6 py]	[BF_4][3]	√	n.d	-	-
[C_{10} py]	[BF_4][4]	•	2.69 ± 0.10	1.76 ± 0.41	29.5 ± 10.0
[C_{14} py]	[BF_4][4]	•	n.d	-	-

(V_{max} is the maximum reaction velocity (mMs^{-1}), K_m is the half saturation constant (mM), V_o is the initial reaction velocity (mMs^{-1}), n.d, indicates complete inhibitory and the parameters cannot be measured; -, indicates the parameters cannot be calculated; [3] indicates single phase; and [4] indicates biphasic system; √ indicates a water miscible ionic liquid; • indicates a water immiscible ionic liquid).

As with imidazolium and quarternary ammonium ionic liquids, the pyridinium ionic liquid containing dicyanamide anion, [C_4(3pic)] [$N(CN)_2$] tended to inhibit the activity of LAB completely, confirming that the dicyanamide anion is not a suitable anion to be use with LAB assay.

Complete inhibition was also observed in the presence of [C_6py] [TFA] and [C_8(3pic)] [linoleate] (Table 7.13). Low residual activity was observed in the assay containing [C_4(3pic)] [AOT] and [C_8(3pic)] [AOT], but the rate could not be determined. In addition, [C_6(3pic)] [AOT] decreased the activity of LAB by 37 % compared to the control. The activity was also decreased in the presence of [C_4py] [NTf_2] by 11.6 % (Table 7.13). As for the tetrafluoroborate anion, [BF_4], complete inhibition was observed in the presence of [C_6py] [BF_4] and [C_{14}py] [BF_4] and [C_{10}py] [BF_4] only allowed 27 % activity compared to the control.

7.4.5 Effect of Piperidinium and Pyrrolidinium Ionic Liquid on LAB Activity

The last two groups of ionic liquids tested consist of six ionic liquids based on piperidinium cation and three ionic liquids of pyrrolidinium cation and the ionic liquids structures are shown in Appendix A.7.5 (page 256). Among the six piperidinium ionic liquid tested, only [$N_{1,6}$ pip] [NO_3] caused complete inhibition towards LAB activity and some residual activity was observed in the presence of [$N_{1,6}$ pip] [Br], [$N_{1,6}$ pip] [C_1OSO_3] and [$N_1C_1OC_3$ pip] [NTf_2], decreasing the activity by 18, 19 and 60 % respectively compared to the control. The activity was too low in the assays containing [$N_{1,4}$ pip] [$C_2H_6PO_4$] and [$N_{1,4}$ pip] [Cl] (Table 7.14).

As with imidazolium, quaternary ammonium and pyridinium ionic liquids, the pyrrolidium ionic liquid containing dicyanamide anions ([$N_{1,4}$ pyrr] [$N(CN)_2$]) also inhibited the activity of LAB completely. This result further indicates that the dicyanamide anion is not a suitable anion for LAB. The other two pyrrolidinium ionic liquids ([$N_{1,4}$ pyrr] [AOT] and [$N_{1,4}$ pyrr] [NTf_2]) decreased the activity by 5.4 and 16 %, respectively (Table 7.14).

Table 7.14 Activity of LAB in the presence of piperidinium and pyrrolidinium ionic liquids.

PIPERIDINIUM					
Cation	**Anion**	**Miscibility**	V_o [mMs^{-1}] x 10^{-4}	V_{max} [mMs^{-1}] x 10^{-4}	K_m [Mm] x 10^{-2}
Control			3.70 ± 0.095	1.59 ± 0.47	24.6 ± 11.0
[$N_{1,4}$ pip]	$[C_2H_6PO_4]^3$	√	+	+	+
[$N_{1,4}$ pip]	$[Cl]^3$	√	+	+	+
[$N_{1,6}$ pip]	$[Br]^3$	√	2.87 ± 0.10	1.397 ± 0.21	23.34 ± 5.64
[$N_{1,6}$ pip]	$[NO_3]^3$	√	n.d	-	-
[$N_{1,6}$ pip]	$[C_1OSO_3]^3$	√	2.99 ± 0.21	1.062 ± 0.08	18.17 ± 2.28
[$N_1C_1OC_3$ pip]	$[NTf_2]^4$	•	1.46 ± 0.05	0.995 ± 0.06	14.07 ± 1.76
PYRROLIDINIUM					
Cation	**Anion**	**Miscibility**	V_o [mMs^{-1}] x 10^{-4}	V_{max} [mMs^{-1}] x 10^{-4}	K_m [Mm] x 10^{-2}
Control			3.70 ± 0.09	1.59 ± 0.47	24.6 ± 11.0
[$N_{1,4}$ pyrr]	$[N(CN)_2]^3$	√	n.d	-	-
[$N_{1,4}$ pyrr]	$[AOT]^4$	•	3.50 ± 0.10	2.03 ± 0.13	27.5 ± 6.6
[$N_{1,4}$ pyrr]	$[NTf_2]^4$	•	3.09 ± 0.18	1.42 ± 0.12	22.4 ± 3.1

(V_{max} is the maximum reaction velocity (mMs^{-1}), K_m is the half saturation constant (mM), V_o is the initial reaction velocity (mMs^{-1}), n.d, indicates complete inhibitory and the parameters cannot be measured; -, indicates the parameters cannot be calculated; [3] indicates single phase; and [4] indicates biphasic system; √ indicate a water miscible ionic liquid; • indicate a water immiscible ionic liquid).

7.5 Discussion

A method developed by Rehmann *et al.* (2012) was suitable to screen a large number of ionic liquids, mainly to 'minimize the amount of ionic liquids and to maximize the experimental throughput' (Rehmann *et al.*, 2012). In addition, this method has the capability of measuring the initial rate of substrate conversion, and also to estimate Michaelis-Menten parameters *via* non-linear regression analysis. A total of 106 different ionic liquids were tested. From this number, only 13 ionic liquids stimulated the activity of LAB, whereas 50 others caused complete inhibition. This enzyme showed residual activity in the presence of 19 other ionic liquids, and the activity was lower than the control. There was very minimal activity of LAB in the presence of 24 ionic liquids which was too low to estimate the kinetic parameters.

One important finding that emerged from this study is that the water immiscible ionic liquids were more suitable for LAB than water miscible ionic liquids. This result has confirmed earlier observation by De Los Rios *et al.* (2007) in which they observed

that the enzymatic activity of lipase in the presence of water immiscible ionic liquids was higher than in water miscible ionic liquids (De Los Ríos *et al.*, 2007). This could be explained by the direct interaction of water miscible ionic liquids on the active sites of the enzyme, which disrupts the electron transfer within the enzyme, thus reducing the catalytic activity.

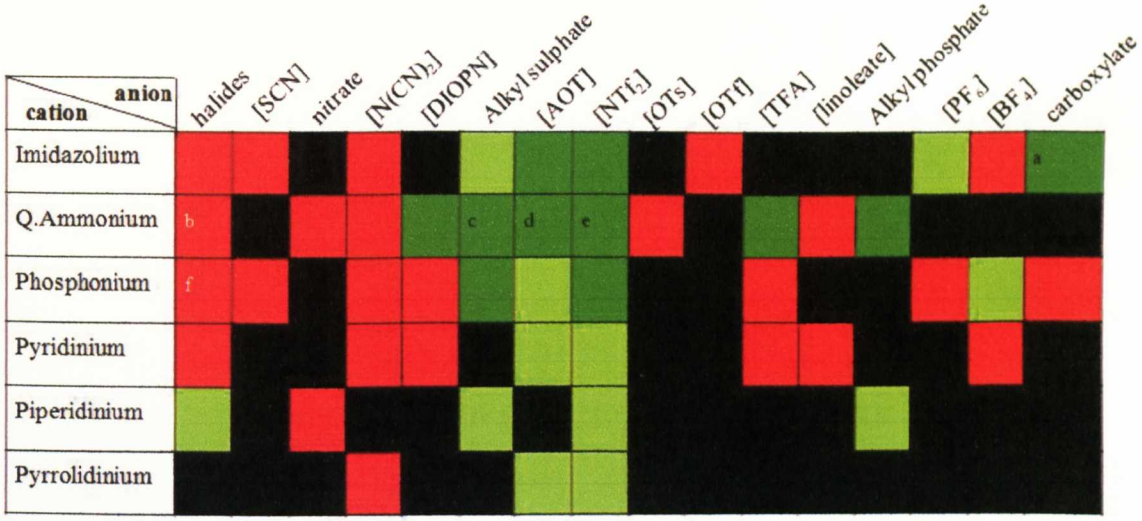
The effect of water miscible ionic liquids on LAB stability may also be explained by the effect of the kosmotropicity of the ions according to the Hofmeister series (Zhang and Cremer, 2006). Enzyme stability is usually promoted by the combination of kosmotropic anions and chaotropic cations, whereas kosmotropic cations and chaotropic anions tend to destabilize enzymes (Zhao, 2005). This trend was noted for kosmotropic C_6mim^+ and C_8mim^+ with the combination of the chaotropic BF_4^- anion (Zhao, 2005) which deactivated LAB in the current experiments (Table 7.5). The kosmotropicity of ions depends on the *B*-coefficients (Zhao, 2005), and although numerous studies of the Hofmeister effects have been reported, *B*-coefficients of the cations used in the current experiments are not available. Thus, the detailed analysis of the Hofmeister effects of each individual ionic liquid used in this study could not be performed. The measurement of *B*-coefficients for individual ions is quite challenging (Zhao, 2006). In order to establish a better understanding of the Hofmeister effects on LAB, it can thus be suggested that the analysis of *B*-coefficients of the ions could be performed in future studies. In this study, most of the ionic liquids containing halides, carboxylates, alkyl phosphate, alkyl sulphate, [SCN], $[N(CN)_2]$, [OTf], $[BF_4]$, $[NO_3]$ and [linoleate] were water miscible, and the kosmotropicity of most of these anions has already been studied previously (Zhao, 2005; 2006). For example, most of the ionic liquids containing carboxylates and alkyl sulphate anions supported LAB activity, since the kosmotropicity of these anions were higher than the others (Zhao, 2005). On the other hand, most of the ionic liquids containing chaotrope anions including halides, [SCN], $[N(CN)_2]$, [OTf], $[BF_4]$, $[NO_3]$, [OTs] and [linoleate] deactivated LAB completely (Table 7.15). Anions such as halides and dicyanamides bind the type 2 and type 3 copper atoms of laccase which disrupts the electron transfer between these copper atoms for the catalytic activity of LAB. This results in the inhibition of the enzyme (Giarfreda *et al.*, 1999). [SCN] is also a strong enzyme inhibitor (Zhao *et al.*, 2005).

However, the stability of the enzyme in ionic liquids does not necessarily depend on the kosmotropicity order (Zhao *et al.*, 2006). For instance, [C_4mim] [L-tartrate] anion was found to be the most promising ionic liquid for LAB, increasing of the activity by more than 90 % compared to the control. Thus, [C_4mim] [L-tartrate] was the best ionic liquid tested. However, this ionic liquid did not follow the kosmotropicity order since the [C_4mim] cation is listed as a kosmotropic cation (Zhao *et al.*, 2006) which is supposed to deactivate the enzyme. Thus, the overall interaction of the enzyme, medium and the substrate (Zhao, 2005) may also need to be taken into consideration. For example, the activity of the enzyme also depends on the amount of ionic liquid. As observed in this study, the activity of LAB decreased as the ionic liquid concentration increased (Fig. 7.4).

Ionic liquids containing [NTf_2] and [AOT] anions have been observed to be the most suitable anions and these ionic liquids mostly formed a biphasic system when in contact with water (Table 7.15). The [NTf_2] anion stimulated LAB activity by increasing the activity by up to 89 % in the presence of the [P_{66614}] cation, 56 % in the presence of [$N_{112}C_1CN$] cation and 22 % in the presence of the [C_4mim] cation. As for the [AOT] anion, the activity was increased up to 28 % in the presence of the [C_2mim] cation and 14 % in the presence of the [$N_{114}C_2OH$] cation. Other potential anions would be alkyl sulphate, since some of the water miscible ionic liquids containing alkyl sulphate anions supported the activity of LAB. For example, [$N_2(C_1OC_2OC_2)_3$] [$CH_3CH_2OSO_3$] and [P_{1888}] [C_1OSO_3].

None of the ionic liquids containing pyridinium, pyrrolidinium and piperidinium cations stimulated the activity of LAB (Table 7.15), which provides further agreement with the studies done by Pham *et al.* (2008) and Zhao *et al.* (2007). Overall, LAB stimulation and inhibition in the presence of anionic liquid must be the result of numerous factors which involve a complex interaction between the enzyme, ionic liquids, ABTS and water.

Table 7.15 The trend for LAB activity in the presence of 16 different anions.



Note: a – most of the carboxylate anions supported LAB activity except for [C_4mim] [acetate]; b – most of the halides deactivated LAB except for [Br] anion; c – Most of the ionic liquids deactivated LAB except for [$N_2(C_1OC_2OC_2)_3$] [$CH_3CH_2OSO_3$] that supported the activity of LAB; d – low residual activity, however, [$N_{114}C_2OH$][AOT] supported LAB activity; e – [$N_{112}C_1CN$][NTf_2] supported the activity; f – most of the ionic liquids deactivated LAB except for [P_{88814}][Br]. – indicates the complete inhibition by most of the ionic liquids; – indicates low residual activity by most of the ionic liquids and – indicates the ionic liquids that supported the activity of LAB; indicates the ionic liquids that are not available in the category.

The current findings add substantial information to a growing body of literature on the compatibility of ionic liquids with laccase activity. Thus, ionic liquids that cause complete inhibition and reduce the activity of laccase have been identified and those that support the activity can be selected. Further investigation and experimentation is needed into the dissolution of lignin in ionic liquids that support the activity of LAB, namely [C_2mim] [AOT], [C_6mim] [AOT], [C_4mim] [NTf_2], [C_4mim] [L-tartrate], [N_{1888}] [DIOPN], [$N_2(C_1OC_2OC_2)_3$] [$CH_3CH_2OSO_3$], [$N_{114}C_2OH$] [AOT], [$N_{112}C_1CN$] [NTf_2], [N_{1888}] [TFA], [P_{88814}] [Br], [P_{1888}] [C_1OSO_3] and [P_{66614}] [NTf_2]. Future trials should assess all ionic liquids that support both LAB (as reported in this thesis) and LTV activity (Rehmann *et al.*, 2012). It can thus be suggested that the compatibility of these ionic liquids to dissolve lignin could be explored in future studies. The cooperation of these ionic liquids and LAB could then be used to depolymerize lignin.

Chapter 8

DISCUSSION AND CONCLUDING REMARKS

8.1 Summary of Results

This thesis aims to study the enzymatic depolymerization of lignin to high value chemicals. As petroleum is becoming more expensive and the supply is reducing, there is an urgent need for a new renewable resource to meet the high world demand. Lignin is the most abundant naturally occurring aromatic resource, with high potential as a renewable feedstock for the production of fine-chemicals production. Currently, the majority of lignin is produced from the pulp and paper industry. However, as lignin is disposed of as a by-product, it is mostly burned as an energy source to feed the process. Therefore the cost of energy consumption for the whole pulping process can be reduced. However, this lignin can be used alternatively for the production of fine chemicals thus offering a lower cost for these chemicals and an increase in profitability. In addition, the use of lignin can also reduce the consumption of fossil resources.

Isolated lignin from the industry is dependent on the source of biomass and the isolation process used. Therefore, the most suitable isolated lignin has to be selected for the task. In this study, the use of sodium lignosulphonate was driven by several factors:

(i) The global production of isolated lignin is currently dominated by the production of lignosulphonate of around 1 million tonnes (Gargulak and Lebo 2000).

(ii) Sodium lignosulphonate is the only isolated lignin that is soluble in water. Since the enzymatic depolymerization of lignin was employed, the

solubility of lignin in water is important for the preliminary study. The reaction in the presence of an enzyme required an aqueous medium for the catalytic activity to take place; therefore the contact between enzyme and lignin could be optimized.

(iii) The sodium lignosulphonate used in this study was supplied by Borregaard Lignotech, which dominates the production of lignosulphonate with a capacity of about 500 000 tonnes lignosulphonates per year (Belgacem and Gandini, 2008; Ek, 2005). Thus the use of lignosulphonate could increase the possibility of dominating the production of fine chemicals from one of the largest lignin suppliers.

Since lignin is a complex aromatic polymer, the depolymerization of this compound was not an easy task. Lignin is not a single well defined biomaterial but more of the combination of different subunits, linkages and functional groups. Therefore, the depolymerization of this complex polymer requires strategies to ensure the depolymerization of the products can be performed. Enzymatic depolymerization of lignin can offer high selectivity, and the conversion of lignin occurs under mild reaction conditions, which is important when striving towards a 'green' process.

Thus, a method was proposed based on the catalytic activity assays of commercial available laccase from *Agaricus bisporus* (LAB) in the presence of 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) for the depolymerization of sodium lignosulphonate as discussed in **Chapter 4**. With LAB as a catalyst, 7.6 % of the sodium lignosulphonates were converted to chemicals which could be identified by gas chromatography mass spectroscopy (GCMS) as monomers. Vanillic acid **(5)** was found to be a major product followed by homovanillyl alcohol **(4)**, vanillin **(2)**, guaiacol **(1)** and acetovanillone **(3)**. However, the product concentrations obtained from the process were too low. Thus, laccase from *Trametes versicolor* (LTV) was employed with the aim to increase the product concentration, under mild reaction conditions as described in **Chapter 5**. After enzymatic depolymerization by LTV, the extracted chemicals were increased by 9.8 % from the total lignin used. Thirteen compounds were observed, and the concentration of the products, namely vanillic acid **(5)**, homovanillyl alcohol **(4)**, vanillin **(2)**, guaiacol **(1)** and acetovanillone **(3)** were increased (Chapter 5 - Table 5.3, page 94). The other products were identified

as phenol (**6**), 4- methylbenzaldehyde (**7**), catechol (**8**), *p*-toluic acid (**9**), 4-hydroxybenzaldehyde (**10**), tyrosol (**11**), isovanillin (**12**), and 3-hydroxy-1-(4-hydroxy-3-methoxyphenyl) propan-1-one (**13**), and these compounds were absent in the presence of LAB.

The work discussed above was based on the depolymerization of sodium lignosulphonate in the presence of two different laccases (LAB and LTV) and mediated by ABTS. However, there is another possibility that the products formed were influenced by the type of mediator, since laccase have more than 100 possible mediators. Thus, five synthetic mediators were selected to test their effects on the depolymerization of sodium lignosulphonate (**Chapter 6**) by LTV, with the aim to increase the product concentrations. Contrary to expectation, changing the mediators did not improve the process performance (Fig. 8.1). The production of guaiacol (**1**), vanillin (**2**), acetovanillone (**3**), homovanillyl alcohol (**4**) and vanillic acid (**5**) were higher in the LTV-ABTS system than with LAB-ABTS system (Fig. 8.1 a-e). In addition, the product concentrations were much lower when LTV was used with the other mediator (TEMPO, HBT, HPI and VLA) (Fig. 8.1). It can thus be suggested that the presence of ABTS as a mediator strongly accelerated the reaction and increased the product concentration attained. In addition, the findings also suggest that the depolymerization could not occur in the absence of mediator. Therefore, a mediator was necessary for every process catalyzed by laccase as discussed earlier (**Chapter 4** and **Chapter 5**). The limiting step in the oxidation of mediators is mainly governed by differences in the redox potential that resulted in different outcomes of the process. Future trials should assess a full optimization of each mediator, including the reaction condition that is necessary to increase the products formation.

The use of enzymatic depolymerization of lignin is limited due to the fact that most of the technical lignins are not soluble in water, except for lignosulphonate. In order to expand the use of the enzymatic process, it would be desirable to find enzyme friendly solvents that can be used to solubilise lignin, since most of the conventional solvents deactivate the enzyme. Thus in this study, the use of 106 ionic liquids was explored and [C_4mim] [L-tartrate] was found to support the activity of LAB, and increase the activity by more than 90 % (**Chapter 7**). It can thus be suggested that

future work should be conducted in order to screen the solubility of this ionic liquid to dissolve lignin.

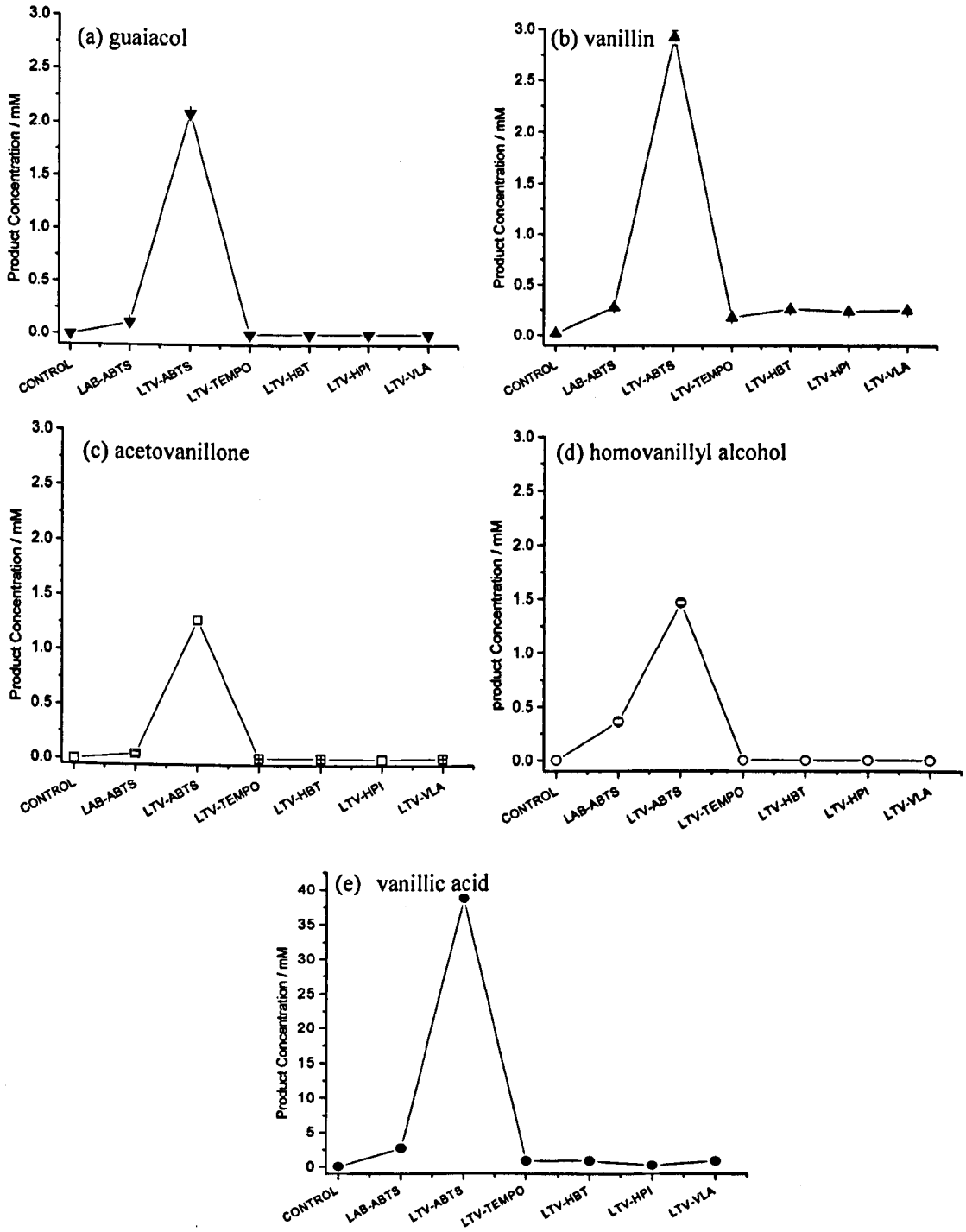


Figure 8.1 Comparison of the products formed after enzymatic treatment of sodium lignosulphonate in the presence of LAB and LTV (with different mediator, namely ABTS, TEMPO, HBT, HPI and VLA). (a) guaiacol (▼), (b) vanillin (▲), (c) acetovanillone (□),(d) homovanillyl alcohol (○) and (e) Vanillic acid (●). The reaction was conducted under identical reaction conditions (30 °C for 6 h). Fractionation was applied and the aqueous ethyl acetate extract was redissolved in DCM and analyzed by GCMS. Each product was quantified based on the authentic standard. The data represents the mean of three replicates. Standard error was less than 1 %.

8.2 Improvement of the Process

This study has thrown up many questions in need of further investigation both from the process and analytical point of view. In order to improve the process, a number of future studies using the same experimental setup are required as follows:

(i) Cooperation with glucose oxidase

There is one question that arises, whether or not laccase alone can degrade lignin efficiently. In nature, there are numerous enzymes involved in the process. According to the study by Green (1977), the low efficiency of depolymerization by laccase may be caused by the production of quinone intermediates following route B in Fig. 8.2.

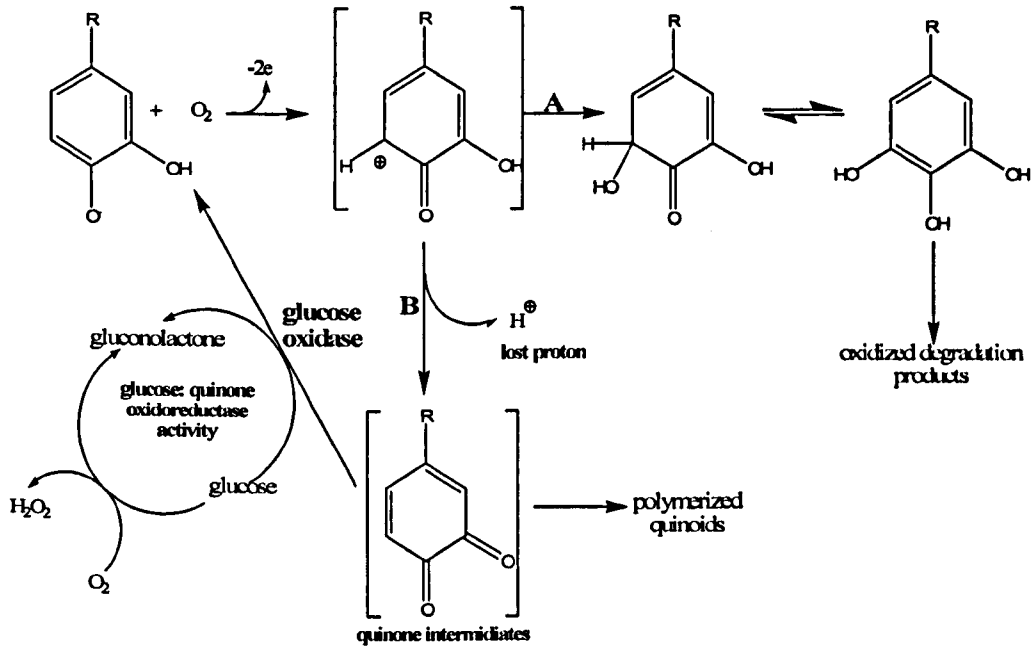


Figure 8.2 Schematic flow diagram of the activity of glucose: quinone oxidoreductase which transforms quinone intermediates to its original form by the action of glucose oxidase. This will reduce the production of polymerized quinoids (adapted from Green, 1977)

Spontaneous coupling of the radicals may produce high molecular weight products. In the presence of glucose oxidase, radicals and quinones which are produced from the laccase reaction can be reduced (Szklarz and Leonowicz, 1986), since the

hydrogen acceptors of glucose oxidation require quinones or radicals following the mechanism proposed by Green (1977). Therefore, the addition of glucose oxidase might improve the depolymerization process and more products may be produced following route A in Fig. 8.2. Therefore, it would be interesting to assess the effect of glucose oxidase on the degradation cycle.

(ii) Introduction of an inducer to improve enzyme production

Commercially available laccase is generally produced with low purity, which explains the low activity of such laccase (Osma *et al.*, 2010), which in turn results in low catalytic efficiency for lignin depolymerization. However, a reasonable approach to tackle the issue would be by improving the isolation technique. By adding an inducer into the cultures used for laccase production, laccase activity can be increased as found by Palmieri *et al.* (2000). In their study, the activity of laccase from *Pleurotus ostreatus* was increased by 50-fold by the addition of 150 μM copper sulphate. In the specific case of LTV, both veratryl alcohol and copper sulphate have proved to increase the activity of about 24-fold higher than those obtained without the inducer (Dominguez *et al.*, 2007). The activity was also by far higher than in the medium containing either copper sulphate or veratryl alcohol alone (Fig. 8.3).

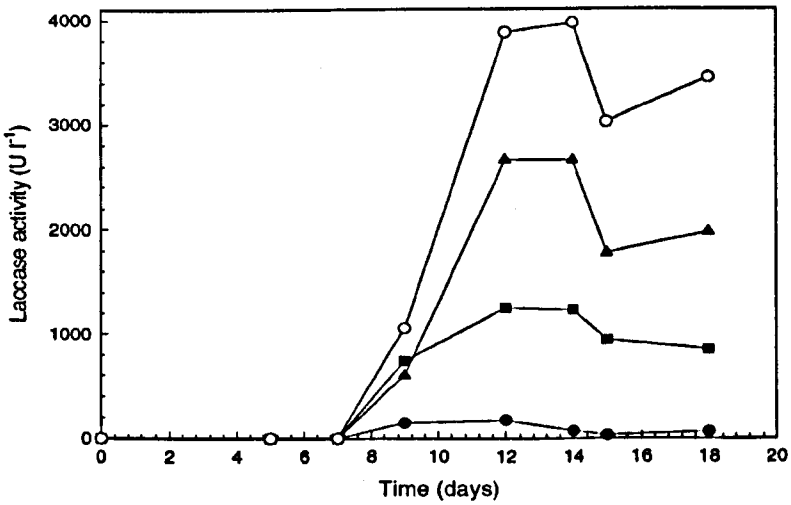


Figure 8.3 Evolution of LTV activity in the absence (•) and presence of inducers: veratryl alcohol (■), copper sulphate (▲) and veratryl alcohol plus copper sulphate (○) taken from Dominguez *et al.* (2007).

It would be interesting to assess the effects of an inducer towards laccase activity. Thus, future studies involving the addition of an inducer to the laccase production culture is fully recommended. In the increase of laccase activity, the rate of lignin depolymerization can also be increased.

(iii) Production of ABTS dication

Earlier in Chapter 2 (Section 2.4.1.1) was pointed out that the oxidation of ABTS by laccase produces a cation radical ($ABTS^{+\bullet}$) and this is followed by the formation of dication ($ABTS^{2+}$). According to Bourbonnais *et al.* (1998) $ABTS^{+\bullet}$ only reacts with phenolic structures whereas $ABTS^{2+}$ is responsible for the oxidation of non-phenolics. Since most of the products formed in this study were phenolic compounds, there is a high possibility that the production of $ABTS^{+\bullet}$ was higher than $ABTS^{2+}$. In order to produce $ABTS^{2+}$, an electrolysis cell could be employed to electrochemically generate the dication as suggested by Bourbonnais *et al.* (1998) (Fig. 8.4). In their study, veratryl alcohol was successfully converted to veratraldehyde (Fig. 8.4) by bulk electrolysis of veratryl alcohol and ABTS at 900 mV (Bourbonnais *et al.*, 1998). It is suggested that the association of this factor should be investigated in a future study, and more information on the dication could contribute to a greater degree of understanding of lignin depolymerization, and improve the product formation.

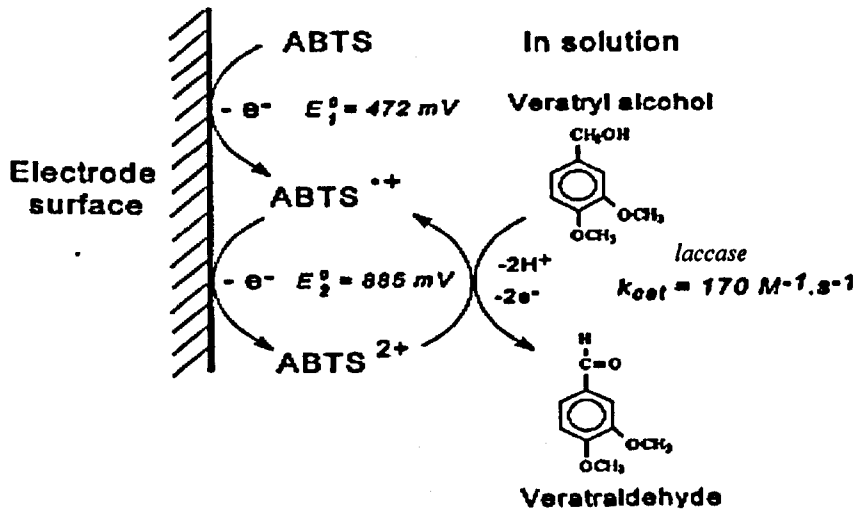


Figure 8.4 Redox catalysis of veratryl alcohol and ABTS taken from Bourbonnais *et al.* (1998).

(iv) Alternative enzymes for the production of chemicals from lignin

A future study investigating the effect of manganese peroxidase (MnP) and lignin peroxidase (LiP) on lignin depolymerization would be very interesting. Several attempts have been made previously to study the potential of MnP and LiP to degrade lignin (Forester *et al.*, 1988; Warishi *et al.*, 1991; Hofrichter *et al.*, 1998). The use of MnP from *Lentinus edodes* was first explored by Forester *et al.* (1998) for the degradation of spruce ball-milled lignin in the presence of glutathione, and vanillin and protocatechuic acid were formed as products. Depolymerization of $^{14}C_{\beta}$-labelled synthetic hardwood lignin has also been demonstrated using LiP from *Phanerochaete chrysosporium*. This produced low molecular weight products as low as 170 although the identity of this compound was not mentioned (Hammel and Moen, 1991). Recently, however, not many attempts have been made to depolymerize lignin using LiP and MnP, perhaps due to the high cost of these peroxidase enzymes as depicted in **Chapter 2** (Table 2.1 – Page 27). Studies of MnP and LiP production and purification techniques at lower cost are needed to improve the use of these enzymes on a larger scale. The degradation potential of MnP and LiP makes these enzymes attractive for biological applications especially in lignocellulosic processing. However, considerably more work will need to be done to improve the stability of peroxidase enzymes since they are highly dependent on H_2O_2 for their catalytic activity (Bloois *et al.*, 2010).

(v) Alternative biomass for the production of chemicals

Much interest has been focused on lignin as a primary source of value-added chemicals, since lignin offers such a great advantage from an economic point of view and is also the most abundant renewable aromatic feedstock. As reported earlier in this thesis, the development of a depolymerization process was a big challenge. The conversion of just 9.8 % of the total lignin added does require a new alternative to replace this complex aromatic polymer. Other than lignin, suberin and tannin have high potential as renewable feedstocks. These compounds are less complex than lignin, and may offer a simple process with high conversion yields. However, the

limited occurrence of these compounds might be a major drawback, compared to the availability of lignin from industry.

Suberin is a biopolymer that occurs naturally in oak cork (up to 50 % w/w) and has a cross-linked aliphatic-aromatic structure (Fig. 8.5) which plays an important role as a hydrophobic barrier (Gandini, 2008). In the study by Conde *et al.* (1997), various compounds were produced after the extraction of cork from *Quercus suber* with methanol-water after 24 h of treatment. The products include gallic acid, protocatechuic acid/aldehyde, aesculetin, vanillic acid, caffeic acid, vanillin, scopoletin, ferulic acid, coniferaldehyde and sinapaldehyde (Conde *et al.*, 1997). The discovery of these compounds proved that cork could be a possible renewable resource for the production of fine-chemicals. However, since cork is usually harvested every 9 to 12 years in limited places such as the western Mediterranean, therefore its use for large scale production of chemicals might be limited.

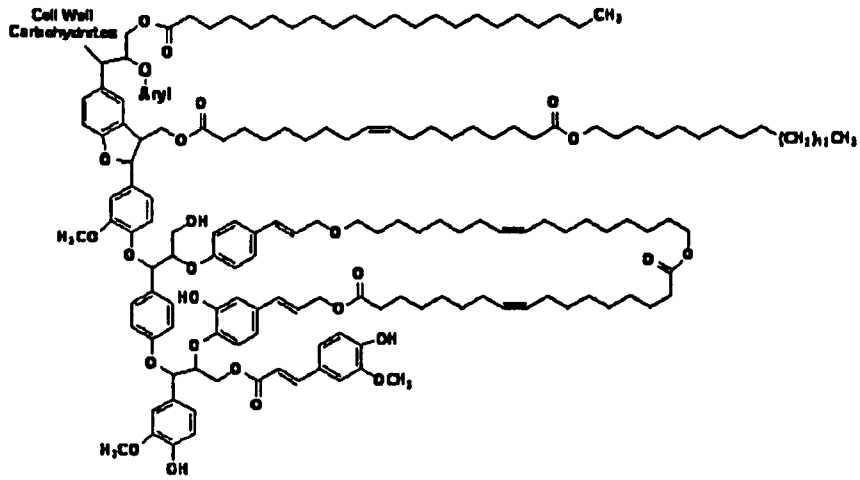


Figure 8.5 A partial view of the structure of suberin taken from Silva *et al.* (2005).

Tannins are naturally occurring plant polyphenols and are produced commercially from wood and bark of *Schinopsis sp.* trees for the production of formaldehyde wood adhesives (Tondi and Pizzi, 2009). Recently, Mensah *et al.* (2012) studied the potential of laccase from *Pleurotus ostreatus* for the degradation of tannin in cocoa pod husks. The treatment has successfully degraded 66 % of tannins. Their study

indicated the potential of laccase to degrade tannins and could be a benchmark for further experimental trials for the production of chemicals.

It can thus be concluded that the sources of biomass for the production of chemicals could be expanded. However, it has to be noted that the study using suberin and cork would need considerably more work since it is still unclear whether these materials could be as cost competitive as lignin.

8.3 Improvement of Analytical Methods

It is known that the structure of lignin is totally dependent on the distribution of its moieties namely *p*-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) (Grabber *et al.*, 1997). The delignification process is then dependent on the ratio of moieties, and the high S/G ratios in wood would increase the rate of delignification (del Rio *et al.*, 2005). As mentioned earlier (**Chapter 4** and **Chapter 5**), the structure of lignosulphonate is mostly build from the guaiacyl (G) derivatives (Matsushita and Yasuda, 2005), however the depolymerization of technical lignin offers a great challenge since the lignin is not pure (Gosselink, 2011). Thus, it would be interesting to assess the chemical structure of sodium lignosulphonate that could contribute towards a better understanding of the interaction and capability of laccase to degrade this complex polymer. However, this study would require significant research effort since the depolymerization of lignin produces a complexity of compounds which could be a major drawback for the analytical techniques. The combination of analytical techniques such as pyrolysis - gas chromatography mass spectroscopy (Py-GCMS), thioacidolysis and 2D-NMR could possibly tackle the issue.

(i) Pyrolysis - gas chromatography mass spectroscopy (Py-GCMS)

The combination of pyrolysis with GCMS enables the direct analysis of unvolatile compounds such as lignin. The current study reported in this thesis was unable to characterize aqueous and solid fractions by GCMS, due to their insolubility in dichrolormethane (DCM). Thus, Py-GCMS could be an answer to this problem. Pyrolysis is able to analyze solid samples and breaks apart large complex molecules

into smaller and more volatile fragments by applying heat up to 550 °C (del Rio *et al.*, 2005). These fragments are then separated by gas chromatography (GC) and characterized by mass spectroscopy (MS) to obtain their structural information by fingerprint analysis.

(ii) Thioacidolysis

Another method that could be deployed is the use of the thioacidolysis method to depolymerize the aqueous fraction after enzymatic treatment with laccase. In this technique, the sample is treated with boron trifluoride in dioxanethanethiol solution (Rolando *et al.*, 1992) as depicted in Fig. 8.6. This causes a selective cleavage of β-O-4 and other types of linkages, including β-5, β-β, β-1 *etc.* The linkages are shown in Chapter 2 – Fig. 2.4. The monomeric product (Fig. 8.6a) is substituted with the thioethyl groups, thus can be analyzed by GCMS after silylation (Brunow, 2001). Dimeric products (Fig. 8.6b) can then be analyzed after removal of the sulphonate group with Raney-nickel (Shah *et al.*, 1948).

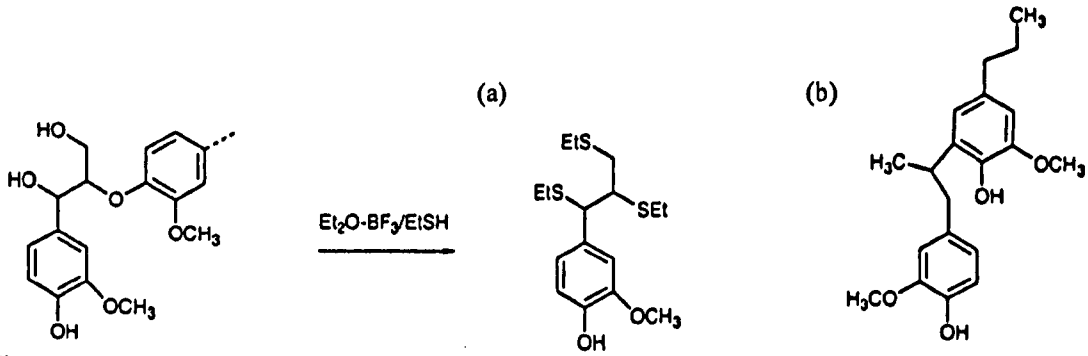


Figure 8.6 Thioacidolysis method to form (a) monomeric product and (b) dimeric product, taken from Brunow (2001).

