Evaluation of combined fungal-wet oxidation pre-treatment of radiata pine wood chips for energy conservation

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Abstract

Biofuel production from lignocellulose has recently been gaining attention. One major problem of using lignocellulosic materials for the production of biofuel is the low accessibility of cellulose to enzymes and microorganisms. Therefore, pre-treatment of lignocellulose is a critical step in biofuel production from such materials. Of the pre-treatments, fungal treatment has become an important process due to its low-energy demands and selective degradation of lignin and hemicellulose. This capability comes from the unique enzymatic systems, cellulolytic and ligninolytic enzymes, especially in white rot fungi. The low-energy demand of fungal pre-treatment has generated interest in studying the applicability of fungal pre-treatment for biofuel production from woody materials. The most significant drawback of fungal pre-treatment is the lengthy time required for the process. Combining fungal pre-treatment with other pre-treatment methods might reduce the time necessary for the whole process to operate. It can also introduce cost-effectiveness. Thus, combining fungal pre-treatment with other physical and chemical methods has been recently contemplated. The applicability of the combination of fungal with other pre-treatment methods has been considered in a number of recent publications. To be commercially attractive, both energy demand and processing time should be reduced. In terms of energy demand reduction, combined fungal physico-chemical pre-treatment has been effective. However, the lengthy time taken for the whole process has not been significantly improved upon.

Wet oxidation is a physico-chemical pre-treatment method which uses pressurised subcooled water and pure oxygen or air to provide an oxidative media. This method has the potential to fractionate insoluble organic contents to simple molecules like water and carbon dioxide. However, the high-energy demand for the process makes this method inefficient for biofuel production from lignocellulosic biomasses.

The aim of this work was to study the effect of prior fungal pre-treatment on wet oxidation pre-treatment in terms of required energy reduction. The study started with an investigation on sole fungal pre-treatment of radiata pine wood chips by two New Zealand white rot fungi. The effect of

this kind of pre-treatment on the chemical and physical properties of radiata pine wood chips was investigated. This led to a better understanding of the effectiveness of each fungal strain on selective lignin degradation and cellulose loss.

To have a perspective on the combined fungal-wet oxidation pre-treatment of the woody biomass, single wet oxidation pre-treatment of radiata pine wood chips under different operating conditions was also studied. A simplified kinetic model for delignification of radiata pine wood chips pre-treated by wet oxidation was investigated. Based on the results on fungal pre-treatment, one of the fungal strains, which has a better performance, was chosen to pre-treat the biomass required for the combined method.

Fungally treated wood chips were placed into wet oxidation reactor to find out the effect of the combined method on energy reduction for the whole process as well as its impact on the physical and chemical properties. A simple energy estimation showed that combination of the fungal pre-treatment with wet oxidation was able to reduce energy demand up to 70 % while the cellulose loss increased up to 40 % when compared to sole wet oxidation pre-treatment.

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Certification by Co-Authors

The undersigned hereby certify that:

- the above statement correctly reflects the nature and extent of the PhD candidate's contribution to this work, and the nature of the contribution of each of the co-authors; and
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Chapter 1
Introduction and literature review

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1.1 Background

There is growing interest in finding alternative forms of energy, mostly driven by depletion of crude oil and concerns over greenhouse gas emissions from fossil fuels. Lignocellulosic materials, such as wood residuals, paper residuals and crop residuals, (the most abundant renewable bioresource in the world), are some of the promising materials for the production of biofuel. There has been much investigation into producing biofuel from lignocelluloses as they are cheap and do not interrupt human food sources in favour of biofuel production [2].

In physical and chemical pre-treatment methods, either an external mechanical, thermal or chemical deriving force is used to deconstruct the compact structure of lignocellulose. A number of promising chemical and physical pre-treatment methods exist [3], but they often suffer from high capital cost, corrosion problems, high-energy demands, and final discharges may have a negative impact on the environment. Fungal pre-treatment with selective lignin degrading fungi has gained attention due to low-energy requirements and minimal environmental footprint [4]. However, the most important drawback of fungal pre-treatment is the low rate of biomass hydrolysis yielding a requirement for large process volumes. Recently, there have been several research projects focusing on pre-treating biomass using different kinds of fungi for biofuel production [5-13]. The majority of work has been carried out on white rot fungi, due to its ability to selectively degrade lignin. The lengthy time required for a sole fungal pre-treatment has been reported as the main disadvantage of this technology. This has resulted in interest in combining fungal pre-treatment with other processes, in search of a combination which leads to a cost-effective process.

1.2 Lignocellulosic biomass

Generally depending on hardwood or softwood, lignocellulose consists of cellulose (40–47 %), hemicellulose (25–35 %), lignin (16–31 %), extractives (1-8 %), forming a highly complex structure in woody materials [14].

Cellulose, with the general formula $(C_6H_{10}O_5)_a$, the main component of lignocellulose, is a linear biopolymer which is made of D-glucose monomers connected by β-(1, 4)-glycosidic bonds (Fig. 1.1.b).

Hemicellulose, with the general formula $(C_5H_4O_8)_b$, is a branched biopolymer comprising of pentoses (β-D-xylose, α-L-arabinose), hexoses (β-D-mannose, β-D-glucose, α- galactose) and/or orgonic acids (α-D-glucuronic, α-D-4-O-methyl-galacturonicanda-D-galacturonicacids) [15]. The major hemicelluloses in softwood are galactoglucomannan, glucomannan and arabinoglucuronoxylan, while, in hardwood the main hemicellulose is xylan (Fig. 1.1.a) [16].

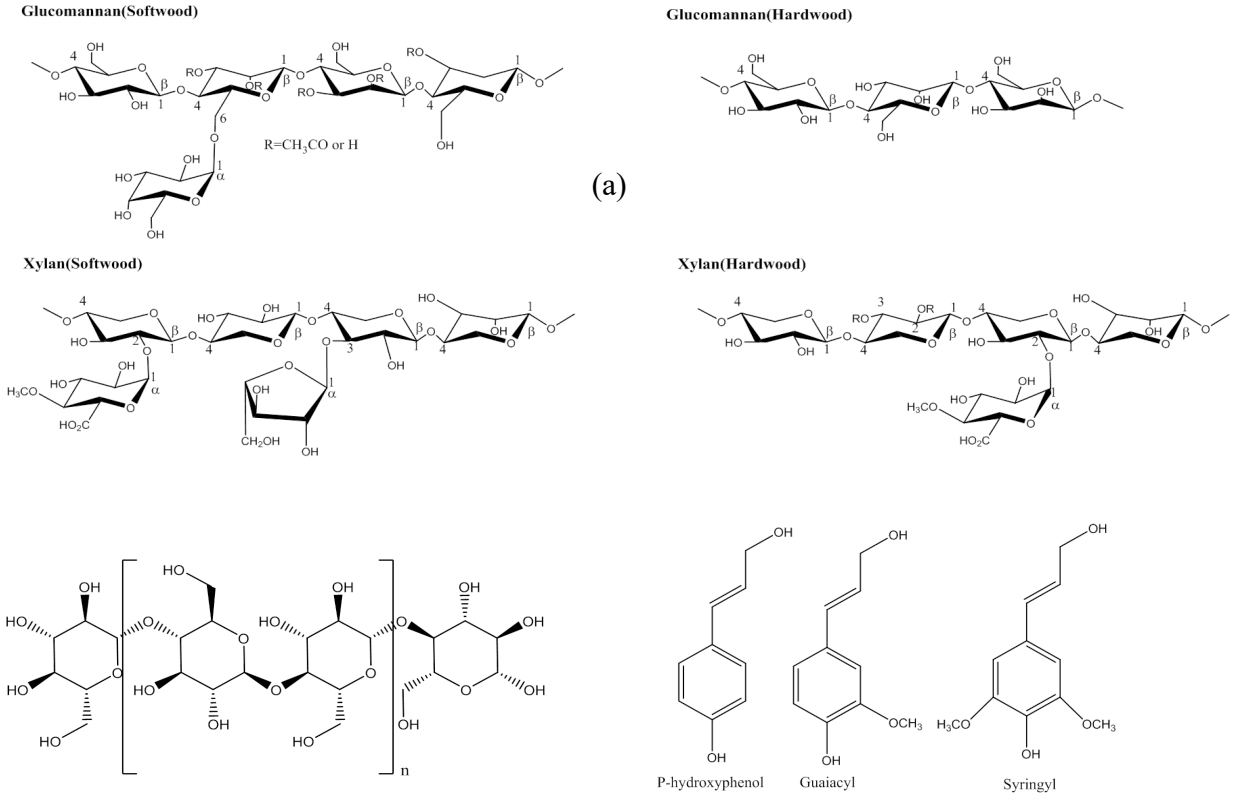

Figure 1.1.1 Schematic structure of (a) hemicellulose, (b) cellulose and (c) forming units of lignin

Lignin with the general formula $(C_9H_{10}O_3\ (OCH_3)_{0.9–1.7})_c$, is an aromatic polymer formed from phenylpropanoid precursors. Syringyl, guaiacyl and p-hydroxy phenol are the main units of a lignin macromolecule (Fig. 1.1.c). Lignin is covalently connected to hemicellulose, making a very complex 3D structure. The most important roles of lignin in lignocellulose are: protection

against degradation, water transport between plant stems and strengthening the mechanical structure of the plant. The composition of lignocellulosic material varies from one biomass to another. Table 1.1 [17-29] shows the composition of several woody materials used in biofuel production.

Table 1.1 Various lignocellulosic biomass compositions (% dry mass)

Lignocellulosic biomass	Cellulose	Hemicellulose				Lignin		References
Barley hull	33.6	30.5	6.1	0.6	-	19.3	-	[17]
Barley straw	33.8	21.9	-	-	-	13.8	-	[18]
Corn cobs	33.7	31.9	-	-	-	6.1	-	[18]
Corn stover	38.3	21	2.7	2.1	-	17.4	-	[19]
Cotton stalks	14.4	-	14.4		-	21.5	-	[18]
Wheat straw	30.2	18.7	2.8	0.8	-	17	-	[20]
Rice straw	31.1	18.7	3.6	-	-	13.3	-	[21]
Rye straw	30.9	21.5	-	-	-	22.1	3.2	[22]
Oat straw	39.4	27.1	-	-	-	17.5	-	[18]
Soya stalks	34.5	24.8	-	-	-	9.8	-	[18]
Sunflower stalks	42.1	29.7	-	-	-	13.4	-	[18]
Switchgrass	39.5	20.3	2.1	2.6	-	17.8	4	[23]
Sugarcane bagasse	43.1	31.1	-	-	-	11.4	-	[24]
Sweet sorghum bagasse	27.3	13.1	1.4	-	-	14.3	-	[25]
Forage sorghum	35.6	18.4	1.8	-	-	18.2	-	[25]
Olive tree pruning	25	11.1	2.4	1.5	0.8	16.2	2.2	[26]
Spruce	43.8	6.3	-	-	14.5	28.3	0.53	[27]
Oak	45.2	20.3	-	-	4.2	21	3.3	[27]
Radiata Pine (D Don)	45.3	6.4	1.5	2.1	12.2	Total Lignin: 26.8		[28]
10-year-old pine tree	38.2	8.5	-	4.3	11.3	29.5	4.9	[29]

Woody materials have very complex structures with highly crystalline cellulose protected by lignin and hemicellulose. One of the problems of using lignocellulosic materials in the production of biofuel is the low accessibility of cellulose to enzymes and microorganisms, due to the rigid association of cellulose with a three-dimensionally complex lignin biopolymer. These barriers, hemicellulose and lignin, lead to conversion of up to 20 % only of the native cellulose (not rearranged or destroyed by any pre-treatment processes) to sugars [2, 15, 30].

Hence, breaking down lignin and hemicellulose walls before any digestion process to enhance the biofuel production is definitely required[31].

Cellulose is the major carbon source for microorganisms to produce biofuel from woody materials in fermentation and anaerobic digestion processes.

This introduces the pre-treatment process which is nowadays considered a prominent and critical means for economic production of biofuel from lignocellulosic materials (Fig. 1.2).

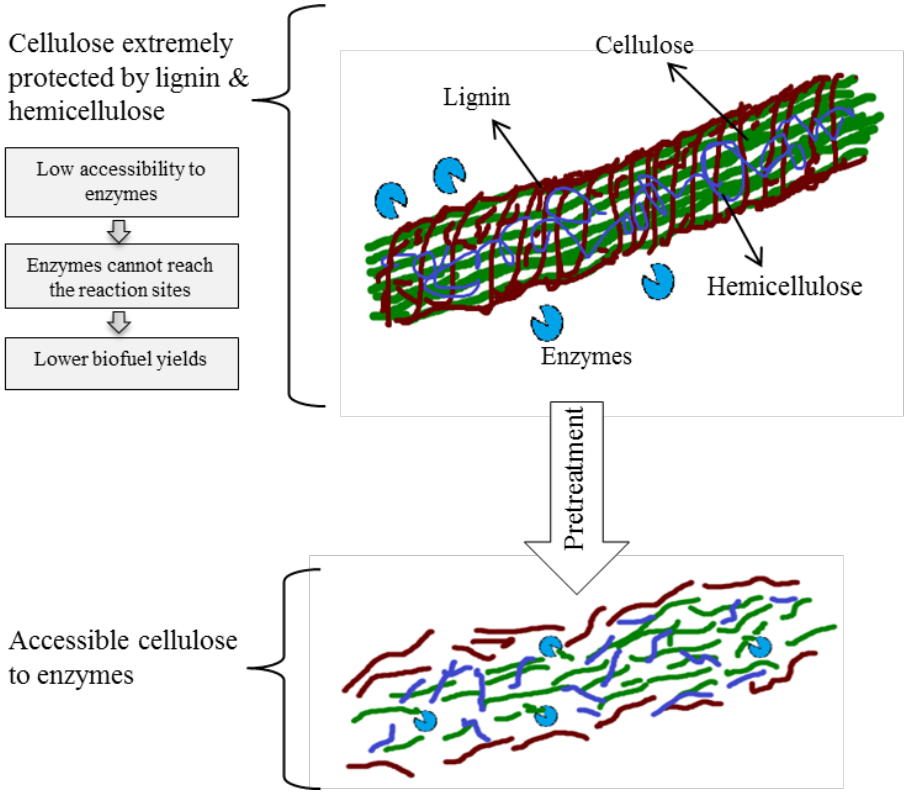

Figure 1.2 Schematic role of pre-treatment

1.3 Pre-treatment methods

1.3.1 Physical pre-treatment

Size reduction, decreasing the degree of polymerisation and crystallinity of lignocellulose are the most common outcomes of mechanical pre-treatment. Also, physical pre-treatments such as milling, grinding and freezing can increase the surface area of pre-treated biomass. Production

of toxic materials during mechanical pre-treatment is generally negligible, which is another important feature of this pre-treatment method.

Ball milling and wet-disc milling are two common physical pre-treatments. The most important disadvantage of milling is high-energy requirement. It has been reported the wet-disc milling can reduce energy requirement comparing to other milling pre-treatment methods, but on the other hand, ball milling has been reported to produce a higher glucose and xylose yield after enzymatic hydrolysis [15]. A study by da Silva et al. [32] compared wet-disc milling and ball milling in the pre-treatment of sugarcane bagasse. Glucose yield and xylose yield for ball milling were 79 % and 72 % respectively while for wet-disc milling the yields were 49 % and 37 % for glucose and xylose respectively [32].

Extrusion is another common physical method. During extrusion, woody materials face shearing, mixing and heating. This results in size reduction of lignocellulose as well as depolymerisation [33]. Hjorth et al. [34] pre-treated five agricultural biomass types with an extruder to enhance biogas production. Methane yield considerably increased by 18–70 % and 9–28 % for 28 and 90 days of digestion respectively [34]. Extrusion pre-treatment was used to pre-treat rice straw in biogas production and the extruded pre-treated biomass increased the methane yield. Methane yield of extruded biomass was 32 % and 72 % greater than milled and untreated rice straw respectively [33].

Freeze pre-treatment has been recently studied as a new method. The principle behind this method is that during the freeze-thaw process, the bulk hydration layer surrounding macromolecules in wood cell might be disrupted [35]. Formation of crystals during freezing also generates a breaking force. This force breaks down some structure of lignocellulosics [36]. Rice straw was pre-treated by freezing to enhance enzymatic conversion [37]. Enzymatic digestibility of the pre-treated straw increased significantly from 48 % to 84 %. Hydrolysis of pre-treated rice straw with cellulase and xylanase after 48 h yielded 417 g/kg and 139 g/kg of

substrate respectively, while under the same conditions hydrolysis of untreated rice straw with cellulase and xylanase converted 227 g/kg and 94 g/kg of substrate respectively [37]

Microwave pre-treatment has been also used to pre-treat lignocellulosic materials. In microwave pre-treatment, woody materials are irradiated with microwaves for a short time period. The advantages of microwave pre-treatment over conventional heating processes are the short process time and lower energy demand [38]. Microwave pre-treatment is also effective in lignin and hemicellulose removal. Microwave pre-treatment is often used in combination with other pre-treatment methods. In a study completed by Cheng et al. [38], rice straw was treated with microwaves assisted with alkali pre-treatment for hydrogen production [38]. The pre-treated biomass generated 43 % of the theoretical hydrogen yield [38]. In another study, rice straw was solely pre-treated by microwaves [39] . Based on the experimental data, microwave intensity, irradiation time and substrate concentration were reported as the main factors controlling enzymatic yield of pre-treated materials. After 24 min irradiation with microwaves of intensity 680W and with substrate concentration 75 g/L, the total saccharification after an enzymatic hydrolysis by cellulase increased by 30 % [39].

1.3.2 Chemical pre-treatment

In chemical pre-treatment, (which includes acid pre-treatment, alkaline pre-treatment, organic-solvent and ionic-liquid pre-treatment), a chemical agent or solvent is used to break down wood components. Although chemical pre-treatment can be extremely effective at deconstruction, the production of toxic materials, carbohydrate loss and the high cost of the process are common disadvantages.

1.3.2.1 Acid pre-treatment

In acid pre-treatment of lignocellulosics, an acid hydrolyses structural carbohydrates and decomposes lignin. Lignin removal and a high yield of sugars are the most common advantages of using acid as a pre-treatment process. However, the high cost of equipment which will withstand acid corrosion as well as formation of toxic components especially under

concentrated acid, and carbohydrate loss are major disadvantages. Generally, the acid strength has a direct effect on pre-treatment efficiency [40]. Particle size, retention time, acid concentration, liquid to solid ratio and temperature are the most important parameters controlling the acid pre-treatment process [40].

The efficiency of the acid pre-treatment, the combined severity factor (R_0) is described as below. R_0 is an index to estimate the effect of pre-treatment temperature, time and pH on the effectiveness of the process [40]:

$$\log R_0 = \log\left(t \,.\, e^{(T-100)/14.75}\right) - pH \quad (1)$$

Where t is the retention time in minutes and T is the hydrolysis temperature in ºC.

Severity factor is a useful tool for the design of the experiment. It can be possible to use severity factor to minimise the total number of experiments [41, 42]. This function can be used to describe lignin removal from a lignocellulose undergone acid pre-treatment [41]. Sulphuric acid is the most common acid which has been used for lignocellulosic pre-treatment. Rice straw was pre-treated with 1 % (w/w) sulphuric acid over a 1–5 min retention time at 160–180 ºC [43]. Enzymatic hydrolysis of pre-treated rice straw led to the high sugar yield of 83 % [43]. A mixture of acetic acid and sulphuric acid was used to pre-treat sugarcane bagasse [44]. In the results of this pre-treatment, hemicellulose was efficiently removed (up to 90 %), and cellulose loss during pre-treatment was less than 15 %.

1.3.2.2 Alkaline pre-treatment

The main benefit of alkaline pre-treatment is efficient lignin and hemicellulose removal as well as its ability to increase the surface area for the reaction site for further hydrolyses [15]. A long process time and the irrecoverable formation of salt have been reported as the drawbacks of this method [45]. Alkaline solvent causes degradation of ester and glycosidic side chains which leads to structural change and degradation of lignin, cellulose swelling and partial decrystallisation of cellulose [40, 46, 47]. Sodium hydroxide is the major alkaline solvent

which has been used. Temperature, time, liquid-solid ratio and sodium hydroxide concentration are the parameters affecting the treatment [40]. Soybean was treated by sodium hydroxide at room temperature and after enzymatic hydrolysis the glucose yield was 65 % and hemicellulose removal was up to 46 % [48]. Pre-treatment of costal Bermuda grass with sodium hydroxide for 15 min resulted in a total sugar yield of 71 % [49].

1.3.2.3 Organic-solvent pre-treatment

Pre-treatment by organic solvents can efficiently remove lignin and hemicellulose. However, problems with solvent recovery, the high cost (in some cases) as well as flammability and volatility can be considered its drawbacks. Organic solvents can break down internal bonds between lignin and hemicellulose [15, 40]. This means cellulose is more accessible to enzymes and there is more surface area, therefore more reaction sites for enzymes are provided by lignin removal [50]. Temperature, retention time and solvent concentration are some of the parameters affecting the pre-treatment [40]. Organic solvents such as methanol, ethanol, acetone, ethylene and N-methylmorpholine-N-oxide (NMMO or NMO) have been used to pre-treat different lignocellulosic materials [15]. Araque et al. used acetone-water to pre-treat Radiata Pine [28]. The pre-treated material produced at optimised conditions of 195 ºC, 5 min, and pH 2, led to 99 % ethanol yield [28]. Pre-treatment with an organic solvent, N-methylmorpholine-N-oxide (NMMO or NMO), has recently been investigated for ethanol production from sugarcane bagasse, cotton, softwood and hardwood. Teghammar et al. [51] used NMMO to pre-treat spruce for biogas production. Their results showed that the pre-treatment improved methane yields by 400–1200 % from untreated materials which was 47–89 % of the theoretical biogas yield, and solvent recovery was about 98 % [51].

1.3.2.4 Ionic liquid pre-treatment

Acid and alkaline solvents are the first and second generations of solvents for lignocellulosic pre-treatment. The third generation of solvents to pre-treat lignocellulose are ionic liquids (ILs) which have gained more attention recently [52]. ILs are organic salts that are liquids at low

temperatures. ILs can significantly change biomass structure dissolving lignocellulose components and breaking down bonds between cellulose, hemicellulose and lignin in woody materials. One of their disadvantages is that they become more viscous during the process, creating difficulty in use [15]. Another difficulty of ILs is that most are toxic to hydrolytic enzymes [52]. They are also expensive. The most commonly applied ILs for pre-treatment lignocellulose are: 1-butyl-3-methylimidazolium chloride, 1-butyl-3-methylimidazolium hexafluorophosphate, 1-butyl-3-methylimidazolium acetate, 1-benzyl-3-methylimidazolium chloride, 1-butyl-1-methylpyrrolidinium chloride, 1-butyl-3-methylimidazolium methylsulfate, *N*, *N*-dimethylethanolammonium groups, 1-ethyl-3-methylimidazolium groups and 1, 3-dimethylimidazolium groups [52].

In one study switchgrass was treated by 1-ethyl-3-methylimidazolium acetate ([C_2MIM][OAc]) as the ionic liquid [23]. After enzymatic hydrolysis, the pre-treated biomass had a glucan yield of 96 % which was an increase of 17 fold [23]. Destruction of biomass structure is one of the features of ionic liquid pre-treatment. Oil palm fronds were pre-treated by BMIMCI (ionic liquid), and scanning electron microscope (SEM) images show that the pre-treated biomass had significant destruction on its surface structure, which is an accomplishment for enzymatic digestion [53].

1.3.2.5 Ozonolysis

Ozone gas as a powerful oxidant can decompose lignin and hemicellulose. It can also disrupt cellulose in lignocellulosic materials. Good solubility in water and no toxic by-products are the most prominent advantages of ozone gas pre-treatment [15]. Lignin is oxidised into low molecular weight soluble components such as acetic acid and formic acid. The high amount of ozone gas required for the process and the fact that it is an expensive process are the common disadvantages of ozonolysis pre-treatment [54]. Ozone concentration, type of lignocellulose, air/ozone flow rate and moisture content are parameters which affect ozonolysis [40]. Wheat and rye straw were pre-treated with ozone by Cubero et al. [22]. Ozonolysis improved

enzymatic hydrolysis yields of wheat and rye straw up to 89 % and 57 % respectively, while for the untreated wheat and rye straw the enzymatic hydrolysis yields were 29 % and 16 % respectively [22].

1.3.3 Physico-chemical pre-treatment

In physico-chemical pre-treatment, lignin and hemicellulose are removed and cellulose is disrupted by changing the operating conditions (pressure and temperature) in the presence or absence of a chemical. Of the physico-chemical pre-treatments, steam explosion, ammonia fibre explosion, carbon dioxide explosion, and wet oxidation (WO) are common examples.

1.3.3.1 Steam explosion pre-treatment

This method involves the breakdown of molecules by steam and shearing due to abrupt depressurising of a pre-pressurised system. This method consists of two steps. First lignocelluloses are exposed to pressurised steam (20–50 bar, 160–270 ºC) for a few seconds. Then they are suddenly depressurised to atmospheric pressure. It is cost effective (in most cases) [15, 30, 55] and it causes destruction of lignin and hemicellulose. However, partial destruction of cellulose during the process, incomplete degradation of lignin and the production of toxic by-products are disadvantages. Particle size, temperature and retention time are the most important factors which impact on the efficiency of the pre-treatment. Steam explosion associated with sodium hydroxide, and hydrogen peroxide pre-treatment of paper tube residuals were investigated by Teghammar and co-workers [31] for biogas enhancement. They found that steam explosion pre-treatment had the better result and improved the biogas production by 103 % in comparison with untreated paper tubes.

1.3.3.2 Ammonia fibre explosion (AFEX) pre-treatment

This process involves pre-treatment of biomasses with ammonia and, like steam explosion, exposure to high pressure (~2MPa) and moderate temperature (60-120 ºC) followed by a rapid decompression. Lignin removal, no inhibitor production and surface area increase are its benefits. Inefficiency for biomass with high-lignin-content and the need for high pressure are

its disadvantages [15, 30, 55-57]. The most important parameters which affect AFEX are ammonia loading, moisture content, retention time and temperature [15, 47]. Various studies have been conducted to find out the optimal condition for AFEX pre-treatment. In one such study, the optimum conversion of glucan and xylan from *Poplus nigra* was achieved at a biomass to ammonia loading of 2:1, 233 % moisture and temperature of 180 ºC for 30 min of pre-treatment [58]. In another study, Murnen and colleagues used almost the same optimal conditions (biomass to ammonia loading of 2:1, temperature of 160 ºC, 233 % moisture and 5 min of reaction time) to pre-treat *Miscanthus x giganteus* [59]. Under these conditions after enzymatic hydrolysis for 168 h, the conversions of glucan and xylan were about 96 % and 81 % respectively [59].

