

**BIOLIGNINOLYSIS: DEGRADATION OF
IONIC LIQUID DERIVED LIGNIN BY
*RHODOCOCCUS***

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DECLARATION

I certify that this thesis represents my own work, unless otherwise stated. This work was carried out between November 2011 and September 2014 mostly in Level 6, Flowers Building, Imperial College London under the supervision of Prof Tony Cass, Dr. Jason Hallett and Dr. James Wilton-Ely. All external contributions and any information derived from other sources have been acknowledged accordingly.

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Patiently, then, persevere...

Verily, with hardship there is relief...

In remembrance of Dr. Judit Nagy

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ABSTRACT

There has been much recent interest in using ionic liquids for processing lignocellulosic biomass. While cellulose has an acknowledged application in generating biofuels, it would be valuable to use the abundant lignin present as well. *Rhodococcus* has been reported previously to degrade lignin. Therefore, it is attractive to consider a scheme in which an ionic liquid is also used to enhance the microbial breakdown of lignin (bioligninolysis). By using vanillic acid as model compound (Chapter 3), results showed that *Rhodococcus* UKMP-5M is able to degrade vanillic acid as a sole carbon source at 10mM concentration to give the highest growth rate. An oxygen-dependent reaction degrades vanillic acid into protocatechuic acid and formate in a previously undescribed metabolic pathway.

In Chapter 4, GC-MS demonstrated guaiacol as the major product of lignin degradation and the lignin degradation assay indicates that the treatment of lignin with ionic liquids assist the lignin degradation despite some ionic liquids showing a toxicity effect on the cells. Toxicological studies (Chapter 5) demonstrated different ionic liquids show varying toxicity to the bacteria. By using classical disk diffusion test in screening 16 different ionic liquids, it was revealed that the toxicity is correlated with the size of the ionic alkyl chain; however, the carbon atom count, not the structure or the distribution of those atoms in the cation, correlates directly with the toxicity. The strongest link was discovered with pH effects rather than with structure in the toxicity of acidic ionic liquids. We also propose that the octanol-water partition coefficient (K_{ow}) has the controlling impact on the toxicity of ionic liquids. Bacterial growth curves exhibited three different trends: a complete inhibition, an increase in toxicity with an increase of ionic liquid concentration and the extension of the lag phase due to bacterial adaptation.

This study established that *Rhodococcus* UKMP-5M could adapt and grow in the presence of ionic liquids with 1-ethyl-3-methylimidazolium acetate, [Emim][OAc] as the most promising candidate in designing an ionic liquid-facilitated system of bioligninolysis.

CONFERENCES

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LIST OF ABBREVIATIONS

[BF ₄]	-	tetrafluoroborate
[C ₄ C ₄ im]	-	dibutylimidazolium
[C ₆ C ₁ im]	-	1-hexyl-3-methylimidazolium
[C ₆ C ₂ im]	-	1-hexyl-3-ethylimidazolium
[C ₇ CO ₂]	-	octanoate
[C ₈ SO ₄]	-	octylsulfate
[Cl]	-	chloride
[Bmim]	-	1-butyl-3-methylimidazolium
[Emim]	-	1-ethyl-3-methylimidazolium
[Hbim]	-	1-butylimidazolium
[Hmim]	-	1-hexyl-3-methylimidazolium
[HSO ₄]	-	hydrogen sulfate
[MeSO ₄]	-	methyl sulfate
[Moemim]	-	1-methoxyethyl-3-imidazolium
[NTf ₂]	-	bis(trifluoromethylsulfonyl)imide
[OAc]	-	acetate
[OBu]	-	butyrate
[Omim]	-	1-octyl-3-methylimidazolium
[OTf]	-	trifluoromethanesulfonate
[PF ₆]	-	hexafluorophosphate
[TEA]	-	triethylammonium
[TMGC ₇]	-	diheptyltetramethylguanidinium
[TMGC ₁₂]	-	didodecyltetramethylguanidinium
[TOS]	-	tosylate
μl	-	microlitre
AFEX	-	ammonia fibre expansion
Ba(OH) ₂	-	barium hydroxide
BTEX	-	a mixture of benzene, toluene, ethylbenzene and xylene
CaO	-	calcium oxide (lime)
CH ₄	-	methane
CO ₂	-	carbon dioxide

d	-	doublet (NMR peaks)
dd	-	doublet of doublets (NMR peaks)
DDVA	-	dehydrodivanillic acid
<i>dhaA</i>	-	haloalkane dehalogenase
DIPEA	-	N,N' - diisopropylethylamine
DMSO-d6	-	deuterated dimethyl sulfoxide
DNP	-	2,4-dinitrophenol
DPPH	-	1,1-diphenyl-2-picrylhydrazyl
DyP	-	dye decolorizing peroxidase
<i>fdh</i>	-	formaldehyde dehydrogenase
FWS	-	US Fish and Wildlife Service
g	-	gram(s)
GGE	-	guaiacylglycerol- β -guaiacyl ether
h	-	hour(s)
H ₂ O ₂	-	hydrogen peroxide
K _{ow}	-	octanol-water partition coefficients
L	-	litre(s)
LiP	-	lignin peroxidase
m	-	metre(s)
mAU	-	mili-absorbance units
Mbp	-	mega base pairs
MCPB	-	4-(4-chloro-2-methylphenoxy)butanoic acid
MeCN	-	acetonitrile
MIC	-	minimal inhibitory concentration
min	-	minute(s)
ml	-	millilitre(s)
mM	-	milimolar
mm	-	milimetre(s)
MnP	-	manganese peroxidase
MTBE	-	methyl tert-butyl ether
NaCl	-	sodium chloride
NADH	-	nicotinamide adenine dinucleotide
NaOH	-	sodium hydroxide

NIST	-	National Institute of Standards and Technology
OD	-	Optical Density
Pb	-	lead
R&D	-	Research and Development
RDX	-	Research Department explosive (nitroamine)
rRNA	-	ribosomal RNA
s	-	singlet (NMR peaks) / second(s)
SciDavis	-	Scientific Data Analysis and Visualization software
SDBS	-	Spectral Database for Organic Compounds
THF	-	tetrahydrofolate
TNP	-	2,4,6-trinitrophenol
TNT	-	trinitrotoluene explosive
UV-vis	-	Ultraviolet-visible
VP	-	versatile peroxidase

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CHAPTER 1

INTRODUCTION

1.1 Background

In this millennium, lignocellulose has increased its stock significantly; a rising concern over sustainable sources of energy and the need to mitigate climate change has led to a focus on biofuels as one possible solution. By 2030, the United States of America (USA) aims to reach an annual consumption of 60 billion US gallons ($\sim 227 \times 10^9$ litres) of biofuel and the European Union (EU) has set a target of 25% of transportation fuel coming from biofuel.¹ Labelled as a second generation of biofuel, lignocellulosic biomass has been identified as the major resource to fulfil this demand.^{2, 3} Besides its role as a biofuel source, lignocellulose is also recognised as a future chemical and material feedstock in the biorefinery. This renewable resource could offset the demand for petroleum-based chemicals with biobased chemicals comprising around 9% of the total chemical industry in the near future.⁴ The initial strategy of exploiting lignocellulose is mainly centred at present on its cellulose and hemicellulose components. Due to its recalcitrant properties, lignin, the third major component of lignocellulose, is treated as waste. Most of the time, lignin is burned for heat and electricity generation. However, lignin is believed to have a potential worth of more than \$242 billion in market value.⁵ This lucrative untapped market has inspired much research and development (R&D) activity as an efficient lignin breakdown route (bioligninolysis) is the key to utilisation of lignin in the biorefinery.

Based on the concept of crude oil fractionation, the biorefinery involves the production of various bioproducts from different biomass feedstocks in one systematic scheme using a combination of inter-disciplinary technologies. The ultimate goal is the omnivorous utilisation of biomass in a range of fuels,

energy, materials and chemicals. It is anticipated that the integration of biomaterial production along side biofuels will maximise profits and capitalise the whole system efficiently. This will thus ensure the stability of the bioprocess concept and attract investors and industry in general.

1.2 Lignocellulose

An understanding of the lignocellulosic matrix is required in order to grasp the challenges associated with its breakdown. However, it should be noted that the detailed structure is subject to the nature of both source species and geography. For instance, a study demonstrated that the same plant species in different locations could have variable percentages of the constituent components.⁶

1.2.1 Structure of lignocellulose

Lignocellulose comprises three primary components; cellulose, hemicellulose and lignin (Figure 1.1). There are also small amounts of pectin, terpenic oils, proteins, fatty acids as well as inorganic matter that complete the structure⁷.

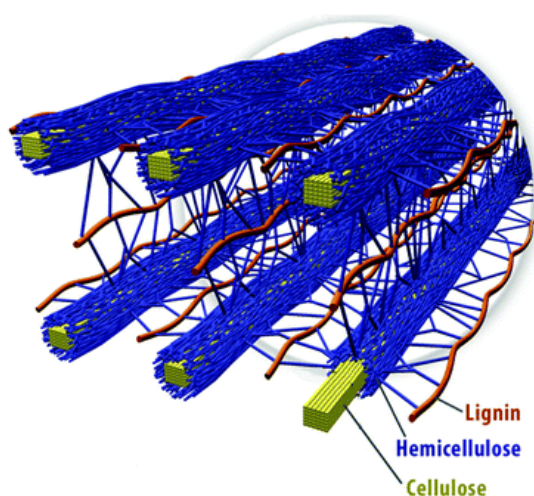


Figure 1.1 The arrangement of lignocellulosic primary components.⁸ Reproduced by permission of The Royal Society of Chemistry.

Cellulose is the most abundant component, taking up to 30 to 60% of the total structure dry weight.⁹ This homo-polysaccharide is made of linear chains of D-glucose with a degree of polymerization that varies between 500 to 25,000 monomeric units.¹⁰ Linked through β -1-4-glucosidic bonds, these chains are also inter- and intra-molecularly connected through hydrogen bonding and van der Waals interactions to form microfibrils.¹¹ Consecutive glucose units along the chains are rotated by 180° making cellobiose the repeating dimer unit. The microfibrils are in turn rigidly packed to form semi-crystalline fibres.¹² However, there are some disordered areas, which are known as amorphous regions. These are easier to degrade, unlike most regions that are resistant to hydrolysis.

The second polysaccharide component is hemicellulose, which comprises approximately 20 to 40% of the total dry weight of lignocellulose structure⁹. It is a complex heterogeneous polymer made of various pentoses (xylose and arabinose) and hexoses (mannose, glucose and galactose).⁹ In addition, uronic acids, ferulates and coumarins can also be incorporated in the hemicellulose structure.¹³ As hemicellulose is a short, branched polymer with a degree of polymerization less than 200 monomeric units,¹⁰ it is easier to hydrolyse compared to cellulose.⁹ It has a hydrophilic structure and acts as a 'glue' between cellulose and lignin.¹² In hardwood, xylan is more common¹⁴ with heteroxylan the most abundant form. Heteroxylan consists of xylose as the backbone with other compounds including acetate, arabinose and glucose. The xylan layer is formed through the hydrogen bonding with cellulose and covalent bonding with lignin.¹⁵ On the other hand, softwood has a higher composition of heteromannan, which contains several monomers including mannose, galactose and glucose.¹⁵

The third component is lignin, which contributes around 10 to 25% of the lignocellulose dry content.¹⁶ It is a complex cross-linked heteropolymer as shown in Figure 1.2. This insoluble aromatic polymer is highly irregular¹⁴ with no repeated subunits. This characteristic makes enzymatic degradation of lignin extremely difficult. At the molecular level, lignin consists of phenyl propane units such as p-coumaryl alcohol, synapyl alcohol and coniferyl

alcohol. These monolignols are incorporated into lignin as phenylpropanoids labelled hydroxyphenyl (H), syringyl (S) and guaiacyl (G).¹⁴ Softwoods commonly have a higher fraction of G whereas hardwoods and grasses mainly contain G + S. Most of the time, grass has the lowest lignin content and softwood has the highest.¹¹ Older plants tend to have higher lignin content in the cell walls to enhance rigidity and strength. Lignin has a high polarity due to the presence of various functional groups including hydroxyl, methoxyl and carbonyl.¹⁷ Even though scientists have described lignin to some extent, the irregular structure of lignin complicates matters and the understanding of lignin still has many unknowns.¹⁸ This complexity of structure is due to random polymerisation, which is driven by free phenolic radical coupling reactions under chemical, rather than rigid biochemical control¹⁹. This well-accepted lignin biosynthesis model has been challenged but the organised-mechanisms suggested do not fit the absolute structural control over lignification.^{19, 20}

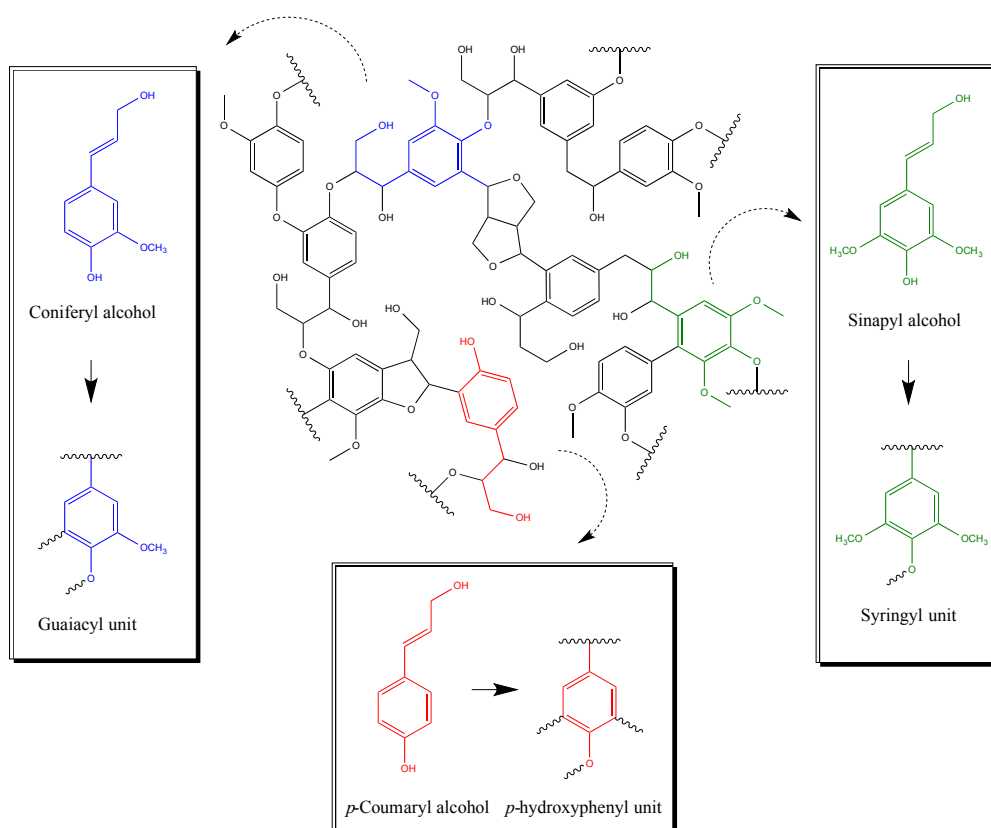


Figure 1.2 The complex structure of lignin.^{21, 22} Adapted by permissions of AIMS Press and The Royal Society of Chemistry.

1.2.2 Pretreatment of lignocellulose

Due to the complex structure of lignocellulose, pretreatment is essential in order to increase the yield and effectiveness of its bioprocessing. Pretreatment is important in breaking down the lignin 'shield' to recover the desired polysaccharides and lignin removal prior to this process is essential as it may inhibit the glycosidase enzymes during hydrolysis.²³ It has been reported that cellulases can non-specifically adsorb to the lignin resulting in low productivity of substrate hydrolysis.²⁴

The three main categories of pretreatment are physical, chemical and biological. There are certain criteria^{11, 25} that are desirable in selecting a pretreatment process. Pretreatment should meet most of these criteria in order to be a viable option for industrial scale operation. The process should preserve the lignin for further use down the biorefinery line. Pretreatment that is able to densify the lignin without a binding agent could help downstream processing and increase the biomass durability for long term storage. Pretreatment should minimise the production of inhibitors, which will affect the downstream process. The process should also consume minimal energy, be cost-effective and use less hazardous chemicals. Parameters such as temperature and pressure should be manipulated to result in low energy utilisation. Recyclable catalysts and/or solvent along with full use of pretreated products and by-products are considered to be advantageous for the process. The most vital criterion is the ability of the pretreatment to adapt to a large-scale process, 2000 tons or more of biomass daily.

Physical pretreatment usually involves mechanical processing such as milling and extrusion. An improvement in hydrolysis rates in the following stage is achieved through an increase in the surface area and improved accessibility. Mechanical processes are also normally incorporated in refining lignocellulose prior to most of other pretreatment methods. Another physical method is irradiation by gamma rays, electron beams or microwave radiation, which reduce the crystallinity of cellulose. The major drawback of most physical pretreatment is that they are highly energy intensive processes.

The second category of pretreatment is chemical pretreatment, which can be under acidic, alkaline or neutral conditions. Acidic conditions are normally achieved by introducing concentrated or diluted acid to treat the lignocellulose. This method works best in hydrolysing hemicellulose, leaving the cellulose for enzymatic conversion.²⁶ On the other hand, alkaline pretreatment degrades ester and glycosidic side chains, altering lignin structure and partial decrystallization of cellulose and hemicellulose solvation, assisting the following hydrolysis stage.^{27, 28} Organosolv pretreatment, which use organic acids, can work in both neutral and acidic conditions though the latter are preferable.²⁵ The latest approach to be employed is ionic liquid pretreatment, which can also take place in either neutral or acidic environments. This technology is favourable as it is labelled environment friendly. However, due to its status as a relatively untried technology, a lack of understanding of the mechanism is thought to be a potential barrier to the scale up process.

Another class of pretreatments is biological pretreatment. This method uses lignin-degrading enzymes such as peroxidases and laccases typically from fungi or bacteria.²⁵ Alternatively, consortia of bacteria,²⁹ which could be isolated from soil or even the guts of wood digesting creatures like termites and gribbles can be used.³⁰ Biological pretreatment is operated under mild conditions but the process is relatively slow. However, rapid advancements in biotechnology could make this pretreatment commercially viable. A genetically engineered strain of yeast, *Saccharomyces cerevisiae* is able to carry out saccharification and fermentation simultaneously by producing extracellular endoglucanase and β -glucosidase, degrading cellulose to glucose and subsequent fermentation to ethanol.³¹ In addition, some studies have engineered the yeast to degrade hemicellulose pentoses, xylose and arabinose, increasing the yield of ethanol.³²⁻³⁴

A combination of physical and chemical pretreatment could compensate for their inherent disadvantages. These physico-chemical technologies include steam explosion, ammonia fibre expansion (AFEX), liquid hot water (LHW), microwave-chemical and supercritical CO₂

pretreatments. Steam explosion, AFEX and supercritical CO₂ use the same concept; first, the lignocellulose is treated with high pressure followed by a drastic drop in pressure, triggering an explosive decompression that disrupts the lignocellulose structure.^{25, 26}

Table 1 summarizes the pros and cons of all of these pretreatment technologies. Even though it is a relatively new technology, the use of ionic liquids is very promising due to the greater yields and environmentally friendly advantages.^{35, 36} Further work should focus on cutting the overall cost and increasing the efficiency of the process. A multidisciplinary approach by combining this ionic liquids' pretreatment with biotechnological tools is one of the suggested strategies.

Table 1 List of pretreatment technologies.^{25, 26, 37}

Classification	Methods	Description	Advantages	Disadvantages
Physical	Milling	Milling and grinding, particle size (0.2–2 mm)	-No chemicals used -Scalable	-Poor sugar conversion -High energy
	Extrusion ³⁸	Screw speed up to 350 rpm, 80 °C, 40% moisture content	-Low temperature -No washing/conditioning -No effluent -Short processing time -Continuous runs possible -Low capital	-High energy -Frequent replacement of screws
	Irradiation	Microwave 680 W, 24 min	-High selectivity -Low energy -Short processing time	-High cost of reactor -High risk -Low yield
Chemical	<u>Acidic conditions</u>			
	-Dilute sulfuric acid (H ₂ SO ₄)	140–190 °C, 0.4–2 % H ₂ SO ₄ , 1–40 min	-Xylose is produced during pretreatment	-High cost of reactor -Difficult to control the reaction conditions -Toxic degradation product(s) -Expensive downstream process
	-Concentrated acids	Hydrochloric acid/ phosphoric acid/ nitric acid/ formic acid etc.	-Effective on softwood -Some are able to depolymerize cellulose without enzymes	-Corrosion -Acid recovery is costly

-Organic acids	130–190 °C, 50–90 mM of acetic acid/ fumaric acid/ maleic acid etc.	-High yield -Low energy	-Acid recovery is costly -High water usage in downstream process
-Organosolv	Methanol/ ethanol/ acetone/ ethylene glycol etc. 195 °C, 5 min	-Produces pure lignin stream -High yield	-High risk due to high pressure and flammability -Volatile solvent
-Ionic liquids	1-butyl-3-methyl hydrogen sulphate, [Bmim][HSO ₄]/ 1-ethyl-3-methylimidazolium hydrogen sulphate, [Emim][HSO ₄]	-High yield -Recyclable ionic liquid -Environmental friendly	-High cost -Relatively untried technology
<u>Alkaline conditions</u>			
-Sodium hydroxide (NaOH)	1.5% of NaOH, 144 h, 20 °C	-Lignin solubilisation	-Large usage of water -Longer residence time
-Lime (CaO)	25–160 °C, 120 min to weeks	-Cost-effective system	-Energy intensive -Longer residence time -Lime recovery is difficult
-Wet oxidation	>120 °C, less than 30 min	-Dry to dry process -No toxic residues	-High cost -Lignin oxidation makes it less dense

	<u>Neutral condition</u>			
	-Ozonolysis	Room temperature, ozone sparging	-Effective lignin removal -Atmospheric condition (low energy)	-High cost -Loss of lignin due to cleavage during pretreatment
	-Ionic liquids	1-ethyl-3-methyl, acetate [Emim][OAc]/ 1-ethyl-3-methylimidazolium chloride, [Emim][Cl]	-High yield -Recyclable ionic liquid -Environmental friendly	-High cost -Relatively untried technology
	<u>Enzymes</u>			
	-Fungi	White-/ brown-/ soft-rot fungi	-Mild conditions -Low energy	-Slow process -Large space requirement
	-Bacteria	<i>Paenibacillus macerans</i> / guts bacteria of termite and gribble / bacterial consortia ²⁹ etc.	-Mild conditions -Low energy	-Slow process -Large space requirement
Physico-chemical	Steam explosion	<i>Physical + Chemical</i> Explosive decompression + Steam and SO ₂ <i>Condition</i> 180–210 °C, 1–120 min	-Works well with both hardwood and grass biomass	-Incomplete destruction of lignocellulose

AFEX	<i>Physical + Chemical</i> Explosion + Anhydrous ammonia <i>Condition</i> 100–140 °C, 30–60 min, 60–100% moisture content	-Recyclable ammonia -Dry to dry process	-Inefficient for hardwood
Liquid-hot water (LHW)	<i>Physical + Chemical</i> High pressure + Water <i>Condition</i> 160–220 °C, 15 min	-Simple system -No external chemical	-Large usage of water -Loss of hemicellulose in water stream
Microwave-chemical ³⁹	<i>Physical + Chemical</i> Irradiation + acid/alkali/H ₂ O ₂ <i>Condition</i> 2450 MHz, 300 W, 60 min	-Production of crystalline xylose	-High risk
Supercritical CO ₂	<i>Physical + Chemical</i> Explosion + Carbonic acid <i>Condition</i> 112–165 °C, 10–60 min, 0–73% moisture content	-Safe chemical -Non-flammable -No waste stream	-High process cost

1.2.3 Bioproducts

The main and most well-established bioproduct is ethanol. The sugars broken down from the polymer of cellulose and hemicellulose are fermented and converted into ethanol. The production of bioethanol as a fuel can be traced back to 1894 in Germany and France⁴⁰ and Brazil has been using this bioproduct since 1925.⁹ However, in the early 1900s, the cost of bioethanol was substantially greater than that of fossil fuels, thus relegating this fuel to relative obscurity until the oil crisis in the 1970s shifted interest back to bioethanol.⁴⁰ Nowadays, Brazil is one of the largest producers of bioethanol with a yield of 6641 litres per hectare.⁴¹ Sugarcane and corn are the main sources of bioethanol in Brazil and USA, respectively, while Europe uses wheat and barley. All of these starch-based feedstocks are considered the first generation of biofuel. However, there is an on-going debate surrounding the use of this feedstock, which also impacts on the cost of food.⁴² The demand for biofuels is expected to increase in the future and the first generation of biofuels is generally deemed unsustainable.² Lignocellulose, labelled as the second generation of biofuels, should be able to supply this projected growth with the support of research and development (R&D). In 2013, the world's first lignocellulose-based bioethanol plant was built in Italy by Beta Renewables.⁴³

Besides biofuels, purified cellulose is used in the production of wood-free paper, cellophane, membranes, explosives and water-soluble gums. A derivative of cellulose, cellulose acetate, is the substrate for photographic film.²⁶ Lyocell is the first textile fibre made of cellulose.⁴⁴ Moreover, the production of cellulose derived commodity chemicals is well-established. Through fermentation, glutamic acid (~1.7 billion kg/year), citric acid (~1.6 billion kg/year) and lysine (~0.9 billion kg/year) are produced annually.²⁶ Several companies including Cargill and Dow Chemical Company have been converting glycerol into a high value propylene glycol.⁴⁵

The production of lactic acid in the industry is mainly based on food-based carbohydrates. The controversial practice of using food commodities as chemical resources has shifted research towards increasing the efficiency of lactic acid production from lignocellulose. Theoretically, cellulose extracted from lignocellulose can replace starch as a source of glucose, producing glucose fermented products like vitamins, xanthan and organic acids. Genencor and Du Pont have patented a cost-effective fermentative route to 1,3-propanediol. In fact, this process is superior to the petroleum based process because it reduces greenhouse gases (GHG) emissions by 20% and consumes 40% less energy.⁴⁶ Dow Chemical Company and Solvay are looking at the possibility of converting biobased glycerol into epichlorohydrin for epoxy resin production.⁴⁵

Hemicellulose is commercially used in the production of films, coatings and hydrogels.^{47, 48} The most abundant monomer of hemicellulose, xylose, is the precursor of xylitol and furfural. If these components can be synthesized, it will be highly beneficial to the biorefinery system. While xylitol is an additive in the formulation of toothpaste and chewing gum and works as an antimicrobial agent, furfural is used in the production of varnishes and pesticides.⁴⁹ Moreover, xylitol is marketable in the pharmaceutical industry due to its anticariogenic properties. Xylitol is not only useful in dental treatment but has been shown to be effective in treating diabetes and osteoporosis.⁵⁰ A recent study has revealed the potential of hemicellulose as a profitable commercial substrate for lactic acid production.⁵¹

1.3 Lignin

Unlike cellulose and hemicellulose, lignin's recalcitrance has hindered its development as a biomaterial. Cellulose and hemicellulose, collectively known as holocellulose, are considered more valuable and well understood compared to lignin. The global pulp and paper industry is believed to produce approximately 50 million tons of lignin every year. Unfortunately, lignin is normally burned to generate heat and electricity as an integrated power supply for the factory. A life cycle assessment revealed that this strategy is deemed counter-productive as lignin would be better used to replace acrylic as the binding agent in a biorefinery system.⁵² The conversion of lignin into chemicals is more efficient in term of mass and atom economy.⁷ Besides, the use of lignin as a chemical substrate could double the income compared to its use in power generation.⁵³

1.3.1 Lignin as a biomaterial

Instead of generating heat and electricity, it is possible to treat the lignin with sulfonic acid to produce liginosulfonate. 1.2 million tons of liginosulfonate are produced every year, which corresponds to 10% of the kraft lignin waste in the pulp industry.⁵⁴ Half of the liginosulfonate production is applied as an admixture in concrete formulation.⁵⁵ Apart from this use, it is also used as a stabilizer and a dispersant.⁵⁴ Several studies have demonstrated the potential of liginosulfonate as a substrate in the production of pesticides, vanillin, dyes, emulsifiers, and industrial cleaners.⁵⁶

However, lignin offers huge potential as a biomaterial either as lignin itself or its aromatic degradation products. Adding value to lignin is vital to increase the viability of a lignocellulose-based biorefinery. Promising studies have shown a mixture of lignin with protein or starch could achieve some desired traits to such composites like water-resistance, better mechanical properties, an increase in thermal stability and improved tensile strength.⁵⁷⁻⁵⁹ By incorporating lignin into polyhydroxyalkanoates (PHA), the biodegradability

of this polymer can be increased while improving its thermal stability.^{56, 60} A study has also demonstrated that lignin could increase the adhesive strength of epoxy resins.⁶¹ In addition, polymer blending of lignin with polyolefin or vinyl polymer is being studied for its compatibility as a UV and thermal stabiliser.⁵⁶ Lignin could also be employed as a filler in natural and synthetic rubbers.⁶²

Since lignin is built of aromatic structures, the degradation (valorisation) of lignin could potentially produce many highly valued, low molecular weight chemicals. In Norway, the company Borregaard converts woody biomass into cellulose and lignosulfonate, which is further processed into vanillin.⁵³ Vanillin is the world's most consumed flavouring agent, with a market value of USD 228 million.⁶³ The largest demand for aromatic chemicals comes in the form of benzene, toluene and xylene, collectively known as BTX. This group of chemicals is 60% of the total volume of the aromatic-chemical industry.⁶³ If lignin is able to share a portion of this lucrative market with petroleum feedstocks, the lignocellulose biorefinery is likely to become more economically viable. Findings indicate the valorisation of lignin could involve the extraction of guaiacol, syringol, vanillin, vanillic acid and syringaldehyde.^{36, 64} However, a significant amount of research is needed to establish a competitive industrial-scale lignin depolymerisation process.

1.3.2 Degradation of lignin

As discussed earlier, the structure of lignin is very complex and our current understanding is limited. It is therefore a major challenge to target lignin degradation and product separation. The structural variability of lignin among different plant species means every research finding is usually species-specific and cannot be generalised. The reaction pathway involved is strongly dependent on the exact composition of the chemical structure of the lignin.⁶⁵ However, the prospect of lignin degradation as an alternative feedstock in the chemical synthesis industry is huge and attracts a lot of interest both from industry and academia. This situation is shown by the number of papers on lignin depolymerisation published in recent times (Figure 1.3).

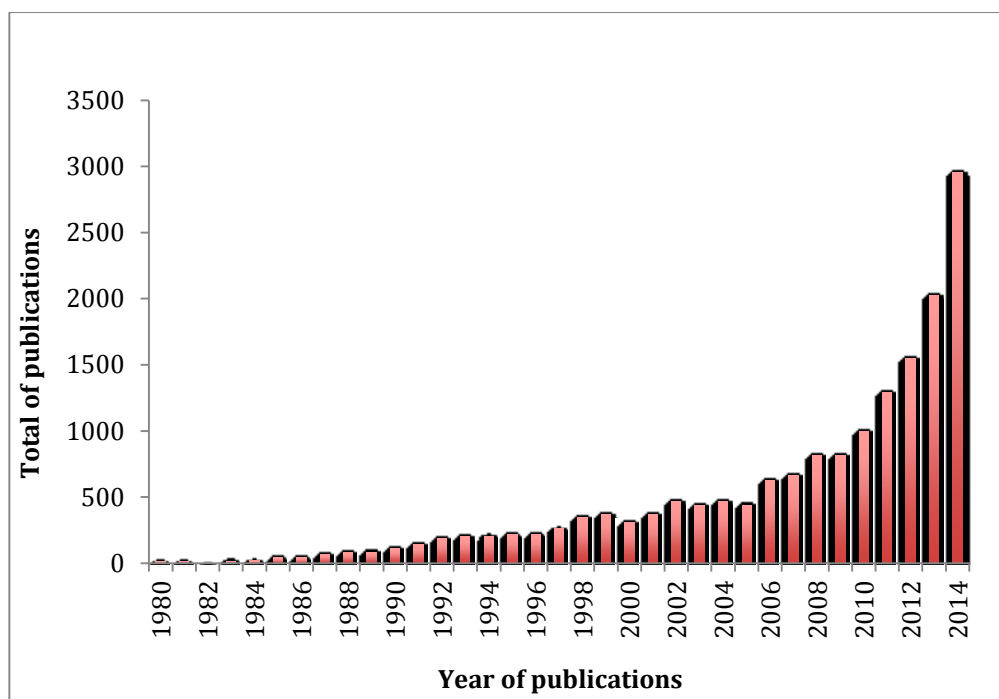


Figure 1.3 Number of published items on the topic of lignin depolymerisation based on a Web of Science search.

Lignin can be depolymerised either chemically or enzymatically. Optimising the reaction parameters is the key to efficient depolymerisation of lignin. Higher temperatures tend to fully degrade the lignin into coke and gases,⁶⁶ which is usually undesirable. The main goal of lignin depolymerisation is to breakdown the lignin and to recover the valuable aromatic monomers.

1.3.2.1 Chemical approaches

Chemical approaches include acid/base catalysed, oxidative or ionic liquid catalysed depolymerisation. Sodium hydroxide (NaOH) is commonly used as a strong base, cleaving lignin monomer linkages as well as functional groups attached to the aromatic rings.⁶⁶ Strongly basic conditions prevent the reformation of lignin polymers. However, due to the energy intensive nature of the reaction, it is hard to control by-product formation and this complicates the

downstream processing stages. On the other hand, formic acid has been used along with ethanol in the acid catalysed process, depolymerising lignin before deoxygenating intermediates methoxyphenol and catechol into stable phenol derivatives such as guaiacol, syringol and a range of alkyl phenols illustrated in Figure 1.4.⁶⁵

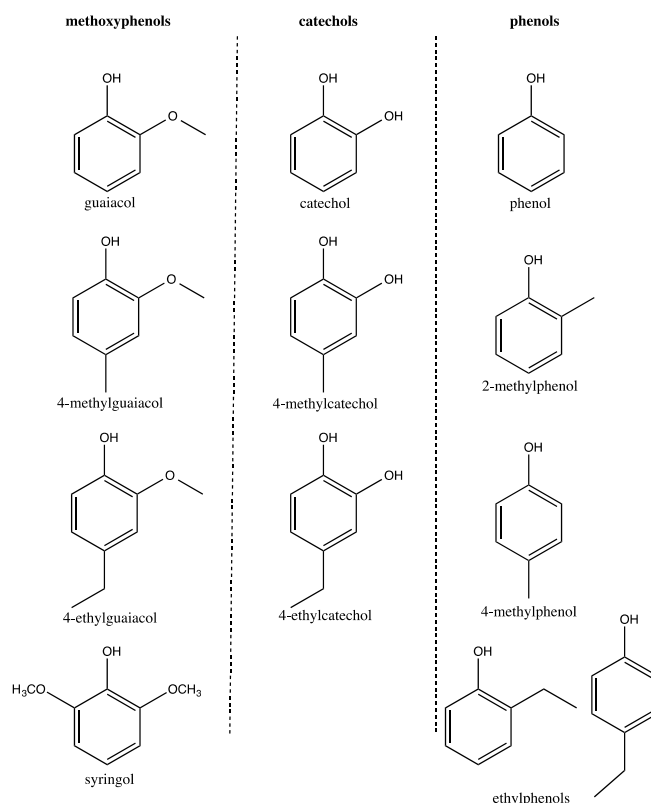


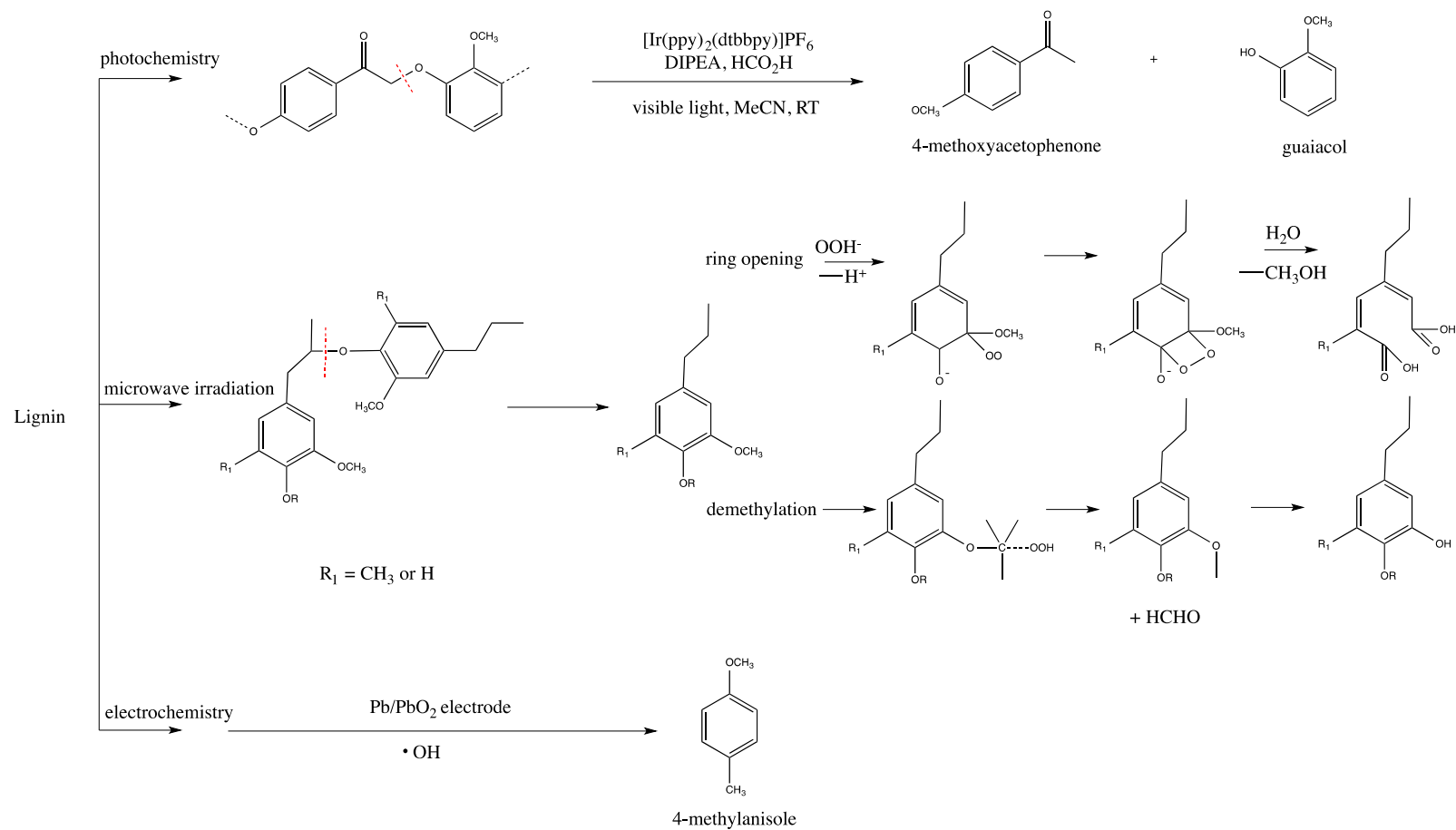
Figure 1.4 Lignin degradation products identified by gas chromatography (GC) as reported in the literature.⁶⁵

Lignin oxidation targets the large number of hydroxyl groups in lignin.⁶⁶ Oxidising agents such as metal oxides and hydrogen peroxide are used to valorise lignin into aromatic aldehydes and carboxylic acids.⁶⁷ This depolymerisation process has a high degradation rate but is low yielding. The re-condensation of degraded lignin impacts negatively on the yield and this issue needs to be tackled in order to increase the efficiency of the oxidative approaches.

Physical chemistry methods have been used in an effort to increase the yield of lignin degradation. As shown in Figure 1.5, several studies have reported the application of physical technologies to oxidation processes including photochemistry, electrochemistry and microwave irradiation.⁶⁸ High yields of 4-methoxyacetophenone (88%) and guaiacol (89%) have been obtained from a photochemical lignin degradation.⁶⁹ This room-temperature reaction involved selective benzylic oxidation followed by photoredox catalysis towards the β -O-4 linkages. A different study demonstrated the degradation of lignin via electrochemistry to produce 4-methylanisole as the main product⁷⁰ using a Pb/PbO₂ electrode. Meanwhile, another study revealed that a microwave assisted oxidation process is able to reduce the oxidation temperature, oxidation dosage and reaction time.⁷¹

Ionic liquids are a class of relatively new solvents in the biomass arena that are frequently labelled 'green', largely due to their extremely low volatility. Studies have demonstrated the ability of these solvents in deconstructing lignin model compounds. 1-butyl-3-methylimidazolium hydrogen sulphate, [Bmim][HSO₄] and 1-hexyl-3-methylimidazolium chloride, [Hmim][Cl] are among the most frequently used ionic liquids in these studies.^{72, 73} Lignin partial deconstruction and structural alteration by ionic liquids has also been reported.^{74, 75} Ionic liquids work both as the catalyst and the solvent in these reactions and the results obtained from these studies are very promising. However, this technology is relatively new and extensive work is required to make it a feasible option. This technology will be discussed in detail in Section 1.5.