Thus, the combination of enzymatic depolymerization of sodium lignosulphonate with further treatment using the thioacidolysis method could increase both product formation and analytical efficiency. It is suggested that the association of these methods is investigated in future studies.

(iii) 2D-NMR

2D-NMR could be an efficient technique to provide chemical information about the structure of the product macromolecules and also could be used to characterize the

structure of lignin. The heteronuclear single quantum coherence (HSQC) spectra consists of the correlation of ^{13}C-NMR and ^{1}H-NMR, which expand the information that is limited by the use of either ^{13}C NMR or ^{1}H NMR alone.

The combination of analytical observation using 2D-NMR could assist further understanding of the products formed and the selective degradation by laccase as discussed earlier in **Chapter 5**. However, due to time limitations, no further attempts were made to use this technique. Future studies on 2D-NMR are strongly recommended.

8.4 Economic Considerations

An economic process is dependent on the raw materials and the process, aiming to provide low processing cost for high profitability. Lignin is referred to as a low value by-product from the pulp and paper and biorefineries industry. The value of lignin may vary depending to the process of isolation and purification involved. According to Gosselink (2011), the lignin prices range from 50 – 750 €/tonne (Fig. 8.7), and lignosulphonate is the second lowest price lignin at values ranging from 250 – 350 €/tonnes. As proved earlier in this thesis, lignosulphonates have successfully been used to produce fine chemicals, which currently offer the highest market price, more than 1000 €/tonnes. The products formed would fall into the phenol derivatives market as depicted in Fig. 8.7.

However, by using lignosulphonate as the feedstock, technologies are needed to remove the sulphonated groups present in the reaction medium. As reported in this thesis, no sulphonated groups were observed in the detectable chemicals. However, sulphonated derivatives might be present in the bulk aqueous fraction (this fraction may also contain unreacted lignin). There is still no method available to date to remove the sulphonated group. Thus, future work could be established to find a suitable method that could be optimized, and reduce the processing cost. High grade lignin such as organosolv could possibly be the solution since these polymers is not chemically modified, thus increasing the purity of the lignin. However, the price for

high grade lignin is higher than lignosulphonates (Fig. 8.7) and is not available commercially, which limits the future scope for large scale use.

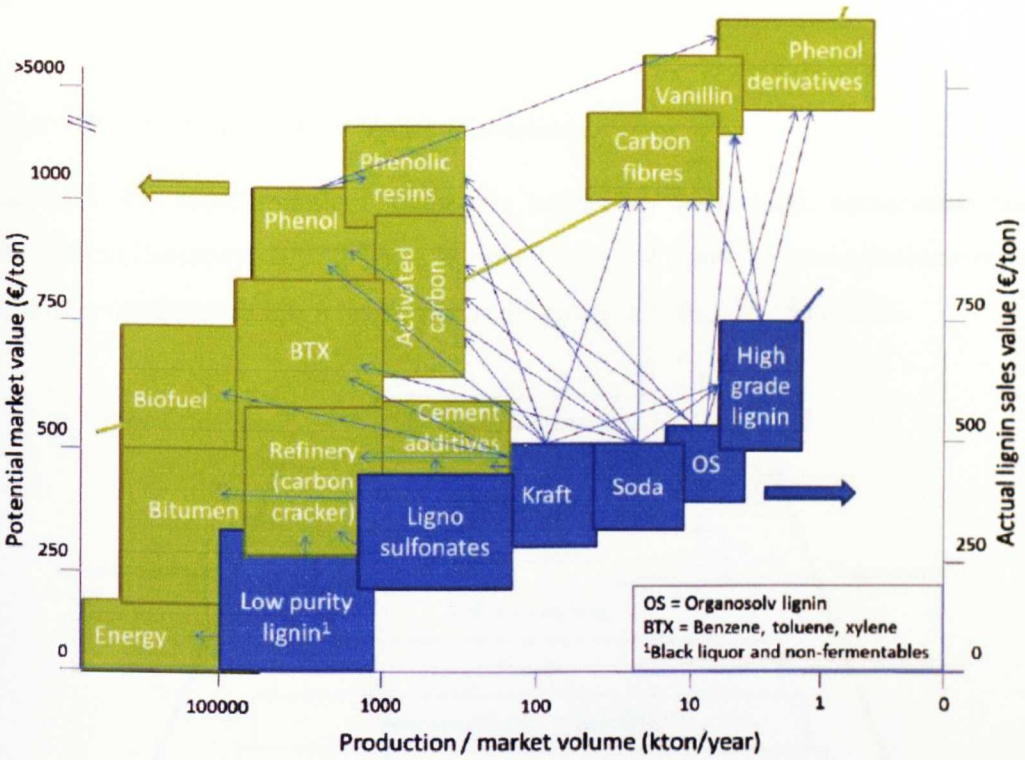


Figure 8.7 The market value of lignin and its potential products taken from Gosselink (2011).

The processing cost could possibly be reduced by employing low purity lignin such as Distiller's Dried Grains with Solubles (DDGS) which is a by-product formed from first generation bioethanol production, and lignin dissolved in black liquor which is currently used to generate energy in the pulp mill. These materials may provide the opportunity to establish a low cost renewable feedstock that could be converted to high value chemicals alongside lignosulphonates. However, as discussed earlier, further investigation and experimentation to remove sulphur and others impurities such as crude fibres, silicates, ash, protein and other compounds originating from the raw material are strongly recommended. The cost of the process should also be competitive with the use of high grade lignins.

It can thus be concluded that lignin offer low cost feedstock that can be converted to high value chemicals. However, considerably more work needs to be done to enable the development of a cost effective process.

8.4.1 Production of High Value Chemicals

Lignin is the most abundant renewable source for high value compounds such as aromatics (Holladay, 2007). Thus, the conversion of lignin to lower molecular weight aromatic compounds has a bright future to produce valuable chemicals.

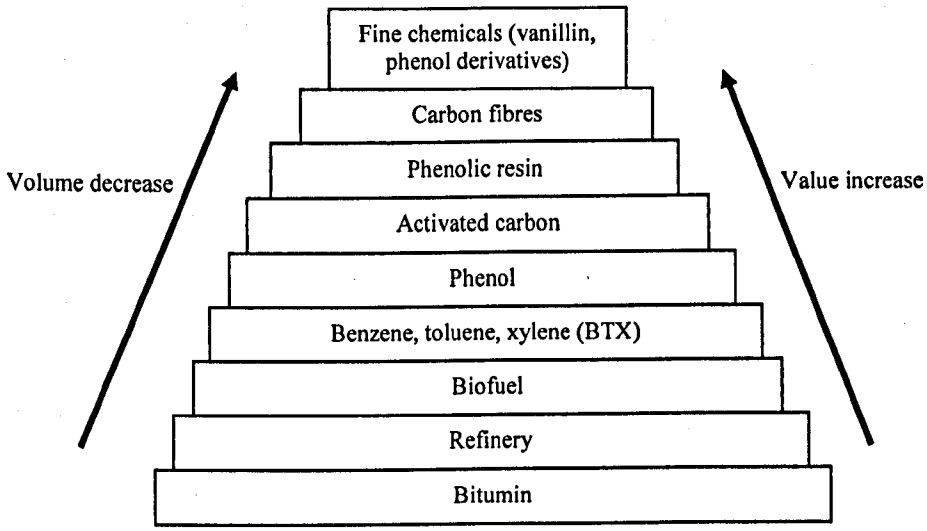


Figure 8.8 Potential lignin applications taken from Gosselink (2011)

Fine chemicals such as vanillin and phenol derivatives have more value than other lignin application as depicted in Fig. 8.7 and Fig. 8.8 (Gosselink, 2011), however, the volume of fine chemicals produce from lignin is lower than the others. The production of high value chemicals in the current study has shown the potential of lignin as source of value added chemicals. The high market value of these chemicals especially vanillin is based on its wide application in food industry as the flavour constituent of vanilla (da Silva *et al.*, 2009), and in cosmetic industry as the flavouring agent in perfume. Other than that vanillin could also be used as a chemical precursor for pharmaceutical industry, antioxidant additive, *etc.* (Cerrutti *et al.*, 1997; Villar *et al.*, 1997). Other than vanillin, phenol and some of its derivatives also offers wide range of application. Most of the phenol is used for the production of bisphenol-

A as an ingredient for polycarbonate, phenolic resin and to produced nylon fibers (Gosselink *et al.*, 2011; Holladay *et al.*, 2007).

Table 8.1 summarized the prices and uses of chemicals produced in the current studies subjected to the prices by Sigma Aldrich (UK). It has to be noted that the price is based on the small quantities production by Sigma Aldrich, and the prices may be lower in bulk quantities. Homovanillyl alcohol has the highest market price with £15.96 per gram followed by tyrosol (£10.24/ g) and vanillin (£7.35/ g). Wide applications of fine chemicals listed in table X have verified that these compounds offer a great deal of opportunity in term of economical point of view. High demand of these compounds may contribute towards further increment of the price, thus increase the opportunity of lignin as a renewable source of fine chemicals.

Table 8.1 Current market price for aromatic chemicals produced in current study (source: www.sigmaaldrich.com). N/A represents a compound without available market price and the uses could not be identified.

compounds	prices (£ /g)	Application	Reference
guaicol	0.04	Food industry, perfumery, personal care products, *etc.*	Rhodia (2008)
vanillin	7.35	Cosmetic industry, chemical precursor in pharmaceutical industry, antioxidant additive, *etc.*	Cerrutti *et al.*, 1997; Villar *et al.*, 1997
acetovanillone	0.53	Anti asthmatic, anti inflammatory, ingredient in whisky	Brown (2011)
vanillic acid	0.732	Flavouring agent, as an intermediate production of vanillin from ferrulic acid	Lesage-meesen *et al.*, 1996
homovanillyl alcohol	15.96	Pharmaceutical as antioxidant, food industry	Conde *et al.*, 2009
phenol	0.604	Production of bisphenol-A and other phenol derivatives	Gosselink *et al.*, 2011; Holladay *et al.*, 2007
4-methylbenzaldehyde	0.0445	Intermediate in pharmaceutical industry, dyes, perfume and agrochemicals	www.chemicalland21.com
catechol	0.189	Antioxidant for perfume and essential oil, oxidizing agent, synthesis of adhesives, paper, ink, *etc.*	Environment Canada, 2008
***p*-toluic acid**	1.36	Intermediate for polymer stabilizers, pesticides, light sensitive compounds, animal feed supplements, *etc.*	www.chemicalland21.com

4-hydroxybenzaldehyde	0.282	Pharmaceutical industry, aromatizer, pesticide, electroplating and liquid crystal industry.	www.chemnet.com
tyrosol	10.24	Pharmaceutical as antioxidant	Giovannini *et al.*, 1999
isovanillin	0.667	Pharmaceutical, cosmetic industry, agrochemical and food industry.	Maliverney , 1997
3-hydroxy-1-(4-hydroxy-3-methoxyphenyl)propan-1-one	N/A	-	-

8.5 Consideration for a Large Scale Depolymerization Process

As an impact of findings that have been described in this thesis, large scale polymerization process could be considered. Following this, flow sheeting is an essential task for scaling up. The flow sheet is a link and shows the layout of each unit operation to produce the final product from the raw material. The process flow sheet in Fig. 8.9 describes details of the flow process through streams and equipment.

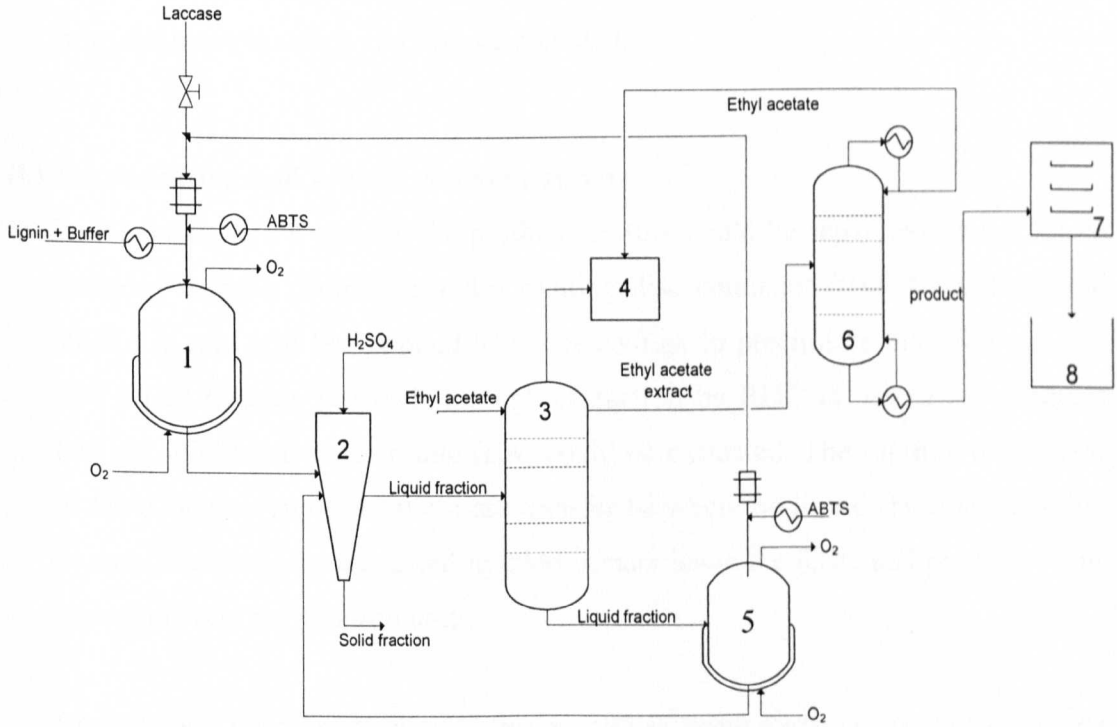


Figure 8.9 Process flow sheet. **1**: batch reactor; **2**: centrifuge; **3**: rotating disc contactor (RDC); **4**: solvent recovery facility; **5**: second unit of batch reactor; **6**: distillation column; **7**: dryer; **8**: storage tank

In order to achieve the final products, it can thus be suggested that four stages of the main unit operation should considered, as outlined below:

(a) Feed supply and reactor system

The process involves the adjustment of the temperature of each feed including sodium lignosulphonate, ammonium acetate buffer and ABTS. Laccase from *Trametes versicolor* (LTV) will be added in a liquid form (solubilized LTV in ammonium acetate buffer). The LTV storage will be facilitated by a cooling system down to -18 °C. The temperature will be increased gradually for the feed preparation up to 25 °C by a heat exchanger. The feed for ABTS and lignin in the buffer will also be facilitated by the heat exchanger to maintain the stream temperature at 30 °C. ABTS and lignin solution will be mixed in a batch reactor and the reaction will be started with the addition of LTV. The reaction will be conducted at a constant stirrer speed for 6 h at 30 °C. Bubble aeration system will be used to delivers the amount oxygen (O_2) or air into the reactor. The expulsion of bubbles can cause a mixing action to occur. The O_2 or air is then purge out from the reactor, which can also be recycled.

(b) Fractionation and solvent recovery system

In the fractionation system, the product mixture could be separated into different fractions using a centrifuge and a rotating disc contactor (RDC). Concentrated sulphuric acid will be supplied to the centrifuge to precipitate the solid residue. The liquid fraction proceeds through extraction by RDC in which the products that are soluble in ethyl acetate (EA) could be extracted. The rotation of the disc in the contactor enhances the mass transfer between the liquid fraction and ethyl acetate. The products extracted in ethyl acetate leave the RDC and proceed to the separation and purification unit.

Even though the extraction may successfully separate ethyl acetate and the liquid fraction, a solvent recovery unit is necessary to further remove the remaining liquid fraction in the ethyl acetate, thus it could be reused in the contactor.

(c) Liquid fraction recovery system

The remaining compound in the liquid fraction that is not soluble in ethyl acetate proceeds to the second unit of batch reactor and the process will be repeated as in the first unit of the reactor.

(d) Product separation and purification system

The distillation column could be used to separate the mixture of products from ethyl acetate. The remaining solvents could be recycled and proceed to the solvent recovery facility. The separated products will then be dried and stored before shipment.

8.6 Concluding Remarks

The aim of the study described in this thesis was to explore the potential of lignin as a renewable feedstock for the production of fine chemicals under mild reaction conditions. Lignin is available at relatively low cost; therefore it is economically feasible to use it in the production of value added chemicals.

This thesis has successfully described the potential of sodium lignosulphonate to be converted to fine chemicals. The result shows the conversion of 9.8 % sodium lignosulphonates to 13 different aromatic compounds. Vanillic acid was found to be a major product. LTV-ABTS were found to be the most suitable enzyme-mediator systems for sodium lignosulphonate depolymerization. This study has revealed basic information of laccase as a potential enzyme for the depolymerization of sodium lignosulphonate and also its mediator system. Development of the enzymatic depolymerization technique needs to be further established in order to produce an efficient method for high lignin conversion. In this study, the enzymatic conversion of lignin was found to be highly dependent on several factors that need to be taken into consideration including:

(i) **The type of lignin** – the isolation method and the source of biomass influence the mass distribution of the lignin.

(ii) **The type of laccase** – the depolymerization process was highly influenced by the activity of this enzyme.

(iii) **The type of mediator** – also playing an important role in products formation, since laccase was totally dependent on its mediators for depolymerization to take place.

Finally, the results presented in this thesis will contribute to new knowledge to increase the use of lignin in the future. Biological routes have been proved to offer much 'greener' processes for the production of value-added chemicals. However, it can be concluded that 'chopping up' lignin is not an easy task. Clearly, the complex evolutionary puzzle regarding lignin depolymerization still needs to be resolved to develop a better process for the high yield production of fine chemicals.

9. REFERENCES

Abdul-Sada, A. A. K., A. M. Greenway, K. R. Seddon & T. Welton (1993) A fast atom bombardment mass spectrometric study of room-temperature 1-ethyl-3-methylimidazolium chloroaluminate(III) ionic liquids. Evidence for the existence of the decachlorotrialuminate(III) anion. *Organic Mass Spectrometry,* 28, 759-765

Abdul-Sada, A.K., P.W. Ambler, P.K.G. Hodgson, K.R. Seddon, N.J. Stewart (1995) World patent 9521871 to BP Chemical Ltd

Adler, E. (1977) Lignin chemistry—past, present and future. *Wood Science and Technology,* 11, 169-218.

Alexandre, G. & R. Bally (1999) Emergence of a laccase-positive variant of Azospirillum lipoferum occurs via a two-step phenotypic switching process. *FEMS Microbiology Letters,* 174, 371-378.

Ander, P., K. Eriksson, H.Yu (1983) Vanillic acid metabolism by *Sporotrichum pulverulentum*: evidence for demethoxylation before ring-cleavage. *Archives of Microbiology,* 136, 1-6

Ander, P. & K. Messner (1998) Oxidation of 1-hydroxybenzotriazole by laccase and lignin peroxidase. *Biotechnology Techniques,* 12, 191-195

Andreasson, L.E., R. Branden & B. Reinhammar (1976) Kinetics studies of *Rhus vernicifera* laccase. Evidance for multi-electron transfer and an oxygen intermediate in the reoxidation reaction. *Biochimica et Biophysica Acta,* 438, 370-379

Annele, H. (1994) Lignin-modifying enzymes from selected white-rot fungi: production and role from in lignin degradation. *FEMS Microbiology Review,* 13, 125-135

Annunziatini, C., P. Baiocco, M. F. Gerini, O. Lanzalunga & B. Sjögren (2005) Aryl substituted *N*-hydroxyphthalimides as mediators in the laccase-catalysed oxidation of lignin model compounds and delignification of wood pulp. *Journal of Molecular Catalysis B: Enzymatic,* 32, 89-96

Antai, S. P. & D. L. Crawford (1981) Degradation of Softwood, Hardwood, and Grass Lignocelluloses by Two Streptomyces Strains. *Appllied Environmental and Microbiology,* 42, 378-380.

Ardon, O., Z. Kerem & Y. Hadar (1998) Enhancement of lignin degradation and laccase activity in *Pleurotus ostreatus* by cotton stalk extract. *Canadian Journal of Microbiol,* 44, 676-680

Arends, I. W. C. E., Y. Li, R. Ausan & R.A. Sheldon (2006) Comparison of TEMPO and its derivatives as mediators in laccase catalysed oxidation of alcohols. *Tetrahedron,* 62, 6659-6665

Argyropoulos, D.S. & S.B. Menachan (1997) Advances in biochemical engineering technology. *Springler-Verlag,* 57, 127

ASPO (2009) The Association for the Study of Peak Oil and gas. Newsletter No. 100. http://aspoireland.files.wordpress.com/2009/12/newsletter100_200904.pdf (accessed November 2012)

Baldrian, P. & J. Gabriel (2002) Copper and cadmium increase laccase activity in *Pleurotus ostreatus. FEMS Microbiology Letters,* 206, 69-74

Baldrian, P. (2004) Purification and characterization of laccase from the white-rot fungus *Daedalea quercina* and decolorization of synthetic dyes by the enzyme. *Applied Microbiology and Biotechnology,* 63, 560-563

Baldrian, P. (2006) Fungal laccases – occurrence and properties. *FEMS Microbiology Reviews,* 30, 215-242

Baiocco, P., A.M. Barreca, M. Fabbrini,C. Galli & P. Gentili (2003) Promoting laccase activity towards non-phenolic substrates: a mechanistic investigation with some laccase-mediator systems. *Organic & Biomolecular Chemistry,* 1, 191-197

Barahona, D., P. H. Pfromm & M. E. Rezac (2006) Effect of water activity on the lipase catalyzed esterification of geraniol in ionic liquid [bmim][PF_6]. *Biotechnology and Bioengineering,* 93, 318-324

Baumann, M. D., A. J. Daugulis & P. G. Jessop (2005) Phosphonium ionic liquids for degradation of phenol in a two-phase partitioning bioreactor. *Applied Microbiology and Biotechnology,* 67, 131-137

Belgacem, M. N., A. Blayo & A. Gandini (2003) Organosolv lignin as a filler in inks, varnishes and paints. *Industrial Crops and Products,* 18, 145-153

Belgacem, M.N. & A. Gandini (2008) Lignin as components of macromolecular materials. *Elsevier,* 243-271

Bender, M.H. (2000) Potential conservation of biomass in the production of synthetic organics. *Resources, Conservation and Recycling,* 30, 49-58

Bisswanger, H. (2008) Enzyme Kinetics: Section 2.1–2.5. *Enzyme Kinetics.* Wiley-VCH Verlag GmbH & Co. KGaA.

Blanch, H. W. & D. S.Clark (1995) Biocehmical Engineering. *Academic Press, New York.*

Blanchard, L. A., D. Hancu, E. J. Beckman & J. F. Brennecke (1999) Green processing using ionic liquids and CO_2. *Nature,* 399, 28-29

Bloois, E., D. E. T. Pazmiño, R.T. Winter & M.W. Fraaije (2010) A robust and extracellular heme-containing peroxidase from *Thermobifida fusca* as prototype of a bacterial peroxidase superfamily. *Appllied Microbiology and Biotechnology,* 86, 1419–1430

Bollag, J.M. & A. Leonowicz (1984) Comparative Studies of Extracellular Fungal Laccases. *Applied and Environmental Microbiology,* 48, 849-854.

Bourbbonnais, R. & M.G. Paice (1990) Oxidation of non-phenolic substrates. An expanded role for laccase in lignin biodegradation. *FEBS Letters,* 267, 99-102

Bourbonnais, R. & M. G. Paice (1992) Demethylation and delignification of Kraft pulp by Trametes versicolor laccase in the presence of 2,2'-azinobis-(3-ethylbenzthiazoline-6-sulphonate). *Applied Microbiology and Biotechnology,* 36, 823-827.

Bourbonnais, R., M. Paice, I. Reid, P. Lanthier & M. Yaguchi (1995) Lignin oxidation by laccase isozymes from Trametes versicolor and role of the mediator 2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonate) in Kraft lignin depolymerization. *Appied and. Environmental. Microbiology,* 61, 1876-1880.

Bourbonnais, R., M. Paice, B. Freiermuth, E. Bodie & S. Borneman (1997) Reactivities of Various Mediators and Laccases with Kraft Pulp and Lignin Model Compounds. *Applied and Environmental Microbiology,* 63, 4627-4632.

Bourbonnais, R., D. Leech & M. G. Paice (1998) Electrochemical analysis of the interactions of laccase mediators with lignin model compounds. *Biochimica et Biophysica Acta,* 1379, 381-390

Branchi, B., C. Galli & P. Gentili (2005) Kinetics of oxidation of benzyl alcohols by the dication and radical of ABTS. Comparison with laccase-ABTS oxidations: an apparent paradox. *Organic and Biomolecular Chemistry,* 3, 2604-2614

Brown, A. (1985) Review of ligninin biomass. *Journal of Application Biochemistry,* 7, 371-387

Brown, S. (2011) What is inside scotch whisky. http://www.drbunsen.org/whats-inside-scotch-whisky (accessed July 2013)

Brunow, G. (2001) Methods for reveal the structure of lignin. *Biopolymers,* 3, 89-116

Buckeye Tecnologies Inc. (2009) University of Florida IFAS, Buckeye technologies and Myriant announce next generation cellulosic ethanol research and demonstration plant. http://www.bkitech.com/ir_newsrelease.aspx?reqid=1300158 (accessed November 2012)

Buswell, J. A. & E. Odier (1987) Lignin Biodegradation. *Critical Reviews in Biotechnology,* 6, 1-60

Camarero, S., S. Sarkar, F. J. Ruiz-Dueñas, M. a. J. Martínez & Á. T. Martínez (1999) Description of a Versatile Peroxidase Involved in the Natural Degradation of Lignin

That Has Both Manganese Peroxidase and Lignin Peroxidase Substrate Interaction Sites. *Journal of Biological Chemistry,* 274, 10324-10330

Canas, A. I. & S. Camarero (2010) Laccases and their natural mediators: Biotechnological tools for sustainable eco-friendly processes. *Biotechnology Advances,* 28, 694-705

Castillo, P. Z., M. L.Villalonga-Santana, J. Tamayo-Cortés, G. Rivera-Muñoz & S. Solís-Pereira (2012) Purification and characterization of laccase from Trametes hirsuta Bm-2 and its contribution to dye and effluent decolorization. *African Journal of Biotechnology,* 11, 3603-3611

Cathala, B., B. Saake, O.Faix & B. Monties (2003) Association behaviour of lignins and lignin model compounds studied by multidetector size-exclusion chromatography. *Journal of Chromatography A,* 1020, 229-239

Cerrutti, P., S.M. Alzamora, S.L. Vidales (1997) Vanillin as an antimicrobial for producing shelf-stable strawberry puree. *Journal of Food Sciene,* 62, 608-610

Cervini, M. (2005) Image: *Agaricus bisporus.* http://www.funghiitaliani.it (accessed July 2013)

Chakar, F.S. & A.J. Ragauskas (2004) Review of current and future softwood Kraft lignin process chemistry. *Industrial crops and products,* 20, 131-141

Chemicalland21. *p*-tolualdehyde. http://www.chemicalland21.com (accessed July 2013)

Chemicalland21. *p*-toluic acid. http://www.chemicalland21.com (accessed July 2013)

Chemnet. 4-hydroxybenzaldehyde. http://www.chemnet.com (accessed July 2013)

Chen, Q., M. N. Marshall, S.M. Geib, M. Tien & T. L. Richard (2012) Effects of laccase on lignin depolymerization and enzymatic hydrolysis of ensiled corn stover. *Bioresource Technology,* 117, 186–192.

Cheng, G., M.S. Kent, L.H.P. Varanasi, D. Dibble, R. Arora, K.Deng, K. Hong, Y.B. Melnichenko, B. A. Simmons & S. Singh (2012) Effect of ionic liquid treatment on the structures of lignins in solutions: molecular subunits released from lignin. *Langmuir,* 28, 11850–11857

Chiappe, C., E. Leandri, B.D. Hammock & C. Morisseeau (2007) Effect of ionic liquids on epoxide hydrolase-catalyzed synthesis of chiral 1,2 diols. *Green Chemistry,* 9, 162-168

Chin J.T., S.L. Wheeler & A.M. Klibanov (1994) On protein solubility in organic solvents. *Biotechnology and Bioengineering,* 44, 140-145

Clark, J. H. (2007) Green chemistry for the second generation biorefinerysustainable chemical manufacturing based on biomass. *Journal of Chemical Technology and Biotechnology,* 82, 603-609.

Cohen, R., Y. Hadar & O. Yarden (2001) Transcript and activity levels of different *Pleurotus ostreatus* peroxidases are differentially affected by Mn^{2+}. *Environmental Microbiology,* 3, 312-322.

Cole, J. L., P. A. Clark & E. I. Solomon (1990) Spectroscopic and chemical studies of the laccase trinuclear copper active site: geometric and electronic structure. *Jounal of American Cheemical Society,* 112, 9534-9548.

Conde, E., E. Cadahía, M.C. García-Vallejo, B. F. de Simon, & J.R.G. Adrados (1997) Low Molecular Weight Polyphenols in Cork of *Quercus suber. Journal of Agricultural Food Chemistry,* 45, 2695-2700

Conde, E., C. Cara, A. Moure, E. Ruiz, E. Castro, H. Dominguez (2009) Antioxidant activity of the phenolic compounds released by hydrothermal treatments of olive tree pruning. *Food Chemistry* , 114, 806RLI2

Couto, S. R. & J.L.T. Herrera (2006) Industrial and biotechnology applications of laccases: a review. *Biotechnology Advances,* 24, 500-513

Cull, S.G., J.D. Holbrey, V. Vargas-Mora, K.R. Seddon, G.J. Lye (2000) Room temperature ionic liquids as replacements for organic solvents in multiphase bioprocess operations. Biotechnology Bioengineering, 69, 227 - 233

Crestini C. & D.S. Argyropoulos (1998) The early oxidative biodegradation steps

of residual kraft lignin models with laccase. *Bioorganic and Medical Chemistry,* 6, 2161ygidua

d'Acunzo, F., P. Baiocco & C. Galli (2003) A study of the oxidation of ethers with the enzyme laccase under mediation by two N-OH-type compounds. *New Journal of Chemistry,* 27, 329-332

da Silva, E.A.B., M. Zabkova, J.D. Araújo, C.A. Cateto, M.F. Barreiro, M.N. Belgacem, A.E. Rodrigues (2009) An integrated process to produce vanillin and lignin-based polyurethanes from Kraft lignin. *Chemical Engineering Research and Design,* 87, 1276, eri

Dashtban, M., H. Schraft, T. A. Syed, W. Qin (2010) Fungal biodegradation and enzymatic modification of lignin. *International Journal of Biochemical and Molecular Biology,* 1, 36-50

Dean, J.A. (1992) Lange's handbook of chemistry. *McGraw Hill, Inc New York,* 14th Edition

Deetlefs, M. K.R. Seddon & M. Share (2006) Predicting physical properties of ionic liquids. *Physical Chemistry Chemical Physics,* 8, 642-649

De Los Ríos, A. P., F. J. Hernández-Fernández, F. A. Martínez, M. Rubio & G. Víllora (2009) The effect of ionic liquid media on activity, selectivity and stability of *Candida antarctica* lipase B in transesterification reactions. *Biocatalysis and Biotransformation,* 25, 151-156.

del Rio, J.C., A. Gutierrez, M. Hernando, P. Landin, J. Romero & A.T. Martinez (2005) Determining the influence of eucalypt lignin composition in paper pulp yield using Py-GC/MS. *Journal of Analytical and Applied Pyrolysis,* 74, 110–115.

Demirbas, A. (2005) Potential applications of renewable energy sources, biomass combustion problems in boiler power systems and combustion related environmental issues. *Progress in Energy and Combustion Science,* 31, 171–192

Diego, T., P. Lozano, S. Gmouth, M. Vaultier & J.L. Iborra (2005) Understanding structure-stability relationships of *Candida antartica* lipase B in ionic liquids. *Biomacromolecules,* 6, 1457-1464

Dodor, D.E., H.M. Hwang & S.I.N. Ekunwe (2004) Oxidation of anthracene and benzo[a]pyrene by immobilized laccase from *Trametes versicolor. Enzyme and Microbial Technology,* 35, 210-217.

Domínguez, A., J. Go´mez & M. Lorenzo (2007) Enhanced production of laccase activity by *Trametes versicolor* immobilized into alginate beads by the addition of different inducers. *World Journal of Microbiology and Biotechnology,* 23, 367–373

Domínguez, A., O. Rodríguez, A. P. M. Tavares, E. A. Macedo, A. Longo & A. Sanromán (2011) Studies of laccase from *Trametes versicolor* in aqueous solutions of several methylimidazolium ionic liquids. *Bioresource Technology,* 102, 7494–7499

Dunford, H.B. & J.S. Stillman (1976) On the function and mechanism of action of peroxidases. *Coordination Chemical Revision,* 19, 187-251

Durán, N., M. A. Rosa, A. D'Annibale & L. Gianfreda (2002) Applications of laccases and tyrosinases (phenoloxidases) immobilized on different supports: A review. *Enzyme and Microbial Technology,* 31, 907-931

Durao, P., Z. Chen, A. Fernandes, P. Hildebrandt, D. Murgida, S. Todorovic, M. Pereira, E. Melo & L. Martins (2008). Copper incorporation into recombinant CotA laccase from *Bacillus subtilis* : characterization of fully copper loaded enzymes. *Journal of Biological Inorganic Chemistry,* 13, 183-193

Earle M. J. & K. R. Seddon (2002) Ionic Liquids: Green Solvents for the Future. *Clean Solvents.* Washington, DC: American Chemical Society.

Eggert, C., U. Temp, J. F. D. Dean & K.E. L. Eriksson (1996) A fungal metabolite mediates degradation of non-phenolic lignin structures and synthetic lignin by laccase. *FEBS Letter,* 391, 144-148.

Ek, M. (2005) The status of applied lignin research, Report No. 2, Processum Technology

Park AB. *In*: L. Kouisni, Y. Fang, M. Paleologou, B. Ahvazi, J. Hawari, Y. Zhang & X. Wang. *Cellulose Chemistry and Technology*, 45, 515-520

Elegir, G., S. Daina, L. Zoia, G. Bestetti & M. Orlandi (2005) Laccase mediator system: Oxidation of recalcitrant lignin model structures present in residual Kraft lignin. *Enzyme and Microbial Technology*, 37, 340-346

Endo, K., K. Hosono, T. Beppu & K. Ueda (2002) A novel extracytoplasmic phenol oxidase of Streptomyces: its possible involvement in the onset of morphogenesis. *Microbiology*, 148, 1767-1776

Environment Canada (2008) Screening Assessement for 1,2-Benzenediol (Catechol). http://www.ec.gc.ca/ese-ees (accessed July 2013)

Eriksson, K. E. L. (1990) Biotechnology in the pulp and paper industry. *Wood Science and Technology*, 24, 79-101.

Fabbrini, M., C. Galli & P. Gentili (2002) Comparing the catalytic efficiency of some mediators of laccase. *Journal of Molecular Catalysis B: Enzymatic*, 16, 231-240

Fabbrini, M., C. Galli, P. Gentili & D. Macchitella (2001) An oxidation of alcohols by oxygen with the enzyme laccase and mediation by TEMPO. *Tetrahedron Letters*, 42, 7551-7553.

Finch, K.B.H., R. M. Richards, A. Richel, A.V. Medvedovici, N. G. Gheorghe, M. Verziu, S.M. Coman & V. I. Parvulescu (2012) Catalytic hydroprocessing of lignin under thermal and ultrasound conditions. *Catalysis Today*, 196, 3-10

Forrester, I.T., A. C. Grabski, R. R. Burgess & G. F. Leatham (1988) Manganese, Mn- dependent peroxidases, and the biodegradation of lignin. *Biochemical and Biophysical Research Communications*, 157, 992–999

Fort, D. A., R. C. Remsing, R. P. Swatloski, P. Moyna, G. Moyna & R. D. Rogers (2007) Can ionic liquids dissolve wood? Processing and analysis of lignocellulosic materials with 1-n-butyl-3-methylimidazolium chloride. *Green Chemistry*, 9, 63-69.

French, A.D. (2000) *In* S. Kung, S. Yang. Discoveries in plant biology. *World Scientific*, 3,164-166.

Fujita, K., M. Forsyth, D. R. MacFarlane, R. W. Reid & G. D. Elliott (2006) Unexpected improvement in stability and utility of cytochrome c by solution in biocompatible ionic liquids. *Biotechnology and Bioengineering*, 94, 1209-1213.

Fujita, K., D.R. MacFarlane, M. Forsyth (2005) Protein solubilising and stabilizing ionic liquids. *Chemical Communication*, 38, 4804-4806

Galli, C. & P. Gentilli (2004) Chemical messengers: mediated oxidations with the enzyme laccase. *Journal of Physical Organic Chemistry*, 17, 973-977.

Gandini, A. (2008) Polymers from Renewable Resources: A Challenge for the Future of Macromolecular Materials. *Macromolecules*, 41, 9491-9504

Gardiol, A.E., R.J. Hernandez & B.R. Harte (1998) Device for detecting oxygen with oxidase. *United State Patent*, 5, 804-401

Gargulak, J.D., S.E. Lebo (2000) Commercial use of lignin-based materials. *In*: W.G.Glasser, R.A. Northey, T.P. Schultz . Lignin: Historical, Biological, and Materials Perspectives. *ACS Symposium Series. American Chemical Society*, 307

Gianfreda L, F. Xu & J.M. Bollag (1999) Laccases: A useful group of oxidoreductive enzymes. *Bioremediation Journal*, 3, 1-25.

Gierer, J. (1980) Chemical aspects of Kraft pulping. *Wood Science and Technology*, 14, 241-266

Giovannini, C., E. Straface, D. Modesti, E. Coni, A. Cantafora, M. De Vincenzi, W. Malorni & R. Masella (1999) Tyrosol, the Major Olive Oil Biophenol, Protects Against Oxidized-LDL-Induced Injury in Caco-2 Cells. *Journal of Nutrition*, 129, 1269-77

Givaudan, A., A. Effosse, D. Faure, P. Potier, M.-L. Bouillant & R. Bally (1993) Polyphenol oxidase in *Azospirillum lipoferum* isolated from rice rhizosphere:

Evidence for laccase activity in non-motile strains of *Azospirillum lipoferum*. *FEMS Microbiology Letters*, 108, 205-210

Glenn, J. K. & M. H. Gold (1985) Purification and characterization of an extracellular Mn(II)-dependent peroxidase from the lignin-degrading basidiomycete, *Phanerochaete chrysosporium*. *Archives of biochemistry and biophysics*, 242, 329-341

Glenn, J.K., L. Akileswaran & M.H. Gold (1986) Mn(II) oxidation is the principal function of the extracellular Mn_peroxidase from *Phanerochaete chrysosporium*. *Archives of Biochemistry and Biophysics*, 251, 688-696

Gold, M.H., M. Kuwahara, A.A. Chiu & J.K. Glenn (1984) Purification and characterization of an extracellular H2O2 requiring diarylpropane oxygenase from the white rot Basidiomycete, *Phanerochaete chrysosporium*. *Archives of Biochemistry and Biophysics*, 234, 353-362

Goncalves, M. L. F. C. & W. Steiner (1996) Purification and Characterization of Laccase from a Newly Isolated Wood-Decaying Fungus. *Enzymes for Pulp and Paper Processing*. American Chemical Society, 655, 258 - 266

Goodell B., K. Yamamoto, J. Jellison, M. Nakamura, T. Fujii & K. Takabe (1998) Laccase immunolabelling and microanalytical analysis of wood degraded by *Lentinus edodes*. *Holzforschung*, 52, 345fors

Gordon, C.M., J.D. Holbrey, A.R. Kennedy & K.R. Seddon (1998) Ionic liquids crystals: hexafluorophosphate salts. *Journal of Material Chemistry*, 8, 2627-2636

Gosselink, R.J.A., E. de Jong, B. Guran & A. Abächerli (2004) Co-ordination network for lignin—standardisation, production and applications adapted to market requirements (EUROLIGNIN). *Industrial Crops and Products*, 20, 121–129

Gosselink, R.J.A. (2011) Lignin as a renewable aromatic resource for the chemical industry. *Ph.D Thesis*. Wageningen University, Netherlands

Grabber, J.H., J. Ralph, R.D. Hatfield & S. Quideau. (1997) *p*-Hydroxyphenyl, guaiacyl, and syringyl lignins have similar inhibitory effects on wall degradability. *Journal of Agricultural Food Chemistry*, 45, 2530-2532

Green, F. (1977) Significance of glucose oxidase in lignin degradation. *Nature*, 268, 78–80

Grűnewald, H. (1976) Handbook of Chemistry and Physics. Herausgegeben von R. C. Weast. CRC Press, *Angewandte Chemie*, 88, 195-195.