1.3.3.3 Carbon dioxide explosion pre-treatment

Supercritical carbon dioxide pre-treatment in some ways is similar to steam explosion and AFEX pre-treatment [47]. It requires lower temperatures than steam explosion pre-treatment and it is more cost-effective than AFEX [47, 60]. In addition, it can increase the accessible surface area and produces almost no inhibitory components. However, it cannot greatly change or modify , and similar to the other explosive methods, high pressure is needed [15]. Supercritical CO_2 has a liquid-like density while showing gas properties. It is known that CO_2 forms carbonic acid when dissolved in water which increases hydrolysis rate [15, 45]. Carbon dioxide molecules are comparable in size with water and ammonia molecules. Therefore, they can penetrate pores which are accessible to water and ammonia. This can disrupt cellulose and hemicellulose [46]. There are several key parameters for CO_2 explosion pre-treatment. Temperature, pressure, retention time, moisture content and biomass/CO_2 ratio are the most studied parameters affecting the process [61, 62]. Alfalfa was pre-treated by this method at a pressure of 5.6 MPa, and 75 % of theoretical glucose was converted after 24 h of enzymatic hydrolysis [63]. Kim and co-workers pre-treated aspen wood and southern yellow pine by carbon dioxide explosion [64]. Untreated aspen wood and yellow pine when subjected to

enzymatic hydrolysis released 14 % and 13 % of the theoretical sugar yield respectively. After enzymatic hydrolysis, treated biomass at 73 % moisture, 3100 psi and 165 ºC for 30 min gave a final reducing sugar yield of 85 % and 28 % of the theoretical maximum respectively [64].

1.3.3.4 Wet oxidation pre-treatment

WO, involving oxidation under high temperature (~125–320 ºC) and moderate to high pressure (5–200 bar), is useful for the treatment of hazardous and toxic materials as well as for pre-treatment of lignocellulosic materials [65]. WO has the potential to fractionate woody materials into a solubilised hemicellulose fraction and a solid cellulose-rich fraction with minimum inhibitor formation. The crystalline structure of cellulose is opened up during WO pre-treatment, the hemicellulose is solubilised and the lignin is decomposed to water and carbon dioxide. Although pre-treatment of lignocellulose by WO effectively opens up the structure of these materials and removes lignin, the operating conditions (high temperature and pressure) can lead to a non-cost-effective process for the production of biofuel. Furthermore, during WO pre-treatment as the time and operating conditions increase, unfavourable cellulose loss might happen [24, 66-70]. A generalised and simplified reaction with acetic acid as the rate-limiting intermediate is shown in Fig. 3 introduced by Li et al. [71].

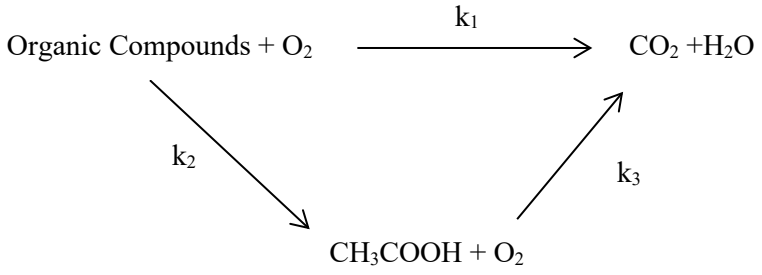

Figure 1.3 Simplified reaction and pathway for WO

In WO the main steps of the process are:

- Mass transfer of oxygen from the gas phase to liquid
- Hydrolysis of solids
- Reactions happening in the liquid phase

Understanding the reaction mechanism is important for the development of kinetic models. In WO, radicals are conducting the reaction [71]. In the absence of initiators, free radicals are formed by the reaction of oxygen with the weakest C-H bond of the organic compound. The wet air oxidation mechanism is described as follows:

$$RH + O_2 \rightarrow R\bullet + HO_2\bullet \quad (2)$$

$$RH + HO_2\bullet \rightarrow R\bullet + H_2O_2 \quad (3)$$

$$R\bullet + O_2 \rightarrow ROO\bullet \quad (4)$$

$$H_2O_2 + M \rightarrow 2HO\bullet \quad (5)$$

Where M can be either a homogenous or heterogeneous species

$$ROO\bullet + RH \rightarrow R\bullet + ROOH \quad (6)$$

WO treatment to remove wood extractives from pulp mill sludge has been investigated by Baroutian et al. [68, 72-74]. WO removed 99.8 % of the measured resin acids at 220 ºC and 20 bar after 20 min. They reported increased acetic acid concentration by approximately 2g/L after 120 min of WO. In another study Klinke et al. [67] used alkaline (sodium carbonate) WO to pre-treat wheat straw. It was reported that after an enzymatic convertibility with a mixture of cellulase and β-glucosidase, alkaline WO increased monosaccharide sugar production and less inhibitory compounds were generated [67]. The lignin removal was about 62 % and cellulose recovery was 96 % [67]. Banerjee et al. [69] treated rice husk by WO and at optimum operating conditions (180 ºC and 5 bar). After 15 min of treatment, the lignin removal reported was 89 % and hemicellulose solubilisation was 70 % [69]. WO was also applied to produce ethanol from woody yard waste [75]. The optimised operating condition was at 185 ºC, 12 bar for 15 min. Lignin removal, cellulose and hemicellulose recovery were 49 %, 91 % and 72 % respectively. Ayeni et al. [76] in another study pre-treated Shea tree and sawdust by alkaline (hydrogen peroxide) WO. At the same operating conditions (185 ºC, 12 bar for 15 min), 17 % of lignin was removed and cellulose and hemicellulose recovery were 52 % and 58 % respectively [76].

1.3.4 Fungal pre-treatment

Physical pre-treatment requires substantial energy and input, chemical pre-treatment involves chemical addition. Fungal pre-treatment has an advantage of low-energy and chemical use. Microorganisms such as fungi (brown rot fungi, white rot fungi and soft rot fungi) are capable of degrading lignocellulose. Fungi are any member of the eukaryotic group (any organism having its fundamental cell unit, nucleus, enclosed within membranes) which are neither animals nor plants. Of these microorganisms, white rot fungi are well known to degrade lignin due to their lignolytic systems.

1.3.4.1 White rot fungi

White rot fungi cause white rot on various woody plants and they are ubiquitous in nature.

1.3.4.1.1 Lignin removal

White rot fungi are well known for lignin degradation. White rot fungi have a unique enzymatic system including a wide range of cellulolytic and ligninolytic enzymes which are essential for degradation of woody materials [77]. The strong degradation ability of white rot fungi comes from intense oxidative activity and low substrate specificity of their ligninolytic enzymes [77, 78] This very important feature makes white rot fungi selectively degrade lignin. Lignin degradation is attributed to three major enzymes, lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase [79]. However, not all white rot fungi secrete all of these enzymes. Although white rot fungi degrade all parts of wood, their preference to selectively degrade lignin is an important feature of their potential in pre-treatment of lignocellulose to produce biofuel. Reid [80] pre-treated aspen wood with *Meulius tremellosus* for 8 weeks, and lignin removal was 52 % based on dry weight. *Phanerochaete chrysosporium* has been widely studied. Its effectiveness on lignin removal of woody materials has been reported in many works. Shi and co-workers investigated the effect of *Phanerochaete chrysosporium* on cotton stalks under two different culture conditions [81]. In Table 1.2 lignin removal for different white rot fungi on different biomass is shown [10, 81-86].

Table 1.2 Effect of various white rot fungi on lignin removal for different biomasses

Strains	Biomass	Time of pre-treatment	Lignin removal (%)	References
Phanerochaete chrysosporium	Cotton stalks	14 days	19 (SMC)[1] 35 (SSC)[2]	[81]
Phanerochaete chrysosporium	Rice straw	7 days 14 days 28 days	17 21 32	[82]
Ganoderma austral	Radiata pine	140 days 360 days	28 35	[83]
Ceriporiopsis subvermispora	Radiata pine	91 days 196 days	31 46	[83]
Ceriporiopsis subvermispora	Corn stover	21 days	32	[84]
Ceriporiopsis subvermispora	Rubberwood	28 days 63 days 91 days	19 37 45	[85]
Trametes versicolor	Rubberwood	28 days 63 days 91 days	13 27 34	[85]
Trametes versicolor	Rice straw	28 days	37	[86]
Pleurotus ostreatus	Rice straw	56 days	41	[86]
Ceriporia lacerata	Japanese red pine	56 day	13	[10]
Stereum hirsutum	Japanese red pine	58 days	15	[10]
Polyporus brumalis	Japanese red pine	56 days	12	[10]

[1] Submerged cultivation
[2] Solid-state cultivation

Lignin degradation of 19 % and 35 % was reported for submerged cultivation and solid-state cultivation respectively. In another study, Bak and co-workers used *Phanerochaete chrysosporium* to produce ethanol from rice straw [82]. After 7, 15, and 30 days, lignin removal from the rice straw was measured at 17 %, 21 %, and 32 % respectively [82].

1.3.4.1.2 Crystallinity modification by white rot fungi

It is believed that the degree of polymerisation and crystallinity are significant parameters which affect the cellulose hydrolysis rate of lignocellulose [55, 87]. However, crystallinity itself might not well explain all of the resistance of woody materials to degradation. Generally, the amorphous fraction of woody materials is related to hemicellulose and lignin, and cellulose is almost considered crystalline [88]. For almost all white rot fungi, it has been reported that at early stages of pre-treatment the crystallinity of treated woody materials increases. Lack of lignin and hemicellulose or partially decreasing lignin and hemicellulose portions (in the amorphous part) during fungal pre-treatment may lead to an increase in the crystallinity of treated materials [89]. In work done by Nazarpour et al., rubberwood was biologically pre-treated by two white rot fungi, *Ceriporiopsis subvermispora* and *Trametes versicolor* between 30 to 90 days [85]. Crystallinity increased for both strains during 30 to 60 days of pre-treatment. *Ceriporiopsis subvermispora* increased the crystallinity of rubberwood with an increasing trend from 43 % to 67 % after 90 days of pre-treatment. Although the final crystallinity of the pre-treated biomass by *Trametes versicolor* was higher than untreated materials, *Trametes versicolor* decreased the crystallinity of rubberwood from 61 % (60 days) to 51 % for 90 days of pre-treatment [85]. Table 1.3. shows the crystallinity change of different lignocellulose exposed to various white rot fungi [7, 10, 82, 85, 90-95].

In another study by Bak and co-workers the crystallinity of pre-treated rice straw by *Phanerochaete chrysosporium* increased slightly over a 15 day pre-treatment from 54 % to 60 % [82]. Xu and co-workers [90] used *Irpex lacteus* to pre-treat corn stover. The crystallinity of pre-treated corn stover increased from 63 % for untreated to 72 % after 60 days of pre-treatment [90]. Three white rot fungi (*Ceriporia lacerata*, *Stereum hirsutum*, and *Polyporus brumalis*) were used to pre-treat Japanese red pine [10]. The crystallinity of treated biomass in comparison with the untreated one did not change significantly, there was a slight decrease from 68 % to 66 % during 8 weeks of pre-treatment [10].

Table 1.3 Change in crystallinity index for different biomasses pre-treated by various white rot fungi

Strains	Lignocellulose	Pre-treatment Time	Crystallinity Index (%)	References
Ceriporiopsis subvermispora	Rubberwood	Untreated 30 days 60 days 90 days	43.1 52.4 65.8 66.7	[85]
Trametes versicolor	Rubberwood	Untreated 30 days 60 days 90 days	43.1 55.5 61.2 51.7	[85]
Phanerochaete chrysosporium	Rice straw	Untreated 15 days	54.5 60	[82]
Irpex lacteus	Corn stover	Untreated 60 days	63 72	[90]
Ceriporia lacerata	Japanese red pine	Untreated 56days	68 66	[10]
Pycnoporus sangineus	Red maple	Untreated 21 days 42 days 63 days 84 days	40 40 41 42 39	[7]
Irpex lacteus	Red maple	Untreated 21 days 42 days 63 days 84 days	40 40 40 39.2 38	[7]
Trametes hirsuteas	Corn stalk	Untreated 7 days 21 days 28 days	44 47 52 51	[91]
Trametes velutina	Poplar	Untreated 28 days 56 days 84 days 112 days	40.7 41.7 41.3 40.4 40.6	[92]
Phanerochaete sordida *Pycnoporus species* *Schizophyllum commune*	Green hemp	14 days	88 (66)[1] 87 88	[93]
Trametes hirsuteas	Parthenium	7 days	A slight decrease 43.14 to 41.07	[94]
Phanerochaete chrysosporium	Wheat straw	Untreated 7 days	44 40	[95]

[1] Number in parenthesis presents untreated crystallinity

Hastrup et al. [7] used two white rot fungi, *Irpex lacteus* and *Pycnoporus sangineus*, to evaluate crystalline cellulose modification in red maple over 12 weeks. Due to probable simultaneous

degradation of all wood components, there was not any significant alteration in the treated wood.

After 12 weeks of pre-treatment, wood crystallinity changed from 40 % (untreated) to 39 % and 38 % for *Pycnoporus sangineus* and *Irpex lacteus* respectively. However, both strains showed a mild increase in the crystallinity from week 3 to week 9. At week 9 the crystallinity was 42 % and 39 % for *Pycnoporus sangineus* and *Irpex lacteus* respectively [7].

1.3.4.1.3 Application of white rot fungi in biofuel production

Low-energy demand, no toxic by-products, and being sustainable are the important features of fungal pre-treatment [96]. *Phanerochaete chrysosporium*, so far, has been the most efficient white rot fungi in lignin removal from woody materials [15]. In Table 1.4 different white rot fungi have been studied for different lignocelluloses in biofuel production [5, 10, 81, 84-86, 90, 97-101].

Table 1.4 Effect of several white rot fungi on sugar and biofuel yield (based on treated biomass) for different biomasses

Strains	Lignocellulose	Time of pre-treatment	Biofuel yield (%)	Sugar yield (%)	References
Ceriporiopsis subvermispora	Corn stover	18 -35 days	58	57.67	[84] and [97]
Ceriporiopsis subvermispora	Japanese cedar	28–56 days	Up to 35[1]	-	[5]
Ceriporiopsis subvermispora	Rubberwood	90 days	-	28[2]	[85]
Phanerochaete chrysosporium	Beech wood	90 days	-	9.5	[98]
Phanerochaete chrysosporium	Cotton stalks	14 days	Reduced ethanol yield	Reduced glucose yield	[81]
Pycnoporus cinnabarinus	Wheat straw	35 days	-	55	[99]
Pleurotus ostreatus	Rice straw	60 days	-	33[3]	[86]
Pleurotus ostreatus	Rice hull	60 days	-	39	[100]
Trametes versicolor	Rubberwood	90 days	-	16[4]	[85]
Coriolus versicolor	Bamboo residues	-	-	37	[101]
Stereum hirsutum,				21	
Polyporus brumalis	Japanese red pine	56 days	-	15	[10]
Ceriporia lacerata				15	
Irpex lacteus	Corn stover	60 days	-	66	[90]

[1] of the theoretical yield based on holocellulose
[2] increased in sugar yield in comparison with the untreated biomass
[3] based on holocellulose (total sugars)
[4] increased in sugar yield in comparison with the untreated biomass

Wan et al. used *Ceriporiopsis subvermispora* to pre-treat corn stover for ethanol production [84, 97]. Cellulose loss was less than 6 % during 18 days of pre-treatment while lignin removal was about 32 % which indicates the ability of this white fungus to selectively degrade lignin over cellulose and hemicellulose. The pre-treated 5mm corn stover had an overall sugar yield of 58 %, 62 %, and 67 % for 18, 28, and 35 days respectively. The highest ethanol yield for the mentioned biomass was about 58 % for 35 days pre-treated corn stover [84, 97]. Zhang et al. [101] used *Coriolus versicolor* for the pre-treatment of bamboo residues. Total sugar yield after hydrolysis was 37 % [101]. Sawada et al. [98] used a fungal strain (*Phanerochaete chrysosporium* as the fungal pre-treatment) prior to steam explosion pre-treatment of beech wood. After 90 days of fungal pre-treatment, the maximum saccharification of 10 % was at day 28 of pre-treatment [98].

Hatakka compared alkali pre-treatment with fungal pre-treatment using different white rot fungi including *Pleurotus ostreatus, Pleurotus*, *Pycnoporus cinnabarinus* and *Ischnoderma benzoinum* for wheat straw pre-treatment [99].Amongst the studied white rot fungi, *Pycnoporus cinnabarinus* had greater sugar yield and converted 55 % of the pre-treated residue to reducing sugars, while only 12 % of the untreated biomass was converted to reducing sugars. After an alkali pre-treatment, only 41 % of wheat straw was converted to sugars by enzymatic saccharification [99].

Taniguchi et al. compared four strains of fungi (*Phanerochaete chrysosporium, Trametes versicolor, Ceriporiopsis subvermispora,* and *Pleurotus ostreatus*) on rice straw, and *Pleurotus ostreatus* has been reported to yield the least degraded amount of cellulose during the pre-treatment [86]. Amirta et al. used the white rot fungus *Ceriporiopsis subvermispora* to pre-treat Japanese cedar for biogas production and the methane yield was 35 % of the theoretical yield, based on holocellulose content [5].

1.3.4.2 Brown rot fungi

In contrast with the white rot fungi, brown rot fungi massively attack holocellulose (cellulose and hemicellulose) with little modification to lignin. They decompose holocellulose with a sharp decrease in the degree of polymerisation. It is claimed that in the beginning of degradation the enzymes for decaying are not involved [102]. Wood cells are made up of several layers; the most external layer, the primary wall and three secondary layers S_1 (outer secondary), S_2 (middle secondary) and S_3 (inner secondary)[103]. Earlier investigation showed that degradation by brown rot fungi occurred in an inner layer of the wood cell wall (the S_2), and the layer next to hyphae (S_3) remained almost unchanged [104]. Cowling suggested that an agent smaller than enzymes must be involved in wood degradation because of the size of enzymes which are larger than pores in the cell wall [105]. Brown rot fungi are claimed to use hydrogen peroxide (H_2O_2) which is produced during the breakdown of hemicellulose. As hydrogen peroxide is a small molecule, it can rapidly disperse through wood cells causing degradation of wood. As a result of this type of decay, the wood colour is changed to brown. It is hypothesised that destruction of holocellulose is a result of free-radical reactions. The hydroxyl radical is the most important species involved, be obtained through the Fenton reaction[12]:

$$H_2O_2+Fe^{2+}+H^+ \rightarrow H_2O+Fe^{3+}+HO\bullet \quad (7)$$

Brown rot fungi use both enzymatic degradation as well as non-enzymatic degradation [106]. Brown rot fungi produce endoglucanases to cleave the β-l,4-glucosidic linkages, and β-glucosidases to hydrolyse cellobiose or other short oligosaccharides [106]. Brown rot fungi can produce Endo-1-4-β-glucanase [106]. Brown rot fungi also produce the enzymes which are needed for breaking down hemicellulose, endo-xylanases and β-xylosidases. So far, only one brown rot fungus has been found to secrete cellobiohydrolase [106]. Similar to white rot fungi, the brown rot fungus *Coniophora puteana* has been reported to produce cellobiohydrolase [107].

Generally, not all brown rot fungi are able to degrade crystalline cellulose. It seems that brown rot fungi increase wood crystallinity at the beginning of decay, followed by a drop in crystallinity. The reason for the early increase in crystallinity has been explained by researchers as at the early stages of degradation brown rot fungi use the non-crystalline cellulose and hemicellulose, which causes an increase in crystallinity. Wood type (softwood or hardwood) might affect this trend for brown rot fungi [7]. This trend was also reported by Howell and co-workers who used *Gloeophyllum trabeum* to evaluate temporal changes in pine wood [108]. The crystallinity of untreated wood was about 42 % and the crystallinity of treated biomass increased until week 2. The decrease in the crystallinity index started after week 2 and continued to week 12 when the crystallinity of wood was 20 % [108]. Hastrup and co-workers used hardwood (red maple) and the common crystallinity change by brown rot fungi (an increase in crystallinity followed by decrease in crystallinity) was not observed, the total decrease in the crystallinity of wood was 3.5 % [7]. As an explanation, brown rot fungi may be better acclimatised to softwood rather than hardwood; their mechanism to degrade lignocellulose might lead to a faster degradation of non-crystalline cellulose and hemicellulose in softwood rather than hardwood [7].

Amongst brown rot fungi, *Gloeophyllum trabeum* is one of the most studied strains, for biofuel production or other applications. In one study aspen was pre-treated by *Gloeophyllum trabeum* over 16 weeks [13]. The best case was obtained after 2 weeks of pre-treatment, where 72 % of cellulose yielded to glucose which was 51 % of original glucan [13]. In another study corn stover was treated by *Gloeophyllum trabeum* for enzymatic hydrolysis. The pre-treatment resulted in cellulose conversion of up to 60 % based on theoretical yield [109]. Ray and co-workers used two types of brown rot fungi *Coniophora puteana* and *Postia placenta* to pre-treat radiata pine for 35 days. Under mild conditions, brown rot pre-treatment was found to convert up to 70 % of cellulose to glucan [12]. In another interesting study, a brown rot fungus, *Neolentinus lepideus*, was reported to convert 5-carbon sugar xylose in hemicellulose to

ethanol with yields of 0.3, 0.33 and 0.34 g of ethanol per gram of xylose under aerobic, oxygen-limited and anaerobic conditions respectively [110]. In another study, *Gloeophyllum trabeum* was used for saccharification of corn stover [111]. Both suspended culture and solid-state fermentation were applied. Only solid-state culture was reported to be effective, with up to 40 % of lignocellulose was degraded in 9 days of pre-treatment. Both fungus and fibre were subjected to anaerobic fermentation and 11 % of corn stover was converted into reducing sugars. The maximum ethanol yield was 3.3 g of ethanol/100 g fibre [111].

1.3.4.3 Soft rot fungi

Savory [112] initially suggested the name "soft rot fungi" for these kinds of fungi, as the surface of degraded wood by soft rot fungi becomes softer than sound wood, [106]. Since all types of decay result in softening of wood, the name "soft" may not seem appropriate for these kinds of fungi. However, all experts in this field have accepted the term and currently use it [106]. By possessing a full range of cellulolytic enzymes including endo1, 4-glucanase, exo-1, 4-β-glucanase and 1, 4-β-glucosidase, soft rot fungi can completely degrade cellulose [106]. Other researchers have reported the ability of soft rot fungi to produce laccase , which has lignin degrading capability [113].

Generally, soft rot fungi attack wood in two ways, denoted as type-I and -II attack , respectively [114]. In a type-I attack, cavities are formed in the S_2 layers of wood cell walls, while in type-II attack general erosion of wood cell walls occurs in S_3 layer outwards. The S_3 layer of the wood cell wall is highly lignified and due to the enzymatic system provided by soft rot fungi, these strains preferably degrade cellulose and hemicellulose. Morphological studies have shown that there is another type of attack by soft rot fungi, denoted as type III [114]. Type-III attack happens after cavity formation and general degradation by hyphae.

Amongst all soft rot fungi, *Aspergillus niger*, *Penicillium chrysogenum* and *Trichoderma reesei* have been used most for pre-treatment of lignocellulosic materials [115], [116] and [117]. Cheng and Liu [118] used *Trichoderma reesei* to enhance hydrogen production from pre-

treatment of corn stalk. The cumulative hydrogen volume produced from the pre-treated biomass was twice as much as hydrogen produced from raw materials [118].

1.3.5 Combination of fungal and other pre-treatment methods

Although fungal pre-treatment may potentially provide a cost-effective solution for biofuel production, the low rate of hydrolysis and the time-consuming nature of the process have made this method commercially inapplicable. A combination of fungal pre-treatment with other pre-treatment methods has shown potential for significant improvements to overall process productivity.

1.3.5.1 Combined fungal-acid pre-treatment

Table 1.5 shows the effect of combined fungal-acid pre-treatments on biofuel and sugar yields [8, 9, 119-121]. Reduction of the inhibitor toxic by-products and their consequent environmental impact is one of the important benefits of combined acid-fungal pre-treatment. Fungal pre-treatment prior to acid pre-treatment has this ability to decrease the severity of chemical pre-treatment.[119]. Ma and co-workers combined fungi with dilute acid (sulphuric acid) to pre-treat water hyacinth and they declared an improvement in ethanol production from 0.146g/g dry matter to 0.192g/g dry matter [119]. Kuhar and co-workers used different types of fungi to pre-treat wheat straw over 10 days. The results showed that by combining acid and fungal pre-treatment the yield of sugars increased while production of inhibitors significantly decreased [9]. In another study, oil palm empty fruit bunches were pre-treated by a combined phosphoric acid-fungus treatment [8]. The combined pre-treatment increased the ethanol yield more than fungal pre-treatment alone but less than acid pre-treatment alone. Enzymatic hydrolysis of untreated biomass resulted in only 14 % of the theoretical ethanol yield while for fungal and acid pre-treatments alone the percentages of the theoretical ethanol yields were 28 % and 90 % respectively. An ethanol yield of 63 % was obtained from the combined method. The effects of fungal pre-treatment on the acid load and the reduction of inhibitory products were not reported.

Severe acid pre-treatment prior to fungal pre-treatment might negatively affect the combined pre-treatment [4]. As an explanation, severe acid pre-treatment can partially deconstruct cellulose and the other polysaccharides, resulting in more accessible components for fungal growth. During fungal pre-treatment, this may lead to increase cellulose consumption in anabolic processes and a consequent lower sugar yield [122]. Studies on the combination of fungal and acid pre-treatments have focused on the effectiveness of the combined process. The environmental impact, changing energy demands needed for the whole process and inhibitory by-products are issues which have not been considered in detail within the surveyed literature.

Table 1.5 Effects of the combination of fungal pre-treatment prior to acid pre-treatment to improve biofuel/ sugar yields (based on treated biomass)

Strain	Type of acid	Biomass	Operating Conditions	Sugar yield	Biofuel yield	Significance(s)	References
Echinodontium taxodii (WR)	Sulphuric acid (0.25 %)	Water hyacinth	15–60 min (acid, sole & combined), 25–100 ºC 10 days (fungal), 28 ºC	Sole acid: 112.9–322.3 mg/g Combined: 208.8–366.0 mg/g	Sole acid: 0.146 g/g of dry matter Combined: 0.196 g/g of dry matter	- Combined method increased sugar yield 1.13–2.11 times more than acid pre-treatment	[119]
Fungal isolate RCK-1	Sulphuric acid (0.5–4.5 %)	Wheat straw	45–60 min (acid), 121 ºC 10 days (fungal), 37 ºC	Untreated: 30.27 g/L Combined: 40.82 g/L	Untreated: 0.48 g/g Combined: 0.54 g/g	- A decrease in acid load from 3.5 % to 2.5 % - A reduction in inhibitor production from 1.31 g/L to 0.63 g/L	[9]
Pleurotus floridanus (WR)	Phosphoric acid	Oil palm empty fruit bunches	300 min (acid), 50 ºC 28 days (fungal), 31 ºC	-	Untreated: 4.1 g/L Sole fungal: 6.8 g/L Combined: 20.3 g/L Sole acid: 21.8 g/L	- Acid pre-treatment increased ethanol yield from both untreated and fungally treated biomass	[8]
Phanerochaete chrysosporium (WR)	Sulphuric acid (0.1–5 %)	Glycyrrhiza uralensis	30–180 min (acid), 25–100 ºC 21 days (fungal), 28 ºC	Sole acid: 20.98–49.34 mg/g Combined: 50–192.07 mg/g	-	- Sugar yield from the combined method was 1.08–1.71-fold higher than acid pre-treatment alone	[120]
Phanerochaete chrysosporium (WR), Cerioporiopsis pannocinta (BR)	Sulphuric acid (1–4 %)	Energy cane	10 days, (fungal), 20–22 ºC) Two-step acid hydrolysis 24 h at room temperature and 15 min at 121 ºC	-	Untreated: 15 mg/L Sole fungal: 1266 mg/L Combined (2 % acid): 3055 mg/L	- Both brown and white rot fungi caused similar ethanol yields - Combined pre-treatment produced higher ethanol yield in comparison with control but less compared to 3 % acid alone - Ethanol production from combined (1 %, 2 %) acid-fungal pre-treatment is comparable with (3 %) acid pre-treatment alone	[121]

1.3.5.3 Combined alkali-fungal pre-treatment

The common disadvantage for both fungal and alkali pre-treatment is the lengthy time of the process. The combination of fungal and alkali pre-treatment has not been reported as greatly effective in reducing the process time. In general, the long process time needed for a sole fungal pre-treatment can be slightly improved by a further alkali pre-treatment [92, 122-128]. However, Zhong and co-workers[123] reported that an alkali pre-treatment of fungal-pre-treated biomass can shorten the process time to 15 days. While 60 days of a sole fungal pre-treatment is required to reach a satisfactory sugar yield [123]. As with acid pre-*treatment*, a severe alkali pre-treatment prior to fungal pre-treatment can negatively affect the fungal pre-treatment, as severe chemical conditions might degrade cellulose which would be used as fungal substrate, and supress lignin degradation by fungi [4, 122, 125]. Hatakka and co-workers combined fungal pre-treatment using *Pleurotus ostreatus* with an alkali treatment (NaOH) to pre-treat wheat straw [99]. The results showed no significant improvement in the combined pre-treatment [99].