Inspired by the oil cracking concept, thermal degradation of lignin has been identified as a lignin depolymerisation alternative. Lignin has broader temperature range of decomposition (200 - 500 °C) and is a slower process compared to cellulose and hemicellulose. The wide range of temperature is due to the presence of various oxygen functional groups with different thermal stabilities.⁷⁶ Depending on the type of plant used, a large selection of high value compounds can be obtained from this pyrolysis methods, including guaiacol, syringol, vanillin, various alkyl phenols and non-condensable gases

Figure 1.5 Physical chemistry approaches of lignin degradation.⁶⁹⁻⁷¹

which are primarily carbon dioxide (CO₂) and methane (CH₄).^{76, 77} However, as in all lignin degradation methods, the product separation and purification needs to be addressed before this method becomes commercially viable.

1.3.2.2 Bioligninolysis: The enzymatic approach

Due to lignin's complex heteropolymer structure and its recalcitrant nature, lignin-degrading microorganisms are limited to a specialized group of fungi and bacteria. There is still a lack of knowledge of ligninolytic enzymes and, compared to cellulases, our understanding lags behind.²² However, thorough research has shed more light in the bioligninolytic field.

The two known groups of ligninolytic enzymes are laccases and peroxidases. Laccases are multi copper oxidases found in fungi and bacteria.⁷⁸ These enzymes are versatile, capable of oxidising aromatic phenols and amines. Coupled with mediators (Figure 1.6), they are able to oxidise non-phenolic aromatic structures. Their biological role and their significance in lignin degradation are still poorly understood.²²

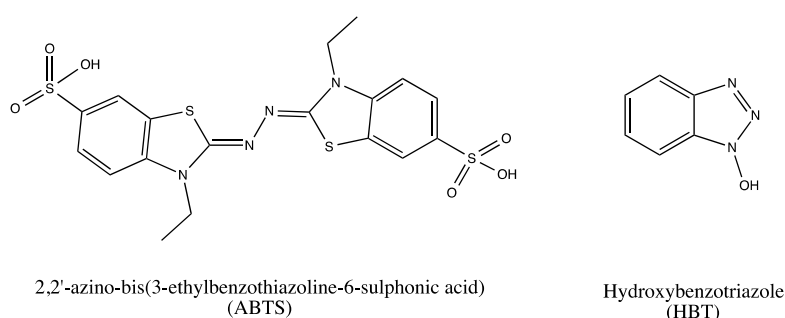


Figure 1.6 Examples of mediators in the laccase-mediator system (LMS).

On the other hand, there are several types of lignin degrading peroxidases. Lignin peroxidase (LiP), the first isolated ligninolytic enzyme, has a high-redox potential. This heme-containing peroxidase has a distinctly low optimum pH near pH 3.⁷⁸ LiP can oxidize both phenolic and non-phenolic

lignin units.²² The second type is manganese peroxidase (MnP). This enzyme catalyses Mn^{2+} oxidation to Mn^{3+} with hydrogen peroxide as the oxidizing agent. Then, a Mn^{3+} - chelator complex is formed with organic acids such as oxalate or malonate, to catalyse lignin oxidation.⁷⁸ Unlike LiP, MnP is not able to attack non-phenolic compounds.²² A unique type of ligninolytic enzyme, versatile peroxidase (VP) is able to catalyse lignin with a dual mechanism as used in both LiP and MnP.⁷⁸ This hybrid is able to oxidize both phenolic and non-phenolic compounds.²² Dye decolorizing peroxidase (DyP) was only discovered recently. This ligninolytic enzyme is prominent in bacteria⁷⁹ and shares no sequence or structural similarity with other known peroxidases.²²

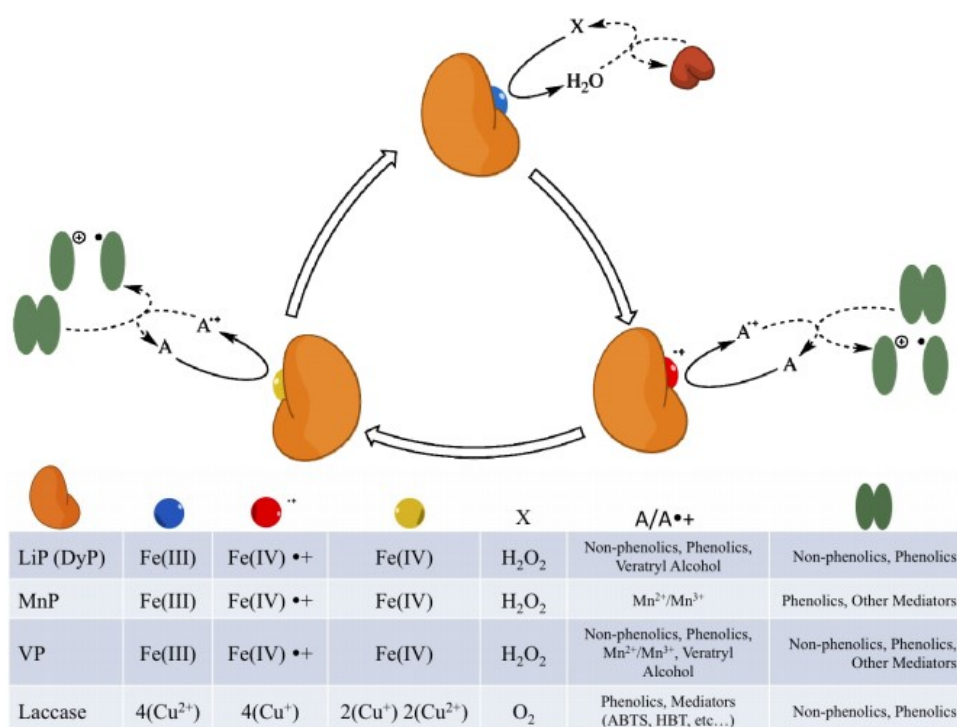


Figure 1.7 The catalytic cycle of ligninolytic peroxidases and laccases. They differ in their oxidizing substrate (X), their target reducing substrates/mediators (A) and their electron accepting metal co-factors (coloured circles).²² Reproduced by permission of the AIMS Press.

Lignin degrading microbes are commonly found in fallen trees and forest litter, composting the plant material in order to continue the carbon cycle in the ecosystem. Filamentous fungi belong to the phylum of

Basidiomycota and are well known for their ability to depolymerise lignin efficiently. Two major groups of lignin degrading fungi are white rot fungi and brown rot fungi. *Phanerochaete chrysosporium* is one of the most well-studied white rot fungi.²² In fact, the first ever isolated lignolytic enzyme, lignin peroxidase is produced by this species.⁷⁸ Some white rot fungi such as *Ceriporiopsis subvermispora* are selective lignin degraders, decomposing lignin without significant loss of cellulose and hemicellulose.⁸⁰ Other white rot fungi that are able to depolymerise lignin include the edible oyster mushroom, *Pleurotus ostreatus*⁸¹ and colourful polypore mushroom, *Trametes versicolor*.⁸²

Brown rot fungi are able to modify the lignin structure through extensive oxidative demethylation.⁷⁸ The majority of altered lignin remains in the wood structure, marked by a brown discolouration. Lignin degrading brown rot fungi represent less than 10% of Basidiomycota, and include *Laetiporus portentosus*, *Postia placenta* and *Serpula lacrymans* among a few.⁷⁸

Bioligninolysis by bacteria involves partial deconstruction by modifying and solubilising the lignin structure, unlike fungi that are capable of complete degradation.⁸³ Mostly soil bacteria, they mineralize low molecular weight components, which originate from fungally degraded lignin.^{78, 82} Although most studies on bacterial degradation of lignin use model compounds, with technology and assay development, recent studies have demonstrated the ability of bacteria to degrade lignin into vanillin,⁸⁴ protocatechuic acid,⁸⁵ β -aryl ethers⁸⁶ and an unidentified ketone.⁸⁷

Ligninolytic bacteria can also be found in the Proteobacteria class. *Sphingomonas paucimobilis*⁸⁸ and *Pseudomonas fluorescens*⁸⁶ belong to α -proteobacteria and γ -proteobacteria respectively. In the Firmicutes phylum, *Bacillus sp.* and *Paenibacillus sp.* are capable of decomposing lignin.⁸⁸ Although little is known about ligninolysis in Archae, laccase has been isolated from *Haloferax volcanii*⁸⁹ and a laccase-encoding gene is found in *Haloterrigena turkmenica* and *Halorubrum lacusprofundi*.⁸⁸ However, the most well-studied lignin degrading bacteria belong to the phylum of Actinobacteria.

*Rhodococcus jostii*⁸⁴ and *Streptomyces viridosporus*²² are members of this filamentous bacteria group.

1.4 Rhodococcus

The *Rhodococcus* genus has shown a promising ligninolytic capability. These bacteria have been extensively studied as biotechnological tools for bioremediation and biotransformation. Along with their versatile metabolic pathways and ability to survive in adverse environments,⁹⁰ thanks to their capacity to acquire a range of diverse catabolic genes and robust cellular physiology,⁹¹ *Rhodococcus* is a suitable candidate to be integrated in a biorefinery system.

1.4.1 Taxonomy of *Rhodococcus*

The genus of *Rhodococcus* belongs to the phylum of Actinobacteria. It is classified in the family of Nocardiaceae in the suborder of the Corynebacterineae together with other closely related bacteria, *Mycobacteria* and *Corynebacteria*. The early classification of *Rhodococcus* was based on cell wall composition.⁹² However, the development of 16S rRNA sequence analysis as a taxonomical tool redefined Actinomycete classification. Some strains were reclassified into the genus of *Mycobacterium* while others were merged or become obsolete. Currently, there are 53 species of *Rhodococci*, which have been recognised and validated.⁹³

The term '*Rhodococcus*' was first used by Zopf in 1891. Later in 1971, this word was revived to classify strains that resembled but did not belong to the established genera of *Nocardia*, *Corynebacterium* and *Mycobacterium*.^{94, 95} Recently, the usage of '*Rhodococcus*' is debatable since technically it was first used to represent a group of green algae in 1884.⁹⁶ The lack of parallel taxonomical guidelines between the botanical, zoological and bacteriological fields has caused this confusion.⁹⁶

1.4.2 Physiology and morphology

Rhodococcus are aerobic, gram-positive actinomycetes. Labelled as nocardioform, these bacteria have a unique morphology, mycelial growth with fragmentation into rod or coccoid shapes.⁹⁴ Although their name indicates the cells are likely to be cocci, they display a range of pleomorphic shapes from short rods to filaments (Figure 1.8). Colour wise, they produce pale red or orange pigmentation whence these bacteria obtain part of their name. *Rhodo* and *coccus* originated from the Greek words *rhodon* and *kokkos* respectively translated as 'red or rose' and 'grain or seed' referring to their common colour and shape.

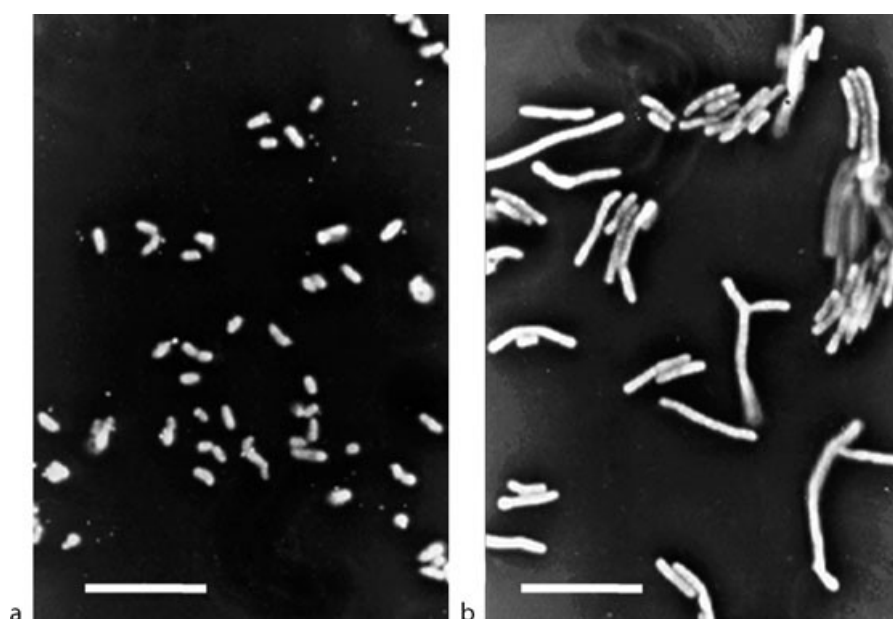


Figure 1.8 Morphology of *Rhodococcus* for a) short rods and cocci and b) branched filaments. Negative phase contrast. Bar represents 10 μ m.⁹⁵ Reproduced by permission of Springer.

Generally, they are able to tolerate and metabolize hydrophobic compounds assisted by their distinct hydrophobic cell walls containing the presence of mycolic acids,⁹⁴ with a length between 34 and 64 carbon atoms. They have been reportedly isolated from various environmental sources including soil, fresh water, saltwater and polluted sites. *Rhodococcus* are well

known for their ability to survive in extremely harsh conditions, from the sub-zero temperature of the arctic⁹⁷ to the high temperatures of volcanic hot springs.⁹⁸ They have also been found to persist in high pH, dry environments, restricted nutrients and highly toxic environments.

1.4.3 Genomic diversity

Recently, the genomic information on *Rhodococcus* has been expanded extensively. For bacteria, they possess a very large genome, up to 9.7 Mbp, consisting of large linear plasmids and smaller circular plasmids.⁹⁹ These plasmids, the most diverse part of the genome, act as a reservoir for the wide range of essential catabolic functions.¹⁰⁰ In term of sequencing, several strains have their complete genome sequence published including *R. jostii*, *R. opacus*, *R. erythropolis* and the pathogenic *R. equi*.⁹⁹

A significant degree of gene redundancy is consistent with this genome size. Multiple gene homologues possessed by *Rhodococcus* ensure metabolic flexibility and high tolerance to harsh environments, two desirable traits as biotechnological tools. Some strains were reported to harbour plasmids that display resistance towards cadmium, arsenite and chloramphenicol.^{91, 92}

In terms of gene regulation, *Rhodococcus* have been reported to exhibit both positive regulator and repressor mechanisms. It is possible that co-regulation of genetically unlinked transcriptional units might be common.⁹¹ Notably, multiple gene homologues are not singularly clustered and different strains have several homologous transcriptional units separated by non-homologous sequences comprising direct and inverted repeats.⁹¹

The genetic material in *Rhodococcus* is linked to their metabolic versatility, which in turn is the reason for their capability for occupying various niches. These bacteria have been isolated in different adverse environments such as high-level nuclear waste plumes,¹⁰¹ medieval graves,¹⁰² arctic soils¹⁰³

and deep-sea sediments.¹⁰⁴ Through adaptation, evolution and/or induction, certain genes will become dominant.⁹¹ Studies on isolates from different countries revealed a complete conservation of their biodegradative genes.⁹¹

A wide range of metabolic diversity in physiologically robust cells can often confer substantial advantages. *Rhodococcus* are reported to have adopted a strategy of hyper-recombination associated with a large genome.⁹¹ The high frequency of recombination in various *Rhodococcus* strains might contribute to the flexibility of their genome and the ability to acquire new genes and alter enzymatic activities.¹⁰⁵ With all of these features, it is unsurprising that *Rhodococcus* is capable of catabolic diversity.

1.4.4 Catabolic versatility

The ability of *Rhodococcus* to metabolise 'difficult' substrates, particularly ones noted for their toxicity and persistence, is remarkable. Substrates such as haloalkanes¹⁰⁶ and cyanides¹⁰⁷ are broken down by *Rhodococcus* and used as carbon and nitrogen sources respectively. Several groups of enzymes have been identified to be responsible in regulating metabolism of these bacteria including dehalogenases, oxygenases and peroxidases.⁹⁰

1.4.4.1 Rhodococcus Enzymology

One of the common classes of metabolic enzymes found in *Rhodococcus* are the dehalogenases, enzymes that break down halogenated compounds. For example, *R. rhodochrous* encodes a haloalkane dehalogenase (*dhaA*) gene that exhibits a wide specificity enzyme, degrading many primary and secondary haloalkanes.⁹⁵ Another study showed that *R. opacus* GM-14 utilized an unusually broad range of haloaromatic compounds like chlorobenzene and dichlorobenzene as carbon sources.¹⁰⁸

As for oxygenases, these enzymes oxidize substrates by incorporating oxygen atoms, derived from dioxygen into the compound. There are two

major groups of oxygenases; the monooxygenases incorporating a single atom of oxygen and the dioxygenases transferring both atoms of an oxygen molecule to the substrate. A very common monooxygenase in *Rhodococcus* is the heme-containing cytochrome P450 monooxygenase. These enzymes consist of protein complexes in which the reaction with oxygen occurs when electrons are transferred to the active site from a donor protein.⁹⁵ Through cleavage of ether bonds, these enzymes degrade alkyl ethers, aralkyl ethers and dibenzyl ethers. This activity enables *Rhodococcus* to degrade methyl tert-butyl ether (MTBE),¹⁰⁹ famously used as gasoline additive as well as herbicides, such as 4-(4-chloro-2-methylphenoxy)butanoic acid (MCPB)¹¹⁰ and atrazine.¹¹¹

In terms of dioxygenases, *Rhodococcus* possesses a wide diversity of the aromatic hydrocarbon ring-hydroxylating dioxygenases. This large range of enzymes ensures catabolism of many aromatic substrates. A study has revealed two biphenyl dioxygenases are induced for the degradation of biphenyl and polychlorinated biphenyl by *Rhodococcus jostii* RHA1.¹¹² Some strains have also been reported to acquire multiple copies of extradiol dioxygenases, which are responsible for ring cleavage.¹¹³

In 2011, peroxidases were identified in *R. jostii* RHA1.^{114, 115} Specifically known as dye-decolorizing peroxidase (DyP), this enzyme was named from its ability to oxidise a large number of synthetic dyes.⁷⁹ Initially discovered in the fungi *Thanatephorus cucumeris*, this enzyme is also responsible for degrading lignin.^{79, 114} It has a unique bifunctional activity catalysing both oxidative and hydrolytic reactions. The DyP enzyme family is classified into four different sub-families based on their phylogenetic differences.⁷⁹ *R. jostii* RHA1 contains both DyPA and DypB enzymes with DypB being mainly responsible for ligninolytic activity.¹¹⁴

1.4.4.2 Degradation of substrates

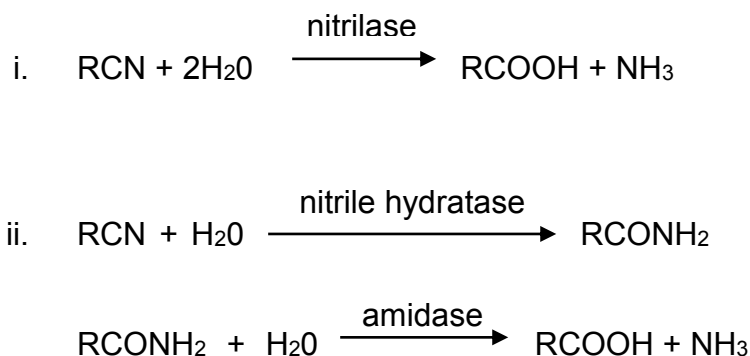
The ability of *Rhodococcus* to catabolise a large range of compounds; short and long chain alkanes, halogenated and nitro-substituted compounds as well as heterocyclic and polycyclic compounds, is well documented. The diversity of substrates degraded by *Rhodococcus* is impressive; from highly toxic compounds like cyanide,¹¹⁶ to persistent synthetic compounds like plasticizers¹¹⁷ and even problematic explosive compounds such as RDX^{118, 119} and TNT.¹¹⁹

In the light of *Rhodococcus*' ability in utilizing a wide range of organic compounds, these bacteria are renowned for degrading many aromatic hydrocarbons including phenolic compounds. These xenobiotics include chlorophenol, nitrophenol, resorcinol and cresol.¹²⁰ Likewise, these bacteria degrade creosote comprising various phenolic compounds, which is commercially used as wood preservative.⁹⁰ They are also capable of degrading other aromatic compounds like BTEX, the gasoline additive, made up of a mixture of benzene, toluene, ethylbenzene and xylene.^{90, 121}

Rhodococcus is also able to utilize halogenated compounds as a sole carbon source. Halogenated compounds are used commercially as solvents, pesticides and fire retardants; they are stable and hard to degrade.⁹⁰ *Rhodococcus* dehalogenate these compounds either by reduction or hydroxylation reactions.¹²² Two separate studies demonstrated the ability of *R. erythropolis* to dehalogenate and grow on 3-chlorobenzoate¹²³ and di-, tri- and tetrachlorobenzene.¹⁰⁶ Moreover, the ability to degrade a wide range of fluorinated compounds has been demonstrated by *R. opacus*, *R. erythropolis* and *R. corallines*.¹²⁴

Rhodococcus can also catabolise nitrogen-containing compounds like 2,4-dinitrophenol (DNP) and 2,4,6-trinitrophenol (TNP) as its sole nitrogen source.¹²⁵ It is worth noting that more than a quarter of the nitrile-converting microorganisms available in public collections are *Rhodococcus* strains.¹²⁶ These actinomycetes have several distinct features to ensure effective nitrile

degradation, such as nitrile-permeable cell walls and a diversity of encoded genes of nitrilase and nitrile hydratase.⁹⁰ These enzymes are responsible for the nitrile hydrolysis routes shown below:



The list of *Rhodococcus* degraded nitrogen-containing compounds also includes acrylonitrile, acetonitrile, propionitrile, acetamide and the explosive, TNT.^{119, 127, 128}

Besides nitrile degradation, owing to their ability in desulfurization, *Rhodococcus* is able to degrade benzothiophene (BT) and dibenzothiophene (DBT).⁹⁰ A pilot study revealed that *R. erythropolis* is able to purify heavy gas oil by removing 40% of the sulfur and nitrogen components.¹²⁹ This could lead to a cleaner gas release from factories burning the fossil fuels.

1.4.4.3 The Central Pathway of Aromatic Hydrocarbon Degradation

As illustrated in Figure 1.9, the catabolic pathway for aromatic hydrocarbon substrates starts with a peripheral pathway, followed by a central pathway. Subject to the particular substrate involved, there are different routes of peripheral pathways adopted by *Rhodococcus*. The central pathways taken thereafter are induced by a limited number of peripheral pathway products. Ultimately, the final product of these central pathways is processed in the citrate cycle to generate energy.

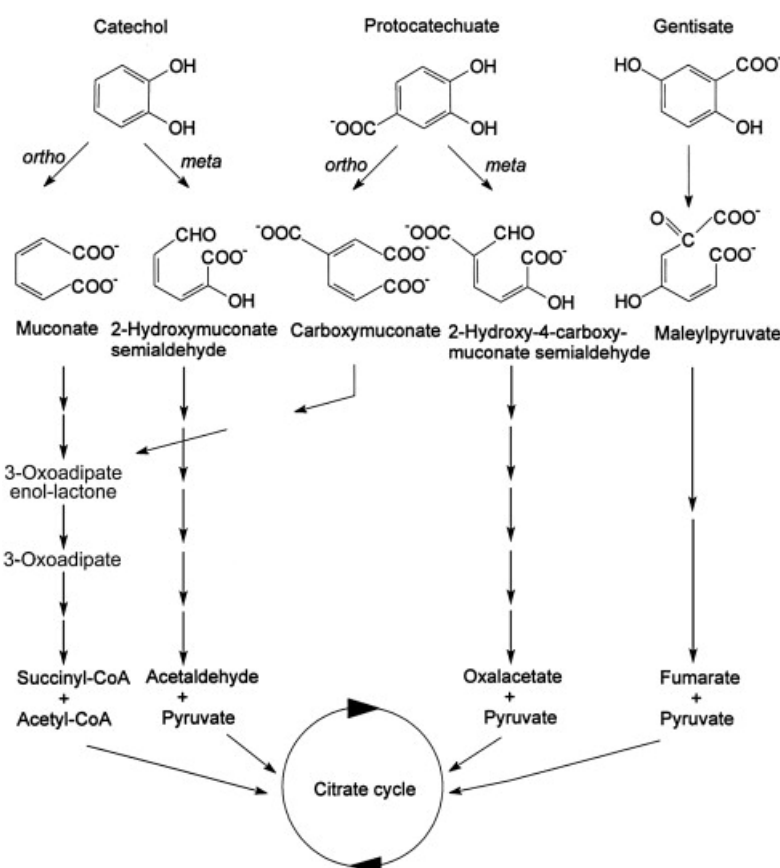


Figure 1.9 Central pathways of aromatic compound catabolism in *Rhodococcus*.⁹⁰
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Prior to the central pathway, the catabolism of aromatic compounds in the peripheral pathways involves several steps including the action of oxygenase enzymes, which lead to dihydroxylated benzene derivatives and initiates the central pathways. The initial metabolite could be catechol, protocatechuate or gentisate. The dioxygenases of these metabolites play central roles in the degradation of aromatic compounds since they catalyse the chemically challenging aromatic ring-cleavage reaction. Ultimately, these metabolites are transformed into pyruvate, then acetyl coenzyme A, before entering the citrate cycle.⁹⁰

Rhodococcus are able to catabolise polyaromatic hydrocarbons with three or four benzene rings such as pyrene, anthracene, fluoroanthene and chrysene.⁹⁰ A study reported the ability of these bacteria to uptake fluorene as

a sole carbon source.¹³⁰ In addition, it has the ability to degrade the complex and recalcitrant aromatic structure of dyes¹³¹ and lignin.⁸⁷ However, the catabolism pathway of polymeric aromatic substrate is more complex. In addition, *Rhodococcus* have been demonstrated to utilize various aromatic carboxylic acids, for example benzoate, phthalate, terephthalate and hydroxybenzoates. Salicylate, also known as o-hydroxybenzoate, is catabolised via the gentisate pathway (Figure 1.9). The same pathway is also used to catabolise polychlorinated biphenyls (PCBs).⁹⁰

Based upon these reported activities, it is clear that the use of *Rhodococcus* is very promising for challenging applications in microbial biotechnology. The combination of aromatic degradation, dehalogenation, denitrogenation and desulfurization offers a plethora of opportunities in industry with the ultimate goal of integrating these bacteria into the biorefinery and the generation of multiple products.

1.4.5 Applications of *Rhodococcus*

1.4.5.1 Environmental applications of *Rhodococcus*

The first attempt at using *Rhodococcus* as a bioremediation agent was recorded in the late 1990s in treating oil-contaminated land.¹³² In the study, *R. rhodochrous* was found to be the most efficient oil degrader as compared to *Pseudomonas sp.*, *Nocardia sp.* and *Streptomyces sp.*

Besides that, the ability of *R. chlorophenilicus* to degrade chlorophenol has prompted the development of a bioremediation process for the removal of chlorinated phenolics from soil.¹³³ This demonstration showed a relatively good mineralization of pentachlorophenol over a period of 200 days. The bioremediation activity is efficient in both sandy and peaty soils suggesting that the bacteria were not hindered by the presence of high organic matter content in soils. This strain has also been successfully applied in the remediation of chlorophenol-containing groundwater.¹³⁴

A promising effect has been reported involving the bioaugmentation of *Rhodococcus* to degrade diesel fuel pollutants.¹³⁵ Several studies showed positive effects for *Rhodococcus* on the biodegradation of petrochemicals and polychlorinated biphenyls in soil and sediments.^{121, 136, 137} Field studies showed very good results in the partial removal of heavy fuel oil spilled from an oil tanker on the cobble beach on the coast of northern Spain in 2002.¹³⁸ Indigenous microbial consortia containing *Rhodococcus* were used in this study.

Mixed cultures containing more than one strain of *Rhodococcus* have been successfully used in field trials in treating contaminated soils affected by fuel, crude oil and oily wastes.¹³⁹⁻¹⁴² In general, contaminated soils are treated with mixed culture, mineral fertilizers and bulking agents to stimulate the bioremediation process. The combination of bioaugmentation and biostimulation are essential for the effective clean up of hydrocarbon contaminated soils. Another proposed method is biosorption where bacteria are used to clean up heavy metal pollution. *R. opacus* has been shown to be effective in this capacity in lead, copper and chromium adsorption.¹⁴³

1.4.5.2 Industrial applications

Apart from bioremediation, *Rhodococcus* also has huge potential in the chemical industry. In fact, Nitto Chemistry Industry Company in Japan has incorporated *R. rhodochrous* in the production of acrylamide, estimated at over 30 000 tons annually.¹⁴⁴ This industrial application stimulates further research into the potential of *Rhodococcus* in assisting the production of various amides including vitamins, nicotinamide and the antimycobacterial agent, pyrazinamide.^{144, 145} These studies gave significant results, showing both high yields and specificity. It seems that the development of recombinant DNA technology has pushed the boundaries of *Rhodococcus* potential to a new level.¹⁴⁶

The biotransformation of *Rhodococcus* cells or enzymes has been widely reported in the literature. Examples include the production of epoxides from gaseous alkenes,¹⁴⁷ novel production of medically valuable sec-cedrenol,¹⁴⁸ synthesis of muconic acids¹⁴⁹ and the potential of cholesterol oxidases, produced by *Rhodococcus*, in the food and drug industries.¹⁵⁰ One recent study demonstrated the application of *R. rubropertinctus* to produce a pharmaceutical intermediate by transforming an LS1 ester into an LP1 ester, a synthetic intermediate for platelet inhibitor production (Figure 1.10).¹⁵¹

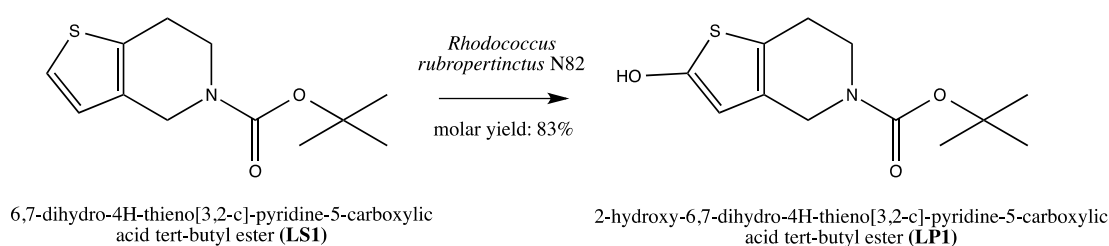


Figure 1.10 Biotransformation of a pharmaceutical intermediate by *R. rubropertinctus*.

The prospect of *Rhodococcus* applications in the pharmaceutical industry is not just limited to biotransformation. They also produce wide spectrum antibiotics. Aurachin and lariatins have been isolated from *R. erythropolis* and *R. jostii* respectively. Aurachin, a quinolone-based antibiotic exhibits a strong antibiotic activity towards both high and low GC content gram-positive bacteria¹⁵² whereas lariatins shows specific inhibition towards *Mycobacteria*.¹⁵³

Rhodococcus could also play important roles in the fruit industry. *R. rhodochrous* are able to delay the ripening of climacteric fruit by modulating the Yang Cycle, responsible for fruit ripening.¹⁵⁴ Another interesting piece of research demonstrated *R. fascians* could degrade limonin, a bitter tasting compound in fruit juices,¹⁵⁵ potentially leading to the flavour enhancement of bitter fruit juices.

Additionally, *Rhodococcus* is also known to produce biosurfactants, which are better alternatives than synthetic surfactants, potentially being more biodegradable and less toxic. Unlike the relatively simple chemical structure of the synthetic version, biosurfactants are more complex. Thus, their physicochemical properties are more flexible. Most reported research has used trehalose lipid surfactants (Figure 1.11) produced by *R. erythropolis* along with several other species such as *R. opacus* and *R. ruber*.¹⁵⁶ These biosurfactants have huge potential as stabilizers of oleophilic emulsions and bases for creams, oily films and pastes in the food, pharmaceutical and cosmetic industries. One study even looked at the possibility of treating oil spills in the Arctic with *Rhodococcus*-produced biosurfactants.¹⁴¹

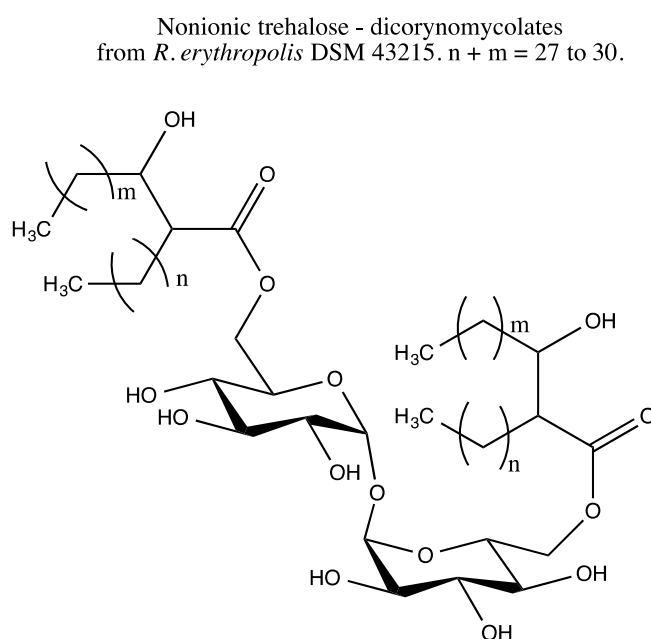


Figure 1.11 Example of trehalose lipid surfactants produced by *Rhodococcus* sp.

Furthermore, *R. erythropolis* are able to produce a bioflocculant, which can trigger flocculation of a wide range of suspended solids. The application of this product is beneficial to wastewater treatment, downstream processing and food production. An optimisation analysis revealed the ability of *R. erythropolis* as bioflocculant in treating dye wastewater with a 94% decolourisation rate.¹⁵⁷

Nevertheless, development of cheaper and more efficient large-scale processes with high yields is vital in order to make the application of *Rhodococcus* in industry highly competent and economically attractive. The intensive on-going research and technology development is likely to ensure that this achievable target is realised in near future. The great current interest in applying these versatile bacteria within the biorefinery concept also plays a significant role in promoting their industrial use. *Rhodococcus* looks set to become one of the lead bioprocess agents in the commercialization of a bacterially-integrated biorefinery.

1.5 Ionic liquids

The prospects for using ionic liquids in lignocellulose pretreatment have been highlighted earlier in this chapter. This new generation of solvents consist of organic salts with (normally) bulky ions, which lead to poorly organised structure and so remain in liquid form at low to moderate temperatures.¹⁵⁸ An ionic liquid consists of a pair of ionic species, cation (positively charged) and anion (negatively charged). At the moment, there is no universal guideline for annotating the name of ionic liquids. The usually lengthy name of ionic liquids is either written in the form of chemical formula or in the form of abbreviations. For example, 1-butyl-3-methylimidazolium hexafluorophosphate can be written as [C₄C₁im][PF₆] or [Bmim][PF₆] (*colour match for visual aid). The list of abbreviations of ionic liquids used in this study is provided in Table 2 (Section 2.1.2).

Although ionic liquids were initially reported in 1914 as molten salts,¹⁵⁹ this discovery did not prompt any significant interest at the time. Their physicochemical properties are tuneable by appropriate modification of the anion and cation species. Different combinations of both can result in changes in melting point, polarity, conductivity and viscosity. This flexibility is advantageous since it can be tailored to the desired application for example to increase the rate of reaction. In contrast to conventional solvents, which are usually volatile organic compounds (VOCs), ionic liquids display negligible vapour pressure and high thermal stability, leading to their association with the green solvent's tag.

1.5.1 Green solvents claims

Public awareness of climate change and the demand for environmentally friendly options has led to a greener approach to research in many fields. This trend has stimulated an increase in ionic liquid research. Nevertheless, the claim that ionic liquids are green solvents is often deemed controversial and debatable.¹⁶⁰ The counter view questions the greenness of the synthesis

of ionic liquids, which sometimes uses the very solvents that they are replacing. Strengths, weaknesses, opportunities and threats (SWOT) analysis showed that the preparation of some ionic liquids undermine their 'green' credentials.¹⁶¹

On top of that, the toxicity and persistence of ionic liquids have raised concerns among the chemistry community. Though there is a lack of risk in terms of air pollution, unlike for VOCs, there is a risk of spillage in water or soil along with the release of waste effluent negatively impacting the environment. As an emerging solvent, this new territory needs to be thoroughly investigated and the benefits considered along with the potential issues. It should be noted that the potential for the reuse of ionic liquids does help counter the VOC use in their preparation.

1.5.1.1 The toxicity of ionic liquids

Recent studies on the toxicity of ionic liquids have investigated many different classes of biological models; from enzyme inhibition to cells and bacteria, complex organisms like plants as well as both vertebrate and invertebrate animals. Ionic liquids were able to induce apoptosis in HeLa¹⁶² cells while different levels of toxicity were displayed in cytotoxicity tests of ionic liquids with MCF-7 (human breast cancer),¹⁶³ LoVo (colon adenocarcinoma)¹⁶⁴ and S2 (*Drosophila melanogaster* cell culture).¹⁶⁵ Ionic liquids also inhibit the enzymatic activity of acetylcholinesterase, which could cause an adverse effect on the neuronal process of organisms.¹⁶⁶

Ionic liquids also exhibit toxicity towards both gram-positive (*Staphylococcus aureus*) and gram-negative bacteria (*Vibrio fischeri*). Some ionic liquids have a wide spectrum of antibacterial properties like 1-hexyl-3-methylimidazolium chloride, [Hmim][Cl] and 1-butyl-3-methylimidazolium bis(trifluoromethylsulfonyl)imide, [Bmim][NTf₂]. The toxicity of these ionic liquids is not limited to bacteria as similar toxicity has been observed in fungi (*Candida tropicalis*) and yeast (*S. cerevisiae*).^{167, 168}

In addition, ionic liquids showed different degrees of toxicity towards invertebrates including the earthworm (*Eisenia foetida*), a nematode (*Caenorhabditis elegans*) and a crustacean (*Daphnia magna*). Significant toxicity is seen in aquatic organisms if exposed to ionic liquids with very long alkyl chain. DNA damage and mortality was observed in a related study involving the zebrafish (*Danio rerio*), goldfish (*Carassius auratus*) and carp (*Cyprinus carpio*).¹⁶⁷ On the other hand, rats or mice, which are normally used for toxicity testing as representative mammals, were also reported to show possible teratogenic effects with ionic liquids.¹⁶⁹ Phytotoxicity testing revealed growth inhibition by ionic liquids of a targeted higher plant, rice (*Oryza sativa*) as well as algae (*Ulva lactuca*).¹⁶⁷

Further research is needed in this area since the mechanisms of toxicity of ionic liquids are still poorly understood. Due to their structural similarities, the mode of action for ionic liquid toxicity is believed to be similar to that of detergents, disrupting the membrane of cells. The toxicity of ionic liquids is dependent on the exposed organisms as some are hardier and able to resist better than others. In addition, some bacteria are capable to adapt and survive. A thorough screening could help to select the best combination of ionic liquids and biological system with a view to achieving an integrated industrial application while minimizing the environmental risks.

1.5.1.2 Ionic liquids guideline

There is no doubt that ionic liquids are able to assist in making a chemical process greener but it does not mean that it will always be the ideal green solvent for all processes. The 12 principles of green chemistry provide useful guidelines to ensure that a process is relatively safe and a better alternative than the original route.^{160, 161} They are as follows:

1. Prevent waste.
2. Design safer chemical products.

3. Design less hazardous chemical syntheses.
4. Use renewable feedstock.
5. Use catalysts, not stoichiometric reagents.
6. Avoid chemical derivatives.
7. Maximize atom economy.
8. Avoid auxiliary substances if possible
9. Increase energy efficiency.
10. Design chemicals and products that degrade after use.
11. Analyse in real time to prevent pollution.
12. Minimize the potential for accidents.

The first principle is to minimize and control chemical waste. Ionic liquids are normally recyclable and can promote selective reactions. Thus, most reactions in which they are used automatically abide by this principle. This principle is also related to another rule regarding maximising the atom economy. The reaction should thus incorporate all materials into the final product. Choosing an ionic liquid with high selectivity and high yield can avoid the production of waste.