Hammel, K.E. & M.A. Moen (1991) Depolymerization of a synthetic lignin in-vitro by lignin peroxidase. *Enzyme Microbiol Technology*, 13, 15-18

Hatakka, A. (1994) Lignin-modifying enzymes from selected white-rot fungi: production and role from in lignin degradation. *FEMS Microbiology Reviews*, 13, 125-135

Hawkins, S., J. Samaj, V. Lauvergeat, A. Boudet & J. Grima-Pettenati (1997) Cinnamyl Alcohol Dehydrogenase: Identification of New Sites of Promoter Activity in Transgenic Poplar. *Plant Physiology*, 113, 321-325

Heinfling, A., F. J. Ruiz-Dueñas, M. J. Martínez, M. Bergbauer, U. Szewzyk & A. T. Martínez (1998) A study on reducing substrates of manganese-oxidizing peroxidases from Pleurotus eryngii and Bjerkandera adusta. *FEBS Letters*, 428, 141-146.

Heitner, C., D. Dimmel & J. Schmidt (2011) Lignin and Lignans: Advances in Chemistry. *CRC Press*.

Hergert, H.L. & E. K. Pye (1992) Recent history of organosolv pulping. *Solvent pulping symposium*, 9 - 26

Hernandez Fernaud, J. R., A. Carnicero, F. Perestelo, M. Hernandez Cutuli, E. Arias & M. A. Falcon (2006) Upgrading of an industrial lignin by using laccase produced by Fusarium proliferatum and different laccase-mediator systems. *Enzyme and Microbial Technology*, 38, 40-48

Higuchi, T. (1990) Lignin biochemistry: Biosynthesis and biodegradation. *Wood Science and Technology*, 24, 23-63

Himmel, M. E., S.Y. Ding, D. K. Johnson, W. S. Adney, M. R. Nimlos, J. W. Brady & T. D. Foust (2007) Biomass Recalcitrance: Engineering Plants and Enzymes for Biofuels Production. *Science,* 315, 804-807

Hinckley, G., V. V. Mozhaev, C. Budde & Y. L. Khmelnitsky (2002) Oxidative enzymes possess catalytic activity in systems with ionic liquids. *Biotechnology Letters,* 24, 2083-2087

Hiner, A. N. P., E. L. Raven, R. N. F. Thorneley, F. Garcia-Cánovas & J. N. Rodriguez-López (2001) Mechanisms of compound I formation in heme peroxidases. *Contributions from the 10th Intenational Conference on Bioinorganic Chemistry,* 91, 27-34

Hofrichter, M., K. Scheibner, I. Schneegaß & W. Fritsche (1998) Enzymatic Combustion of Aromatic and Aliphatic Compounds by Manganese Peroxidase from *Nematoloma frowardii. Appllied and Environmental Microbiology,* 64, 399-404

Hofrichter, M. (2002) Review: lignin conversion by manganese peroxidase (MnP). *Enzyme and Microbiol Technology,* 30, 454-466

Holladay, J.E., J.J. Bozell, J.F. White & D. Johnson (2007) Top Value-Added Chemicals from Biomass. *Pacific Northwest National Laboratory (PNNL) and the National Renewable Energy Laboratory (NREL),* 1-77

Holbrey, J. D. & K. R. Seddon (1999) Ionic Liquids. *Clean Technologies and Environmental Policy,* 1, 223-236.

Hossain, S.M. & N. Anantharaman (2006) Activity enhancement of ligninolytic enzymes of *Trametes versicolor* with baggase powder. *African Journal of Biotechnology,* 5, 189-194

Huang, Z., L. Dostal & J. P. Rosazza (1993) Mechanisms of ferulic acid conversions to vanillic acid and guaiacol by *Rhodotorula rubra. Journal of Biological Chemistry,* 268, 23954-23958

Huddleston, J.G., H.D. Willauer, R.P. Swatloski, A.E. Visser & R.D. Rogers (1998) Room temperature ionic liquids as novel media for 'clean' liquid-liquid extraction. *Chemical Communication,* 16, 1675-1766

Huijgen, W.J. J., J. H. Reith & H. den Uil (2010) Pretreatment and Fractionation of Wheat Straw by an Acetone-Based Organosolv Process. *Industrial and Engineering Chemical Research,* 49, 10132–10140

Hullo, M.F., I. Moszer, A. Danchin & I. Martin-Verstraete (2001) CotA of Bacillus subtilis Is a Copper-Dependent Laccase. *Jounal of Bacteriology.,* 183, 5426-5430.

Hüttermann, A., C. Herche & A. Haars (1980) Polymerisation of Water-Insoluble Lignins by Fomes annosus. *Holzforschung,* 34, 64-66

Hüttermann, A., C. Mai & A. Kharazipour (2001) Modification of lignin for the production of new compounded materials. *Applied Microbiology and Biotechnology,* 55, 387-384

Ingram, L. O. & J. B. Doran (1995) Conversion of cellulosic materials to ethanol. *FEMS Microbiology Reviews,* 16, 235-241

Jaworski, A. W. & C. Y. Lee (1987) Fractionation and HPLC determination of grape phenolics. *Journal of Agricultural and Food Chemistry,* 35, 257-259

Jebens, A., H. Janshekar & K. Yokose (2012) Lignosulphonates http://chemical.ihs.com/CEH/Public/Reports/671.5000 (accessed November 2012)

Jham, G. N., S. A. Fernandes, C. F. Garcia & A. A. D. Silva (2002) Comparison of GC and HPLC for the quantification of organic acids in coffee. *Phytochemical Analysis,* 13, 99-104

Jia, S., B. J. Cox, X. Guo, Z. C. Zhang & J. G. Ekerdt (2011) Hydrolytic Cleavage of β-O-4 Ether Bonds of Lignin Model Compounds in an Ionic Liquid with Metal Chlorides. *Industrial and Engineering Chemistry Research, 50,* 849–855

Johannes, C. & A. Majcherczyk (2000) Natural Mediators in the Oxidation of Polycyclic Aromatic Hydrocarbons by Laccase Mediator Systems. *Appllied and Environmental Microbiology,* 66, 2524-528

Johannes, T.W., R.D. Woodyer & H. Zhao (2006) High-throughput screening methods developed for oxidoreductases. *In*: Reymond, J. Enzyme assays. *Wiley-VCH*, Page 77-92

Juan, F. & Z. Huaiyu (2008) Optimization of synthesis of spherical lignosulphonate resin and its structure characterization. *Chinese Journal of Chemical Engineering*, 16, 407-410

Kader, F., B. Roverl, M. Girardin & M. Metche (1996) Fractionation and identification of the phenolic compounds of Highbush blueberries (*Vaccinium corymbosum, L.*). *Food Chemistry*, 55, 35-40

Karen Whimsy (2013) Tree clipart: Beech tree. http://karenswhimsy.com/tree-clipart.shtm (accessed July 2013)

Kawai, S., M. Nakagawa & H. Ohashi (1999) Aromatic ring cleavage of a non-phenolic [beta]-O-4 lignin model dimer by laccase of *Trametes versicolor* in the presence of 1-hydroxybenzotriazole. *FEBS Letters*, 446, 355-358

Kawai, S., T. Umezawa & T. Higuchi (1988) Degradation mechanisms of phenolic [beta]-1 lignin substructure model compounds by laccase of *Coriolus versicolor. Archives of Biochemistry and Biophysics*, 262, 99-110

Khan, A. W. & R. P. Overend (1990) An oxygenase enzyme system for *Trametes versicolor. FEMS Microbiology Letters*, 66, 215-220

Kilpeläinen, I., H. Xie, A. King, M. Granstrom, S. Heikkinen & D. S. Argyropoulos (2007) Dissolution of Wood in Ionic Liquids. 55, 9142-9148.

Koch, V. R., L. L. Miller & R. A. Osteryoung (1976) Electroinitiated Friedel-Crafts transalkylations in a room-temperature molten-salt medium. *Journal of American Chemical Society*, 98, 5277-5284

Kolb, M., V. Sieber, M. Amann, M. Faulstich & D. Schieder (2012) Removal of monomer delignification products by laccase from *Trametes versicolor. Bioresource Technology*, 104, 298-304

Koschorreck, K., S. Richter, A. Ene, E. Roduner, R. Schmid & V. Urlacher (2008) Cloning and characterization of a new laccase from *Bacillus licheniformis* catalyzing dimerization of phenolic acids. *Applied Microbiology and Biotechnology*, 79, 217-224

Kragl, U., M. Eckstein & N. Kaftzik (2002) Enzyme catalysis in ionic liquids. *Current Opinion in Biotechnology*, 13, 565-571

Kubisa, P. (2004) Application of ionic liquids as solvents for polymerization processes. *Progress in Polymer Science*, 29, 3-12

Kuhad, R., A. Singh & K.E. Eriksson (1997) Microorganisms and enzymes involved in the degradation of plant fiber cell walls. *Biotechnology in the Pulp and Paper Industry*, 45-125

Kulys, J. & R. Vidziunaite (2005) Kinetics of laccase-catalysed TEMPO oxidation. *Journal of Molecular Catalysis B: Enzymatic*, 37, 79-83

Kunamneni A, F.J. Plou , A. Ballesteros & M. Alcalde (2007) Laccases and their applications: A patent review. *Recent Patents on Biotechnology*, 2, 10-24.

Kuwahara, M., J.K. Glenn, M.A. Morgan & M.H. Gold (1984) Separation and characterization of two extracellular H sub (2) O sub (2)-dependent oxidases from lignolytic cultures of *Phanerochaete chrysosporium. FEBS Letters*, 169, 247-250

Lancaster, M (2000) Green chemistry: an introductory text. *Education in chemistry*, 37, 40-42

Lau, R.M., M.J. Sorgedrager, G. Carrea, F.V. Rantwijk, F. Secundo& R.A. Sheldon (2004) Dissolution of *candida antartica* lipase B in ionic liquids: Effects on structure and activity. *Green Chemistry*, 6, 483–487

Laufer, Z., R. P. Beckett & F. V. Minibayeva (2006) Co-occurrence of the Multicopper Oxidases Tyrosinase and Laccase in Lichens in Sub-order Peltigerineae. *Ann Bot*, 98, 1035-1042

Lavoie, J.M., W. Bare & M. Bilodeau (2011) Depolymerization of steam-treated lignin for the production of green chemicals. *Bioresource Technology*, 102, 4917-4920

Lee, I.Y., K.H. Jung, C.H. Lee & Y.H. Park (1999) Enhanced production of laccase in *Trametes vesicolor* by the addition of ethanol. *Biotechnology Letters,* 21, 965-968

Leonowicz, A., A. Matuszewska, J. Luterek, D. Ziegenhagen, M. Wojtas-Wasilewska, N.-S. Cho, M. Hofrichter & J. Rogalski (1999) Biodegradation of Lignin by White-rot Fungi. *Fungal Genetics and Biology,* 27, 175 - 185

Leonowicz, A., G. Szklarz & M. Wojtas-Wasilewska (1985) The effect of fungal laccase on fractionated lignosulphonates (peritan Na). *Phytochemistry,* 24, 393-396

Lesage-Meessen, L., M. Delattre , M. Haon , J.F. Thibault , B.C. Ceccaldi , P. Brunerie, M. Asther (1996) A two-step bioconversion process for vanillin production from ferulic acid combining *Aspergillus niger* and *Pycnoporus cinnabarinus. Journal of Biotechnology,* 50, 107-13

Li, K., F. Xu & K.E. L. Eriksson (1999) Comparison of Fungal Laccases and Redox Mediators in Oxidation of a Nonphenolic Lignin Model Compound. *Applied and Environmental Microbiology,* 65, 2654-2660

Li, C., Q. Wang & Z.K. Zhao (2008) Acid in ionic liquids: an efficient system for hydrolysis of lignocellulose. *Green Chemistry,* 10, 177-182

Liu, J.F., G.B. Jiang, & J. Å. Jonsson (2005) Application of ionic liquids in analytical chemistry. *TrAC Trends in Analytical Chemistry,* 24, 20-27

Lora, J.H. & W.G. Glasser (2002) Recent industrial application of lignin: A sustainable alternative to nonrenewable materials. *Journals of Polymers and the Environment,* 10, 39-48

Lozano, P., T. d. Diego, J.-P. Guegan, M. Vaultier & J. L. Iborra (2001) Stabilization of chymotrypsin by ionic liquids in transesterification reactions. *Biotechnology and Bioengineering,* 75, 563-569

Lu, F. & J. Ralph (2010) *In:* R. Sun. Lignin cereal straw as a resources for sustainable biomaterials and biofuels (chemistry, extractives, lignins, hemicelluloses and celluloses). *Elsevier,* 169

Lutz-Wahl, S., E. M. Trost, B. Wagner, A. Manns & L. Fischer (2006) Performance of d-amino acid oxidase in presence of ionic liquids. *Journal of Biotechnology,* 124, 163-171

Lynd, L., H.-J. Ahn, G. Anderson, P. Hill, D. Sean Kersey & T. Klapatch (1991) Thermophilic ethanol production investigation of ethanol yield and tolerance in continuous culture. *Applied Biochemistry and Biotechnology,* 28-29, 549-570.

Lynd, L. R., P. J. Weimer, W. H. van Zyl & I. S. Pretorius (2002) Microbial Cellulose Utilization: Fundamentals and Biotechnology. *Microbiology andMolecular Biology Reviews,* 66, 506-577

Mabee, W.E., D.J. Gregg, C. Arato, A. Berlin, R. Bura, N. Gilkes, O. Mirochnik, X. Pan, E.K. Pye, J.N. Saddler (2006)Updates on Softwood-to-Ethanol Process Development. *Applied Biochemistry and Biotechnology,* 129, 55-70

Madzak, C., M. C. Mimmi, E. Caminade, A. Brault, S. Baumberger, P. Briozzo, C. Mougin & C. Jolivalt (2006) Shifting the optimal pH of activity for a laccase from the fungus *Trametes versicolor* by structure-based mutagenesis. *Protein Engineering Design and Selection,* 19, 77-84

Mansur, M., T. Suarez, J. Fernandez-Larrea, M. Brizuela & A. Gonzalez (1997) Identification of a laccase gene family in the new lignin-degrading basidiomycete CECT 20197. *Appllied Environmental and Microbiology,* 63, 2637-2646

Majcherczyk, A. & A. Hüttermann (1997) Size-exclusion chromatography of lignin as ion-pair complex. *Journal of Chromatography A,* 764, 183–191

Majcherczyk, A., C. Johannes & A. Hüttermann (1998) Oxidation of aromatic alcohols by laccase from *Trametes versicolor* mediated by the 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) cation radical and dication. *Applied Microbiology and Biotechnology,* 51, 267-276

Maliverney, C. (1997) Process for the preparation of isovanillin US patent: 5,648,552

Maragoni, A.G. (2003) Enzyme kinetics: a modern approach. *A John Wiley and Sons Inc.*

Marjasvaara, A., J. Jänis & P. Vainiotalo (2008) Oxidation of a laccase mediator ABTS as studied by ESI-FTICR mass spectrometry. *Journal of Mass Spectrometry,* 43, 470-477

Matsushita, Y. & S. Yasuda (2005) Preparation and evaluation of lignosulphonates as a dispersant for gypsum paste from acid hydrolysis lignin. *Bioresource Technology,* 96, 465-470

Mensah, C.A., N.A. Adanafio, K. Amaning-Kwarteng & F.K. Rodrigues (2012) Reduced tannin content of laccase-treated cocoa (*Theobroma cocoa*) pod husk. International *Journal of Biological Chemistry,* 6, 31-36

Mester, T. & J. A. Field (1997) Optimization of manganese peroxidase production by the white rot fungus *Bjerkandera sp.* strain BOS55. *FEMS Microbiology Letters,* 155, 161-168

Mikolasch, A. & F. Schauer (2009) Fungal laccases as tools for the synthesis of new hybrid molecules and biomaterials. *Applied Microbiology and Biotechnology,* 82, 605-624

Minussi, R.C., G.M. Pastore & N. Dura'n (2002) Potential applications of laccase in the food industry. *Trends in Food Science & Technology,* 13, 205–216

Minussi, R.C., G.M. Pastore & N. Dura'n (2007). Laccase induction in fungi and laccase/N–OH mediator systems applied in paper mill effluent. *Bioresource Technology,* 98, 158-164

Moniruzzaman, M. & T. Ono (2012) Ionic liquid assisted enzymatic delignification of wood biomass: A new 'green' and efficient approach for isolating of cellulose fibers. *Biochemical Engineering Journal,* 60, 156– 160

Morozova, O., G. Shumakovich, S. Shleev & Y. Yaropolov (2007) Laccase-mediator systems and their applications: A review. *Applied Biochemistry and Microbiology,* 43, 523-535.

Mosier, N., C. Wyman, B. Dale, R. Elander, Y. Y. Lee, M. Holtzapple & M. Ladisch (2005) Features of promising technologies for pretreatment of lignocellulosic biomass. *Bioresource Technology,* 96, 673-686

Murugesan, S. & R. J. Linhardt (2005) Ionic Liquids in Carbohydrate Chemistry - Current Trends and Future Directions. *Current Organic Synthesis,* 2, 437-451

Nierop, K. (2007) Molecular soil organic matter transformations. http://staff.science.uva.nl/~knierop/research.html (accessed November 2012)

Nugroho, P. E., T. Kudanga, L. Østergaard, J. Rencoret, A. Gutierrez, J.C. Del Rio, J. Ignacio Santos, L. Nieto, J. Jimenez-Barbero, A. T. Martinez, J. Li, G. Gellerstedt, S. Lepifre, C. Silva, S.Y. Kim, A. Cavaco-Paulo, B. Seljebaakken Klausen, B. F. Lutnaes, G. S. Nyanhongo & G. M. Guebitz (2010) Polymerization of lignosulphonates by the laccase-HBT (1-hydroxybenzotriazole) system improves dispersibility. *Bioresource Technology,* 101, 5054-5062

Octavio, L.C., P. P. Ma., C. I. B. R. J. Ricardo & V. Ortega Francisco (2006) Laccases. *Advances in Agricultural and Food Technology,* 323-340

Orth, A.B. & M. Tien (1995) Biotechnology of lignin degradation. *Springer-Verlag,* 287-302

Osma, J.F., J. L. Toca-Herrera & S. Rodr'ıguez-Couto (2010) Uses of Laccases in the Food Industry. *Enzyme Research,* 1- 8

Othmer, K (2009) Encyclopedia of Chemical Technology. *John Wiley and Sons,* 26, 836-920

Palma, C., A. T. Martínez, J. M. Lema & M. J. Martínez (2000) Different fungal manganese-oxidizing peroxidases: a comparison between *Bjerkandera sp.* and *Phanerochaete chrysosporium. Journal of Biotechnology,* 77, 235-245

Palmer, J. M., P. J. Harvey & H. E. Schoemaker (1986) The role of Peroxidases, Radical Cations and Oxygen in the Degradation of Lignin [and Discussion]. *Mathematical and Physical Sciences,* 321, 495-505

Palmieri, G., P. Giardina, C. Bianco, B. Fontanella & G. Sannia (2000) Copper Induction of Laccase Isoenzymes in the Ligninolytic Fungus *Pleurotus ostreatus. Applied and Environmental Microbiology,* 66, 920 - 924

Pan, X., A. Claudio, N. Gilkes, D. Gregg, W. Mabee, K. Pye, Z. Xiao, X. Zhang & J. Saddler (2005) Biorefining of Softwoods Using Ethanol Organosolv Pulping: Preliminary Evaluation of Process Streams for Manufacture of Fuel-Grade Ethanol and Co-Products. *Biotechnology and Bioengineering*, 90, 473-481

Pandey, M. P. & C. S. Kim (2011) Lignin Depolymerization and Conversion: A Review of Thermochemical Methods. *Chemical Engineering & Technology*, 34, 29-41

Park S. & R.J. Kazlauskas (2001) Improved preparation and use of room temperature ionic liquids in lipase-catalyzed enantio- and regioselective acylations. *Journal of Organic Chemistry*, 66, 8395-8401.

Park, S. & R. J. Kazlauskas (2003) Biocatalysis in ionic liquids - advantages beyond green technology. *Current Opinion in Biotechnology*, 14, 432-437

Paszcynskia, A., V. Huynh & R. Crawford (1986) Comparison of ligninase-I and peroxidase-M2 from the white rot fungus *Phanerochaete chrysosporium. Archives of Biochemistry and Biophysics*, 244, 750-756

Patrick, F., G. Mtui, A. M. Mshandete, G. Johansson & A. Kivaisi (2009) Purification and characterization of a laccase from the basidiomycete *Funalia trogii* (Berk.) isolated in Tanzania. *African Journal of Biochemistry*, 3 (5), 250-258

Peacock River Ranch (2012) A pine tree for the newly weds.
http://peacockranch.com/blog/?p=143 (accessed November 2012)

Pecina, R., P. Burtscher, G.Bonn & O. Bobleter (1986) GC-MS and HPLC analyses of lignin degradation products in biomass hydrolyzates. *Fresenius' Journal of Analytical Chemistry*, 325, 461-465

Pellinen, J. & M. Salkinoja-Salonen (1985) High-performance size-exclusion chromatography of lignin and its derivatives. *Journal of Chromatography*, 328, 299-308

Pham, T. P. T., C.W. Cho, J. Min & Y.S. Yun (2008) Alkyl-chain length effects of imidazolium and pyridinium ionic liquids on photosynthetic response of *Pseudokirchneriella subcapitata. Journal of Bioscience and Bioengineering*, 105, 425-428

Plechkova, N. & K. Seddon (2007) Applications of ionic liquids in the chemical industry. *Chemical Society Reviews*, 37, 123-150

Polaskova, M., R. Cermak, V. Verney, P. Ponizil, S. Commereuc, M.F.C. Gomes, A.A.H. Padua, P. Mokrejs & M. Machovsky (2013) Preparation of microfibers from wood/ionic liquid solutions. *Carbohydrate Polymers*, 92, 214–217

Potthast, A., T. Rosenau, C. L. Chen & J. S. Gratzl (1996) A novel method for conversion of benzyl alcohols to benzaldehydes by laccase-catalysed oxidation. *Journal of Molecular Catalysis A: Chemical*, 108, 5-9

Potthast, A., T. Rosenau, H. Koch & K. Fischer (1999) The Reaction of Phenolic Model Compounds in the Laccase-Mediator System (LMS) – Investigations by Matrix Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF-MS). *Holzforschung*, 53, 175-180

Prasetyo N. E., T. Kudanga, L. Østergaard, J. Rencoret, A. Gutiérrez, J.C. del Río, S. J. Ignacio, L. Nieto, J. Jiménez-Barbero, A.T. Martínez, J. Li, G. Gellerstedt, S. Lepifre, C. Silva, S.Y. Kim, A. Cavaco-Paulo, B. Seljebakken Klausen, B.F. Lutnaes, G.S. Nyanhongo, G.M. Guebitz (2010) Polymerization of lignosulphonates by the laccase-HBT (1-hydroxybenzotriazole) system improves dispersibility. *Bioresource Technology*, 101, 5054-5062

Pu, Y., N. Jiang & A. J. Ragauskas (2007) Ionic Liquid as a Green Solvent for Lignin. *Journal of Wood Chemistry and Technology*, 27, 23 - 33

Pulp and Paper Manufacture (1987) Alkaline Pulping, vol. 5. *Tappi Press*, Atlanta.

Pye, E.K. & J.H. Lora (1991) The Alcell process: A proven alternative to Kraft pulping. *TAPPI*, 74, 113-118

Ragauskas, A. J., C. K. Williams, B. H. Davison, G. Britovsek, J. Cairney, C. A. Eckert,

W. J. Frederick, Jr., J. P. Hallett, D. J. Leak, C. L. Liotta, J. R. Mielenz, R. Murphy, R. Templer & T. Tschaplinski (2006) The Path Forward for Biofuels and Biomaterials. *Science,* 311, 484-489

Rancano, G., M. Lorenzo, N. Molares, S. Rodriguez Couto & M. Á. Sanroman (2003) Production of laccase by *Trametes versicolor* in an airlift fermentor. *Process Biochemistry,* 39, 467-473

Rehmann, L., E. Ivanova, J. L. Ferguson, H.Q.N. Gunaratne, K.R. Seddon, & G.M. Stephens (2012) Measuring the effect of ionic liquids on laccase activity using a simple, parallel method. *Green Chemistry,* 14, 725-733

Reiss, R., J. Ihssen & L. Thony-Meyer (2011) *Bacillus pumilus* laccase: a heat stable enzyme with a wide substrate spectrum. *BMC Biotechnology,* 11, 9

Reknes, K. (2004) The performance of a new lignosulphonate plasticizing admixture and its sensitivity to cement composition. *29th Conference on Our World in Concrete and Structures.* http://cipremier.com/100029056 (accessed November 2012)

Renganathan, V., K. Miki & M. H. Gold (1985) Multiple molecular forms of diarylpropane oxygenase, an H2O2-requiring, lignin-degrading enzyme from Phanerochaete chrysosporium. *Archives of Biochemistry and Biophysics,* 241, 304-314

Renganathan, V., M.H. Gold (1986) Spectral characterization of the oxidized states of lignin peroxidase, an extracellular heme enzyme from the white rot basidiomycete *Phanerochaete chrysosporium. Biochemistry,* 25, 1626-1631

Rittstieg, K., A. Suurnakki, T. Suortti, K. Kruus, G. M. Guebitz & J. Buchert (2003) Polymerization of Guaiacol and a Phenolic β-O-4-Substructure by *Trametes hirsuta* Laccase in the Presence of ABTS. *Biotechnology Progress,* 19, 1505-1509

Rhodia (2008) Guaiacol (GPS Safety Summary). http://www.rhodia.com/en/binaries/GPS_2011 (accessed July 2013)

Roberts, E. A. H., R. A. Cartwright & M. Oldschool (1957) The phenolic substances of manufactured tea. I.—Fractionation and paper chromatography of water-soluble substances. *Journal of the Science of Food and Agriculture,* 8, 72-80

Roberts, E. A. H. & D. M. Williams (1958) The phenolic substances of manufactured tea. III.—ultra-violet and visible absorption spectra. *Journal of the Science of Food and Agriculture,* 9, 217-223

Roberts, V.M., V. Stein, T. Reiner, A. Lemonidou, X. Li, J.A. Lercher (2011) Towards quantitative catalytic lignin depolymerization.*Chemistry,* 17, 5939-5948

Rodríguez, O., R. O. Cristóvão, A. P. M. Tavares, E.A. Macedo (2011) Study of the alkyl chain length on laccase stability and enzymatic kinetic with imidazolium ionic liquids. *Applied Biochemistry and Biotechnology,* 164, 524–533

Rogers, R.D., K.R. Seddon & S. Volkov (2002) Green industrial applications of ionic liquids. *Oxford University Press,* 361

Rogalski, J., A. Dawidowicz & A. Leonowicz (1990) Purification and immobilization of the inducible form of extracellular laccase of the fungus Trametes versicolor. *Acta Biotechnologica,* 10, 261-269

Rogers, R.D. & K.R. Seddon (2002) Ionic liquids industrial applications to green chemistry. ACS Symposium Series 818. *Oxford University Presss,* 2-458

Rolando, C., B. Monties, & C. Lapierre (1992) Thioacidolysis. *In:* Methods in Lignin Chemistry (C.W. Dence & S.Y. Lin) *Springer-Verlag,* 334–349

Sakakibara, A (1983) Chemical structureof lignin related mainly to degradation of products. *Research Tokyo UNI Publisher,* 12-33

Sanchez-Amat, A., P. Lucas-Elío, E. Fernández, J. C. García-Borrón & F. Solano (2001) Molecular cloning and functional characterization of a unique multipotent polyphenol oxidase from Marinomonas mediterranea. *Biochimica et Biophysica Acta (BBA) - Protein Structure and Molecular Enzymology,* 1547, 104-116

Sarkanen, S., D.C. Teller, J. Hall & J.L. McCarthy (1981) Lignin: Associative effects among organosolv lignin components. *Macromolecules,* 14, 434-442

Schlosser, D., R. Grey & W. Fritsche (1997) Patterns of ligninolytic enzymes in Trametes versicolor . Distribution of extra- and intracellular enzyme activities during cultivation on glucose, wheat straw and beech wood. *Applied Microbiology and Biotechnology,* 47, 412-418

Schoemaker, H.E.& K. Piontek (1996) On the interaction of lignin peroxidase with lignin. *Pure and Applied Chemistry,* 68(11), 2089-2096

Schäfer, A., B. Fischer, H. Paul, R. Bosshard, M. Hesse, M. Viscontinif (1992) Electrospray-Ionization Mass Spectrometry: Detection of a radical cation present in solution: New results on the chemistry of (tetrahydropteridinone)-metal complexes. *Helvetica Chimica Acta,* 6, 1955 - 1964

Sealey, J., A.J. Ragauskas & T.J. Elder (1999) Investigations into Laccase-Mediator Delignification of Kraft Pulps. *Holzforschung,* 53, 498-502

Seddon, K. R. (1997) Ionic Liquids for Clean Technology. *Journal of Chemical Technology & Biotechnology,* 68, 351-356

Sgalla, S., G. Fabrizi, S. Cacchi, A. Macone, A. Bonamore & A. Boffi (2007) Horseradish peroxidase in ionic liquids: Reactions with water insoluble phenolic substrates. *Journal of Molecular Catalysis B: Enzymatic,* 44, 144-148

Shah, K.H., B.D. Talak & K. Venkataraman (1948) Raney Nickel reduction - Part I. http://repository.ias.ac.in/53674/1/137_PUB.pdf (accessed November 2012)

Shipovskov, S., H.Q.N. Gunaratne, K.R. Seddon, G. Stephens (2008) Catalytic activity of laccases in aqueous solutions of ionic liquids. *Green Chemistry,* 10, 806-810

Shleev, S., P. Persson, G. Shumakovich, Y. Mazhugo, A. Yaropolov, T. Ruzgas & L. Gorton (2006) Interaction of fungal laccases and laccase-mediator systems with lignin. *Enzyme and Microbial Technology,* 39, 841-847

Silva, S. P. , M. A. Sabino, E. M. Fernandes, V. M. Correlo, L. F. Boesel & R. L. Reis (2005) Cork: properties, capabilities and applications. *International Materials Reviews,* 50, 435 - 365

Sjöström, E. (1993) Wood chemistry: fundamentals and application. *Academic Press,* 71

Stark, K., N. Taccardi, A. Bosmann & P. Wasserscheid (2010) Oxidative depolymerization of lignin in ionic liquids. *Chem Sus Chem,* 3,719–723

Szklarz, G. & A. Leonowicz (1986) Cooperation between fungal laccase and glucose oxidase in the degradation of lignin derivatives. *Phytochemistry,* 25, 2537-2539

Smook, G.A (1992) Handbook for Pulp and Paper Technologists, *Angus Wilde Publications,* 11, 163–183

Solano, F., P. Lucas-El, D. López-Serrano, E. Fernández & A. Sanchez-Amat (2001) Dimethoxyphenol oxidase activity of different microbial blue multicopper proteins. *FEMS Microbiology Letters,* 204, 175-181

Srebotnik, E. & K.E. Hammel (2000) Degradation of nonphenolic lignin by the laccase/1-hydroxybenzotriazole system. *Journal of Biotechnology,* 81, 179-188

Sun, Y. & J. Cheng (2002) Hydrolysis of lignocellulosic materials for ethanol production: a review. *Bioresource Technology,* 83, 1-11

Swatloski, R. P., S. K. Spear, J. D. Holbrey & R. D. Rogers (2002) Dissolution of cellose with ionic liquids. *Journal of the American Chemical Society,* 124, 4974-4975

Szklarz, G. & A. Leonowicz (1986) Cooperation between fungal laccase and glucoseoxidase in the degradation of lignin derivatives. *Phytochemistry,* 25, 2537–2539

Takada, D., K. Ehara, & S. Saka (2004) Gas chromatographic and mass spectrometric (GC-MS) analysis of lignin-derived products from *Cryptomeria japonica* treated in supercritical water. *Journal of Wood Science,* 50, 253-259

Tanaka, H., S. Itakura, & A. Enoki (1999) Hydroxyl radical generation by an extracellular low-molecular-weight substance and phenol oxidase activity during wood degradation by the white-rot basidiomycete *Trametes versicolor. Journal of Biotechnology,* 75, 57-70

Tavares, A. P. M., O. Rodriguez & E. A. Macedo (2008) Ionic liquids as alternative co-solvents for laccase: Study of enzyme activity and stability. *Biotechnology and Bioengineering,* 101, 201-207

Tavares' A.P.M., J.A.N. Pereira, A. M. R. B. Xavier (2012) Effect of ionic liquids activation on laccase from *Trametes versicolor*: Enzymatic stability and activity. *Engineering in Life Sciences,* 12, 1–8

Telke, A.A., A.N. Kagalkar, U.B. Jagtap, N.S. Desai, V.A. Bapat & S.P. Govindwar (2011) Biochemical characterization of laccase from hairy root culture of *Brassica juncea L.* and role of redox mediators to enhance its potential for the decolorization of textile dyes. *Planta,* 234, 1137-1149

Thurston, C. F. (1994) The structure and function of fungal laccases. *Microbiology,* 140, 19-26

Tien, M. (1987) Properties of Ligninase from *Phanerochaete Chrysosporium* and their possible applications. *Critical Reviews in Microbiology,* 15, 141-168

Tien, M. & T. K. Kirk (1983) Lignin-Degrading Enzyme from the Hymenomycete *Phanerochaete chrysosporium* Burds.*Science,* 221, 661-663

Tien, M., T. K. Kirk, C. Bull & J. A. Fee (1986) Steady-state and transient-state kinetic studies on the oxidation of 3,4-dimethoxybenzyl alcohol catalyzed by the ligninase of *Phanerocheate chrysosporium* Burds. *Journal of Biological Chemistry,* 261, 1687-1693

Tien M, K. T. (1984) Lignin-degrading enzyme from *Phanerochaete chrysosporium*: Purification, characterization, and catalytic properties of a unique H_2O_2-requiring oxygenase. *Proceedings of the National Academy of Sciences of the United States of America,* 81, 2280-2284

Tondi, G. & A. Pizzi (2009) Tannin-based rigid foams: Characterization and modification. *Industrial Crops and Products,* 29, 356–363

Train, P. M. & M. T. Klein (1991) Hydroprocessing lignin and lignin model compounds: Products, kinetics, and catalystaging. *Fuel Science and Technology International,* 9, 193 - 227

van Bloois, E., D. Torres Pazmiño, R. Winter & M. Fraaije (2009) A robust and extracellular heme-containing peroxidase from Thermobifida fusca as prototype of a bacterial peroxidase superfamily. *Applied Microbiology and Biotechnology,* 86, 1419–1430

Vardar-Sukan, F. (1998) Foaming: Consequences, prevention and destruction. *Biotechnology Advances,* 16, 913-948

Ventura, S.P.M., L. D. F. Santos, J. A. Saraiva & J. A. P. Coutinho (2012) Concentration effect of hydrophilic ionic liquids on the enzymatic activity of *Candida antarctica lipase B. World Journal of Microbiology and Biotechnology,* 28, 2303–2310

Vigneault, A., D. K. Johnson & E. Chornet (2007) Base-Catalyzed Depolymerization of Lignin: Separation of Monomers. *The Canadian Journal of Chemical Engineering,* 85, 906-916

Villar, J.C., A. Caperos & F. GarciaOchoa (1997) Oxidation of hardwood kraft-lignin to phenolic derivatives. Nitrobenzene and copper oxide as oxidants. *Journal of Wood Chemistry,* 17, 259-285

Vishtal, A. & A. Kraslawski (2011) Challenges in industrial applications of technical lignins. *Bioresources,* 6, 3547 - 3568

Wang, Y., R. Vazquez-Duhalt & M.A. Pickard (2003) Manganese-lignin peroxidase hybrid from *Bjerkandera oxidizes* polycyclic aromatic hydrocarbons more actively in the absence of manganese. *Canadian Journal Microbiol,* 49, 675-682

Wariishi, H., L. Akileswaran & M. H. Gold (1988) Manganese peroxidase from the basidiomycete Phanerochaete chrysosporium: spectral characterization of the oxidized states and the catalytic cycle. *Biochemistry,* 27, 5365-5370

Wariishi, H., H. B. Dunford, I. D. MacDonald & M. H. Gold (1989a) Manganese peroxidase from the lignin-degrading basidiomycete Phanerochaete chrysosporium.

Transient state kinetics and reaction mechanism. *Journal of Biological Chemistry,* 264, 3335-3340

Wariishi, H., K. Valli & M. H. Gold (1989b) Oxidative cleavage of a phenolic diarylpropane lignin model dimer by manganese peroxidase from *Phanerochaete chrysosporium. Biochemistry,* 28, 6017-6023

Wariishi H, V. K., Gold MH. (1991) In vitro depolymerization of lignin by manganese peroxidase of Phanerochaete chrysosporium. *Biochemical and Biophysical Research Communications,* 176, 269-275

Wasserscheid, P. (2006) Chemistry: Volatile times for ionic liquids. *Nature,* 439, 797-797

Weast, R.C. (1975) Handbook of chemistry and physics. *CRC Press Cleveland,* 56 Eds.

Wilkes, J.S., M.J. Zaworotko (1992) Air and water stable 1-ethyl-3-methylimidazolium based ionic liquids. *Journal of the Chemical Society, Chemical Communications,* 13, 965-967

Wilkes, J. S., J. A. Levisky, R. A. Wilson & C. L. Hussey (1982) Dialkylimidazolium chloroaluminate melts: a new class of room-temperature ionic liquids for electrochemistry, spectroscopy and synthesis. *Inorganic Chemistry,* 21, 1263-1264

Wilson, N. (2002) Image: *Trametes versicolor.* http://mushroomobserver.org (accessed July 2013)

Xu F, W. Shin, S. Brown, J.A. Wahleithner, U.M. Sundaram, E.I. Solomon (1996) A study of a series of recombinant fungal laccases and bilirubin oxidase that exhibit significant differences in redox potential, substrate specificity, and stability. *Biochimica et Biophysica Acta,* 1292, 303-311

Xu, F., J.J. Kulys, K. Duke, K. Li, K. Krikstopaitis, H.-J. W. Deussen, E. Abbate, V. Galinyte & P. Sshneider (2000) Redox Chemistry in Laccase-Catalyzed Oxidation of N-Hydroxy Compounds. *Applied and Environmental Microbiology,* 66, 2052-2056

Yang, Z. & W. Pan (2005) Ionic liquids: Green solvents for nonaqueous biocatalysis. *Enzyme and Microbial Technology,* 37, 19-28

Yaver, D.S., R.M. Berka, S.H. Brown & F. Xu (2001) *In:* Kunamneni, A. A. Ballesteros, F. J. Plou and M.Alcalde (2007) Fungal laccase – a versatile enzyme for biotechnological applications. *Communicating Current Research and Educational Topics and Trends in Applied Microbiology,* 233-245

Zakis, G.F. (1994) The functional analysis of lignins and their derivatives. *TAPPI Press,* 102

Zavrel, M., D. Bross, M. Funke, J. Büchs & A. C. Spiess (2009) High-throughput Screening for Ionic Liquids Dissolving Lignocellulose. *Bioresource Technology,* 100, 2580 - 2587

Zawodzinski, T. A. J. & R. A. Osteryoung (1987) Aspects of the chemistry of water in ambient-temperature chloroaluminate ionic liquids: 17O NMR studies. *Inorganic Chemistry,* 26, 2920-2922

Zakzeski, J., P.C. Bruijnincx, A.L. Jongerius & B.M. Weckhuysen (2010) The catalytic valorization of lignin for the production of renewable chemicals.*Chemical Reviews,* 110, 3552-3599

Zhang, Y. H. (2008) Reviving the carbohydrate economy via multi-product lignocellulose biorefineries. *Journal of Industrial Microbiology and Biotechnology,* 35, 367-375

Zhang, Y. & P.S. Cremer (2006) Interactions between macromolecules and ions: the Hofmeister series. *Current Opinion in Chemical Biology,* 10, 658–663

Zhao, H. (2005) Effect of ions and other compatible solutes on enzyme activity, and its implication for biocatalysis using ionic liquids. *Journal of Molecular Catalysis B: Enzymatic,* 37, 16–25

Zhao, H., O. O. Z. Song, A. L. Sims, T. E. Person, R. A. Lawal, L. A. Holley (2006) Effect of kosmotropicity of ionic liquids on the enzyme stability in aqueous solutions. *Bioorganic Chemistry,* 34, 15–25

Zhao, H. (2006) Are ionic liquids kosmotropic or chaotropic? An evaluation of available

thermodynamic parameters for quantifying the ion kosmotropicity of ionic liquids. *Journal of Chemical Technology and Biotechnology,* 81, 877–891

Zhao, D., M. Wu, Y. Kou & E. Min (2002) Ionic liquids: applications in catalysis. *Catalysis Today,* 74, 157-189.