Fungal pre-treatment conducted prior to alkali pre-treatment might enhance cellulose digestibility [126]. It can also reduce the production of inhibitors which are *toxic* for subsequent fermentation [126]. Yu and co-workers used *Irpex lacteus* and sodium hydroxide as a combined pre-treatment. Fungal pre-treatment improved delignification and xylan loss during alkali pre-treatment. Also, biologically pre-treated material which was subjected to alkali pre-treatment showed better glucan digestibility [124]. Table 1.6 shows the effect of combined fungal-alkali pre-treatment on biofuel/sugar yield improvement [92, 122-128].

Table 1.6 Effects of combinations of different alkali pre-treatments and fungal pre-treatments on biofuel/ sugar yield(based on treated biomass)

Strain	Type of alkaline	Biomass	Operating Conditions	Sugar yield	Biofuel yield	Significance(s)	References
Irpex lacteus	Sodium hydroxide (0.25 M)	Cornstalks	30 min ,30 ºC (alkali) 15–60 days, 28 ºC (fungal)	alkaline:425.7 mg/g combined: 458.7 mg/g	-	- long time needed for biological pre-treatment compensated by alkaline pre-treatment - fungal pre-treatment increased the sugar yield in comparison with alkaline pre-treatment alone	[129]
Trametes velutina	Sodium hydroxide (1 % w/v)	Poplar	180 min, 75 ºC (alkali) 28–112 days, 28 ºC (fungal)	fungal: ~12 % of original cellulose combined: 38.8 % of original cellulose	-	- combination of alkaline and fungal pre-treatment reduces the recalcitrance of biomass conversion to sugars	[92]
Pleurotus ostreatus	Sodium hydroxide (2 % w/v)	Wheat straw	10 min, 115 ºC (alkali) 7–35 days, 28 ºC (fungal)	untreated: 12 % (of original straw) fungal: 35 % (of original straw) alkali: 41 % (of original straw) combined: 37 % (of original straw)	-	- combined pre-treatment did not improve sugar yield in comparison with either method alone	[125]
Panus (Lentinus) tigrinus	Ammonia (5 %)	Birch and pine wood	10 min, 165 ºC (alkali) 14 days, 26 ºC (fungal)	-	-	- lignin degradation decreased in the combined process	[122]
Ceriporiopsis subvermispora	Sodium hydroxide	Wheat straw	60 min, 50 ºC (alkali) 7–21 days, 28 ºC (fungal)	untreated: ~36 % fungal: 69 %	untreated: 35 % combined: 62 %	- combination of mild alkali pre-treatment and fungal pre-treatment did not produce inhibitors for the fermentation process	[126]
Gloeophyllum trabeum	Sodium hydroxide (25 % w/w)	Radiata pine	300 min, 180 ºC, (alkali) 28–84 days 27 ºC (fungal)	untreated: 83 % combined: 77 %	untreated: ~160 mL/ kg wood combined: ~90 mL/ kg wood	- combined process did not increase both sugar yield and subsequent ethanol yield	[127]
Echinodontium taxodii	Sodium hydroxide (0.0016 %) and oxygen peroxide (3 %)	Corn straw	1140 min, 25 ºC (alkali) 15 days, 25 ºC (fungal)	alkali:40.3 % combined: 50.7 %	-	- fungal pre-treatment significantly improved enzymatic hydrolysis of alkali pre-treated biomass	[128]

1.3.5.4 Combined explosion-fungal pre-treatment

Steam explosion pre-treatment prior to fungal pre-treatment might decrease the time of pre-treatment needed compared to fungal pre-treatment alone. Taniguchi and co-workers [130] combined steam explosion with four white rot fungi for the pre-treatment of rice straw. They reported that steam explosion reduced the time of fungal pre-treatment from 60 days to 36 days, and this subsequently led to lower cellulose loss compared to fungal pre-treatment alone. However, severe steam explosion pre-treatment can cause condensation of acid-insoluble lignin with other carbohydrates. Formation of these components can mask subsequent enzymatic hydrolysis and fermentation [98]. Subsequent fungal pre-treatment after a steam explosion can also disrupt the network of formed lignin due to steam explosion pre-treatment [4, 130].

In an interesting study, Vaidya and co-workers used one white rot fungi (*Trametes versicolor*) and three brown rot fungi (*Coniophora puteana, Antrodia xantha* and *Oligoporus placenta*) to pre-treat steam exploded wood (SEW) [131]. Enzymatic hydrolysis of SEW and fungally-treated SEW gave 0.5 g glucose/l and 5 glucose/l respectively. This means post-fungal treatment of SEW for both brown and white rot fungi increased the sugar yield 12–25 % compering to sole steam explosion treatment [131].

Sawada and co-workers combined fungal pre-treatment using *Phanerochaete chrysosporium* with steam explosion to pre-treat beech wood. The highest efficiency was obtained after 28 days fungal pre-treatment, followed by steam explosion pre-treatment at 215 ºC for 6.5 min [98]. Pre- or post-fungal pre-treatment can affect sugar and biofuel yields. Steam explosion as the first treatment step of pre-treatment can break down lignin-hemicellulose bonds which may help decrease the time of subsequent fungal pre-treatment [132]. Almost all studies related to combined steam explosion/fungal pre-treatment have reported a significant increase in sugar yield when compared to a sole pre-treatment [98, 130, 132-134].

The combination of AFEX with fungal pre-treatment also showed an improvement in sugar yield from rice straw [135]. Balan et al. [135] pre-treated mushroom spent straw by AFEX. Fungal pre-treatment (*Pleutorus ostreatus*) caused a reduction in operating condition severity for AFEX. The combined pre-treatment converted 15 % more glucan than AFEX pre-treatment alone [135]. Table 1.7 shows the effect of explosion methods in combination with fungi on sugar/biofuel yields of different lignocellulose [98, 130, 132-134].

1.3.5.5 Combined fungal-organic-solvent pre-treatment

A combination of fungal pre-treatment and an organic solvent can reduce the severity of a sole organic-solvent pre-treatment [11]. Fungal pre-treatment prior to organic-solvent pre-treatment can increase solvent accessibility to biomass. This might make this method more cost-effective than a single pre-treatment [11]. Monrroy and co-workers [11] used the combination of fungal pre-treatment with ethanol-water pre-treatment. They found that this combination led to lower process severity with the same ethanol production when compared to a single pre-treatment. The severity factor in organic-solvent processes is described by H factor which relates the time and temperature [11]. The improved results of combined pre-treatment has been confirmed by the study completed by Itoh and co-workers [136], who pre-treated beech wood by combining four strains of fungi with ethanol as the organic solvent. In another study Baba and co-workers used *Ceriporiopsis subvermispora* as the fungal strain, and ethanol as the organic solvent to pre-treat Japanese cedar. The combined process increased sugar yield seven times over that found when using ethanol pre-treatment alone [6]. In another study, radiata pine wood chips were treated by a combined method using ethanol and *Gloeophyllum trabeum* [127]. The combined process produced 210 mL/kg ethanol which is 72 % of the theoretical ethanol yield [127].

Table 1.7 Effects of the combination of explosion methods and fungal pre-treatments to improve biofuel/ sugar yields(based on treated biomass)

Strain	Biomass	Operating Conditions	Sugar yield	Biofuel yield	Significance(s)	References
Pleurotus ostreatus	Rice straw	Steam explosion: 1.5 MPa, 1 min Fungal: 25 ºC, 48–72 days	Fungal treatment for 48 days: ~22 % conversion Steam explosion prior to fungal for 48 days: ~33 % conversion	-	Steam explosion prior to fungal pre-treatment significantly decreased time needed for sole biological pre-treatment	[130]
Phellinus baumii	Corn stalk	Steam explosion: 0.8–1.7 MPa, 1 min Fungal: 28 ºC, 21 days	Sole steam explosion: ~130 g/kg Combined: 313 g/kg	-	Steam explosion prior to fungal pre-treatment can improve fungal degradation of biomass	[132]
Phanerochaete chrysosporium	Beech wood	Steam explosion: 170–230 ºC, 0–10 min Fungal: 37 ºC, 98 days	Steam explosion: 67.5 % (saccharification) Combined (28 days for fungal): 76 % (saccharification)	-	The combined method caused a significant increase in sugar yield	[98]
Trametes versicolor	Wheat straw	Steam explosion: 0.8 MPa Fungal: 30 ºC, 40 days	-	-	Steam explosion increased biodegradation of lignin, sole fungal pre-treatment degraded 31 % of lignin while the combined process degraded 73 % of lignin	[133]
Lentinula edodes	Sawtooth oak (90 %), corn and bran (10 %)	Steam explosion: 1–3 MPa Fungal: 120 days	Sole fungal: 3.2 g/100 g Combined: 5.7 g/ 100g (2 MPa)	Sole fungal: 10 g/ 100g Combined: 15.9 g/ 100g (2 MPa)	Steam explosion increased ethanol and sugars yield of spent shiitake mushroom medium (fungal pre-treated)	[134]

1.3.5.6 Combined hot water extraction-fungal pre-treatment

Wan and Li [137] combined hot water extraction (HWE)and liquid hot water (LHW) pre-treatment to explore their effects on fungal pre-treatment by *Ceriporiopsis subvermispora* for different biomass (corn stover, wheat straw, and soybean straw). HWE had little or no effect on fungal degradation while LHW improved fungal degradation, removing 37 % of lignin.

1.3.5.7 Combined ultrasonic-fungal and hydrogen peroxide-fungal pre-treatment

Ultrasound can open up α-O-4 and β-O-4 linkages in lignin. It can also cause oxidation of hydroxyl groups by the formation of radicals and hydrogen peroxide, which leads to an increase in fibre wall porosity [138]. Ultrasound pre-treatment prior to fungal pre-treatment can improve fungal degradation rates of biomass [4]. Rice hull was pre-treated by a combined fungal/hydrogen peroxide process. The combined pre-treatment resulted in higher lignin removal and a higher sugar yield in comparison with the results from sole pre-treatment [100]. Kadimaliev and co-workers used ultrasound to enhance fungal pre-treatment of beech wood and pine. Ultrasound increased fungal degradation [139]. The significant effect of ultrasound on fungal degradation is reported in a work by Yu and co-workers. They used ultrasound to accelerate lignin removal by fungal strains on rice hull. The result was in agreement with the work of Kadimaliev and co-workers. Also, ultrasound increased hemicellulose and cellulose destruction, resulting in a higher sugar yield [100].

1.4 Conclusions

Being environmentally friendly, having low-energy demands and no harmful impact on the environment are advantages of fungal pre-treatment for biofuel production from lignocelluloses. Selective degradation of lignin is one of the important features of white rot fungi. However, the length of time needed for fungal pre-treatment is the main drawback of using fungi to pre-treat woody materials. Therefore, investing in fungal pre-treatment for biofuel production might not be commercially viable.

A combination of fungal pre-treatment with other chemical/physical pre-treatment methods has shown an improvement in sugar and biofuel yields. Due to the very low amount of energy needed for fungal pre-treatment, a combination of fungal pre-treatment with other methods can provide mild operating conditions e.g. in comparison with the conditions needed for a sole chemical pre-treatment. Subsequently, production of toxic by-products under mild operating conditions might be reduced.

White rot fungi can selectively degrade lignin in general. However, this feature varies for different species even for the same species in different regions. New Zealand local white rot fungi are not different from other white rot fungi in the globe, therefore, choosing the proper strains for selective lignin degradation of radiata pine wood chips is the matter of concern and question. Among various local white rot fungi two white rot fungi, which are possibly capable of selective degradation, are chosen. The white strains living on dead wood in forests are suitable for this purpose. Although selective lignin degradation of chosen white fungal strains had been reported in other studies, selective degradation of lignin in pine wood chips needed to be tested.

Wet oxidation can fractionate organic components of lignocellulosic materials into carbon dioxide and water. However, complete wet oxidation is not suitable for pre-treatment of woody biomass, as cellulose will be totally degraded. Therefore, partial wet oxidation pre-treatment might have the ability to destruct the complex structure of woody biomass.

Wet oxidation is not an energy efficient process. However, combination of fungal pre-treatment with wet oxidation can decrease energy consumption for the whole process.

In this study we assume that the chosen fungal strains are able to selectively degrade lignin in pine radiata wood chips. In addition to that, combining fungal pre-treatment with wet oxidation pre-treatment can reduce the energy consumption required for the latter.

Thesis framework

Chapter 1 reviews all available pre-treatment methods for lignocellulosic materials pre-treatment within literatures. The advantages and disadvantages of all possible pre-treatment methods are briefly introduced. This chapter also highlights fungal pre-treatment method as an environmentally friendly method and its combination with other physical and chemical processes. Based on literature reviewed, a combination of fungal and physico-chemical processes can introduce a cost-effective and mild pre-treatment process for woody biomass pre-treatment.

Chapter 2 presents a sole fungal pre-treatment. The main goal of this project is to assess a combined fungal-WO pre-treatment of radiata pine wood chips. To have a better look through a combined process, there is a need to evaluate individually each process involved in the combined process. New Zealand is one of the countries whose residents seriously care about preserving their environment and local green plantations. As international fungal strains might be harmful to the New Zealand agricultural industry and green plantations, two New Zealand native white rot fungi were chosen. The effect of the New Zealand fugal strains on physical and chemical properties of radiata pine wood chips during the fungal pre-treatment are presented. The biomass needed for a combined fungal-WO is also pre-treated at this stage.

Chapter 3 presents a mild sole wet-oxidative pre-treatment of radiata pine wood chips. To have a better understanding of WO of fungally pre-treated biomass, raw radiata wood chips are pre-treated with a high-pressure reactor, which is pressurised with pure oxygen, at different operating conditions. Both pre-treated biomass and liquid phase undergo different physical and chemical analyses to evaluate the effect of the treatment. A kinetic study of delignification of radiata pine wood chips is also proposed in **Appendix**

Chapter 4 is about wet-oxidative pre-treatment of fungally treated biomass. To find out how effective the fungal pre-treatment is over the WO, in terms of energy consumption reduction, a mild wet-oxidative pre-treatment on fungally pre-treated wood chips is carried out.

Both sole wet-oxidative pre-treated biomass and combined WO/fungally pre-treated biomass are compared. The physical and chemical properties of both groups of biomasses are analysed with different methods. A simple energy analysis is carried out to compare the energy demand for both groups of biomasses.

Chapter 5 summarises the thesis. Future work and possibilities are also discussed in this part of the thesis.

Chapter 2

Pre-treatment of radiata pine using two white rot fungal strains *Stereum hirsutum* and *Trametes versicolor*

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2.1 Introduction

The depletion of fossil fuels and consequent greenhouse gas emissions have led to increased research on finding renewable and sustainable forms of energy. Biofuels such as biomethane and bioethanol have been gaining much interest as a form of renewable energy. In biofuel production form lignocellulosic materials cellulose should be selectively separated from other components. Delignification is a key step not only in wood refinery but has always been a critical stage in biomass pulping industry. Lignin liquor which has been used to generate steam in pulp and paper industry for decades now is gaining much more attention as a renewable biomass for several applications [141]. One of the problems of using lignocellulosic materials for production of biofuel is the low accessibility of cellulose to enzymes and microorganisms. To have an efficient sugar yield and consequent biofuel yield from lignocelluloses, breaking down the lignin and hemicellulose wall is required before any digestion or fermentation process [31]. In other words, to have better conversion of cellulose to fermentable sugars, the specific surface area of cell wall polysaccharides must be increased to accelerate enzymatic hydrolysis. Pre-treatment processes can efficiently increase the porosity of lignocelluloses and partly remove lignin and hemicellulose as the main barrier to cellulose conversion. Fungal pre-treatment does not need any additive chemicals and a high-energy demand. Fungi such as white rot fungi, brown rot fungi, and soft rot fungi target lignocellulose as a carbon source. White rot fungi are well known for selective degradation of lignin [4]. Soft rot fungi can also degrade all components of lignocellulosic materials. In comparison with white rot fungi, brown rot fungi generally attack hemicellulose and cellulose with a little modification to lignin. White rot fungi can secrete cellulolytic and ligninolytic enzymes, including LiP, manganese peroxidase (MnP), and laccase [79]. Therefore, they are capable of degrading all parts of wood, with preference for lignin [4, 5, 98] . Selective degradation of lignin is the main advantage of white rot fungi for pre-treatment of lignocellulosic materials. The most significant drawback of fungal pre-treatment is the

lengthy time required for the process. Combining fungal pre-treatment with other pre-treatment methods might reduce the time necessary for the whole process to operate. It can also introduce cost-effectiveness. Thus, combining fungal pre-treatment with other physical and chemical methods has been recently contemplated. Moreover, fungal pre-treatment of woody materials has a low-energy demand, the combination of fungal pre-treatment (post or prior) with other pre-treatment methods might reduce their disadvantages [11, 81, 131, 136].

For example, acid pre-treatment needs high-cost equipment under corrosive condition, high temperature and pressure (with a correspondingly high-energy demand) and produces toxic by-products. Prior or post-fungal pre-treatment may effectively be combined with another pre-treatment method which could then be performed under milder conditions [119].

Two of the main parameters which can influence hydrolysis rate of woody biomass are the degree of polymerisation and crystallinity index [55, 87]. The digestibility of lignocellulosic biomass may increase with a reduction in crystallinity index. The white rot fungi's enzymatic system secretes enzymes which are required for breaking down crystalline cellulose [89]. The crystalline part of wood consists almost entirely of cellulose (crystalline cellulose), while the amorphous part of lignocelluloses comprises lignin and hemicellulose [88]. Degradation by white rot fungi may increase the crystallinity index of lignocellulosic biomass at the beginning of degradation; at the early stage of degradation, lignin and hemicellulose (the amorphous part) appear to be degraded at a higher rate rather than that of cellulose. As more lignin is degraded, more cellulose is exposed to the fungi, which may result in a decrease in crystallinity [89]. However, crystallinity itself might not well explain the wood resistance to degradation as in some it has been reported that a reduction in crystallinity did not lead to an increase in lignocellulose digestibility [88, 142].

In the present study, the effect of fungal pre-treatment with two native strains, *Stereum hirsutum* and *Trametes versicolor* on *Pinus radiata* (radiata pine) were investigated. New

Zealand's dominant plantation type is radiata pine. Radiata pine is a cellulose-rich woody material; around 46 % of this species is cellulose. In addition, radiata pine is a versatile, fast-growing, medium-density softwood, suitable for a wide range of end uses. Consequently, qualities such as being abundant in New Zealand and not affecting human beings' food-supply chain make this lignocellulosic material a favourable feedstock for biofuel production. Researchers have been always looking for a cost-effective and commercial way to convert soft wood into simple sugars for consequent biofuel production [143]. Although the possible fuel yield from soft wood such as radiata pine is high, the conversion of soft wood to simple sugars is still tricky and needed to be tackled [143]. So far, the big improvement according to SCION researches was 26 % using new enzymes and biological conversion [143]. This means using radiata pine for biofuel production is still ongoing and thanks to possible new technology, radiata pine can be a promising feedstock for biofuel production. The rationale for using these two native fungi come from their features, secreting essential enzymes to oxidise lignin in wood. These two strains were also reported to have the appropriate enzymatic system for lignin degradation. Also, *Trametes versicolor* was reported to produce laccase, which causes lignin degradation in lignocellulosic materials [144].

In an interesting study, nine fungal strains fungi including *Trametes versicolor* were tested for their ability to mineralise pentachlorophenol [144]. Although some international strains might have a better result in lignin removal and pre-treatment of woody materials, they could be harmful to New Zealand forestry and agricultural industries.

Trametes versicolor and *Stereum hirsutum* are varieties of white rot fungi which favourably mineralise the deadwood in the forest which means that they cannot be dangerous for a living green plantation. Therefore, their natural presence as part of forest ecosystem may allow them to be used to provide a mild pre-treatment without adversely impacting the native ecosystem.

Although *Trametes versicolor* and *Stereum hirsutum* have been studied for different woody biomass pre-treatment and applications, very few studies considered selective degradation of these two fungal strains for lignocellulosic biomass especially radiata pine. In addition, the effect of fungal pre-treatment by these two white rot fungi on crystallinity change of radiata pine wood chips has not been reported in any other related studies. The purpose of this work was to test the performance of native breed of these two white rot fungi in wood pre-treatment.

2.2 Materials and methods

2.2.1 Fungal strains and biomass preparation

The two New Zealand native strains, *Stereum hirsutum* and *Trametes versicolor* were supplied by Landcare Research – International Collection of Microorganisms from Plants, Auckland, New Zealand. These strains were grown on potato dextrose agar (PDA) plates at 30 ºC for 1 week.

In this experiment, soft wood of radiata pine was used as a woody biomass. It was cut into wood chips size. Media preparation for fungal pre-treatment was based on the method suggested by Keller and co-workers [145].

2.2.2 Fungal pre-treatment

Pre-treatments were conducted in 250 mL Erlenmeyer flasks. Ten grams of radiata pine wood chips were put into each flask. To bring moisture content up to 70 %, 23 mL of distilled water was added to each flask. All flasks were autoclaved at 121 ºC for 30 min to destroy any unexpected microorganisms. The effect of autoclaving on biomass is much less than steam explosion and other heat pre-treatment methods, however the severity of autoclaving can affect cellulose and hemicellulose solubilisation in wood [81, 98] Four pellets of fully grown mycelia (cultivated on PDA, as previously described) were added to the woody biomass and flasks were incubated at 25 ºC for 3 to 7 weeks in solid-state conditions. Every 2 days the

flasks were shaken gently for a better mixing. For each time of pre-treatment different flasks were used, 12 flasks in total were used for weeks 3, 5 and 7 of degradation including treatment by both fungal strains and replication. All experiments were conducted in duplicate and the values are an average of two value obtained by a 95 % confidence level based on t-test.

2.2.3 Analysis of chemical and physical properties of biomass

Structural carbohydrates and lignin of untreated and fungally treated wood chips were tested using the National Renewable Energy Laboratory (NREL) procedure [146, 147]. This protocol includes two steps of hydrolysis using sulphuric acid. In the first step, sugars and the other acid-soluble components were extracted with 72 % (w/w) sulphuric acid at 30 ºC for 1 h. This step was followed by a diluted sulphuric acid 4 % (w/w) at 121 ºC for another 1 h. Generally pine trees contain high amount of extractive depending on their ages, this varies from ~2 % to ~18 % [148]. According to NREL, the large amount of extractives in wood sample can interrupt lignin determination resulting in an unusual high-lignin-content. The biomass in this study contains around 1.5 % extractives. This amount of extractives caused only 3 % difference in lignin determination between extractive free sample and fresh sample which is negligible. Therefore, the results for structural carbohydrates and lignin content were reported based on extractive free according to NREL procedure. Acid-soluble lignin was determined by UV-Vis spectroscopy at 240 nm. Acid-insoluble lignin was quantified by filtration of the residue based on the NREL method. Wood sugars were quantified by high-performance liquid chromatography (HPLC) equipped with a reflective index detector (RID 10A) and an Agilent column (Hi-Plex Pb, 300 × 7.7 mm). Deionised water was used as the mobile phase at a flow rate of 0.4 mL/min. The column and the detector's working temperature was 60 ºC and the injection volume was 10 µL. All analyses were repeated at least in triplicate.

An X-Ray diffractometer (XRD, Bruker D2 Phaser) was used to measure the crystallinity of both the treated and pre-treated woody biomass. Crystallinity (%) is described as follows [149]:

$$\mathrm{CrI} = \frac{\mathrm{I_{002}} - \mathrm{I_{am}}}{\mathrm{I_{002}}} \times 100 \quad (1)$$

Where:

$\mathrm{I_{002}}$: The maximum intensity at $2\theta = 22.8°$

$\mathrm{I_{am}}$: The minimum intensity at $2\theta = 18.4°$

Structural changes of the pre-treated radiata pine chips were appraised by a SEM.

Selective lignin degradation (selectivity value) is one of white rot fungi's most important features. This is defined as the portion of lignin loss to cellulose loss [99, 150].

$$Selectivity\ value = \frac{Lignin\ loss\ at\ the\ time\ of\ pretreatment}{Cellulose\ loss\ at\ the\ time\ of\ preteatment} \quad (2)$$

2.3 Results and discussion

2.3.1 Microstructure analysis

The morphology of the untreated radiata pine chips and fungally pre-treated chips at 3, 5 and 7 weeks of degradation is shown in Fig. 2.1. The untreated biomass had a compact structure with almost not broken surface structure (Fig. 2.1a).