Secondly, it is essential to design biodegradable ionic liquids. Even though ionic liquids are recyclable, this efficiency will reduce after a certain number of cycles. Hence, at the end of their use, they should not persist in the environment, breaking down into harmless substances. The presence of oxygen in the structure could enhance the biodegradability of ionic liquids.¹⁷⁰ By introducing functional polar groups in the alkyl chain, the toxicity of ionic liquids can be reduced and their biodegradation efficiency increased.¹⁷¹ It has been demonstrated that the biodegradability of ionic liquids increases by lengthening the alkyl chain and substituting the side chains with hydroxyl functional groups.¹⁷² The same study also noted the factor of biological variability in the test. The observation of biodegradation is not universal and is to some extent, bacterial specific.

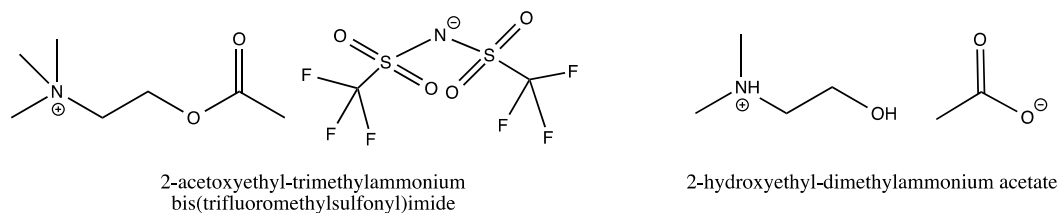


Figure 1.12 Examples of readily biodegradable ionic liquids for *R. rhodochrous*.

For example, *R. rhodochrous* was found to be able to degrade a large range of ionic liquids and achieves complete degradation with some ionic liquids (Figure 1.12).¹⁷³ These studies have shed some light on the design of biodegradable ionic liquids with low toxicity.

In order to comply with green chemistry principles, every step of an ionic liquid's application should be accounted for, both in terms of the chemicals involved as well as the process and the preparation stage. Ionic liquids are generally non-volatile and non-flammable. Any other chemicals used in the synthesis of ionic liquids and the main reactions should be replaced by the least hazardous alternatives, where possible. While maintaining the efficacy of the reaction, the toxicity effect should be taken into consideration in designing the process. As shown in Figure 1.13,¹⁷⁴ the use of an ionic liquid in a Diels-Alder reaction allows the replacement of the conventional lithium perchlorate-diethyl ether mixtures, $\text{Li}[\text{ClO}_4]\text{-Et}_2\text{O}$.

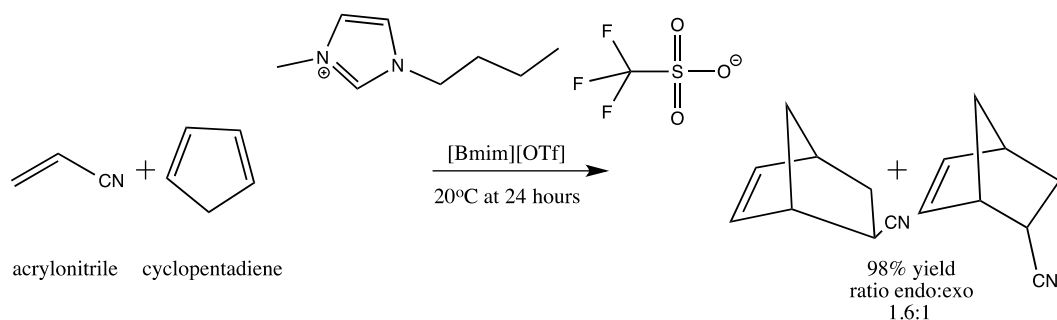


Figure 1.13 Ionic liquids increase yield and selectivity of Diels-Alder reaction.

Since ionic liquids are thermally stable and the reaction occurs under ambient conditions, this option avoids the safety issues associated with

diethyl ether and high-pressure reactions. Additionally, the recyclable ionic liquid generates almost no waste, unlike the conventional system, which generates toxic and potentially explosive lithium perchlorate waste.

Moreover, the principles state that, wherever practicable, the use of stoichiometric reagents, chemical derivatives and auxiliary substances should be limited. Ionic liquids can also play the role of catalysts as well as solvents, rendering auxiliary substances unnecessary. The one-pot synthesis of 2-amino-2-chromenes using the basic ionic liquid, 1-butyl-3-methylimidazolium hydroxide, [Bmim][OH] is a good example of applying these green chemistry principles (Figure 1.14).¹⁷⁵ This compound is of commercial interest as a key component in cosmetics, pigments and biodegradable agrochemicals. Conventionally, the production of this compound involves the presence of organic solvents like ethanol and acetonitrile along with piperidine as catalyst. Thus, the application of an ionic liquid avoids the use of volatile organic solvents, solvent waste and the need for an additional base, all of which present environmental hazards.

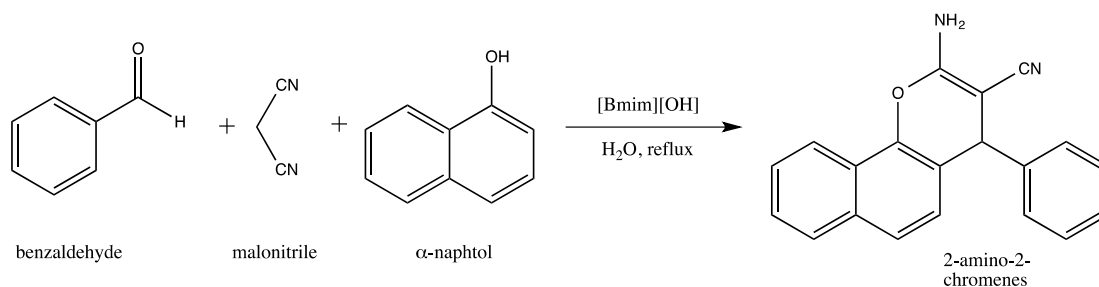


Figure 1.14 The synthesis of 2-amino-2-chromenes.

Energy and feedstocks are also accountable factors in the principles of green chemistry. Ionic liquids have the advantage of increasing the energy efficiency of the process since they work well at ambient pressure and temperature. In addition, the ability of ionic liquids to increase the reaction rate allows for less energy input. As for the feedstocks used, some ionic liquids

are already made from renewable resources. The current interest in the use of ionic liquids in biomass pretreatment could also justify this principle.

The last two principles are that real-time analytical methodologies should be available for the process and that the risk of chemical accidents should be minimised. Whilst ionic liquids are relatively new solvent class, they have been found to be compatible with most spectroscopic techniques making them amenable to *in situ* analysis of the progress of reaction. A major risk associated with VOCs is their flammability and volatility. These factors are eliminated when using ionic liquids, leaving their potential toxicity through spillage the main hazard. These issues are being addressed through the design of biodegradable and chemically safe ionic liquids.

In summary, ionic liquids are often a better option than conventional VOCs but they should not be considered intrinsically 'green solvents'. Their flexibility is the key to modifying them to producing a more environmentally friendly combination of cation and anion. Ionic liquids could provide a greener alternative through a careful design of the process while fulfilling the principles of green chemistry.

1.5.2 Applications of ionic liquids

The ability to tailor their distinctive characteristics has allowed the application of ionic liquids to cover a broad range of fields, including chemistry, biotechnology, pharmaceutical, engineering, electrochemistry among others. These solvents are able to increase the rate of reaction and produce greater yields. In addition, their ability to induce a biphasic system could assist the downstream process. The demand for greener alternatives has also driven research to incorporate ionic liquids in many diverse fields.

By scaling up the process in which ionic liquids are involved, their manufacturing costs will be lowered, possibly making the application of ionic liquids a profitable option in industry. One of the best-known industrial

applications of ionic liquids is the BASIL process developed by the chemical company, BASF.^{159, 176, 177} This acid scavenging method has resulted in a 98% yield alkoxyphenylphosphines compared to 50% yield using the conventional method (Figure 1.15).

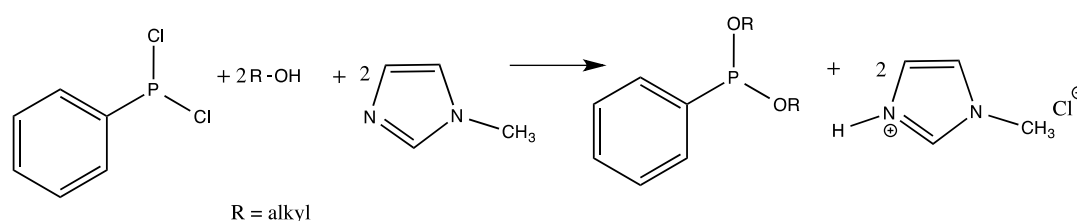


Figure 1.15 Biphaseic Acid Scavenging using Ionic Liquids (BASIL) process.

The use of ionic liquids is also widely patented in the petrochemical industry by BP, ExxonMobil, Chevron and PetroChina.¹⁵⁹ The applications of ionic liquids by these companies range from the refinery process to carbon dioxide removal. Meanwhile, Petronas, a Malaysian petroleum company, is using ionic liquids to remove mercury from natural gas streams.¹⁷⁸

Apart from these applications, ionic liquids are well known in the chemistry field as catalysts. The Eastman Chemical Company used phosphonium ionic liquids along with a Lewis acid catalyst (e.g. triphenyltin iodide) in the isomerisation of 3,4-epoxybut-1-ene to 2,5-dihydrofuran. Unfortunately, after operating from 1996 to December 2004, this plant is now idle due to a declining market for this product. Recently, a novel solvent free benzene oxidation to phenol has been introduced.¹⁷⁹ It involves an ionic liquid as the catalyst along with H_2O_2 as the oxidant. The reaction resulted in a 65% yield of phenol and the ionic liquid can be recycled up to five times. In another example, a Brønsted–Lewis acidic ionic liquid, $[\text{HSO}_3\text{-pmim}]^+ (1/2 \text{ Zn}^{2+})\text{SO}_4^{2-}$ is used in the esterification of 1-octanoic acid to produce 95.4% 1-methyl octanoate.¹⁸⁰

Ionic liquids are also used in biotechnology, especially in biotransformations. Examples of the application of ionic liquids in whole cell biotransformation include bacteria, yeast and fungi as the biotransformation agents. *Lactobacillus kefir* is used to reduce 4-chloroacetophenone with [Bmim][NTf₂] as the solvent. Unlike conventional VOC solvents, some ionic liquids do not damage the cell membrane but enhance the membrane integrity, resulting in high yield and high purity.¹⁸¹ Ionic liquids have also been applied along-side recombinant *Escherichia coli* in the transformation of 2-octanone and benzaldehyde to 2-octanol and mandelic acid respectively.^{182, 183} The yeast, *S. cerevisiae* and the fungus, *Geotrichum candidum* have been reported in the ionic liquid-assisted production of alcohols.¹⁸⁴

Ionic liquids have also been reported to help to stabilise proteins¹⁸⁵ as the hydrogen bond between anion and cation prevents the protein from unfolding. Hydrophobic ionic liquids stabilise enzymes, as ionic liquids do not remove the essential water shell from around them. As a result, the enzymes remain stable, maintaining their three dimensional conformation as the protein activity increases, resulting in higher yield in the processes in which they are employed. Studies have reported that ionic liquids are able to increase the thermal stability, enhance the solubility and provide long-term stabilisation of proteins such as lysozyme and cytochrome c. Ionic liquids also assist the protein crystallization of lysozyme and trypsin. Biphasic systems based on ionic liquids also help aid protein extraction and separation in proteomics studies.

Furthermore, the pharmaceutical industry also gains considerable benefits from the application of ionic liquids.¹⁸⁶ Their tuneable properties allow the flexible design of compatible ionic liquids aiming to increase solubilisation and bioavailability of active compounds. The first firm to produce pharmaceutical intermediates using ionic liquids was The Central Glass Company.¹⁵⁹ Using tetraalkylphosphonium ionic liquids, alkynes are produced in a more efficient way than by using organic solvents. A report was also published on an improved process of tadalafil (used to treat male erectile dysfunction) production in the presence of ionic liquids to replace VOCs as

solvents.¹⁸⁷ The usage of ionic liquids has also been reported in the synthesis of trifluridine (antiviral drug),¹⁸⁸ pravadoline (anti-inflammatory)¹⁸⁹ and L-4-boronophenylalanine (potential antitumor agent).¹⁹⁰

Due to their unique flexibility, innovations incorporating ionic liquid technology have extensive potential in engineering. A carefully selected combination of the cations and anions can result in desirable characteristics that create materials, which are beneficial as performance additives. A recent report demonstrated that phosphonium-based ionic liquids are better lubricant antiwear additives than the commercial ashless additives.¹⁹¹ Branched and long alkyl chains in ionic liquids increase their solubility in oil and thus improve the functionality. The study of ionic liquids as performance additives in water-based printing inks revealed 1% addition of ionic liquids increases the wettability and adhesion of inks, resulting in a better print quality.¹⁹² There is a wide range of products engineered with ionic liquid-additives with promising results such as paints,¹⁵⁹ perfumes,¹⁹³ polymers¹⁹⁴ and surfactants.¹⁹⁵ By modifying the structure of the ionic liquid, they also have potential as antistatic cleaning agents¹⁵⁹ and herbicides.¹⁹⁶

Ionic liquids provide also attractive fundamental properties for electrochemistry. High charge density, tuneable polarity and transport properties along with highly stable thermophysical and electrochemical properties make them of considerable interest in electrochemistry applications. With a wide electrochemical window and negligible vapour pressure, ionic liquids are able to sustain higher voltages before any electrochemical or thermal decomposition. These characteristics make them fitting candidates as electrolytes in energy storage devices, viz. batteries, fuel cells and solar cells.¹⁹⁷ By establishing the viscosity through different pairs of cations and anions, they are able to display a good electrical conductivity, suitable for electroplating and electropolishing. Developed by a university spin off company called Scionix, a choline-chromium (III) derived ionic liquids used this distinctive properties to replace a conventional chromium (IV) process, which is toxic.¹⁵⁹

1.6 Scope of study

Given the need to decrease dependence on petrochemical feedstocks, it is clear that biomass offers a massive potential with lots of research opportunities. *Rhodococcus* and ionic liquids both display unique characteristics that could be applied to a wide range of applications. Their combination is especially promising and has the potential to tackle the challenge faced in the biorefinery, in particular with regards to lignin. However, this novel area of study is in its infancy and requires substantial further research, which has motivated the work described here.

1.6.1 Research overview

Although the use of *Rhodococcus* was the first reported example of ionic liquid whole-cell biocatalysis, there is still a lack of research exploring the compatibility of *Rhodococcus* with ionic liquids. In that initial report, *Rhodococcus* R312 was used to transform 1,3-dicyanobenzene into 3-cyanobenzamide and 3-cyanobenzoic acid.¹⁹⁸ [Bmim][PF₆] was used to replace the need for a volatile organic solvent, creating a biphasic system for the reaction.

Based on the available literature, it appears that most studies have focused on the biotransformation of chemicals. Results obtained from these works showed either similar or better rates of reaction with higher yields compared to the conventional methods. *R. erythropolis* doubled the yield for the reduction of 6-Br- β -tetralone into 6-Br- β -tetralol with a 10-fold decrease of cell viability in the conventional solvent, ethanol, compared to the ionic liquid, 1-ethyl-3-methylimidazolium tosylate, [Emim][TOS].¹⁹⁹

Even if the reaction produced a similar result to the conventional method, the approach involving ionic liquids was often advantageous due to the biphasic system formed, which facilitated the downstream process. *R. erythropolis* was used in the biodesulfurization of diesel fuel in the presence of

[Bmim][BF₄] and [Bmim][PF₆].²⁰⁰ The former exhibited a strong inhibition growth while the later showed a better compatibility, maintaining the desulfurization capacity while the absence of an emulsion will simplify the downstream process. Furthermore, the use of recyclable ionic liquids is more environmentally friendly.

In addition, two separate studies recorded the isolation of *R. erythropolis* originating from salt marsh²⁰¹ and marine²⁰² ecosystems, which are tolerant to 1M [Emim][Cl]. Based on these findings, both studies proposed the possibility of using these isolates in the bioremediation of ionic liquid spills. The biodegradability of ionic liquids was also studied using *R. rhodochrous* as the biodegradation agent. Interestingly, this species was able to degrade almost all of the ionic liquids tested in the study.¹⁷³ This paper has also set a precedent for future toxicity studies of *Rhodococcus* in ionic liquids.

In the context of the role played by ionic liquids in the bioprocess, these chemicals have also been used as a solvent to selectively extract cellulose²⁰³ and hemicellulose.²⁰⁴ They have also been tested as an extraction solvent and catalyst in biodiesel production.²⁰⁵ While cellulose and hemicellulose have been employed in a lot of industrial applications, especially biofuels and biomaterials; companies like BASF have licensed various patents related to cellulose dissolution,¹⁵⁹ lignin development in the bioprocess still lags behind. Research on lignin as a bioprocess feedstock need to be prioritised as this potentially valuable compound could facilitates a viable and profitable biorefinery system.

In addition to the recently adopted role of ionic liquids in biomass pretreatment, *Rhodococcus* has also been applied in processing biomass. *R. jostii* RHA1 has been shown to possess Dyp B enzymes that function as a lignin degrader.^{114, 115} The same species was demonstrated to break down lignin into vanillin.⁸⁴ *R. opacus* DSM1069 is able to transform organosolv lignin into lipids.²⁰⁶ These illustrate the capability and suitability of *Rhodococcus* as a bioligninolytic agent, which could potentially be integrated into a biorefinery system.

1.6.2 Significance of study

In order to ensure a viable lignocellulose biorefinery system, the potential of lignin as a biomass feedstock needs to be realised. The efficiency of lignin degradation is still a challenge and the present study will try to tackle this issue. The potential of *Rhodococcus* as a bioligninolytic agent and ionic liquids as the media for biocatalysis and lignocellulose pretreatment could provide a novel combination to increase the efficiency of lignin degradation. Using lignin derived from the ionic liquid-pretreatment of lignocellulose, this study could offer a viable option for a lignocellulosic biorefinery system.

The interdisciplinary knowledge of biotechnology in *Rhodococcus* and chemistry in ionic liquids will allow this project to investigate this ligninolytic system from a new perspective. In this research project, the compatibility of the relatively recent solvent class of ionic liquids, to be integrated into the biodegradation of lignin, was investigated.

1.6.3 Aim and objectives

The ultimate aim of this study is to explore the possibility of developing a bioligninolytic system by combining the unique characteristics of both *Rhodococcus* and ionic liquids. The compatibility of *Rhodococcus* biocatalysis in ionic liquids will first need to be analysed. Hence, a list of objectives is outlined in order to achieve this goal:

1. To study the degradation of the lignin model compound, vanillic acid by *Rhodococcus*.
2. To examine the efficiency of *Rhodococcus* as a bioligninolytic agent in an ionic liquids-facilitated system.
3. To investigate the toxicity effect of different ionic liquids on the viability of *Rhodococcus* UKMP-5M.

CHAPTER 2

EXPERIMENTAL DESIGN

2.1 Materials

2.1.1 Bacterial cultures

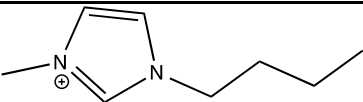
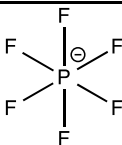
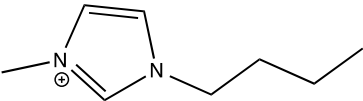
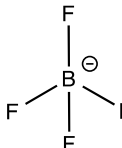
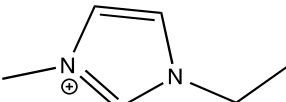
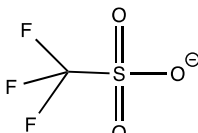
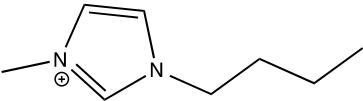
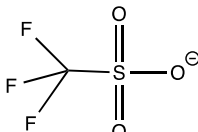
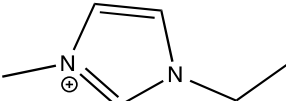
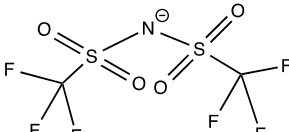
***Rhodococcus* UKMP-5M** was obtained from the Culture Collection Unit, Institute of Bio-IT Selangor, Universiti Selangor (UNISEL), Malaysia. This bacterium was originally isolated from petroleum-contaminated soil, taken from an oil refinery, situated in the state of Malacca, Malaysia.

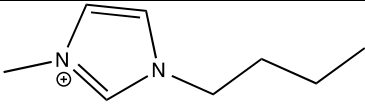
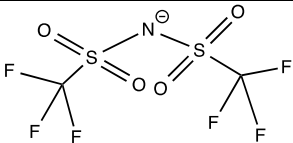
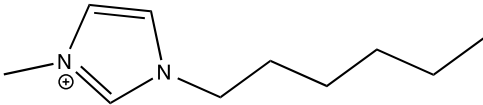
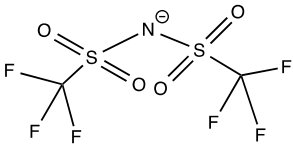
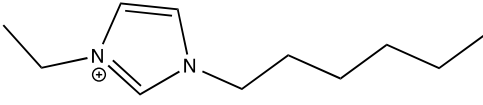
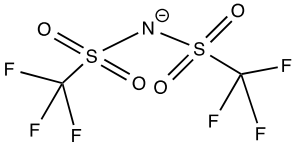
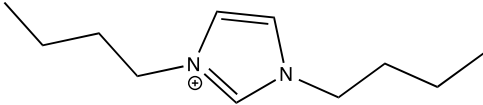
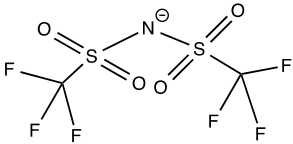
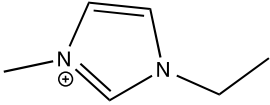
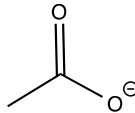
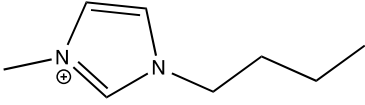
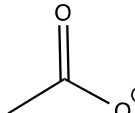
Bacillus subtilis was kindly supplied by Dr. Karen Polizzi (Imperial College London, UK). This culture was used as a negative control in relevant experiments.

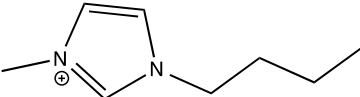
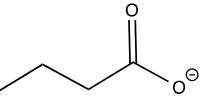
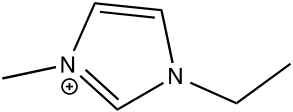
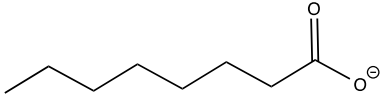
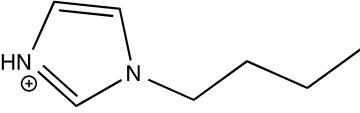
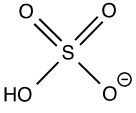
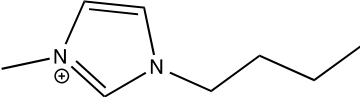
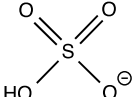
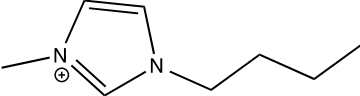
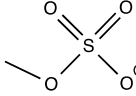
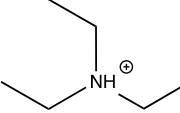
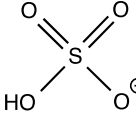
2.1.2 Ionic liquids

A wide range of ionic liquids was used in this study. Apart from 1-butyl-3-ethylimidazolium butyrate, [Bmim][OBU], sixteen of them were generously supplied by members of Dr. Jason Hallett's lab group. These ionic liquids were synthesized in his laboratory in the Department of Chemical Engineering, Imperial College London according to the literature procedures.^{207, 208} Ionic liquids used are listed in Table 2 along with the abbreviations and structures.

Table 2 Structures and abbreviations of the studied ionic liquids.

Ionic liquids	Abbreviation	Cation	Anion
1-butyl-3-methylimidazolium hexafluorophosphate	[Bmim][PF ₆]		
1-butyl-3-methylimidazolium tetrafluoroborate	[Bmim][BF ₄]		
1-ethyl-3-methylimidazolium trifluoromethanesulfonate	[Emim][OTf]		
1-butyl-3-methylimidazolium trifluoromethanesulfonate	[Bmim][OTf]		
1-ethyl-3-methylimidazolium bis(trifluoromethylsulfonyl)imide	[Emim][NTf ₂]		

1-butyl-3-methylimidazolium bis(trifluoromethylsulfonyl)imide	[Bmim][NTf ₂]		
1-hexyl-3-methylimidazolium bis(trifluoromethylsulfonyl)imide	[C ₆ C ₁ im][NTf ₂]		
1-hexyl-3-ethylimidazolium bis(trifluoromethylsulfonyl)imide	[C ₆ C ₂ im][NTf ₂]		
dibutylimidazolium bis(trifluoromethylsulfonyl)imide	[C ₄ C ₄ im][NTf ₂]		
1-ethyl-3-methylimidazolium acetate	[Emim][OAc]		
1-butyl-3-methylimidazolium acetate	[Bmim][OAc]		

1-butyl-3-methylimidazolium butyrate	[Bmim][OBu]		
1-ethyl-3-methylimidazolium octanoate	[Emim][C ₇ CO ₂]		
1-butylimidazolium hydrogen sulfate	[Hbim][HSO ₄]		
1-butyl-3-methylimidazolium hydrogen sulfate	[Bmim][HSO ₄]		
1-butyl-3-methylimidazolium methylsulfate	[Bmim][MeSO ₄]		
triethylammonium sulfate	[TEA][HSO ₄]		

2.1.3 Chemicals

2.1.3.1 General chemicals

Absolute ethanol (GPR Rectapur grade) was purchased from VWR. Sodium hydroxide, hydrochloric acid, tris(hydroxymethyl)aminomethane, glacial acetic acid and trichloroacetic acid were acquired from Sigma Aldrich while concentrated nitric acid, 69% (BDH Analar) was bought from VWR. For NMR spectroscopy, MagniSolv solvents were supplied by VWR.

2.1.3.2 Carbon sources

D-(+)-Glucose ($\geq 99.5\%$, G7021) was purchased from Sigma-Aldrich.

Lignin was either obtained from the pretreatment of lignocellulose (as described in the experiment) or commercially available kraft lignin (370959) by Sigma-Aldrich.

Protocatechuic acid ($\geq 97\%$, 37580) was purchased from Sigma-Aldrich. The structure is illustrated below.

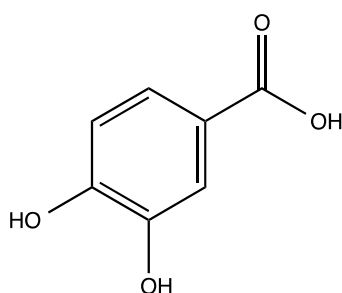


Figure 2.1 The structure of protocatechuic acid.

Sodium formate ($\geq 99\%$, 247596) was purchased from Sigma-Aldrich.

Vanillic acid (97%, H36001) was purchased from Sigma-Aldrich. The structure is illustrated below.

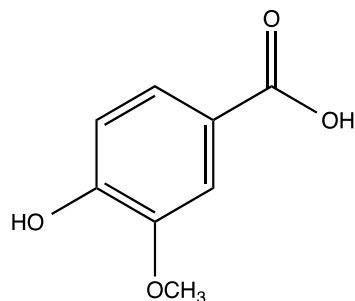


Figure 2.2 The structure of vanillic acid.

Vanillin (99%, V1104) was also purchased from Sigma-Aldrich. The structure is illustrated below.

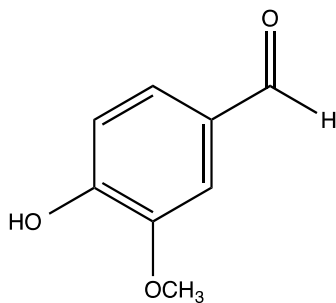


Figure 2.3 The structure of vanillin.

2.1.3.3 Culture media

Water, purified by reverse osmosis ion exchange and carbon filtration in a MilliQ water purification system, was used to prepare these solutions.

LB broth (MILLER) (Cat. No. 1.10285.0500) was purchased from Merck Millipore. 25 g of powder was dissolved in 1 L of distilled water and autoclaved.

LB agar (MILLER) (Cat. No. 1.10283.0500) was purchased from Merck Millipore, 37 g of powder was dissolved in 1 L of distilled water, autoclaved and plated in petri dishes.

Mineral Salts Medium (MSM) was prepared and composed of $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ (1.6 g/L), Na_2HPO_4 (1.4 g/L), $(\text{NH}_4)_2\text{SO}_4$ (0.5 g/L), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.2 g/L), K_2SO_4 (0.5 g/L) and 10.0 ml of trace element containing $\text{Na}_2\text{-EDTA}$ (12.0 g/L), $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ (0.4 g/L), $\text{MnSO}_4 \cdot 7\text{H}_2\text{O}$ (0.4 g/L), $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (0.1 g/L), $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (2.0 g/L), CaSO_4 (1.0 g/L) and $\text{NiSO}_4 \cdot 6\text{H}_2\text{O}$ (0.1 g/L). The MSM was neutralised to pH 7 prior to autoclaving.

2.1.4 Apparatus

Autoclave Prestige Medical Classic (12 L) was used to sterile media and instruments. The conditions were 121 °C for 22 min at 1.05 atm.

Centrifuge ALC Refrigerated Centrifuge PK120R.

Freeze dryer Heto Drywinner

Gas Chromatography / Mass Spectrometry (GC-MS) Agilent HP6890 GC coupled to a Micromass Autospec mass spectrometer. A BPX 5 column, 30 mm x 0.25 mm, with electron impact ionization and Helium as the carrier gas at 1 ml/min, was used. 50 °C was the initial temperature and held for 0.5 min, gradually raised by 10 °C per min up to 300 °C, maintained for 2 min.

Incubator LTE Scientific IP60 (Incubator) and Jeio Tech SI-900R (Incubated Shaker)

Nuclear Magnetic Resonance (NMR) spectra were recorded on Bruker Avance III HD 400MHz NMR spectrometers running TopSpin 3.2 software for ^1H and/or ^{13}C . Proton spectra were measured at room temperature at a frequency of 400MHz with a spectra width of 8000Hz (centred on 6ppm) and 32768 data points. A 30° pulse was employed along with a 1s delay. 16 transients were collected. Data were processed using 16384 data points and an exponential multiplication of 0.3Hz. Chemical shift values are reported in parts per million (ppm) and coupling constants (J-values) are given in Hertz.

UV-Vis Spectrophotometer Perkin Elmer LAMBDA 20

Varioskan Flash Multimode Reader Thermo Scientific was used for the experiments involving microplates.

2.2 Methods

2.2.1 Culture Maintenance

Cultures were maintained on LB agar plates. Prior to growth in liquid culture, a loop of a bacterial colony was added to 10 ml of LB Broth in a Falcon centrifuge tube and incubated at 30 °C with shaking (160 rpm) until it reached an OD₆₀₀ of 0.5 (typically 24 h).

2.2.2 Ionic liquid synthesis

All ionic liquids except for the [Bmim][OBU] were supplied by members of Dr. Jason Hallett's lab group. [Bmim][OBU] was produced in a three stage process. Firstly, 57.5 g of [Bmim][MeSO₄] was diluted with 5 ml of deionized water in a three-necked round-bottomed flask fixed with a thermometer and dropping funnel. 2 drops of aqueous sulphuric acid (5M) were added. While stirring vigorously, the solution was heated at 175 °C for 3 h to boil off the generated methanol. After the solution dropped to room temperature, 50 ml of deionized water and decolorizing charcoal were added, mixed and stirred overnight at 50 °C. The slurry was filtered through silica gel and dried by rotary evaporation followed by a high vacuum, to produce a colourless highly-viscous product. The product was verified as [Bmim][HSO₄] by NMR spectroscopy with the chemical shifts as listed. ¹H NMR (400 MHz, DMSO-d₆): 9.13 (s, 1H), 7.37 (t, J = 2 Hz, 1H), 7.32 (t, J = 2 Hz, 1H), 4.00 (t, J = 7 Hz, 2H), 3.76 (s, 3H), 1.67 - 1.55 (m, 2H), 1.16 - 1.03 (m, 2H), 0.68 (t, J = 7 Hz, 3H).

In the second stage, 1.0 g (1 equivalent) of barium hydroxide octahydrate, Ba(OH)₂·8H₂O was added to 10 ml deionized water. The solution was heated at 70 °C for 1 h. In a separate flask, 1 equivalent of [Bmim][HSO₄] was diluted with an equal volume of deionized water. This solution was slowly dropped into the hot aqueous Ba(OH)₂ solution over 5 min, resulting in the formation of a white precipitate. The mixture was heated at 70 °C for another hour and the cooled to

room temperature and refrigerated for 30 min to assist precipitation. The filtered solution was 1-butyl-3-methylimidazolium hydroxide, [Bmim][OH].

The final stage was the addition of butyric acid to [Bmim][OH] until it reached pH of 7. Rotary evaporation was used to remove the water followed by filtration of any remaining solid material. The product, [Bmim][OBU] was dried under high vacuum at 50 °C. The product was observed as a pale yellow foul smelling viscous liquid and its structure confirmed by NMR spectroscopy with chemical shift as listed. ¹H NMR (400 MHz, DMSO-d₆): δ 10.42 (1H, s), 7.82 (1H, t, J = 2 Hz), 7.73 (1H, t, J = 2 Hz), 4.20 (2H, q, J = 8 Hz), 3.86 (3H, s), 1.75 (4H, m, J = 8 Hz), 1.44-1.32 (4H, m), 0.85 (3H, t, J = 8 Hz), 0.78 (3H, t, J = 8 Hz). ¹³C NMR (100 MHz, DMSO-d₆): δ 175.1, 137.4, 123.5, 121.9, 49.0, 41.4, 35.5, 32.0, 20.1, 19.7, 15.2, 14.6.

2.2.3 Substrate utilization by *Rhodococcus*

2.2.3.1 Growth on carbon substrates

An inoculum of *Rhodococcus* was introduced into MSM with three different carbon sources, vanillic acid (3 and 20 mM), vanillin (3 and 20 mM) and for the control sample, glucose (20 mM). Each of these samples was run in triplicate and incubated at 30 °C for 48 h. The optical density value (OD₆₀₀) was recorded every 12 h during this time.

2.2.3.2 Optimisation of vanillic acid degradation

The same method as described in Section 2.2.3.1 was followed with the concentration of vanillic acid ranging from 1 mM to 20 mM. The OD₆₀₀ was measured after 60 h of incubation.

2.2.3.3 NMR analysis

Inocula were grown in 10 ml of MSM in the incubator (160 rpm, 30 °C) with vanillic acid, protocatechuic acid and sodium formate as the carbon sources. Samples were collected under the assumption that the extracellular enzymes had degraded the substrate and so the extracellular product was analysed. Details of the extracellular enzymes were discussed in Section 4.3. Samples (1ml) were taken at specific intervals or, alternatively, 10ml samples were collected for one-off sampling, centrifuged (4000 rpm, 30 min) and the supernatant was freeze-dried. Samples were dissolved in the NMR spectroscopy solvent (D₂O or DMSO-d₆ as detailed below) and filtered prior to the NMR spectroscopy analysis.

Deuterated NMR solvents, D₂O and DMSO-d₆ were used as required. DMSO-d₆ was used for NMR spectroscopy analysis in the synthesis of ionic liquids whereas D₂O was used in the sampling analysis of the vanillic acid and lignin degradation assays. Chemical shifts are reported downfield of tetramethylsilane, in units of ppm (referenced against the HDO residual peak at 4.80 ppm [¹H] or dimethyl sulfoxide, DMSO-d₆ with the chemical shift at 2.50 ppm [¹H] / 39.50 ppm [¹³C]).

In the oxygen dependence studies, the sample was exposed to the anaerobic atmosphere by purging the oxygen with nitrogen. A 1 ml sample was withdrawn and the culture anaerobically incubated for 12 h. A second 1 ml sample was taken out for analysis. Then, oxygen was introduced back into the system and the culture was incubated an additional 12 h. The third sample (1 ml) was taken and all three samples were freeze-dried prior to the NMR spectroscopy as described in Section 2.1.4.

2.2.4 Lignin degradation by *Rhodococcus*

2.2.4.1 Lignin pretreatment with ionic liquids

Miscanthus x giganteus (grass) was used as the biomass source. The ionic liquid, ([Hbm][HSO₄] or [TEA][HSO₄] (as described in Section 4.2, 4.4 and 4.5 accordingly) was mixed with water in the ratio of 4:1 (w/w). The homogenous solution was added into the air-dried biomass. The sample was incubated in the oven for desired pretreatment temperature and time. The sample was then washed with 40 ml absolute ethanol in a 50 ml Falcon centrifuge tube. This was well-mixed and left at room temperature for 1 h. Then, sample was centrifuged (50 min, 4000 rpm) and the supernatant was collected. This washing step was repeated at least four times. Next, the ethanol was evaporated from the liquid with a parallel evaporator at 40 °C. After that, water was added in the ratio of 3:1 water : ionic liquid (w/w) and centrifuged for 40 min. Solid lignin was separated by decanting and dried in a vacuum oven at 45 °C.

2.2.4.2 Preparation of nitrated lignin

This process followed proposed literature lignin degradation assay.⁸⁷ 25 mg of lignin and 5 ml of acetic acid were mixed and the insoluble material removed by filtration. 1 ml of concentrated nitric acid was added and stirred for 1 h. 10 ml of purified water was added and the solution was adjusted to pH 7 with 1 M of sodium hydroxide, NaOH. The yellow solution formed was diluted 100-fold with 750 mM Tris buffer (pH 7.4) containing 50 mM sodium chloride, NaCl. This diluted nitrated lignin was used for the lignin degradation assay.

2.2.4.3 Lignin degradation assay

The assay was carried out using a Varioskan Flash Multimode Reader. A 96 well plate reader was used, with each well filled with 40 µl of bacterial culture supernatant and 160 µl of nitrated lignin and the absorbance measured at 430 nm

over time. Each assay was run in triplicate with buffer replacing the nitrated lignin or bacterial supernatant in control samples. Different variables were studied including the comparison of kraft lignin and ionic liquid-derived lignin, the effect of different ionic liquids on the lignin degradation and the effect of various durations of ionic liquids pretreatment on the degradation of lignin. The sample was also analysed on LC-MS for the identification of lignin breakdown product(s).

2.2.5 Toxicity of ionic liquids

2.2.5.1 Disk diffusion test

Rhodococcus UKMP-5M was streaked onto an LB agar plate prior to the test. Filter disks (Whatman) were cut with a hole punch to a size of 7mm in diameter. The ionic liquids were dropped onto the disks. Each disk was placed in the middle of the plate containing the bacterial culture. Plates were incubated inverted (as normal) for 48 h at 30 °C. The ionic liquid-impregnated disk should remain adhered to the plate. The diameter of inhibitory zone was measured in millimetres (Figure 2.4). Each disk diffusion assay was repeated three times.

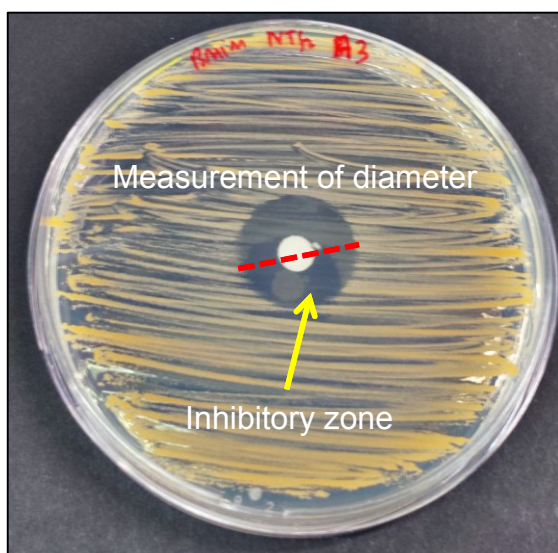


Figure 2.4 The plate of *Rhodococcus* UKMP-5M in the disk diffusion assay. The yellow arrow points to the clear inhibitory zone and the red dashed line indicates the diameter.