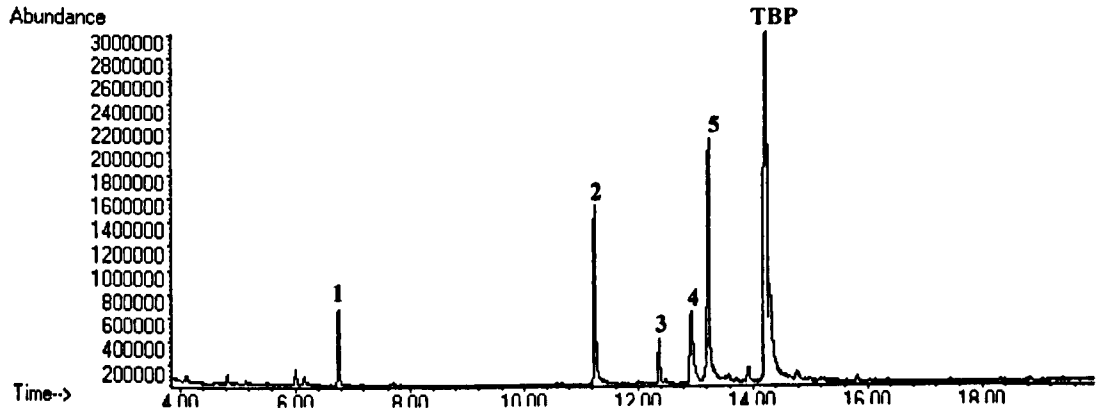
Zhao, D., Y. Liao & Z. Zhang (2007) Toxicity of Ionic Liquids. *Clean - Soil, Air, Water,* 35, 42-48

APPENDICES

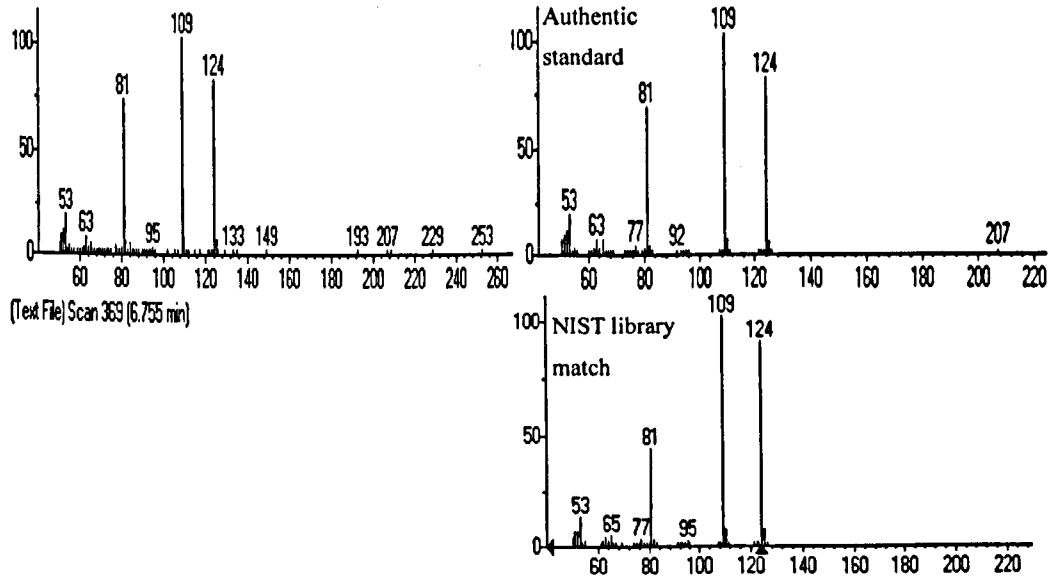
Appendix A.1 GCMS Analysis of Lignin Depolymerization Products (LAB)

The products formed after enzymatic treatment of sodium lignosulphonate by LAB. Sample was incubated at 30 °C for 6 h, shaken at 200 rpm. The sample was evaporated to dryness and the dried sample of ethyl acetate extract fraction was redissolved in DCM. The chromatogram represents the duplicate analysis.

A.1.1 Chromatogram of Aqueous Ethyl Acetate Extract Fraction of the LAB-catalyzed reaction

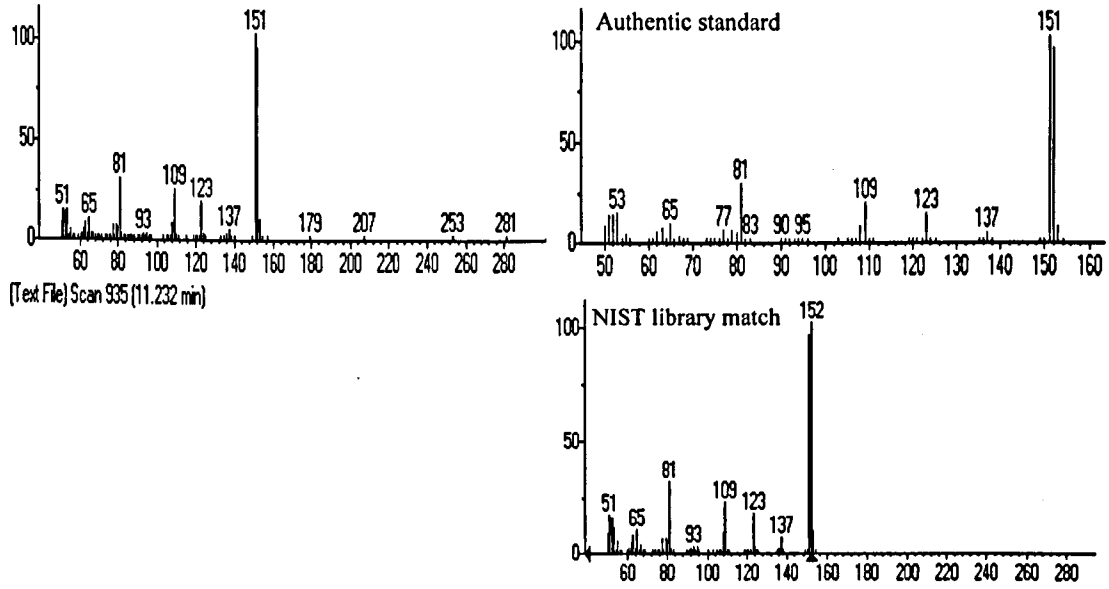


(a) Mass Spectrum of guaiacol (1) and the match with authentic standard



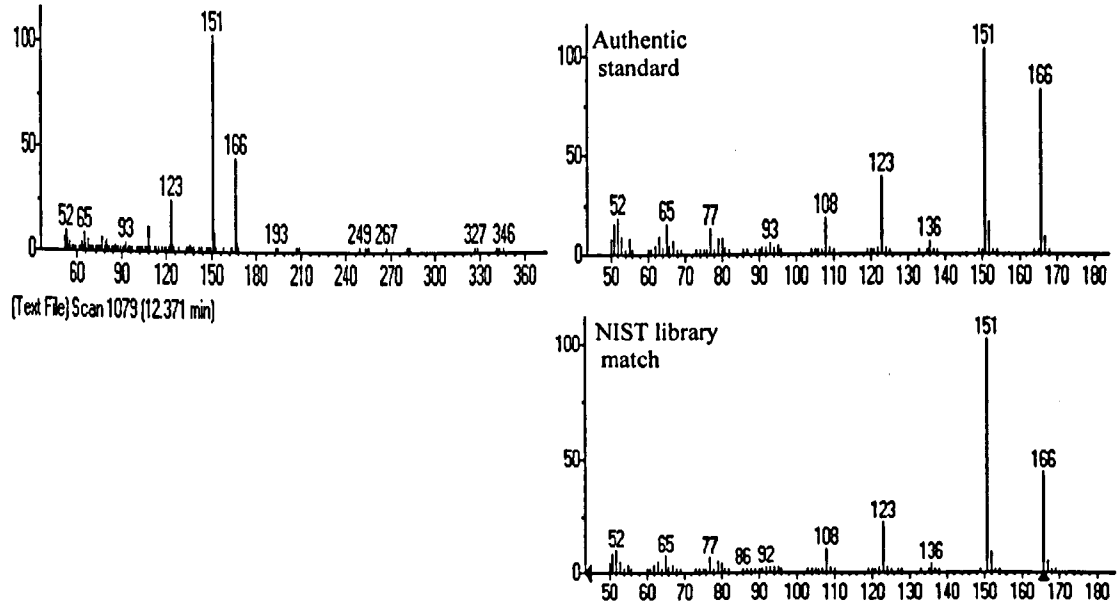
NIST # 20584 **MF:** $C_7H_8O_2$ **MW:** 124 **CAS:** 90-05-1
Name: 2-methoxy phenol (Guaiacol) (1)
The retention time of the standard matched the unknown compound, indicating that the high molecular weight ions (207 of the authentic standard and 133, 149, 193, 207, 229 and 253 of the unknown compound) were due to contaminants.

(b) Mass Spectrum of vanillin (2) and the match with authentic standard



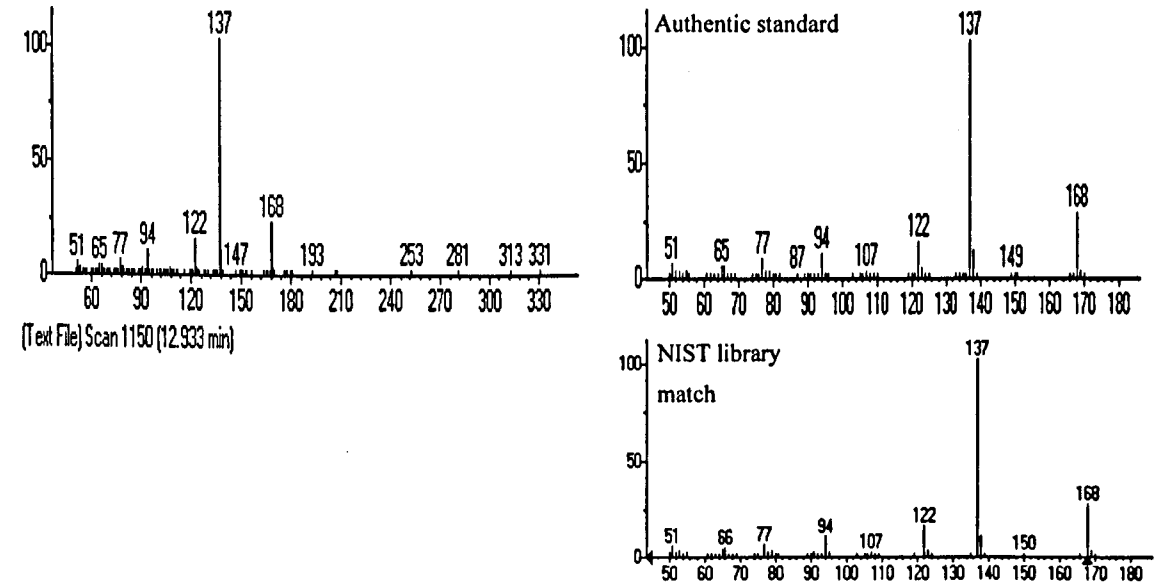
NIST # 227894 MF: $C_8H_8O_3$ MW: 152 CAS: 121-33-5
Name: 4-hydroxy-3-methoxybenzaldehyde (Vanillin) (2)
The retention time of the standard matched the unknown compound, indicating that the high molecular weight ions (179, 207, 253 and 281 of the unknown compound) were due to contaminants.

(c) Mass Spectrum of Acetovanillone (3) and the match with authentic standard



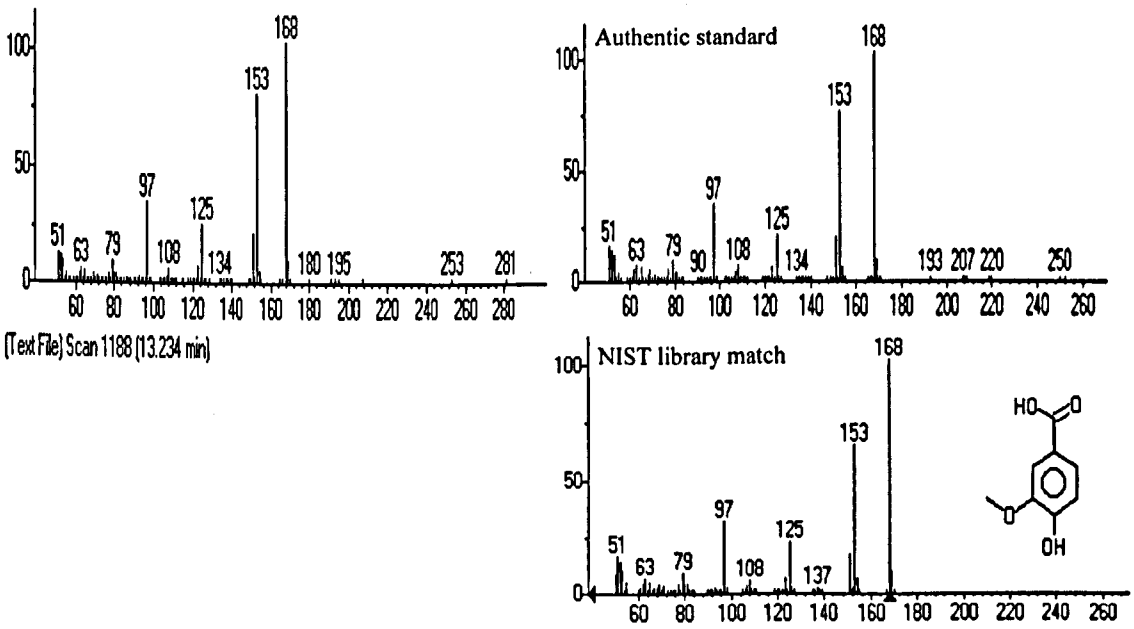
NIST # 352840 MF: $C_9H_{10}O_3$ MW: 166 CAS: 498-02-2
Name: 1-(4-hydroxy-3-methoxyphenyl)-ethanone (Acetovanillone) (3)
The retention time of the standard matched the unknown compound, indicating that the high molecular weight ions (193, 249, 267, 327 and 346 of the unknown compound) were due to contaminants.

(d) Mass Spectrum of Homovanillyl alcohol (4) and the match with authentic standard



NIST # 133524 MF: $C_9H_{12}O_3$ MW: 168 CAS: 2380-78-1
Name: 4-hydroxy-3-methoxyphenylethyl alcohol (Homovanillyl alcohol) (4)
The retention time of the standard matched the unknown compound, indicating that the high molecular weight ions (193, 253, 281, 313 and 331 of the unknown compound) were due to contaminants.

(e) Mass Spectrum of vanillic acid (5) and the match with authentic standard

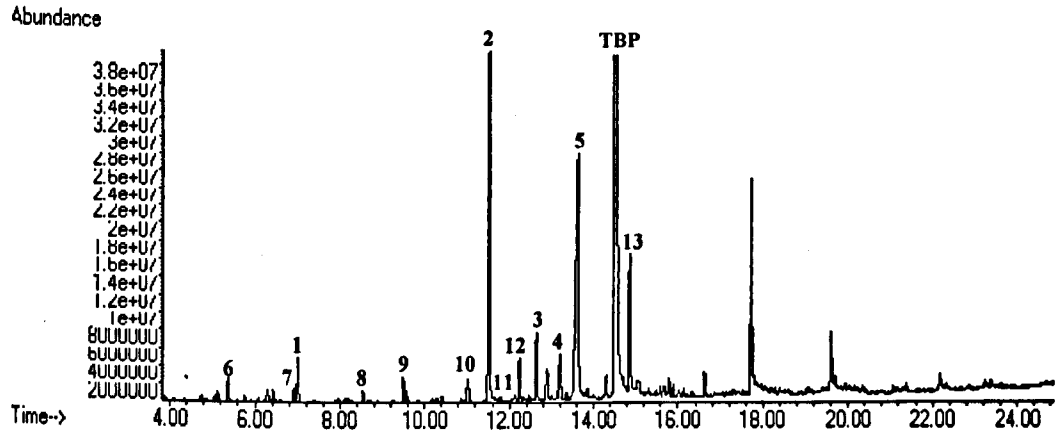


NIST # 6514 MF: $C_8H_8O_4$ MW: 168 CAS: 121-34-6
Name: 4-hydroxy-3-methoxybenzoic acid (Vanillic acid)
The retention time of the standard matched the unknown compound, indicating that the high molecular weight ions (193, 207, 220 and 250 of the authentic standard and 180, 195, 253 and 281 of the unknown compound) were due to contaminants.

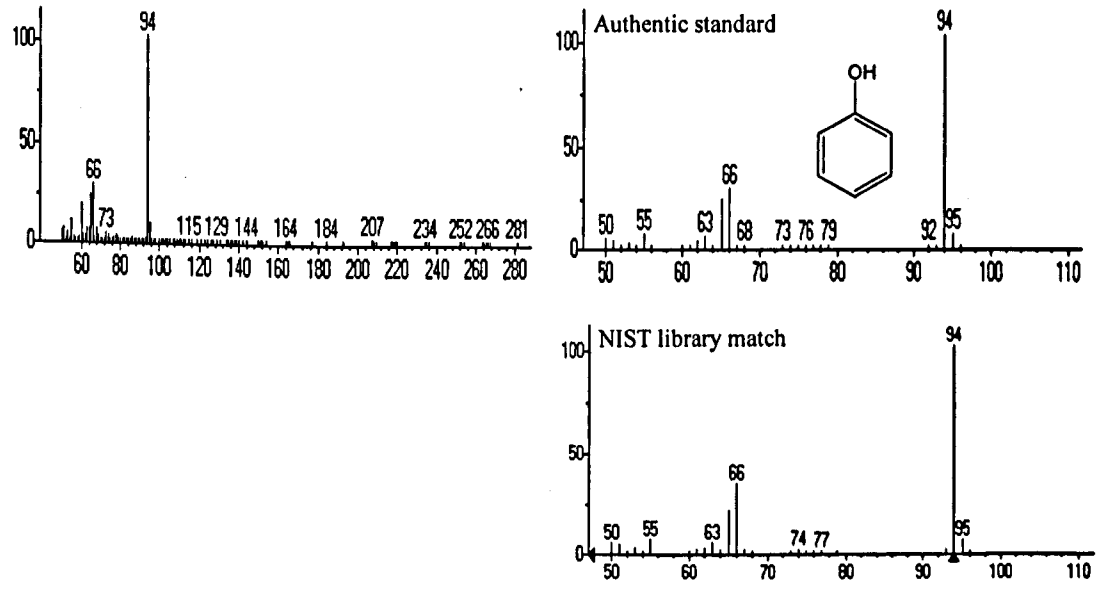
Appendix A.2 GCMS Analysis of Lignin Depolymerization Products (LTV)

The products formed after enzymatic treatment of sodium lignosulphonate by LTV. Sample was incubated at 60 °C for 6 h, shaken at 200 rpm. The sample was evaporated to dryness and the dried sample of ethyl acetate extract fraction was redissolved in DCM. The chromatogram represents the duplicate analysis.

A.2.1 Chromatogram of Aqueous Ethyl Acetate Extract Fraction of the LTV-catalyzed reaction

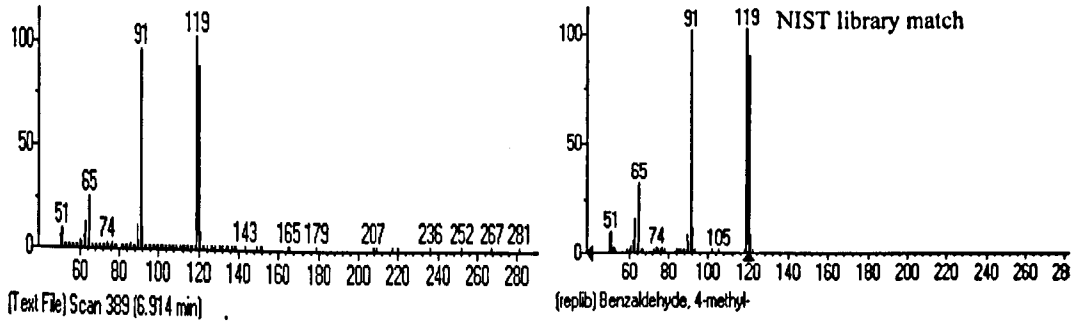


(a) Mass Spectrum of phenol (6) and the match with the NIST library



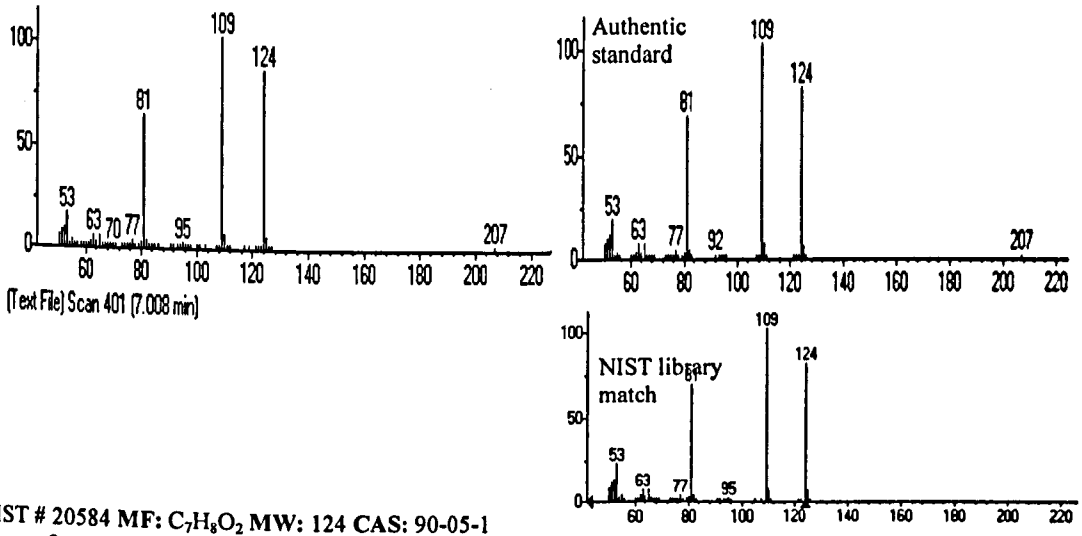
NIST # 221160 **MF:** C_6H_6O **MW:** 94 **CAS:** 108-95-2 **Name:** Phenol
The retention time of the standard matched the unknown compound, indicating that the high molecular weight ions (115, 129, 144, 164, 184, 207, 234, 252, 266 and 281of the unknown compound) were due to contaminants.

(b) Mass Spectrum of 4-methylbenzaldehyde (7) and the match with the NIST library



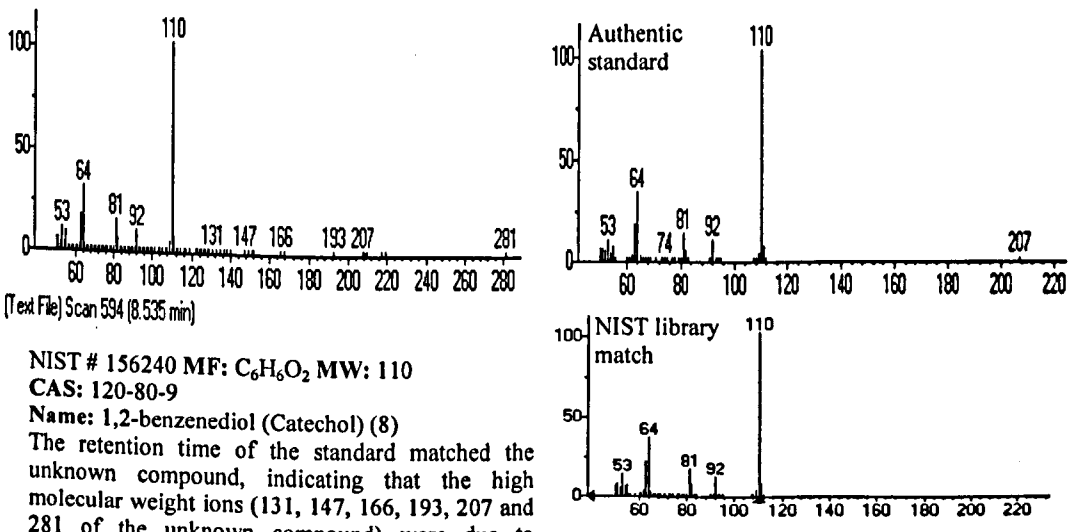
NIST # 109891 **MF:** C_8H_8O **MW:** 120 **CAS:** 104-87-0 **Name:** 4-methylbenzaldehyde
The peaks at 143, 165, 179, 207, 236, 252, 267 and 281may be baseline contaminants.

(c) Mass Spectrum of guaiacol (1) and the match with the authentic standard



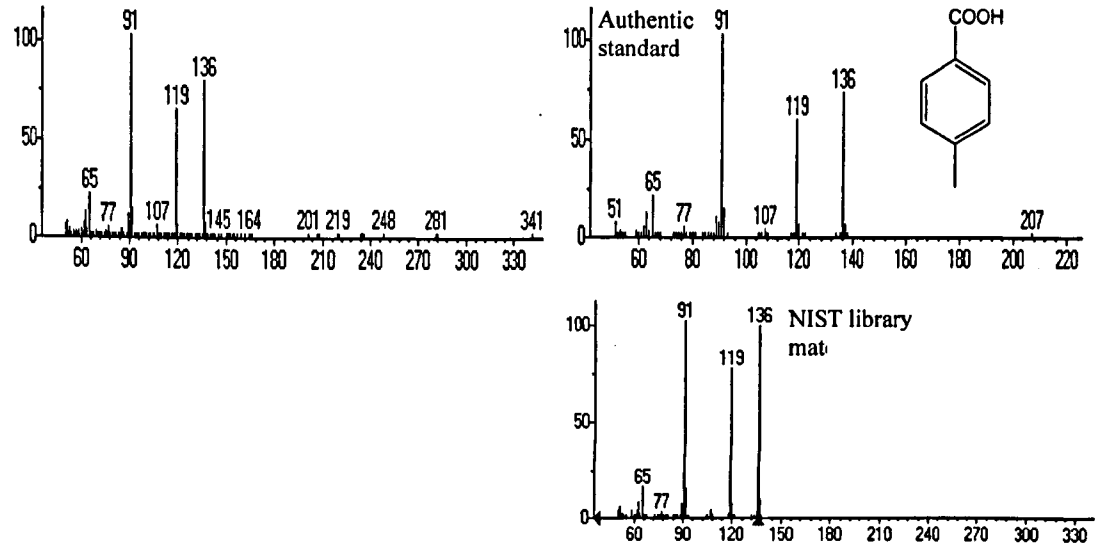
NIST # 20584 **MF:** $C_7H_8O_2$ **MW:** 124 **CAS:** 90-05-1
Name: 2-methoxy phenol (Guaiacol) (1)
The retention time of the standard matched the unknown compound, indicating that the high molecular weight ions (207 of the authentic standard and the unknown compound) were due to contaminants.

(d) Mass Spectrum of catechol (8) and the match with the authentic standard



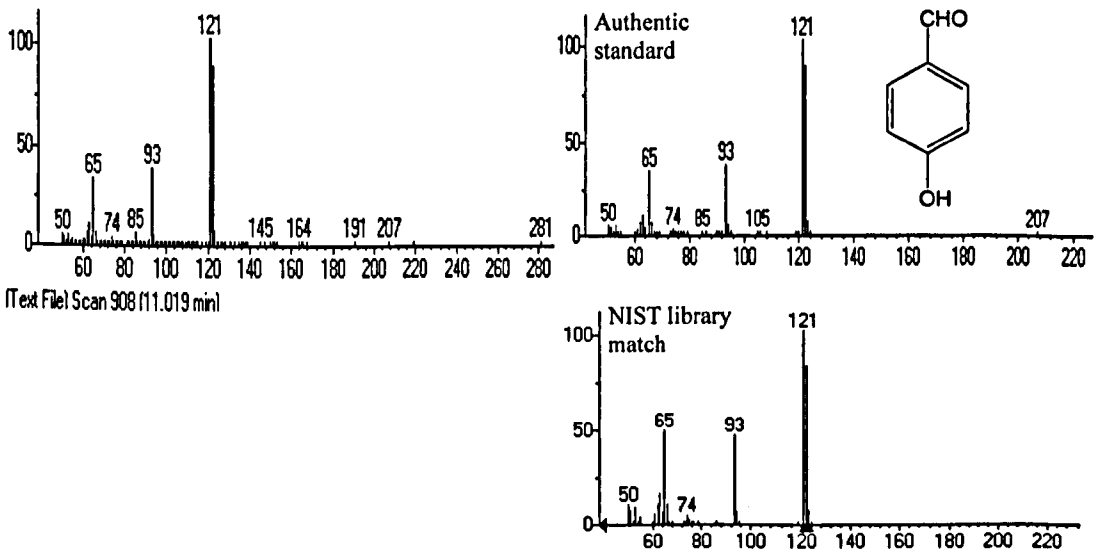
NIST # 156240 **MF:** $C_6H_6O_2$ **MW:** 110
CAS: 120-80-9
Name: 1,2-benzenediol (Catechol) (8)
The retention time of the standard matched the unknown compound, indicating that the high molecular weight ions (131, 147, 166, 193, 207 and 281 of the unknown compound) were due to contaminants.

(e) Mass Spectrum of *p*-toluic acid (9) and the match with the authentic standard



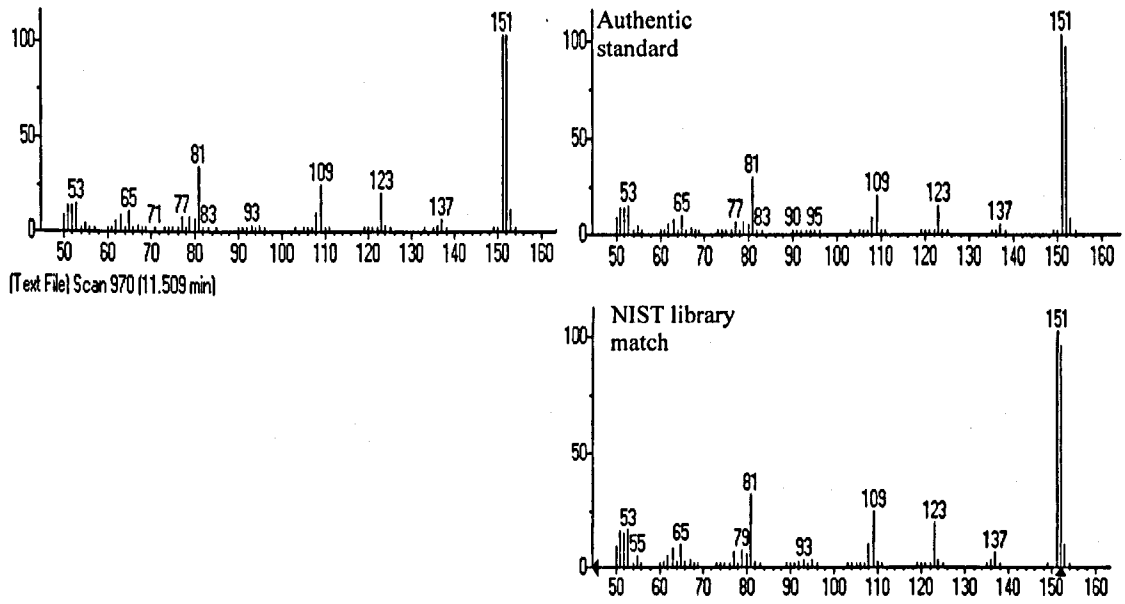
NIST # 21058 MF: $C_8H_8O_2$ MW: 136 CAS: 99-94-5
Name: 4-methylbenzoic acid (*p*-toluic acid) (9)
The retention time of the standard matched the unknown compound, indicating that the high molecular weight ions (207 of the authentic standard and 145, 164, 201, 219, 248, 281 and 341 of the unknown compound) were due to contaminants.

(f) Mass Spectrum of 4-hydroxybenzaldehyde (10) and the match with the authentic standard



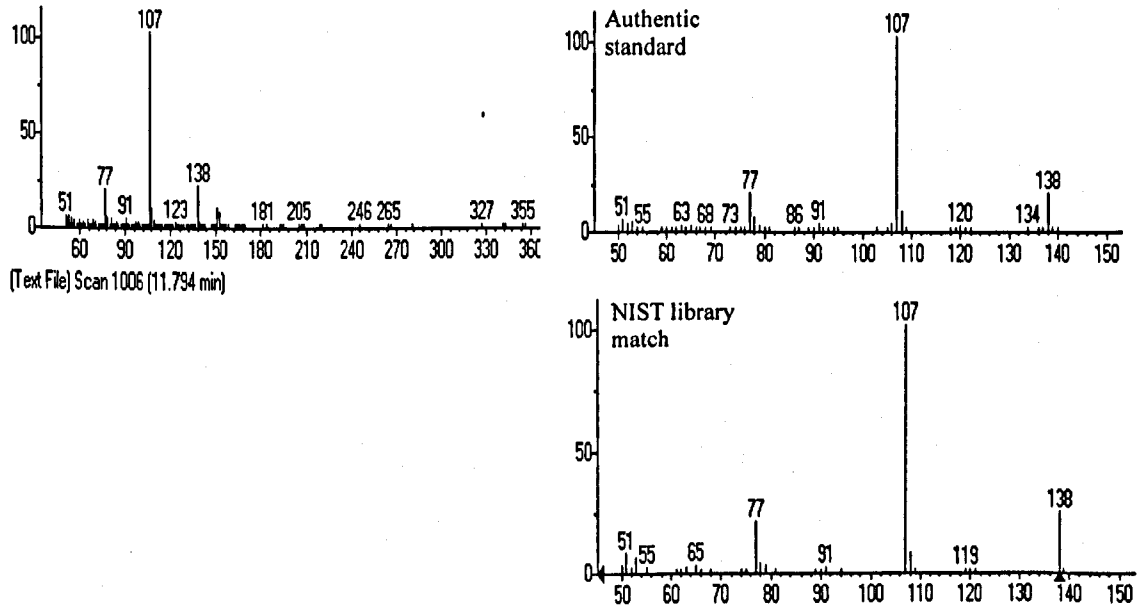
NIST # 135511 MF: $C_7H_6O_2$ MW: 122 CAS: 123-08-0
Name: 4-hydroxybenzaldehyde (10)
The retention time of the standard matched the unknown compound, indicating that the high molecular weight ions (207 of the authentic standard and 145, 164, 191, 207, and 281 of the unknown compound) were due to contaminants.

(g) Mass Spectrum of vanillin (2) and the match with the authentic standard



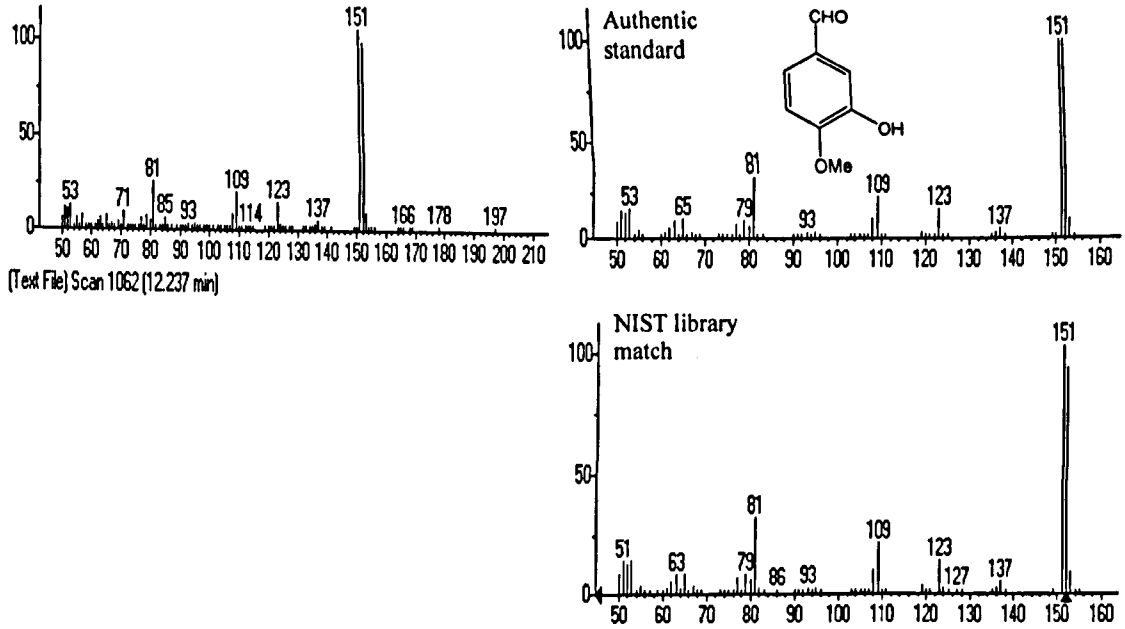
NIST # 227894 **MF:** $C_8H_8O_3$ **MW:** 152 **CAS:** 121-33-5
Name: 4-hydroxy-3-methoxybenzaldehyde (vanillin) (2)
The retention time of the standard matched the unknown compound.

(h) Mass Spectrum of tyrosol (11) and the match with the authentic standard



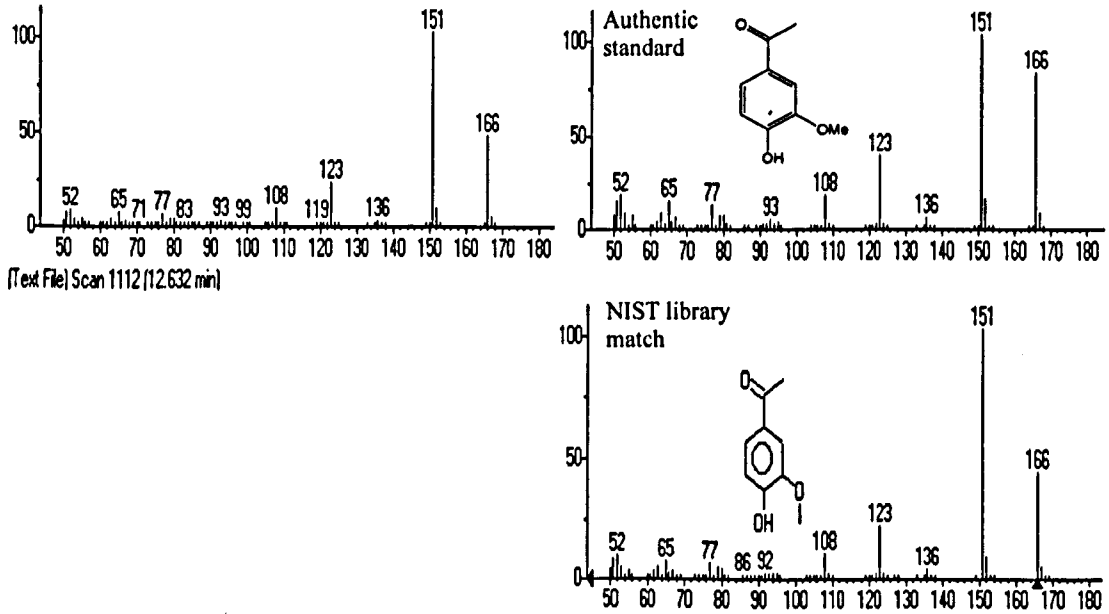
NIST # 92403 **MF:** $C_8H_{10}O_2$ **MW:** 138 **CAS:** 501-94-0
Name: 4-(2-hydroxyethyl)phenol (tyrosol) (11)
The retention time of the standard matched the unknown compound, indicating that the high molecular weight ions (181, 205, 246, 265, 327 and 355 of the unknown compound) were due to contaminants.