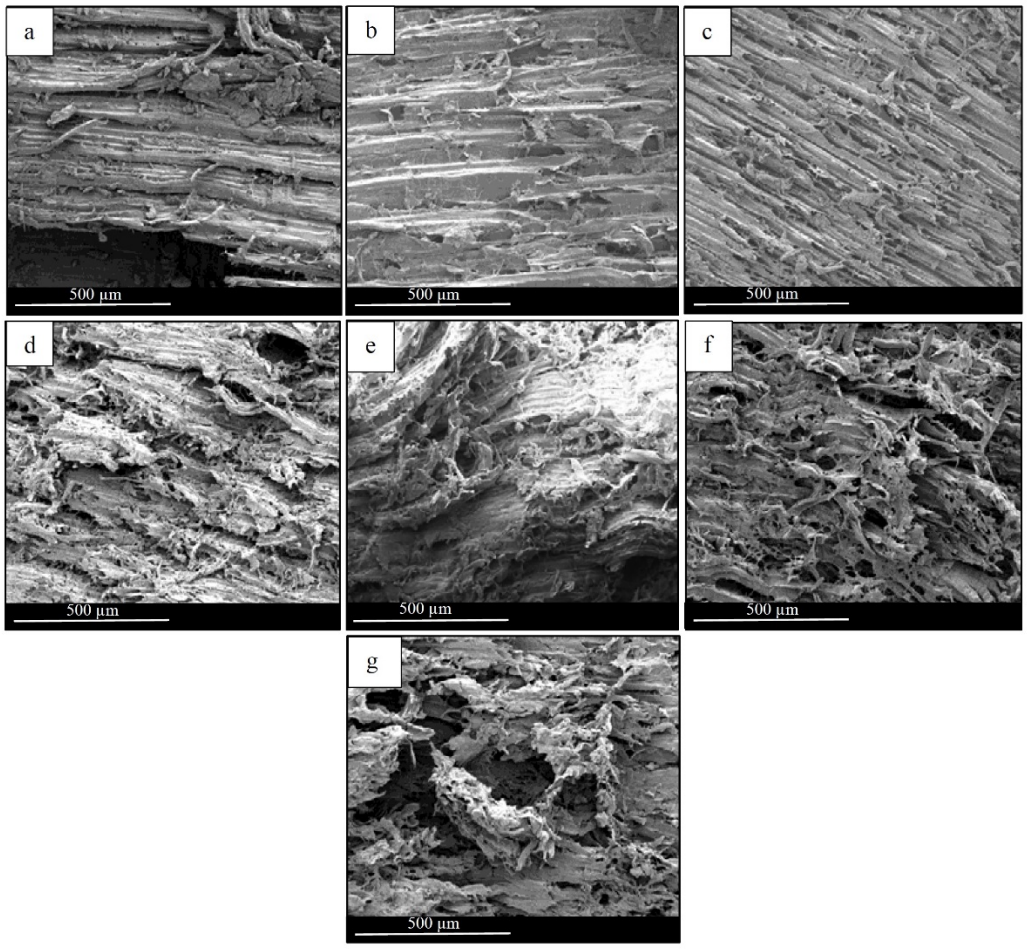

Figure 2.1 SEM images of untreated and treated radiata pine chips

(a) untreated radiata pine chips (b) 3-week degradation by *Trametes versicolor* (c) 3-week degradation by *Stereum hirsutum* (d) 5-week degradation by *Trametes versicolor* (e) 5-week degradation by *Stereum hirsutum* (f) 7-week degradation by *Trametes versicolor* (g) 7-week degradation by *Stereum hirsutum*. The magnitude of all images is 250X.

As pre-treatment time increased, the surface of treated samples became rough and more cracks and pores appeared on the surface of pre-treated biomass for both strains (Fig. 2.1b–2.1g). This is also important in cellulose digestibility by hydrolysing enzymes [151]. SEM images show that in the week 3 of pre-treatment for both strains, the biomass still has an almost intact surface structure, but in comparison with the untreated biomass, the surface porosity has slightly increased. While at weeks 5 and 7 of degradation, the treated biomass contained more porous surface structure. This can be related both to more lignin removal and lignocellulose network breaking down caused by the fungal strains [85].

2.3.2 Effect of fungal pre-treatment on chemical and physical properties

The effects of pre-treatment with the two fungal strains are shown in Table 2.1.

Table 2.1 Component losses during pre-treatment by both strains

Strain		Total cellulose loss (%)	Total hemicellulose loss (%)	Total lignin loss (%)	Selectivity value
Trametes versicolor	Week 3	5 ±0.29	5 ±0.80	16 ±1.55	3.2 ± 0.52
	Week 5	7 ±1.35	20 ±2.75	22 ±1.10	3.1 ±0.73
	Week 7	9 ±1.68	23 ±0.33	22 ±1.25	2.5 ±0.35
Stereum hirsutum	Week 3	9 ±0.02	9 ±2.71	16 ±5.01	1.7 ± 0.54
	Week 5	13 ±1.00	14 ±1.28	19 ±3.80	1.4 ±0.17
	Week 7	13 ±0.80	14 ±0.48	22 ±3.43	1.6 ±0.41

As illustrated in Fig. 2.2(a), the decrease in lignin between untreated and treated materials is significant. Total lignin in untreated wood decreased from 34 % to 25 % in 5-week degraded wood using both *Trametes versicolor* and *Stereum hirsutum*. Both strains significantly decreased total lignin up to week 5 of pre-treatment ($p < 5$ %). However, total lignin was not significantly decreased ($p > 5$ %) from week 5 to week 7 of degradation for either strain ($p > 5$ %).

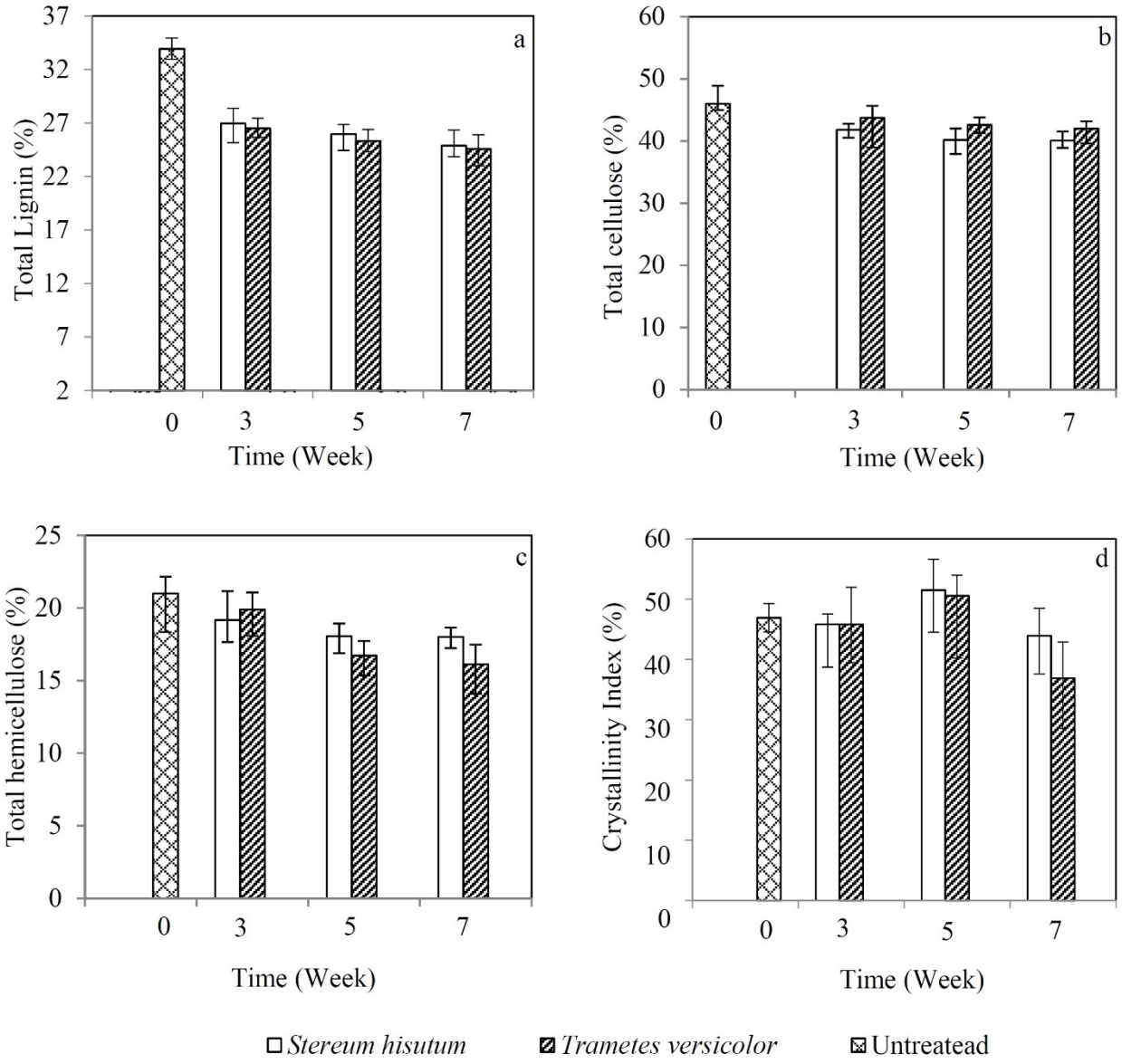

Figure 2.2 Total lignin, cellulose, hemicellulose and crystallinity index (%) of radiata pine treated by *Trametes versicolor* and *Stereum hirsutum*

After 3 weeks of degradation, *Trametes versicolor* significantly decreased cellulose from 46 % to 43 % ($p < 5$ %). Comparing the 5-week and 7-week degraded wood chips by *Trametes versicolor* to untreated biomass, cellulose decreased from 46 % to 42 % and 41 % respectively ($p < 5$ %).

The results also indicate that cellulose did not change significantly from week 3 of degradation to week 5 and from week 5 to week 7 of degradation ($p > 5$ %). The results for *Stereum hirsutum* showed that cellulose significantly degraded from 46 % (untreated) to 41

% and 40 % for 3-week degraded and 5,7-week degraded wood chips respectively ($p < 5$ %). However, there was not any significant cellulose change between 3-week degraded, 5-week degraded and 7-week degraded biomass ($p > 5$ %).

From untreated to week 3 of degradation, there was not any significant decrease in hemicellulose for both fugal strains ($p > 5$ %). While from untreated to week 5 and 7 of degradation for both strains there was a significant decrease from 22 % (untreated) to 18 % (week 5 and 7) for *Stereum hirsutum* and from 22 % (untreated) to 17 % and 16 % (week 5 and 7) for *Trametes versicolor*. Also, hemicellulose did not significantly decrease for both strains from week 5 to week 7 of degradation ($p > 5$ %). This indicates the preference of this strain for lignin degradation at the early stages of degradation. Over time, *Trametes versicolor* degraded more cellulose than lignin. The other strain, *Stereum hirsutum*, at weeks 3, 5 and 7 of pre-treatment, had selective lignin degradation ratios of 1.7, 1.4 and 1.6, respectively. The results for selective lignin degradation showed that *Trametes versicolor* degrades radiata pine selectively at the early stages of degradation and became non-selective as pre-treatment time increased. *Stereum hirsutum* showed a low selectivity value, which means cellulose loss might be higher than lignin loss. Regarding selective lignin degradation, therefore, *Trametes versicolor* provides a better pre-treatment at the beginning of the treatment compared to *Stereum hirsutum*.

2.3.3 Crystallinity index

The crystallinity index for untreated pine chips was 47 %. After 5 weeks of degradation, the crystallinity index did not significantly increase for both *Trametes versicolor* and *Stereum hirsutum*.

At week 7 of degradation, the crystallinity index decreased from 47 % (untreated) to 44 % and 37 % for *Trametes versicolor* and *Stereum hirsutum*, respectively ($p < 5$ %). Analysis of the results showed that crystallinity changes for both strains were insignificant from 3-week

degraded biomass to week 5 of degradation ($p > 5$ %). From week 5 of pre-treatment to week 7 of degradation, there was a statistically significant change in crystallinity for both strains ($p < 5$ %, $p = 1$ %). For both studied strains, the crystallinity index and lignin removal results indicate that the majority of lignin degradation occurred at the beginning of the pre-treatment. The crystallinity data for untreated and fungally treated pine chips are shown in Fig. 2.1(d).

The results for crystallinity index, lignin loss, and cellulose loss indicate that the studied strains were degrading lignin the most at the earlier stage of pre-treatment. At week 7, a decrease in crystallinity index shows that after 7 weeks of degradation, more cellulose may be exposed to the fungal strains. While the degradation rate of the crystalline part (cellulose) increases against the degradation rate of the amorphous part (lignin and hemicellulose), the crystallinity index decreases. If the rate of lignin degradation is higher than that of other parts of the biomass, the crystallinity index is increasing through the pre-treatment and vice versa. This increasing trend is expected since the degradation rate of the crystalline part is less than amorphous part (including lignin)[85].

This result for the crystallinity index is in agreement with literature [85]. In their research [85] they used different types of white rot fungi (including *Trametes versicolor*) to pre-treat rubberwood. The crystallinity index for untreated biomass in their study was 43 %. After 30 and 60 days of pre-treatment, the crystallinity index had respectively increased to 55.5 % and 61 %. A decreasing trend was observed after day 60; crystallinity decreased to 51.7 % after day 90 of pre-treatment.

2.4 Conclusions

Trametes versicolor showed a better result in selective lignin degradation at the early stages of degradation compared to *Stereum hirsutum*. With almost the same lignin removal for both fungal strains, *Stereum hirsutum* caused more cellulose loss during pre-treatment. XRD analysis showed that both strains decreased the crystallinity index after 7 weeks of

degradation. Because of the long-time requirement for fungal pre-treatment, the combination of fungal pre-treatment with other pre-treatment methods may be beneficial. The information provided in this study may help in further developing this energy-saving and sustainable pre-treatment method for biofuel production.

Chapter 3

Hydrothermal pre-treatment of radiata pine wood chips under a mild oxidative condition

3.1 Introduction

For decades fossil fuels have been the main sources of energy, as they still are. Concerns over greenhouse gas mitigation and the consequence of which, environmental effects such as global warming, have led to more investigation of other sustainable and renewable sources of energy. Recently, biofuel production from lignocellulosic materials has gained much more attention. Lignocellulosic biomasses are abundant all around the globe and might be considered promising raw materials for biofuel production. This can provide the potential to produce a low-cost biofuel from such materials [24, 152, 153]. However, the low accessibility of hydrolysing enzymes to cellulose due to very rigid association with lignin and hemicellulose presents an important challenge to produce biofuel from lignocellulosic materials. As its obvious consequence, only a little portion of the accessible cellulose might be converted to biofuel. In other words, without any destruction and rearrangement of native cellulose, conversion of cellulose to biofuel from lignocellulose-based materials would be minor. This makes the role of a pre-treatment process more significant.

Pre-treatment can increase the surface area of cell walls leading to enzymatic hydrolysis acceleration [45]. During the pre-treatment processes lignin and hemicellulose can be partially removed, which is important to maximise the conversion of cellulose to biofuel [31, 45]. There are different pre-treatment methods, mainly divided into physical, chemical, physico-chemical and biological techniques. In physical pre-treatment, an external mechanical force causes size reduction of woody materials leading to an increase of surface area with almost no production of toxic by-products. Physical pre-treatment including milling also has a high-energy demand, which is considered to be its disadvantage [1, 15, 32]. In chemical pre-treatment, a chemical agent or solvent, whether acidic, basic, organic or ionic liquid, is used to open up the complex structure of lignocelluloses. Chemical pre-treatment processes are generally effective for lignin removal and deconstruction of lignocelluloses.

However, production of toxic by-products and carbohydrate loss can be considered cons [15, 22, 23, 28, 40-47, 49-54, 154]. Low-energy demand and no toxic by-products are two of the important features of biological pre-treatment. Pre-treatment with white rot fungi can provide a unique potential to pre-treat lignocellulose biomasses due to selective degradation of lignin by these kinds of fungi. However, the long time for the pre-treatment and low-hydrolysis rate are some of the issues that still need to be tackled for commercial interests. Steam explosion, AFEX, carbon dioxide explosion and WO pre-treatments are physio-chemical pre-treatment methods. In these processes, changing the operating condition in the presence or absence of a chemical can result in disrupting of the complex structure of lignocelluloses [15, 24, 30, 31, 45, 47, 55-57, 59, 60, 65-70, 72-74]. It can effectively remove lignin and increase the biofuel conversion. However, the partial cellulose removal and production of toxic by-products are its drawbacks.

WO is a physico-chemical pre-treatment carried out under a temperature between (120–320 ^{o}C) and pressure (5–200 bar). In WO, the presence of radicals conducts the reaction [71]. In the absence of initiators, free radicals are formed by reaction of oxygen with the weakest C-H bond of the organic compound. The wet air oxidation mechanism can be described as follows:

$$RH + O_2 \rightarrow R\bullet + HO_2\bullet \quad (1)$$

$$RH + HO_2\bullet \rightarrow R\bullet + H_2O_2 \quad (2)$$

$$R\bullet + O_2 \rightarrow ROO\bullet \quad (3)$$

$$H_2O_2 + M \rightarrow 2HO\bullet \quad (4)$$

M can be either a homogenous or heterogeneous species

$$ROO\bullet + RH \rightarrow R\bullet + ROOH \quad (5)$$

A generalised and simplified reaction with acetic acid as the rate-limiting intermediate is shown in Fig. 3.1 introduced by Li et al. [71]

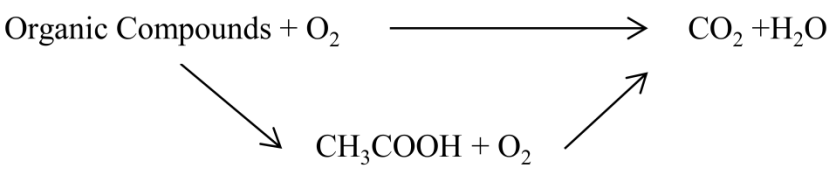

Figure 3.1 Simplified reaction and pathway for wet oxidation

WO has this potential to solubilise and fractionate insoluble content to carbon dioxide and water [67, 69, 155]. This feature is important for treatment of viscous slurries [68]. In addition, WO has the potential to pre-treat lignocelluloses into a solubilised fraction and a cellulose-rich fraction [152, 156-159]. Although WO may have a higher capital cost than the other pre-treatment methods, the operating cost can be comparable with other pre-treatment methods [69]. The operating costs include power to compress the oxygen and fuel costs for increasing the enthalpy of incoming stream to the operating temperature [160, 161].Table 3.1 shows a number of WO-related studies for pre-treatment of woody materials. This process can provide a unique potential to pre-treat woody materials for an enhanced biofuel production.

Radiata pine is the dominant plantation timber in New Zealand. This softwood contains around 40–45 % cellulose associated with 30–35 % lignin[14, 28, 162-165]. Its high level of cellulose makes this lignocellulose a promising biomass for biofuel production. However, its high lignin portion is also an important barrier to using this kind of woody material in the biofuel industry.

As mentioned earlier, WO has been applied to different biomass with a range of acidic or alkaline pH [24, 67, 155, 156, 166, 167]. As far as the literature reviewed, few studies have focused on oxidative hydrothermal pre-treatment of radiata pine. In addition to that, the effect of an initial pH on WO pre-treatment of radiata pine has not been reported.

Table 3.1 Pre-treatment of different lignocelluloses by wet oxidation

Lignocellulose	Operating Condition	Companion of other chemical(s)	Lignin removal (%)	Hemicellulose removal	Enzymatic Convertibility (g/kg) /Sugar yield	Biofuel yield	Ref
Sugar bagasse	195 ºC, 13 bar, 10 min	2 g of Na_2CO_3	-	-	Untreated: 152 Treated: 670	-	[152]
Rice hulls	195 ºC, 13 bar, 10 min	2 g of Na_2CO_3	-	-	Untreated: 73 Treated: 392		[152]
Peanut shells	195 ºC, 13 bar, 10 min	2 g of Na_2CO_3	-	-	Untreated: 104 Treated: 171		[152]
Cassava stalks	195 ºC, 13 bar, 10 min	2 g of Na_2CO_3	-	-	Untreated: 103 Treated: 432		[152]
Corn Stover	195 ºC, 12 bar, 15 min	2 g/L Na_2CO_3	60	80	-	~87 % of theoretical	[157]
Corn Stover	195 ºC, 12 bar, 15 min	0.5 mL/L H_2SO_4 96 %	45	96	-	~78 % of theoretical	[157]
Rice husk	185 ºC, 5 bar, 15 min	-	89	69	-	-	[69]
Mixtures of clover and ryegrass	195 ºC, 12 bar, 10 min	-	69	-	-	93 %	[159]

Furthermore, there is no report of a mild WO pre-treatment of such material. In this study, the effect of WO pre-treatment on radiata pine wood chips under different operating conditions, temperature: 150–190 ºC and pH of 5, 7 and 11 at a constant oxygen pressure of 20 bar was studied. The effect of reaction time under a neutral pH condition on the formation of volatile fatty acids was also studied. The aim of this work was to study the effect of pre-treatment on physical and chemical properties, crystallinity change, and lignin and hemicellulose removal.

3.2 Materials and methods

3.2.1 Raw material

Radiata pine, as lignocellulosic material prepared locally, was cut into chip size. The wood chips were oven dried at 105 ºC for 6-8 h. Some untreated wood chips were taken for related analyses. The moisture content of feedstock was around 5%.

3.2.2 Wet oxidation pre-treatment

WO was carried out in a 1L Parr Reactor (a 4540 high-pressure reactor equipped with a 4848 controller; Parr Instrument Company, US) with a maximum working volume of 600 ml. A schematic diagram of the reactor is shown in Fig. 3.2. Wood chips (10 g) were well mixed with 300 mL of deionised water in the reactor. To provide an initial pH 5–11, both formic acid ($C_2H_2O_2$) and sodium carbonate (Na_2CO_3) were used individually.

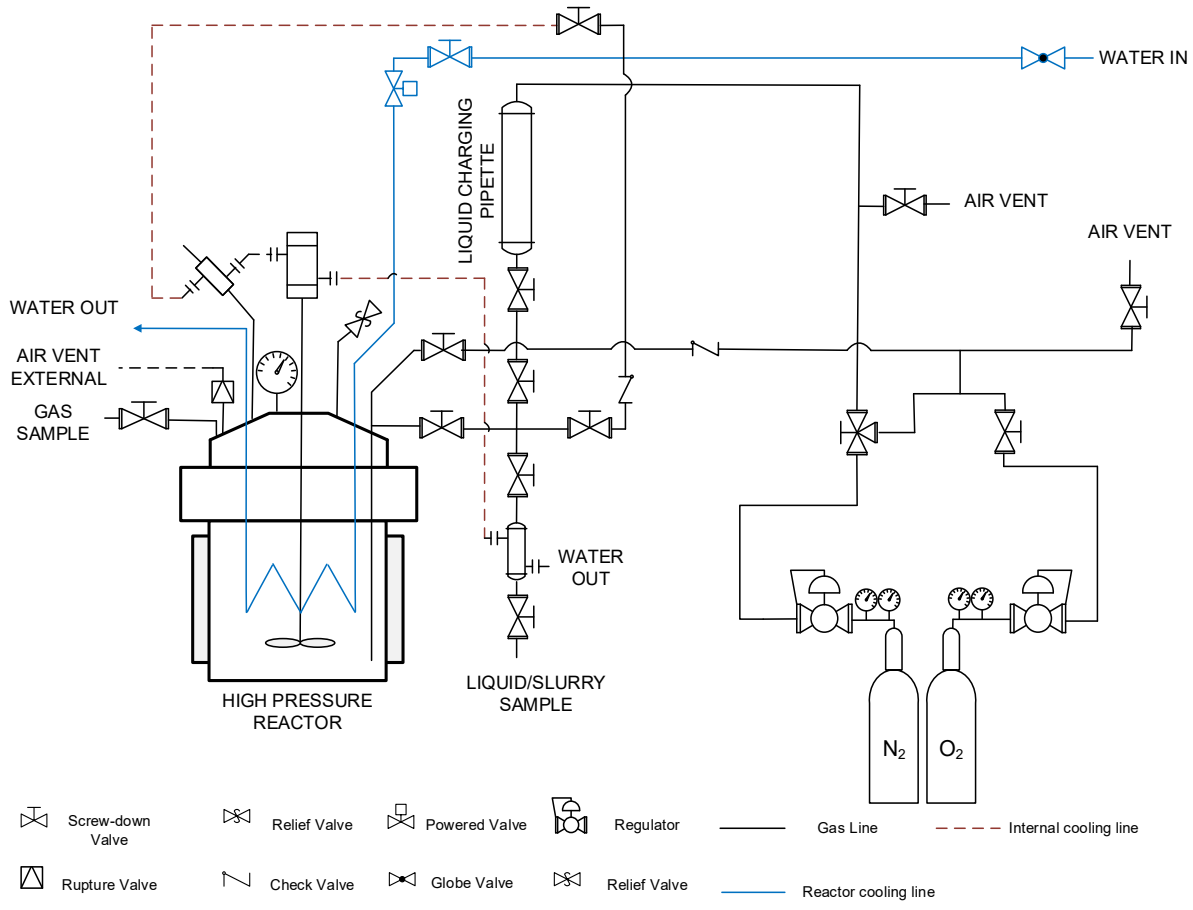

Figure 3.2. Schematic diagram of the processing for wet oxidation of radiata pine chips

Suspended materials were added to the reactor and sealed completely to avoid any leakage.

For each run, the reactor was pressurised at 20 bar with pure oxygen. This provided an approximate stoichiometric oxygen requirement. The reactor was then heated to between 150–190 ºC. The reaction was then carried out for 5–10 min. The stirring speed was 400 rpm. The reactor was immediately cooled to room temperature after that, the solid phase was separated from the liquid phase by vacuum filtration. Both solid and liquid phases were collected for further analyses.

3.3 Analysis

Both untreated and wet-oxidative treated materials were subjected to a two-step acid hydrolysis to find out lignin and structural carbohydrates contents based on NREL procedure [146]. This protocol includes two steps of hydrolysis using sulphuric acid. At the first step, sugars and the other acid-soluble components were extracted by 72 % (w/w) sulphuric acid at 30 ºC for 1 h. This step was followed by a diluted sulphuric acid 4 % (w/w) at 121 ºC for another 1 h. Acid-soluble lignin was determined by UV-Vis spectroscopy (Shimadzu-UV-2550, Japan) at 240 nm. Acid-insoluble lignin was quantified by filtration of the residue base on NREL method. Wood sugars were quantified by HPLC (Shimadzu-20A, Japan) equipped with a reflective index detector (RID 10A, Shimadzu, Japan) and an Agilent column (Hi-Plex Pb, 300 × 7.7 mm). Deionised water was used as the mobile phase at a flow rate of 0.4 mL/min. The column and the detector's working temperature were 60 ºC and injection volume was 10 µL. All analyses were repeated in triplicate, at least.

An XRD (Bruker D2 Phaser, Germany) was used to measure the crystallinity index of both treated and pre-treated woody biomass. Crystallinity index (%) is described as [149]:

$$\mathrm{CrI} = \frac{I_{002} - I_{am}}{I_{002}} \times 100 \quad (6)$$

Where:

I_{002}: The maximum intensity at $2\theta = 22.8°$

I_{am}: The minimum intensity at $2\theta = 18.4°$

Selective lignin removal/partial removal (selectivity value), which is the portion of lignin loss to cellulose loss, can be described as follows:

$$\text{Selectivity value} = \frac{\text{Lignin loss at the time of pre-treatment}}{\text{Cellulose loss at the time of pre-teatment}} \quad (7)$$

This parameter can be used to optimise the pre-treatment's operating conditions which possibly leads to minimum loss of cellulose with a higher portion of lignin loss.

To determine the quantity of all formed volatile fatty acids during WO in liquid phase, a gas chromatographic analysis was carried out. –A Shimadzu GC2010 with an AOC20i autosampler equipped with a flame ionisation detector (FID) and an Agilent DB-FFAP column (30 m × 0.53mm × 0.5 µm) was used. Working conditions were as follows: injection volume 2 µL, split temperature of 170 ºC, injection mood was splitless with the pressure of 24.9 kPa and the total flow as 5 mL/min. The column temperature was 2 min at 40 ºC and with the ramp of 10 ºC/min to 180 ºC held for 24 min (total time was 40 min), column flow was 5 mL/min. FID temperature was 190 ºC with hydrogen flow rate of 40 mL/min, air flow rate 400 ml/min and helium as the backup gas with the flow rate of 30 mL/min.