2.2.5.2 Bacterial growth test

A 2% inoculum of *Rhodococcus* UKMP-5M was cultured in MSM in a Falcon centrifuge tube. 20 mM of glucose was supplied as a carbon source along with 0, 0.5, 1, 2 or 4 % (v/v) of ionic liquid. The total volume of sample was 10 ml. Cultures were incubated in 160 rpm, 30 °C and the OD₆₀₀ measured at time intervals.

CHAPTER 3

DEGRADATION OF LIGNIN MODEL COMPOUND

3.1 Introduction

Due to the complex nature of lignin's structure, simple and well-characterised model compounds of lignin have been used instead to study potential mechanisms of lignin degradation. Phenol-based compounds are normally used, especially those which are known intermediates in other lignin degradation pathways.²⁰⁹ These building blocks of lignin are mostly connected by ether linkages. As illustrated in Figure 3.1, these compounds range from dimeric units such as guaiacylglycerol- β -guaiacyl ether (GGE) and dehydrodivanillic acid (DDVA), to simple monomers like benzoic acid, veratric acid and vanillic acid. Previous studies have been able to probe the mechanism of lignin degradation through other means by using model compounds.²¹⁰

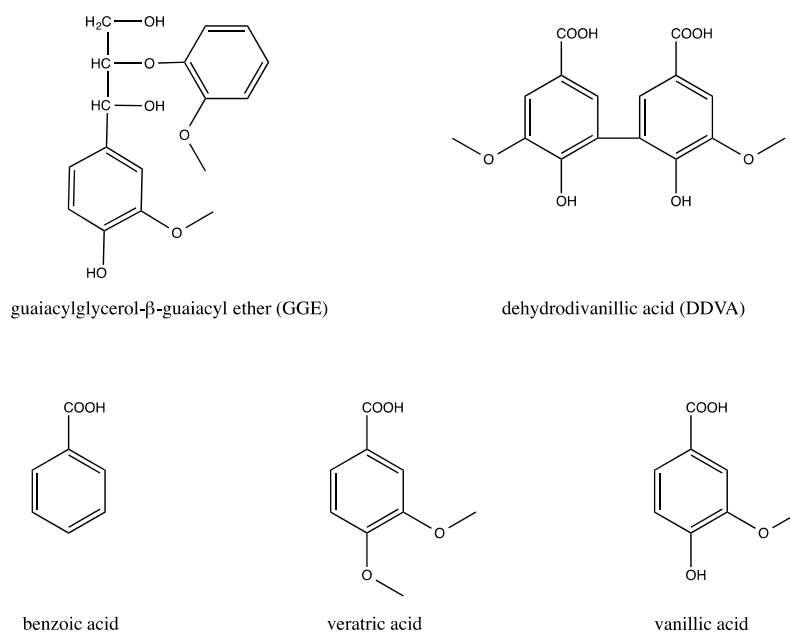


Figure 3.1 Examples of lignin model compounds.

3.2 Degradation of lignin based compounds

In the preliminary screening stage, vanillic acid and vanillin were used as lignin model compounds. Vanillic acid was selected, as it is a well-established product of lignin degradation and has been elucidated as a metabolic product of several prokaryotes.²¹⁰⁻²¹² It can be abundantly found as a component of solubilised lignin biomass.²¹² Vanillic acid is part of the vanillin catabolic pathway of *Rhodococcus*,²¹³ thus the comparison between both vanillin and vanillic acid as substrates for *Rhodococcus* will establish the utility of these model compounds for this study. The growth of *Rhodococcus* UKMP-5M with vanillic acid and vanillin as the sole carbon source was observed during a 48 h period. The optimum glucose concentration, 20 mM was used as the control. Two different concentrations were selected as the substrates, 20 mM following the concentration of glucose and 3 mM as suggested by a literature report of lignin decomposition by actinomycete *Nocardia* sp., which is a closely related species to *Rhodococcus*.²¹⁴ Figure 3.2 showed the results of growth curves using both vanillin and vanillic acid as carbon and energy sources.

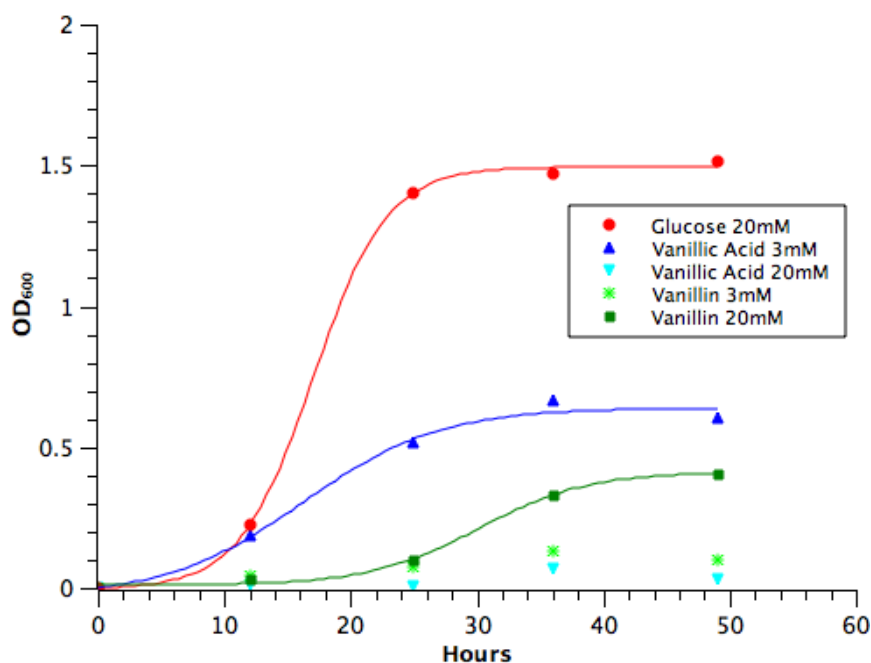


Figure 3.2 Growth curves of *Rhodococcus* UKMP-5M on vanillic acid, vanillin and glucose. Cells were grown at 30 °C in MSM with substrate compounds as sole carbon and energy sources.

This experiment demonstrated that *Rhodococcus* UKMP-5M has the ability to degrade both vanillic acid and vanillin, taking up these substrates as the sole carbon source for biomass production. Compared to the control, vanillic acid and vanillin showed a slower growth rate of *Rhodococcus* UKMP-5M. There is no significant bacterial growth observed in either 20 mM vanillic acid or 3 mM vanillin. A possible reason is that 20 mM vanillic acid is toxic to the growth of the bacteria whereas 3 mM vanillin is too little to permit significant growth. A study demonstrated the growth of *Saccharomyces cerevisiae* in culture media was enhanced by the presence of low concentration of vanillin (1 mM) but this became toxic at higher concentrations (6 mM). In our study, no growth was observed in 3 mM vanillin while 20 mM gave some bacterial growth.²¹⁵

Recently, the ability of *Rhodococcus* sp. to use 1mM vanillic acid and vanillin as the sole carbon source has been reported in *Rhodococcus opacus* D360²¹⁶ and *Rhodococcus jostii* RHA1.²¹³ It is worth noting that the ability to use vanillic acid as a sole carbon source is not exclusive to *Rhodococcus*. Vanillic acid metabolic activity has also been reported in other actinomycetes, *Nocardia* sp.²¹⁴ and *Streptomyces* sp.^{212, 217} along with *Bacillus subtilis*.²¹⁷

Figure 3.2 suggests that vanillic acid gives a higher growth rate for *Rhodococcus* UKMP-5M as compared to vanillin. The lag phase is extended in vanillin, suggesting that vanillin is more toxic than vanillic acid and it takes longer for *Rhodococcus* UKMP-5M to adapt and grow in the presence of this substrate. The lag phase is the period where bacteria are growing in size but not in population, adapting themselves to the growth conditions. The present results are in agreement with a similar study held with *R. jostii* RHA1; vanillin but not vanillic acid inhibited the growth of this bacterial strain at higher concentrations with no growth observed in 10 mM vanillin.²¹³ In a different study using *R. opacus* D360, the strain took 5 days to grow in vanillin, slower than the growth shown in vanillic acid (2 days).²¹⁶ In the same study, however, in R2A media containing soluble starch, *R. opacus* D360 was able to grow in a higher concentration of vanillin, up to 1.0 g/L (7 mM) whereas the same bacteria could only tolerate up to 1 mM of

vanillic acid with no growth observed in 1.0 g/L, which is equal to 6 mM concentration.²¹⁶

The toxicity of vanillin could be due to the aldehyde moiety and/or vanillin's hydrophobicity, which have been linked to its antimicrobial properties.²¹⁸ Aldehydes are good electrophiles and could form reaction products with biological compounds such as lipids, proteins and nucleic acids.²¹⁹ Vanillin has been studied for its role in controlling food spoilage, inhibiting the growth of yeasts with the minimum inhibitory concentration (MIC) as low as 13 mM.²¹⁸ Meanwhile, the inhibitory concentration, IC_{50-24h} of vanillin in *E. coli* is 3 mM.²¹⁹ It has been reported that aromatic aldehydes in general may be toxic to *R. jostii* RHA1²¹³ and the same could possibly be applied to our strain of *Rhodococcus*.

3.3 Optimisation of the degradation process

Since it has been established that *Rhodococcus* UKMP-5M has a higher growth rate in vanillic acid than vanillin, the effect of the degradation of vanillic acid in various concentrations was investigated. The growth of *Rhodococcus* UKMP-5M in vanillic acid was conducted in different concentrations in a range of 1 to 20 mM. The optical density (OD) value of bacterial culture was measured via UV-spectrophotometer after 48 h. The results are illustrated in Figure 3.3.

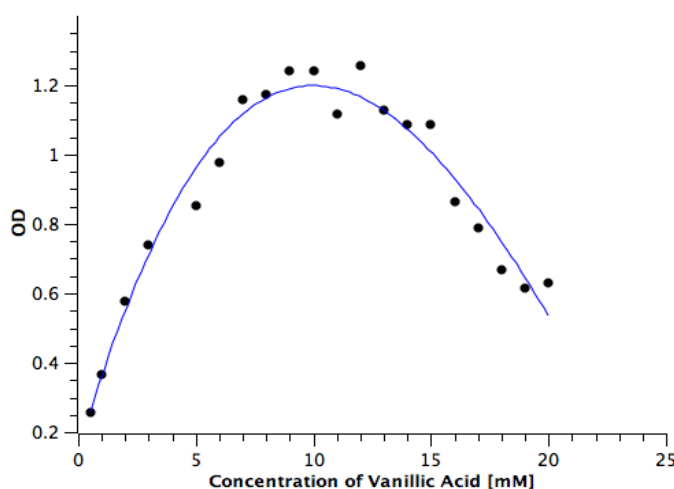


Figure 3.3 The growth of *Rhodococcus* UKMP-5M in various concentrations of vanillic acid.

A concave curve is observed in this graph; the growth increases with an increase of vanillic acid concentration, followed by a parabolic effect observed in the range of 6 to 14 mM before a gradual decrease of growth associated with the increase of concentration of this substrate. Based on the above figure, *Rhodococcus* UKMP-5M gives a maximum growth rate in 10 mM vanillic acid with the first notable inhibitory effect being observed when exposed above 15 mM vanillic acid. The shape of this curve derives from the nature of balancing the roles of substrates as nutrient and toxin. The substrate supplies a carbon source to the bacteria and increases the growth rate. However, there will be a point where the increase of toxicity outweighs this role as a nutrient. This explains the decreasing trend of growth rate with the increase of toxicity.

This bacterial strain showed a better tolerance towards vanillic acid compared to other studies reported in the literature. Vanillic acid showed signs of inhibition in the growth of *Staphylococcus aureus* and *Lactobacillus paraplantarum* at a concentration of 6 mM while the growth of *Lactobacillus coryniformis* starts to show an inhibition effect at a concentration of 1.5 mM.²²⁰ One possible toxicity mechanism of this compound is due to the hyperacidification of cell membranes. Studies on phenolic acids demonstrated that their dissociation causes hyperacidification, altering the cell membrane potential and disrupting the ATPase pump.²²⁰⁻²²² The lack of an outer membrane in *S. aureus* and *Lactobacillus* sp. could cause the reported susceptibility of these bacteria to this irreversible membrane alteration effect.²²⁰ This could explain the better tolerance of *Rhodococcus* to vanillic acid, which could be due to its robust cell wall containing mycolic acids.²²³ *Rhodococcus* has several defence and adaptation mechanisms. For example, *Rhodococcus* is able to produce biosurfactants, increasing cell surface hydrophobicity and altering membrane fluidity.^{223, 224}

3.4 Understanding the metabolic pathway

Since vanillic acid degradation by *Rhodococcus* has been demonstrated by the growth curve in Figure 3.2, the mechanism of degradation was next studied. ¹H

NMR spectroscopy was used as the main method in identifying the compounds involved in the degradation of vanillic acid. Prior to the NMR analysis, a sample of culture containing vanillic acid was collected and freeze-dried in order to remove water and concentrate the compounds (as detailed in the Chapter 2: Experimental Design). The NMR spectrum for the sample collected at the beginning of the experiment is displayed in Figure 3.4.

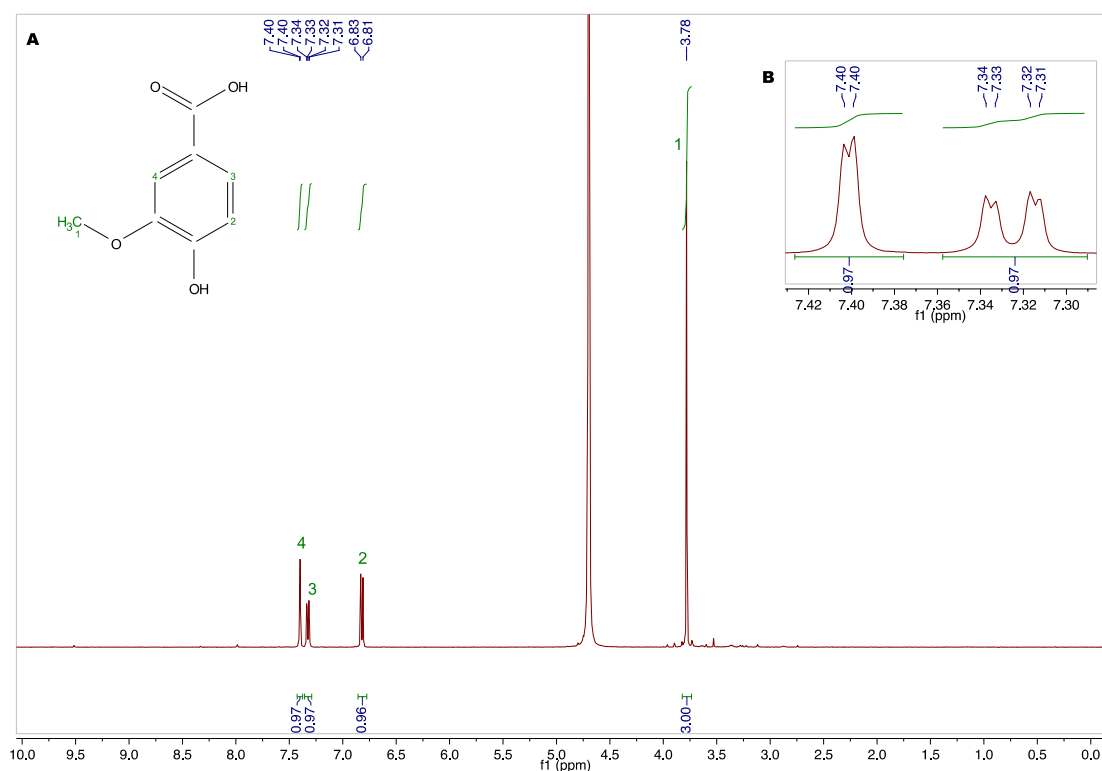


Figure 3.4 ^1H NMR spectrum of vanillic acid at 0 h. Insert spectrum highlights the peaks between 7.0 ppm to 7.5 ppm.

The proton assignments were made based on chemical shifts in similar compounds and the spin coupling patterns, as detailed below.

^1H NMR (400MHz, D_2O): δ 7.40 (d, J = 2Hz, 1H, CH ring), 7.33 (dd, J = 8Hz, 1H, CH ring), 6.82 (d, J = 8Hz, 1H, CH ring), 3.78 (s, 3H, CH_3) ppm.

Figure 3.4 confirms that vanillic acid is the only organic compound present in the media and hence the sole carbon source. The noise between 4.0 to 3.0 ppm could be attributed to the presence of Na₂-EDTA in the MSM media.

In order to determine the metabolic pathway that is taking place during the degradation of vanillic acid by *Rhodococcus* UKMP-5M, samples were collected over a time course of 48 h and are shown in Figure 3.5.

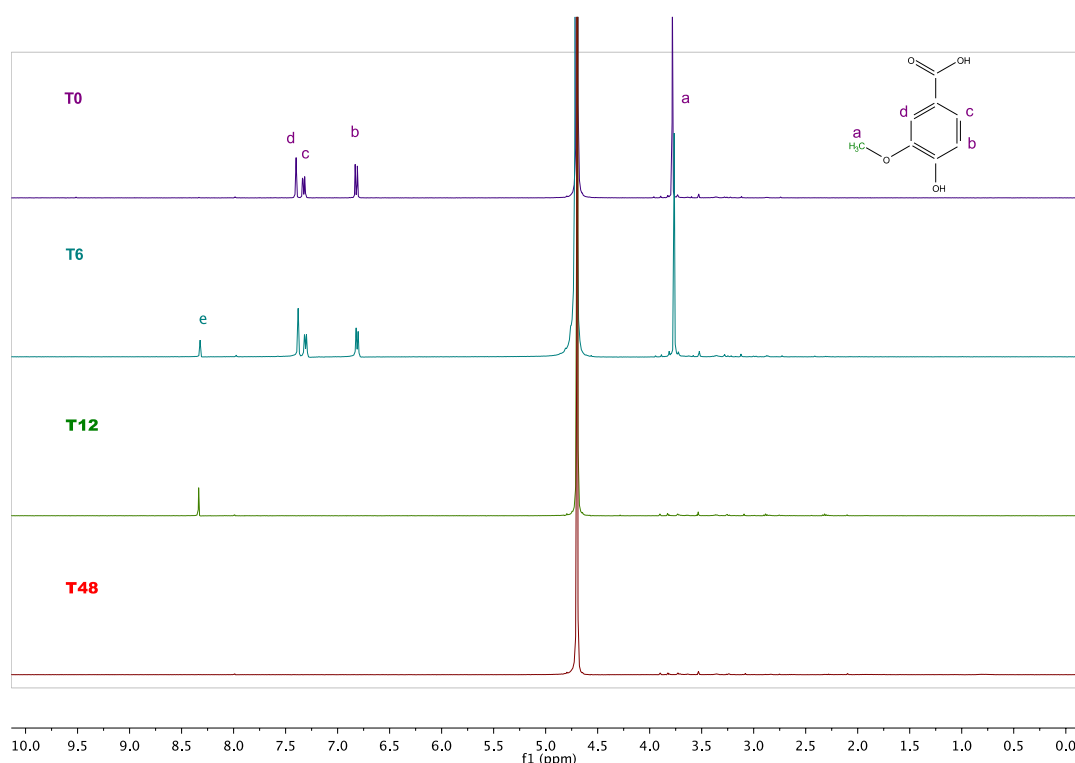


Figure 3.5 ¹H NMR spectra of the degradation of vanillic acid by *Rhodococcus* UKMP-5M at the interval times of 0, 6, 12 and 48 h.

Figure 3.5 shows the stacked NMR spectra of culture supernatants from *Rhodococcus* UKMP-5M in vanillic acid. There is a clear reduction of peak intensity assigned for vanillic acid throughout the time with a complete disappearance at 12 h, confirming that vanillic acid is completely metabolized and disappears from the media after 12 h. After 6 h, there is a singlet peak (labelled 'e') emerging at 8.3 ppm, which also completely disappears after 48 h. The

hypothesis is that 'e' is a signal from an intermediate that is accumulated and then degraded. In order to confirm this, a fed batch experiment was performed.

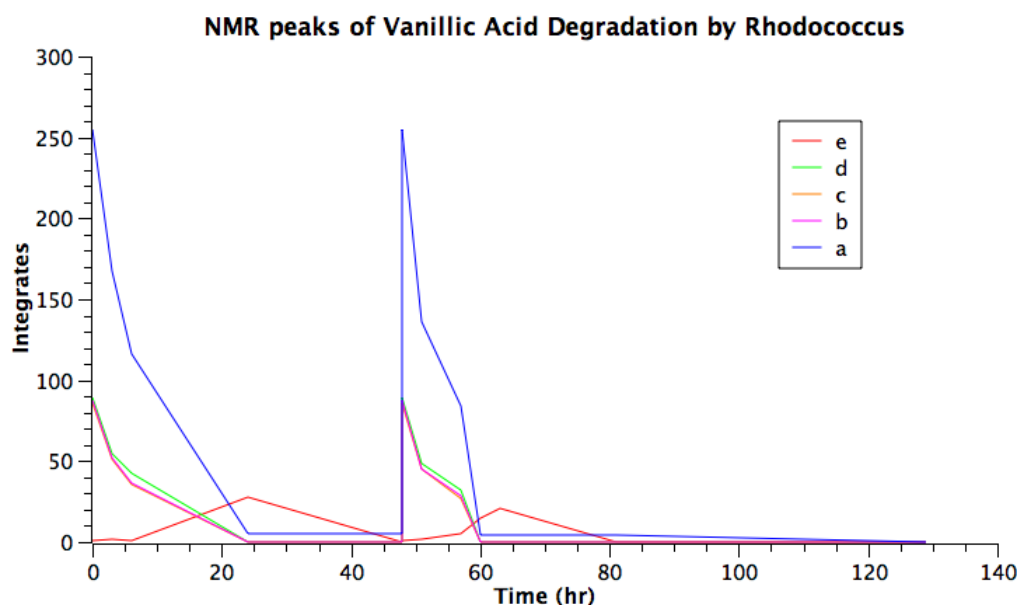


Figure 3.6 Chemical shift changes of the ^1H NMR spectra vs time for vanillic acid degradation. Refer to Figure 3.5 for the peak assignments.

As shown in Figure 3.6, the intensity of peak 'e' increases while vanillic acid concentration decreases. After 48 h, once peak 'e' has disappeared, the culture was fed with vanillic acid again and the same trend occurred only the time span was shorter to complete the process. This suggests that peak 'e' is an intermediate compound. One possible explanation for the shorter time of second cycle is that the bacterial cells no longer used vanillic acid to grow, instead solely regulating a vanillic acid metabolism as resting cells. Alternatively, it is possible that the regulated enzyme has been induced, thus, the degradation of vanillic acid becomes faster.

This peak is probably due to the formation of formate considering that vanillic acid is the substrate used. While attempting to identify compound 'e', it was found that this compound is extremely soluble in deuterium oxide (D_2O) as NMR solvent but not in other solvents including deuterated dimethyl sulfoxide (DMSO-d_6). Attempts to identify compound 'e' using mass spectrometry failed,

which could be due to its low molecular weight. The chemical shift of 8.3 ppm is slightly shifted upfield compared to the value for the formate ^1H NMR peak of 8.5 ppm in the SDBS database.²²⁵ However, these values are sufficiently close to be the same species as the chemical shift observed is also dependent upon the cations present. The peak assigned to formate in the database is coupled with sodium and DMSO- d_6 was used as the reference solvent.²²⁵ Formate seems the most plausible fit for compound 'e' and is also a reasonable metabolic intermediate.

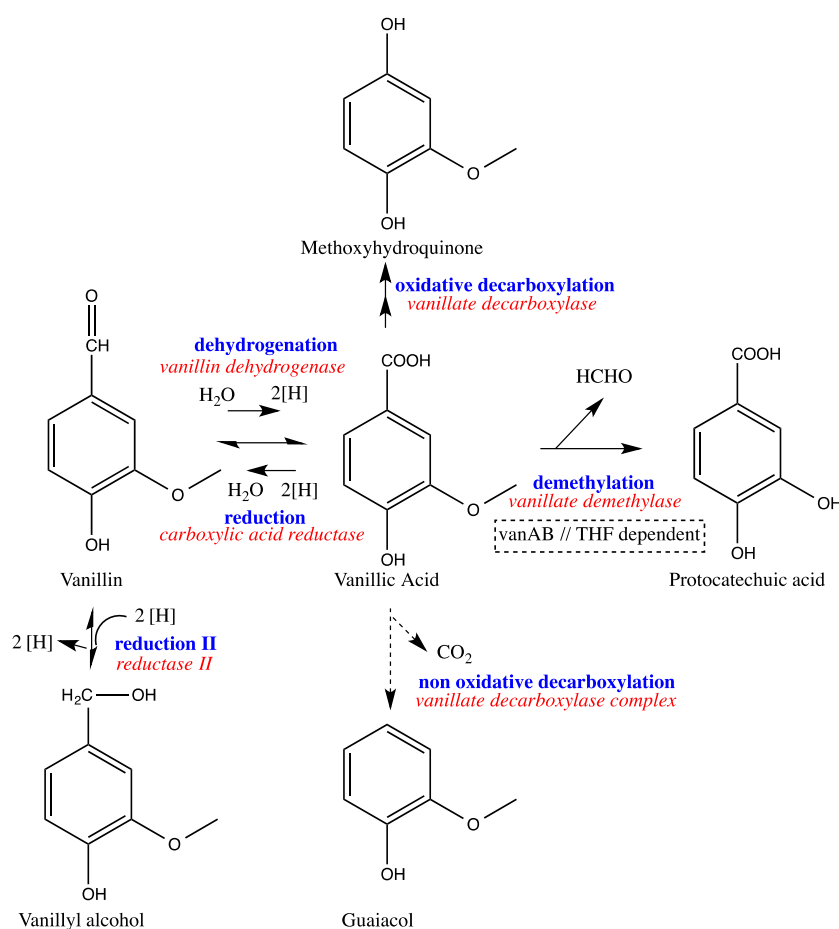


Figure 3.7 The microbial degradation pathway for vanillic acid as reported in the literature.^{216, 217, 226-229} The dashed arrow is the reaction with uncharacterised enzyme. The dashed box is the responsible gene cluster responsible for the reaction. The bold blue text indicates the enzyme reaction while italic red text denotes the identified enzymes.

There are four plausible routes for microbial degradation of vanillic acid as reported in the literature (refer to Figure 3.7). For instance, *Streptomyces sp.* and

Bacillus subtilis degrade vanillic acid to produce guaiacol²¹⁷ whereas *Nocardia* sp. metabolise vanillic acid to give 69% yield of guaiacol and 11% vanillyl alcohol.²²⁹ In addition, formaldehyde is released by *Burkholderia cepacia* while degrading vanillic acid to protocatechuic acid.²²⁸ Moreover, *R. jostii* RHA1 also produces protocatechuic acid from vanillic acid degradation.²²⁹ In fungi, protocatechuic acid and methoxyhydroquinone are the common intermediates prior to ring cleavage of phenolic compounds.^{209, 211} Likewise, *R. opacus* has also been reported to produce protocatechuic acid and methoxyhydroquinone as vanillic acid degradation metabolites.²¹⁶

It is also possible for bacteria to possess parallel pathways. It has been reported that *Streptomyces* sp. simultaneously decarboxylates vanillic acid to guaiacol and degrades it via the protocatechuate pathway in an equimolecular ratio.²¹⁷ In addition, a different published study showed *Nocardia* NRRL 5646 has the ability to concurrently transform vanillic acid into two products via two different metabolic pathways, decarboxylation and reduction to form guaiacol and vanillin respectively.²²⁹

No protocatechuic acid or formaldehyde was detected in this study. However, due to the presence of formate, the most likely pathway for vanillic acid degradation by *Rhodococcus* UKMP-5M would be the demethylation of vanillic acid to produce protocatechuic acid and possibly formaldehyde, which is then immediately oxidised into formate. This intermediate metabolite would thus have been completely consumed in Krebs cycle. If the rate of consumption for protocatechuic acid is faster than formic acid, the former would be unable to accumulate sufficiently for NMR detection. It is also possible that formaldehyde has a shorter half-life and so the six hours interval gap of sampling is too long to detect the presence of formaldehyde.

In order to confirm this proposed scheme, *Rhodococcus* UKMP-5M was fed with protocatechuic acid and sodium formate. Figure 3.8 and Figure 3.9 showed the spectra of the degradation of protocatechuic acid and sodium formate respectively.

The complete disappearance of the peaks after 48 h in Figure 3.8 (*Spectrum C*) suggests the complete degradation of protocatechuic acid by *Rhodococcus* UKMP-5M. This is consistent with a study of vanillic acid degradation by *R. opacus* DSM 1069 and PD630. Monitored by HPLC, both strains were able to fully consume protocatechuic acid.²³⁰ The bacterial degradation mechanism of protocatechuic acid has been described in the literature. An ortho cleavage of protocatechuic acid between C3 and C4 and meta cleavage between C4 and C5 have both been observed in *Pseudomonas sp.*²³¹ A different mechanism was observed in *Bacillus sp.* through oxidative meta cleavage between C2 and C3.²³² These ring opened-products will then finally produce pyruvate, which enters the Krebs cycle.

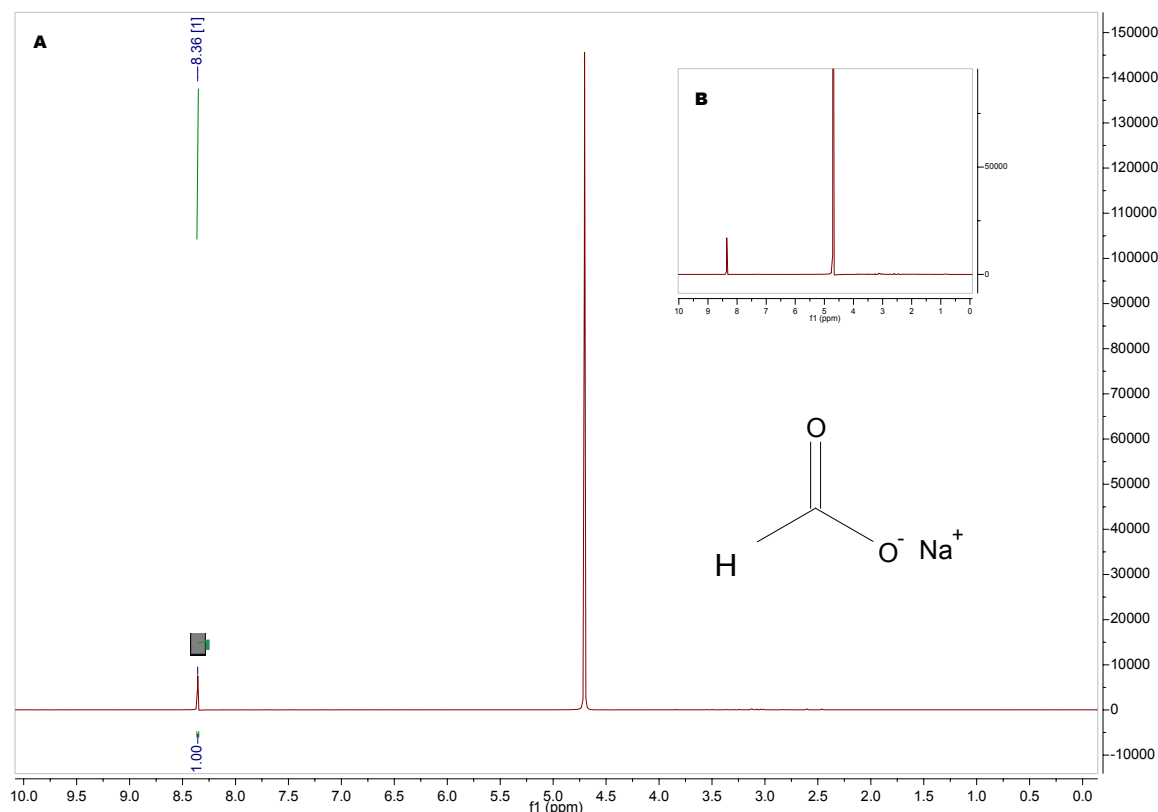


Figure 3.9 ^1H NMR spectra of the degradation of sodium formate by *Rhodococcus* UKMP-5M at different stages: a) 0 h; b) 48 h.

Although the assigned peaks for protocatechuic acid had completely disappeared after 48 h, the same occurrence was not observed with sodium formate. Unlike the initial hypothesis of formate degradation by *Rhodococcus* UKMP-5M, Figure 3.9 suggests that this strain is not able to degrade sodium formate. This is actually consistent with one study reported in literature involving *R. jostii*. In the study, two strains of this species, RHA 1 and DSM 44719 showed no growth when sodium formate was introduced as the sole carbon source.²³³ In contrast to our study, *R. erythropolis* were able to grow in sodium formate.²³⁴ Genomic studies showed the presence of formate dehydrogenase gene in *R. erythropolis*.²³⁵ Nevertheless, it is worth noting that the peak assigned for formate is at 8.3 ppm, which is identical to the one observed after 6 h during the degradation of vanillic acid (Figure 3.5).

One possible explanation for the inability of *Rhodococcus* UKMP-5M to metabolise sodium formate could be that this involves a cometabolism reaction, which requires the presence of protocatechuic acid to induce formate degradation. In order to test this hypothesis, *Rhodococcus* UKMP-5M was introduced with protocatechuic acid and sodium formate as combined substrates. The NMR spectra are exhibited in Figure 3.10 below.

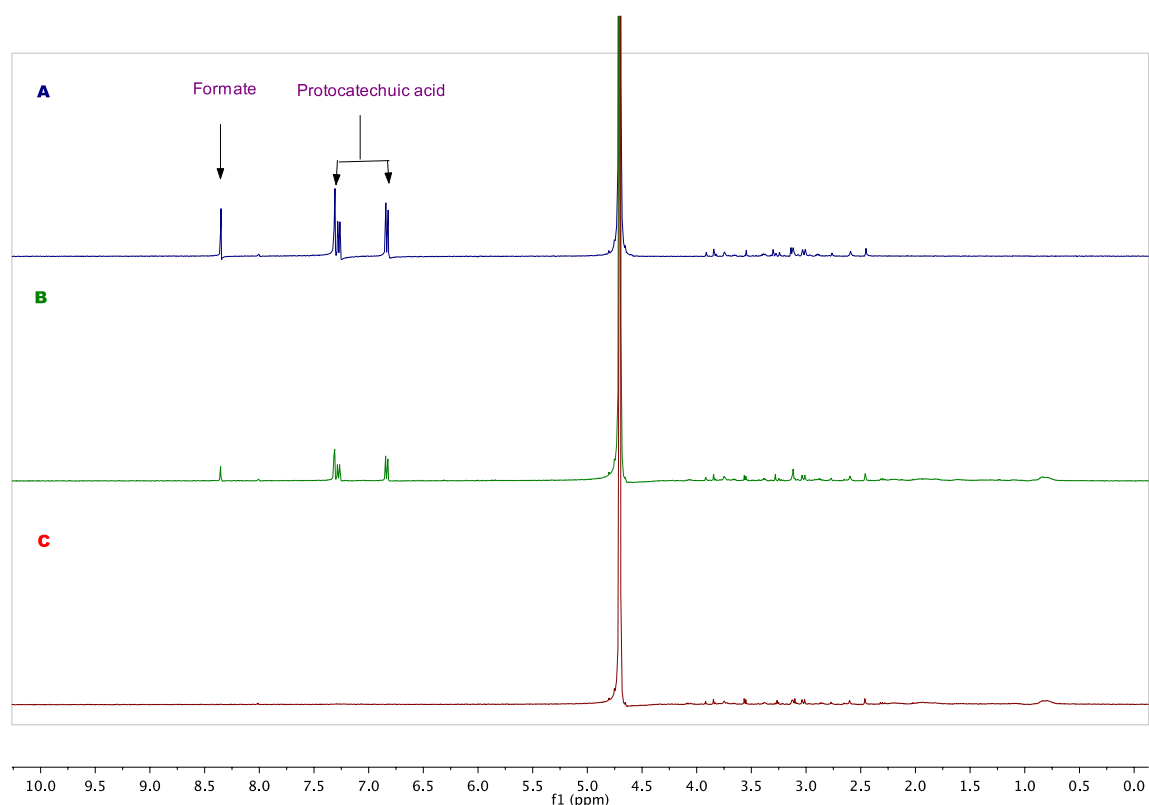


Figure 3.10 ^1H NMR spectra of the degradation of combined substrates by *Rhodococcus* UKMP-5M at different stages: a) 0 h; b) 48 h; c) 96 h.

Based on Figure 3.10, all of the peaks had disappeared after 96 h. This indicates complete degradation by *Rhodococcus* UKMP-5M, which suggests that co-metabolism occurred. Sodium formate was only degraded in the presence of protocatechuic acid. Protocatechuic acid has been demonstrated to be an inducer in vanillic acid degradation previously.²³⁶

Co-metabolism of aromatic compounds by *Rhodococcus* species is well documented in the literature. The capacity of *Rhodococcus* AM 144 in degrading aniline into catechol was greatly enhanced in the presence of glucose as a co-substrate. Glucose provides reducing power through regeneration of NADH and NADPH cofactors to assist oxygenative transformation reaction, transforming aniline into catechol.²³⁷ *Rhodococcus* also possesses cometabolism in degrading chlorinated aromatic compounds. Studies have demonstrated the co-metabolism capacity of *Rhodococcus* R04 and S7 in degrading 2-chlorobenzoic acid²³⁸ and 2-chlorobenzoate²³⁹ respectively in the presence of glucose.

A study of the degradation of polycyclic aromatic hydrocarbons (PAHs) revealed co-metabolism as an important feature in attacking wide range of PAH compounds. No activity was observed when PAHs were introduced as individual substrates but once naphthalene vapours were introduced as a co-substrate, *Rhodococcus* sp. was able to degrade phenanthrene, anthracene and fluorine.²⁴⁰ Cometabolism is common to microbes as a mechanism to transform toxic compounds or potential nutrient sources such as nitrogen or sulphur-containing compounds²³⁷.

In a separate study with *R. UKMP-5M*, the degradation of cyanide formed formate, which was then fully consumed by this strain.¹⁰⁷ The authors also mention that the formation of formate in cyanide degradation is not observed in other cyanide degradation studies, suggesting that this could be a novel route by the *Rhodococcus* strain.²³³ Instead of formamide as the conventional final product of cyanide degradation, this substrate was broken down into formate and ammonia by *Rhodococcus* UKMP-5M.¹⁰⁷ Although this is not mentioned by the authors, it is possible that the bacteria produce formamidase to regulate this reaction.

Based on literature precedent, an oxygenase enzyme is likely responsible for the degradation of the aromatic ring.²⁴¹ Thus, the oxygen dependence of the vanillic acid degradation was investigated to further our understanding of the degradation mechanism. The protocol was detailed in Section 2.2.3.3.

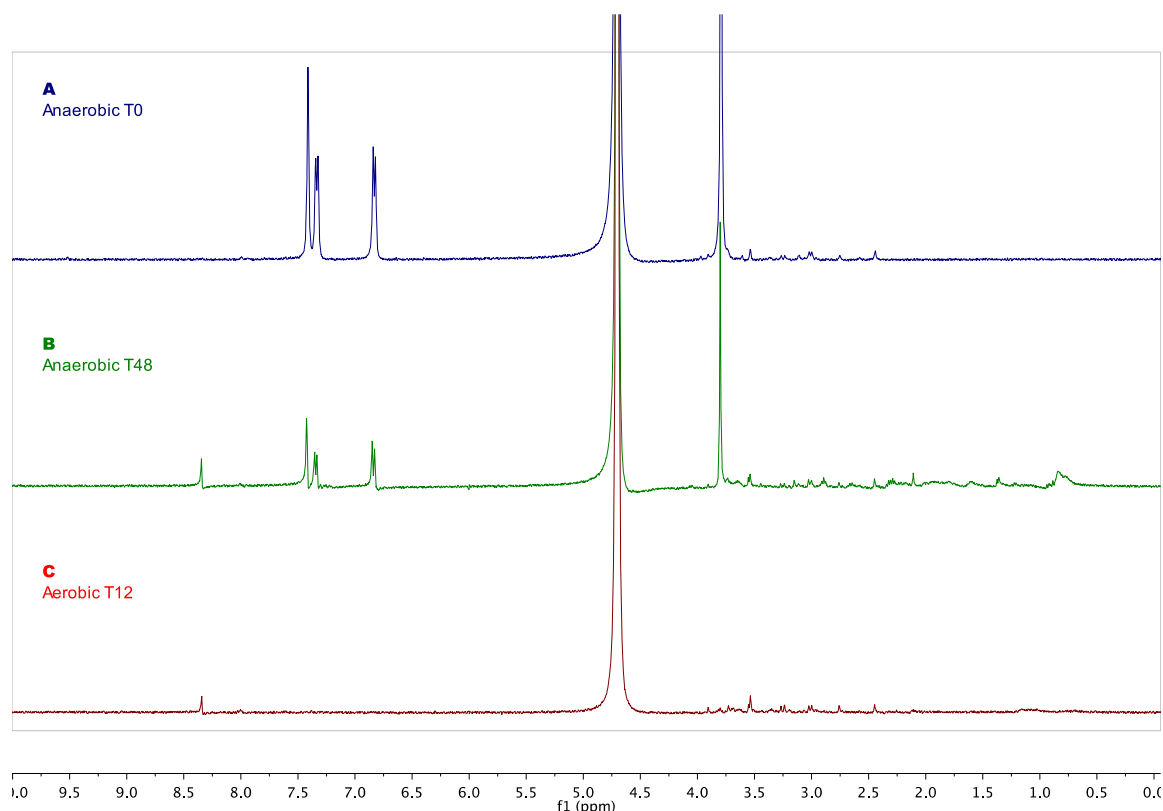


Figure 3.11 ^1H NMR spectra of the degradation of vanillic acid by *Rhodococcus* UKMP-5M at different stages: a) anaerobic conditions; b) anaerobic conditions after 48 h; c) reintroduction of aerobic conditions after 12 h.