(i) Mass Spectrum of isovanillin (12) and the match with the authentic standard



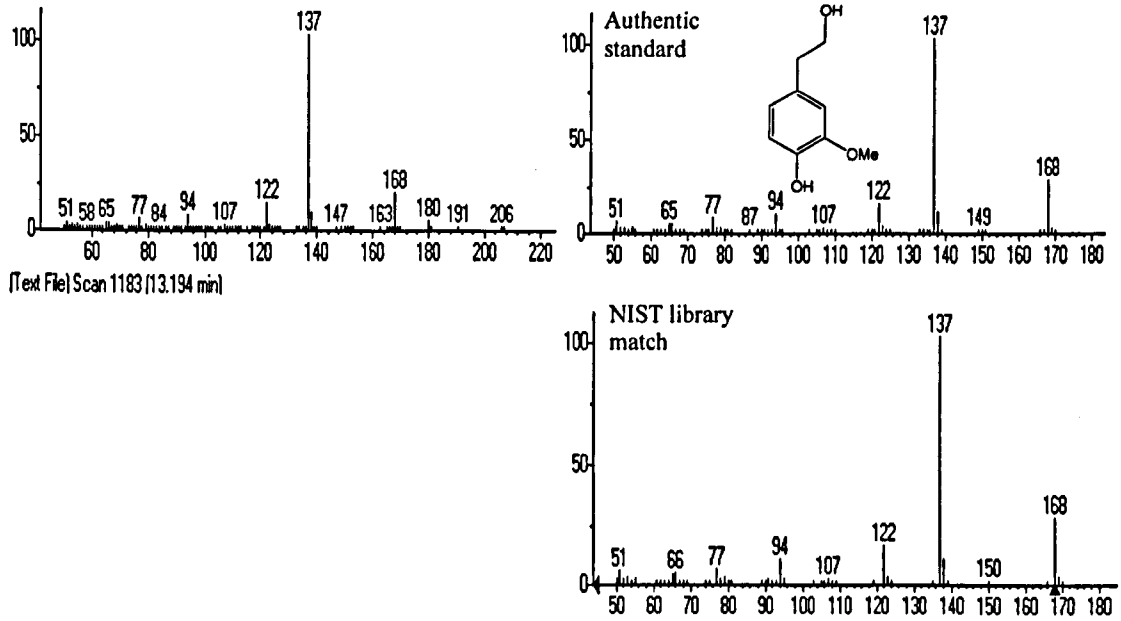
NIST # 229150 MF: $C_8H_8O_3$ MW: 152 CAS: 621-59-0
Name: 3-hydroxy-4-methoxybenzaldehyde (isovanillin) (12)
The retention time of the standard matched the unknown compound, indicating that the high molecular weight ions (166, 178 and 197 of the unknown compound) were due to contaminants.

(j) Mass Spectrum of acetovanillone (3) and the match with the authentic standard



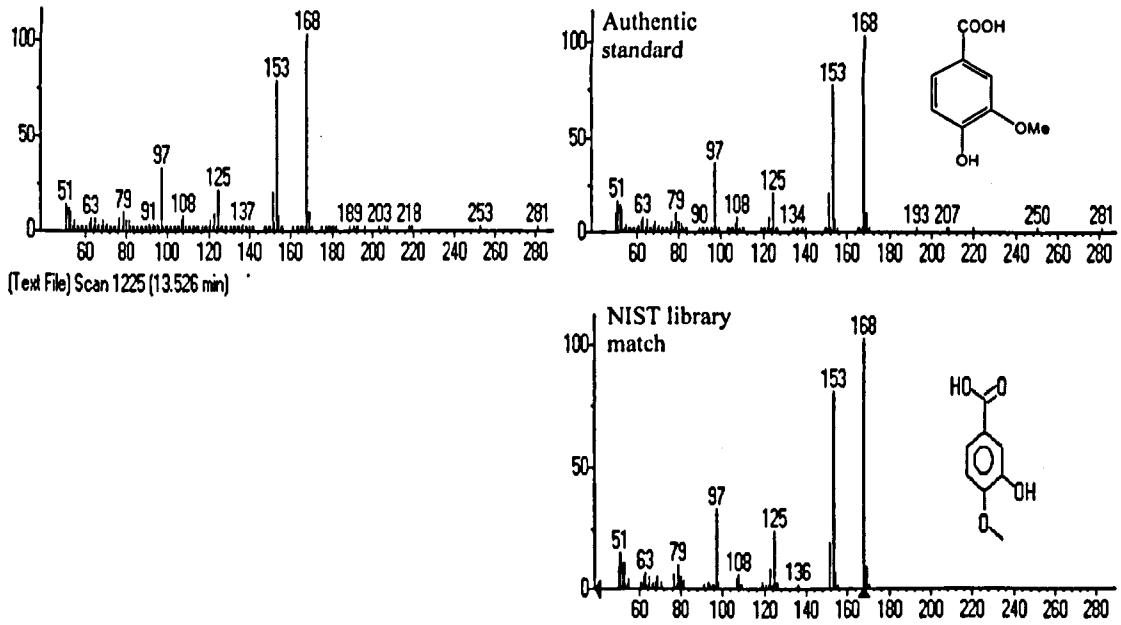
NIST # 352840 MF: $C_9H_{10}O_3$ MW: 166 CAS: 498-02-2
Name: 1-(4-hydroxy-3-methoxyphenyl)-ethanone (Acetovanillone) (3)
The retention time of the standard matched the unknown compound.

(k) Mass Spectrum of homovanillyl alcohol (4) and the match with the authentic standard



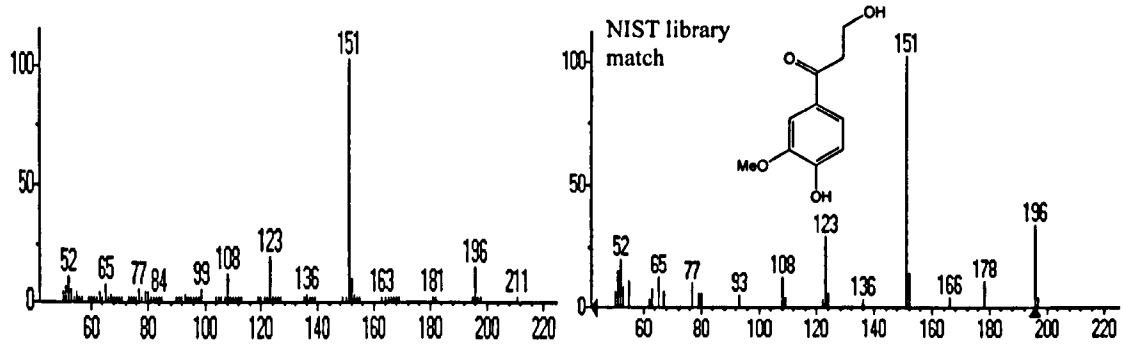
NIST # 133524 MF: $C_9H_{12}O_3$ MW: 168 CAS: 2380-78-1
Name: 4-hydroxy-3-methoxyphenylethyl alcohol (Homovanillyl alcohol) (4)
The retention time of the standard matched the unknown compound, indicating that the high molecular weight ions (180, 191 and 206 of the unknown compound) were due to contaminants.

(l) Mass Spectrum of vanillic acid (5) and the match with the authentic standard



NIST # 6514 MF: $C_8H_8O_4$ MW: 168 CAS: 121-34-6
Name: 4-hydroxy-3-methoxybenzoic acid (Vanillic acid) (5)
The retention time of the standard matched the unknown compound, indicating that the high molecular weight ions (193, 207, 250 and 281 of the authentic standard and 189, 203, 218, 253 and 281 of the unknown compound) were due to contaminants.

(m) Mass Spectrum of 3-hydroxy-1-(4-hydroxy-3-methoxyphenyl)propan-1-one (13) and the match with the NIST library

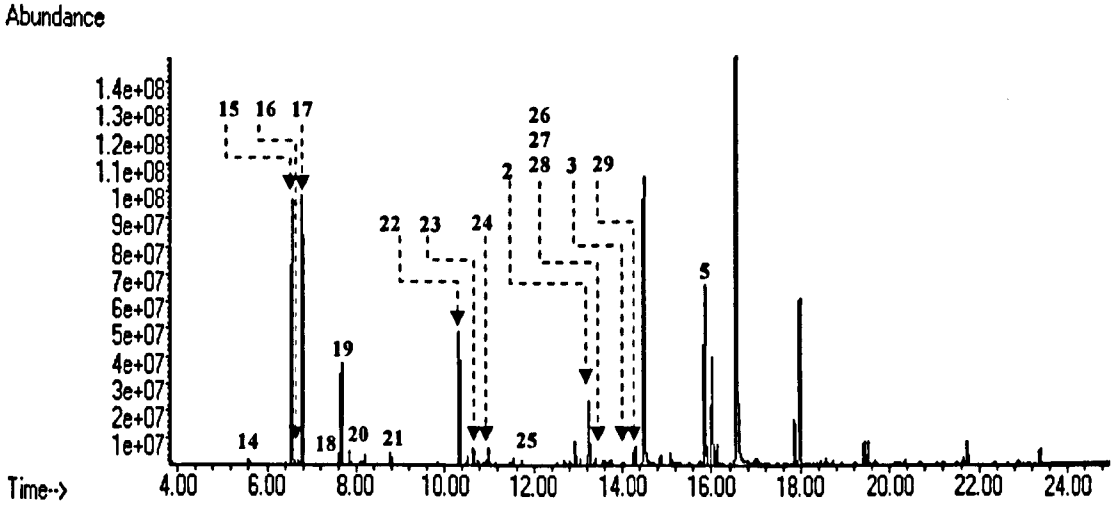


NIST # 8701MF: $C_{10}H_{12}O_4$ MW: 196 CAS: 2196-18-1
Name: 3-hydroxy-1-(4-hydroxy-3-methoxyphenyl)propan-1-one (13)
This seems to be reasonable match. However, in the absence of a standard the identification remains to be confirmed.

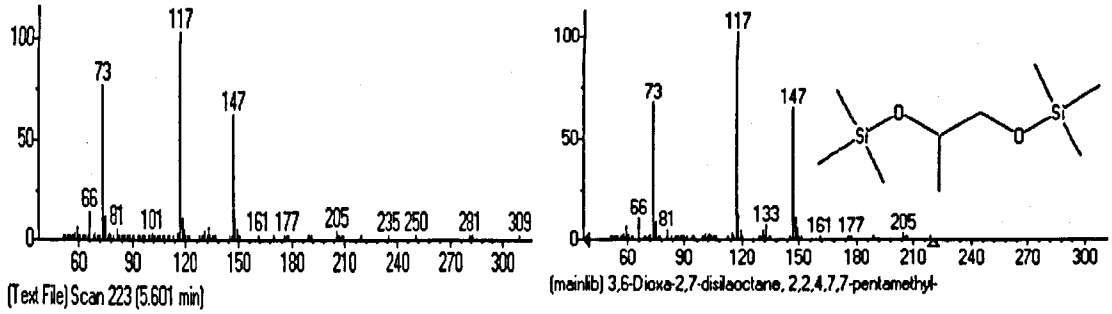
Appendix A.3 GCMS Analysis of Lignin Depolymerization Products after Derivatization (LTV)

The products formed after enzymatic treatment of sodium lignosulphonate by LTV. Samples were incubated at 60 °C for 6 h, shaken at 200 rpm. Fractionation was applied and the dried sample of ethyl acetate extract was derivatized by adding acetonitrile (1 ml), trimethylchlorosilane (TMCS) (10 µl) and bistrimethylsilyltrifluroacetamide (BSTFA) (600 µl). The reaction vessel was closed and heated at 70 °C for 1 h.

A.3.1 Chromatogram of Aqueous Ethyl Acetate Extract Fraction of the LTV-catalyzed reaction after derivatization

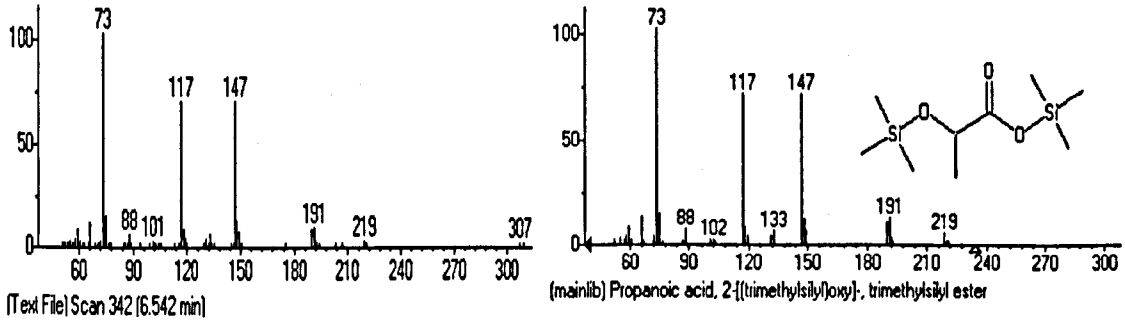


(a) Mass Spectrum of propane-1,2-diol (14) and the match with the NIST library



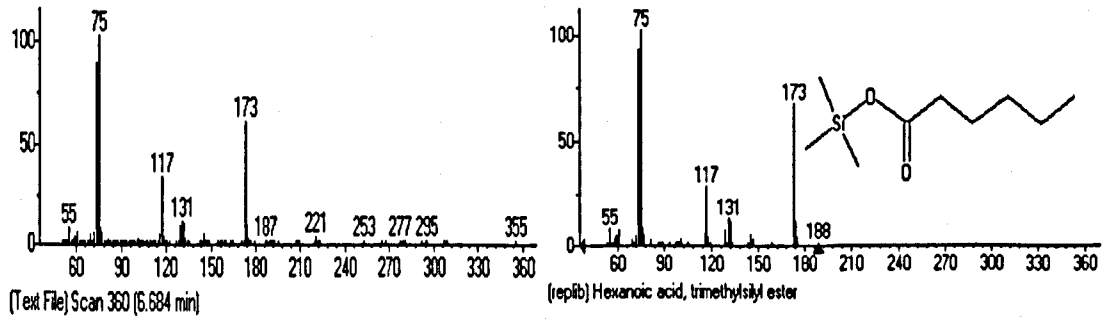
NIST # 333033 MF: $C_9H_{24}O_2Si_2$ MW: 220 CAS: 17887-27-3
Name: 2,2,4,7,7-Pentamethyl-3,6-dioxa-2,7-disilaoctane
This seems to be reasonable match. However, in the absence of a standard the identification remains to be confirmed.

(b) Mass Spectrum of 2-hydroxypropanoic acid (15) and the match with the NIST library



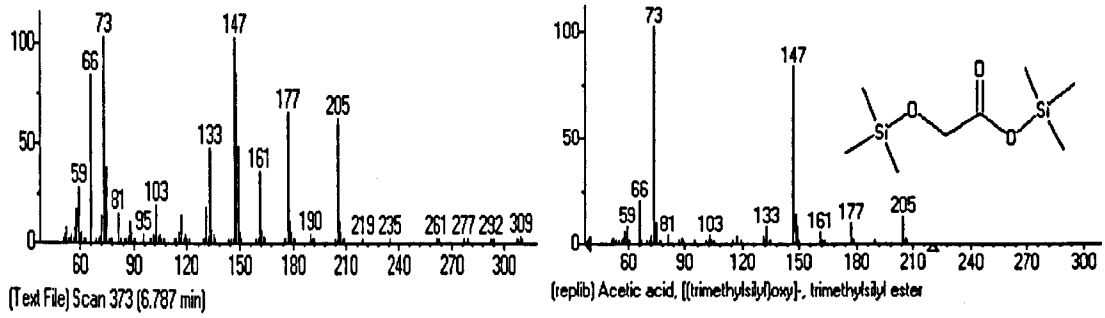
NIST # 78865 MF: $C_9H_{22}O_3Si_2$ MW: 234 CAS: 17596-96-2
Name: Propionic acid, 2-(trimethylsiloxy)-, trimethylsilyl ester
This seems to be reasonable match. However, in the absence of a standard the identification remains to be confirmed.

(c) Mass Spectrum of hexanoic acid (16) and the match with the NIST library



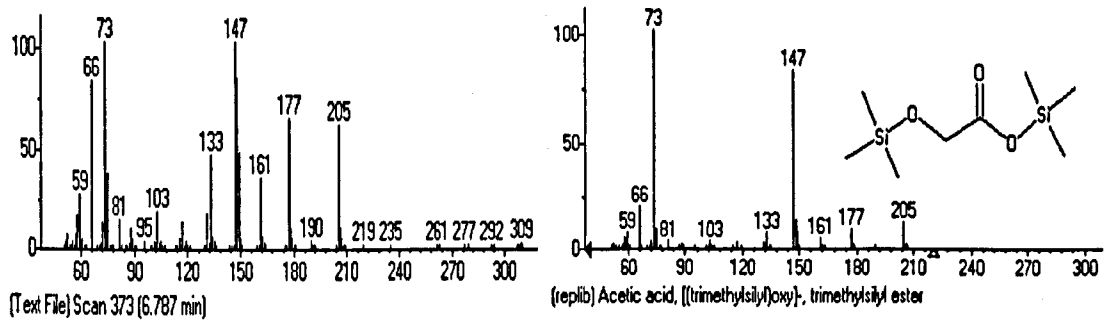
NIST # 71645 MF: $C_9H_{20}O_2Si$ MW: 188 CAS: 14246-15-2
Name: Hexanoic acid, trimethylsilyl ester
The unknown compound has a good match with the NIST library, indicating that the high molecular weight ions (221, 253, 277, 295 and 355) were due to contaminants.

(d) Mass Spectrum of 2-hydroxyacetic acid (17) and the match with the NIST library



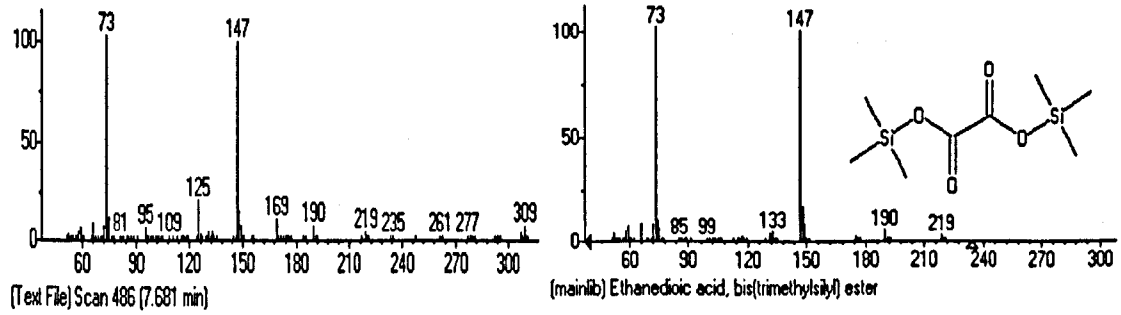
NIST # 78836 MF: $C_8H_{20}O_3Si_2$ **MW: 220 CAS: 33581-77-0**
Name: Acetic acid, [(trimethylsilyl)oxy]-, trimethylsilyl ester
The closest match is shown, but it is clear that this is not a correct identification. The mass spectrum cannot be interpreted.

(e) Mass Spectrum of 2-hydroxybutanoic acid (18) and the match with the NIST library



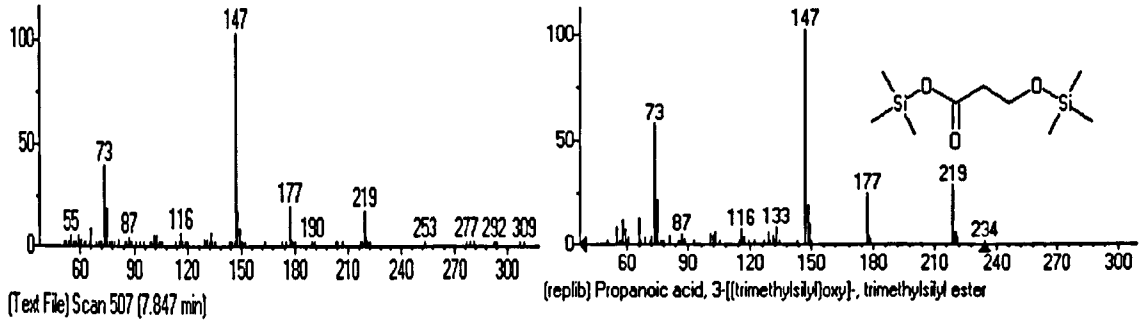
NIST # 78836 MF: $C_8H_{20}O_3Si_2$ **MW: 220 CAS: 33581-77-0**
Name: Acetic acid, [(trimethylsilyl)oxy]-, trimethylsilyl ester
The closest match is shown, but it is clear that this is not a correct identification. The mass spectrum cannot be interpreted.

(f) Mass Spectrum of oxalic acid (19) and the match with the NIST library



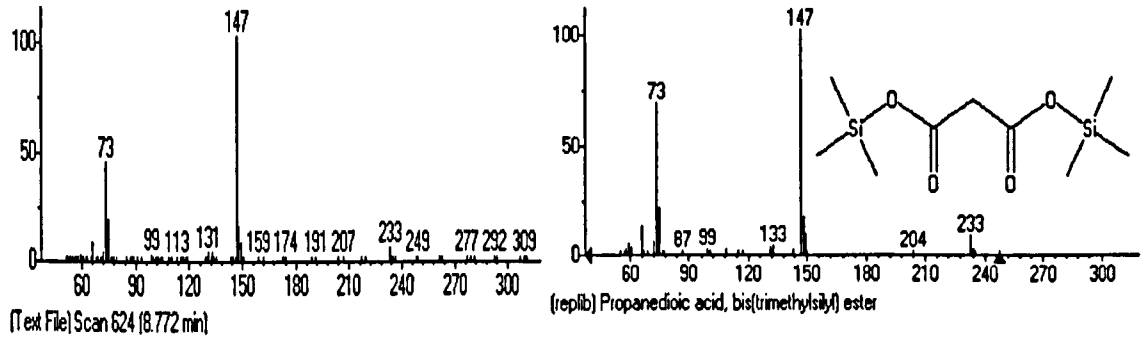
NIST # 352455 MF: $C_8H_{18}O_4Si_2$ **MW: 234 CAS: 18294-04-7**
Name: Oxalic acid, bis(trimethylsilyl) ester
The closest match is shown, but it is clear that this is not a correct identification. The mass spectrum cannot be interpreted.

(g) Mass Spectrum of 3-hydroxypropanoic acid (20) and the match with theNIST library



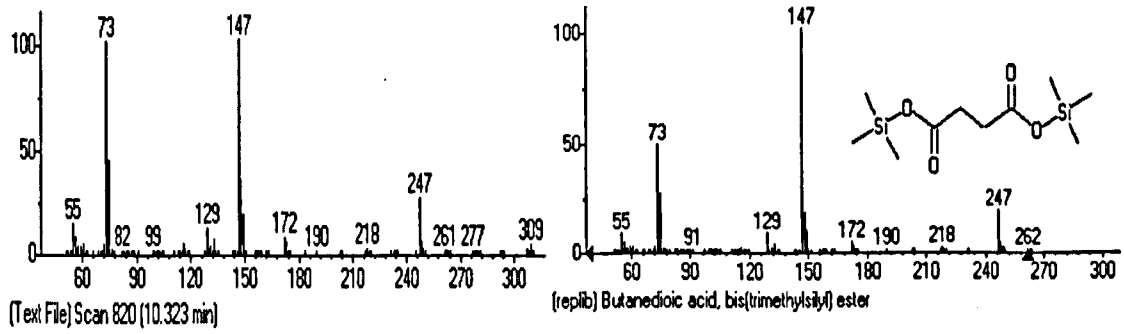
NIST # 281712 MF: $C_9H_{22}O_3Si_2$ MW: 234 CAS: 55162-32-8
Name: Propanoic acid, 3-[(trimethylsilyl)oxy]-, trimethylsilyl ester
There is a poor match between the compound and the library mass spectra and this identification cannot be confirmed. The high molecular weight ions (253, 277, 292 and 309) were due to contaminants.

(h) Mass Spectrum of malonic acid (21) and the match with the NIST library



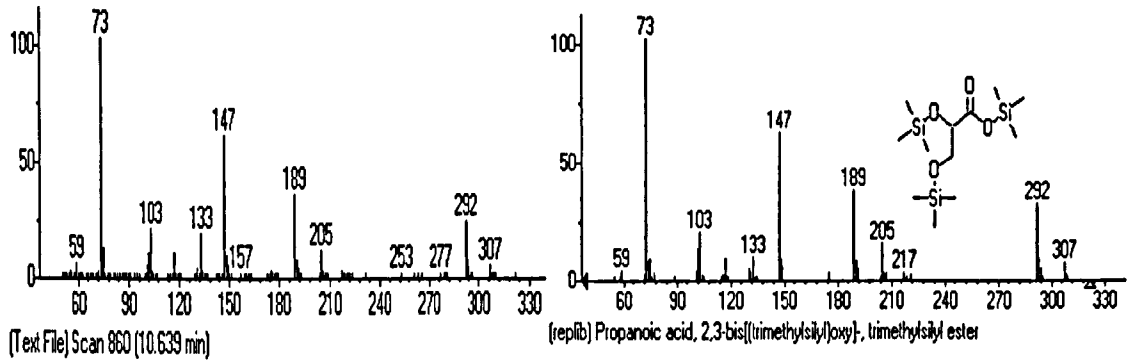
NIST # 78892 MF: $C_9H_{20}O_4Si_2$ MW: 248 CAS: 18457-04-0
Name: Malonic acid, bis(trimethylsilyl) ester
The closest match is shown, but it is clear that this is not a correct identification. The mass spectrum cannot be interpreted.

(i) Mass Spectrum of succinic acid (22) and the match with from the NIST library



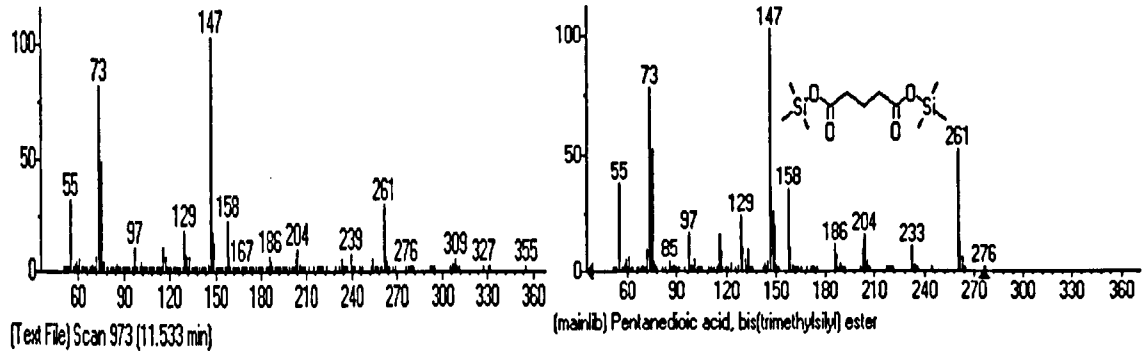
NIST # 331692 MF: $C_{10}H_{22}O_4Si_2$ MW: 262 CAS: 40309-57-7
Name: Succinic acid, di(trimethylsilyl) ester
There is a poor match between the compound and the library mass spectra and this identification cannot be confirmed.

(j) Mass Spectrum of 2,3-dihydroxypropanoic acid (23) and the match with the NIST library



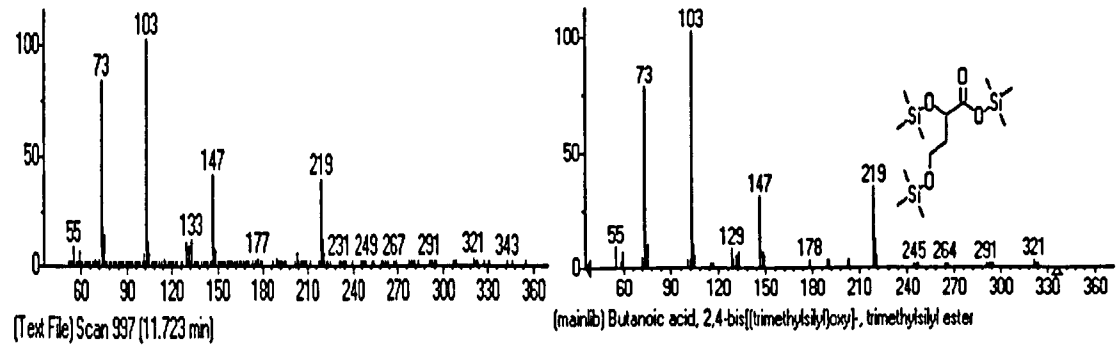
NIST # 71911 MF: $C_{12}H_{30}O_4Si_3$ MW: 322 CAS: 38191-87-6
Name: Propanoic acid, 2,3-bis[(trimethylsilyl)oxy]-, trimethylsilyl ester
This seems to be reasonable match. However, in the absence of a standard the identification remains to be confirmed.

(k) Mass spectrum of glutaric acid (24) and the match with the NIST library



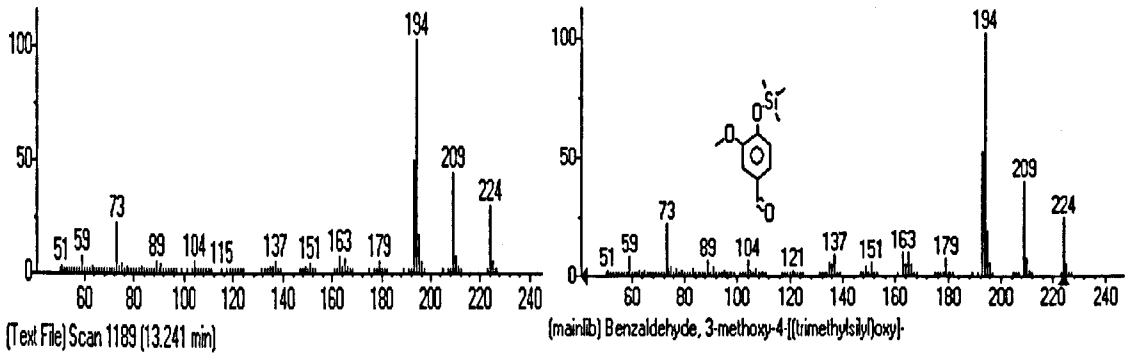
NIST # 332906 MF: $C_{11}H_{24}O_4Si_2$ MW: 276 CAS: 55494-07-0
Name: Glutaric acid, di(trimethylsilyl) ester
This seems to be reasonable match. However, in the absence of a standard the identification remains to be confirmed.

(l) Mass spectrum of 2,4-dihydroxybutanoic acid (25) and the match with the NIST library



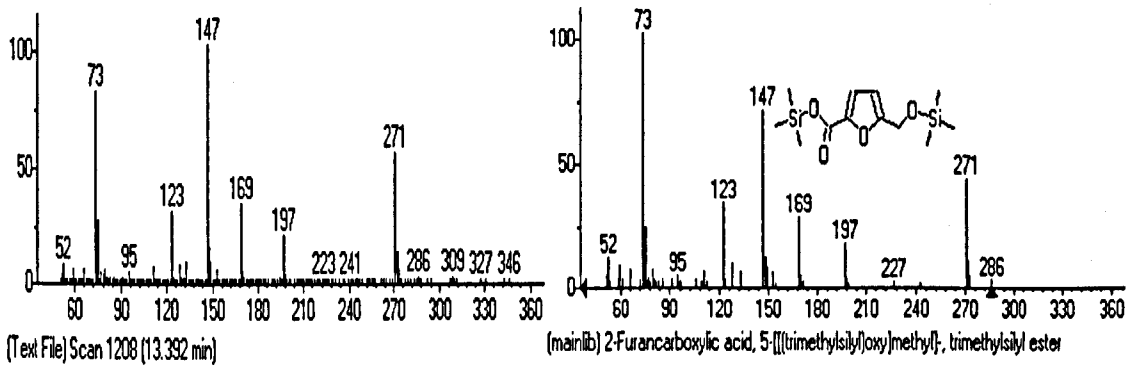
NIST # 15577 MF: $C_{13}H_{32}O_4Si_3$ MW: 336 CAS: 55191-52-1
Name: Butanoic acid, 2,4-bis[(trimethylsilyl)oxy]-, trimethylsilyl ester
There is a poor match between the compound and the library mass spectra and this identification cannot be confirmed.

(m) Mass spectrum of vanillin (2) and the match with the NIST library



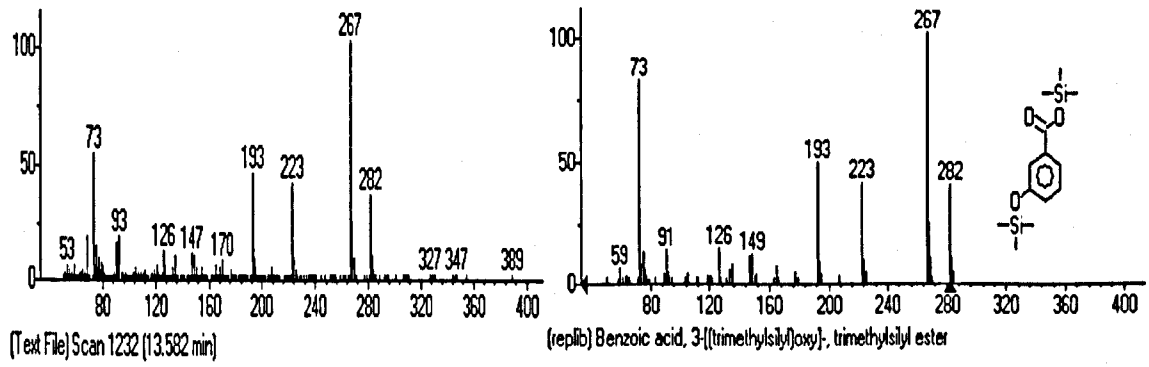
NIST # 352847 MF: $C_{11}H_{16}O_3Si_3$ **MW: 224 CAS: 6689-43-6**
Name: Trimethylsilyl vanillin

(n) Mass spectrum of 5-(hydroxymethyl)furan-2-carboxylic acid (26) and the match with the NIST library



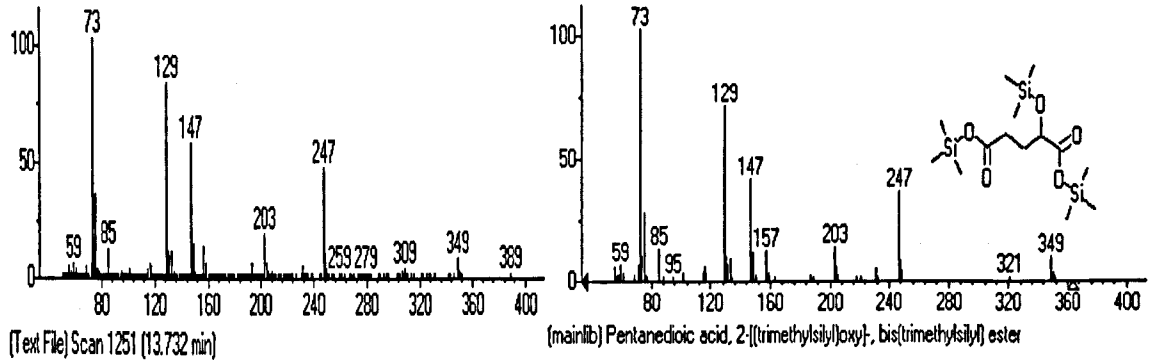
NIST # 30956 MF: $C_{12}H_{22}O_4Si_2$ **MW: 286 CAS: 55517-40-3**
Name: 2-Furancarboxylic acid, 5-[[(trimethylsilyl)oxy]methyl]-, trimethylsilyl ester
This seems to be reasonable match. However, in the absence of a standard the identification remains to be confirmed. The high molecular weight ions (309, 327 and 346) were due to contaminants.

(o) Mass spectrum of 3-hydroxybenzoic acid (27) and the match with the NIST library



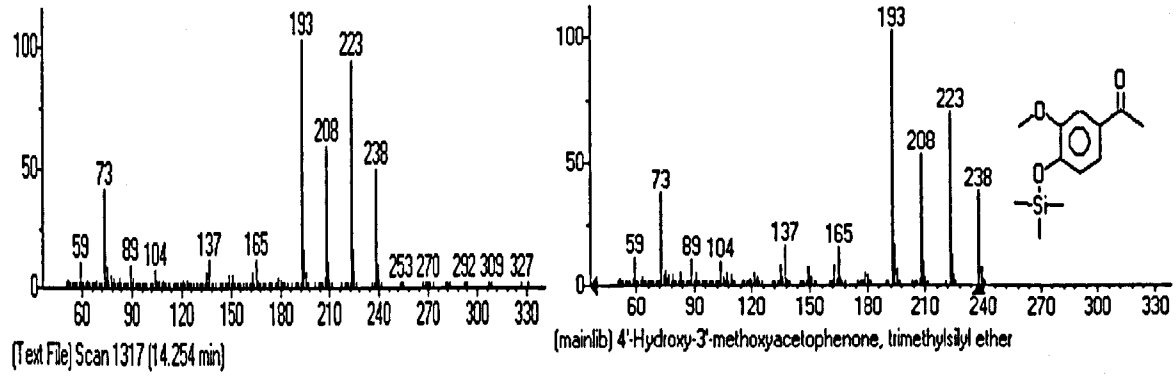
NIST # 30895 MF: $C_{13}H_{22}O_3Si_2$ **MW: 282 CAS: 3782-84-1**
Name: Benzoic acid, m-(trimethylsiloxy)-, trimethylsilyl ester
This seems to be reasonable match. However, in the absence of a standard the identification remains to be confirmed. The high molecular weight ions (327, 347 and 389) were due to contaminants. See also figure A.3.1(r) for 4-hydroxybenzoic acid.

(p) Mass spectrum of 3-hydroxypentanedioic acid (28) and the match with the NIST library



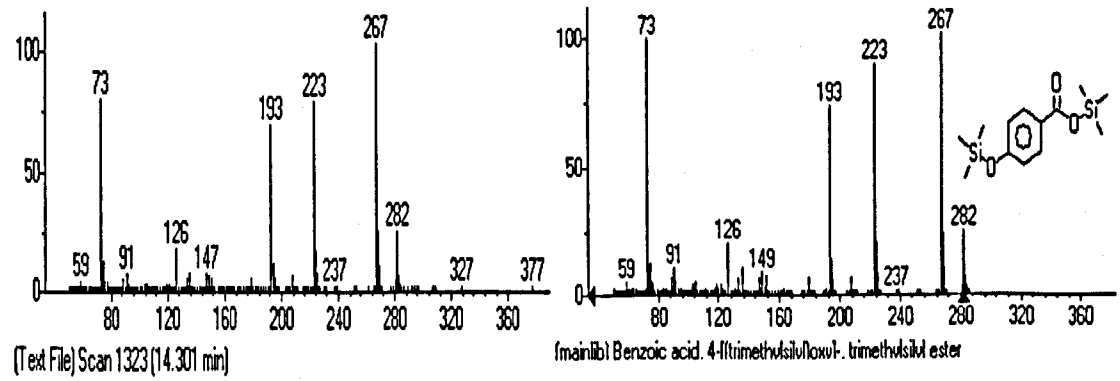
NIST # 30803 MF: $C_{14}H_{32}O_5Si_3$ **MW: 364 CAS: 55530-62-6**
Name: Pentanedioic acid, 2-[(trimethylsilyl)oxy]-, bis(trimethylsilyl) ester
This seems to be reasonable match. However, in the absence of a standard the identification remains to be confirmed.

(q) Mass spectrum of acetovanillone (3) and the match with the NIST library



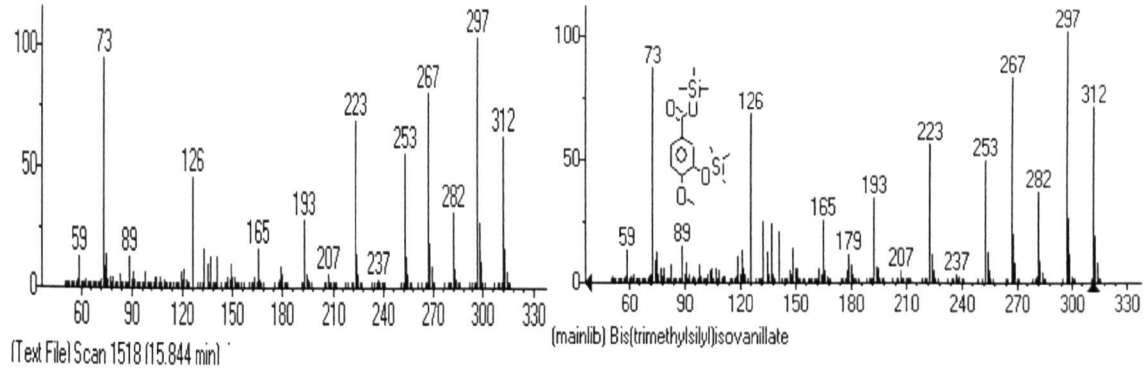
NIST # 352842 MF: $C_{12}H_{18}O_3Si$ **MW: 238**
Name: 4'-Hydroxy-3'-methoxyacetophenone, trimethylsilyl ether
The unknown compound has a good match with the NIST library, indicating that the high molecular weight ions (253, 270, 292, 309 and 327) were due to contaminants.