3.4 Results and discussions

3.4.1 Effect of pre-treatment on the formation of volatile fatty acids

Fig. 3.3 presents the formation of acetic acid, propionic acid and butyric acid, which are the three main volatile fatty acids formed in WO process, under neutral pH conditions and different reaction times. Both reaction time and temperature play important roles in volatile acid concentration. Comparing the results, temperature has a more significant impact on acid

concentrations. As shown in the graph, at low temperature (150 °C), and the early stages of reaction (before 10 min) there is no significant change in concentration of all presented volatile acids (p-value>5%). As the time of reaction increases, from 10 min on, the concentration of acetic acid starts increasing significantly (p-value<5 %) compared to 2 and 5 min of reaction. The most significant increase in acetic acid concentration happened at 40 min for all tested temperatures (p-value<<5 %). However, at 190 °C there is no significant increase in acid concentration from 2 to 10 min and significant change starts from 20 min, compared to 2 min of reaction. This also shows the importance of reaction temperature as, at higher temperature, more biomass is converted to acetic acid over the time of reaction than the other by-products at the early stages of reaction. Generally, according to acetic acid concentration, production of acetic acid increases as both time and the temperature of reaction increase. Acetic acid is more resilient, compared to other volatile fatty acids, to converting to carbon dioxide and water at the milder temperature of WO. Acetic acid is also known as the most refractory pollutant and it is difficult to convert into the other components [168-171]. This is why, as time and temperature increase, the concentration of acetic acid increases. Formation of propionic acid during the WO had some similarities to the acetic acid formation pattern. At 150 °C, after 2 min of reaction there was not a significant increase in propionic acid concentration. In other words, at 150 °C, concentration of propionic acid was constant after 2 min of reaction. At 170 °C, the significant increase in propionic acid concentration happened after 10 min of reaction compared to the concentration at 2 min. The result for the propionic acid also indicates that as the temperature of the reaction increases, more biomass can be converted into propionic acid. For example, at 170 and 190 °C at 40 min of reaction, there is an 82 % and 243 % increase respectively in propionic acid concentration compared to 150 °C of reaction. Generally, at a higher temperature of WO

reaction (temperature above 200 °C) there is a possibility of conversion of propionic acid to acetic acid and other by-products [168, 169].

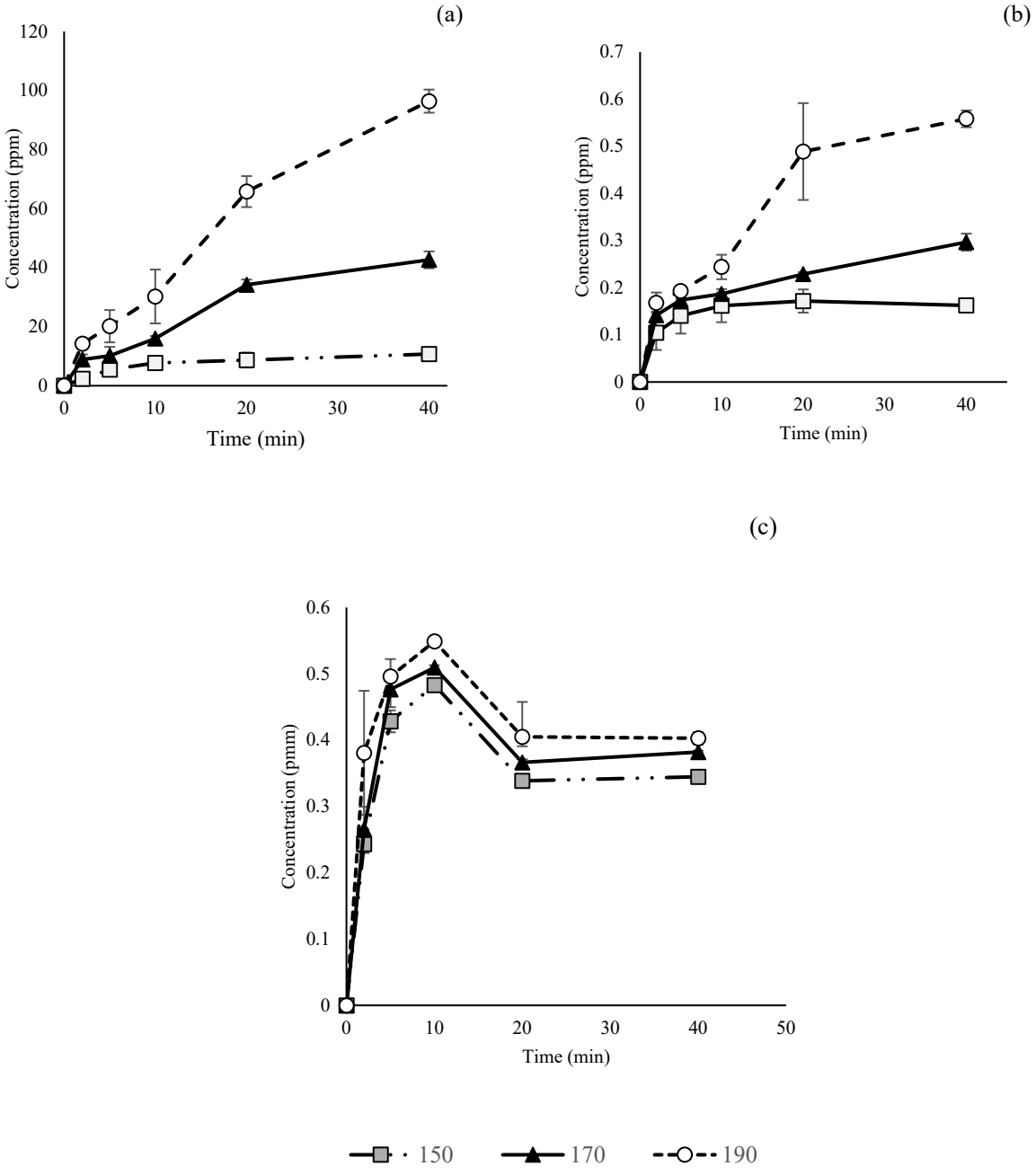

Figure 3.3 Concentration of volatile fatty acid over different time of reaction at neutral pH, (a) acetic acid, (b) propionic acid and (c) butyric

Unlike acetic acid resistance to degradation in WO, which is sometimes called the end of oxidation reaction [164], butyric acid can be converted into other products even at mild

temperatures [165, 166]. The maximum concentration of butyric acid is at 10 min of reaction for all temperatures. After 10 mins, there was a significant decrease (p-value<5 %) in butyric acid concentration, which can be related to conversion of butyric acid to the other WO by-products. From 20 to 40 min of reaction for all reaction temperatures, there were no significant changes in butyric acid concentrations (p-value>5 %).

3.4.2 Effect of pre-treatment on chemical properties of wood chips

Temperature and time both have significant effects on cellulose, lignin and hemicellulose removal. As presented in Table 3.2, hemicellulose at all reaction temperatures and pHs is more degraded compared to lignin and cellulose. At pH 11, for all temperatures and reaction times, lignin degradation was more than its value at the other initial pH conditions.

Table 3.2 Effect of different operating conditions on chemical properties

Time of reaction (min)	pH	Temperature (°C)	Total cellulose loss (%)	Total lignin loss (%)	Total hemicellulose loss (%)	Selectivity value
5	5	150	18±1.2	30±2.1	67±1.3	1.7±0.2
		170	19±0.1	40±0.4	74±3.0	2.2±0.0
		190	32.0±0.2	53.4±1.0	93±0.0	1.7±0.0
	7	150	24.9±0.6	35.6±0.7	72.1±1.0	1.4±0.0
		170	29.8±0.8	44.3±2.2	90±0.3	1.4±0.0
		190	36.2±0.7	66±0.2	97.7±0.0	1.8±0.0
	11	150	16.5±1.4	49.3±9.0	71.1±6.8	3.1±0.8
		170	14.8±0.7	42.6±0.7	70.7±1.0	2.9±0.2
		190	25±0.0	58.5±0.4	81.8±0.7	2.3±0.0
10	5	150	23.6±4.3	40.0±2.0	78±5.2	1.8±0.4
		170	26.0±3.4	57.2±3.3	90.5±2.5	2.2±0.1
		190	35.1±2.4	71.0±0.1	ND*	2.0±0.1
	7	150	29.1±1.9	41.2±0.7	78.9±5.2	1.4±0.0
		170	33.2±0.0	51.3±2.8	91.6±0.0	1.5±0.0
		190	43.6±1.5	70.2±1.8	98.4±0.0	1.6±0.0
	11	150	25.5±0.0	49.0±9.5	62.7±5.0	1.9±0.5
		170	26.3±1.6	50±0.3	68.5±3.3	1.9±0.1
		190	33.0±1.7	65.7±4.8	91.9±0.4	1.9±0.2

*Not detected

Interestingly, at pH 11, cellulose and hemicellulose degradation were almost less than the other pHs for all temperatures. this is because, at basic pH, hemicellulose and cellulose degradation might be suppressed, while the WO reaction is shifted to degrade more lignin [172].

This can indicate that neutral pH might cause more cellulose and lignin removal, which is not appropriate as more cellulose might be lost during the pre-treatment. Likewise, increasing reaction time also has a negative effect on cellulose degradation and the loss of other wood components. For example, at pH 5 after 5 min of reaction there was 18 % cellulose loss, while at the same temperature and pH condition, for 10 min of reaction, cellulose loss was around 24 %. This trend was also shown under other pH conditions. Comparing pH 5, 7 and 11, at the same reaction time, the minimum cellulose loss happened at pH 11 while the maximum lignin loss occurred at pH 11. Accordingly, the maximum selective lignin oxidation may be found at pH 11. As the reaction temperature increased, both lignin and cellulose removal increased. Although increasing reaction temperature can improve partial lignin removal, cellulose loss increased which is not favourable. The appropriate operating conditions regarding minimum cellulose loss and maximum lignin removal might be found at pH 11 and 5 min of reaction. At pH 11, 5 min of reaction and 150 °C, as the selectivity value has no certain value (±0.8), it may be acceptable to consider temperature 170 °C as the maximum value for the selectivity which was 2.9. In this operating condition, cellulose loss is around 15 % which is less than both temperatures 150 and 190 °C.

Fig. 3.4 shows the percentage of wood components in treated biomass under different operating conditions. Neutral pH, compared to pH 5 and 11, at all reaction temperatures and times showed more mass loss. Pre-treatment at alkaline pH resulted in the minimum cellulose loss, as in Figs. 3.4 (c) and (d). Furthermore, alkaline pH and higher temperature, showed the highest lignin degradation, as shown in Figs. 3.4 (e) and (f). The results for hemicellulose, in

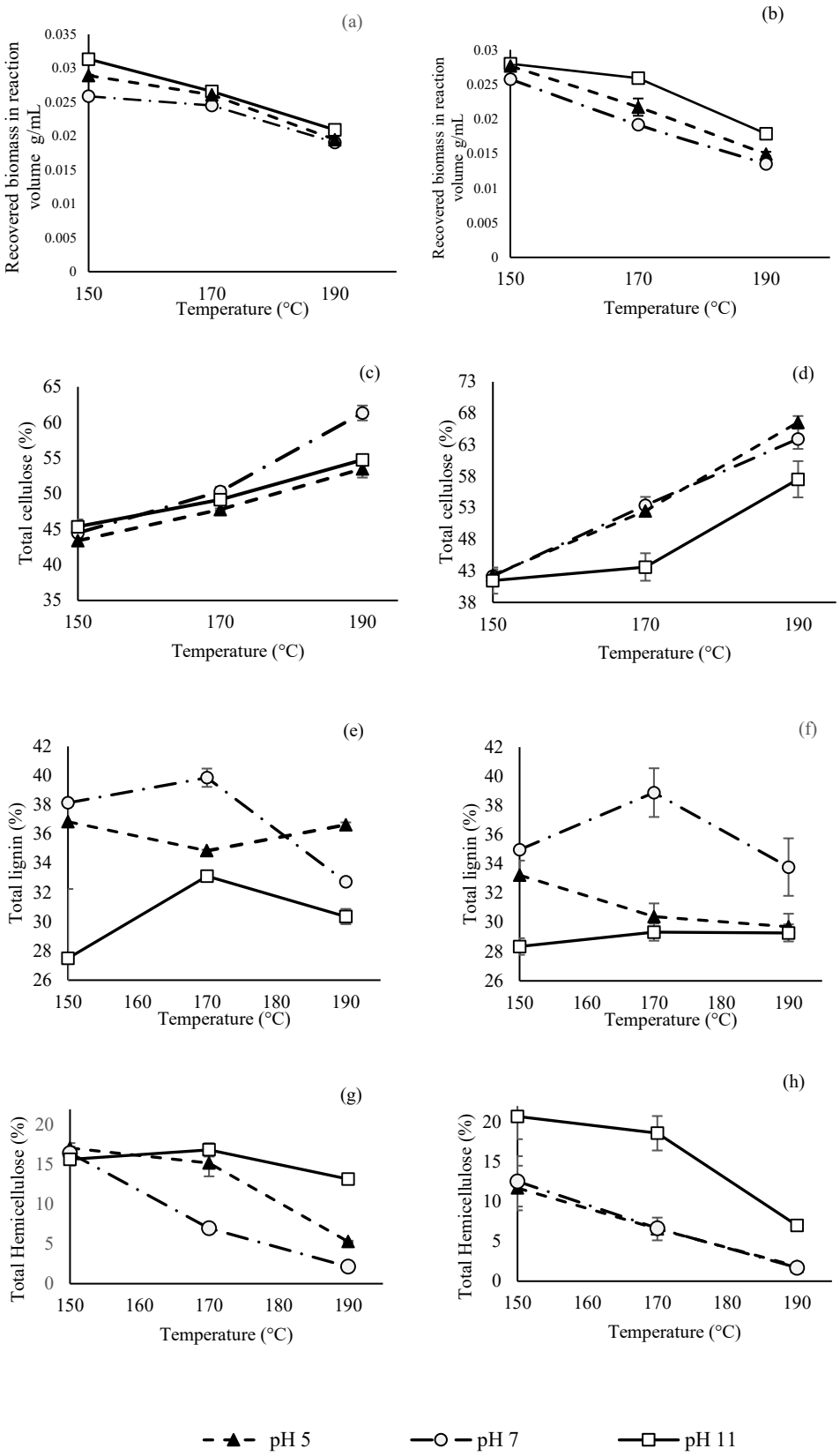

Figure 3.4 Properties' composition of treated biomass at different operating conditions, (a), (c), (e) and (g) after 5 min (b), (d), (f) and (h) after 10 min of pre-treatment.

Figs. 3.4 (g) and (h), indicated that at neutral and acidic pHs, almost all hemicellulose was degraded after 10 min of reaction. This can show that from all wood components, hemicellulose is more vulnerable to oxidation than lignin and cellulose (hemicellulose decreased to 1.7 % in the recovered biomass at maximum degradation).

If there is a need to extract hemicellulose from lignocellulosic waste by an oxidative hydrothermal process, time of reaction is extremely important; a shorter reaction time must be chosen. Initial pH also had a significant effect on total lignin and cellulose degradation.

After 10 min of reaction at 170 °C there was no significant change ($p > 5$ %) between remained cellulose at pH 5 and 7 (52% and 53% for pH 5 and 7 respectively). However, at both pHs 5 and 7, treated biomass after 10 min of reaction had significantly more recovered ($p < 5$ %) cellulose comparing to pH 11 which was 43 %.

Under these operating conditions, the percentage of lignin in treated biomass was also bigger than lignin at pH 11. Therefore, both pH 5 and 7 might not be sensible suggestions for cellulose recovery in WO of lignocellulosic biomass, as the rates of cellulose and lignin oxidation were high. Interestingly, the amount of total lignin at pH 7 for both reaction times was significantly higher than two other reaction temperatures. This can be explained by the condensation of lignin to solid biomass during the reaction [173, 174]. Condensation reaction can lead to a stable linkage and this might result in increasing molecular size of liberate lignin, which previously was freed in WO reaction, leading to precipitation of this component [173, 174].

At 190 °C and pH 5 for 10 min of reaction and pH 7 for 5 min of degradation, the maximum cellulose content in treated biomass was produced. However, these are not favourable as the maximum selective oxidation did not happen under these operating conditions. Theoretically,

the best answer can be found after optimisation of operating conditions for maximum selective lignin oxidation and minimum cellulose loss.

3.4.3 Effect of pre-treatment on crystallinity

Statistical analysis showed that temperature had the most significant effect on the crystallinity index of treated biomass comparing to time and initial pH (Fig 3.5). Increasing the reaction temperature had a distinct effect on changing the crystallinity index. This can be described by the effect of reaction temperature on lignin to cellulose portion of the treated biomass.

Lignin and some portion of cellulose are considered the amorphous parts of any lignocellulosic materials. Higher temperatures may cause more oxidation of lignin, a decrease in the amorphous portion, and an increase in crystallinity index. The maximum increase in crystallinity index occurred at pH 7, 190 °C (24 % increase). Lignin condensation might also have an impact on the results for the crystallinity index. Higher temperatures and lower pH can increase the lignin condensation on treated biomass.

Decreasing pH, and reaction temperature, increases lignin precipitation [175]. This can indicate that condensation of lignin at lower pH caused an unusual decrease in the crystallinity index of the treated biomass. As the portion of lignin, which is a part of non-crystalline biomass, increased, the crystallinity index decreased.

3.5 Evaluation of process energy requirement

In oxidative hydrothermal reaction, compressed hot water is used. It is assumed that the whole energy required for the process is the energy to bring water pressure and temperature from ambient to operating conditions. It is assumed there is no energy loss after reaching the desired operating condition; the process energy loss is almost assumed to be nil. The rationale for that is the reactor is completely sealed with very thick insulation and the temperature drop after reaching the desired temperature is very slow (almost 5 °C per 20 min).

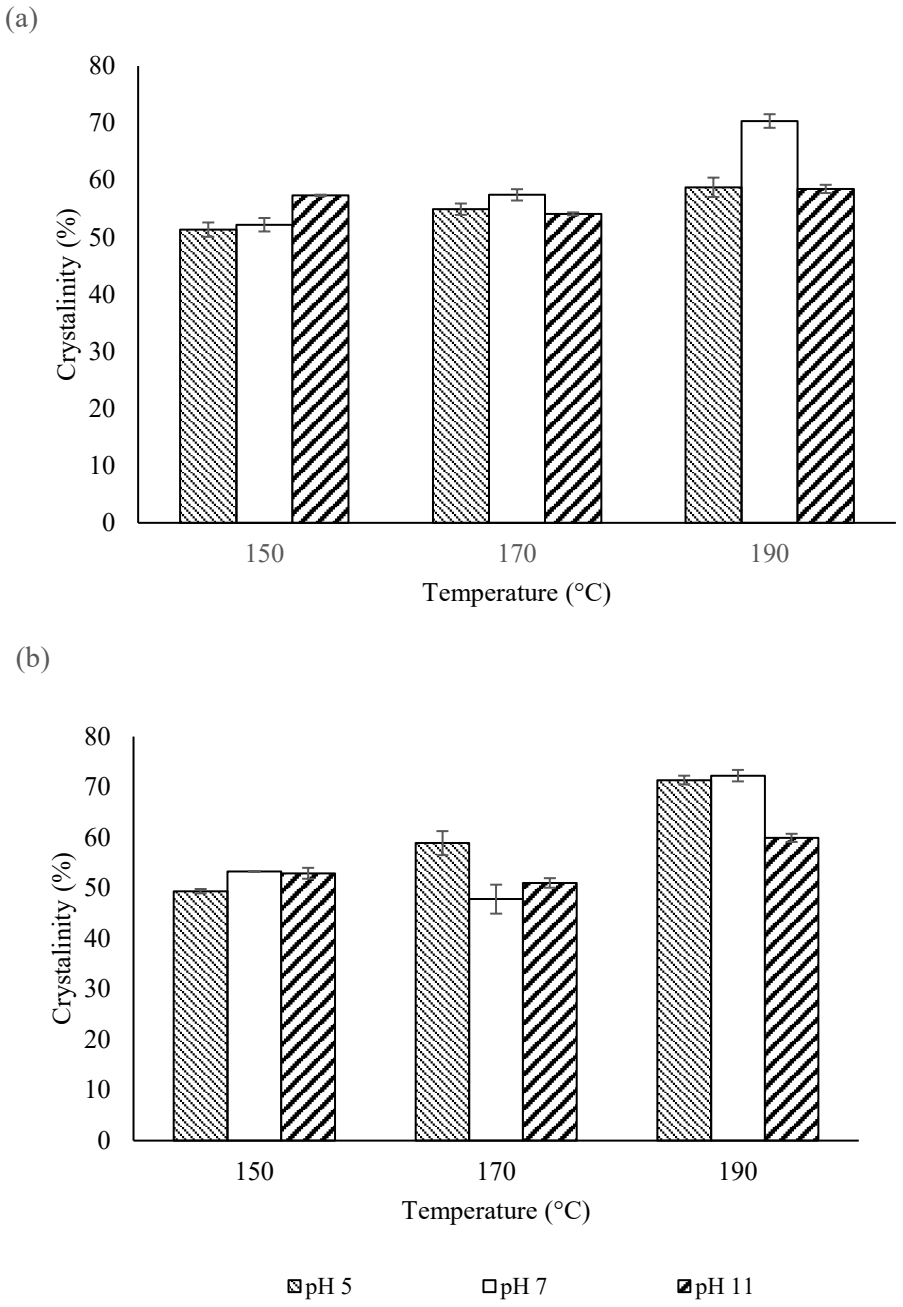

Figure 3.5 Crystallinity of treated radiate pine wood chip at different operating conditions. (a) 5 min, (b) 10 min

Although the WO is an exothermic process, the portion of energy needed to bring the water temperature and pressure to the operating conditions is much more than the energy produced during the process. Another assumption which was made is the mechanical power to mix the reaction media was not considered. However, for a large-scale process mechanical power is much more significant, which is required to be precisely measured. Therefore, the above-

mentioned simplification can provide a rough estimate of the energy consumption of the process. Considering the assumption, subcooled water enthalpy is considered the main energy required for the process. In addition to that, water enthalpy was assumed to be constant for different initial pH conditions. By reading the enthalpy and density at the operating conditions from any thermodynamic table, the energy required for the process was calculated. Table 3.3 presents the energy requirement to process the woody biomass for cellulose, lignin and hemicellulose removal under different operating conditions.

Table 3.3 Energy consumption for wet oxidation of radiata pine biomass under different operating conditions

Time (min)	T (°C)	pH	Energy consumption for lignin removal (kJ/g)	Energy consumption for cellulose removal (kJ/g)	Energy consumption to remove hemicellulose (kJ/g)
5	150	5	281.2±17	234.5±10	235.6±3
		7	468.5±19	165.0±12	184.3±24
		11	173.9±12	260.9±17	194.4±12
	170	5	319.9±5	250.9±9	187.3±3
		7	250.5±15	151.2±14	109.1±5
		11	280.1±16	329.0±20	220.5±11
	190	5	178.3±5	153.5±19	110.8±9
		7	120.1±17	134.7±9	100.0±5
		11	148.8±9	197.9±6	152.0±11
10	150	5	303.4±10	175.1±8	139.4±12
		7	277.0±15	139.5±13	138.9±7
		11	176.5±2	160.9±9	341.0±21
	170	5	141.8±5	175.3±12	108.2±4
		7	177.1±14	175.3±9	105.2±2
		11	184.2±12	173.4±5	249.1±18
	190	5	106.1±12	139.3±12	95.9±8
		7	108.2±3	110.9±3	98.5±12
		11	120.9±6	148.5±10	114.8±5

By calculating the amount of cellulose, hemicellulose and lignin removal at the operating conditions, energy consumption per gram of each component of lignocellulose (cellulose,

hemicellulose and lignin) was obtained, by dividing the calculated energy at that operating condition to gram of mass removal. Energy assessment indicated that at a milder treatment temperature, the basic condition might be more energy efficient. For example, at 150 °C, as the pH increased, the energy consumption of lignin removal decreased due to an increase in lignin removal at the constant temperature. The reason for that is as pH increased at temperature 150 °C, more lignin was removed from samples while the initial energy to bring the reaction media to the desired operating condition was almost the same.

Under the same operating conditions, energy consumption for cellulose had a different pattern. At 150 °C and 10 min of oxidation, energy consumption for cellulose was at its maximum in acidic pH. At the same pH and reaction times, when reaction temperature increased, the energy consumption decreased. As the activation energy decreased by increasing the reaction temperature, the rate of oxidation increased more at a higher temperature and more biomass was consumed. In addition, total energy to bring the water condition from 150 °C to 190 °C at 20 bar varies from 633 kJ/kg to 807 kJ/kg while lignin removal changes from 0.57[1] g to 2.0 g or for cellulose, from 0.62 g to 1.96 g. This can explain the decrease of energy consumption per gram of biomass while the reaction temperature increased. Cellulose, hemicellulose and lignin removal were obtained from difference between the weight of each component in the untreated biomass and treated biomass.

1- The results for amount of each component in the untreated or treated biomass were obtained from HPLC and UV-vis analyses in agreement with NREL procedure.

3.6 Conclusion

A mild WO pre-treatment can partially remove lignin in woody biomass with a probable minimum cellulose loss. Both results for chemical properties and energy estimation indicated that there is an optimised operating condition for energy consumption and minimum cellulose loss. At the early stages of reaction and at a lower reaction temperature, the crystallinity index did not significantly change. As the reaction temperature increased, significant changes occurred at 190 °C and pH 7 which was a 24 % increase in the crystallinity index. Selectivity value is a factor to find out the optimum condition for minimum cellulose loss and maximum lignin removal. Based on the results found in this study, the basic operating conditions may have a better result in the case of minimum cellulose loss. At an alkaline condition and early stages of reaction the most selective lignin degradation, with minimum cellulose loss, happened. At this operating condition (pH 11, 5 min of reaction and 170 °C), selective lignin degradation was 2.9.

Chapter 4
Combined fungal-hydrothermal pre-treatment of radiata pine wood chips

4.1 Introduction

Concerns regarding the consequences of using fossil fuel and its significant impacts on global warming and the environment have been a matter of discussion for decades. This has led to attempts to use new and sustainable forms of energy. Biofuels from organic waste, especially from lignocellulosic material, have been studied; however, lignocellulosic materials which are used should not interrupt human being or animal food chains. This is considered one of the challenges of using such materials. Another issue with lignocellulosic materials is due to their chemical structures. Lignocelluloses, generally, consist of cellulose, hemicellulose and lignin. Cellulose is covered with hemicellulose and lignin with a complex structure. In addition to that, cellulose in woody biomass is also incorporated in fibre branches with an almost crystalline structure. This makes cellulose a difficult target to hydrolysing enzymes. Therefore, if lignocellulosic biomasses are the feed for any biofuel production, the destruction of cellulose or partial lignin removal is essential before introducing any other processes.