As shown in Figure 3.11, the peaks assigned for vanillic acid were still present after 48 h under anaerobic conditions. However, once oxygen was supplied and the aerobic conditions were restored, vanillic acid completely disappeared after 12 h. This experiment demonstrated that vanillic acid degradation is an oxygen dependent process. This is in agreement with one study conducted with *Streptomyces* sp. According to this study, demethylation is strictly dependent on the presence of the oxidising agent, hydrogen peroxide, which results in the formation of protocatechuate.²⁴²

It is noteworthy that the formate peak emerged after 48 h under anaerobic conditions. One possible explanation is that oxygen is not completely removed in the culture, thus a vanillic acid degradation process still occurs but at a very slow rate. However, vanillic acid degradation under anaerobic conditions has been demonstrated by *Pseudomonas* PN1²³⁶ albeit at a slower rate than under aerobic

conditions. This aerobic species has been considered as a facultative anaerobe by some. *Pseudomonas* PN1 is able to degrade vanillic acid under both aerobic and anaerobic conditions with protocatechuate being identified as the intermediate product. Furthermore, formaldehyde was detected under aerobic conditions but not under the anaerobic conditions of vanillic acid degradation by *Pseudomonas* PN1.

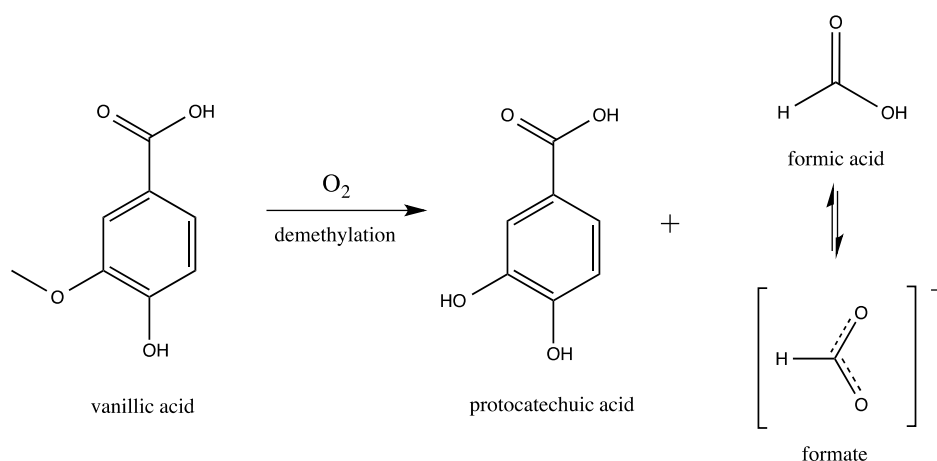


Figure 3.12 The proposed metabolic pathway of vanillic acid degradation. It is likely that the production of formate is via formaldehyde as the initial product of demethylation.

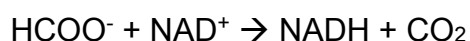
Summarising the data collected at this stage, a proposed metabolic degradation of vanillic acid is illustrated in Figure 3.12. This oxygen dependent reaction involves demethylation of vanillic acid into protocatechuic acid with formic acid reversibly forming via deprotonated formate.

Based on the literature, there are two different types of vanillate demethylase (refer to Figure 3.7), which have been characterised for the demethylation of vanillic acid. The cluster genes of *vanA* and *vanB* have been identified from *Rhodococcus jostii* RHA1. The *vanA* gene and *vanB* gene encode the oxygenase and reductase components respectively. This cluster is predominantly cotranscribed from a promoter upstream of *vanA*. However, among the completed *Rhodococcus* genome in GenBank, this similar set of

genes can only be found in *Rhodococcus opacus* B4 while *vanA* gene is missing from the pathogenic *Rhodococcus equi* and *Rhodococcus erythropolis*.²¹³

On the other hand, a tetrahydrofolate (THF) dependent demethylation enzyme has been identified in *Pseudomonas paucimobilis*.³³⁵ Even though this reaction was identified under aerobic conditions, it is typically present under anaerobic conditions. Characterisation of the *Sphingobium* sp. SYK-6 strain revealed that the methyl group of vanillic acid is transferred to THF, which is later utilised as a carbon source.³³⁶

The production of formate as a vanillic acid degradation product has not been reported before, suggesting that the degradation of vanillic acid by *Rhodococcus* UKMP-5M has a different possible route. The presence of formate is the source of the NADH generator²⁴³ as shown in the equation below:



It is believed that formaldehyde dehydrogenase is responsible for converting formaldehyde into formate. The presence of the formaldehyde dehydrogenase (*fdh*) gene in *Rhodococcus* UKMP-5M strengthens this theory (genome sequence to be published).

The NADH that is being produced will be incorporated into the cellular mechanism. In respect to bioligninolysis, NADH has been reported to stimulate the cleavage of β -aryl ethers. A study of *Pseudomonas paucimobilis* SYK-6 revealed the role of NADH as a cofactor for the membrane bounded β -aryl ether cleaving enzyme.²⁴⁴ Apart from that, NADH is involved in the bacterial degradation of aromatic compounds as shown in Figure 3.13.

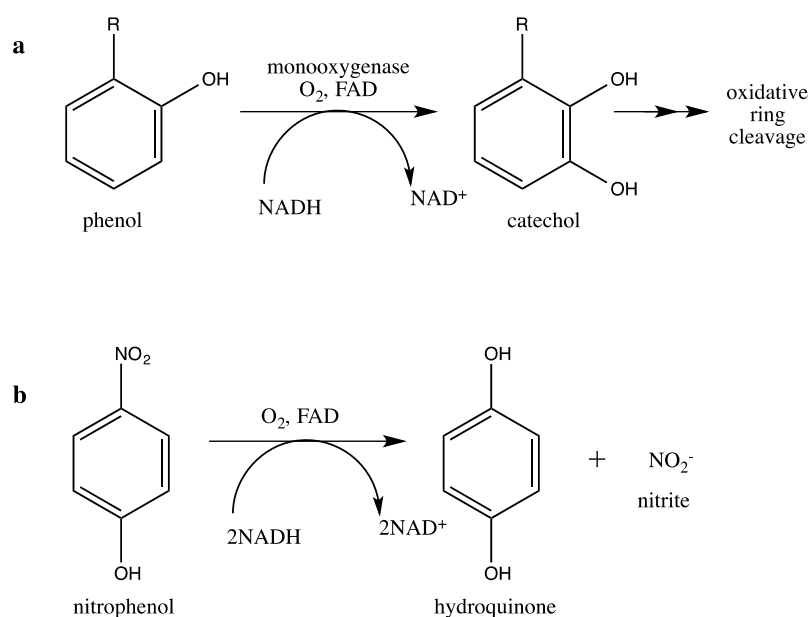


Figure 3.13 NADH-dependent reaction in the bacterial degradation of aromatic compound.

An ortho-hydroxylation process of phenol by FAD-dependent monooxygenases is assisted by NADH to produce catechol (Scheme A, Figure 3.13). Likewise, nitrite removal is also an NADH-dependent reaction as illustrated in Scheme B, Figure 3.13.

With respect to *Rhodococcus*, it was demonstrated that *Rhodococcus* BPG-8 integrates NADH in a protective mechanism to prevent the autooxidation of the toxic intermediate, 1,2,4-benzenetriol (Figure 3.14). This reaction stabilises 1,2,4-benzenetriol to allow catalysis by meta cleavage enzymes.

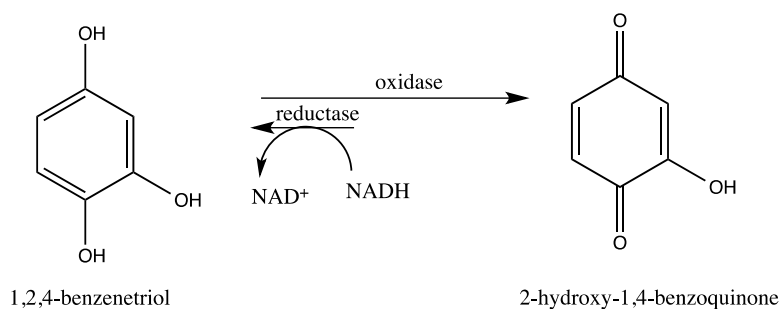


Figure 3.14 Protective mechanism of *Rhodococcus* BPG-8.

Based on the literature,^{84, 114, 213, 217, 242, 245-247} protocatechuic acid is then fed into the β -ketoadipate pathway. The integration of vanillic acid degradation into the ligninolysis pathway is elucidated in Figure 3.15. Lignin can be depolymerised by peroxidase to form dimers; for example β -aryl ethers. As reported in the literature,¹¹⁴ DypB is the enzyme that is responsible for degrading lignin in *R. jostii* RHA1, which is possibly the same mechanism followed by *Rhodococcus* UKMP-5M. However, the potential involvement of other enzymes in disrupting the lignin structure should not be dismissed. Esterase activity could be the source of some of the lignin fraction such as vanillin and coniferyl alcohol subunits.²⁴²

Oxidative cleavage targeting β -O-4 lignin linkages, which is caused by DypB peroxidase, will result in the formation of guaiacol and vanillin.¹¹⁴ Next, vanillin dehydrogenase will transform vanillin into vanillic acid. Vanillic acid could also be produced from ferulic acid. Then, vanillic acid is further degraded into protocatechuic acid via demethylation by vanillate demethylase.²¹³ Protocatechuic acid (also known as 3,4-dihydroxybenzoic acid) will subsequently be processed through the β -ketoadipate pathway. Protocatechuate 3,4-dioxygenase will catalyse the ortho cleavage for ring fission.²⁴⁶ The final product of this pathway, succinyl-CoA and acetyl-CoA will trigger the Krebs cycle, generating energy and releasing carbon dioxide to complete this system.

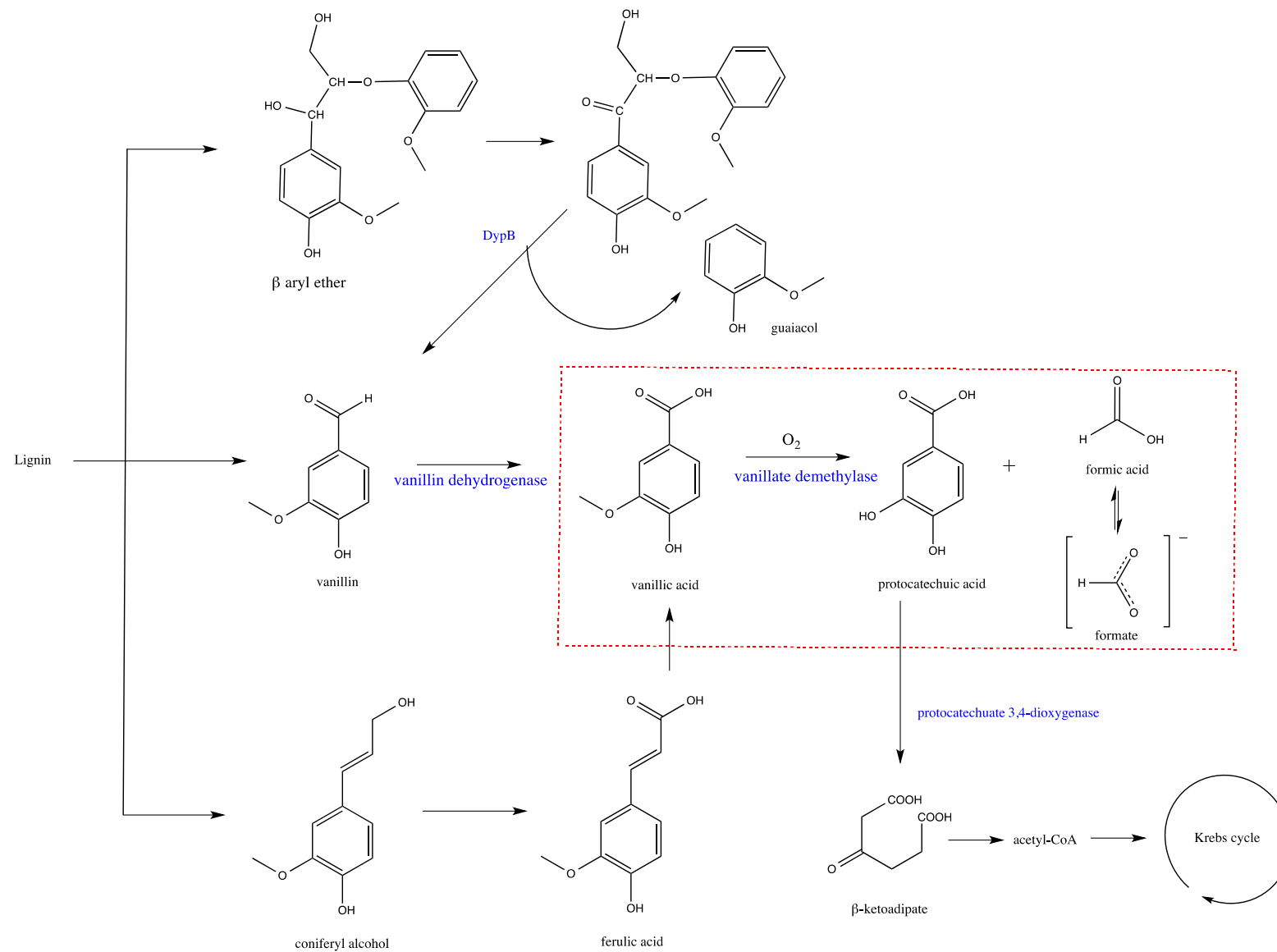


Figure 3.15 Lignin catabolic pathway involving vanillic acid degradation.^{84, 114, 213, 217, 242, 245-247}

3.5 Conclusion

The ability of *Rhodococcus* UKMP-5M to degrade vanillic acid has been demonstrated and the integration of this process in ligninolysis has been shown. The ability to degrade this hydroxylated phenolic compound in general should be a good indicator of its ability to breakdown lignin, a polymer with abundance of phenolic hydroxyl groups connected by ether linkages.

The understanding of the metabolic pathway of vanillic acid degradation and its integration in bioligninolysis is very beneficial. This knowledge can be exploited, for example in producing the valuable vanillin metabolite. Through the deletion of vanillin dehydrogenase gene, vanillin is accumulated by *R. jostii* RHA1.⁸⁴ The mutant strain can give a high yield of this precious bioproduct, which is the world's most popular flavouring agent.²⁴⁸

Furthermore, this study provided insight into a possible new route of vanillic acid degradation by *R. UKMP-5M*. Further study is required to elucidate the mechanism involved, which results in the production of formate instead of formaldehyde. Nevertheless, this study has achieved the main objective in establishing the ability of *R. UKMP-5M* to degrade lignin model compounds and the pathway responsible for this degradation process.

CHAPTER 4

LIGNIN DEGRADATION

4.1 Introduction

Microbial lignin degradation has been studied by several assays, each with their own pros and cons. The available assays have evolved through the development of new technology, which results in the design of better and more reliable assays.

One of the basic methods is by growing the microorganism on agar plates.²⁴⁹ Lignin containing agar is used to incubate the culture. After the culture is scraped off with a razor, the surface is stained green with ferric chloride – potassium ferricyanide reagent. The surface in which lignin has been degraded will remain colourless. This method is inexpensive but the incubation period is long and the result is qualitative. Furthermore, the reagent is mainly used to indicate the presence of phenols. Thus, the result is a non-specific representation of lignin dephenolisation, either by degradation through breakdown of lignin molecule or of phenol oxidation leading to dephenolisation.

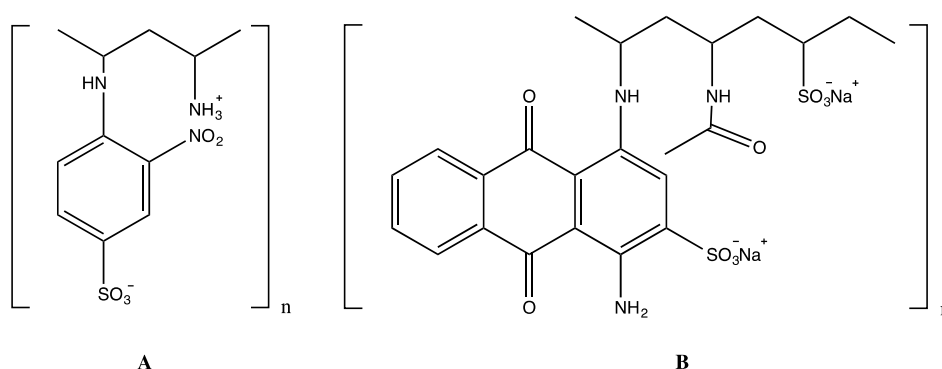


Figure 4.1 The structure of polymeric dyes: A) Poly Y and B) Poly B.

There is another method, which could also use the solid agar plate or liquid media, known as the decolourisation assay. By using polymeric dyes (**Figure 4.1**) as the lignin model compound, the degradation of the dye is observed as a clear zone in a plate assay or the reduction of absorbance relative to a standard in a liquid media assay respectively.²⁵⁰ The advantages of this method are that polymeric dyes are cheap, commercially available, stable and of low toxicity. The spectrophotometric application in liquid media assay allows a rapid and quantitative measurement. However, a poor correlation between the ability to decolourise the dyes and to degrade lignin has been reported.^{251, 252}

The use of radiolabelled lignin is considered one of the more advanced methods. The sensitivity and specificity of this method has improved the understanding of mechanisms in lignin degradation.^{253, 254} Degradation of carbon-14 lignin is monitored through the release of $^{14}\text{CO}_2$, which allows for quantitative measurement. However, the production of carbon dioxide is relatively slow and the preparation of ^{14}C lignin is cumbersome.²⁵³ As a result, this assay is not well suited for large-scale screening or kinetic studies.

An alternative to whole cell assays is to focus on ligninolytic enzymes such as peroxidases²⁵⁵ and laccases.²⁵⁶ This enzyme assay works on the principle of the observation that degradation of the substrates will result in a change of absorbance. Guaiacol and 2,4-dichlorophenol are two of the lignin model compounds that have been used in enzymatic assays.²⁵⁵ Although this method allows a rapid screening, it is not lignin-specific and could lead to a false positive result. This method is a good complementary one for an established ligninolytic reaction.

In addition, there is a colorimetric assay for lignin, which relies on its reaction with diazotized sulfanilic acid (**Figure 4.2**).²⁵⁷ A UV-Spectrophotometer is used to measure the absorbance. The reduction of lignin concentration will result in the reduction of absorbance intensity. By comparing the value with a standard curve, the quantitative measurement is obtained. Thus, this is a very useful method in quantifying the remaining amount of lignin recovered after the

incubation period. Even though this method is rapid and reliable, it is laborious and the incubation period can take up to 72 h depending on the microbes studied.

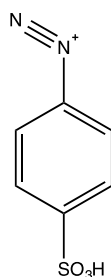


Figure 4.2 The structure of diazotized sulfanilic acid.

In 2010, new assays have been reported which allow a high throughput screening of potential lignin degrading microorganisms.²⁵⁸ These assays incorporate a spectrophotometer and are able to run in either a fluorescence or a UV-vis assays. These assays use lignin that is labelled with a fluorophore or is nitrated and run in 96-well microplates. The degradation of lignin will result in the release of labelled phenols, which results in an increase in fluorescence or UV-vis absorbance. These reactions are illustrated in Figure 4.3.

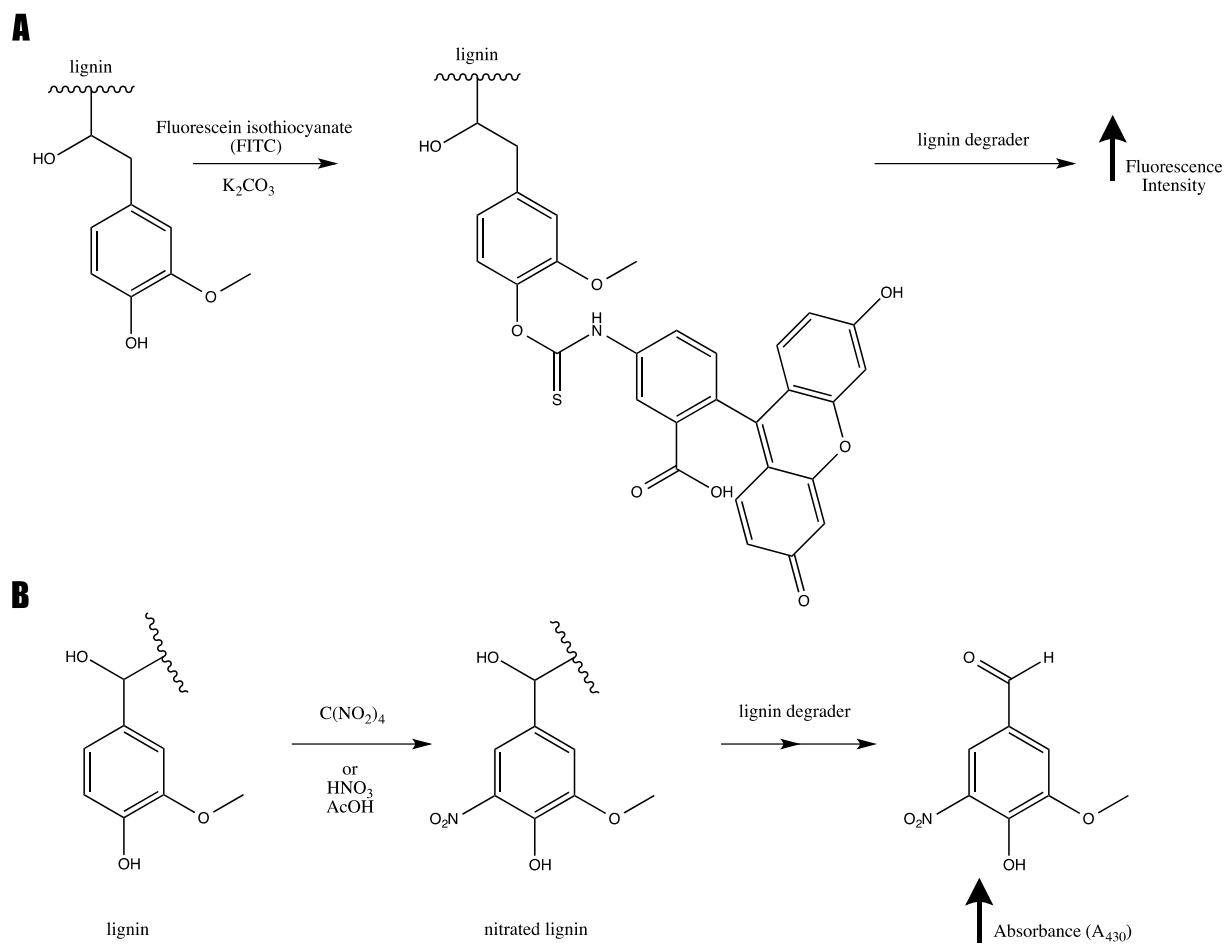


Figure 4.3 Schemes of spectrophotometric assays for lignin degradation.

4.2 Lignin degradation assay

In order to determine the ability of *Rhodococcus* UKMP-5M to degrade lignin, the nitrated lignin degradation assay was used, as it is a convenient, rapid and a high throughput method.²⁵⁸ Two types of lignin were collected from different sources; the ionic liquid pretreated lignin and kraft lignin. The ionic liquid pretreated lignin was derived from *Miscanthus x giganteus* (grass) and treated with 1-butylimidazolium hydrogen sulfate, [Hbim][HSO₄] for 22 h. The results of the assay are shown in Figure 4.4.

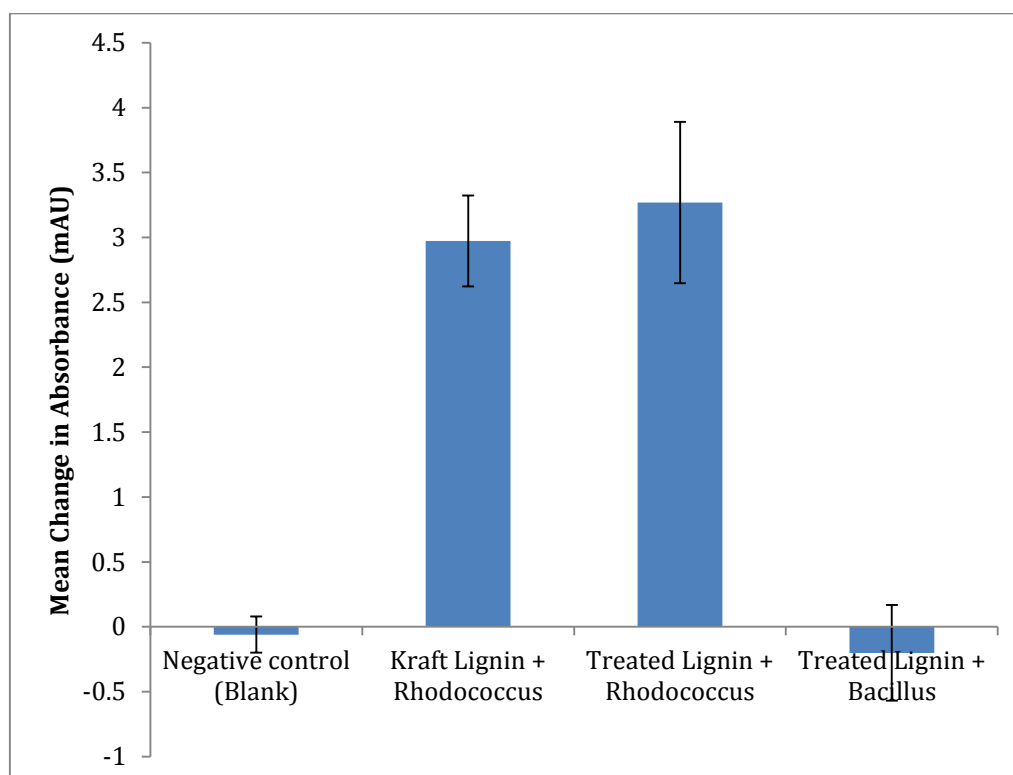


Figure 4.4 Nitrated lignin degradation assay (430nm, 20 min). Change in absorbance of different nitrated lignin samples following incubation with *Rhodococcus* UKMP-5M for 20 min. The negative controls either in the absence of *Rhodococcus* or in the presence of a *Bacillus* sp. known to not degrade lignin. ($n=3$)

This assay demonstrates the ability of *Rhodococcus* UKMP-5M to degrade lignin, as shown by the change of absorbance. *Bacillus* sp., a non-lignin degrading bacteria, was used as one negative control and the other contained

lignin without bacterial culture (blank). Both of these showed no change in absorbance, which indicates no spontaneous ligninolytic activity is taking place. Despite Figure 4.4 showing a higher mean change of absorbance in the degradation of lignin pretreated by ionic liquid (3.0 mAU) as compared to the kraft lignin (3.2 mAU), a statistical analysis, t-test revealed that this difference is not considered to be statistically significant. A further study will be discussed in Section 4.4.

Apart from that, *Rhodococcus* UKMP-5M showed better ligninolysis performance as compared to a similar study with the same assay documented in the literature.²⁵⁸ *Rhodococcus* RHA1 and *R. erythropolis* showed a smaller change of absorbance, 1.1 mAU and 2.7 mAU respectively. *Streptomyces viridosporus*, belongs to the same group of actinomycete, gave 1.0 mAU change of absorbance. However, the same study also showed that this ligninolytic ability is strain-specific. The same species of bacteria can give a very different value of absorbance change; *S. coelicolor* M110 gave rise to a significantly large change of absorbance (13.1 mAU) as compared to *S. coelicolor* ISP 5233 (2.7 mAU) and *S. coelicolor* M1190 (0.3 mAU). Fungi showed a big change of absorbance, especially on softwood-derived lignin. For example, *Phanerochaete chrysosporium* demonstrates high ligninolytic ability (261 mAU) whereas a moderate value of change in absorbance was observed in *Lepista nuda*, *Serpula lacrymans* and *Paxillus involutus* with values of 16.6, 12.4 and 2.3 mAU respectively. The ability of fungi in degrading lignin is well established²⁵³ and thus a significantly high value is expected.

4.3 Product identification

Since the ability of *Rhodococcus* UKMP-5M to degrade lignin has been established, the breakdown product(s) needed to be identified. Gas chromatography – mass spectrometry (GC-MS) was used for this purpose. Supernatants of the sample were collected after 48 h of incubation. The sample was centrifuged (5 min, 10 000 rpm) and the supernatant was cooled on ice. Then, supernatant was then treated with 50 μ l of cooled trichloroacetic acid (1 mg/ml) and left on ice for 3 min. This sample was centrifuged and the supernatant was analysed by GC-MS. The GC-MS procedure is detailed in Chapter 2: Experimental design and the result is shown in Figure 4.5.

The largest peak is shown at a retention time of 3.51 min. According to the NIST mass spectral library,²⁵⁹ the fragmentation pattern of this peak is consistent with the presence of guaiacol as the most abundant degradation product. In order to confirm this, a commercial standard of guaiacol was run through the same procedure and the result is shown in Figure 4.6. The consistent peak with an almost identical fragmentation pattern was observed, thus verifying guaiacol as the major product of lignin degradation by *Rhodococcus* UKMP-5M.

Guaiacol has previously been reported as a lignin degradation product. Several studies have shown the production of guaiacol from the degradation of lignin model compounds. For instance, guaiacol is formed through the degradation of ferulic acid^{212, 260} and vanillic acid²¹² by *Rhodotorula rubra* and *Streptomyces* D7 respectively. *Arthrobacter*, a member of actinomycetes, are able to degrade the β -O-4 substructure of lignin into guaiacol along with vanillic acid, vanillyl alcohol and 3-methoxyhydroquinone.²⁶¹

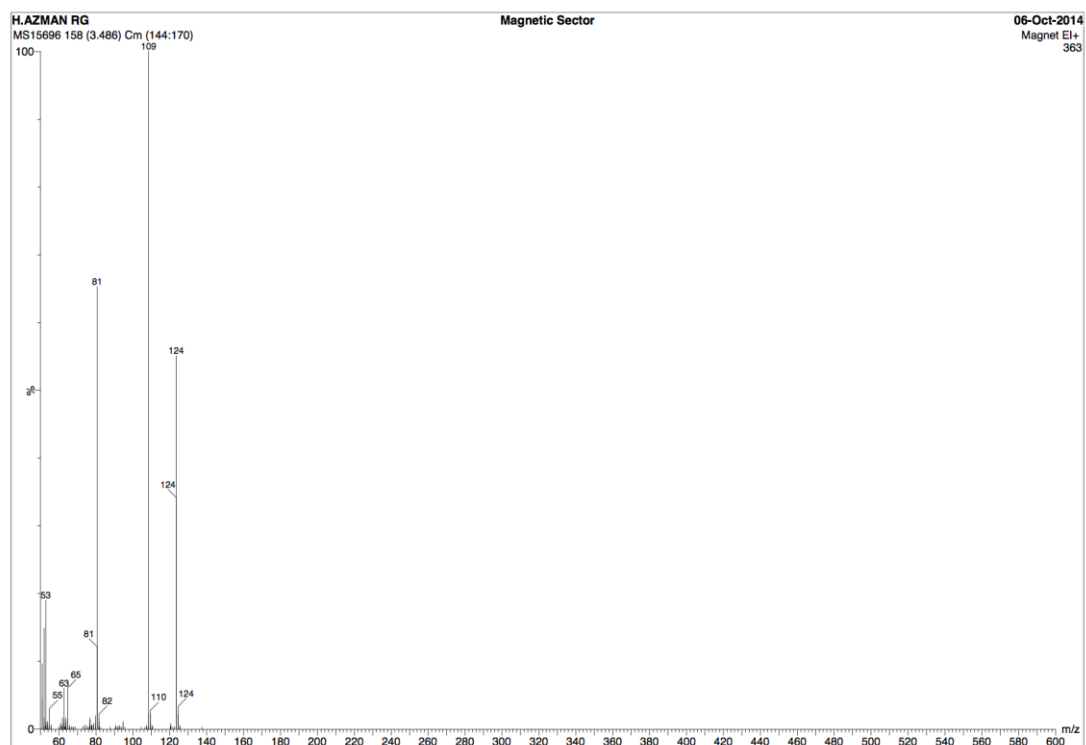
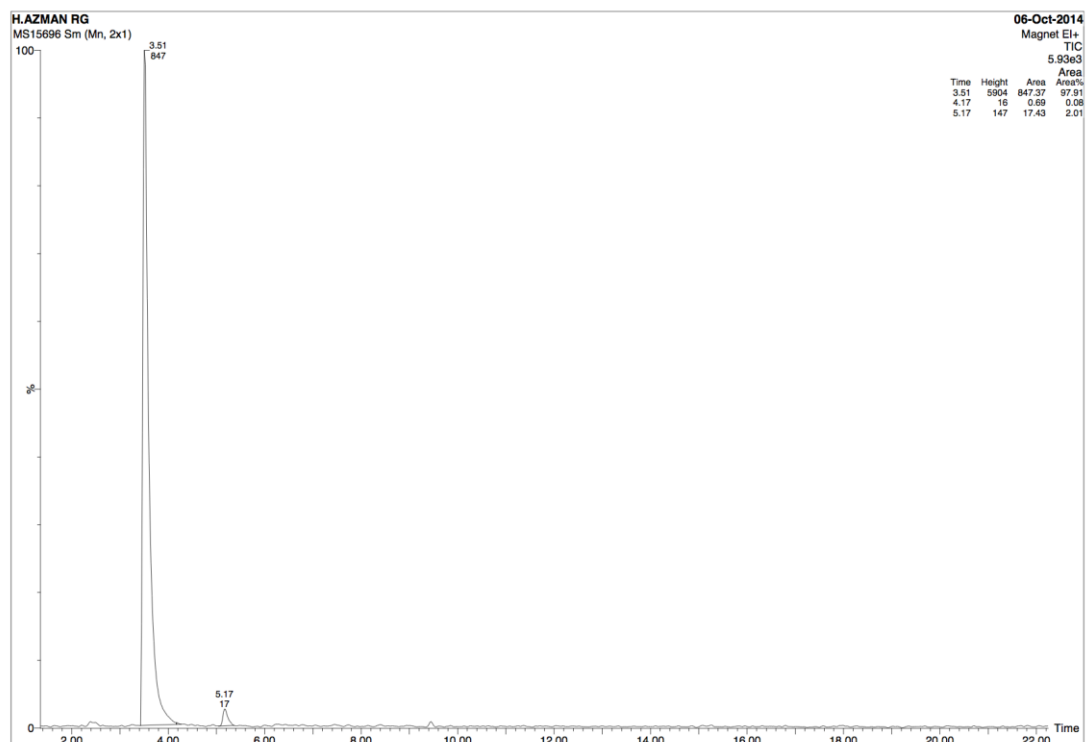


Figure 4.5 GC-MS analysis of sample from lignin degradation by *Rhodococcus* UKMP-5M after 48 h. Total ion chromatogram of the culture supernatant (above) and MS spectrum of the peak at 3.51 retention time.

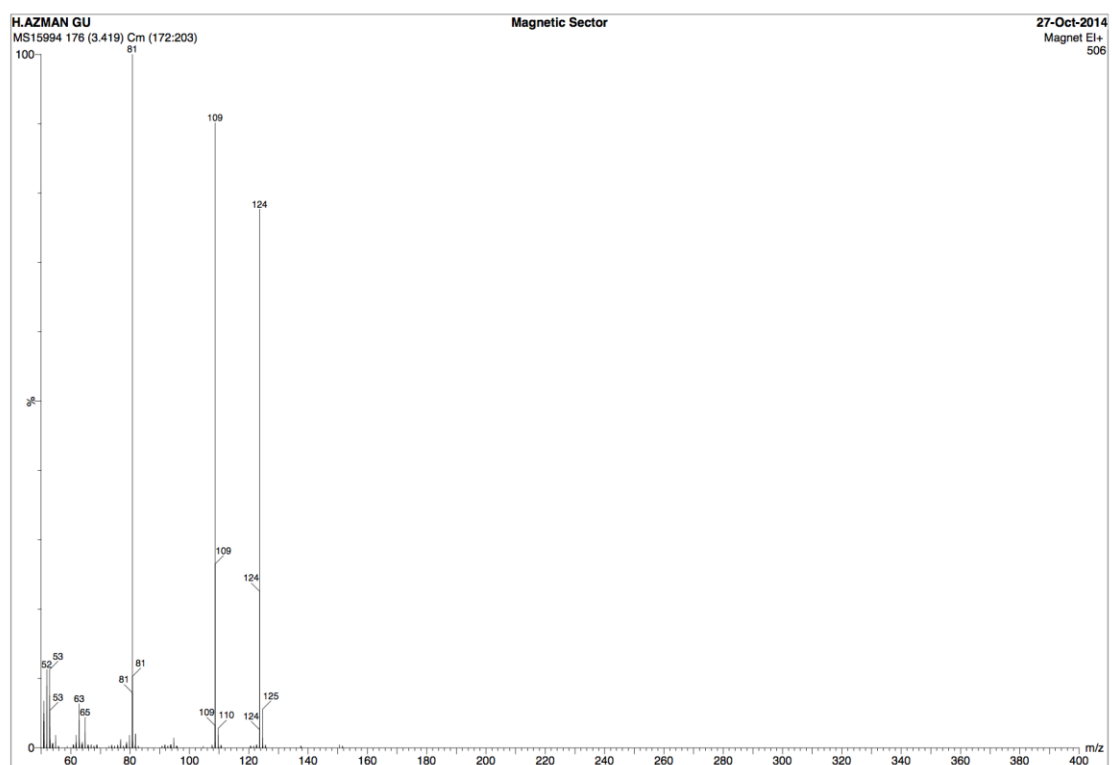
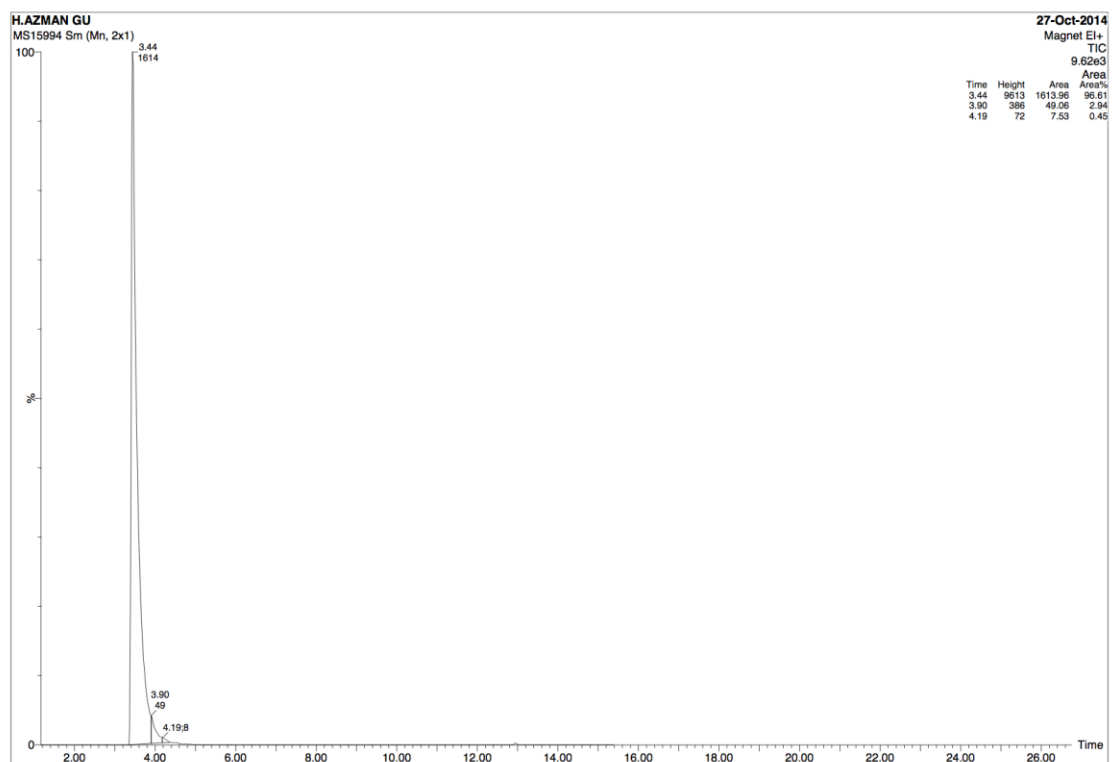


Figure 4.6 GC-MS analysis of standard guaiacol. *Total ion chromatogram of the sample supernatant (above) and MS spectrum of the peak at 3.44 retention time.*

Based on the literature, there are two possible routes for guaiacol formation through lignin degradation. The degradation of β -aryl ethers, which are depolymerised from lignin, will result in the cleavage of β -O-4 bonds to produce vanillin and the remaining structure is further degraded into guaiacol and glycolaldehyde as illustrated in Figure 4.7.^{114, 242} The ligninolytic enzyme, Dyp B peroxidase is possibly responsible for this mechanism¹¹⁴.