(r) Mass spectrum of 4-hydroxybenzoic acid (29) and the match with the NIST library



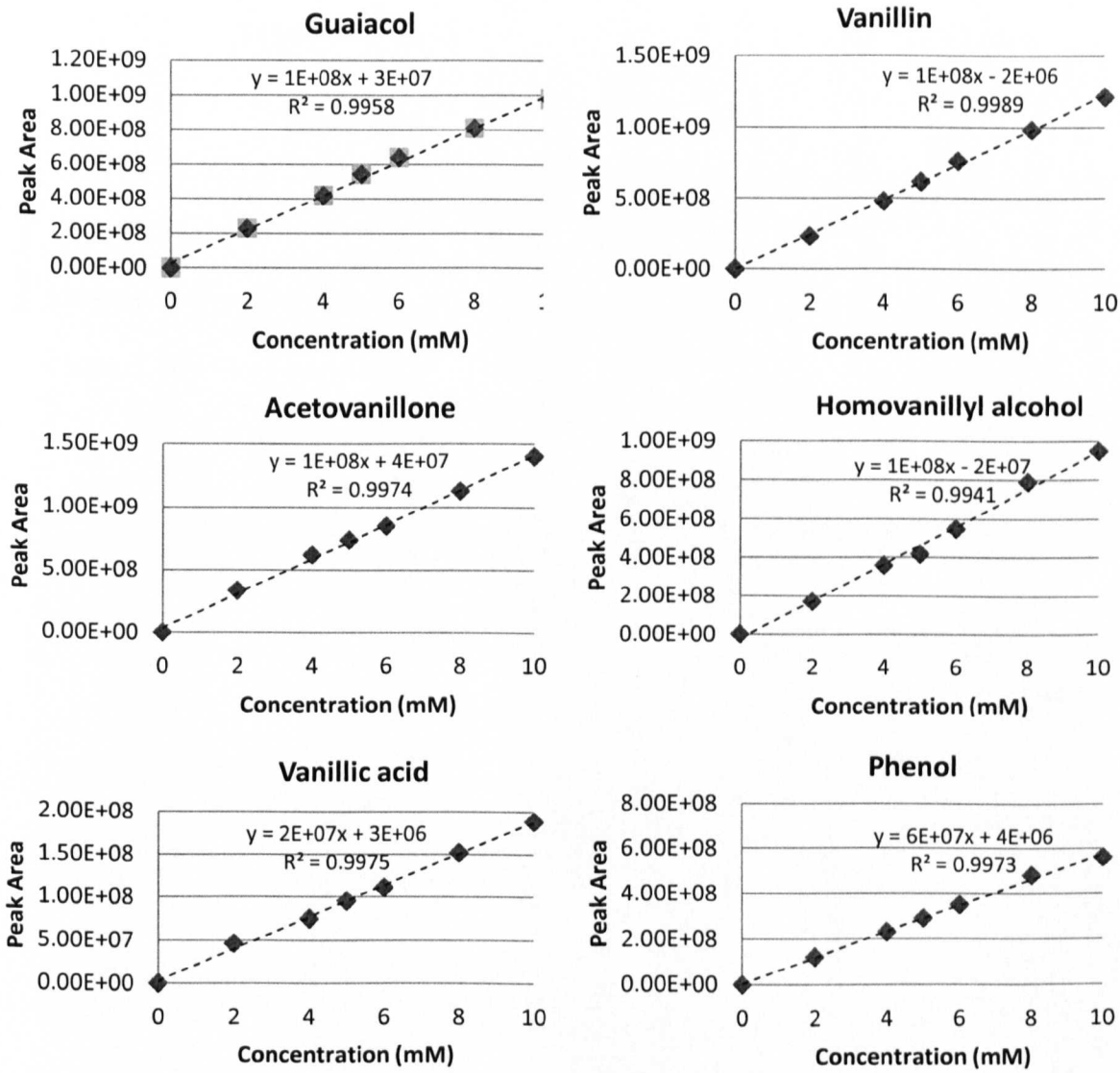
NIST # 352451 MF: $C_{13}H_{22}O_3Si_2$ **MW: 282 CAS: 2078-13-19**
Name: Benzoic acid, p-(trimethylsiloxy)-, trimethylsilyl ester
The unknown compound has a good match with the NIST library, indicating that the high molecular weight ions (327 and 377) were due to contaminants.

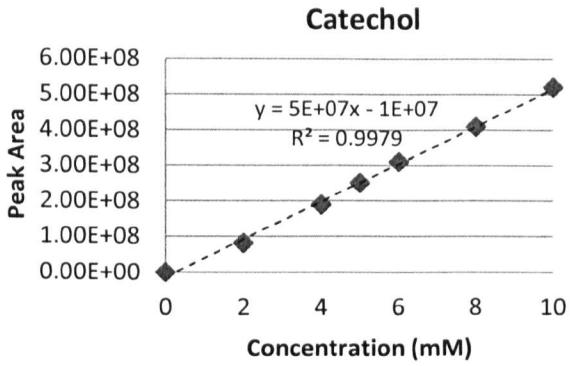
(s) Mass spectrum of <u>vanillic acid</u> (5) and the match with the NIST library

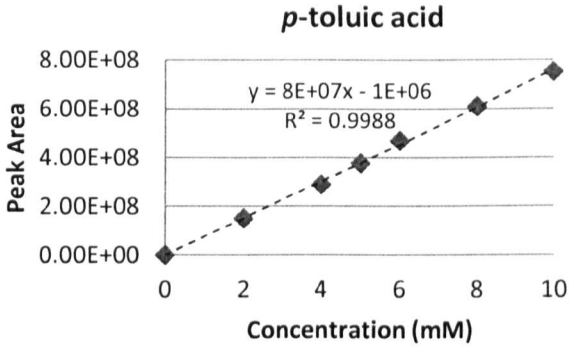


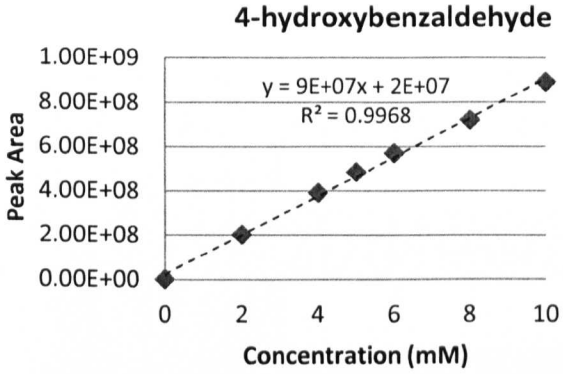
NIST # 352853 **MF:** $C_{14}H_{24}O_4Si_2$ **MW:** 312 **CAS:** 68595-68-6
Name: Bis(trimethylsilyl)isovanillate
The unknown compound has a good match with the NIST library.

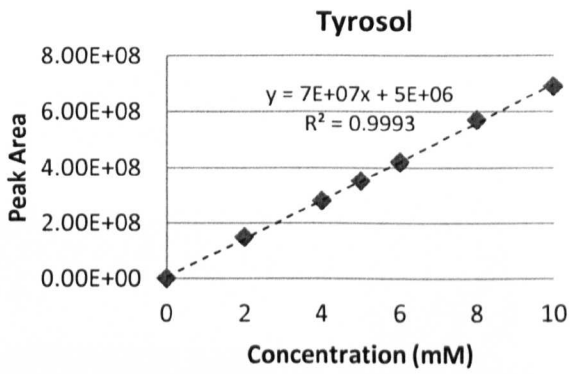
A.3.2 GCMS standard calibration curve

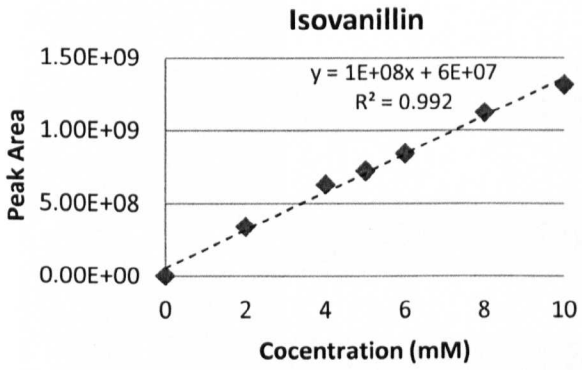



Catechol
y = 5E+07x - 1E+07
R² = 0.9979
Peak Area
Concentration (mM)


p-toluic acid
y = 8E+07x - 1E+06
R² = 0.9988
Peak Area
Concentration (mM)


4-hydroxybenzaldehyde
y = 9E+07x + 2E+07
R² = 0.9968
Peak Area
Concentration (mM)


Tyrosol
y = 7E+07x + 5E+06
R² = 0.9993
Peak Area
Concentration (mM)


Isovanillin
y = 1E+08x + 6E+07
R² = 0.992
Peak Area
Cocentration (mM)

Appendix A.4 HPLC Analysis of Lignin Depolymerization Products

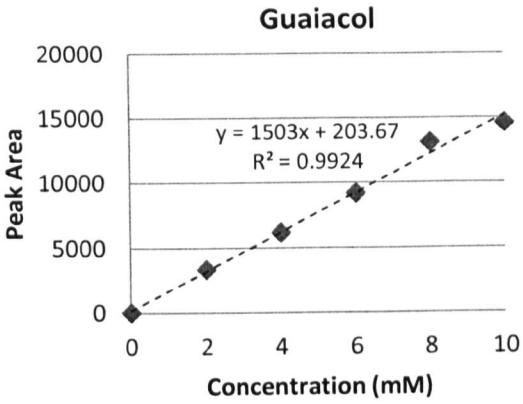
The identification of the products was confirmed *via* the retention times of the authentic standards. The sample peak areas are proportional to the amount of the compound in the sample. Therefore, the peak areas were used with the calibration curves to quantify the amount of the compound in the sample. Calibration curves were generated by analyze the standard with the concentration varies from 2 to 10 mM.

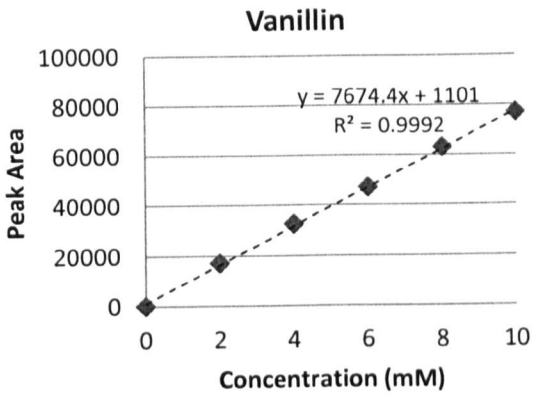
A.4.1 HPLC authentic standard peak area

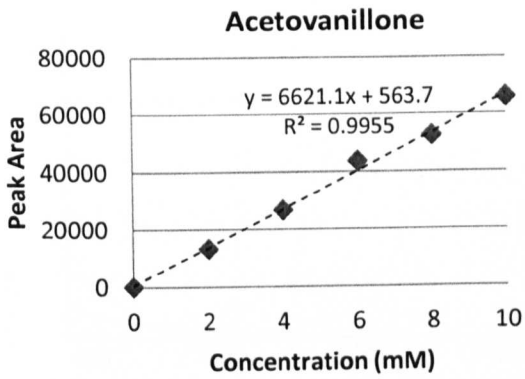
Compound		Concentration (mM)	Peak Area	Retention Time (RT) (min)	Mean RT (min)
1	Homovanillyl alcohol	0	0	0	6.21
		2	7568	6.217	
		4	14379.4	6.208	
		6	21206.1	6.214	
		8	27058.7	6.21	
		10	34708.7	6.203	
2	Vanillic acid	0	0	0	9.69
		2	5122.1	9.741	
		4	9200	9.706	
		6	20104	9.648	
		8	24429.4	9.673	
		10	21751.8	9.729	
3	Vanillin	0	0	0	11.29
		2	17070.5	11.336	
		4	32596.4	11.375	
		6	47161.8	11.31	
		8	63096.1	11.245	
		10	76913.2	11.186	
4	Guaiacol	0	0	0	12.89

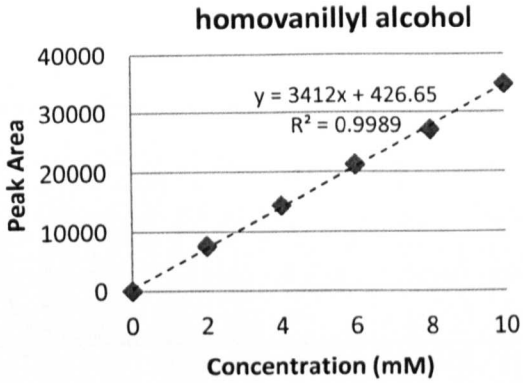
		2	3334	12.863	
		4	6151.2	12.896	
		6	9151.4	12.901	
		8	13083.3	12.909	
		10	14592.6	12.91	
5	Acetovanillone	0	0	0	14.35
		2	13271.5	14.347	
		4	26760.7	14.274	
		6	43657.1	14.368	
		8	52616.5	14.369	
		10	65708.9	14.393	

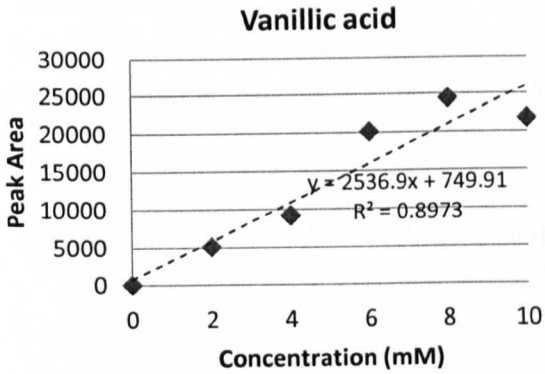
A.4.2 HPLC standard calibration curves











Sample was incubated at 30 °C for 2 h, shaken at 200 rpm. The sample was evaporated to dryness and redissolved in DCM. The chromatogram represents the duplicate analysis.

A.5.1 The Oxidation of Vanillin (2)

Products of vanillin have been identified as 2-methoxyhydroquinone (**30**), acetovanillone (**3**), vanillic acid (**5**).

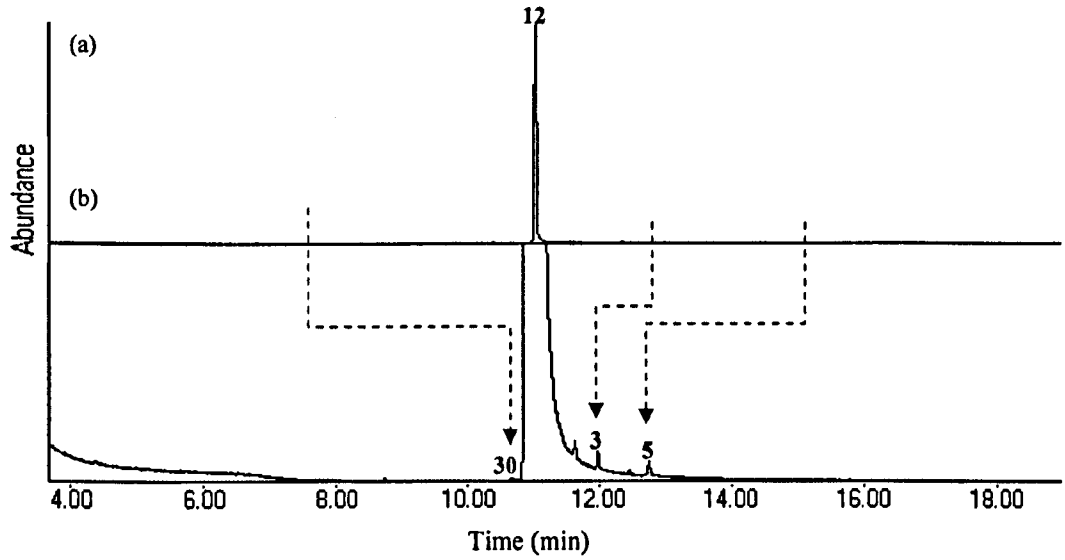
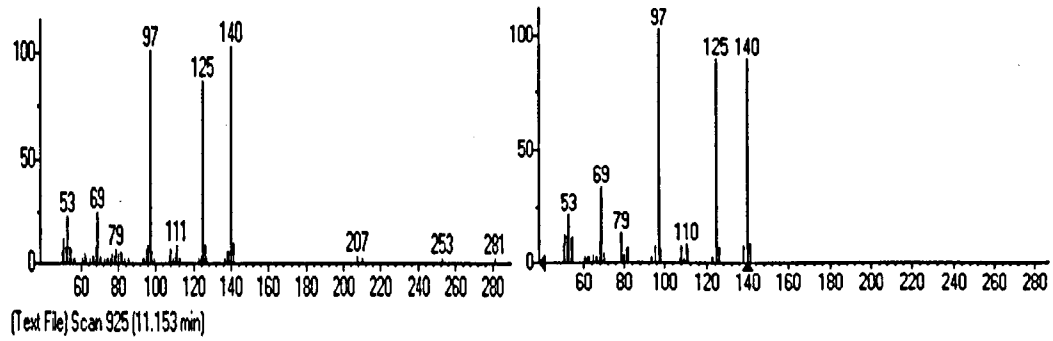


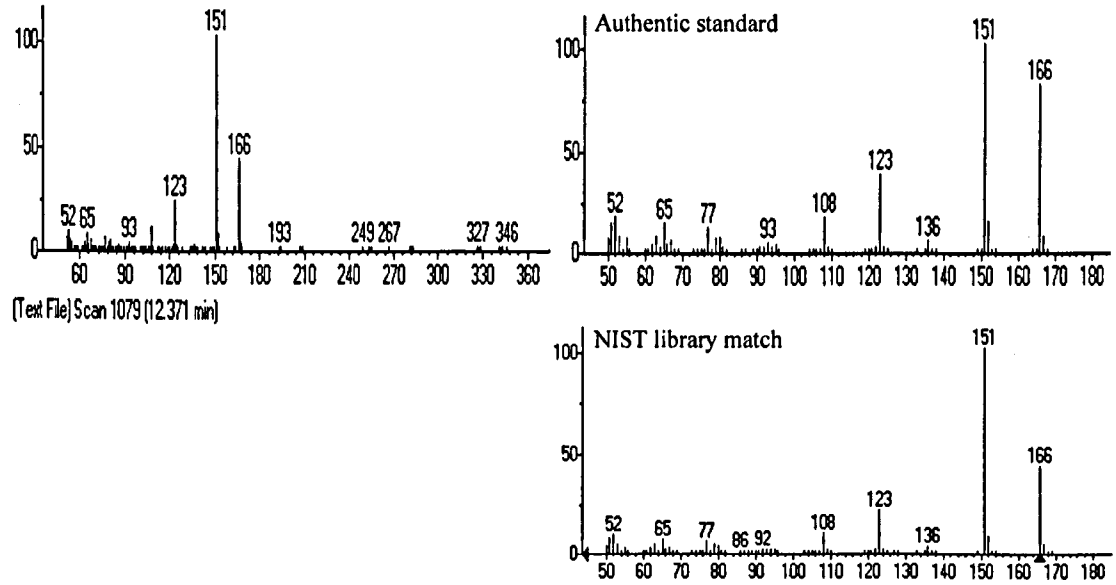
Figure A.6.1 The gas chromatograms of products formed after enzymatic treatment of vanillin by LTV. (a) The authentic standard of vanillin (5 mM) (b) the products formed after enzymatic treatment of vanillin (20 mM) by LTV in the presence of ABTS.

(a) Mass Spectrum of 2-Methoxyhydroquinone (30) and the match from the NIST library



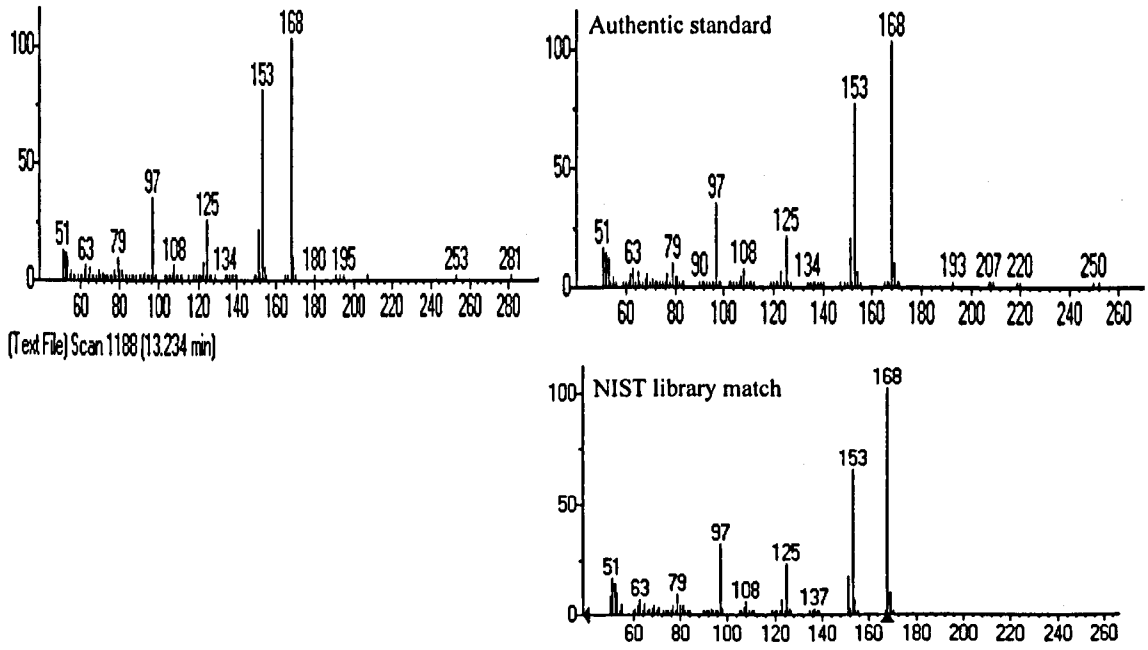
NIST # 113416 **MF:** $C_7H_8O_3$ **MW:** 140 **CAS:** 824-46-4 **Name:** 2-Methoxy hydroquinone
The peaks at 207, 253 and 281 may be baseline contaminants.

(b) Mass Spectrum of Acetovanillone (3) and the match with the authentic standard



NIST # 352840 **MF:** $C_9H_{10}O_3$ **MW:** 166 **CAS:** 498-02-2
Name: 1-(4-hydroxy-3-methoxyphenyl)-ethanone (Acetovanillone) (3)
The retention time of the standard matched the unknown compound, indicating that the high molecular weight ions (193, 249, 267, 327 and 346) were due to contaminants.

(c) Mass Spectrum of Vanillic acid (5) Peak and the match with authentic standard



NIST # 6514 **MF:** $C_8H_8O_4$ **MW:** 168 **CAS:** 121-34-6
Name: 4-hydroxy-3-methoxybenzoic acid (Vanillic acid) (5)
The retention time of the standard matched the unknown compound, indicating that the high molecular weight ions (193, 207, 220 and 250 of the authentic standard and 180, 195, 253 and 281 of the unknown compound) were due to contaminants.

A.5.2 The Oxidation of Acetovanillone (3)

Products of acetovanillone have been tentatively identified as 2-methoxyphenyl acetate **(31)**, 4-acetyl-2-methoxyphenyl acetate **(32)**, 1-(2,6-dihydroxy-4-methoxyphenyl)-ethanone **(33)**, 4-methoxy-3-(4-methoxycarbonylphenoxy)-benzoic acid, methyl ester **(34)**.

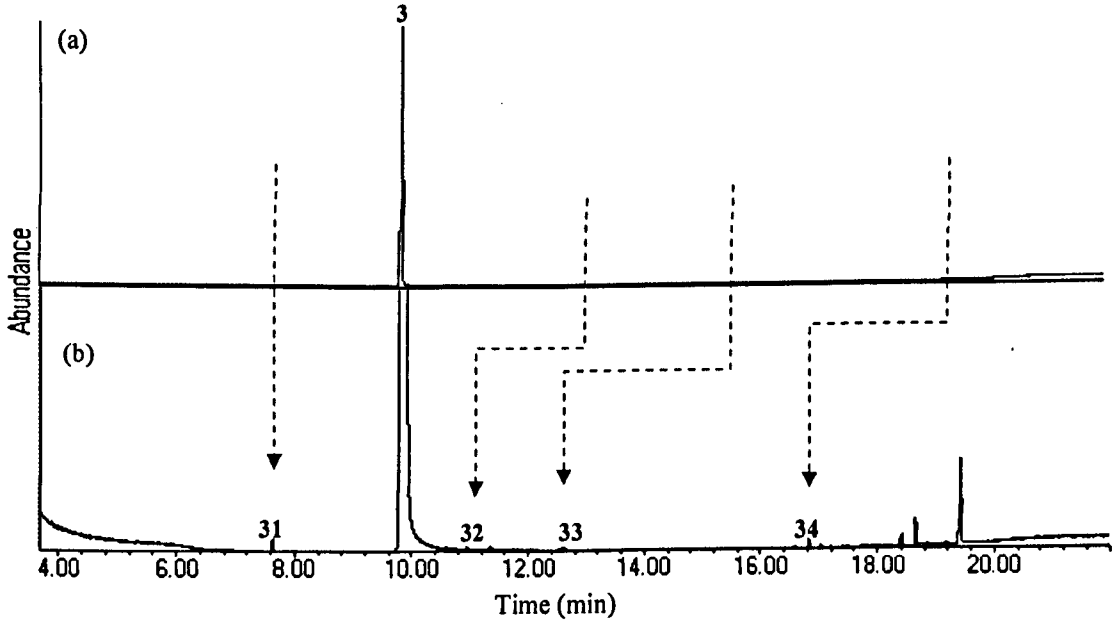
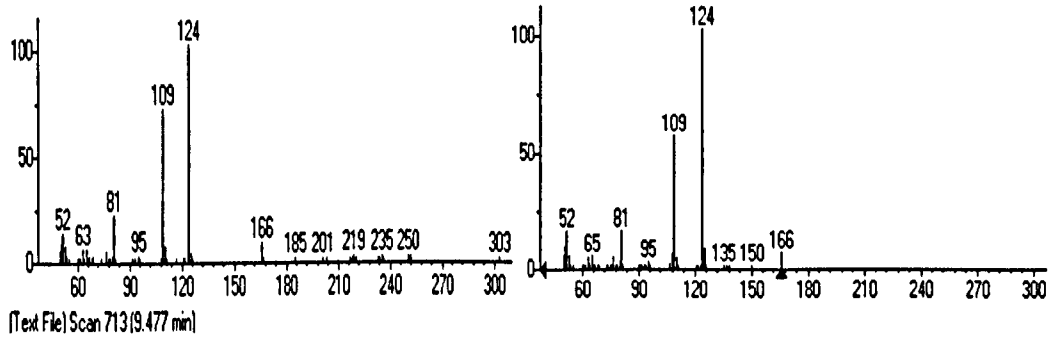


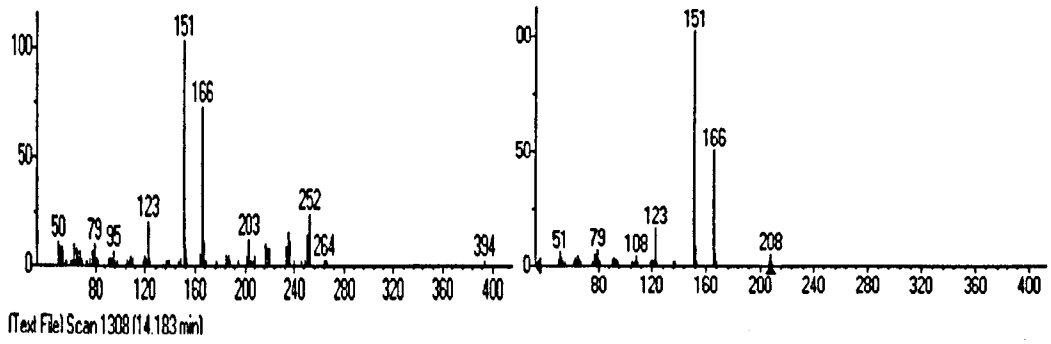
Figure A.6.2 The GCMS chromatograms of products formed after enzymatic treatment of acetovanillone by LTV. (a) The authentic standard of acetovanillone (5 mM) (b) the products formed after enzymatic treatment of acetovanillone (20 mM) by LTV in the presence of ABTS.

(a) Mass Spectrum of 2-methoxyphenyl acetate (31) and the match from the NIST library



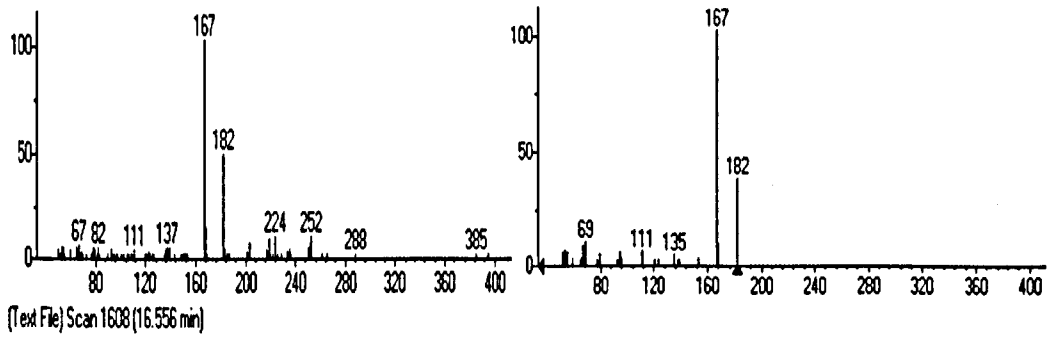
NIST # 6297 **MF:** $C_9H_{10}O_3$ **MW:** 166 **CAS:** 613-70-7
Name: 2-Methoxyphenyl acetate (31)
There is a poor match between the compound and the library mass spectra and this identification cannot be confirmed.

(b) Mass Spectrum of 4-acetyl-2-methoxyphenyl acetate (32) and the match from the NIST library



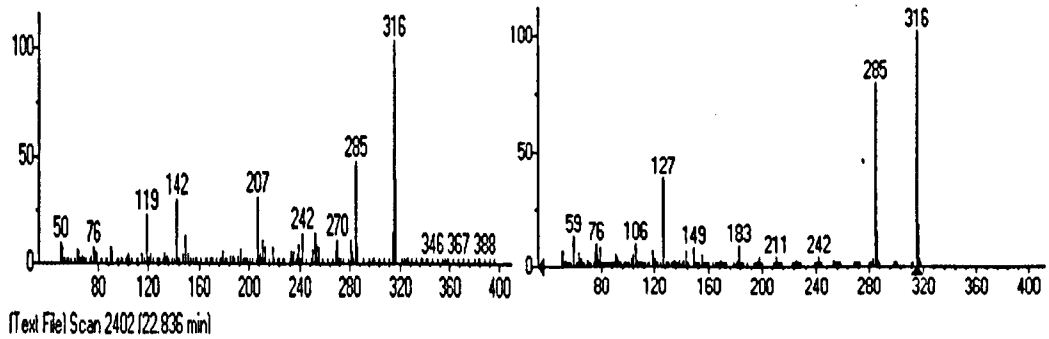
NIST # 118040 **MF:** $C_{11}H_{12}O_4$ **MW: 208 CAS: 54771-60-7**
Name: 4-Acetyl-2-methoxyphenyl acetate (32)
There is a poor match between the compound and the library mass spectra and this identification cannot be confirmed.

(c) Mass Spectrum of 1-(2,6-dihydroxy-4-methoxyphenyl)-ethanone (33) and the match from the NIST library



NIST # 32711 **MF:** $C_9H_{10}O_4$ **MW: 182 CAS: 7507-89-3**
Name: 1-(2,6-dihydroxy-4-methoxyphenyl)-ethanone (33)
There is a poor match between the compound and the library mass spectra and this identification cannot be confirmed.

(d) Mass Spectrum of 4-methoxy-3-(4-methoxycarbonylphenoxy)-benzoic acid, methyl ester (34) and the match from the NIST library



NIST # 267390 **MF:** $C_{17}H_{16}O_6$ **MW: 316 CAS: 5566-15-4**
Name: 4-methoxy-3-(4-methoxycarbonylphenoxy)-benzoic acid, methyl ester (34)
The closest macth is shown, but it is clear that this is not a correct identification. The mass spectrum cannot be interpreted.

A.5.3 The Oxidation of Guaiacol (1)

Products of guaiacol have been identified as 1-hydroxy-3,5,6-trimethoxyxanthone **(35)**, 4-4'-biguaiacol **(36)**.

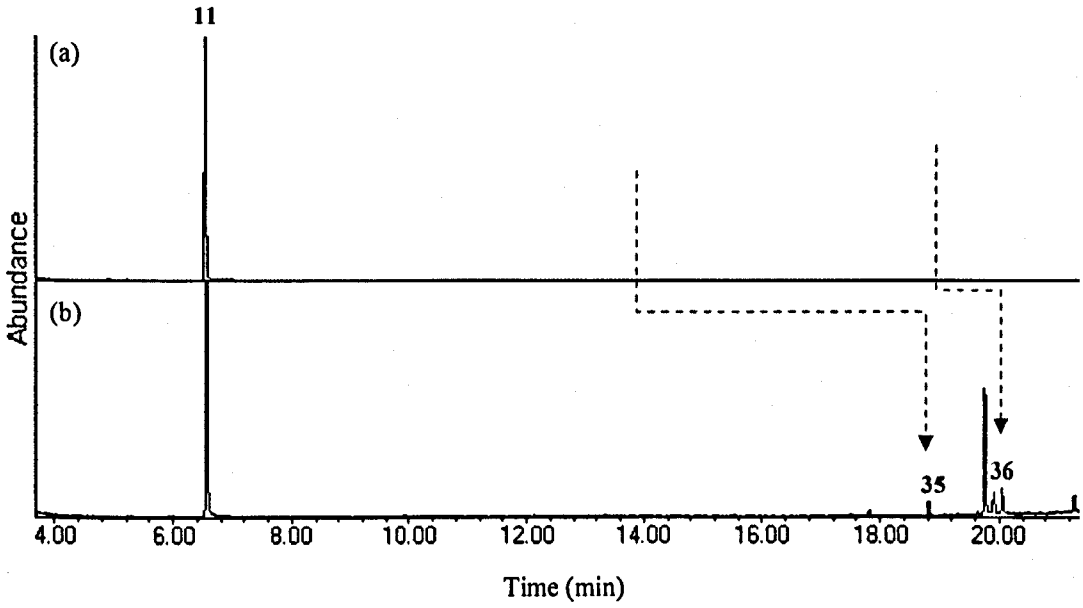
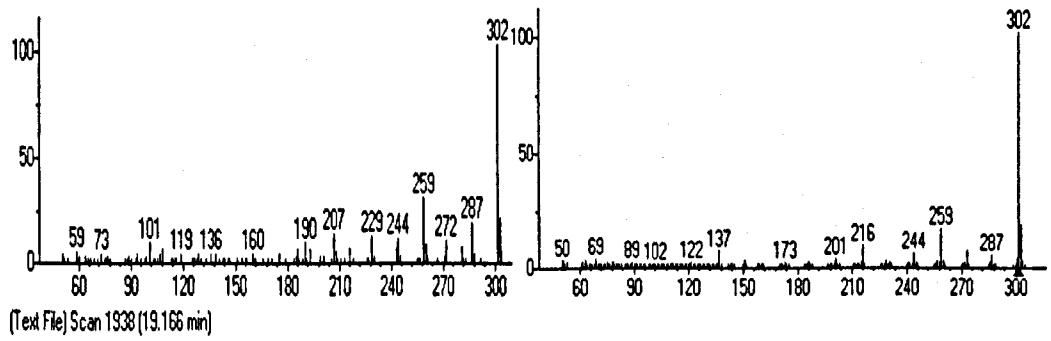


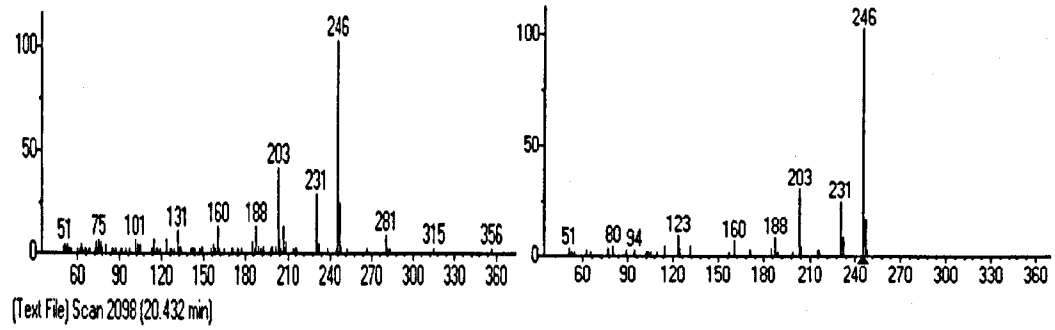
Figure A.6.3 The GCMS chromatograms of products formed after enzymatic treatment of guaiacol by LTV. (a) The authentic standard of guaiacol (5 mM) (b) the products formed after enzymatic treatment of guaiacol (20 mM) by LTV in the presence of ABTS.

(a) Mass Spectrum of 1-hydroxy-3,5,6-trimethoxyxanthone (35) and the match from the NIST library



NIST # 14453 **MF:** $C_{16}H_{14}O_6$ **MW:** 302 **CAS:** 4090-62-4
Name: 1-hydroxy-3,5,6-trimethoxyxanthone (35)
The closest macth is shown, but it is clear that this is not a correct identification. The mass spectrum cannot be interpreted.

(b) Mass Spectrum of 4-4'-biguaiacol (36) and the match from the NIST library



NIST # 100607 **MF:** $C_{14}H_{14}O_4$ **MW:** 246 **CAS:** 4433-09-4
Name: 4-4'-biguaiacol (36)
This seems to be reasonable match. However, in the absence of a standard, the identification remains to be confirmed.

A.5.4 The Oxidation of Vanillic Acid (5)

Products of vanillic acid have been identified as guaiacol **(1)**, 2-methoxyhydroquinone **(30)**, vanillin **(2)**, and methyl vanillate **(37)**.

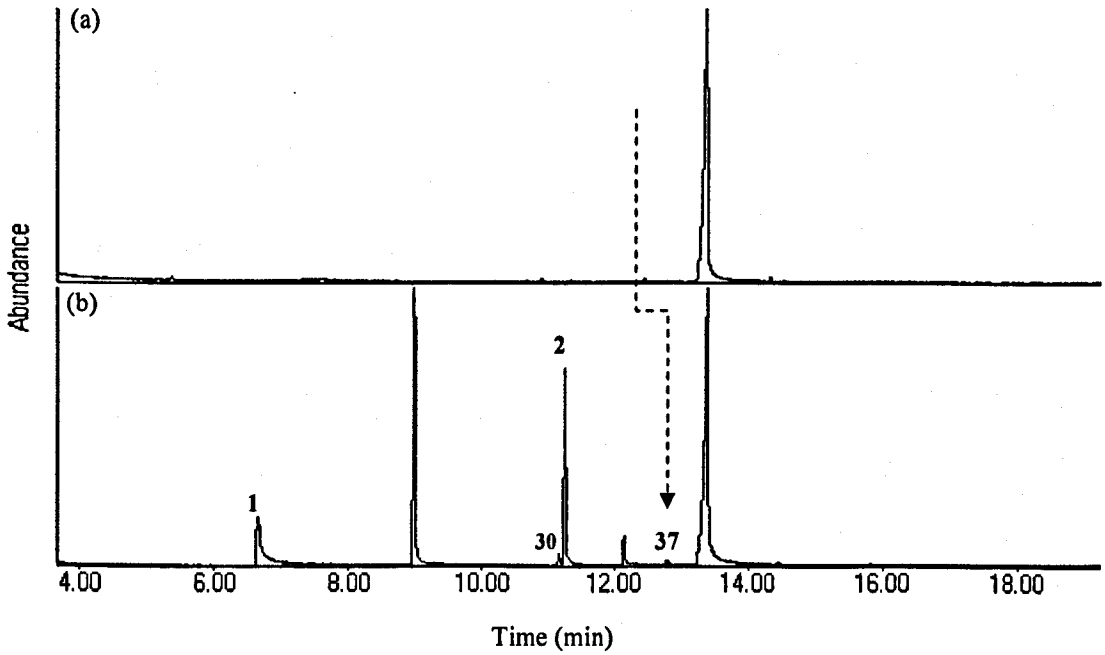
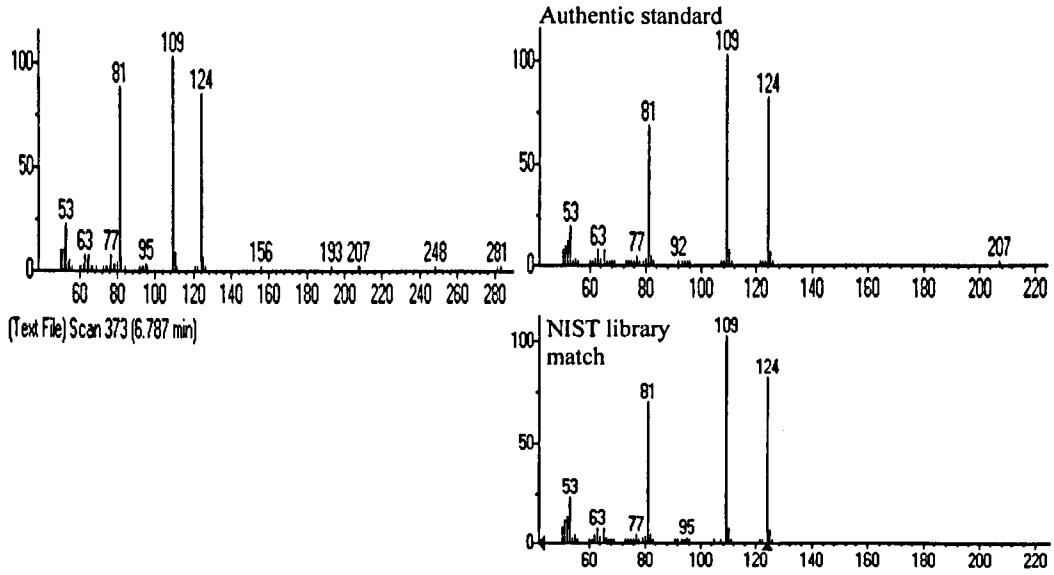


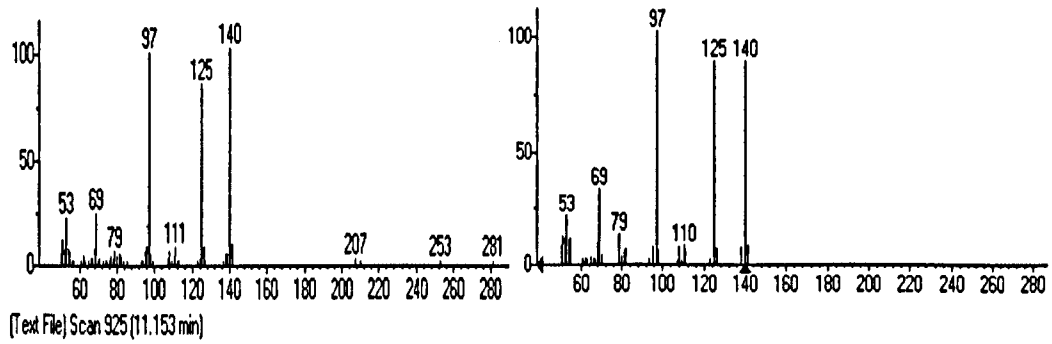
Figure A.6.4 The GCMS chromatograms of products formed after enzymatic treatment of vanillic acid by LTV. (a) The authentic standard of vanillic acid (5 mM) (b) the products formed after enzymatic treatment of vanillic acid (20 mM) by LTV in the presence of ABTS.