Pre-treatment can be categorised into physical, chemical, physico-chemical and biological pre-treatments. All these pre-treatment methods have their own pros and cons. In physical pre-treatment methods, lignocellulosic materials will be crushed into small pieces, with the application of an external mechanical force. This causes size reduction and a possible increased surface area with mostly nontoxic by-product production. However, its disadvantage is high-energy demand [1, 15, 32]. In chemical pre-treatment, a chemical agent is used to break down the complex structure of woody materials. Effective partial lignin removal and deconstruction of lignocelluloses can be considered its advantages. But production of toxic by-products and unfavourable structural carbohydrate loss are downside of this pre-treatment method [15, 22, 23, 28, 40-47, 49-54, 154]. In biological pre-treatment, microorganisms such as white, brown or soft rot fungi degrade lignocellulosic materials. This

provides potential to pre-treat woody biomass using a low amount of energy and an environmentally friendly process, especially if using white rot fungi. White rot fungi can selectively degrade lignin with minimum cellulose loss. However, the lengthy pre-treatment time needs to be reduced to enable commercial interest.

In physico-chemical -chemical pre-treatment in the presence of a chemical agent, accompanied by a change in operating conditions, lignin removal and cellulose destruction can be achieved. Steam explosion, AFEX, carbon dioxide explosion and WO pre-treatments are the examples of this method of pre-treatment [15, 24, 30, 31, 45, 47, 55-57, 59, 60, 65-70, 72-74]. Generally, these methods are effective in partial lignin removal and increasing post-biofuel conversion, yet cellulose degradation, some toxic by-product production and high-energy demand (in some cases) can be counted as their disadvantages.

As mentioned previously, fungal pre-treatment can introduce a low-energy demand process with almost no toxic by-products harmful for the environment. But the long time needed for the process should be tackled. The combination of fungal pre-treatment with other pre-treatment methods might provide a potential solution for both the lengthy time required for fungal pre-treatment and the energy inefficiency of the other physico-chemical pre-treatment methods for example, fungal treatment combined with acid, alkali, steam and ammonia explosion and hot water extraction pre-treatment [98, 130, 132-134]. The combination of fungal pre-treatment with these physical and chemical pre-treatment methods has been the subject of research. It has been shown that this combined pre-treatment provides an improvement in overall pre-treatment efficiency regarding glucose conversion and energy demand. However, there was not any study in the literature of combined WO and fungal pre-treatment.

WO is a physico-chemical pre-treatment carried out at a temperature between 120–320 ºC and pressure 5–200 bar. In WO, the radicals conduct the reaction [71]. In the absence of initiators, free radicals are formed by reaction of oxygen with the weakest C-H bond of the organic compound. The wet air oxidation mechanism can be described as follows [71]:

$$RH + O_2 \rightarrow R\bullet + HO_2\bullet \quad (1)$$

$$RH + HO_2\bullet \rightarrow R\bullet + H_2O_2 \quad (2)$$

$$R\bullet + O_2 \rightarrow ROO\bullet \quad (3)$$

$$H_2O_2 + M \rightarrow 2HO\bullet \quad (4)$$

$$ROO\bullet + RH \rightarrow R\bullet + ROOH \quad (5)$$

Where M can be either a homogenous or heterogeneous carbonaceous species.

A generalised and simplified reaction with acetic acid as the rate-limiting intermediate was introduced by Li et al. [71] and is shown in Fig 4.1.

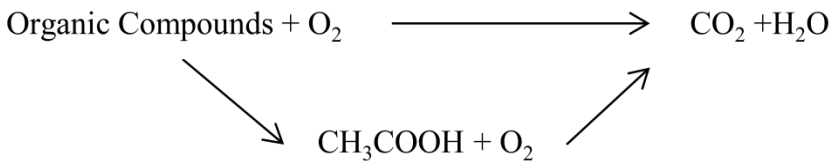

Figure 4.1 Simplified reaction pathway for wet oxidation [71]

WO has the potential to solubilise and fractionate the insoluble carbonaceous content to carbon dioxide and water [67, 69, 155]. This feature is important for treatment of viscous slurries [68]. In addition, WO has the potential to pre-treat lignocelluloses into a solubilised fraction and a cellulose-rich fraction [152, 156-159]. Although WO may have more capital cost than the other pre-treatment methods, the operating cost can be comparable with other pre-treatment methods [69]. The operating costs include power to compress the oxygen and fuel cost for increasing the enthalpy of the incoming stream to the operating temperature [160, 161]. Despite the high-energy demand for WO, its combination with fungal pre-

treatment may improve energy demand for the whole process as well as decrease the time of pre-treatment for fungal pre-treatment alone.

With the hope of providing a combined method which has less energy demand compared to single WO pre-treatment, fungally pre-treated radiata pine wood chips were pre-treated by WO at different operating conditions. Radiata pine is a fast-growing medium-density softwood and is abundant in New Zealand. Qualities such as fast growth and not interrupting human food-supply chains make this lignocellulose source suitable for a wide range of end uses.

In this work, the effect of WO pre-treatment on fungally pre-treated radiata pine wood chips under different operating conditions, temperature: 150–190 ºC and pH of 5, 7 and 11 at a constant oxygen pressure of 20 bar was studied. The effect of WO pre-treatment on physical and chemical properties of woody biomass were also studied. To find out the impact of fungal pre-treatment on energy demand reduction for WO pre-treatment, a rough energy calculation was also applied.

4.2 Materials and methods

4.2.1 Fungal strain and biomass preparation

As previously presented in research into fungal pre-treatment of radiata pine wood chips with two New Zealand local fungal strains [140], amongst the two New Zealand local white rot fungi, pre-treatment with *Trametes versicolor* for 3 weeks showed a better result when it is compared to the other strains named *Stereum hirsutum.* Therefore, the biomass needed for the combined pre-treatment method (fungal-WO), is firstly pre-treated by the white fungal strain, *Trametes versicolor*.

The New Zealand native strain *Trametes versicolor* was supplied by Landcare Research – International Collection of Microorganisms from Plants, Auckland, New Zealand. This strain was grown on PDA plates at 30 ºC for 1 week.

In this experiment, radiata pine soft wood was used as a woody biomass. It was cut into wood-chip size (approximately 1 cm^3). Media preparation for fungal pre-treatment was based on the method suggested by Keller and co-workers [145].

4.2.2 Fungal pre-treatment

Pre-treatments were conducted in 250 mL Erlenmeyer flasks. Ten grams of radiata pine wood chips were put into each flask. To bring the moisture content up to 70 %, 23 mL of distilled water was added to each flask. All flasks were autoclaved at 121 ºC for 30 min to destroy any unexpected microorganisms. The effect of autoclaving on biomass is much less than steam explosion and other heat pre-treatment methods; however, the severity of autoclaving can affect cellulose and hemicellulose solubilisation in wood [81, 98]. Four pellets of fully grown mycelia (cultivated on PDA, as previously described) were added to the woody biomass and flasks were incubated at 25 ºC for 3 weeks. Every 2 days the flasks were shaken gently for better mixing.

4.2.3 Raw material

To prepare enough woody biomass for this stage (WO), about 1 kg of wood chips were pre-treated with the white rot fungus, and around 50-60 Erlenmeyer flasks were used.

The woody biomass was pre-treated by the New Zealand native strains *Trametes versicolor*. After pre-treatment, all biomass was oven dried at 105 ºC for 6–8 h and collected to make a united biomass.

4.2.4 Wet oxidation pre-treatment

WO was carried out in a 1L Parr reactor (a 4540 high-pressure reactor equipped with a 4848 controller; Parr Instrument Company, US) with a maximum working volume of 600 ml. A

schematic diagram of the reactor is shown in Fig 4.2. Wood chips (10 g) were slurried with 300 mL of deionised water in the reactor. To provide an initial pH of 5–11, both acetic acid ($C_2H_2O_2$) and sodium carbonate (Na_2CO_3) were added separately. Suspended materials were supplied into the reactor and sealed completely to avoid any leakage. For each run, the reactor was pressurised at 20 bar with pure oxygen.

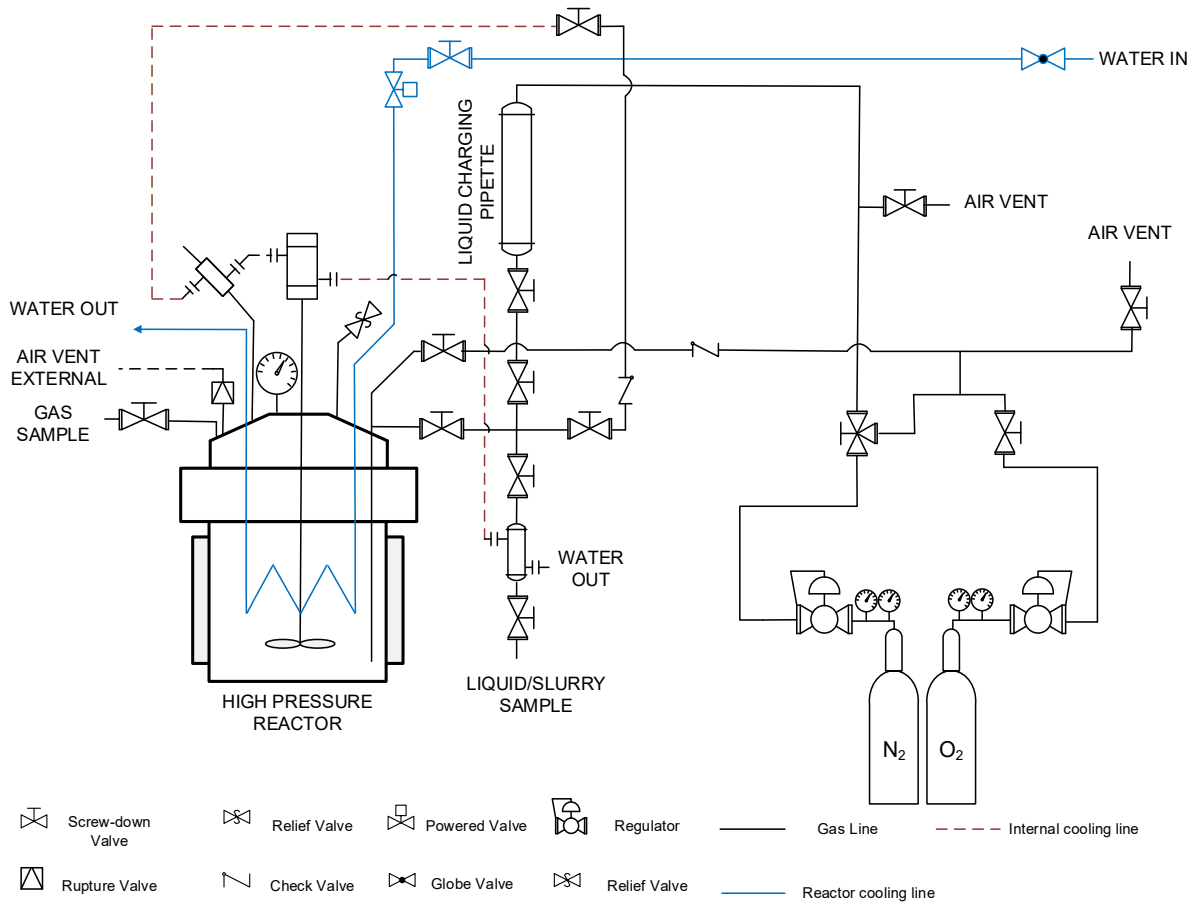

Figure 4.2 Schematic diagram of wet oxidation processing of radiata pine chips

This provided an approximately stoichiometric oxygen environment. The reactor was then heated up to between 150–190 ºC. The reaction was then carried out for 5–10 min. The reactor was then immediately cooled down to room temperature. After that, the solid phase was separated from the liquid phase by vacuum filtration. Both solid and liquid phases were collected for further analyses.

4.3 Analysis

Both non- and wet-oxidative treated materials were subjected to a two-step acid hydrolysis to find out their lignin and structural carbohydrate contents based on the NREL procedure [146]. This protocol includes two steps of hydrolysis using sulphuric acid. In the first step, sugars and other acid-soluble components were extracted by 72 % (w/w) sulphuric acid at 30 ºC for 1 h. This step was followed by treatment with diluted sulphuric acid of 4 % (w/w) at 121 ºC for another 1 h. Acid-soluble lignin was determined by UV-Vis spectroscopy (Shimadzu-UV-2550, Japan) at 240 nm. Acid-insoluble lignin was quantified by filtration of the residue based on the NREL method. Wood sugars were quantified by HPLC (Shimadzu-20A, Japan) equipped with a reflective index detector (RID 10A, Shimadzu, Japan) and an Agilent column (Hi-Plex Pb, 300 × 7.7 mm). Deionised water was used as the mobile phase at a flow rate of 0.4 mL/min. The column and the detector's working temperature were 60 ºC and the injection volume was 10 µL. All analyses were repeated in triplicate, at least.

An XRD (Bruker D2 Phaser, Germany) was used to measure the crystallinity of both treated and pre-treated woody biomass. Crystallinity, CrI (%) is described as [149]:

$$\mathrm{CrI} = \frac{I_{002} - I_{am}}{I_{002}} \times 100 \quad (6)$$

Where:

I_{002}: The maximum intensity at $2\theta = 22.8°$

I_{am}: The minimum intensity at $2\theta = 18.4°$

Selective lignin removal/partial removal (selectivity value), which is the portion of lignin loss to cellulose loss, can be described as follows:

$$\text{Selectivity value} = \frac{\text{Lignin loss at the time of pre-treatment}}{\text{Cellulose loss at the time of preteatment}} \quad (7)$$

This parameter can be used to optimise the pre-treatment's operating conditions which possibly leads to minimum loss of cellulose with a higher portion of lignin loss.

4.4 Results and discussion

4.4.1 Effect of the pre-treatment on chemical properties and energy requirement evaluation

The combination of two different pre-treatment methods may effectively improve some expected disadvantages of a single pre-treatment such as energy consumption. Nevertheless, the concern over mass loss, which in lignocellulosic materials is cellulose loss, is raised. Since the biomass here was first pre-treated with white rot fungi, an increase in mass loss compared to WO alone was expected. For WO alone, based on the results, the best operating conditions which led to maximum selectivity value were at alkaline conditions and for a shorter time of reaction. Table 4.1 shows the effect of combined pre-treatment on the chemical properties of the wood chips.

The same as for WO pre-treatment alone, the alkaline operating conditions at a mild temperature of 170 °C and a shorter reaction temperature led to better results for the selectivity value. As was presented in Table 3.2, in the last chapter, for the WO pre-treatment alone cellulose loss varied from 18 to 43 % for different operating conditions. Yet, for the combined method cellulose losses compared to single WO pre-treatment increased by 0.3 % (pH 5, 170 °C and 5 min of reaction) to 15 % (pH 5, 190 °C and 10 min if reaction) at the same operating conditions for WO alone. Also, lignin removal, for the combined method compared to WO alone, was not significantly changed ($p>5$ %). Therefore, the higher amount of cellulose loss and insignificant change in lignin removal caused a lower selectivity value compared to the WO pre-treatment alone. Higher temperature and longer reaction time had more effect on chemical properties of treated biomass than the pH.

Table 4.1 Effect of combined pre-treatment on chemical properties of radiata pine wood chips

Time of reaction (min)	pH	Temperature (°C)	Total cellulose loss (%)	Total lignin loss (%)	Total hemicellulose loss (%)	Selectivity value
5	5	150	23±5.4	22±2.2	69±10.4	0.9±0.1
		170	19.3±2.2	33.5±3.4	75.5±15.2	1.7±0.2
		190	32.5±2.3	58.8±2.5	88.1±0.2	1.8±0.0
	7	150	26.0±2.6	36.8±3.1	56.4 ±4.1	1.4±0.1
		170	31.2±4.2	46.3±3.4	90.8±0.5	1.5±0.0
		190	38.8±0.8	56.1±1.2	93.8±0.5	1.5±0.1
	11	150	27.2±3.6	32.1±6.1	54.4±3.4	1.2±0.3
		170	20.8±4.7	48.6±2.1	56.6±1.3	2.3±0.4
		190	27.7±0.5	58.5±0.4	69.8±0.7	2.0±0.1
10	5	150	24.7±3.5	34.5±2.8	57.9±2.2	1.3±0.4
		170	35.1±2.4	54.8±1.3	83.2±3.1	1.5±0.0
		190	49.9±1.8	76.0±0.2	97.6±1.9	1.5±0.0
	7	150	38.4±0.2	39.6±3.4	56.3±4.2	1.0±0.1
		170	21.1±3.1	31.6±1.3	78.6±1.3	1.5±0.1
		190	44.5±4.8	70.7±5.4	96.4±10.2	1.6±0.0
	11	150	26.3±2.1	37.1±3.8	45.9±4.2	1.4±0.1
		170	28.2±2.5	50.5±6.5	60.3±6.3	1.8±0.1
		190	35.7±3.1	70.2±4.2	81.8±5.3	2.0±0.0

Hemicellulose degraded more compared to lignin and cellulose regardless of operating conditions. At a basic pH of 11, cellulose and hemicellulose removal was less than at the other pHs.

Similar to WO alone, for combined pre-treatment at basic pH, cellulose and hemicellulose degradation were supressed while the WO shifted to more lignin degradation [172]. Like WO pre-treatment alone, acidic and neutral pHs were expected to have more cellulose loss, which is not desired. Basic pH provided a better result with less cellulose loss and more lignin degradation for the combined pre-treatment. As was mentioned earlier, in the previous chapter, the major amount of the energy required for the oxidative hydrothermal reaction is used to bring water from ambient conditions to the operating conditions. It was assumed, as

the reactor was covered with a thick insulation, that there was no energy loss after reaching operating conditions. Enthalpy of pure water at those operating conditions was taken as the energy needed for the process. At each operating condition the amount of cellulose, lignin and hemicellulose was quantified by the NREL method.

Increasing the pH enhanced the lignin removal in WO pre-treatment. Other related studies show that lignin removal is higher under alkaline conditions [167, 176]. Faster decomposition of organic components under alkaline conditions is the reason why lignin removal is higher under basic media [177].

Increasing reaction temperature has a significant effect on lignin, cellulose and hemicellulose removal. Reaction rate is higher under higher reaction temperature. Higher formation rate of free radicals at higher reaction temperature can be the reason of higher lignin, cellulose and hemicellulose removal[71].

Table 4.2 presents results for the energy requirements for the fungally pre-treated biomass. The results indicate that a short fungal pre-treatment showed an improvement in energy consumption for lignin, cellulose and hemicellulose removal in post-WO pre-treatment of radiata pine wood chips. The combination of fungal pre-treatment with WO pre-treatment showed an improvement in energy consumption although it increased cellulose loss.

The energy consumption for lignin removal for this combined pre-treatment was reduced from 4 to 331 kJ/g and from 21 to 157 kJ/g for 5- and 10-min reaction time respectively, compared to WO pre-treatment alone. Combination of fungal and WO pre-treatment showed a reduction of energy consumption ranging between 4-70 % for lignin removal. On the other hand, there was an increase in cellulose as it mentioned before between 0.3 % to 15 % when compared to wet oxidation alone. Although losing cellulose is not favoured for the pre-

treatment method of lignocellulosic biomass, the considerable decrease in lignin removal energy consumption can be taken into consideration.

Table 4.2 Energy consumption calculations for wet oxidation of fungally pre-treated radiata pine biomass under different operating conditions

Time (min)	T (°C)	pH	Energy consumption for lignin removal (kJ/g)	Energy* reduction (%)	Energy Consumption for cellulose removal (kJ/g)	Energy* reduction (%)	Energy consumption to remove hemicellulose (kJ/g)	Energy* reduction (%)
5	150	5	220.2±12	21	160.2±6	31	98.3±2	58
		7	136.8±15	70	145.6±8	12	129.8±11	29
		11	130.1±20	24	139.2±10	46	137±9	29
	170	5	155.5±10	51	217.5±9	14	99.2±6	47
		7	121.4±9	52	140.4±14	7	102.3±5	6
		11	115.3±6	58	203.5±5	39	141.2±8	35
	190	5	104.2±8	41	141.3±9	7	105.2±4	4
		7	115.7±13	4	119.0±2	11	92.2±7	8
		11	109.4±11	26	166.3±5	16	135.2±11	9
10	150	5	146.2±13	55	153.0±7	12	131.3±4	5
		7	127.3±5	54	100.2±11	28	136.8±6	1
		11	136.2±3	22	138.9±7	13	160.9±11	39
	170	5	102.5±5	27	112.3±3	36	100.1±4	7
		7	124.6±8	30	136.1±8	22	91.2±2	13
		11	104.2±11	43	140.2±9	19	135.2±5	45
	190	5	79.2±5	25	87.5±8	37	91.2±6	4
		7	87.0±3	20	97.4±3	11	97.0±3	1
		11	87.7±6	28	108.1±5	27	109±11	7

*Energy reduction compared to wet oxidation pre-treatment alone

Generally, a higher reaction temperature and longer reaction time can cause more cellulose loss. In addition to that, combining two methods of pre-treatment might have a significant effect on mass loss. Accordingly, results for this combined method confirmed that more cellulose loss is inevitable in all reaction conditions compared to the sole WO pre-treatment. According to results both for the combined and single WO pre-treatment methods, the higher temperature and reaction time resulted in a greater decrease in energy consumption for lignin

removal (except pH7). As mentioned, this resulted in more cellulose loss which is not favourable. Likewise, WO pre-treatment alone at 170 °C and pH 11 for 5 min of reaction showed the maximum selectivity value. All in all, the combined method (fungal-WO pre-treatment) had the maximum selectivity value at the milder operating conditions and shorter reaction time (170 °C and 5 min of reaction). At these operating conditions (pH 11, 170 °C and 5 min of reaction), energy consumption for lignin removal was calculated to be around 115 kJ/g. This was 280.1 kJ/g for WO alone at the same operating conditions. This means the combined method decreased the energy usage by around 60 % compared to the WO pre-treatment alone at this specific operating condition, which led to the maximum selectivity value. At this operating condition, cellulose loss for the combined method was around 21 % while for the WO method cellulose loss was around 15 % which means a 6 % absolute increase in cellulose loss. As the energy required for both WO pre-treatment alone and the combined method are almost the same, the higher amount of lignin removal causes the less energy consumption per gram of mass. The biomass in the WO alone was treated only by the hydrothermal processing while in the combined pre-treatment there have been two stages for the lignin, cellulose and hemicellulose removal. Therefore, a decrease in energy consumption for lignin removal in the combined method was expected.

Thermal hydrolysis is an energy-consuming process which therefore cannot be a suitable option for lignocellulose pre-treatment. Fungal pre-treatment might be considered a less energy-consuming process since the process can be conducted even at room temperature. This provides a potential to decrease the energy requirements for the WO process. Mass loss, especially cellulose loss in a process like this, is also a drawback and it needs to be taken into account. The reason this point is raised is that the longer the reaction time is, the more cellulose is exposed to reaction and degradation. Therefore, a decrease in cellulose loss in a

combined process cannot be achieved. But the significant decrease in energy consumption, with minimum cellulose loss, compared to WO pre-treatment alone, might be favourable.

The results for this combined method showed that with an increase in cellulose loss varying between 0.3 to 15 %, a significant energy reduction of up to 70 % is achievable compared to WO pre-treatment alone. Fig. 4.3 presents the percentage of different chemical components in pre-treated biomass. Alkaline pH at higher temperatures showed more cellulose and lignin degradation compared to the other pHs. On the other hand, neutral and acidic pHs showed more hemicellulose degradation than basic pH. This means if there was a need to recover hemicellulose, the alkaline pH could provide a better result. For all reaction conditions for the combined method the results showed a significant increase ($p< 5$ %) in cellulose degradation compared to WO pre-treatment alone. As the lignocellulosic biomass, here, was first pre-treated by a white rot fungus, the higher amount of cellulose degradation is expected in comparison to sole WO pre-treatment. However, results indicated that more lignin was removed, and a significant amount of cellulose remained in the biomass, this varies between 1.7 g to 3.9 g for the combined pre-treatment and for the single wet oxidation pre-treatment it varies between 2.5 g to 3.9 g. This is because both parts of the combined method oxidise more lignin. As white rot fungi preferably oxidise more lignin than cellulose, lignin is further exposed to the free radicals generated during the following WO process.

As more lignin was removed, the percentage of the remaining cellulose in the treated biomass was higher. Accordingly, as the complex structure of woody biomass was opened more in the first fungal stage of the combined method, more cellulose might be exposed to the free radicals in the WO step of the pre-treatment. This is also in agreement with the results presented in Table 4.2. As the WO pre-treatment is the dominant step of pre-treatment in the combined method, some results might follow a similar trend compared to WO only pre-treatment method. Here, the time and temperature of the reaction also affected the chemical

components in the pre-treated biomass more than initial pH. For example, after 10 min of reaction and temperature of 190 °C, changing the initial pH from acidic to alkaline had no significant change on the percentage of cellulose in pre-treated biomass.

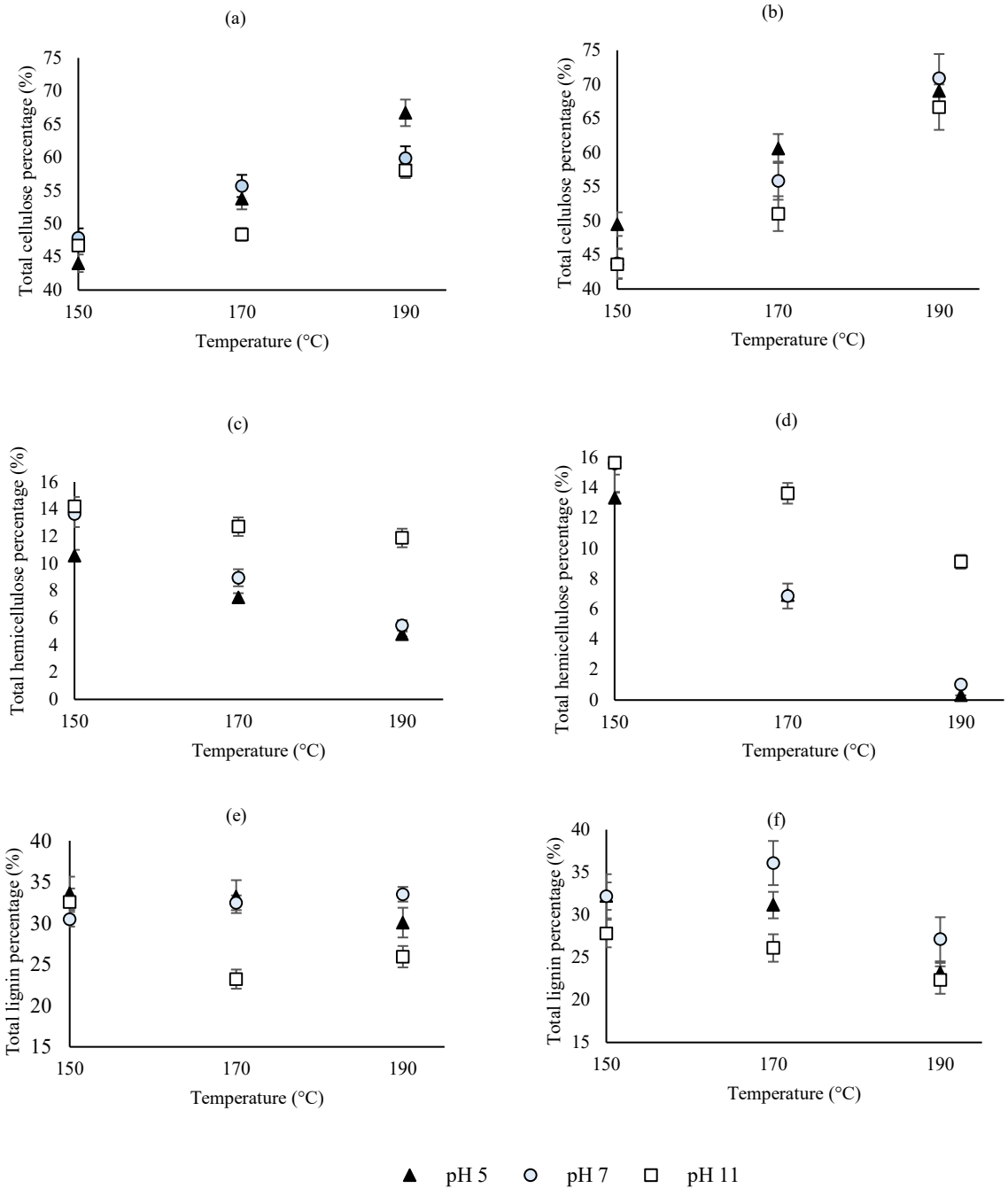

Figure 4.3 Cellulose, Hemicellulose, and Lignin composition of treated biomass at different operating conditions, (a), (c) and (e) after 5 min (b), (d) and (f) after 10 min of combined pre-treatment.