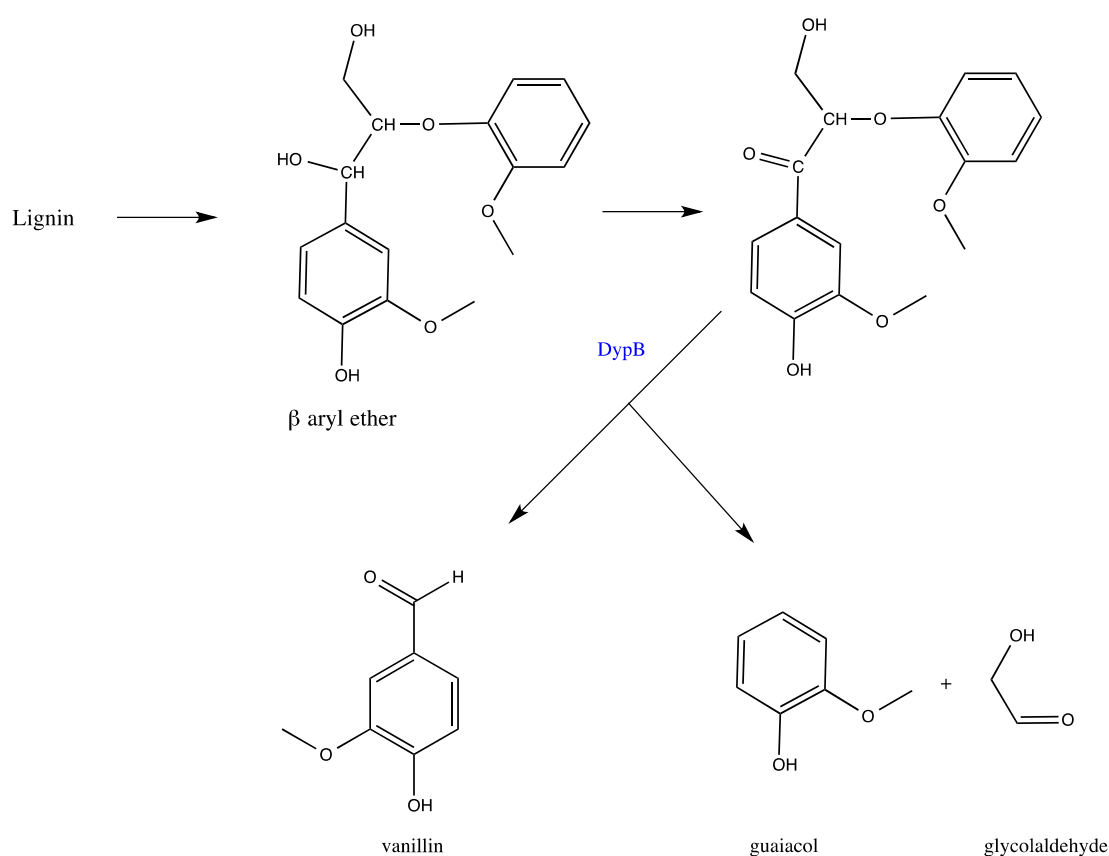


Figure 4.7 Possible lignin degradation pathway by *Rhodococcus* UKMP-5M.

Dyp B is a member of the dye-decolorizing peroxidases. Its enzymatic role in lignin degradation was first discovered in *Rhodococcus jostii* RHA1. The catalytic activity is enhanced by the presence of Mn^{2+} ions.¹¹⁴ Whilst the mechanism is similar to typical heme-peroxidases in plants, its molecular structure is different.⁷⁸ As illustrated in Figure 4.8, The heme cofactor is located in between of two ferredoxin-like domains.¹¹⁵ Unlike the usual heme-peroxidases

that are typically comprised of α -helical secondary structure, these domains are made of peripheral α -helices and anti-parallel β -pleated sheets.^{78, 115} Dyp enzymes only contain a proximal histidine instead of the common pair of distal and proximal histidine.⁷⁸ Moreover, Dyp B contains a distal channel and a propionate pocket for solvent access.¹¹⁵ This distal channel is exclusive to Dyp B and D only and possibly provides the entrance for hydrogen peroxide during the catalytic cycle.⁷⁸ The propionate pocket is where the binding site for Mn^{2+} is potentially located.¹¹⁵ There is also a hydrophobic groove found in the protein surface, a proposed binding site for kraft lignin and long-range electron transfer site for bulky substrates similar to the one observed in LiP and VP peroxidases.^{78,}

115

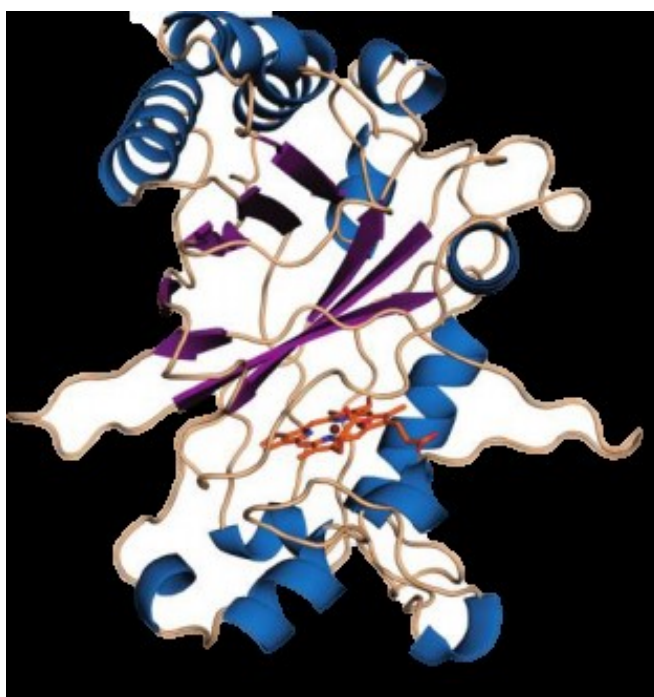


Figure 4.8 The structure of Dyp B protein.¹¹⁵ Blue, violet and wheat represents the structure of α -helices, β -sheets and loops respectively. The heme prosthetic group is shown as a stick model with carbon atoms in orange colour. Reproduced by permission of American Chemical Society.

In regards to the catalytic cycle of Dyp B, the mechanism involves a three-step chain of reactions (Figure 4.9).^{78, 114} The native resting state of ferric enzyme [Fe(III)] is oxidized by a hydroxyperoxide compound, which is normally hydrogen

peroxide. This two-electron oxidation forms an oxo-ferryl radical cation intermediate $[\text{Fe(IV)} = \text{OP}^{\bullet+}]$. This compound I is responsible for oxidising the β -aryl ether to produce vanillin and the remaining intermediate is further oxidized by the oxyferryl form of the enzyme (compound II) to yield guaiacol and glycolaldehyde. This oxidation will return Dyp B peroxidase to its resting state in order to restart the cycle.

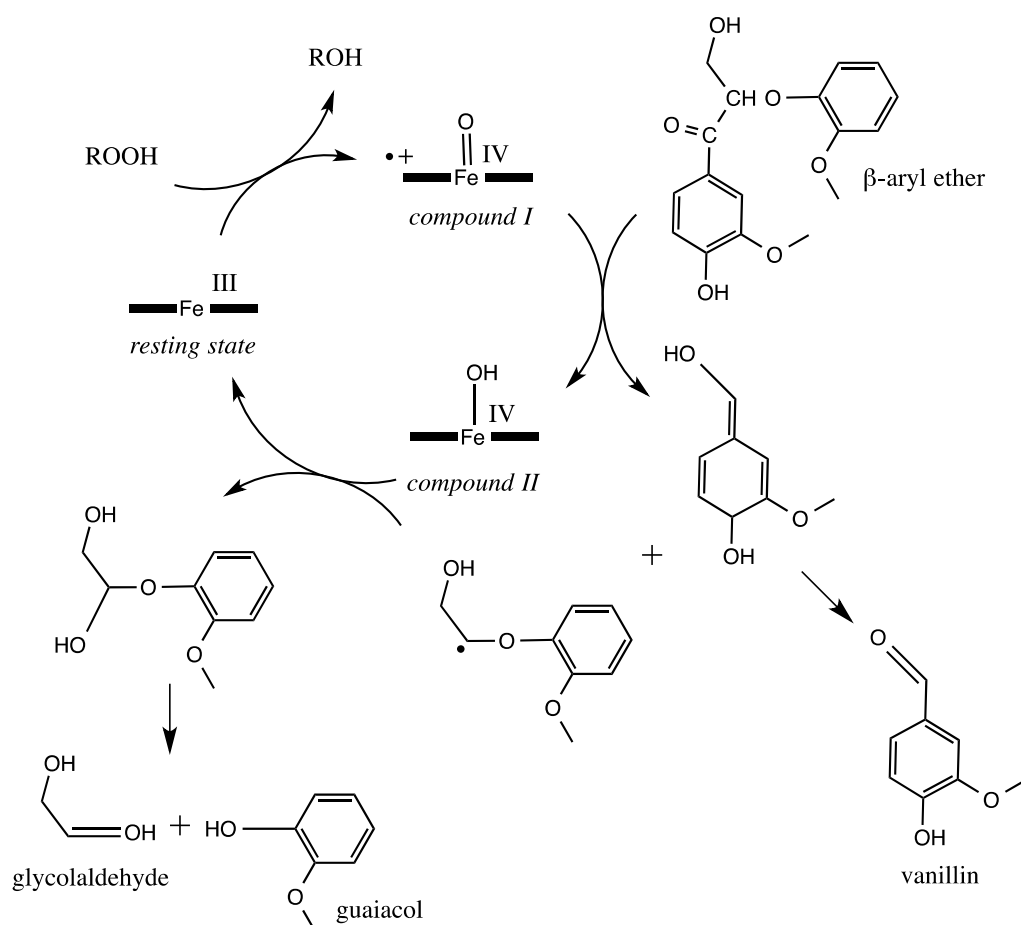


Figure 4.9 Catalytic cycle of Dyp B in degrading the intermediate of lignin, β -aryl ether.¹¹⁴
Adapted with permission by American Chemical Society.

Besides the degradation of the β -aryl ether group, a study has revealed that the degradation of vanillic acid as a lignin model compound could also lead to the formation of guaiacol.^{217, 262} However, this mechanism is exclusive to some species as a study observed the formation of guaiacol in both *Streptomyces sp.* and *Bacillus subtilis* but not in yeast, as vanillic acid was not converted to

guaiacol in *Rhodospiridium kratochvilovae*, *Rhodotorula slooffiae*, *Sporidiobolus johnsonii* and *Sporobolomyces salmonicolor*.²¹⁷ Instead, as discussed in Chapter 3, the degradation of vanillic acid could produce protocatechuate via *o*-demethylation. Then, this compound could be degraded through a β -ketoadipate pathway, followed by the Krebs cycle and, finally, released as carbon dioxide.

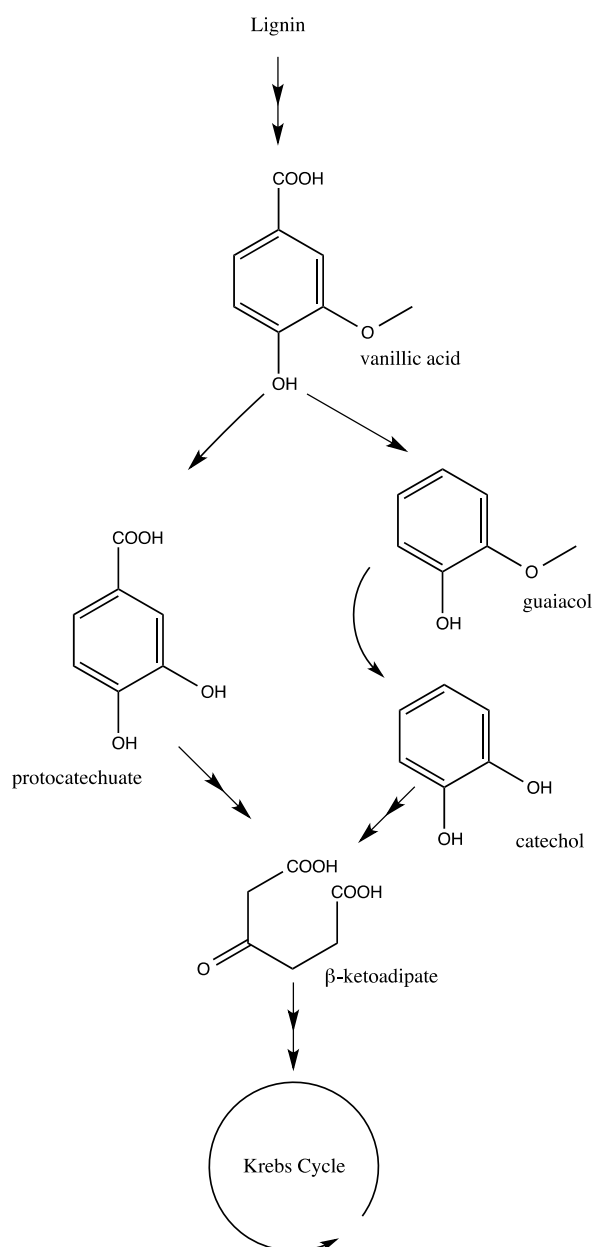


Figure 4.10 The microbial degradation pathway of vanillic acid via β -ketoadipate pathway.

The fact that neither vanillic acid nor protocatechuic acid are observed in the GC-MS analyses suggests that guaiacol *o*-demethylation is the rate limiting step in Figure 4.10. It is possible that the vanillic acid degradation is faster while the slow degradation of guaiacol lead to its accumulation. Guaiacol is then transformed into catechol before entering β -ketoadipate pathway.²¹⁷

It is also worth mentioning that this assay was held without the presence of added hydrogen peroxide. Using the same assay, a previous study reported the ability of *Rhodococcus* RHA1 and *Rhodococcus* sp. 43230 strains to degrade lignin in the absence of hydrogen peroxide. It is possible that laccases were used to catalyse the process, in addition to, or instead of, extracellular peroxidases. Laccases use dioxygen as a co-substrate to generate the catalytic cycle of oxidation as shown in Figure 4.11. Apart from that, laccases are also able to attack the non-phenolic structures of lignin in the presence of mediators, which act as an oxidant mediator between the enzyme and the final substrate. Vanillin and syringaldehyde are some of the effective naturally-occurring mediators during ligninolysis.

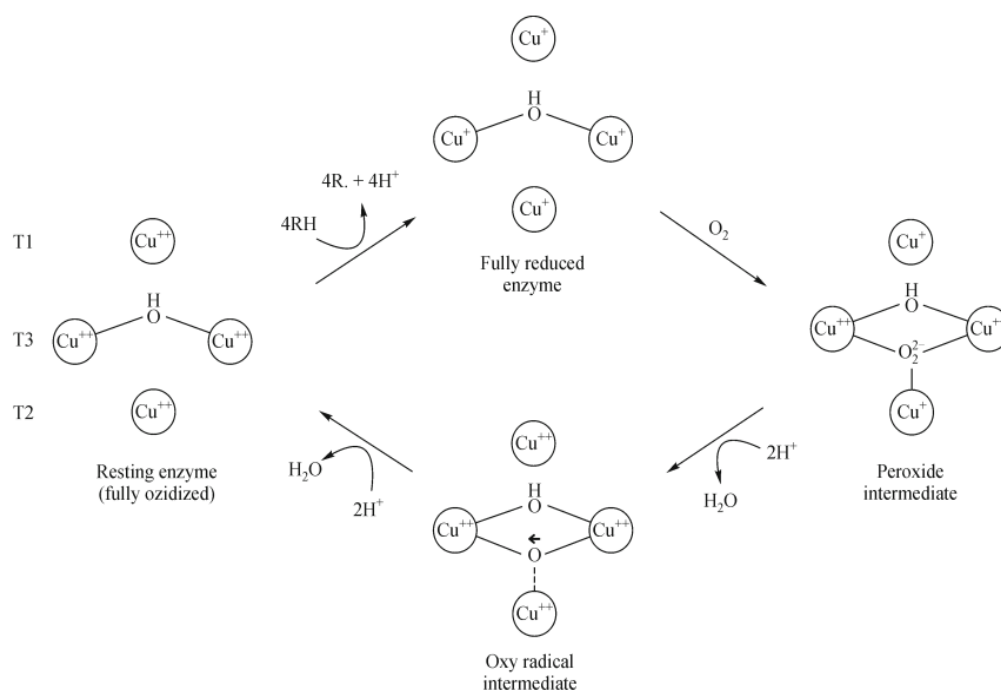


Figure 4.11 A schematic diagram of the catalytic cycle of laccases. Reproduced by permission of Springer.

4.4 Kraft lignin vs Ionic liquid-pretreated lignin

Based on earlier results (refer to Figure 4.4), ionic liquid pretreated lignin has similar rate of lignin degradation with the kraft lignin. In order to further evaluate this, the degradation of both types of lignin against time was investigated. By using the nitrated lignin degradation assay, samples were incubated for 20 minutes time and measurement was collected in a minute time interval. The result is shown in Figure 4.12.

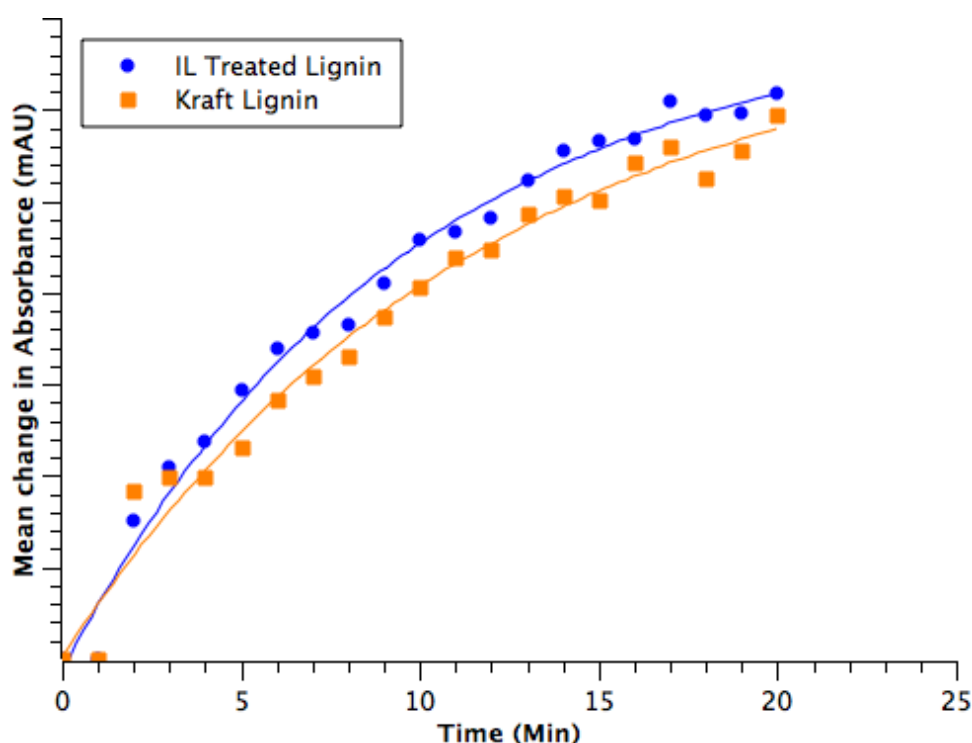


Figure 4.12 Nitrated lignin degradation assay to study the effect of pretreatment to the degradation of lignin by *Rhodococcus* UKMP-5M.

The curves show a typical time course for an enzyme-catalysed reaction where the rate is observed to decrease over time. This is usually due to a combination of factors including substrate depletion, product inhibition and/or enzyme inactivation. In order to compare the rate of degradation of this lignin, the early data points were fitted to a linear regression (Figure 4.13). The early data points were used in order to eliminate the limiting factor described above.

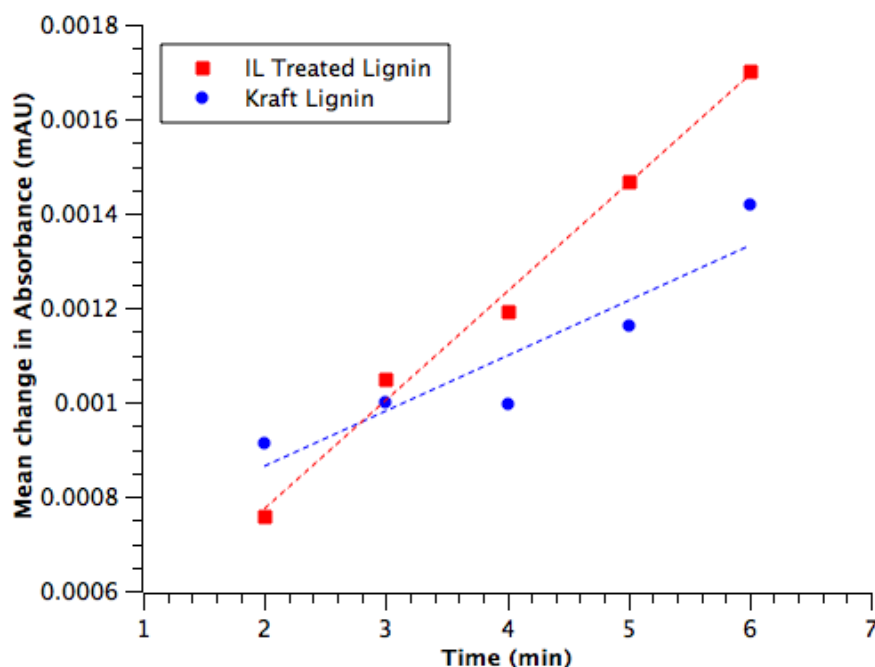


Figure 4.13 Nitrated lignin degradation assay between two types of lignin, fitted to a linear regression by using the SciDavis software.

This graph illustrates that the ionic liquid-treated lignin has a larger slope, indicating a higher bacterial degradation rate than kraft lignin. This observation suggests that lignin pretreatment with ionic liquid assists its degradation by *Rhodococcus* UKMP-5M.

Kraft lignin is a product of kraft pulping, aiming to remove enough lignin to separate cellulosic fibres from one another mainly for paper manufacturing. On the other hand, ionic liquid pretreated lignin is lignin recovered from the pretreatment of lignocellulose. The purpose of the pretreatment is to deconstruct the lignocellulose to obtain the cellulose mainly for biofuel production. In this instance, the kraft process operates under basic conditions whereas an acidic ionic liquid was used for the pretreatment.

The conventional process of kraft lignin production involves the reaction of white liquor comprised of sodium hydroxide and sodium sulfide with wood chips in a digester, a large pressure vessel.²⁶³ The vessel is heated to 120 °C for 2 h.

During this thermal treatment, the hydroxide and hydrosulfide anions react with the lignin causing its fragmentation. The α and β -aryl ether linkages that hold the phenylpropane units together are cleaved and free phenoxide groups are generated along with a concomitant condensation reaction. These free functional groups will increase the hydrophilicity of the lignin and lignin fragments, thus increasing the lignin solubility. The stable carbon-carbon linkages tend to survive the kraft treatment.

Meanwhile, the ionic liquid pretreatment of lignin involves the dissolution of lignocellulose through the introduction of acidic ionic liquids, 1-butylimidazolium hydrogen sulfate, [Hbim][HSO₄]. The process takes place in a 120 °C incubator for 22 h. The protic anions are reactive at elevated temperatures and under acidic conditions and catalyse the hydrolysis of lignin and delignification. Next, a two stage recovery process is initiated through the precipitation of cellulose while lignin remains in solution. After the separation, lignin is then precipitated and recovered.

Table 3 The comparison between kraft lignin and ionic-liquid treated lignin.²⁶³⁻²⁶⁵

Kraft lignin	Mode of comparison	Ionic liquid-treated lignin
Basic, high in pH	pH	Acidic, low in pH
Free phenoxide functional group	Ligninolysis	Partially depolymerise lignin structure
Higher MW	Molecular Weight (MW)	Lower MW
Higher	Degree of polydispersity	Smaller
Heavily cross-linked	Structure	Less cross-linked

These two very contrasting processes resulted in different structures of lignin. As shown in Table 3, ionic liquid-treated lignin has a lower molecular weight compared to kraft lignin. The smaller degree of polydispersity in ionic liquid-treated lignin signifies that lignin has been depolymerized into lower

molecular weight products through the removal of either branched or end-units.²⁶⁵ Due to condensation,²⁶³ kraft lignin is cross-linked, which is less likely to occur in the lignin treated with ionic liquids.²⁶⁵ The simpler nature of the lignin that was pretreated with the ionic liquid could have enhanced the lignin degradation by *Rhodococcus* UKMP-5M. This finding has not been reported before and further study at a molecular level could help in the understanding of the mechanisms involved in the ligninolysis of this deconstructed lignin.

4.5 Time dependence of ionic liquid pretreatment

This study looked at the effect of pretreatment time on the bacterial degradation of lignin. A nitrated lignin assay was used in this study and the samples were collected for every 2 h for the first 12 h followed by the final sample collection after 24 h of pretreatment with the acidic ionic liquid, triethylammonium sulfate [TEA][HSO₄]. Ionic liquids containing sulfate anions are the best solvents for fragmenting lignin and reducing its strength as polymer.²⁶⁶ Studies showed that this type of ionic liquids decrease the β -O-4 aryl ether bond content²⁶⁷ and yield higher amounts of guaiacol as the cleavage product.²⁶⁸ Figure 4.14 displays the result of this time dependent assay.

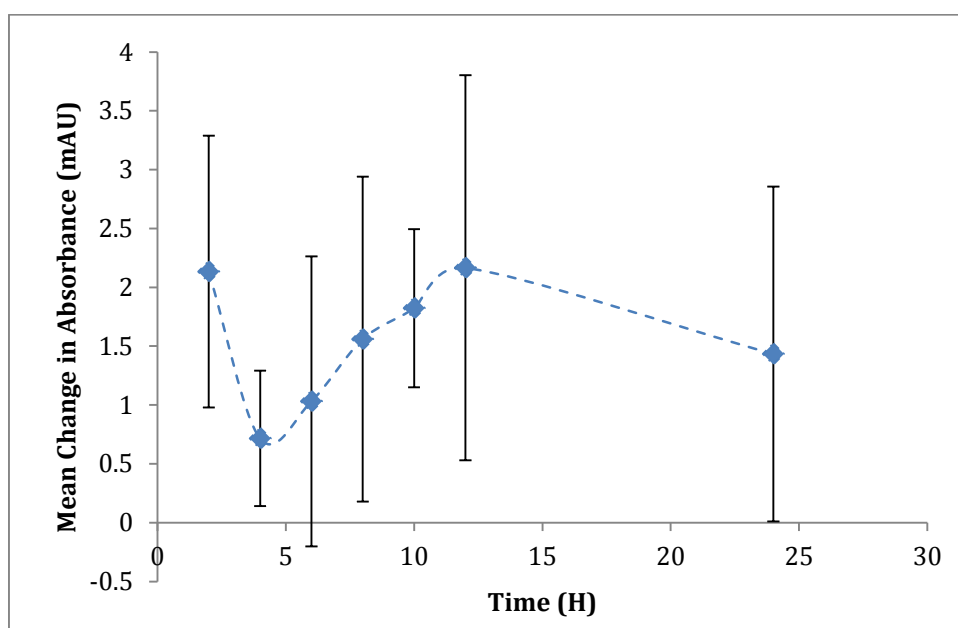


Figure 4.14 Nitrated lignin assay of bacterial degradation of lignin against the time of ionic liquid pretreatment. The dashed line is used for visual aid and does not imply a functional model.

This study signifies that the correct tuning of the ionic liquid pretreatment could assist the degradation of lignin by *Rhodococcus* UKMP-5M. Nevertheless, this graph showed the challenge faced in this experiment. Due to the sensitivity of the assay and the small volume of samples involved, this experiment is prone to experimental errors. Good data consistency and reproducibility are extremely

difficult to achieve as illustrated by the large standard deviation error bar produced in Figure 4.14. This error can be minimised through repeating the process many times in order to achieve higher precision. Additionally, repeating the experiment improved the operator handling skills, which in return increases the accuracy and reduces random error.

Despite the large experimental scatter, this graph appears to show three different phases, a decrease of absorbance intensity in the beginning followed by a constant increase of absorbance for the next 10 h. However, the decreasing trend was observed again after 24 h of the ionic liquid pretreatment. A possible explanation for the high intensity of absorbance after 2 h of pretreatment is due to the incomplete dissolution of lignocellulose. It is likely that 2 h is not enough for a complete removal of hemicellulose attached to lignin. The presence of hemicellulose sugars like xylose and arabinose could provide carbon sources for *Rhodococcus* UKMP-5M. Subsequently, this substrate will drive the ligninolysis ability of the bacteria thus increasing the rate of degradation which is reflected by a higher change of absorbance.

The gradual increase of absorbance change for the next 10 h suggests that the pretreatment of the ionic liquid depolymerised the lignin, allowing a higher degradation rate of bioligninolysis. It is believed that the β -O-4 ether bonds are cleaved at this stage. The longer the ionic liquid pretreatment, the greater the amount of these ether bonds being broken. However, the data collected after 24 h of pretreatment were not consistent with this trend. One possible reason for this is that the lignin at this stage is cross-linked due to the condensation process. At this particular point, the lignin is comprises mostly aromatic groups with more carbon-carbon bonds and fewer functional groups including hydroxyl, ester, ether and aldehyde. The molecular weight of the lignin is higher than lignin from the second phase.

4.6 Lignin degradation assisted by ionic liquids

In this study, the ionic liquids are introduced during the bacterial lignin degradation process and the samples were analysed in triplicate via the nitrated lignin degradation assay. The selection of ionic liquids was based on a preliminary study of the toxicity of the ionic liquids employed. The toxicity of ionic liquids will be discussed in detail in Chapter 5.

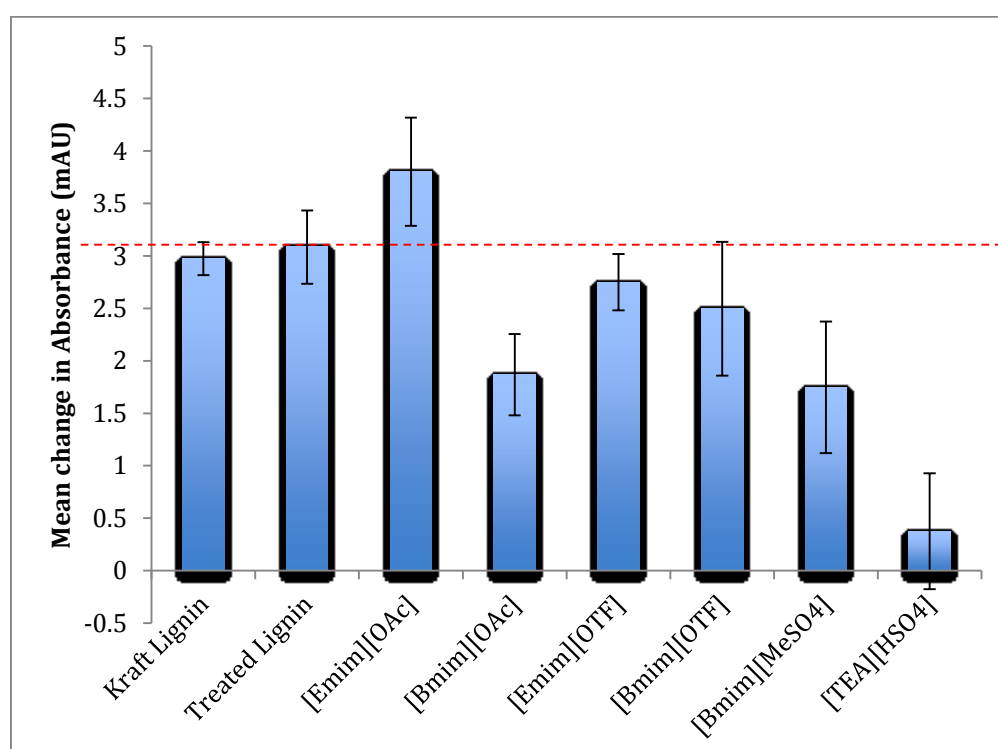


Figure 4.15 The ionic liquid-pretreated lignin degradation by *Rhodococcus* UKMP-5M with 1% ionic liquid. The dashed red line represents the absorbance value of treated lignin, used as visual aid. The error bars represent the experimental replicates (N=3).

Based on Figure 4.15, lignin degradation in the presence of 1% 1-ethyl-3-methylimidazolium acetate, [Emim][OAc] shows the largest change in absorbance whereas the presence of triethylammonium hydrogen sulfate, [TEA][HSO₄] shows the smallest change in absorbance. It is clearly observed that [Emim][OAc] is the only ionic liquid that helps to increase the rate of degradation compared to the control in the absence of an ionic liquid.

The ability of [Emim][OAc] to deconstruct lignocellulose is well established with the reported lignin removal being as high as 65%.⁸ The analysis of residual lignin in [Emim][OAc] exhibited the reduction of β -O-4 aryl ether bond and deacetylation of xylan.²⁶⁷ It has also been reported that [Emim][OAc] catalyses the dehydration of lignin model compounds due to the greater basicity of the anionic acetate and affinity towards water.^{8, 269} This reaction is illustrated in Figure 4.16.

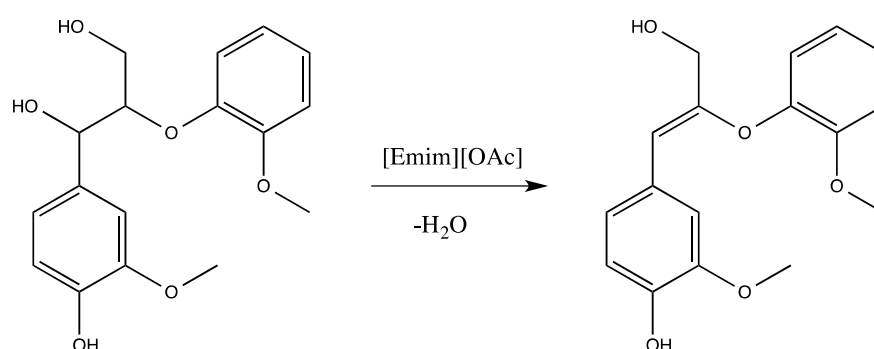


Figure 4.16 Dehydration of guaiacylglycerol- β -guaiacyl ether in an ionic liquid.

Ionic liquids have been demonstrated to increase the rate of other biocatalysis reactions.²⁷⁰ In a study of guaiacol oxidation by peroxidases in the presence of ionic liquids, it was shown that the presence of the ionic liquid enhanced the peroxidase activities of Fe(III)protoporphyrin(IX) chloride (hemin) and microperoxidase-11 (MP-11) whereas cytochrome c (cyt-c) demonstrated a comparable activity to the conventional organic solvent.²⁷¹ In addition, ionic liquids have been reported to increase the thermal stability of horseradish peroxidase (HRP). However, the high concentration of ionic liquids at 25% (w/v) deactivated the enzymes due to a change in the biphasic nature.²⁷²

Whole cell biocatalysis is desirable as it provides both stability and integrated metabolic reactions.^{184, 270} It has additional benefits as compared to the isolated enzymes as this method is cheaper than producing and maintaining the

enzymes. Several studies have showed the advantage of whole cell biocatalysis in yielding a higher rate of product formation.¹⁸⁴

The polarity of ionic liquids determines the mechanisms involved in the whole cell catalysis process.¹⁸⁴ The individual ions of the ionic liquid interact with the charged groups on the substrates and transfer electric charge to (or from) the cell membrane, changing its ionic state and thus altering its permeability. In general, the more hydrophilic the ionic liquid, the faster it facilitates the penetration of the substrate into the cell membrane. On the other hand, more hydrophobic ionic liquids can enhance the water activity around extracellular enzymes, thus promoting enzymatic activity in aqueous phase.

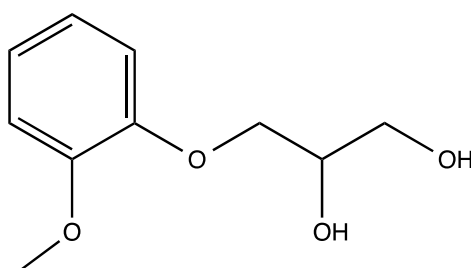
The potential toxicity of the ionic liquids could also play a role in the observed rate of reaction. Both acidic ionic liquids 1-butyl-3-methylimidazolium methyl sulfate, [Bmim][MeSO₄] and [TEA][HSO₄] showed poor ligninolysis. It is possible that the acidic conditions induced by the ionic liquid could affect the degradation activity as well as the viability of the bacteria. Similarly, the chain lengths of substituents on the cation are reported to affect the toxicity of ionic liquids.¹⁷⁰ In this study, Figure 4.15 displays ionic liquids containing two-carbon alkyl chains attached to the cation, 1-ethyl-3-methylimidazolium, [Emim] showed a better rate of lignin degradation than an ionic liquid containing a four-carbon alkyl chains on the cation, 1-butyl-3-methylimidazolium, [Bmim]. The effect of the acidity of the ionic liquids on the growth of *Rhodococcus* UKMP-5M as well as more general measures of ionic liquids' toxicity will be addressed in the next Chapter 5.

4.7 Conclusion

In this chapter, the ability of *Rhodococcus* UKMP-5M in ligninolysis has been established. Guaiacol has been identified as the major product of this particular bacterial lignin degradation, which is potentially catalysed by Dyp B peroxidase and/or extracellular laccases.

As shown in an earlier section of this chapter, guaiacol is a major product in the biodegradation of lignin. It is a fine chemical with a marketable value. It has been regarded as one of the lignin-derived products that could contribute to the economic feasibility of a lignocellulose-based biorefinery.³⁶ Apart from that, guaiacol is also a potential compound of bio-oil fractionation, obtained via biomass pyrolysis.²⁷³ This particular biorefinery thermal process has attracted a lot of interest recently due to its ability to be integrated into readily available petroleum refineries.²⁷³

Guaiacol is used in the food industry as an additive for flavouring and fragrance.^{274, 275} It has distinct organoleptic properties,^{217, 276, 277} which are described as sweet, woody, smoky, medicinal, savoury and meaty. Guaiacol is also used as a precursor in the synthesis of vanillin and eugenol.²⁷⁸ As methoxyphenols, they work as stabilizers in the manufacturing of rubbers and plastics.²⁷⁷ They are also used as anti-skinning agents for paint and varnish.²⁷⁷ Due to their unique odour and antiseptic properties, they are also used in the formulation of detergent, deodorant and other perfumery products.^{274, 277} Guaiacol is also widely used as a pharmaceutical agent. It is used as expectorant in cough syrups in the form of guaifenesin (Figure 4.17).²⁷⁹ In addition, guaiacol is also applied as a disinfectant and sedative especially in dentistry.^{275, 280-282}



IUPAC name: 3-(2-methoxyphenoxy)propane-1,2-diol

Figure 4.17 The chemical structure of guaifenesin, the expectorant drug derived from guaiacol.