(a) Mass Spectrum of Guaiacol (1) and the match with the authentic standard



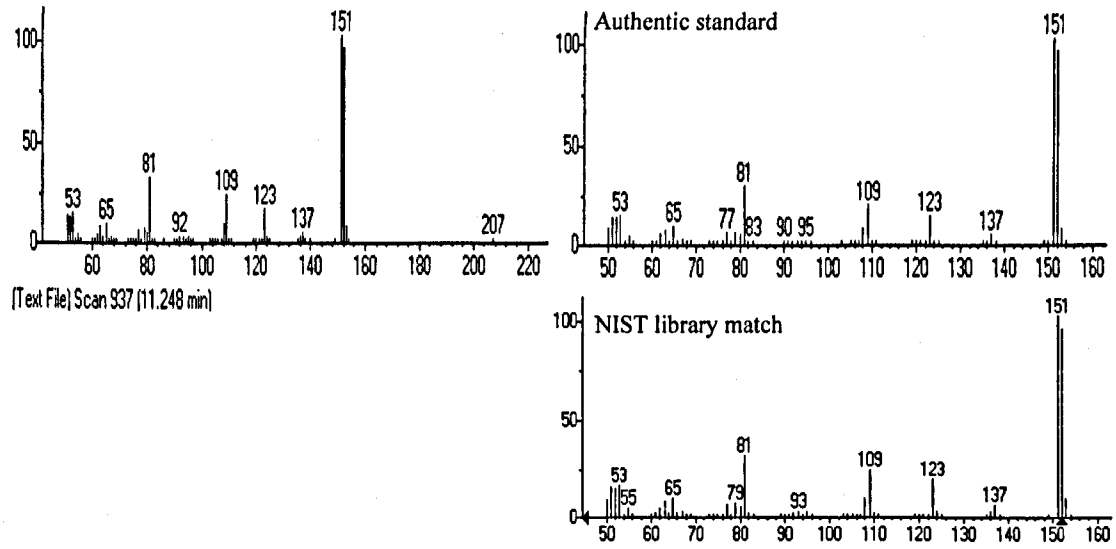
NIST # 20584 **MF:** $C_7H_8O_2$ **MW:** 124 **CAS:** 90-05-1
Name: 2-methoxy phenol (Guaiacol) (1)
The retention time of the standard matched the unknown compound, indicating that the high molecular weight ions (207 of the authentic standard and 156, 193, 207, 248 and 281 of the unknown compound)) were due to contaminants.

(b) Mass Spectrum of 2-Methoxyhydroquinone (30) and the match from the NIST library



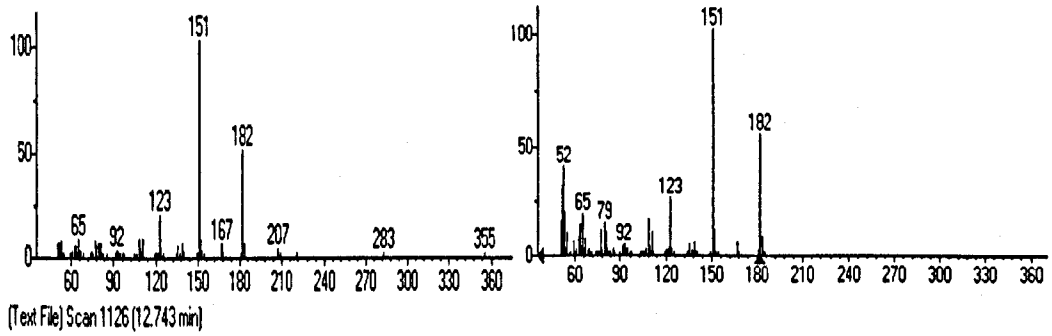
NIST # 113416 **MF:** $C_7H_8O_3$ **MW:** 140 **CAS:** 824-46-4
Name: 2-Methoxy hydroquinone (30)
The peaks at 207, 253 and 281 may be baseline contaminants

(c) Mass Spectrum of vanillin (2) and the match with authentic standard



NIST # 227894 **MF:** $C_8H_8O_3$ **MW: 152 CAS: 121-33-5**
Name: 4-hydroxy-3-methoxybenzaldehyde (Vanillin) (2)
The retention time of the standard matched the unknown compound, indicating that the high molecular weight ions (207 of the unknown compound) were due to contaminants.

(d) Mass Spectrum of Methyl vanillate (37) and the match from the NIST library



NIST # 256165 **MF:** $C_9H_{10}O_4$ **MW: 182 CAS: 3943-74-6**
Name: Methyl vanillate (37)
The closest match is shown, but it is clear that this is not a correct identification. The mass spectrum cannot be interpreted. There is a poor match between the compound and library mass spectra and this identification cannot be confirmed.

A.5.5 The Oxidation of Homovanillyl Alcohol (4)

Products of homovanillyl alcohol have been identified as vanillin (**2**), 2-methoxy-4-propyl phenol (**38**), homovanillic acid (**39**), and 4-hydroxy-3-methoxyphenyl glycol (**40**).

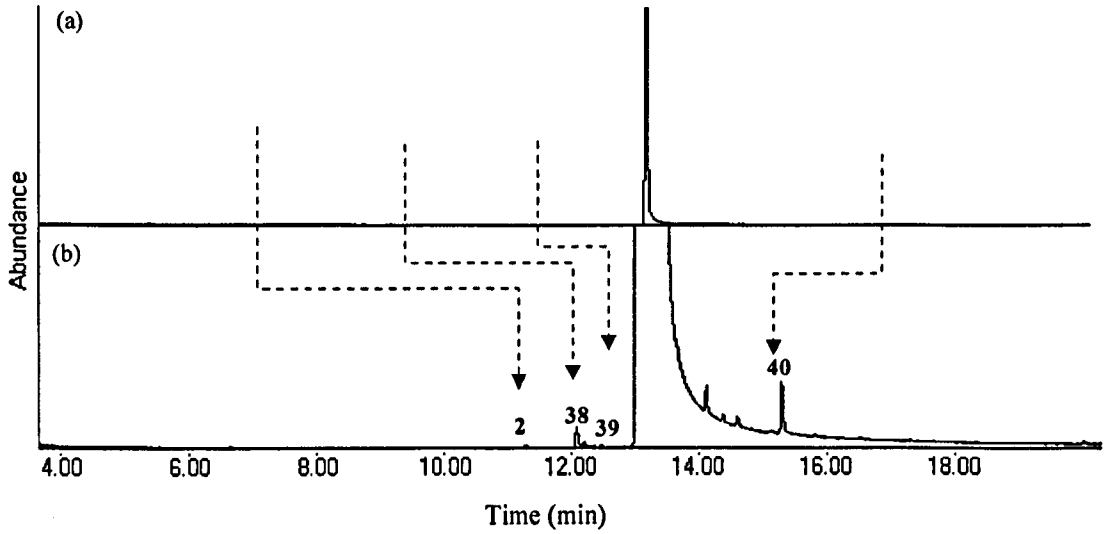
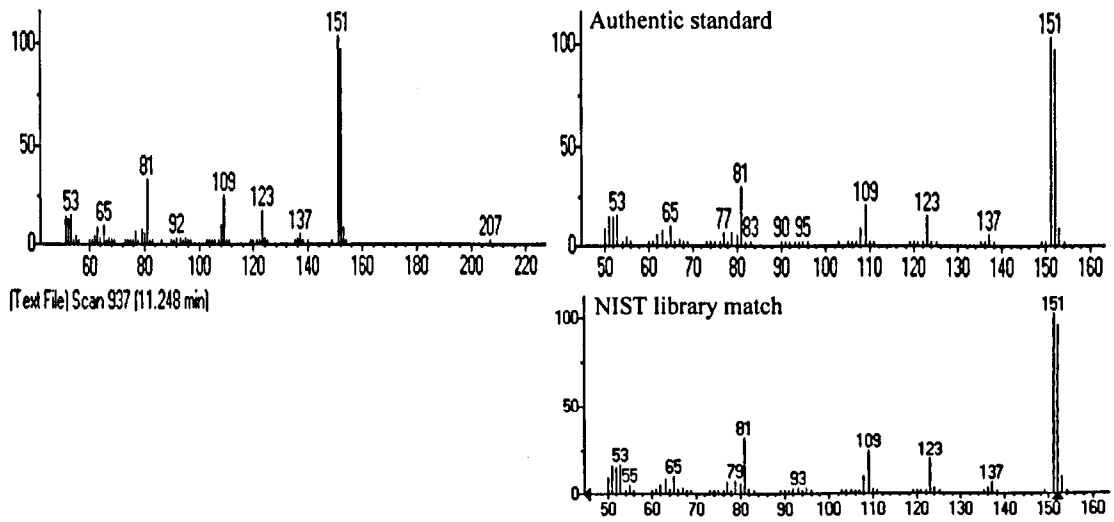


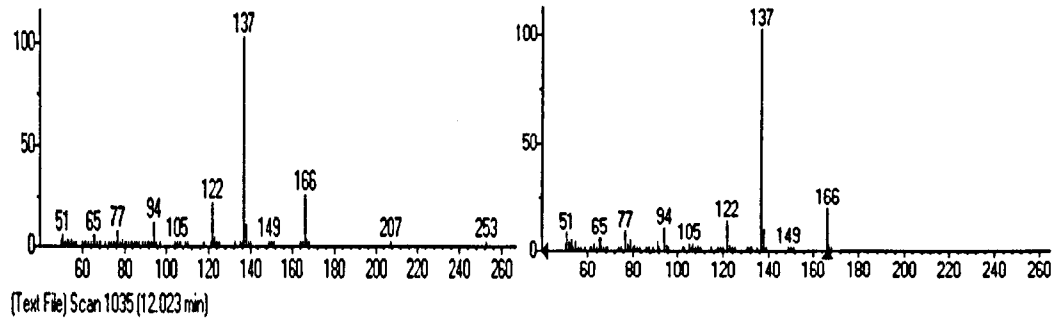
Figure A.6.5 The GCMS chromatograms of products formed after enzymatic treatment of homovanillyl alcohol by LTV. (a) The authentic standard of homovanillyl alcohol (5 mM) (b) the products formed after enzymatic treatment of homovanillyl alcohol (20 mM) by LTV in the presence of ABTS.

(a) Mass Spectrum of vanillin (2) and the match with authentic standard



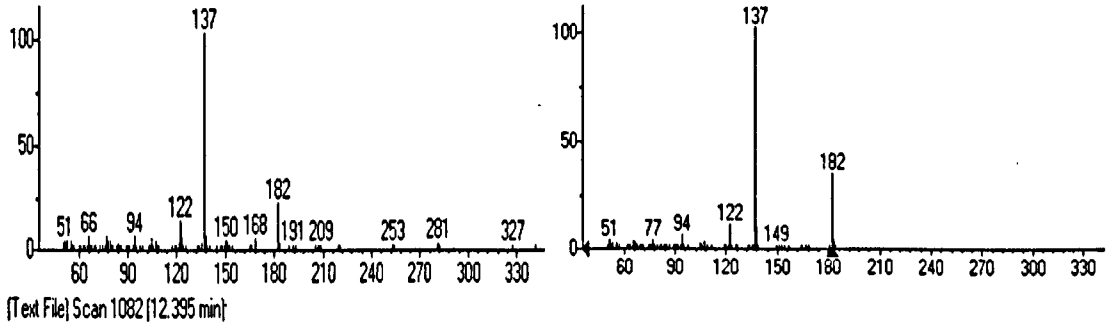
NIST # 227894 MF: $C_8H_8O_3$ MW: 152 CAS: 121-33-5
Name: 4-hydroxy-3-methoxybenzaldehyde (Vanillin) (2)
The retention time of the standard matched the unknown compound, indicating that the high molecular weight ions (207) were due to contaminants.

(b) Mass Spectrum of 2-methoxy-4-propyl phenol (38) and the match from the NIST library



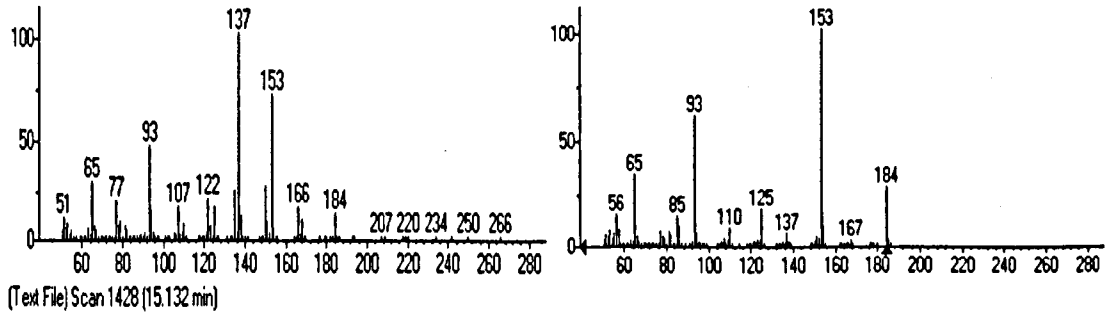
NIST # 135362 **MF:** $C_{10}H_{14}O_2$ **MW:** 166 **CAS:** 2785-87-7
Name: 2-methoxy-4-propyl phenol (38)
This seems to be a reasonable match. However, in the absence of a standard, the identification remains to be confirmed.

(c) Mass Spectrum of Homovanillic acid (39) and the match from the NIST library



NIST # 248367 **MF:** $C_9H_{10}O_4$ **MW:** 182 **CAS:** 306-08-01
Name: Homovanillic acid (39)
There is a poor match between the compound and the library mass spectra and this identification cannot be confirmed.

(d) Mass Spectrum of 4-hydroxy-3-methoxyphenyl glycol (40) and the match from the NIST library

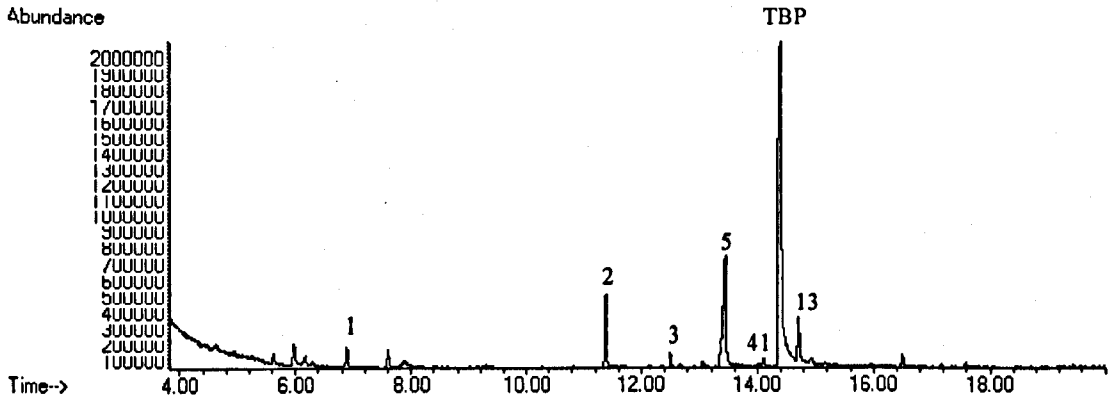


NIST # 126177 **MF:** $C_9H_{12}O_4$ **MW:** 184
Name: 4-hydroxy-3-methoxyphenyl glycol (40)
The closest match is shown, but it is clear that this is not a correct identification. The mass spectrum cannot be interpreted.

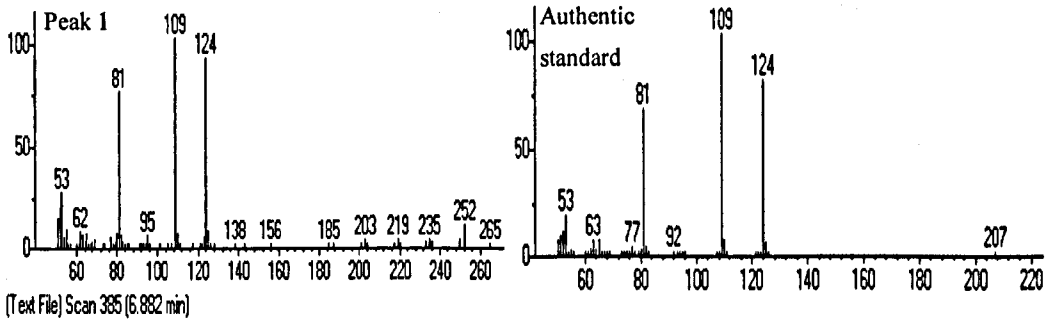
Appendix A.6 Laccase Mediator System

GCMS analysis of the products formed after enzymatic treatment of sodium lignosulphonate by LTV and mediated by different synthetic mediator nominated as: 2,2,6,6-Tetramethylpiperidin-1-yloxy (TEMPO), violuric acid (VLA), 1-hydroxybenzotriazole (HBT) and N-hydroxyphthalimide (HPI)

A.6.1 Chromatogram of Aqueous Ethyl Acetate Extract Fraction of the LTV-catalyzed reaction Mediated by TEMPO

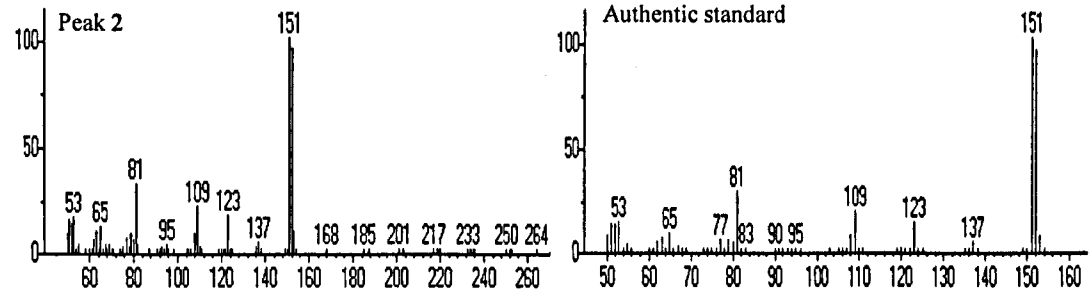


(a) Mass Spectrum of guaiacol (1) and the match with authentic standard



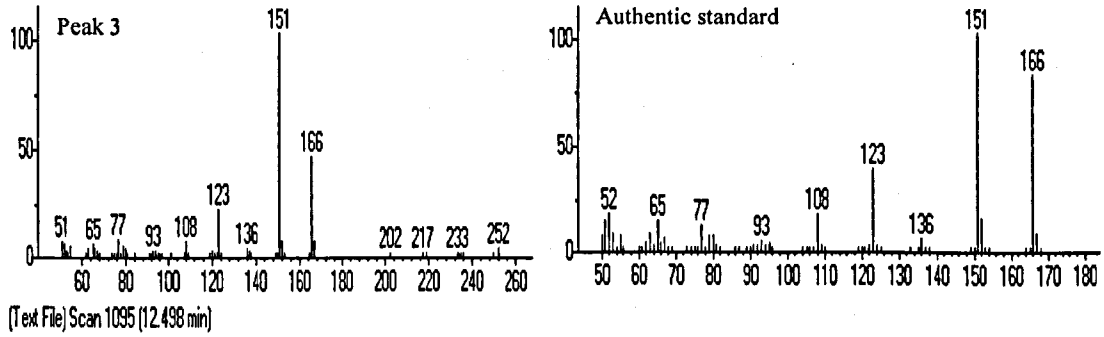
NIST # 20584 **MF:** $C_7H_8O_2$ **MW:** 124 **CAS:** 90-05-1
Name: 2-methoxy phenol (Guaiacol) (1)
The retention time of the standard matched the unknown compound, indicating that the high molecular weight ions (207 of the authentic standard and 138, 156, 185, 203, 219, 235, 252 and 265 of the unknown compound) were due to contaminants.

(b) Mass Spectrum of vanillin (2) and the match with authentic standard



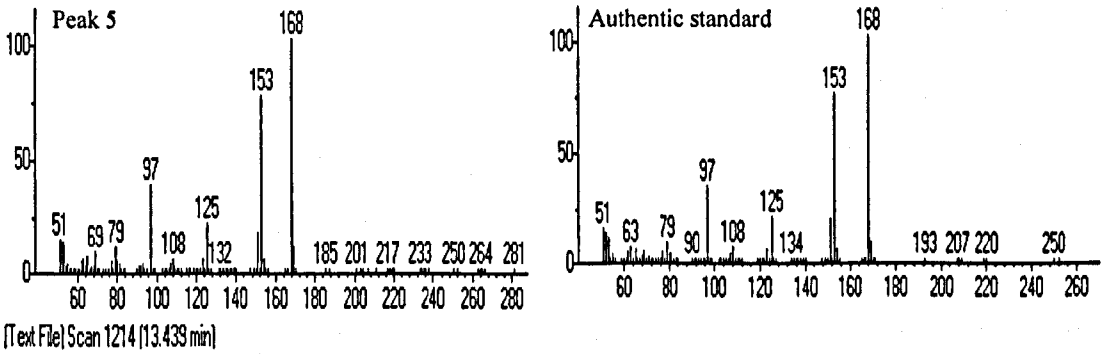
NIST # 227894 **MF:** $C_8H_8O_3$ **MW:** 152 **CAS:** 121-33-5
Name: 4-hydroxy-3-methoxybenzaldehyde (Vanillin) (2)
The retention time of the standard matched the unknown compound, indicating that the high molecular weight ions (168, 185, 201, 217, 233, 250 and 264 of the unknown compound) were due to contaminants.

(c) Mass Spectrum of acetovanilline (3) and the match with authentic standard



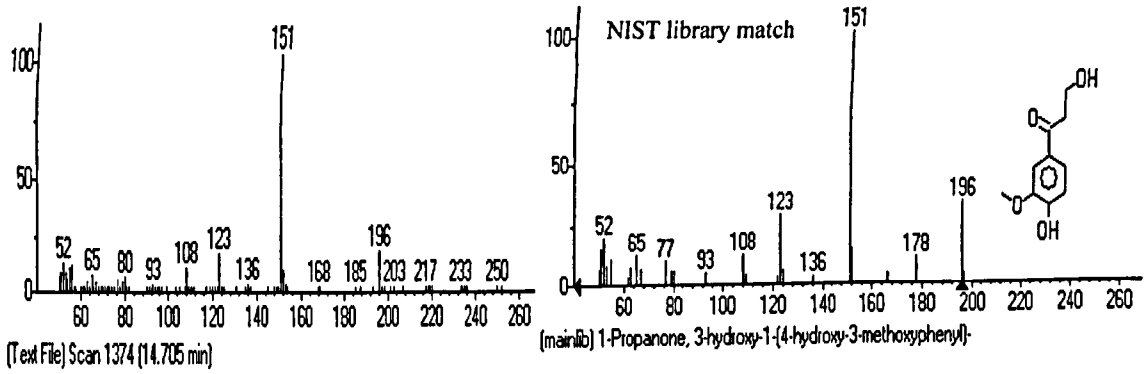
NIST # 352840 **MF:** $C_9H_{10}O_3$ **MW:** 166 **CAS:** 498-02-2
Name: 1-(4-hydroxy-3-methoxyphenyl)-ethanone (Acetovanillone) (3)
The retention time of the standard matched the unknown compound, indicating that the high molecular weight ions (202, 217, 233 and 252) were due to contaminants.

(d) Mass Spectrum of vanillic acid (5) and the match with authentic standard



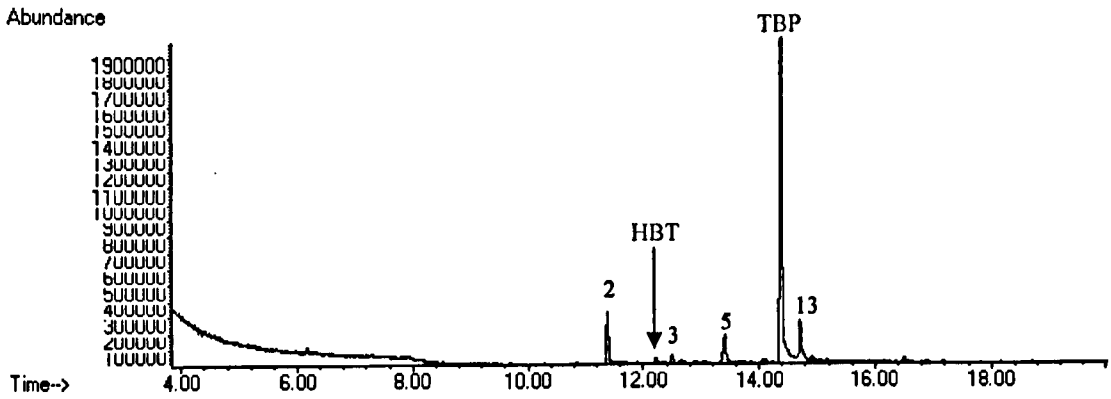
NIST # 6514 **MF:** $C_8H_8O_4$ **MW:** 168 **CAS:** 121-34-6
Name: 4-hydroxy-3-methoxybenzoic acid (Vanillic acid) (5)
The retention time of the standard matched the unknown compound, indicating that the high molecular weight ions (193, 207, 220 and 250 of the authentic standard and 185, 201, 217, 233, 250, 264 and 281 of the unknown compound) were due to contaminants.

(e) Mass Spectrum of 3-hydroxy-1-(4-hydroxy-3-methoxyphenyl)-1-Propanone (13) and the match with the NIST library

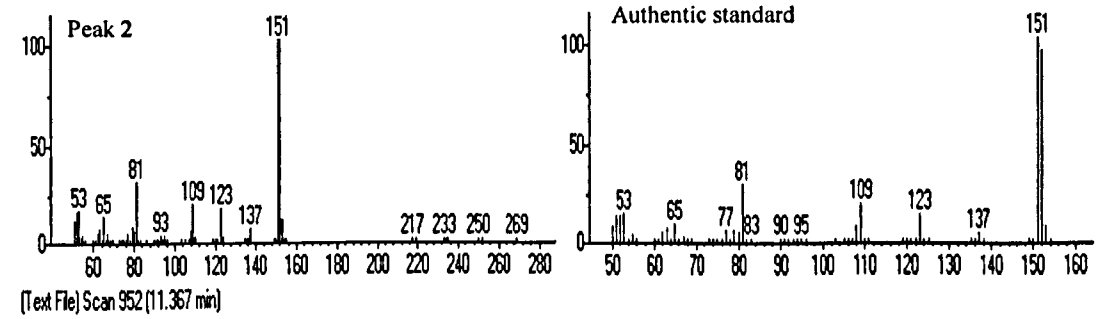


NIST # 8701 **MF:** $C_{10}H_{12}O_4$ **MW:** 196 **CAS:** 2196-18-1
Name: 3-hydroxy-1-(4-hydroxy-3-methoxyphenyl)propan-1-one (13)
This seems to be reasonable match. However, in the absence of a standard the identification remains to be confirmed.

A.6.2 Chromatogram of Aqueous Ethyl Acetate Extract Fraction of the LTV-catalyzed reaction Mediated by HBT

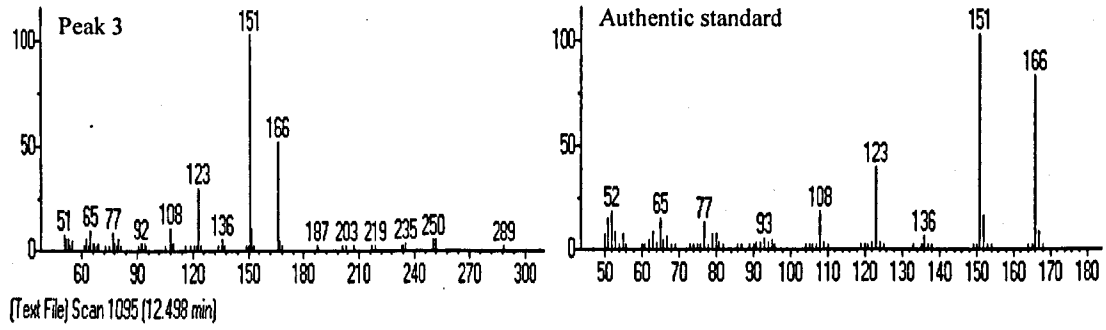


(a) Mass Spectrum of vanillin (2) and the match with authentic standard



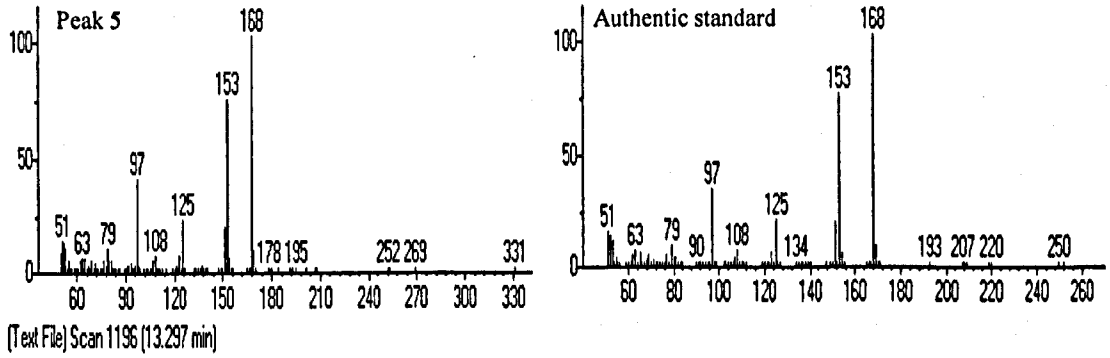
NIST # 227894 **MF:** $C_8H_8O_3$ **MW:** 152 **CAS:** 121-33-5
Name: 4-hydroxy-3-methoxybenzaldehyde (Vanillin) (2)
The retention time of the standard matched the unknown compound, indicating that the high molecular weight ions (217, 233, 250 and 269 of the unknown compound) were due to contaminants.

(b) Mass Spectrum of acetovanillone (3) and the match with authentic standard



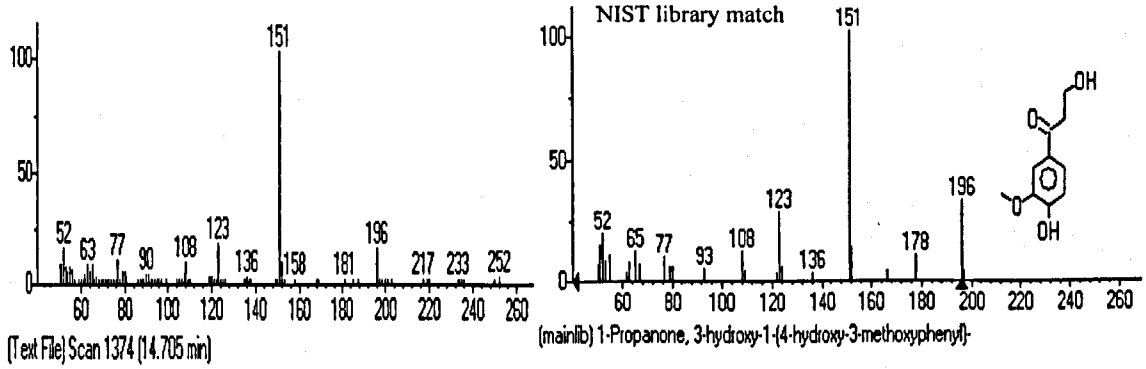
NIST # 352840 **MF:** $C_9H_{10}O_3$ **MW:** 166 **CAS:** 498-02-2
Name: 1-(4-hydroxy-3-methoxyphenyl)-ethanone (Acetovanillone) (3)
The retention time of the standard matched the unknown compound, indicating that the high molecular weight ions (187, 203, 219, 235, 250 and 289) were due to contaminants.

(c) Mass Spectrum of vanillic acid (5) and the match with authentic standard



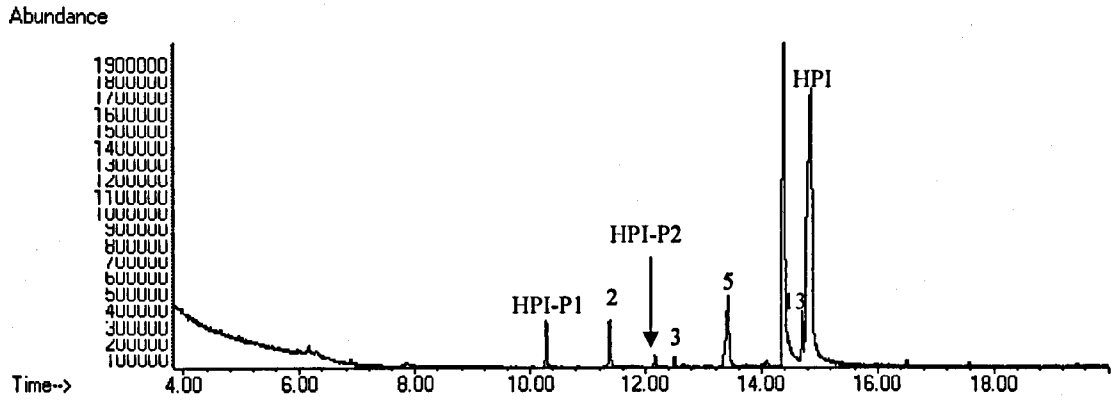
NIST # 6514 **MF:** $C_8H_8O_4$ **MW:** 168 **CAS:** 121-34-6
Name: 4-hydroxy-3-methoxybenzoic acid (Vanillic acid) (5)
The retention time of the standard matched the unknown compound, indicating that the high molecular weight ions (193, 207, 220 and 250 of the authentic standard and 178, 195, 252, 269 and 331 of the unknown compound) were due to contaminants.

(d) Mass Spectrum of Compound 4 Peak and 3-hydroxy-1-(4-hydroxy-3-methoxyphenyl)-1-Propanone (13) from the NIST library

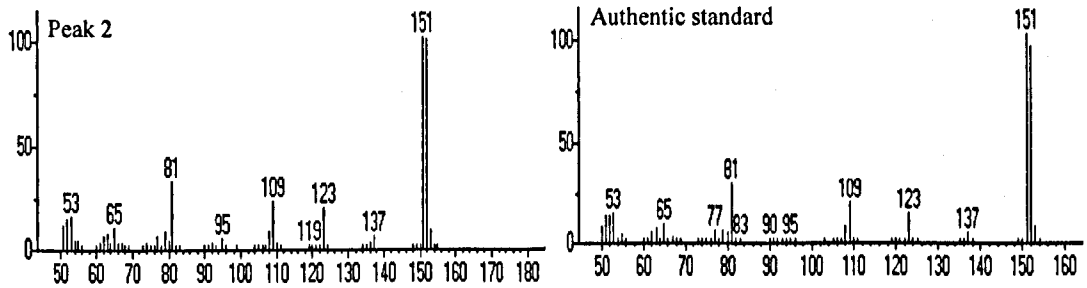


NIST # 8701**MF:** $C_{10}H_{12}O_4$ **MW:** 196 **CAS:** 2196-18-1
Name: 3-hydroxy-1-(4-hydroxy-3-methoxyphenyl)propan-1-one (13)
This seems to be reasonable match. However, in the absence of a standard the identification remains to be confirmed.

A.6.3 Chromatogram of Aqueous Ethyl Acetate Extract Fraction of the LTV-catalyzed reaction Mediated by HPI

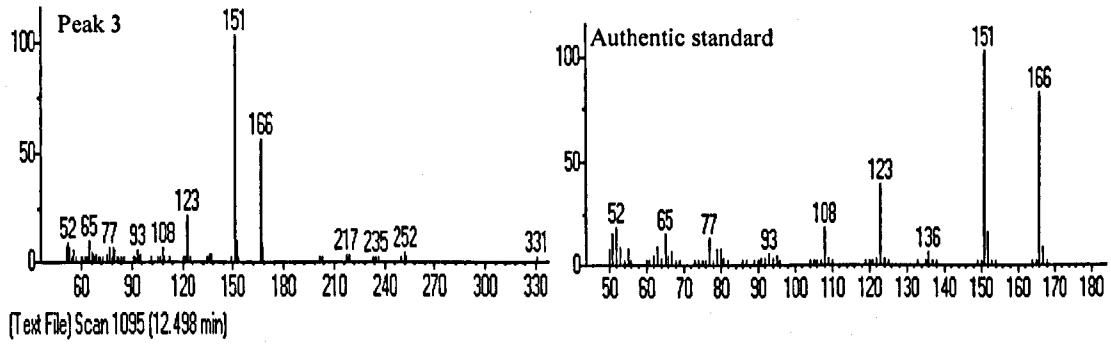


(a) Mass Spectrum of vanillin (2) and the match with authentic standard



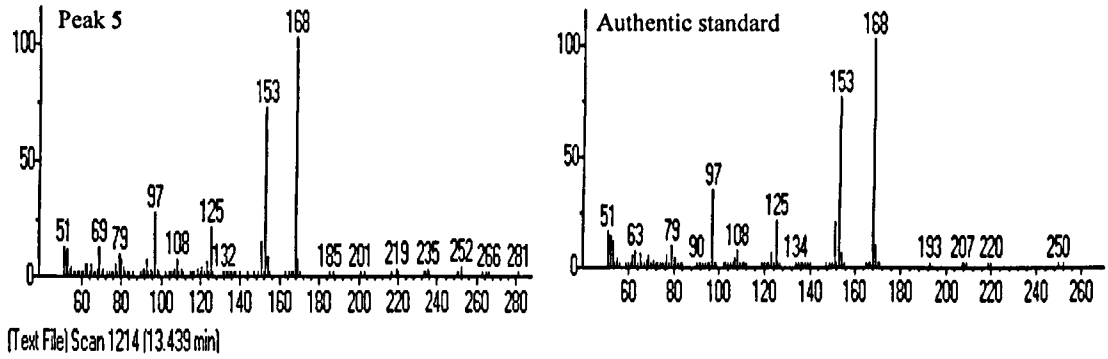
NIST # 227894 MF: $C_8H_8O_3$ MW: 152 CAS: 121-33-5
Name: 4-hydroxy-3-methoxybenzaldehyde (Vanillin) (2)
The retention time of the standard matched the unknown compound.

(b) Mass Spectrum of acetovanillone (3) and the match with authentic standard



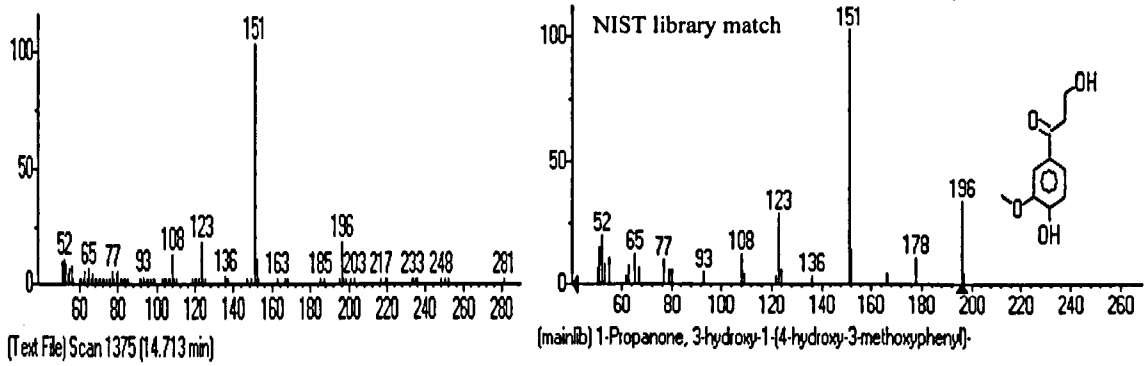
NIST # 352840 MF: $C_9H_{10}O_3$ MW: 166 CAS: 498-02-2
Name: 1-(4-hydroxy-3-methoxyphenyl)-ethanone (Acetovanillone) (3)
The retention time of the standard matched the unknown compound, indicating that the high molecular weight ions (217, 235, 252 and 331) were due to contaminants.