Another similarity is that the longer reaction time and acidic pH caused almost all hemicellulose to be degraded.

4.4.2 Effect of Pre-treatment on Crystallinity index

Like the WO treatment alone, a higher reaction temperature caused a higher crystallinity index. The maximum crystallinity index was at 190 °C and neutral pH both for 5 and 10 min of reaction (72 %). Fig. 4.4 shows the results for the crystallinity index of combined pre-treatment of the woody samples.

At 5 min of reaction for both reaction temperatures of 150 and 170 °C, there were no significant ($p > 5$ %) changes in crystallinity index when compared to the WO pre-treatment alone at the same operating conditions. The higher temperature, 190 °C showed a significant increase in crystallinity index compared to oxidative hydrothermal pre-treatment alone at all pHs except neutral pH, which demonstrated no significant change ($p > 5$ %).

As in the combined method, more cellulose was exposed to degradation (fungal pre-treatment followed by WO), a decrease in crystallinity index compared to WO alone can be expected. But lignin removal might also rise, which can affect the crystallinity index which was not significantly changed. As at alkaline pH the reaction shifts to more lignin degradation [172], an increase in crystallinity index was expected at that pH condition. This trend was seen in the results for the combined method. However, the presence or removal of lignin in the pre-treated biomass is one of the factors affecting the crystallinity index.

Both lignin part and non-crystalline cellulose in lignocellulose affect crystallinity index. Not only does more lignin content in the biomass cause less crystallinity index but also destruction of crystalline cellulose to non-crystalline cellulose may decrease the crystallinity index. Destruction of crystalline cellulose can occur during the reaction as well. Although, at the higher reaction temperature of 190 °C and basic pH (11), cellulose removal was

decreased, crystalline cellulose destruction to non-crystalline part may have happened at a higher rate which caused the crystallinity index to decrease.

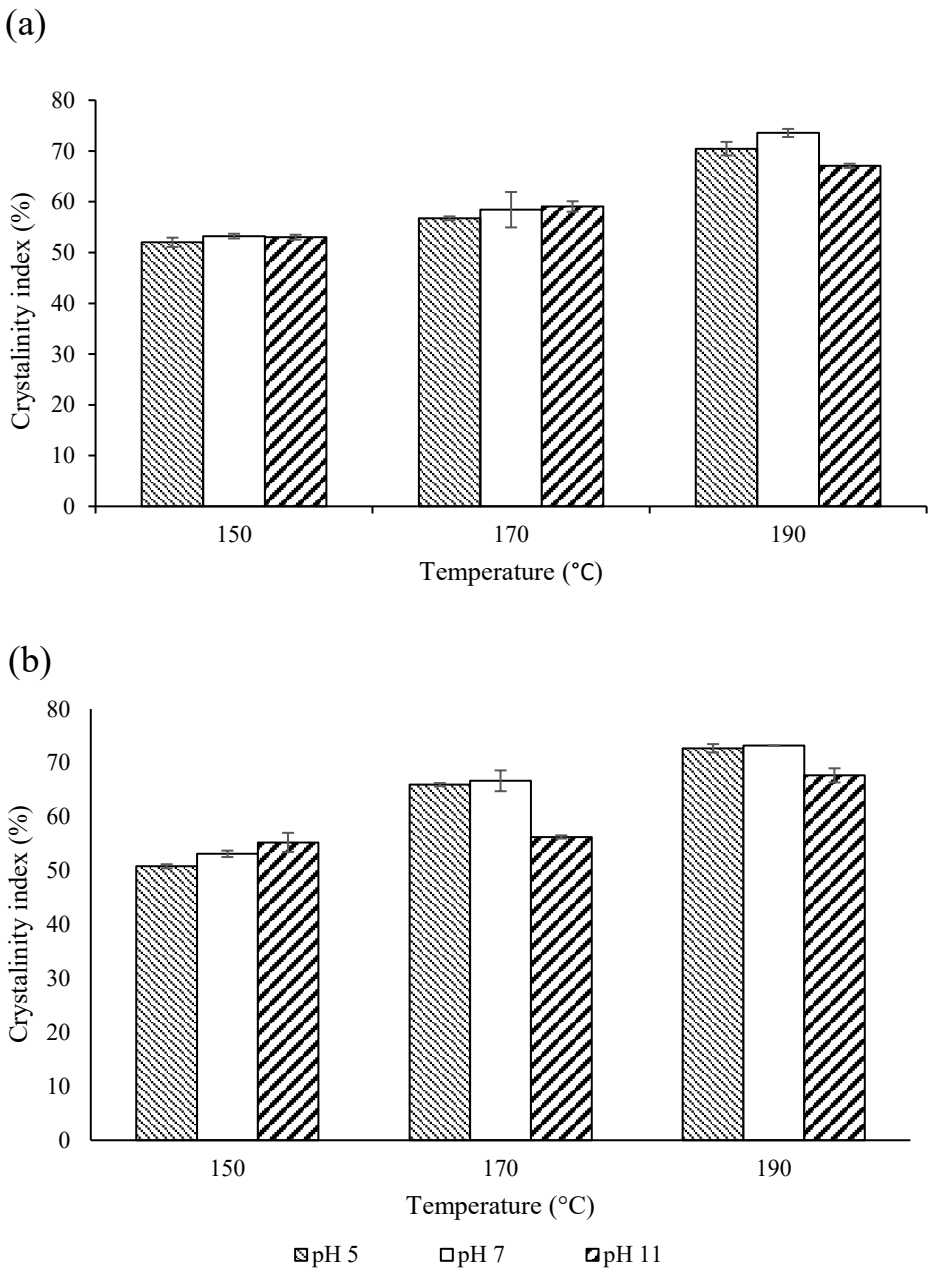

Figure 4.4 Crystallinity of treated radiata pine wood chip at different operating conditions. (a) 5 min, (b) 10 min

This can explain why the crystallinity index has decreased at alkaline pH where it was expected to increase, as lignin removal increased.

4.5 Conclusions

A mild WO pre-treatment of radiata pine wood chips which were first pre-treated with a local New Zealand white rot fungus showed that with an absolute increase in cellulose loss varying between 0.3 to 15 %, the energy required for the whole process decreased by up to 70 % compared to WO pre-treatment alone. XRD analyses showed that there is no significant change between the crystallinity of pre-treated woody biomass with the combined method or WO pre-treatment alone. The maximum selectivity value was at medium temperature, alkaline pH and the shorter reaction time: 170 °C, 11 and 5 min respectively. Selectivity value at this operating condition was 2.3 with 21 % cellulose loss and 50 % lignin removal. An estimation for energy consumption at this operating condition was 115 kJ/g. This means the combination of this fungal strain with WO pre-treatment can provide a potential solution for reducing the high-energy requirements of WO pre-treatment alone.

Chapter 5
Conclusions and future work

This chapter summarises all important discussions from this dissertation and possible future work in this area.

5.1 Conclusions

In this thesis, a combined method of pre-treatment was studied for the pre-treatment of radiata pine wood chips. As the combined method, fungal pre-treatment with New Zealand white rot fungi was coupled with a mild hydrothermal pre-treatment. To achieve this goal, firstly the selected lignocellulosic biomass, New Zealand grown radiata pine, was fungally pre-treated with two native white rot fungi, *Stereum hirsutum* and *Trametes versicolor.* The reasons for choosing the two New Zealand native white rot fungi were:

- New Zealand rules for protection of native plantations restrict the use of international strains as they might be harmful to green plantation and cause changes in the natural native forest ecosystem.
- These two native white rot fungi are naturally known as the factors for mineralising the dead wood in forests.

To find out the effects of fungal pre-treatment, the physical and chemical properties of fungal pre-treatment over 3 to 7 weeks of treatment were analysed.

In addition, to have a better understanding of the effect of hydrothermal pre-treatment on the wood chips, wood chips were hydrothermally pre-treated at different operating conditions, reaction times of 5 and 10 min, pH 5 ,7 and 11 and temperatures of 150 ºC to 190 ºC. Also, the physical and chemical properties of the hydrothermal pre-treatment on different operating conditions were analysed.

Considering both results for sole fungal and sole WO (oxidative hydrothermal) pre-treatment, woody biomass pre-treated by *Trametes versicolor* showed a better result. Therefore, radiata wood chips fungally pre-treated by *Trametes versicolor* were prepared for WO pre-treatment.

A simplified energy assessment was also used to evaluate the energy consumption compared to the combined method and sole WO pre-treatment.

A simplified kinetic model was presented as well, in the appendix of this thesis, to study delignification of the radiata pine wood chips in the WO pre-treatment.

Below is a summary of important conclusions from this research:

1) Fungal pre-treatment of radiata pine wood chips by two New Zealand native white rot fungi, *Stereum hirsutum* and *Trametes versicolor*, from 3 to 7 weeks of degradation showed that these two fungal strains were able to selectively degrade lignin. Comparing both studied fungal strains, *Trametes versicolor* showed a greater selective lignin degradation after 3 weeks of treatment. Although increasing the time of fungal pre-treatment increased lignin degradation, which is favourable, it also caused more cellulose loss. This led to a decrease in selective lignin degradation for both fungal strains. These two native fungal strains also significantly changed the crystallinity index after 7 weeks of pre-treatment. XRD showed that it decreased from 46 % for untreated wood chips to 37 % and 44 % for *Stereum hirsutum*- and *Trametes versicolor*-treated biomass, respectively. Both fungal strains provided a cellulose-rich biomass after 3 weeks of degradation, especially for *Trametes versicolor*.

   SEM images indicated that untreated biomass had a compact structure with an almost non-broken surface structure. The morphology results showed that as time of pre-treatment increased, more cracked surface area appeared in the treated woody biomass. This is also important in cellulose digestibility by hydrolysing enzymes. Considering results for both fungal strains, 3-week biomass pre-treated by *Trametes versicolor* was prepared for the next step of pre-treatment as this fungus showed a better result in lignin removal and less cellulose loss.

2) Partial lignin removal can be achieved by WO pre-treatment at moderate operating conditions. Results for structural carbohydrates analyses and a simple energy estimation showed that an optimised energy consumption and a reduced cellulose loss are achievable. At the early stages of reaction and lower reaction temperature, the crystallinity index did not change significantly. As the reaction temperature increased, the significant change occurred at 190 °C and pH 7 which was a 24 % increase in crystallinity index. Based on the results found in this study, the basic operating conditions may have a better result in terms of minimum cellulose loss. At an alkaline condition and early stages of reaction a higher selective lignin degradation with minimum cellulose loss happened. At pH 11 and 5 min of reaction, selective lignin degradation was 3.2. for this operating condition, lignin removal was around 50 % of its original, while the cellulose loss was 15 %. An energy estimation showed that the higher reaction temperature and shorter reaction time led to a better result for energy consumption, which was around 149 kJ per gram of lignin removal for the above-mentioned reaction condition

3) A mild WO pre-treatment combined with fungal pre-treatment of radiata pine wood chips resulted in an improvement in energy consumption for the whole process. An energy estimation showed that this kind of combined pre-treatment of the lignocellulosic biomass has the potential to decrease the energy required for the process by up to 70 % when compared to the sole WO pre-treatment of radiata pine wood chips. However, as the woody biomass was first pre-treated by the fungal strains, the chemical analyses for structural carbohydrates was higher than the WO pre-treatment alone by up to 40 %. This may not be a matter of concern since the energy saving caused by fungal pre-treatment was quite significant. Selective lignin degradation in this combined pre-treatment method was lower than sole WO. This is

expected since the biomass has been exposed to two steps of pre-treatment, first fungal, followed by a mild WO pre-treatment. XRD analysis showed that the crystallinity index did not change significantly compared to WO pre-treatment.

5.2 Future works

Based on this study, the following recommendations are made:

1) Sugar yield and biofuel yield can be improved by a combination of fungal and other pre-treatment methods. There is still a need to develop a more efficient and effective combined method to make this of commercial interest.

2) A combination of fungal pre-treatment with the other physical and chemical processes may result in a sustainable and cost-effective pre-treatment method. However, only a few studies have investigated the environmental impact and energy balance in combined processes.

3) Severe physico-chemical pre-treatments make cellulose more accessible. This can supress lignin degradation during post-fungal pre-treatment as the accessible cellulose can be consumed by fungi, leading to biofuel/sugar yield reduction. A short fungal pre-treatment prior to physico-chemical pre-treatment may lead to a better result.

4) Production of valuable by-products using pre-treatment methods has not been well studied. Valuable by-products such as organic acids can be subsequently converted into biofuel during fermentation or anaerobic digestion. There is still a need for more research and investigation on operating conditions which maximise the formation of valuable by-products.

5) With regard to recent developments in genetic engineering, developing new selective degrading fungal strains with increased hydrolysis-rate properties might be considered.

6) In this study, only two New Zealand fungal strains were studied for the combined pre-treatment method. The possibility of finding other fungal strains with a potentially better performance is still open.

Chapter 6 Appendix:
Oxidative hydrothermal pre-treatment of radiata pine: A kinetic study

6.1 Introduction

Negative environmental impacts related to fossil fuels have raised the challenge to reduce the amount of environmentally harmful sources of energy. Substitution of some portion of fossil fuels with biofuels can be a possible solution [178]. Currently, biofuels are widely generated using dedicated energy crops, referred to as first generation biomass. This has raised concerns regarding the impact on biodiversity and the competition of land use with food crops [179]. Second generation biomass refers to inedible lignocellulosic biomass and agricultural wastes [180]. These may be the source of cheaper, more environmentally friendly biofuels [2]. Production of biofuels from second generation biomass requires pre-treatment before undergoing any fermentation. This is due to the complex three-dimensional structure of lignocellulosic biomass, as seen in Fig. 6.1. The pre-treatment process partially removes lignin and hemicellulose, and can provide the cellulose portion with an increased accessibility to hydrolysis and a reduction in crystallinity [31, 45, 181].

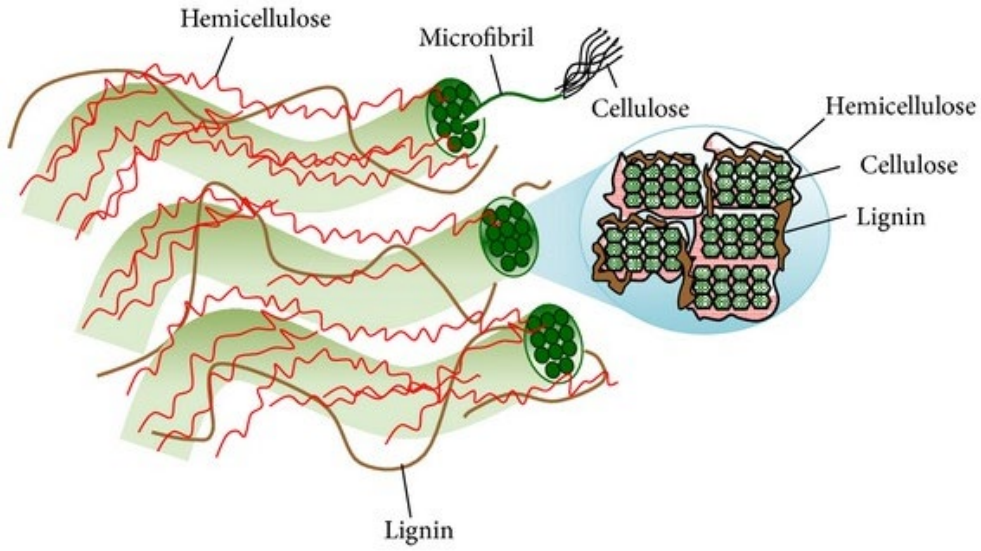

Figure 6.1 Lignocellulosic biomass structure

[182] (Open access article)

This increases the glucose yield significantly, up to 90 % in some cases [183, 184]. Generally, woody lignocellulosic materials consist of cellulose (35–50 %), hemicellulose (20–30 %), lignin (15–35 %), ash and extractives (1–5 %), forming a highly complex three-dimensional structure [185]. In biofuel production from second generation biomass, pre-treatment should selectively degrade lignin and hemicellulose, leaving the highest proportion of cellulose remaining in the pre-treated solid.

Radiata pine is a softwood containing around 40–50 % cellulose and 30–35 % lignin. The high cellulose content makes it a promising biomass for biofuel production [162]. The high-lignin-content of radiata pine is the main barrier for using this biomass in industry. Hydrothermal processing is considered to be a suitable pre-treatment process due to its high delignification yield [186]. Hydrothermal processing is a physio-chemical pre-treatment carried out under temperatures between 120–320 °C and pressures between ~10–200 bar [187, 188]. In oxidative hydrothermal processing, the presence of oxygen free radicals causes the reaction to occur [71]. A generalised reaction pathway with acetic acid as the rate-limiting intermediate, as introduced by Li, Chen [71], is shown in Fig. 6.2.

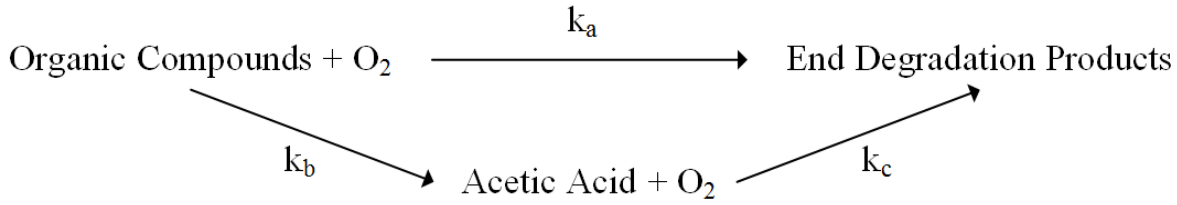

Figure 6.2 A Simplified reaction and pathway for wet oxidation [71]

If the initial concentration of acetic acid is assumed to be zero, then the reaction pathway may be simplified to two parallel first-order equations with kinetics as follows:

$$\frac{A+B}{[A+B]_0} = P_x e^{-k_x t} + P_y e^{-k_y t} \quad (1)$$

Where A represents the organic compounds, B represents acetic acid, $P_{x/y}$ and $k_{x/y}$ are constants representing the portions and rate constants of x and y respectively (these are dependent on k_a, k_b, and $k_{c)}$, and *t* is time (min).

The kinetic study of WO pre-treatment can provide useful information for finding optimal conditions for the process. Degradation of lignocellulosic biomass has been reported to follow the kinetics in Equation 1 as seen in Table 6.1, which briefly presents kinetic models used or developed in previous work. However, in some studies an additional exponential term (hence an additional parallel reaction) has been added in order to increase fit of model, also seen in Table 6.1. These three terms have been referred to in previous literature as: initial, bulk, and residual stages or similarly: fast, medium, and slow regions (moieties). Based on different biomasses and pre-treatment processes, the activation energy for degradation of lignocellulosic components can vary between 10–220 kJ/mol, as seen in Table 6.1.

The overall aims of this study were to: (i) find a model derived from simplified WO kinetics that fit and predict experimental data within the data range with confidence; (ii) estimate activation energies of the different fractions and components of radiata pine; and (iii) find optimum conditions for the WO pre-treatment of radiata pine in order to increase its efficiency for hydrolysis by maximising selectivity criteria.

6.2 Materials and methods

6.2.1 Raw material

Locally sourced radiata pine was used as the lignocellulosic biomass and cut into chips of approximately 1 cm × 1 cm in size. The wood chips were analysed to have a total-solid content of approximately 91.6 %. This was determined by oven-drying samples at 105 ºC for 6–8 h.

The solids were further analysed for composition, and were found to contain 46 ± 2 % cellulose, 35 ± 2 % lignin, and 19 ± 1 % hemicellulose.

Table 6.1 Kinetic models used by previous literature

Kinetic model	Treatment methods	Biomass	Range of activation energies (kJ/mol)	References
One first-order reaction	Organic-solvent	Willow pulp	50–85	[154]
Two parallel first-order reactions	Alkaline oxidation, Autohydrolysis, Organic-solvent	Birch wood, Corncobs, Poplar wood, Wheat straw	10–220	[189-193]
Three parallel first-order reactions	Alkaline oxidation, Organic-solvent	Arundo donax L, Corn stover, Poplar wood	10–150	[192-195]
General power-law reaction	Alkaline oxidation	Softwood pine Kraft pulp	47	[196]
Series of first-order reactions	Autohydrolysis	Sugarcane bagasse	140–160	[197]
Multiple stage/component reaction pathway	Autohydrolysis	Maple wood, Sugarcane bagasse	60–150	[198, 199]

6.2.2 Wet oxidation pre-treatment

WO was carried out in a 1L Parr reactor (a 4540 high-pressure batch reactor equipped with a 4848 controller; Parr Instrument Company, US) with a maximum working volume of 600 ml. A total 10 g of the wood chips were mixed well with 300 mL of deionised water in the reactor and sealed completely to avoid any leakage. For each run, which was repeated twice, the reactor was pressurised to 20 bar with oxygen. The reactor was then heated up to a temperature of 150, 170 or 190 ºC and the reaction was carried out for 2-40 min. The reactor was then immediately cooled down to room temperature. A summary of the experimental conditions used are listed in Table 6.2. The solid phase (suspended solids) was then separated from the liquid phase (solubilised lignocellulosic biomass) by vacuum filtration. Both solid and liquid phases were collected for further analyses.

Table 6.2 Summary of the experimental conditions used

Condition	Value
Volume of water	300 mL
Biomass wet mass	10 g
Oxygen partial pressure	20 bar
pH	7
Temperature range	150–190 °C
Experiment duration	0–40 min

6.3 Analysis

Both untreated and wet-oxidative-treated materials were subjected to two-step acid hydrolysis to determine lignin and structural carbohydrates contents based on the NREL procedure [146]. This procedure includes a two-step hydrolysis using sulphuric acid. Firstly, sugars and the other acid-soluble components were extracted with 72 % (w/w) sulphuric acid at 30 ºC for 1 h. This was followed by dilute sulphuric acid 4 % (w/w) at 121 ºC for another 1 h. Acid-soluble lignin was determined by UV-Vis spectroscopy at 240 nm. Acid-insoluble lignin was quantified by filtration of the residue, based on the NREL method. Wood sugars were quantified by HPLC with a refractive index detection (RID 10A, Shimadzu, Japan) and an Agilent column (Hi-Plex Pb, 300 × 7.7 mm). Deionised water was used as the mobile phase at a flow rate of 0.4 mL/min. The working temperature for the column and the detector was 60 ºC and the injection volume was 10µL. All analyses were at least repeated in triplicate.

6.4 Modelling

The measurements of cellulose, lignin and hemicellulose were expressed in terms of their yield, Y_i, which is defined as follows:

$$Y_i = \frac{C_i \times M}{C_{i0} \times M_0} \tag{2}$$

where subscript letter i is used to denote the three components: cellulose C, lignin L, or hemicellulose H; Y_i is the yield of component i at time t (mass of component i in treated biomass/ mass component i in untreated biomass); C_i is the content of component i in the treated biomass at time t (mass of component i in treated biomass/ mass of treated biomass); C_{i0} is the content of component i in the untreated biomass (mass of component i in untreated biomass/ mass of untreated biomass); M is the mass of treated biomass at time t (grams); and M_0 is the mass of untreated biomass (grams).

As presented by Table 6.1, there are several different kinetic models, from multiple stage/component reaction pathways to simple pseudo-first-order degradation. Many of the reactions investigated take into consideration the partial pressure of oxygen and the pH of the mixture before treatment [193-196]. However, since these variables are kept constant at 20 bar and pH 7 in this study, these can be absorbed into the frequency factor of the reaction rate constant.

Equation 1 can be rewritten as follows:

$$Y_i = \sum_j P_{ij}^T \exp\left(-k_{ij}t\right) \tag{3}$$

where j refers to the different reaction pathways that portions of component i can follow: 'f' for fast-reacting and, 's' for slow-reacting; P_{ij}^T is the maximum fraction of component i involved in reaction pathway j at temperature T (K); k_{ij} is the reaction rate constant of component i for the reaction pathway j and is defined by the Arrhenius equation as follows:

$$k_{ij} = A_{ij} \exp\left(-\frac{E_{A_{ij}}}{RT}\right) \tag{4}$$

where A_{ij} is the frequency factor (min^{-1}) of component *i* for the reaction pathway *j*; E_{ij} is activation energy (kJ/mol) of component *i* for the reaction pathway *j; R* is ideal gas constant 8.314×10^3 kJ/(mol.K) and *T* is absolute temperature (K).

Equation 3 is subject to the constraint:

$$\sum_i P_{ij}^T = 1 \tag{5}$$

Because $Y_i = 1$ at t=0.

In order to fit the models to the experimental data, model parameters were estimated by minimising the sum of squared residuals (SSR) between the experimental data and the fitted model, as follows:

$$\mathrm{SSR} = \sum \left(\mathrm{Y_{i_{exp}}} - \mathrm{Y_{i_{model}}} \right)^2. \tag{6}$$

There are several methods to solve such optimisation problems; however, some of them may yield local minima, which is problematic [200]. This can be overcome by using powerful solvers which find global minima such as the Levenberg-Marquardt method [193, 200]. The 'lsqnonlin' subroutine function in Matlab R15 using the Levenberg-Marquardt method is used in this study.

6.5 Results and discussion

A plot of total suspended solids (TSS) of wet oxidised biomass at different temperature versus time (min) is shown in Fig. 6.3. As temperature and time increases, the percentage of TSS decreases, this indicates that degradation of the biomass is a direct function of these factors. Maximum degradation of approximately 80 % was found at the highest temperature studied of 190°C at 40 min.

6.6 Parameter estimation

The Arrhenius plot of $ln\ k_{ij}$ (ln(min^{-1}), i = C, L, or H, j = f, or s) versus $1/T$ (K^{-1}) for cellulose in Fig. 6.4 a) shows an excellent fit of the fast and slow-reaction rates estimated, with linear regression values (R^2) of 0.995 and 0.993 respectively. Satisfactory fits for the fast and slow-reaction rates were found for lignin and hemicellulose in Figures 6.4 b) and c). For the lignin fast and slow-reaction rates, R^2 values of 0.738 and 0.870 were obtained respectively, hemicellulose yielded R^2 values of 0.825 and 0.806 respectively.