The US Department of Energy, in their report on the potential candidates for biorefinery lignin, classified the potential market value of guaiacol as medium-

high.²⁸³ Camlin Fine Chemicals (CAMFIN), which recently acquired Borregaard, one of the leading companies in the production of lignin-derived guaiacol and vanillin, has launched its new division in guaiacol production in 2013. They estimated the market to be about 5000 metric tonnes per annum with a market realisation of approximately \$5.50 /kg. It was also noted that there is a scalable opportunity in this market as only 4% of the industry segment sales was ventured in the fiscal year of 2014.²⁸⁴

The research and development opportunities (R&D) of guaiacol are still being explored. A recent study has shown that guaiacol has good antioxidant properties.²⁸⁵ The antioxidant activity analysis revealed that it gives a similar percentage inhibition of the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals compared to commercial antioxidants.²⁸⁵ This indicates that guaiacol has a good radical scavenging ability. Besides that, new research has addressed the potential of guaiacol as a component of biobased resin composites.²⁸⁶ Along with phenols and catechols, they formed vinyl ester resins. Interestingly, this composite has comparable thermo-mechanical properties to the commercial petroleum based resins.²⁸⁷ The applications as antioxidants and composites are just a few of the examples that promise good prospects for guaiacol in industry.

Ionic liquid pretreatment has been demonstrated to enhance lignin degradation and the tuning of treatment duration is essential for optimum rate of reaction. In addition, the introduction of [Emim][OAc] could assist the degradation process. Whilst the ionic liquids are shown to enhance the ligninolysis, some of them reduced the rate of reaction, suggesting that some ionic liquids could be toxic to *Rhodococcus* UKMP-5M. It is important to screen the toxicity of ionic liquids to ensure successful integration of the ionic liquid in the bioligninolysis. This will be addressed in the following chapter.

CHAPTER 5

THE TOXICITY OF IONIC LIQUIDS

5.1 Introduction

In the Section 1.5, the 'green' label attached to ionic liquids was discussed. The awareness of the toxicity of ionic liquids has increased recently with the rise in investigations focusing on this issue.¹⁶⁷ Not only are some ionic liquids as toxic as conventional organic solvents but some also exhibit even greater toxicity. However, due to the enormous potential combination of cations and anions in ionic liquids and the individual toxic effects of structures on different organisms, there is still a lack of understanding of the toxicity mechanism of ionic liquids. Ionic liquids display various toxic behaviours; some inhibit biological systems drastically while some demonstrate weak or even no apparent effect.¹⁶⁷ The wide range of physicochemical properties of ionic liquids could account for these different behaviours.

In this chapter, the focus is on the toxicity of different ionic liquids on *Rhodococcus* UKMP-5M. This study is vital in designing an ionic liquid incorporated bioligninolysis process while providing insight into the mechanism of ionic liquids' toxicity on this bacterial strain. There is no standard convention for scaling the hazard of ionic liquids. Therefore, careful selection of the best assessment assay is essential in providing the useful information on toxicity.

The Kirby Bauer disk diffusion susceptibility test is a standard protocol used in clinical laboratories to study the sensitivity of microbes to antibiotics. Due to its rapidity, simplicity and low cost, this method has been applied in testing other toxic chemicals including ionic liquids.^{288, 289} This method was used as the first stage of high throughput toxicity screening for a large range of ionic liquids, both miscible and immiscible in water, with *Clostridium butyricum*²⁸⁸ and

Escherichia coli.²⁸⁹ It involves putting a filter disk containing the test substance in the middle of a bacterial culture plate. The substance will spread on the surface and/or diffuse into the agar and generate a gradient concentration. The culture is incubated under its optimum conditions and the clear inhibitory zone, also known as the “halo zone”, formed will indicate the toxicity through the measurement of its diameter. These data will provide limited quantitative information but they are sufficient for screening purposes and for providing a preliminary insight into the toxicity.

Next, a more quantitative measure of the toxicity of ionic liquids can be made by determining the minimum inhibitory concentration (MIC) and/or minimum bactericidal concentration (MBC). These methods involve the serial dilution of the test substance, which is then incubated with culture broth. The lowest concentration at which there is no visible growth observed will represent the MIC value. The cultures are then transferred to solid media in order to determine the MBC value, which is demonstrated through the lack of recovery growth. MBC value is normally higher than MIC value. These methods are laborious but provide more quantitative data in term of cell viability. MIC methods have been applied in determining the toxicity of ionic liquids on antibiotic resistant bacteria including *Staphylococcus aureus*, *Klebsiella pneumoniae* and *E. coli* where one study used an ampicillin based ionic liquids, which showed good antimicrobial properties against resistant strains.^{290, 291}

In addition, a toxicological study can also use cell viability assays as a measure of potency. There are several types of cell viability assays applicable in determining the toxicity of ionic liquids. For example, a total viable count method can be implemented by counting the colonies formed on a solid culture plate after being exposed to the ionic liquids and the colony forming unit (CFU) count determined. A toxicity study on the influence of ionic liquids on *E.coli* in which this assay was used has been reported.²⁹² Apart from that, a bioluminescence assay has also been applied to assess the toxicity of ionic liquids. This technique involves the luminescence inhibition test of marine bacterium, *Vibrio fischeri* as a tool to analyse the toxicity of ionic liquids. This method has been introduced in

evaluating the toxicity of guanidinium, imidazolium and phosphonium based ionic liquids (structures are shown in Figure 5.1).²⁹³

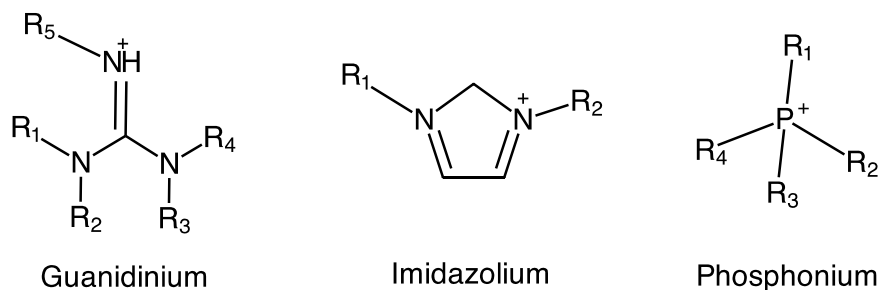


Figure 5.1 Examples of the cation structure of ionic liquids in toxicity evaluation.

Another approach to a cell viability assay is through monitoring the growth of culture through the measurement of the optical density (cell turbidity) using a spectrophotometer at a wavelength of 600nm. This method allows study of the kinetics and provides understanding of the toxicity behaviour towards culture growth, which will be discussed further in the results' section. Based on the optical density (OD) value, an EC₅₀ value can be calculated. EC₅₀ is the effective concentration in which 50% of growth is inhibited relative to the control.¹⁶⁷ This information is useful especially when comparing toxicological data in the literature.

It was decided to use the disk diffusion test as the screening protocol due to the capacity of this assay to rapidly analyse a number of ionic liquids. Subsequently, the measurement of growth rate (assessed by turbidity) provided further understanding of the toxicity behaviour of selected ionic liquids towards *Rhodococcus* UKMP-5M.

5.2 Disk diffusion test

In this study, 16 different ionic liquids (ILs) were selected which covered a range of different cation and anion combinations. These included the widely used group of fluorine-based anions. The bis(trifluoromethylsulfonyl)imide, [NTf₂]⁻ anion was also used in this study and paired with different chain length imidazolium-based cations. A group of acidic ionic liquids was also selected along with various lengths of alkyl-based ionic liquids (Table 4). Except for triethylammonium hydrogen sulfate [TEA][HSO₄], imidazolium cations were used as these are some of the most common cations in ionic liquids, which allowed direct comparison with data available in the literature. The structures of these ionic liquids are shown in Table 2 (Section 1.2.1).

Table 4 The list of ionic liquids used in disk diffusion test.

Anion characteristics	List of ILs
Simple fluorine based ILs	1-butyl-3-methylimidazolium tetrafluoroborate [Bmim][BF ₄]
	1-butyl-3-methylimidazolium hexafluorophosphate [Bmim][PF ₆]
	1-ethyl-3-methylimidazolium trifluoromethanesulfonate [Emim][OTf]
	1-butyl-3-methylimidazolium trifluoromethanesulfonate [Bmim][OTf]
[NTf₂]⁻ based ILs	1-ethyl-3-methylimidazolium bis(trifluoromethylsulfonyl)imide [Emim][NTf ₂]
	1-butyl-3-methylimidazolium bis(trifluoromethylsulfonyl)imide [Bmim][NTf ₂]
	dibutylimidazolium bis(trifluoromethylsulfonyl)imide [C ₄ C ₄ im][NTf ₂]
	1-hexyl-3-methylimidazolium bis(trifluoromethylsulfonyl)imide [C ₆ C ₁ im][NTf ₂]
	1-hexyl-3-ethylimidazolium bis(trifluoromethylsulfonyl)imide [C ₆ C ₂ im][NTf ₂]

ILs with different length of alkyl chain	1-ethyl-3-methylimidazolium acetate [Emim][OAc]
	1-butyl-3-methylimidazolium acetate [Bmim][OAc]
	1-butyl-3-methylimidazolium butyrate [Bmim][OBu]
	1-ethyl-3-methylimidazolium octanoate [Emim][C ₇ CO ₂]
Acidic ILs	Triethylammonium hydrogen sulfate [TEA][HSO ₄]
	1-butyl-3-methylimidazolium hydrogen sulfate [Bmim][HSO ₄]
	1-butyl-3-methylimidazolium methyl sulfate [Bmim][MeSO ₄]

The result of the disk diffusion test of the toxicity of ionic liquids is shown in Figure 5.2. The data have been sorted in order of increasing diameter of inhibition zone from left to the right to assist visualisation for the purpose of comparison and analysis. The larger the inhibition zone corresponds to the greater the toxicity of that particular ionic liquid. The ionic liquid with the largest inhibition zone was found to be [Bmim][HSO₄], while that with the smallest inhibition zone in diameter was [Emim][OAc].

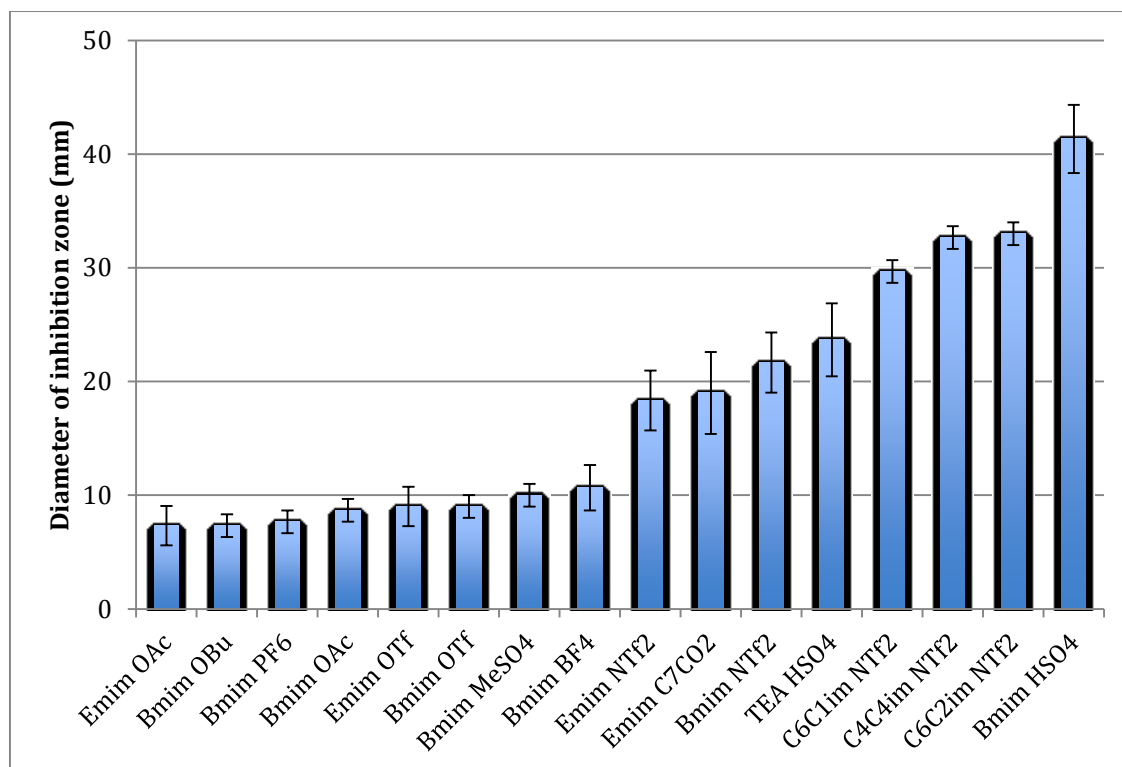


Figure 5.2 Results of the disk diffusion test for *Rhodococcus* UKMP-5M in different ionic liquids.

Recently, [Bmim][HSO₄] has been extensively studied as a potential solvent in various applications (Figure 5.3) due to its acidic nature and less toxic synthetic route compared to other acidic ionic liquids. This ionic liquid has been demonstrated as an effective catalyst for the Prins reaction with more than a 90% yield reported (Figure 5.3).²⁹⁴ The nature of [Bmim][HSO₄], which allows a two-phase downstream process and its recyclability make it a better option than the usual strong protic acid catalysts. It is also a good extractant and catalyst in the oxidative desulfurization of dibenzothiophene (DBT) (Figure 5.3).²⁹⁵ As it is more stable than the flammable and volatile conventional organic solvents, this finding is favourable for the production of ultra low sulfur diesel (ULSD) fuel. Recently, its potential application has been extended to the green leaching to the recovery of gold and silver from ores in aqueous systems along with thiourea and iron (III) sulfate.²⁹⁶

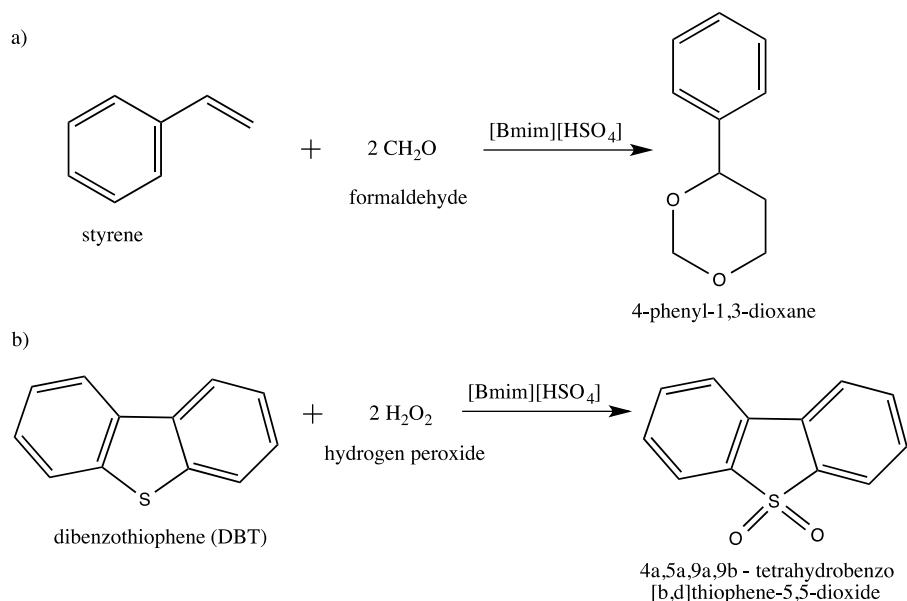


Figure 5.3 Chemical synthetic reactions with [Bmim][HSO₄]. a) Prins reaction for dioxanes production b) Oxidative desulfurization of DBT.

Due to the rise of applications involving [Bmim][HSO₄], it was important to determine the toxicity of this ionic liquid. Even though this study showed its high level of toxicity, [Bmim][HSO₄] has been classified as a non-toxic liquid based on the Acute Toxicity Rating Scale by the US Fish and Wildlife Service (FWS).²⁹⁷ It is worth noting that the growth of bacteria is often sensitive to external constraints including pH and growth substrate concentration. Therefore, a further study is required in order to determine the cause of such toxicity behaviour of [Bmim][HSO₄] in our test. This issue will be discussed in detail in a subsequent Section 5.3.

According to Figure 5.2, the least toxic ionic liquid to *Rhodococcus* UKMP-5M is [Emim][OAc]. This is consistent with a published study, which reported that no cellular toxicity was observed for this ionic liquid on human corneal epithelial (HCE) cells and *Escherichia coli*.²⁹⁸ [Emim][OAc] is widely studied for its role in lignocellulose pretreatment, demonstrating a high selectivity in extracting lignin and as an effective solvent for cellulose dissolution.²⁹⁹ Apart from that, this ionic liquid has been shown to enhance enzyme activities, stabilise protein and increase enantioselectivities in the synthesis of L-phenylalanine, as compared to the conventional organic solvent, acetonitrile.³⁰⁰

The toxicity of the cation with regards to the length of its alkyl chain is well established.^{167, 170, 301} Ionic liquids' toxicity becomes greater with the increasing length of alkyl chain on the cation. This can be partially attributed to the increase of hydrophobicity as the alkyl chain increases in length and thus forms aggregates.³⁰² Consequently, it affects the surface charges of cell membranes,²⁹⁸ mimicking a detergent.³⁰³ In return, the membrane permeability is compromised and this leads to cell disruption.¹⁷⁰ However, it was found that micelle formation only occurs with alkyl chains longer than six carbons.³⁰² Instead of disrupting the lipid bilayer, cation species with shorter alkyl chains are positioned at the water/lipid interface; they aggregate the structure thus affecting the fluidity. This less intrusive mechanism is expressed by lower level of toxicity.^{302, 304}

This trend of toxicity is portrayed in Figure 5.2 where the longer alkyl chains resulted in a larger diameter of inhibition zone observed, indicating an increase in toxicity. [Bmim][OAc] and [Bmim][OTf], both of which possess a four carbon alkyl chain, formed a larger inhibition zone compared to their respective three carbon alkyl chain analogues. A similar pattern is demonstrated by bis(trifluoromethylsulfonyl)imide-based ionic liquids [NTf₂] with the order of toxicity represented in Figure 5.4.

It is interesting to note that an increased number of carbon atoms in the alkyl chain correlates directly with the toxicity without being subjected to other aspects of the structure or the distribution of those atoms in the cation. This finding is illustrated by the similarity of the size of the inhibition zone formed by both the asymmetrical [C₆C₂im][NTf₂] and symmetrical [C₄C₄im][NTf₂]. To the best of our knowledge, the only reported toxicity in respect to atom position was the impact of the central atom in the cation of ionic liquids.³⁰⁵ This aforementioned study could be the first of such toxicity behaviour related to the distribution of carbon atoms in alkyl chain structure. This information could benefit the future design of benign ionic liquids. Nevertheless, further work is required in order to support this claim.

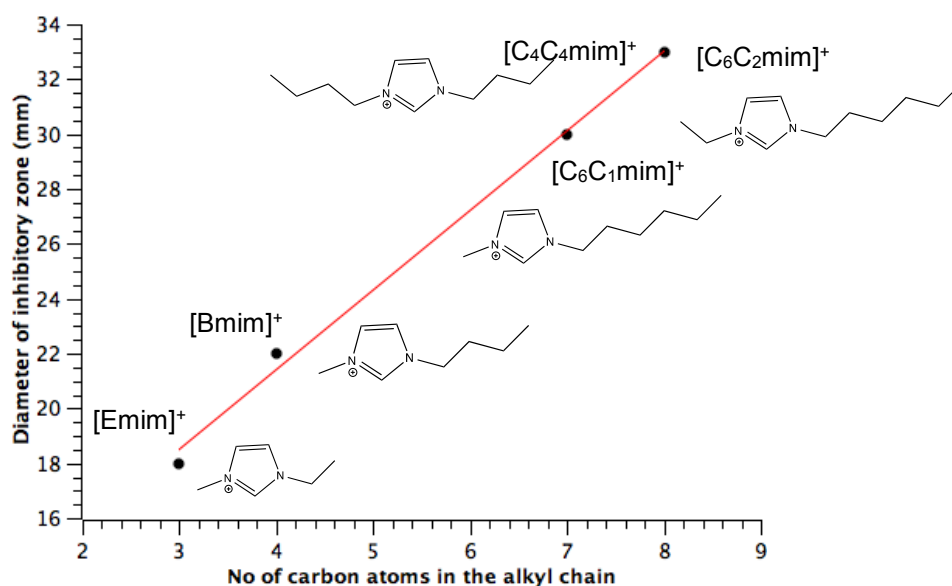


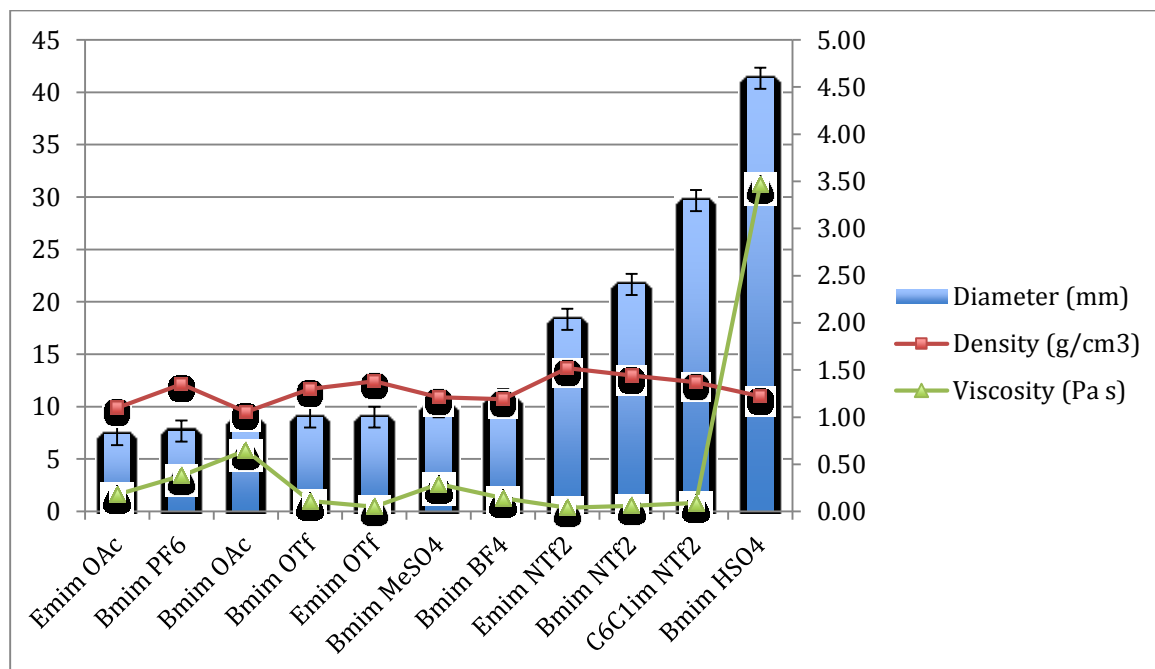
Figure 5.4 The correlation of the number of carbon atoms in the alkyl chain with the diameter of inhibitory zone.

The influence of alkyl chain length on the toxicity of ionic liquids only holds true to a limited extent. There will be a point where the increase of the ionic liquid alkyl chain length decreases its toxicity. This observation was documented by a toxicological study of guanidinium-based ionic liquids; diheptyltetramethylguanidinium iodide, [TMGC₇][I] has a lower level of toxicity than didodecyltetramethylguanidinium iodide, [TMGC₁₂][I].²⁹³ One proposed explanation of this behaviour is that a kinetic process dominates any steric effects and that the uptake of ionic liquids is slowed down due to their large molecular size. Another potential reason is the solubility constraints, which were demonstrated in the toxicity study of the anionic surfactants, alkyl sulfates. An increase in the alkyl chain length of more than 14 carbons reduced their bioavailability and toxicity.³⁰⁶

Whilst the increase of alkyl chain length on the cation is associated with higher viscosity and lower density,³⁰⁷ the link between these properties and toxicity is poorly understood. Based on the limited data³⁰⁸ gathered in Table 5, the correlation of physical properties with the toxicity of ionic liquids is plotted in Figure 5.5.

Table 5 List of ionic liquids' physical properties on selected ionic liquids studied in the disk diffusion test on *Rhodococcus* UKMP-5M.

Ionic liquid	Mean in Diameter of Inhibitory zone (mm)	Density (g/cm ³)	Viscosity (Pa.s)	Octanol-water partition coefficients (K _{ow})
[Emim][OAc]	7	1.10	0.18	<i>n/a</i>
[Bmim][PF ₆]	8	1.35	0.38	0.022
[Bmim][OAc]	9	1.06	0.65	0.001
[Bmim][OTf]	9	1.30	0.11	0.015
[Bmim][BF ₄]	11	1.19	0.14	0.003
[Emim][NTF ₂]	18	1.52	0.04	0.090
[Bmim][NTF ₂]	22	1.44	0.06	0.110
[Bmim][HSO ₄]	41	1.22	3.47	<i>n/a</i>
[Bmim][MeSO ₄]	10	1.21	0.29	<i>n/a</i>
[Bmim][OBU]	7	<i>n/a</i>	<i>n/a</i>	<i>n/a</i>
[Emim][OTf]	9	1.38	0.05	<i>n/a</i>
[Emim][C ₇ CO ₂]	19	<i>n/a</i>	<i>n/a</i>	<i>n/a</i>
[TEA][HSO ₄]	24	<i>n/a</i>	<i>n/a</i>	<i>n/a</i>
[C ₆ C ₁ im][NTF ₂]	30	1.37	0.09	1.400
[C ₄ C ₄ im][NTF ₂]	33	<i>n/a</i>	<i>n/a</i>	<i>n/a</i>
[C ₆ C ₂ im][NTF ₂]	33	1.35	<i>n/a</i>	<i>n/a</i>

**Figure 5.5** Comparison of ionic liquids' physical properties to the toxicity pattern from disk diffusion test on *Rhodococcus* UKMP-5M.

As shown in Figure 5.5, there is no significant relationship observed between toxicity and either the viscosity or density of ionic liquids. This is inconsistent with a published study, which proposed such a relationship between these two properties with toxicity. Ammonium-based ionic liquids with higher densities and viscosities than phosphonium-based ionic liquids exhibited lower toxicities.³⁰⁵ It is likely that this observation in the literature study was limited to the small group studied and is not necessarily applicable in general.

Another attribute that could be involved in the toxicity of ionic liquids is the octanol-water partition coefficient, K_{ow} . Based on the limited data available in the literature, Figure 5.6 was plotted.

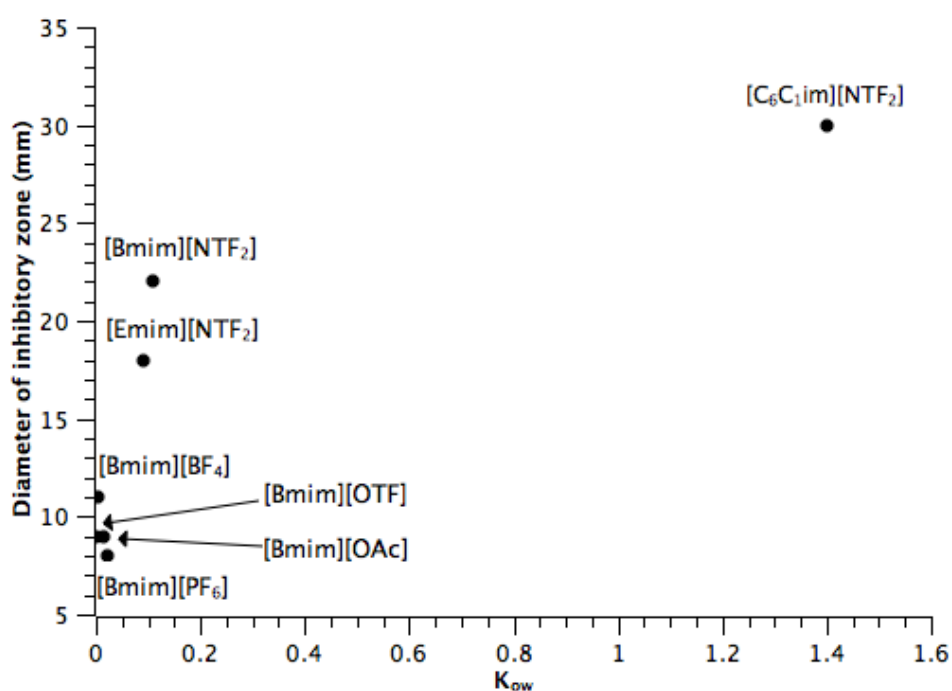


Figure 5.6 The correlation of octanol-water partition coefficient (K_{ow}) and the diameter of the inhibitory zone.

As shown in Figure 5.6 above, the increase of the K_{ow} value will result in a larger inhibitory zone, indicating increased toxicity. The K_{ow} value increases with the extension of alkyl chain length in a homologous series of compounds.³⁰⁹ This positive relationship has also been attributed to increased hydrophobicity. The

rise of hydrophobicity leads to an increase in K_{ow} value and toxicity of ionic liquids.³¹⁰ The same report also stressed that the K_{ow} of ionic liquids is pH and concentration dependent and both factors were taken into account in the next assay, which will be discussed in Section 5.3. Apart from that, it has been documented that the hydrophilic group of ionic liquids with low value of K_{ow} is generally less toxic with a lower potential for accumulation in the environment and living organisms.^{170, 309}

Both cation and anion are responsible for determining the solubility of ionic liquids in water and 1-octanol thus affecting the value of K_{ow} .^{310, 311} Figure 5.7 is plotted in order to understand the correlation of both species on the toxicity of ionic liquids.

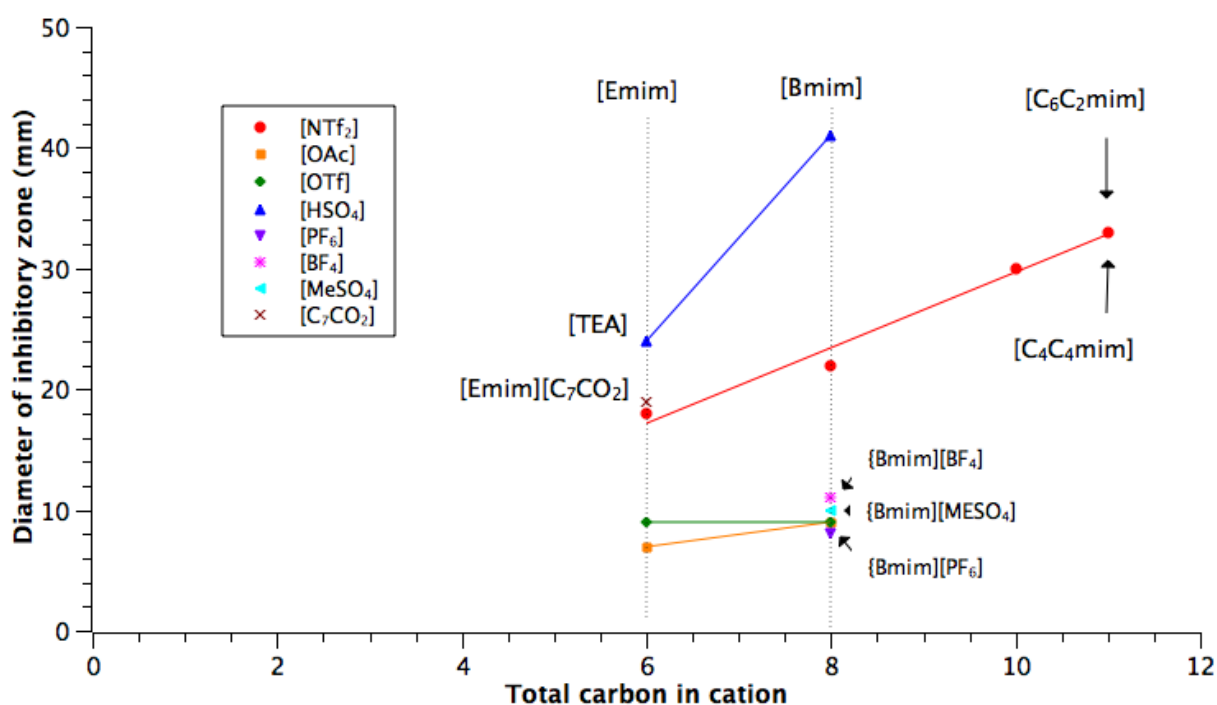


Figure 5.7 The mutual correlation of cation and anion in the toxicity of ionic liquids.

As illustrated in Figure 5.7, the increase in carbon atom count in the cation species will result in the increase of the toxicity of ionic liquids. It is also clear that the anion impacts the toxicity as well by significantly shift the toxicity level higher

or lower than their counterpart with the same cation species. Based on this observation, it is likely that K_{ow} , which is influenced by both cation and anion, has the controlling impact on the toxicity of ionic liquids.

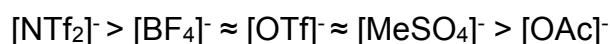
Whilst the impact of the anion on the toxicity of ionic liquids is well recognised, there remains a lack of understanding of the mechanism involved. Recently, there has been a lot of interest in the specific study on the relationship between the anion of ionic liquids and cytotoxicity.³¹²⁻³¹⁵ These studies looked at the effect of ionic liquids on various biological samples including animal cells, bacteria (both beneficial like *Clostridium sp.* and pathogens like *Listeria monocytogenes* and *Escherichia coli*) and plants. Although several studies found no regularity in the relationship between the anion and toxicity,³¹³ several findings have contributed towards our understanding of this aspect.

One of those studies suggested that an increase in the number of fluorine atoms in the anion increases the toxicity of ionic liquids.³¹⁴ This claim is in agreement with the result obtained in the disk diffusion test as illustrated in Figure 5.2. The order is outlined as below with the number of fluorine atoms increasing from left to right.



The mechanism of this toxicity relies on the hydrolytic cleavage, which releases free fluoride ions that could potentially inhibit Na^+K^+ -ATPase on the cell surface.³¹⁶ As a result, cellular processes are disrupted. As an aside, it is worth mentioning that $[PF_6]^-$ has been omitted from this list, as it does not follow the rule in this case. $[Bmim][PF_6]$ is highly viscous as exhibited in Figure 5.5. This limitation affects the reliability of disk diffusion test. Under normal circumstances, $[PF_6]^-$ is placed between $[NTf_2]^-$ and $[BF_4]^-$ as it has a higher fluorine content (six fluorine atoms) than $[BF_4]^-$ (four fluorine atoms).³¹⁴ However, it has been reported that $[BF_4]^-$ are more hydrolysable than $[PF_6]^-$, readily releasing protons to alter the pH conditions.³¹⁷ $[PF_6]^-$ will only be easily hydrolysable at high temperatures. Based on this finding, it is possible that $[BF_4]^-$ is more toxic than $[PF_6]^-$ which is reflected in the disk diffusion test (Figure 5.2).

In addition, different studies have proposed the toxicity of the anion species depends on the chaotropicity as seen by their position in the Hofmeister series.^{315, 318} Disk diffusion tests seem to support this claim as listed in the series below.



Chaotropic agents are known to denature proteins and destabilize biological membranes.³¹⁸ Therefore, the role of the anion as a chaotropic agent is expected, affecting the bilayer membrane via deep penetration into the interior. This action triggers electrostatic interactions between the negative charge of penetrated anion and the headgroup of lipid, disorientating the structure, which result in membrane disruption.³¹⁹

Apart from that, the alkyl chain of the anion possibly affects the toxicity in a similar manner to the cation equivalent. The greater the chain length, the more toxic it becomes for carboxylate anions as depicted in the order below.



There is a possibility that the detergent effect of long alkyl chain anions is similar to that detailed for cations, which increases their toxicity. However, it has been reported that the alkyl chain length gave a small toxicity affect in the anion compared to the cation. The increase of length from methyl sulfate $[\text{MeSO}_4]^-$ to octyl sulfate $[\text{OctSO}_4]^-$ resulted in an approximately 5-fold increase whereas in the cation, there is up to an 130-fold increase in the toxicity from 1-ethyl-3-methylimidazolium, $[\text{Emim}]^+$ to 1-octyl-3-methylimidazolium, $[\text{Omim}]^+$.³²⁰

Nonetheless, there is a study that reported a contradicting observation; no inhibitory zone was observed in ionic liquids with a very long alkyl chains in the anion species.²⁸⁸ This paper suggested a combination of decreased alkyl chain length of cation and increased alkyl chain of anion will result in a non-toxic ionic liquid. Due to the anion species used in this particular study (Figure 5.8), it is

possible that the toxicity is reduced due to the increase of ether linkages in the anion alkyl chain. In cations, the reduction of toxicity level due to the presence of oxygen is well documented. One study reported the choline-based ionic liquids, which contain an oxygen-containing cation, are relatively non-toxic.³²¹ Meanwhile, a different study concluded that the incorporation of ethers corresponds to the decrease of toxicity and increase of biodegradability of ionic liquids.³²² Though it is not highlighted, a study on anion toxicity showed that oxygen-containing trifluoroacetate, $[\text{CF}_3\text{COO}]^-$ is less toxic than other species of anions, $[\text{BF}_4]^-$, $[\text{PF}_6]^-$, $[\text{OTf}]^-$ and $[\text{NTf}_2]^-$.³¹⁴

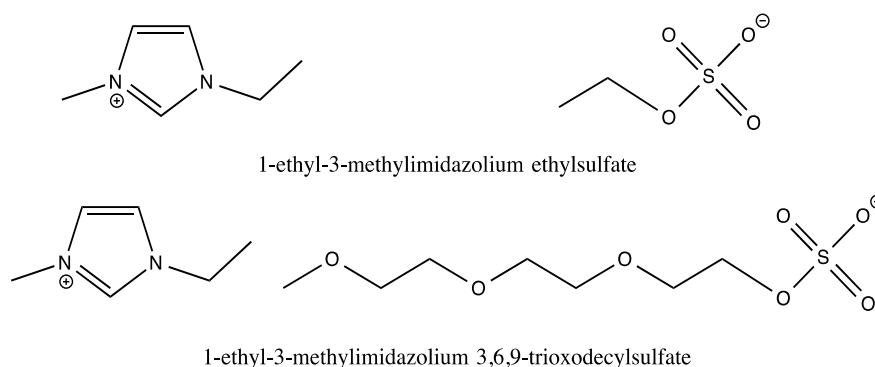


Figure 5.8 ionic liquids containing oxygen-functionalised anions.

Although the disk diffusion test is rapid, simple and reliable, its limitations should be acknowledged. Ionic liquids possessed a wide range of physicochemical properties, which result in different abilities to wet the cellulose-based filter disc.²⁸⁹ In turn, the diffusion of ionic liquids into the culture is affected by the quantity and rate of transport through the agar. Some radii of the inhibition zones formed were not circular, ensuing potential measurement errors. The bacterial growth test is a good follow up test for further confirmation. It should be noted that due to several factors including exposure time and incubation mechanisms, some discrepancies are expected between these two different toxicity assays.

5.3 Bacterial Growth Test

Based on the results obtained from the disk diffusion test, seven different ionic liquids were selected for the bacterial growth test. All of these represented different groups of ionic liquids. These included an ionic liquid, which exhibited the lowest level of toxicity in a disk diffusion test, [Emim][OAc] and its analogues with longer alkyl chain in either cation or anion respectively, [Bmim][OAc] and [Emim][C₇CO₂]. Meanwhile, the water immiscible [Bmim][PF₆] and miscible [Bmim][OTf] were chosen due to their opposing characteristics while [Bmim][NTf₂], another immiscible ionic liquid, was selected for its high level of toxicity in the disk diffusion test. Last but not the least, [Bmim][MeSO₄] was chosen to represent sulfate-based ionic liquids.

By measuring the turbidity (optical density at 600nm), the bacterial growth was monitored accordingly and the results are shown in Figure 5.9. Overall, there are three distinguishing patterns depicted: (1) a complete inhibition of growth at all concentrations tested as observed with [Bmim][NTf₂]; (2) an increase in toxicity as the concentration of the ionic liquids increases with a cut off concentration at which a total inhibition of bacterial growth occurs; (3) an increase in the lag phase period with an increase of the concentration of ionic liquids as illustrated by [Bmim][MeSO₄] and [Bmim][OTf]. This was on occasion accompanied by a decrease in cell density in the stationary phase.