(c) Mass Spectrum of vanillic acid (5) and the match with authentic standard



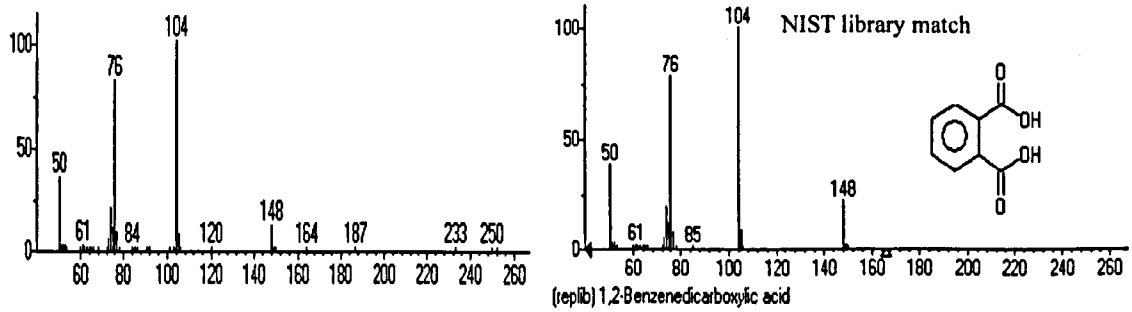
NIST # 6514 **MF:** $C_8H_8O_4$ **MW:** 168 **CAS:** 121-34-6
Name: 4-hydroxy-3-methoxybenzoic acid (Vanillic acid) (5)
The retention time of the standard matched the unknown compound, indicating that the high molecular weight ions (193, 207, 220 and 250 of the authentic standard and 185, 201, 219, 235, 252, 266 and 281 of the unknown compound) were due to contaminants.

(d) Mass Spectrum of 3-hydroxy-1-(4-hydroxy-3-methoxyphenyl)-1-Propanone (13) and match with the NIST library



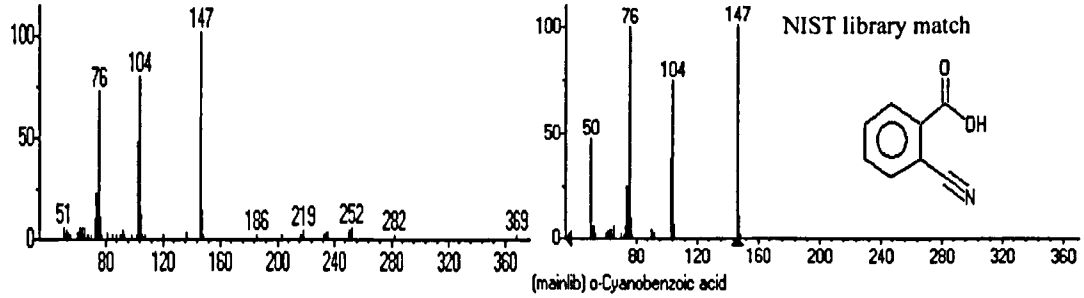
NIST # 8701**MF:** $C_{10}H_{12}O_4$ **MW:** 196 **CAS:** 2196-18-1
Name: 3-hydroxy-1-(4-hydroxy-3-methoxyphenyl)propan-1-one (13)
This seems to be reasonable match. However, in the absence of a standard the identification remains to be confirmed.

(e) Mass Spectrum of 1,2-Benzenedicarboxylic acid (HPI-P1) and match with the NIST library



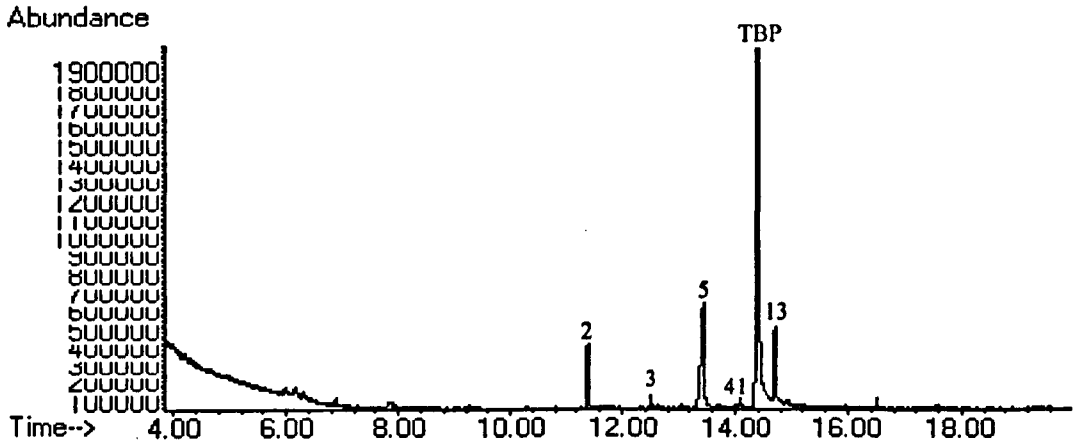
NIST # 290999 **MF:** $C_8H_6O_4$ **MW:** 166 **CAS:** 88-99-3
Name: 1,2-Benzenedicarboxylic acid (HPI-P1)
This seems to be reasonable match. However, in the absence of a standard the identification remains to be confirmed. The high molecular weight ions (120, 164, 187, 233 and 250) were due to contaminants.

(f) Mass Spectrum of o-Cyanobenzoic acid (HPI-P2) and match with the NIST library

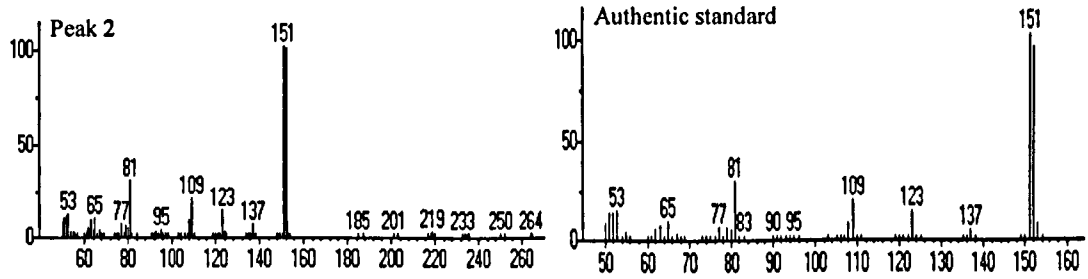


NIST # 134862 MF: $C_8H_5O_2$ MW: 147 CAS: 3839-22-3
Name: o-Cyanobenzoic acid (HPI-P2)
This seems to be reasonable match. However, in the absence of a standard the identification remains to be confirmed. The high molecular weight ions (186, 219, 252, 282 and 369) were due to contaminants.

A.6.4 Chromatogram of Aqueous Ethyl Acetate Extract Fraction of the LTV-catalyzed reaction Mediated by VLA

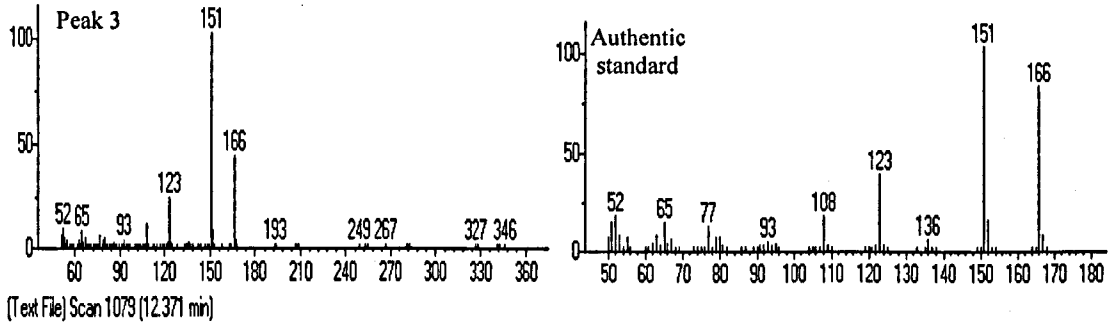


(a) Mass Spectrum of vanillin (2) and the match with authentic standard



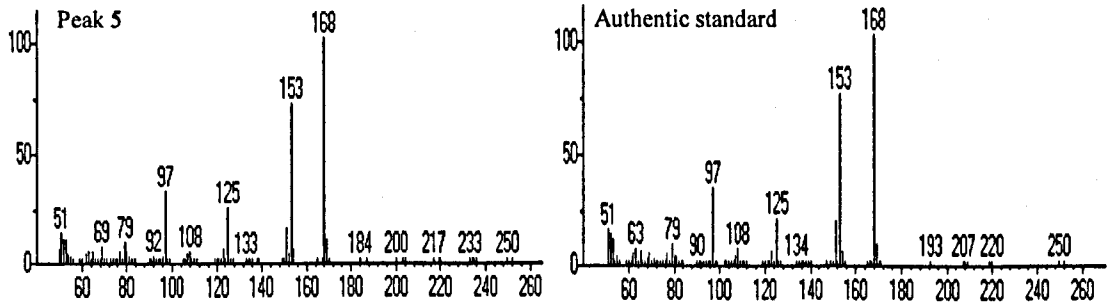
NIST # 227894 MF: $C_8H_8O_3$ MW: 152 CAS: 121-33-5
Name: 4-hydroxy-3-methoxybenzaldehyde (Vanillin) (2)
The retention time of the standard matched the unknown compound, indicating that the high molecular weight ions (185, 201, 219, 233, 250 and 264 of the unknown compound) were due to contaminants.

(b) Mass Spectrum of acetovanilline (3) and the match with authentic standard



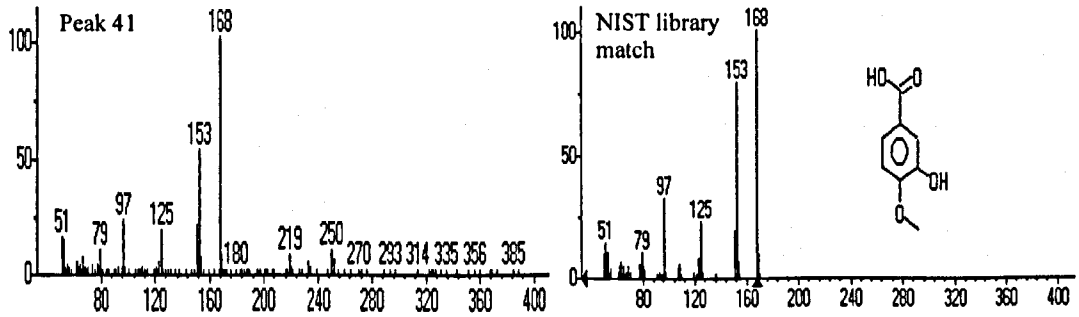
NIST # 352840 **MF:** $C_9H_{10}O_3$ **MW:** 166 **CAS:** 498-02-2
Name: 1-(4-hydroxy-3-methoxyphenyl)-ethanone (Acetovanillone) (3)
The retention time of the standard matched the unknown compound, indicating that the high molecular weight ions (193, 249, 267, 327 and 346) were due to contaminants.

(c) Mass Spectrum of vanillic acid (5) and the match with authentic standard



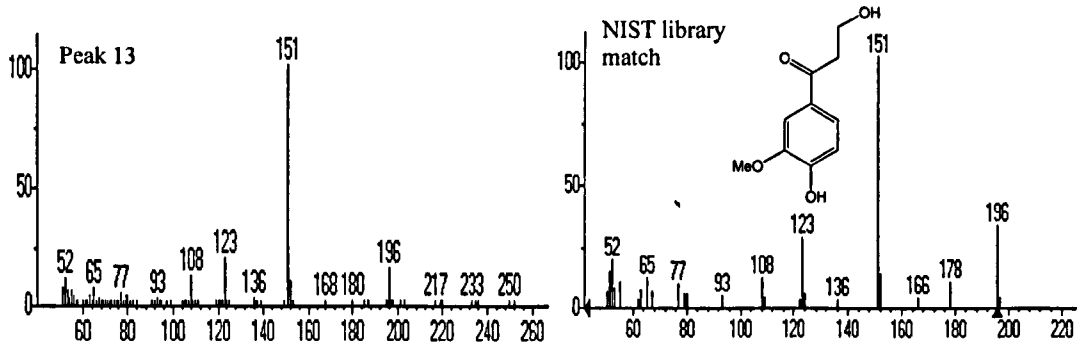
NIST # 6514 **MF:** $C_8H_8O_4$ **MW:** 168 **CAS:** 121-34-6
Name: 4-hydroxy-3-methoxybenzoic acid (Vanillic acid) (5)
The retention time of the standard matched the unknown compound, indicating that the high molecular weight ions (193, 207, 220 and 250 of the authentic standard and 184, 200, 217, 233 and 250 of the unknown compound) were due to contaminants.

(d) Mass Spectrum of isovanillic acid (41) and the match with the NIST library



NIST # 133825 **MF:** $C_8H_8O_4$ **MW:** 168 **CAS:** 645-08-9
Name: 3-hydroxy-4-methoxybenzoic acid (Isovanillic acid) (41)
The retention time of the NIST library matched the unknown compound, indicating that the high molecular weight ions (180, 219, 250, 270, 293, 314, 335, 356 and 385 of the unknown compound) were due to contaminants. However, in the absence of a standard the identification remains to be confirmed.

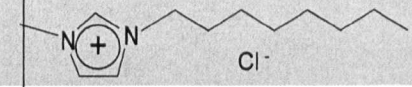
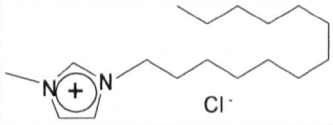
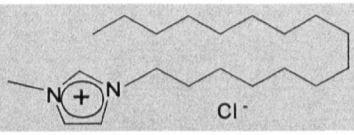
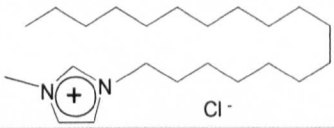
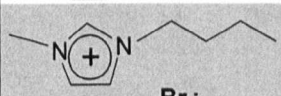
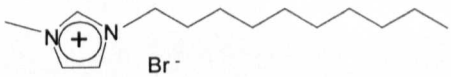
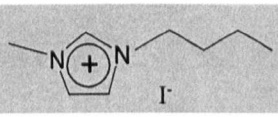
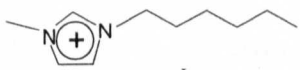
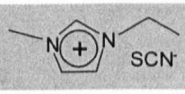
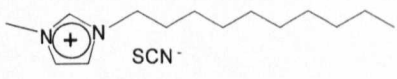
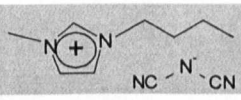
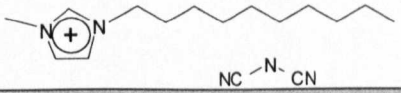
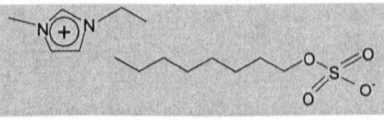
(g) Mass Spectrum of 3-hydroxy-1-(4-hydroxy-3-methoxyphenyl)-1-Propanone (13) and match with the NIST library

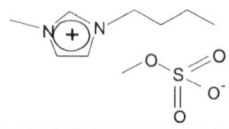
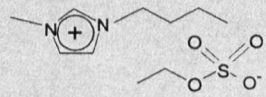
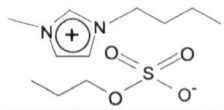
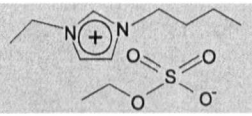
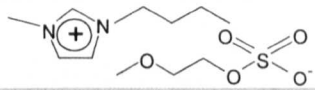
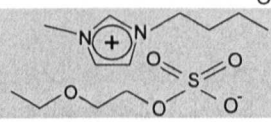
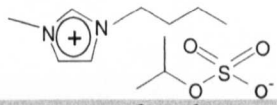
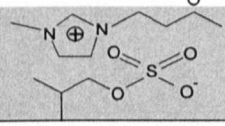


NIST # 8701MF: $C_{10}H_{12}O_4$ MW: 196 CAS: 2196-18-1
Name: 3-hydroxy-1-(4-hydroxy-3-methoxyphenyl)propan-1-one (13)
This seems to be reasonable match. However, in the absence of a standard the identification remains to be confirmed.

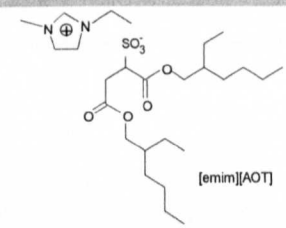
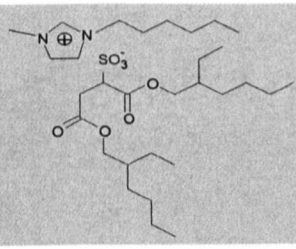
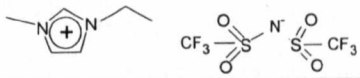
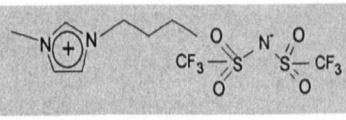
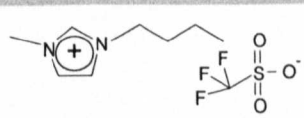
Appendix A.7 List of ionic liquids used in this study

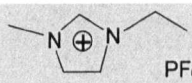
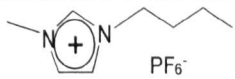
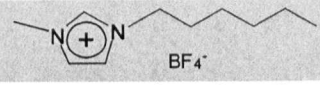
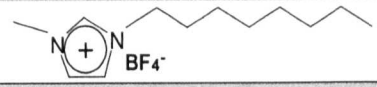
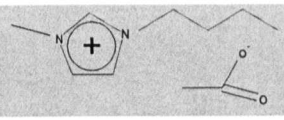
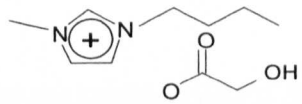
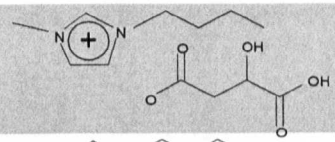
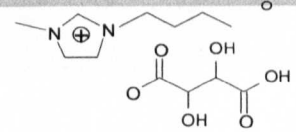
A.7.1 Imidazolium Based Ionic Liquid

IMIDAZOLIUM BASED IONIC LIQUID (Halides anion)				
Cation	**Anion**	**Miscibility**	**Chemical Formula**	**Phase**
[C_1mim]	[Cl]	Water miscible		Liquid
[C_8mim]	[Cl]	Water miscible		Liquid
[C_{13}mim]	[Cl]	Water miscible		Solid
[C_{16}mim]	[Cl]	Water miscible		Solid
[C_{18}mim]	[Cl]	Water immiscible		Solid
[C_4mim]	[Br]	Water immiscible		Liquid
[C_{10}mim]	[Br]	Water immiscible		Liquid
[C_4mim]	[I]	Water miscible		Liquid
[C_6mim]	[I]	Water miscible		Liquid
IMIDAZOLIUM BASED IONIC LIQUIDS (Thiocyanates and dicyanamides anions)				
Cation	**Anion**	**Miscibility**	**Chemical Formula**	**Phase**
[C_2mim]	[SCN]	Water miscible		Liquid
[C_{10}mim]	[SCN]	Water miscible		Liquid
[C_4mim]	[$N(CN)_2$]	Water miscible		Liquid
[C_{10}mim]	[$N(CN)_2$]	Water miscible		Liquid
IMIDAZOLIUM BASED IONIC LIQUID (alkyl sulphate anion)				
Cation	**Anion**	**Miscibility**	**Chemical Formula**	**Phase**
[C_2mim]	[C_8OSO_3]	Water miscible		Liquid

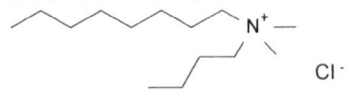
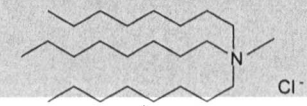
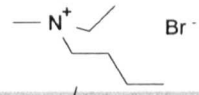
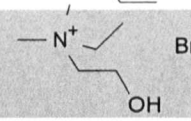
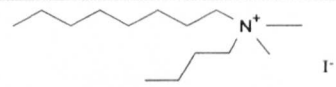
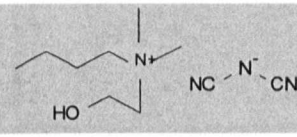
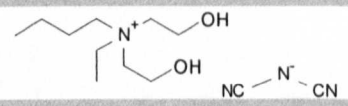
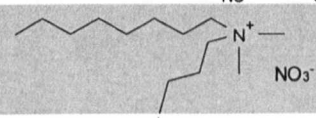
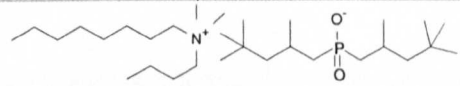
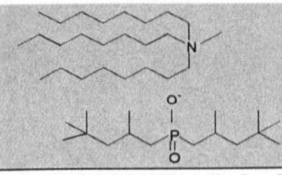
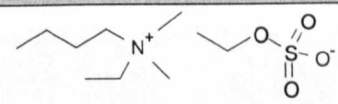
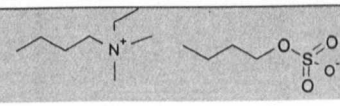
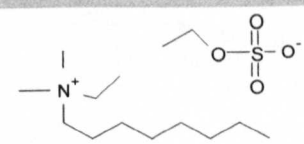
[C_4mim]	[C_1OSO_3]	Water miscible		Liquid
[C_4mim]	[C_2OSO_3]	Water miscible		Liquid
[C_4mim]	[C_3OSO_3]	Water miscible		Liquid
[C_4eim]	[C_2OSO_3]^3	Water miscible		Liquid
[C_4mim]	[$C_1OC_2OSO_3$]	Water miscible		Liquid
[C_4mim]	[$C_2OC_2OSO_3$]^3	Water miscible		Liquid
[C_4mim]	[$C_2(C_1)OSO_3$]	Water miscible		Liquid
[C_4mim]	[$C_3(C_1)OSO_3$]	Water immiscible		Solid

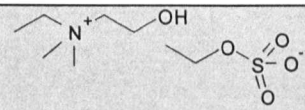
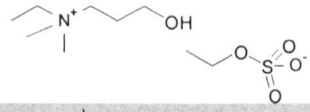
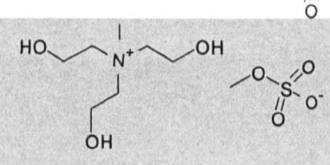
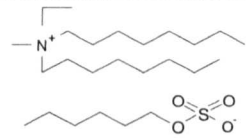
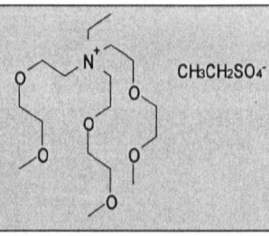
IMIDAZOLIUM BASED IONIC LIQUID ([AOT], [NTf_2] and [OTf] anions)

Cation	Anion	Miscibility	Chemical Formula	Phase
[C_2mim]	[AOT]	Water immiscible		Liquid
[C_6mim]	[AOT]	Water immiscible		Liquid
[C_2mim]	[NTf_2]	Water immiscible		Liquid
[C_4mim]	[NTf_2]	Water immiscible		Liquid
[C_4mim]	[OTf]	Water miscible		Liquid

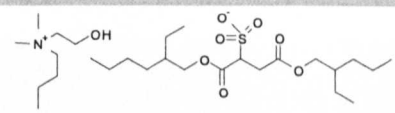
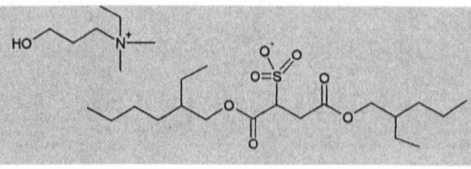
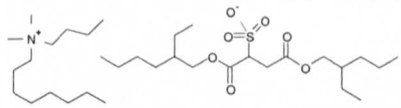
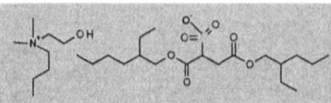
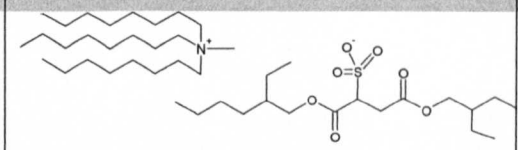
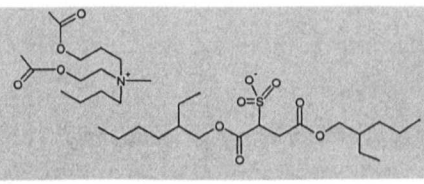
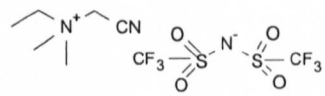
IMIDAZOLIUM BASED IONIC LIQUID ([PF_6] and [BF_4] anions)				
Cation	**Anion**	**Miscibility**	**Chemical Formula**	**Phase**
[C_2mim]	[PF_6]	Water immiscible		Solid
[C_4mim]	[PF_6]	Water immiscible		Liquid
[C_6mim]	[BF_4]	Water miscible		Liquid
[C_8mim]	[BF_4]	Water miscible		Liquid
IMIDAZOLIUM BASED IONIC LIQUID (carboxylates anion)				
Cation	**Anion**	**Miscibility**	**Chemical Formula**	**Phase**
[C_4mim]	[acetate]	Water miscible		Liquid
[C_4mim]	[lactate]	Water miscible		Liquid
[C_4mim]	[DL-malate]	Water miscible		Liquid
[C_4mim]	[L-tartrate]	Water miscible		Solid

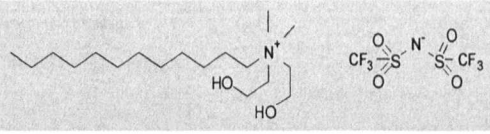
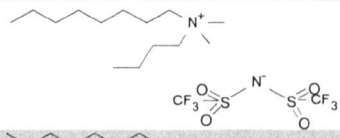
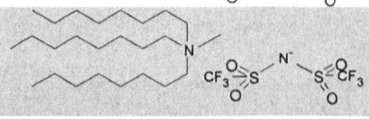
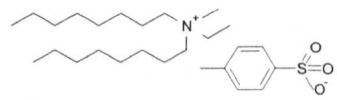
A.7.2 Quaternary Ammonium Based Ionic Liquid

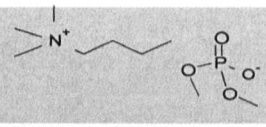
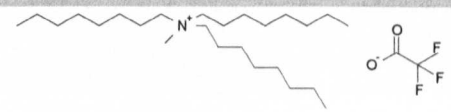
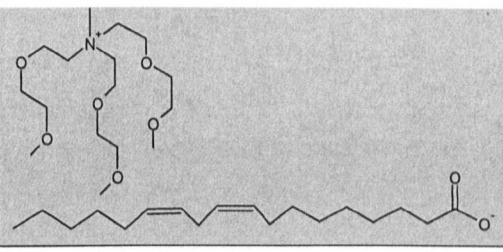
QUATERNARY AMMONIUM BASED IONIC LIQUID (Halides anion)				
Cation	**Anion**	**Miscibility**	**Chemical Formula**	**Phase**
$[N_{1148}]$	[Cl]	Water miscible		Solid
$[N_{1888}]$	[Cl]	Water immiscible		Liquid
$[N_{1124}]$	[Br]	Water miscible		Solid
$[N_{112}C_2OH]$	[Br]	Water miscible		Solid
$[N_{1148}]$	[I]	Water miscible		Solid
QUATERNARY AMMONIUM BASED IONIC LIQUID (Dicyanamides, nitrate, DIOPN anions)				
Cation	**Anion**	**Miscibility**	**Chemical Formula**	**Phase**
$[N_{114}C_2OH]$	$[N(CN)_2]$	Water miscible		Liquid
$[N_{24}(C_2OH)_2]$	$[N(CN)_2]$	Water miscible		Liquid
$[N_{1148}]$	$[NO_3]$	Water miscible		Liquid
$[N_{1148}]$	[DIOPN]	Water immiscible		Solid
$[N_{1888}]$	[DIOPN]	Water immiscible		Liquid
QUATERNARY AMMONIUM BASED IONIC LIQUID (alkyl sulphate anion)				
Cation	**Anion**	**Miscibility**	**Chemical Formula**	**Phase**
$[N_{1124}]$	$[C_2OSO_3]$	Water miscible		Solid
$[N_{1124}]$	$[C_4OSO_3]$	Water miscible		Liquid
$[N_{1128}]$	$[C_2OSO_3]$	Water miscible		Solid

$[N_{112}C_2OH]$	$[C_2OSO_3]$	Water miscible		Liquid
$[N_{112}C_3OH]$	$[C_2OSO_3]$	Water miscible		Liquid
$[N_1(C_2OH)_3]$	$[C_1OSO_3]$	Water miscible		Liquid
$[N_{1288}]$	$[C_6OSO_3]^3$	Water miscible		Solid
$[N_2(C_1OC_2OC_2)_3]$	$[CH_3CH_2OSO_3]^3$	Water miscible		Liquid

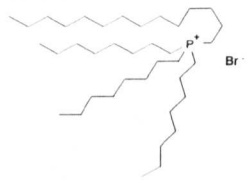
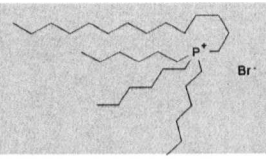
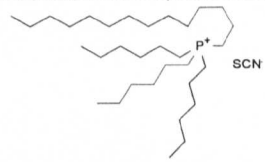
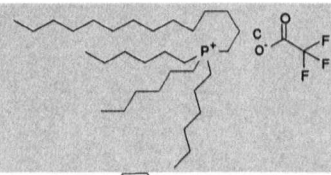
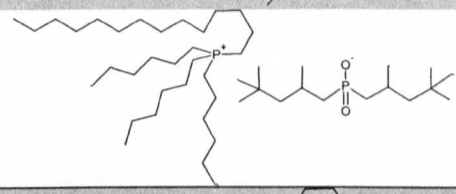
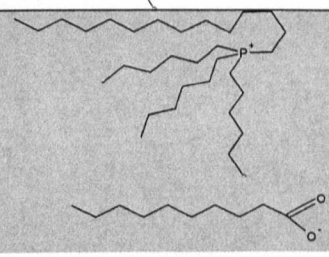
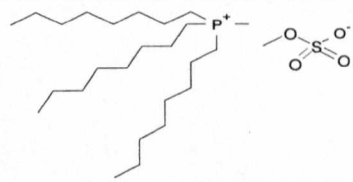
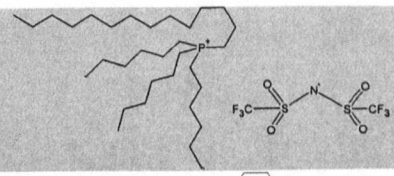
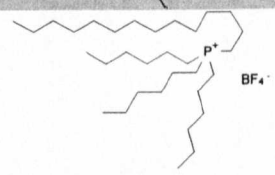
QUATERNARY AMMONIUM BASED IONIC LIQUID ([AOT], [NTf_2] and [OTs] anions)

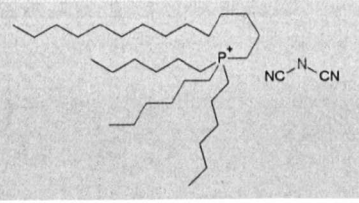
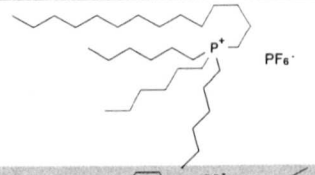
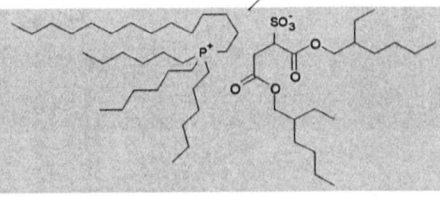
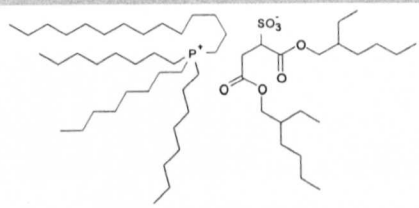
Cation	Anion	Miscibility	Chemical Formula	Phase
$[N_{112}C_2OH]$	[AOT]	Water immiscible		Liquid
$[N_{112}C_3OH]$	[AOT]	Water immiscible		Liquid
$[N_{1148}]$	[AOT]	Water immiscible		Solid
$[N_{114}C_2OH]$	[AOT]	Water immiscible		Liquid
$[N_{1888}]$	[AOT]	Water immiscible		Liquid
$[N_{14}(propylacetate)_2]$	[AOT]	Water miscible		Liquid
$[N_{112}C_1CN]$	$[NTf_2]$	Water miscible		Liquid

$[N_{1112}(C_2OH)_2]$	$[NTf_2]$	Water immiscible		Liquid
$[N_{1148}]$	$[NTf_2]$	Water immiscible		Liquid
$[N_{1888}]$	$[NTf_2]$	Water immiscible		Liquid
$[N_{1288}]$	[OTs]	Water immiscible		Solid

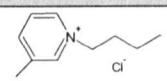
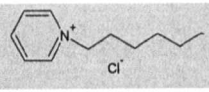
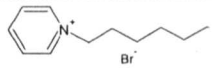
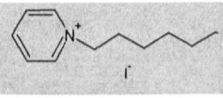
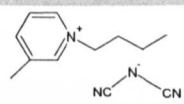
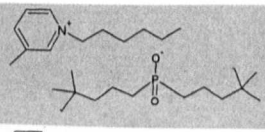
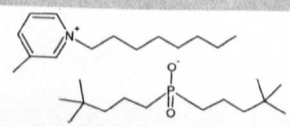
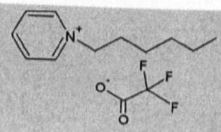
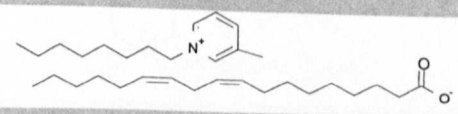
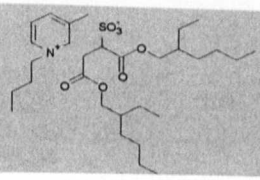
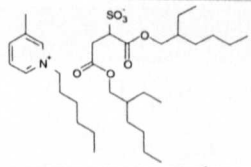
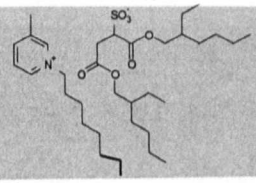
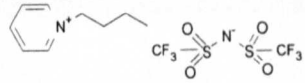
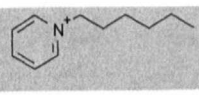
QUATERNARY AMMONIUM BASED IONIC LIQUID (phosphate, TFA and linoleate anions)				
Cation	**Anion**	**Miscibility**	**Chemical Formula**	**Phase**
$[N_{1114}]$	$[C_2H_6PO_4]$	Water miscible		Solid
$[N_{1888}]$	[TFA]	Water immiscible		Liquid
$[N_1(C_1OC_2OC_2)_3]$	[linoleate]	Water miscible		Liquid

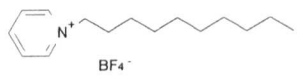
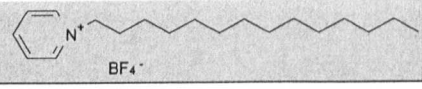
A.7.3 Phosphonium Based Ionic Liquid

PHOSPHONIUM				
Cation	**Anion**	**Miscibility**	**Chemical Formula**	**Phase**
[P_{88814}]	[Br]	Water immiscible		Liquid
[P_{66614}]	[Br]	Water immiscible		Liquid
[P_{66614}]	[SCN]	Water immiscible		Liquid
[P_{66614}]	[TFA]	Water immiscible		Liquid
[P_{66614}]	[DIOPN]	Water immiscible		Liquid
[P_{66614}]	[decanoate]	Water immiscible		Liquid
[P_{1888}]	[C_1OSO_3]	Water miscible		Solid
[P_{66614}]	[NTf_2]	Water immiscible		Liquid
[P_{66614}]	[BF_4]	Water immiscible		Solid

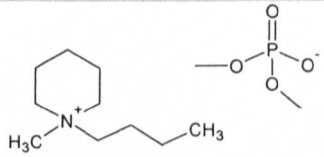
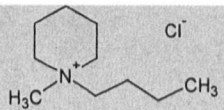
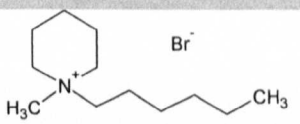
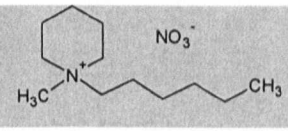
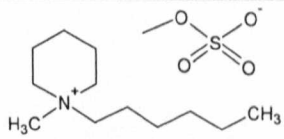
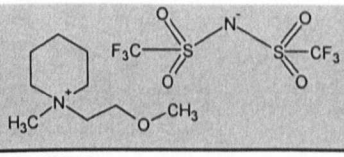
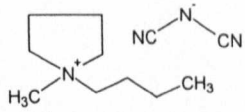
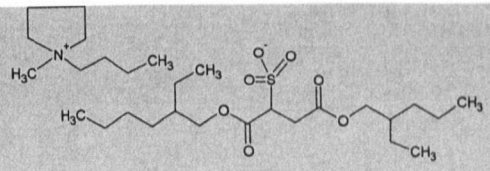
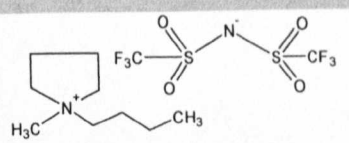
[P_{66614}]	[$N(CN)_2$]	Water immiscible		Liquid
[P_{66614}]	[PF_6]	Water immiscible		Solid
[P_{66614}]	[AOT]	Water immiscible		Liquid
[P_{88814}]	[AOT]	Water immiscible		Liquid

A.7.4 Pyridinium based Ionic Liquid

PYRIDINIUM				
Cation	**Anion**	**Misciblity**	**Chemical Formula**	**Phase**
$[C_4(3pic)]$	[Cl]	Water miscible		Solid
$[C_6\ py]$	[Cl]	Water miscible		Liquid
$[C_6\ py]$	[Br]	Water miscible		Liquid
$[C_6\ py]$	[I]	Water miscible		Solid
$[C_4(3pic)]$	$[N(CN)_2]$	Water miscible		Liquid
$[C_6(3pic)]$	[DIOPN]	Water miscible		Liquid
$[C_8(3pic)]$	[DIOPN]	Water immiscible		Solid
$[C_6\ py]$	[TFA]	Water miscible		Liquid
$[C_8(3pic)]$	[linoleate]	Water miscible		Liquid
$[C_4(3pic)]$	[AOT]	Water miscible		Liquid
$[C_6(3pic)]$	[AOT]	Water immiscible		Liquid
$[C_8(3pic)]$	[AOT]	Water immiscible		Liquid
$[C_4\ py]$	$[NTf_2]$	Water immiscible		Liquid
$[C_6\ py]$	$[BF_4]$	Water miscible		Liquid

$[C_{10}\ py]$	$[BF_4]$	Water immiscible		Solid
$[C_{14}\ py]$	$[BF_4]$	Water immiscible		Solid

A.7.5 Piperidinium and Pyrrolidinium based Ionic Liquid

PIPERIDINIUM				
Cation	**Anion**	**Miscibility**	**Chemical Formula**	**Phase**
$[N_{1,4}\ pip]$	$[C_2H_6PO_4]$	Water miscible		Solid
$[N_{1,4}\ pip]$	[Cl]	Water miscible		Liquid
$[N_{1,6}\ pip]$	[Br]	Water miscible		Liquid
$[N_{1,6}\ pip]$	$[NO_3]$	Water miscible		Liquid
$[N_{1,6}\ pip]$	$[C_1OSO_3]$	Water miscible		Liquid
$[N_1C_1OC_3\ pip]$	$[NTf_2]$	Water immiscible		Liquid
PYRROLIDINIUM				
Cation	**Anion**	**Miscibility**	**Chemical Formula**	**Phase**
$[N_{1,4}\ pyrr]$	$[N(CN)_2]$	Water miscible		Liquid
$[N_{1,4}\ pyrr]$	[AOT]	Water immiscible		Liquid
$[N_{1,4}\ pyrr]$	$[NTf_2]$	Water immiscible		Liquid