The complete inhibition of [Bmim][NTf₂] is not surprising as several studies have demonstrated its high toxicity towards various biological systems including human and animal cell,^{162, 323} enzymes,³²⁴ microbes³²⁰ and plants.³²⁴ Toxicity tests of 2% imidazolium-based ionic liquids paired with [NTf₂]⁻ on *Escherichia coli* showed no growth at all.²⁸⁹ Furthermore, a study reported that *Clostridium sp.* can only grow in 0.1% (v/v) 1-methoxyethyl-3-imidazolium bis(trifluoromethylsulfonyl)imide, [Moemim][NTf₂] with no growth observed at 0.25% and higher concentrations.³¹⁴ Considering the amount of [Bmim][NTf₂] introduced to the *Rhodococcus* UKMP-5M, which is 0.5% to 4% (v/v), the complete inhibition observed is in agreement with the literature. In addition, an

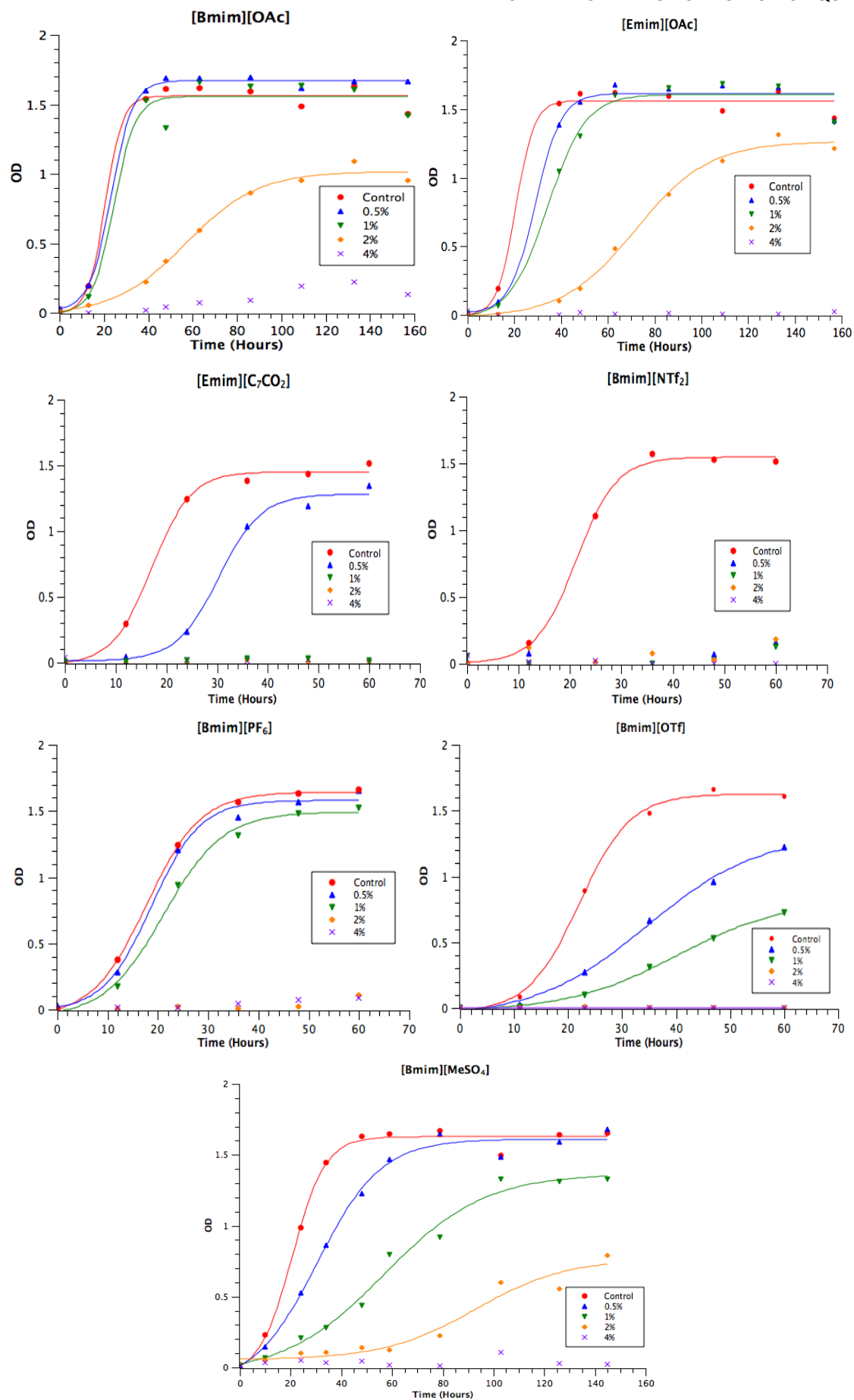


Figure 5.9 The bacterial growth test of *Rhodococcus* UKMP-5M in different concentration of various ionic liquids.

antimicrobial study showed that [Bmim][NTf₂] gave a lower minimal inhibitory concentration (MIC) than [Bmim][BF₄] and [Bmim][MeSO₄] for all of the tested microbes including *Escherichia coli* (gram-negative), *Staphylococcus aureus* (gram-positive) and *Saccharomyces cerevisiae* (yeast). This finding is also consistent with the disk diffusion test results reported in Figure 5.2.

Although the toxicity of [Bmim][NTf₂] is not favourable, the ionic liquid has been demonstrated as a convincing candidate for use in whole cell biocatalysis.³²⁵ This ionic liquid based biphasic system with an aqueous phase, which assists biocatalysis and downstream processes, leads to a higher yield. After 3 h, the asymmetric reduction of 4-chloroacetophenone by *Escherichia coli* gave a 98% of conversion rate in 20% (v/v) [Bmim][NTf₂] while only a 36% conversion rate was found in the organic solvent, 20% (v/v) decane (Figure 5.10).³²⁵ The key to this biocompatibility is the short exposure time to the ionic liquids and/or the use of resting cells, which are not growing but only generating enzymes. In addition, a study has shown that [NTf₂]⁻ can be degraded by *Rhodococcus* albeit less rapidly than the octylsulfate [C₈SO₄]⁻ anion.¹⁷⁰ Due to these features possessed, the yield increases as well as biodegradability, showing that this ionic liquid has a huge potential in biotechnology applications including in a biorefinery.

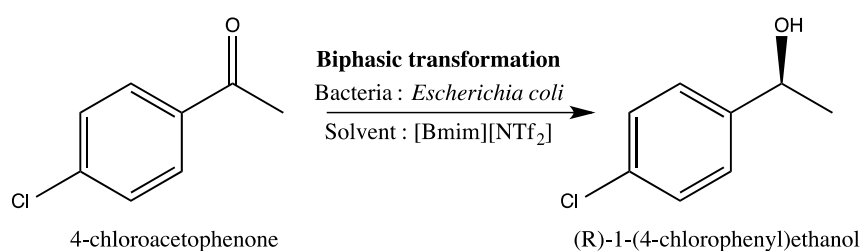


Figure 5.10 Schematic diagram of the whole cell biotransformation using [Bmim][NTf₂].

The second pattern observed in this test is more common in the bacterial growth toxicity test, where an increase of the ionic liquids concentration results in a decrease of bacterial growth, indicating a rise of toxicity level. However, it is observed that the cells showed differing sensitivity to different ionic liquids. Also,

Rhodococcus appears to be more tolerant towards ionic liquids than other bacteria, for example with [Bmim][PF₆] *Clostridium* sp. does not grow at concentrations above 0.25%³²⁶ whilst *Rhodococcus* UKMP-5M can grow at concentrations of 1%. In contrast, some other species have shown a higher tolerance to this ionic liquid such as yeast, *Pischia pastoria*, which has been shown to grow in the presence of up to 10% (v/v) [Bmim][PF₆].³²⁷

As illustrated in Figure 5.9, acetate-paired ionic liquids also follow the same pattern of increased toxicity with increased concentration. In both [Bmim][OAc] and [Emim][OAc], the stationary phase cell density of *Rhodococcus* UKMP-5M in 0.5% and 1% (v/v) concentrations is higher than the control, which is the culture in the absence of ionic liquids. *Rhodococcus* is able to degrade the acetate anion, which is integrated into the central metabolism.¹⁷³ This extra carbon source uptake could be the reason for a higher bacterial growth than the control sample. In addition, throughout the incubation time, the media will normally become acidic. The presence of [OAc]⁻ is able to buffer the pH thus favouring the growth of bacteria.³²⁸

The third pattern exhibited in this test is that an increase in the concentration of some ionic liquids prolongs the lag phase period as clearly demonstrated by [Bmim][OTf] and [Bmim][MeSO₄] in Figure 5.9. This is a sign of bacterial adaptation. The lag phase is a maturation period of cells where no bacterial division has yet taken place. In order to adapt, bacteria slow down their growth rate to acquire tolerance towards toxic components. During this period of lag phase, *Rhodococcus* could change the length of fatty acids and mycolic acids accordingly to maintain their permeability barrier. By tailoring the percentage of branched fatty acid, the degree of fatty acids saturation is controlled.³²⁹

As mentioned previously, the bacterial growth test could provide in depth information on toxicity behaviour to support the results of the disk diffusion test. Examining Figure 5.2, [Bmim][HSO₄] formed the largest inhibition zone. It is essential to determine whether this toxicity effect is caused by the ionic structure or the intrinsic nature of acidic ionic liquids. The bacterial growth test was conducted with both acidic ionic liquids, [Bmim][HSO₄] and [TEA][HSO₄].

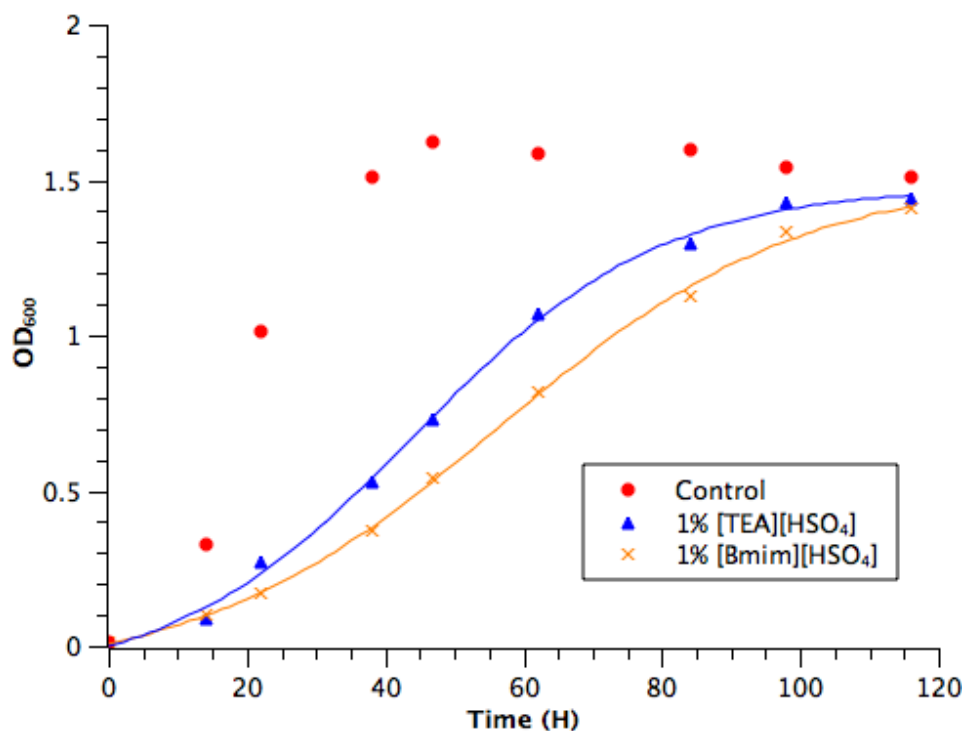


Figure 5.11 The toxicity of neutralised acidic - ionic liquids to *Rhodococcus* UKMP-5M.

While there is no growth observed in acidic ionic liquids (with pH measured in the range of 2 to 4), Figure 5.11 reveals the growth of *Rhodococcus* UKMP-5M in 1% (v/v) of neutralised ionic liquids. Prior to the bacterial incubation, the ionic liquids were adjusted to pH7 through the addition of sodium hydroxide. The results shown in Figure 5.11 indicate that the structure of both [TEA][HSO₄] and [Bmim][HSO₄] is not toxic to the bacteria. Although the lag phase seems longer, the concentration of bacteria in the stationary phase is quite similar to that in the control, which is the culture without the presence of ionic liquid. [TEA][HSO₄] showed a higher growth rate than [Bmim][HSO₄]. This is expected since triethylammonium-based ionic liquids are generally more toxic than imidazolium-based ionic liquids.¹⁶²

In order to further study this issue, an experiment using a bacterial growth test with different concentrations of neutralised ionic liquids was conducted and the results are shown in Figure 5.12.

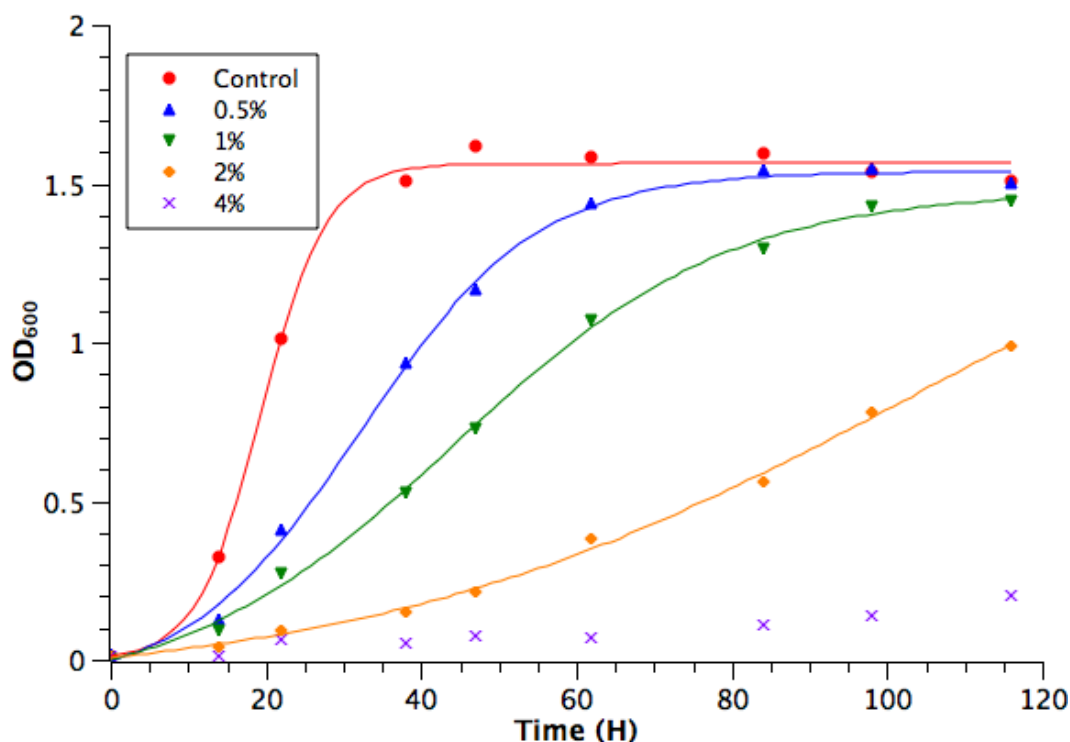


Figure 5.12 The growth of *Rhodococcus* UKMP-5M in the present of neutralised [TEA][HSO₄].

In this figure, the increase in ionic liquid concentration prolongs the lag phase. Whilst this pattern indicates the increase of toxicity with the increase of concentration, significant growth is observed unlike the unneutralised media with acidic ionic liquids. No bacterial growth is observed even in 0.5% (v/v) of unneutralised medium. This supports the hypothesis that the growth inhibition of acidic ionic liquids is mainly due to the pH rather than the concentration. The optimum pH for the growth of *Rhodococcus* is neutral, pH 7.

In a recent study conducted with freshwater and marine fishes, [Bmim][HSO₄] showed a lower median lethal concentration (LC₅₀) than [Bmim][NTf₂], signifying a higher toxicity. Nevertheless, the LC₅₀ value is classified as practically non-toxic according to the acute-toxicity rating scales provided by the US Fish Wildlife Service (FWS).²⁹⁷ To the best of our knowledge, this is the first study suggesting that the toxicity of acidic ionic liquids is due to shifts in the pH, not the ionic components themselves.

5.4 Determining the EC₅₀ and MIC values of ionic liquids

The EC₅₀ value is one of the well-known parameters used in scaling the toxicity of chemical compounds. It is important to determine this value in order to allow a direct comparison with other studies published in the literature. As explained in the introduction (Section 5.1), EC₅₀ can be defined as the effective concentration that reduces 50% of bacterial growth relative to the control. Based on the data shown in Figure 5.9, the value of EC₅₀ is determined by computational simulated data fitting software, SciDAVis. The calculated values are listed in Table 6.

Table 6 Half-maximal effective concentration of ionic liquids after 24 h of incubation (EC₅₀).

Ionic liquids	EC ₅₀ (mM)
[Bmim][PF ₆]	0.97
[Bmim][OAc]	0.84
[Emim][OAc]	0.60
[Bmim][MeSO ₄]	0.56
[Bmim][OTf]	0.46
[Emim][C ₇ CO ₂]	0.34
[Bmim][NTf ₂]	0.26

Based on this table, [Bmim][NTf₂] has the lowest EC₅₀ and the most toxic whereas [Bmim][PF₆] gave the highest value of 0.97 mM. The small value of [Bmim][NTf₂] with 0.26 mM is consistent with the one reported in the toxicity assay of the same ionic liquid towards *Vibrio fischeri*, 0.30 mM and *Escherichia coli*, 0.35 mM.³²⁴ Even though [Bmim][PF₆] is on the opposite end of the EC₅₀ value, this is misleading due to the cut-off point observed in the bacterial growth test (Figure 5.9). This cut-off point caused a lack of data for the fitting, which resulted in a higher value of EC₅₀. It is worth noting that EC₅₀ is time-dependent and species-specific thus the comparison between studies can be difficult. For example, the value is 1.3 mM in a 96 h incubation with the alga, *Selenastrum capricornutum*³³⁰ and 14 mM in an 8 h incubation with *Escherichia coli*.²⁹² As a qualitative comparison, [Bmim][PF₆] gave a smaller value than [Bmim][OTf] in the toxicity study with *S. capricornutum*.

[Bmim][OAc] and [Emim][OAc] are considered to be of low toxicity, with better prospects in various applications. Toxicity assessments against leukemia cells, IPC-81 showed the EC_{50} for [Bmim][OAc] is 50% less toxic than [Bmim][PF₆].³³¹ In addition, the role of [Emim][OAc] in biomass deconstruction and biocatalysis is well established with the use of this solvent resulting in a high recovery of cellulose and lignin³³² and a high conversion rate of yield respectively.³⁰⁰

In order to further analyse the relationship between EC_{50} value and the toxicity, a graph was plotted against the mean in diameter of inhibitory zone, which was obtained from the disk diffusion test described earlier.

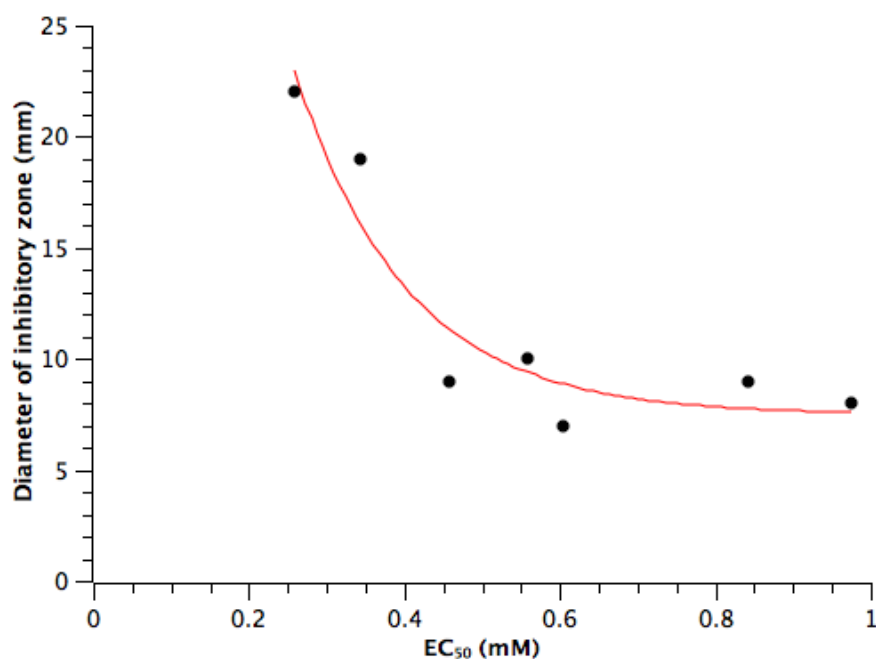


Figure 5.13 The value of EC_{50} against the diameter of the inhibitory zone.

As shown in Figure 5.13, an exponential decay pattern is observed, which indicates the correlation between the value of EC_{50} and the size of the inhibition zone. The inhibition zone is getting smaller as the EC_{50} value decreases. This trend is in excellent agreement with the reported toxicity test of ionic liquids with

anaerobic *Clostridium butyricum*.²⁸⁸ Moreover, this finding also validates the reliability of the disk diffusion test in determining the toxicity of ionic liquids.

Table 7 The comparison of MIC values between microbes.^{167,337}

Ionic Liquid	<i>Rhodococcus</i> UKMP-5M	<i>Escherichia</i> <i>coli</i>	<i>Bacillus</i> <i>subtilis</i>	<i>Enterococcus</i> <i>sp.</i>	<i>Saccharomyces</i> <i>cerevisiae</i>
[Bmim][NTf ₂]	5.0 mg/ml	2.5 mg/ml	2.5 mg/ml	1.2 mg/ml	0.6 mg/ml
[Bmim][MeSO ₄]	40.0 mg/ml	19.7 mg/ml	N/A	10.0 mg/ml	7.4 mg/ml

Besides EC₅₀, minimal inhibitory concentration (MIC) is a parameter normally used to compare the toxicity effect between bacteria. Based on the data available in Figure 5.9, the estimate of the upper limit of the MIC can be determined by the lowest concentration at which there is no significant growth observed for the treated cells compared to the control, at the earliest time point. Table 7 shows that *Rhodococcus* UKMP-5M is more resistant to the ionic liquids with a significantly higher value of MIC compared to other bacteria and yeast (*Saccharomyces cerevisiae*).

5.5 Conclusion

In this Chapter 5, the testing of toxicity of a wide range of ionic liquids towards *Rhodococcus* UKMP-5M has revealed the ability of these bacteria to tolerate ionic liquids. These toxicity assays have also revealed the major role of both cation and anion species in the mechanism of their toxicity. The effect is interconnected between both ions and their physicochemical properties, making the pattern of toxicity more complex and not easily defined.

These assays revealed some novel findings while supporting some of the previously reported observations. An increase in the number of carbon atoms, but not the structure or the distribution of those atoms in the cation side chains, correlates directly with the toxicity. In addition, the relationship between K_{ow} and the toxicity of ionic liquids has been observed with both cation and anion influencing this value and the toxicity of ionic liquids. Besides this, the study on acidic ionic liquids suggests that the primary toxicity is caused by the lowering of pH, not the structure of the ionic liquids. However, a further study is required in order to establish these observations. At the same time, this study is consistent with previous toxicity theories, the increase of toxicity due to the increase of alkyl chain length in the cation or anion, the Hofmeister series and the fluorine content of the anion. Fundamentally, all of this information is useful in the design of less toxic ionic liquids.

CHAPTER 6

CONCLUSION

6.1 Summary of the study

The exploitation of lignocellulose as a sustainable resource for biofuel and biomaterials has been one of the foci of recent research. Bioligninolysis and ionic liquids treatment are two different mechanisms that are used in tackling this aspect. The work described in this thesis was designed to investigate the possibility of incorporating ionic liquids to enhance the degradation of lignin by *Rhodococcus* UKMP-5M. Based on Section 1.6.3, the objectives of this study were to address the ability of these bacteria to degrade a lignin model compound, the integration of ionic liquids into the bioligninolysis process and the toxicity of ionic liquids on the cells. A flow chart (Figure 6.1) shows the outline of this study.

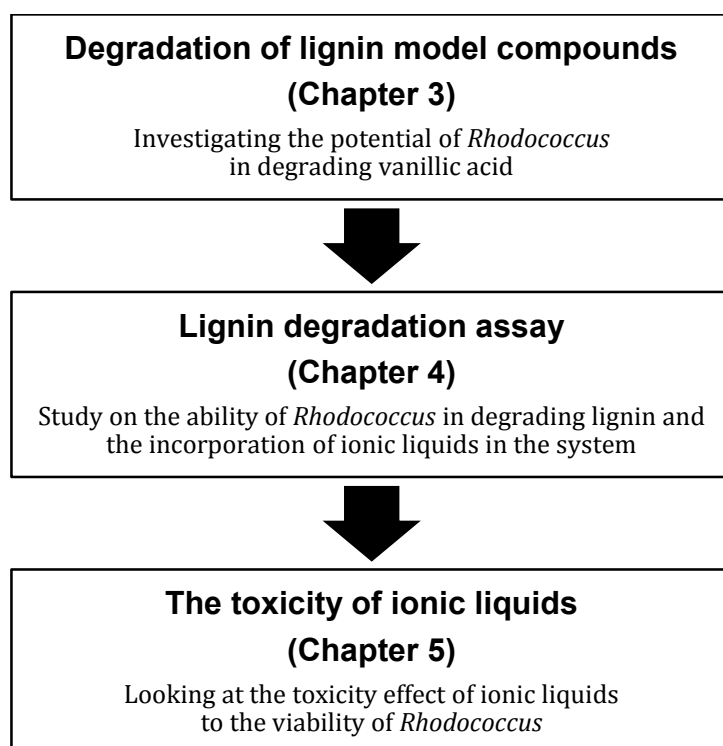


Figure 6.1 The outline of study (Bioligninolysis : Degradation of ionic liquid derived lignin by *Rhodococcus*).

In Chapter 3, the ability of *Rhodococcus* UKMP-5M to degrade vanillic acid as a lignin model compound has been established, revealing a previously undescribed route of vanillic acid metabolism. Instead of formaldehyde, formate was identified which is likely due to the presence of formaldehyde dehydrogenase (*fdh*) in this particular strain. If this is proven true through further research, this strain can be exploited via genetic engineering technology to generate vanillic acid, formic acid and/or as NADP generator. This topic will be discussed briefly in Section 6.3.

The understanding of the metabolic pathway of vanillic acid degradation suggested the possibility of using *Rhodococcus* UKMP-5M as a bioligninolysis agent. The development of a new high throughput lignin degradation assay has driven the study in Chapter 4, assessing lignin degradation while evaluating the potential of ionic liquids to enhance this process. The roles of ionic liquids as a solvent for chemical pretreatment of lignocellulose and in catalysing the degradation of lignin were also investigated. As a result, the incorporation of some ionic liquids into the system has been demonstrated to facilitate the bioligninolysis (e.g., [Emim][OAc]). However, other ionic liquids decrease the efficiency of lignin degradation (e.g., [TEA][HSO₄]).

Therefore, in the following Chapter 5, a study of the toxicity of ionic liquids (if any) towards *Rhodococcus* UKMP-5M was conducted. This toxicological study was conducted using the disk diffusion test and confirmed by a bacterial growth study. Through the determination of EC₅₀ value and its direct correlation with the result obtained via the disk diffusion test, the reliability of the disk diffusion test as the preliminary toxicity screen for ionic liquids has been established. While the toxicity of some ionic liquids inhibits the viability of the studied strain even at low concentrations (e.g., [Bmim][NTf₂]), some ionic liquids have been identified as safe with minimal inhibitory effect towards *Rhodococcus* UKMP-5M (e.g., [Bmim][OAc] and [Emim][OAc]).

Overall, this study has identified [Emim][OAc] as the best potential candidate for bioligninolysis using *Rhodococcus* UKMP-5M. This ionic liquid has demonstrated its ability to increase the efficiency of lignin degradation and to

show minimal toxicity with *Rhodococcus* UKMP-5M. The role of [Emim][OAc] in biomass deconstruction, specifically of lignocellulose, is well established. Studies have shown the pretreatment of lignocellulose with this solvent resulted in a high recovery of cellulose and lignin^{8, 332}. A bioligninolysis system facilitated by [Emim][OAc] in the pretreatment stage and lignin degradation by *Rhodococcus* UKMP-5M is a promising prospect to be developed, which could provide a feasible technology towards a biorefinery based on lignocellulose.

6.2 Research limitations

The first limitation and the biggest challenge for this study was the availability of ionic liquids. Despite 17 different ionic liquids being used in this study, and considering that their syntheses were outsourced, their availability was not such that they were present at the same time. Hence, instead of assessing them in parallel, different batches of ionic liquids were evaluated as they became available, which was less time efficient. Furthermore, careful planning with a small margin of error was vital due to the limited volume of some supplied samples. This limitation has also constrained the flexibility of the study in respect of the structural diversity of ionic liquids available for the study and the accessibility of some ionic liquids.

The second limitation was the strain used in this study, *Rhodococcus* UKMP-5M. The strategy of focusing on a single bacterial strain was effective in terms of cost and time. However, similar to most microbiological studies, the results obtained were strain specific. As mentioned throughout this study, different species demonstrate different behaviours with ionic liquids in regard to both toxicity and bioligninolysis. Nevertheless, the results could provide insight into the mechanisms involved and some potential leads to design future studies based on *Rhodococcus* and its bioligninolysis as well as the study of ionic liquids in this area in general.

Since to the best of our knowledge, this is the first study involving the integration of ionic liquids and *Rhodococcus* in ligninolysis, there was a limited literature to guide the study. The reliability of the disk diffusion test as a toxicity

test for ionic liquids was initially a concern. However, the results were supported by further studies of bacterial growth curves and EC₅₀ determination. In addition, the lignin degradation assay used was only developed recently. Thus, there is not currently a widespread use of this assay and adopting it was a calculated risk, which turned out to be a good decision as it allowed a high throughput study to be performed, although the sensitivity of the assay was an issue, as discussed in Section 4.5.

While acknowledging these limitations, this study has successfully achieved the targeted objectives and demonstrated an operational bioligninolysis system. Nonetheless, there is room for improvement, which will be discussed in the following Section 6.3.

6.3 Future work

6.3.1 Exploiting lignin degradation by *Rhodococcus*

The understanding of the lignin degradation pathway through vanillic acid as a lignin model compound allows the manipulation of the route to generate valuable chemicals such as vanillin and NADH.

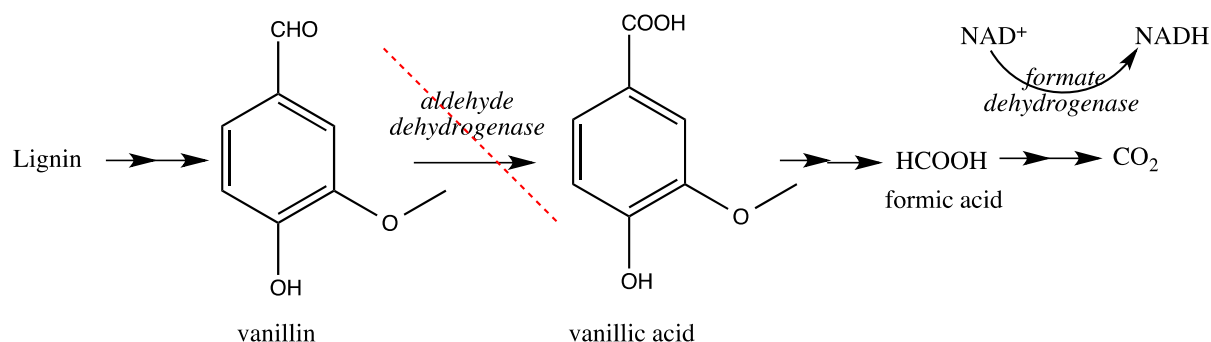


Figure 6.2 Simplified pathway of lignin degradation by *Rhodococcus* UKMP-5M. The dashed red line represents the potential gene knock-out.

As shown in Figure 6.2, the deletion of the aldehyde dehydrogenase gene will result in the accumulation of vanillin. This strategy has been demonstrated by

a study with *Rhodococcus jostii* RHA1, which resulted in maximum yield of 96 mg/L in 144 h of incubation.⁸⁴ Vanillin is well known for its high market value in the food industry.

Moreover, the isolation of formate dehydrogenase enzymes is highly valuable for NADH regeneration. As illustrated in Figure 6.3, this enzyme allows a viable production of chiral compounds, which is a NADH dependent reaction.²⁴³ The by-product of this reaction, CO₂, does not inhibit most dehydrogenase enzymes and so a high yield, high purity product is achievable.

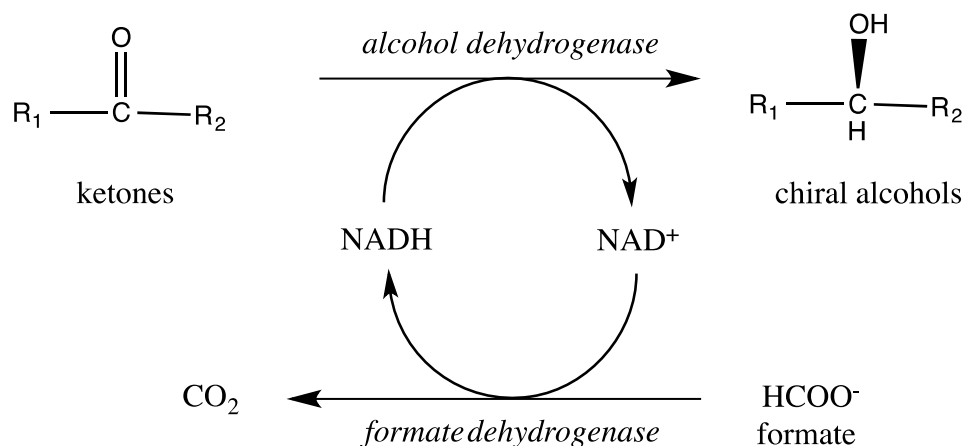


Figure 6.3 A schematic diagram for the synthesis of optically active compounds facilitated by formate dehydrogenase for NADH regeneration.

Prior to genetic manipulation, further molecular study at a genomic and proteomic level is advisable to fully understand the unique lignin degradation route of *Rhodococcus* UKMP-5M. All of these strategies will enhance the feasibility of a lignocellulose-based biorefinery and improve the credibility of *Rhodococcus* as the bioligninolysis agent.

6.3.2 Improving the efficiency of bioligninolysis

This study has demonstrated the ability of ionic liquids to assist the lignin degradation. Investigating the effect of different concentrations of ionic liquids in the system and designing the downstream part are some of the issues that could

be analysed in a following study. A pilot study at a larger scale would be an interesting next step to think of, in order to realise this concept. The input of this pilot study will help in designing a more efficient bioligninolysis system.

Another alternative approach is available to increase the efficiency of bioligninolysis. The development of synthetic biology allows the vast possibilities for modification of living organisms as desired. Researchers have looked into the potential of transgenic plants and recombinant bacteria in improving the bioligninolysis process.^{333, 334}

Lignin bioengineering is a novel strategy developed through the emergence of new synthetic biology tools (*i.e.*, genome bioediting and transgene regulation).³³³ By targeting the tissue-specific promoter (*e.g.*, fibre-specific promoter) and overexpression of transgenes, novel monolignols can be developed without deleterious effect to the plant. These monolignols has desirable traits as they are cleavable, reduce the lignin-polysaccharide interaction, decrease lignin polymerization degree and increase the lignin value.

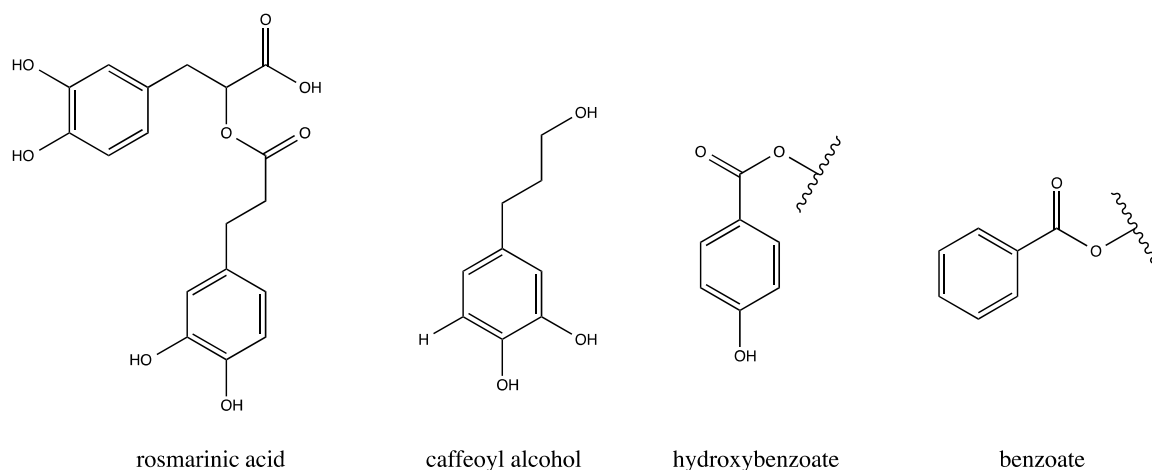


Figure 6.4 The structure of novel monolignols through lignin bioengineering.

As illustrated in Figure 6.4,³³³ rosmarinic acid is an ester-linked hydroxycinnamate, which make it easily cleavable. On the other hand, the β -O-4 polymerization of conventional monolignols with benzene diols (*e.g.* caffeoyl

alcohol) and triols will minimise the cross linkages between lignin and polysaccharide, thus reduce the lignin recalcitrance. By overexpressing monomers like hydroxybenzoate that initiate or terminate lignin chains, the degree of lignin polymerisation will be significantly reduced. Lignin bioengineering also allows the overproduction of valuable moieties such as benzoate. These monolignols could be recovered after pretreatment of lignin and readily used for industrial purposes. This lignin bioengineering approach will increase the value of lignin and its accessibility.

The second strategy in increasing the efficiency of bioligninolysis is by engineering the bacterial genome. DNA recombinant technology is well established. Recently, the recombination of DypB lignin peroxidase in *Escherichia coli* originally isolated from *Rhodococcus jostii* RHA1, claimed to be the first, has been performed.¹¹⁴ This kind of study provides essential information with which to develop recombinant bacteria with a competent bioligninolysis capacity. In respect to lignocellulose, *Rhodococcus opacus* PD360 has been engineered to utilise xylose (a product of hemicellulose hydrolysis) to produce the biofuel precursors, triacylglycerols.³³⁴

6.3.3 Increasing the bacterial resistance to ionic liquids

Through toxicological assays, it has been established that *Rhodococcus* UKMP-5M is tolerant towards some ionic liquids while exhibiting signs of adaptation to others, which is demonstrated by the increased lag phase. There are a few approaches, which can be employed with which to tackle this toxicity issue.

The first suggested approach is to increase the resistance of bacteria to the ionic liquids by random mutagenesis. The bacteria grown in the presence of ionic liquids is then exposed to the UV radiation or chemical mutagens and subcultured. The culture is then exposed to a higher concentration and variants displaying higher resistance can then be selected. This cycle is repeated several times in order to obtain a mutated strain with high resistance to ionic liquids. This approach is laborious but low in cost and easy to conduct as opposed to a genetic engineering study.

The alternative approach is to focus on designing less toxic ionic liquids, which will complement the bioligninolysis system without any inhibitory effect to the bacteria. This approach will require in-depth understanding of the correlation between the structure of ionic liquids and their toxicity. Biodegradability of ionic liquids is also a factor to be taken into account when designing the ionic liquid as this will drive the 'green' technology agenda. This study has provided some insight into the toxicity of ionic liquids, thus a future study with a bigger selection of the structures of both cation and anion structures will complement this study. Further work to support the findings in this study and the relationship between toxicity and biodegradability is needed.

6.4 Closing remarks

Supported by the promising results demonstrated in this study, it is clear that the bioligninolysis system incorporating ionic liquids has significant prospect for delivering the demand for lignocellulose as a biomass resource in the biorefinery concept. This abundant and widely-distributed alternative resource is sustainable in the foreseeable future. Therefore, tackling this issue is a priority and the results of this study should generate further interest in this field. Furthermore, this study has shown that multidisciplinary research is the best way to resolve the issue of using lignocellulose efficiently.

This study provides a platform for future work involving ionic liquids and *Rhodococcus* in various biotechnology applications. The toxicity test offers fundamental information in designing a relevant system to integrate ionic liquids in bioremediation, biotransformation and other whole cell biocatalysis processes. Hopefully, this study will be of use to the science community, especially in the field of bioligninolysis using ionic liquids.

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