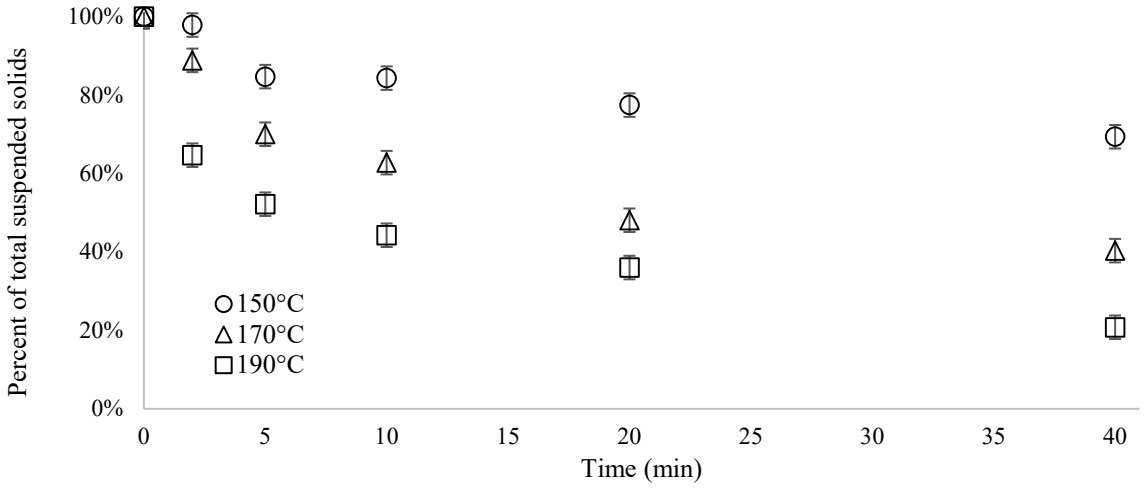

Figure 6.3 Total suspended solids versus time at different temperatures

From the Arrhenius plots, the activation energies ($E_{A_{ij}}$) and frequency factors (lnA_{ij}) were determined as seen in Table 6.3. All the estimated activation energies were in the same range as values from previous literature found in Table 6.1. In previous literature, there were no differences between the fast or slow activation energies. However, the estimated activation energies from this study showed a difference; the fast-reacting portion always had a lower activation energy than the slow-reacting portion. The lower activation energy of the fast-reacting portion increases the relative rate of reaction between the fast-reacting and slow-reacting portion.

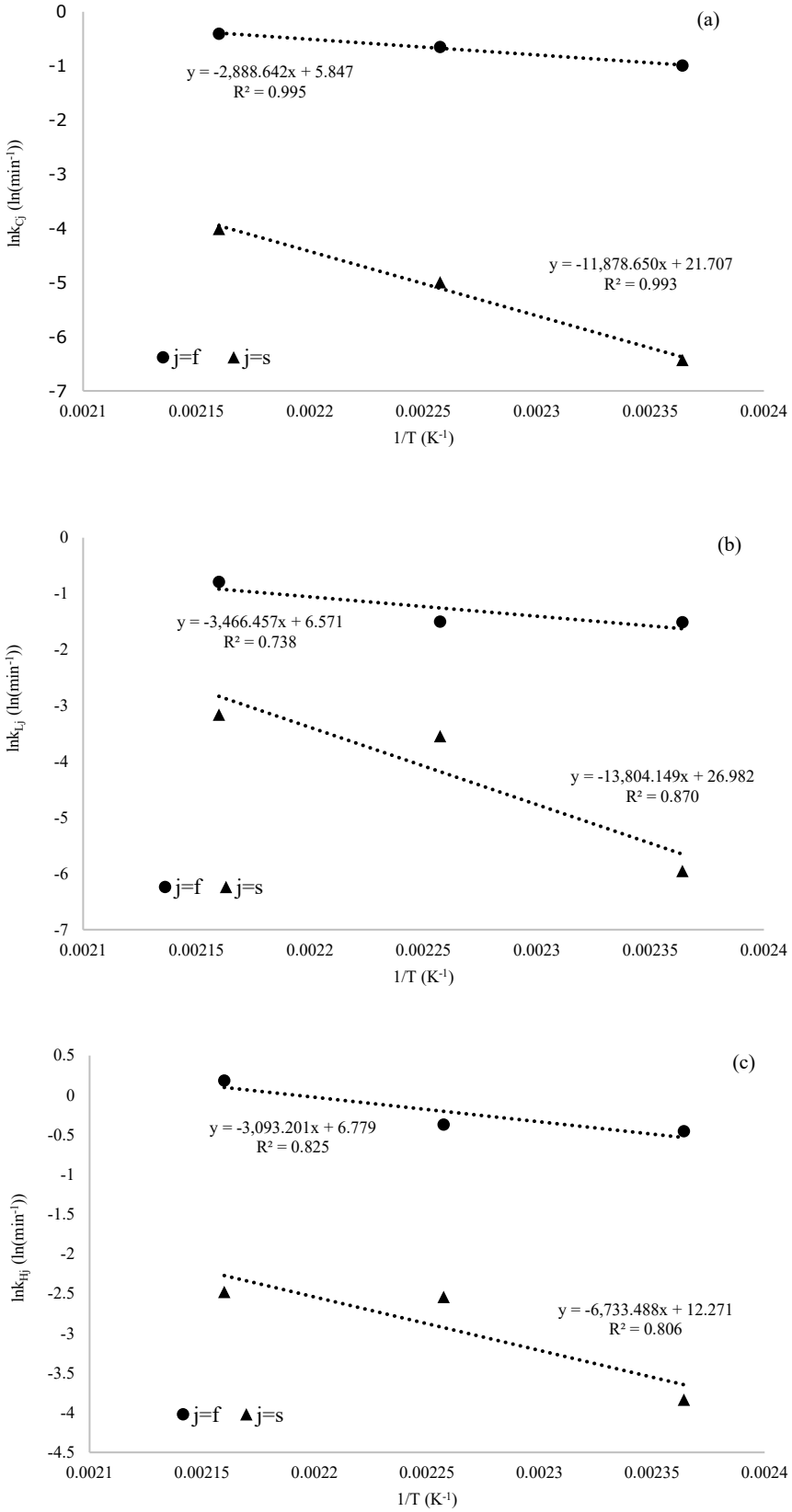

Figure 6.4 lnk_{ij} versus 1/T for: (a) cellulose, (b) lignin, and (c) hemicellulose (f and s for fast and slow region respectively).

Table 6.3 Activation energies and frequency factors for different components, values in parentheses represent standard deviations

Component	Portion (*j*)	$E_{A_{ij}}\left(\frac{kJ}{mol}\right)$	lnA_{ij} (ln (min^{-1})
Cellulose (*i* = C)	f[1]	24.0 (± 1.7)	5.85 (± 0.47)
	s[2]	98.8 (± 8.0)	21.7 (± 2.2)
Lignin (*i* = L)	f[1]	28.8 (± 8.6)	6.57 (± 2.3)
	s[2]	115 (± 22)	27.0 (± 6.0)
Hemicellulose (*i* = H)	f[1]	25.7 (± 5.9)	6.78 (± 1.6)
	s[2]	56.0 (± 14)	12.3 (± 3.7)

[1] Fast-reaction portion

[2] Slow-reaction portion

6.7 Initial yield

At $t = 0$ the summation in Equation 3 results in $P_{ij}^{T} = Y_{ij}^{T}$. This is defined as the initial yield of component *i* for the reaction pathway *j* from raw biomass at temperature T (K) ((mass of component *i* for the reaction pathway *j* / total mass of component *i*) in untreated biomass). Due to the model assumed in Fig. 6.2, the proportions of fast to slow-reacting components are functions of k_a, k_b and k_c, which in turn are a function of temperature due to the Arrhenius equation.

As seen in Table 6.4, all the P_{if}^{T} values found exhibit a positive correlation with temperature. As the temperature increased from 150 °C to 190 °C, P_{Cf}^{T} increased from 0.215 to 0.248, P_{Lf}^{T} increased from 0.100 to 0.329, and P_{Hf}^{T} increased from 0.196 to 0.917. The model shows that as temperature increases, the proportion of fast-reacting to slow-reacting component increases. Cellulose exhibits the least change in proportion while hemicellulose shows the largest. This implies that over the temperature range studied, hemicellulose is the most sensitive to temperature change. Since proportion is a function of k_a, k_b and k_c, which vary with component,

extrapolation out of the temperature range studied (<150°C, or >190°C) cannot be performed with confidence.

Table 6.4 Initial yields of fast and slow portions of different components at different temperatures.

Component *(i)*	Portion (*j)*	*T (°C)*	P_{ij}^{T}
Cellulose (*i* = C)	f[1]	150	0.215
		170	0.231
		190	0.248
	s[2]	150	0.783
		170	0.770
		190	0.750
Lignin (*i* = L)	f[1]	150	0.100
		170	0.294
		190	0.329
	s[2]	150	0.900
		170	0.706
		190	0.664
Hemicellulose (*i* = H)	f[1]	150	0.196
		170	0.654
		190	0.917
	s[2]	150	0.794
		170	0.356
		190	0.081

[1] Fast-reaction portion
[2] Slow-reaction portion

6.8 Goodness of fit

Initially, an additional parallel reaction was considered: medium-rate-reacting portion ‘m,’ in order to increase the fit of the model. However, preliminary studies showed that this model yielded duplicate reaction rates for the medium and slow portions. This indicates that the dual fast and slow-reaction model was sufficient for fitting the data.

Overall fit of the fast and slow model was excellent, with almost all of the predicted values falling within the 95 % prediction intervals as seen in Fig. 6.5. SSR values for each component

were determined. This was to compare the fit of the model with the data, by component. The following SSR values were found: 0.0049, 0.0484, and 0.0278, for cellulose, lignin, and hemicellulose, respectively. Higher SSR values indicate higher accumulation of error. This is consistent with Fig. 6.5, as the only two prediction values that fall outside the 95 % prediction intervals are lignin points.

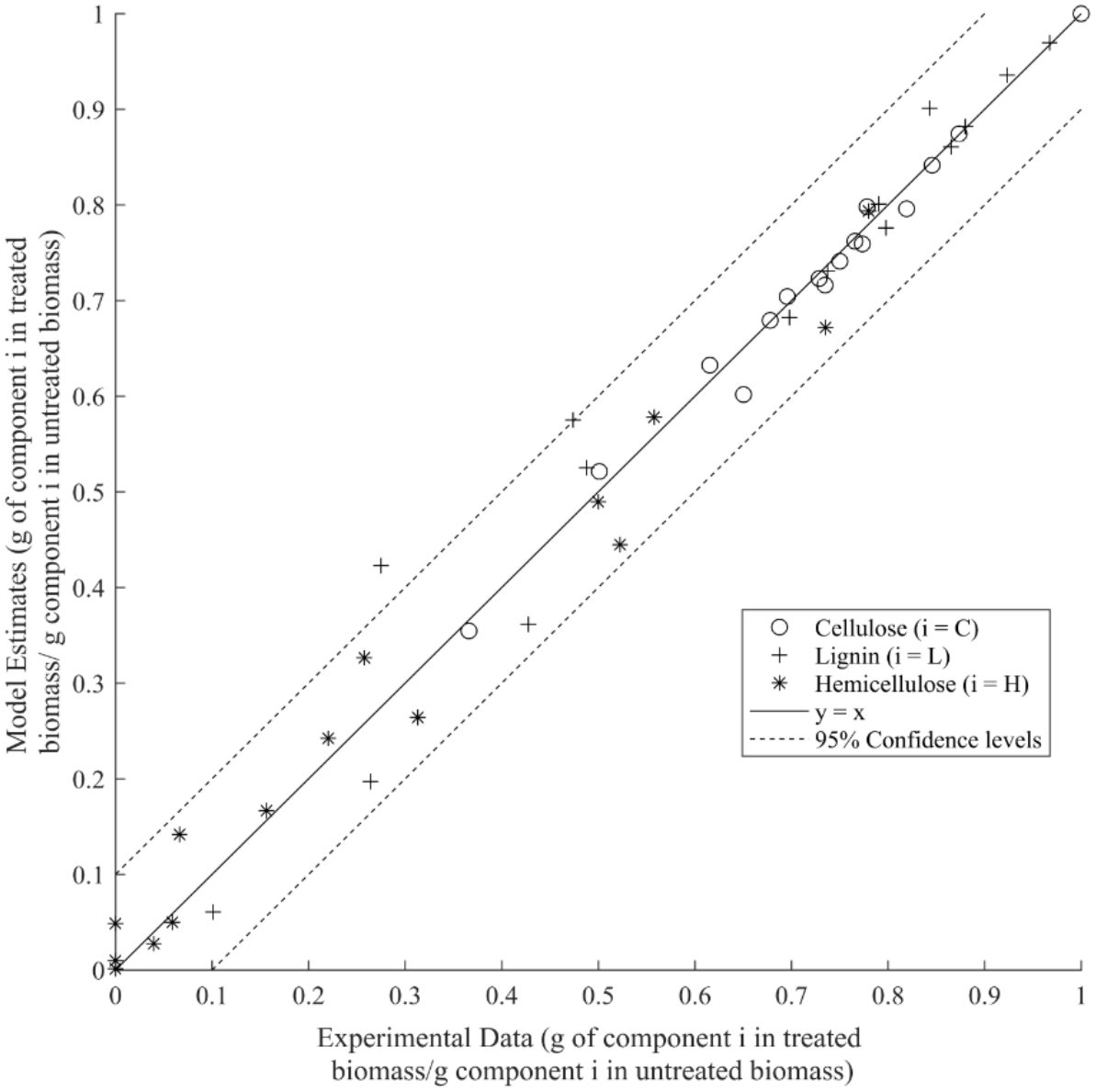

Figure 6.5 Model assessment determined from equations (2) to (6)

6.9 Degradation

6.9.1 Cellulose

Fig. 6.6 (a) shows that as temperature and time increase, so does the degradation of cellulose. The model fits the experimental data very well, with an SSR of 0.0049, the lowest of the three components. At the data point of 170 °C, 40 min, the degradation of cellulose was overestimated, whereas the data points at 150 and 190 °C were predicted within the 95 % confidence intervals of the data, therefore it was concluded that this may be due to experimental error. Maximum cellulose degradation was found at 190 °C at 40 min. Approximately 65 % of the cellulose was degraded by this point.

6.9.2 Lignin

Fig. 6.6 (b) shows that as temperature and time increase, so does the degradation of lignin. At 150 °C the model fits the data very well, however at 170 °C the degradation appears to be overestimated between 0 to 15 min and underestimated from approximately 15 min onwards. At 190 °C, the model overestimates degradation from approximately 7 min onwards. This is reflected by the relatively high SSR (0.0484) of the model for lignin degradation. Maximum lignin degradation was found at 190 °C and 40 min where approximately 90 % of the lignin was degraded.

6.9.3 Hemicellulose

Fig. 6.6 (c) shows that as temperature and time increase, so does the degradation of hemicellulose. The model lies within the 95 % confidence intervals for both 150 and 190 °C; however, the model underestimates the degradation for 170 °C from approximately 15 min onwards. The good fit of the model is shown in the SSR of 0.0278, which is in between cellulose and lignin. Maximum hemicellulose degradation occurred at 170 °C at 40 min and at 190 °C from 20 min onwards. This implies that for complete hemicellulose degradation,

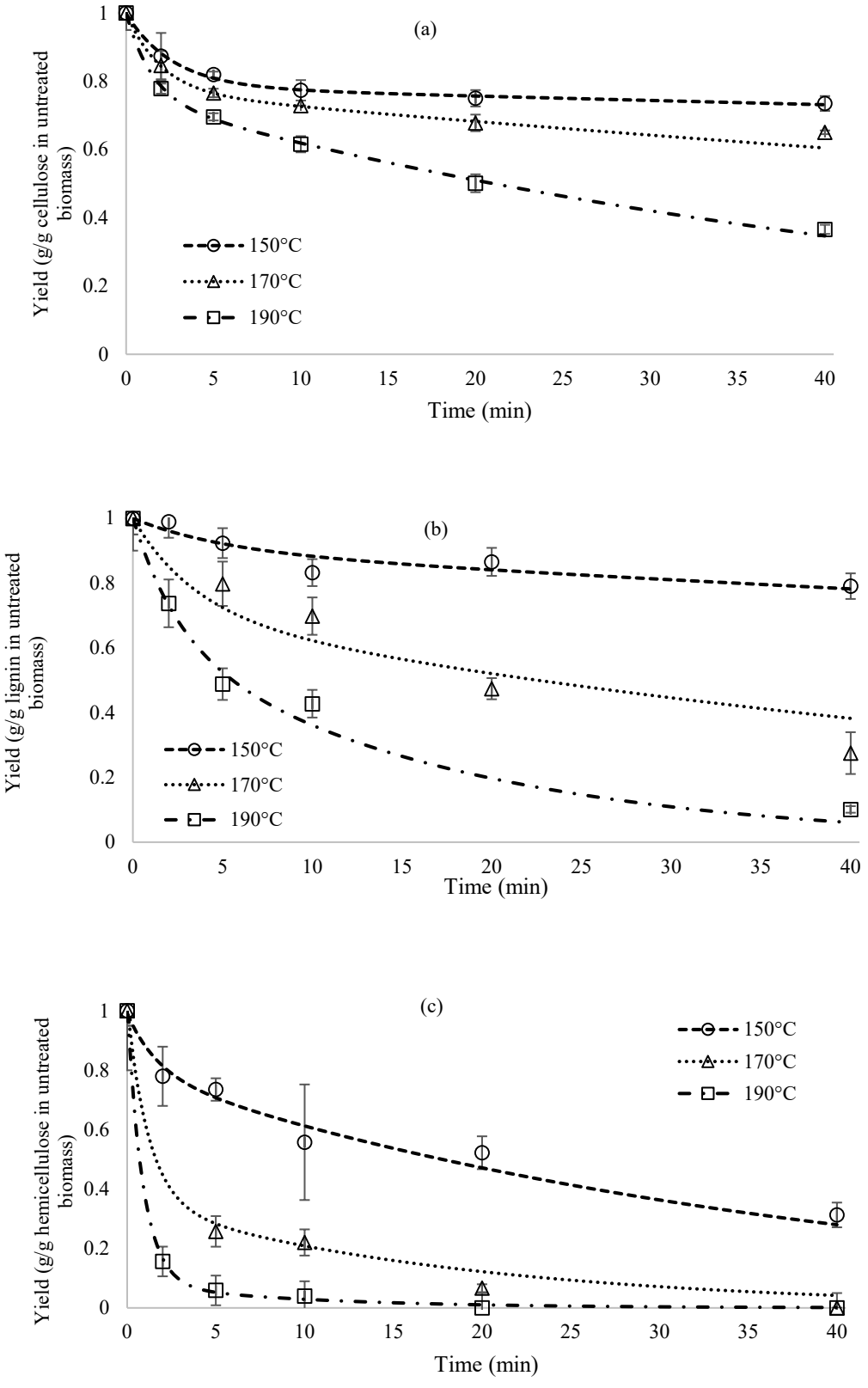

Figure 6.6 Yield versus time for different temperatures for: (a) cellulose, (b) lignin, and (c) hemicellulose. Lines represent value obtained by model

temperatures slightly below 170 °C at 40 min or temperatures between 170 °C and 190 °C for 20 to 40 min may be used.

6.9.4 Optimal operating conditions

From Fig. 6.6, it can be seen that the different components have different degradations and degradation rates at the same temperatures and times. Under the conditions studied, cellulose degrades the least with a maximum degradation of 65 %, followed by lignin at 80 %, and hemicellulose with complete degradation.

Lignin is regarded as the most recalcitrant macromolecule in woody lignocellulosic biomass due to its molecular architecture, where different phenolic units form complex three-dimensional structures [201]. However, due to the structure of woody lignocellulosic biomass seen in Fig. 6.1, cellulose exhibits the highest recalcitrance in WO. The tightly packed microfibrils of cellulose form bundles that are bound together by hemicelluloses and covered by lignin [202, 203]. This reduces chemical accessibility to cellulose therefore increasing its resistance [182]. Hemicellulose is relatively short, branched, and heterogeneous in structure, making it easily hydrolysed compared to cellulose and lignin. It was proposed by Agbor, Cicek [204] that 50 % of hemicellulose should be removed to significantly increase cellulose digestibility [204]. This is used in the following selectivity analysis.

Currently the production of bioethanol from lignocellulosic biomass is dominated by *Saccharomyces cerevisiae* and *Zymomonas mobilis*. The issue with these microorganisms is their relatively narrow substrate range, as they are unable to ferment pentose sugars, only hexose [205]. Therefore, for the current production of bioethanol, degradation of lignin and hemicellulose, while maintaining the cellulose fraction, should be the main concern.

In order to estimate optimum conditions for WO in the range studied, the aforementioned criteria need to be fulfilled and selectivity maxima need to be estimated. The proposed minimum requirement of 50 % for the degradation of hemicellulose was constrained by plotting a three-dimensional plot of 1-Y_H (g/g) versus time (min) and temperature (K). 1-Y_H was plotted instead of Y_H so degradation increased with 1-Y_H. A two-dimensional view can be seen in Fig. 6.7. The black curve indicates 50 % hemicellulose degradation, therefore the surface to the bottom left of the black curve represents sub-optimal conditions. The plot was constructed from 2 min onwards due to the undefined values at t = 0.

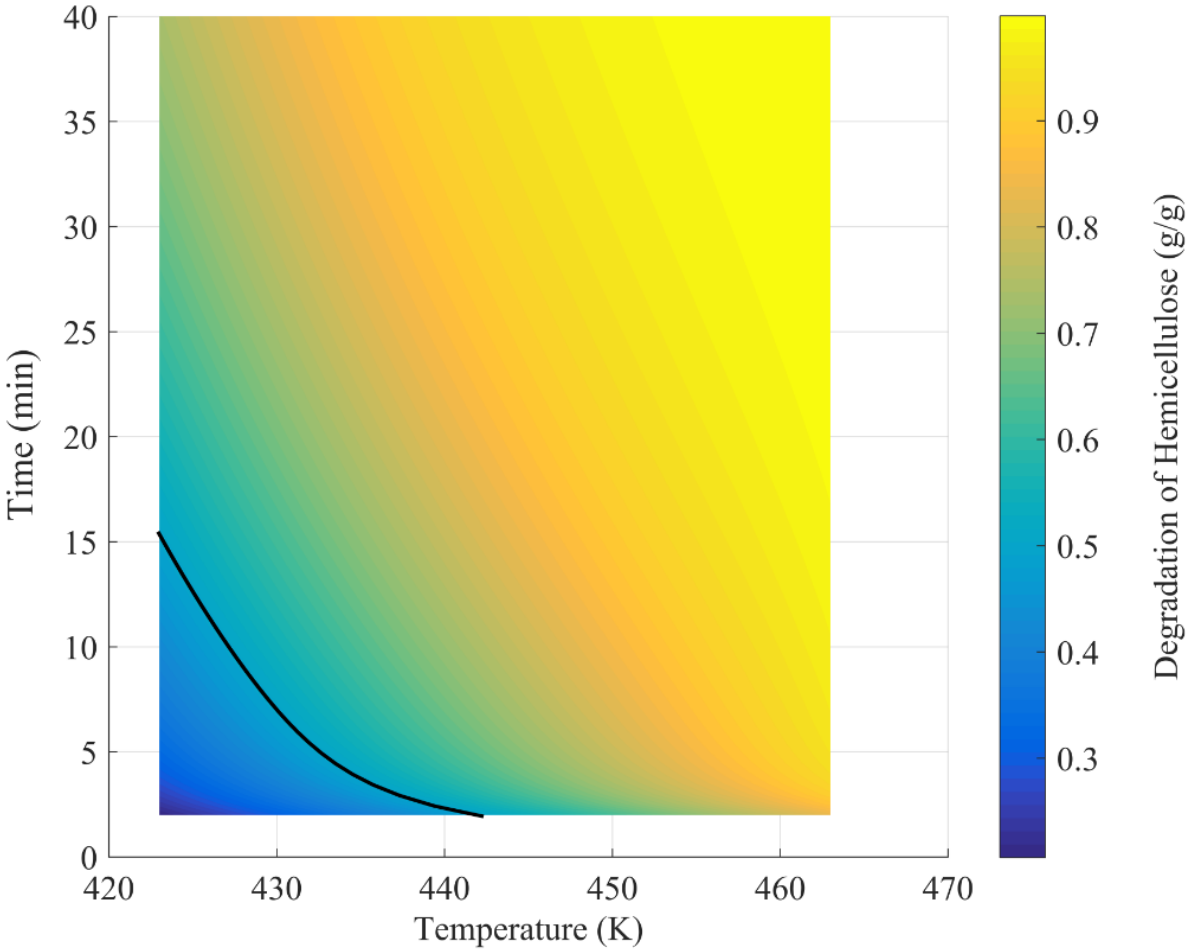

Figure 6.7 Two-dimensional view of a surface plot of hemicellulose degradation versus time and temperature, black curve denotes 50 % hemicellulose degradation

Once the 50 % degradation criteria for hemicellulose was accounted for, the selective degradation of lignin to cellulose needed to be maximised in order to increase chemical accessibility to cellulose, as seen in Equation 7:

$$S_{LC} = \frac{1 - Y_L}{1 - Y_C} \tag{7}$$

A surface plot of S_{LC} (degradation of lignin/ degradation of cellulose) versus time (min) and temperature (K) was constructed, as seen in Fig. 6.8. The black curve was replicated from Fig. 6.7 to Fig. 6.8, representing the region of sub-optimal conditions constrained by the minimum of 50 % hemicellulose degradation. The maximum S_{LC} was found at 190 °C and 12.2 min, where lignin degradation was 1.69 times more than cellulose. This is indicated in Fig. 6.8 as the black triangle. In the pre-treated solid, 59.4 % of initial cellulose, 29 % of initial lignin, and 2.1 % of initial hemicellulose remained. This resulted in the optimal pre-treatment solid being composed of 70.8 % cellulose, 28.2 % lignin, and 1.0 % hemicellulose, by mass. WO at this maximum increased cellulose content in the pre-treated solid by 1.5 times, decreased lignin content by 1.3 times and decreased hemicellulose content by 19 times. The optimum found is constrained by temperature, therefore it can be concluded that there may be more optimal solutions at temperatures higher than 190 °C. Similar optimal conditions were found by Martín, Klinke [24] and Szijártó, Kádár [206] for different substrates [24, 206].

Previous literature emphasises the importance of reducing the amount of degradation of cellulose to increase the final bioethanol yield [186]. Therefore a further constraint was placed in order to reflect this. Optimum temperatures and times were found in terms of amount of cellulose degradation. This is seen in Table 6.5. Fig. 6.8 also has cellulose degradation isolines plotted in blue. For a given percentage cellulose degradation, the surface above the line yields conditions which are sub-optimal (i.e., cellulose degradation is too high). Above 41 % cellulose degradation, the initial maxima found denoted by the black triangle was still valid; however, at

Table 6.5 Final solid properties with maximum SLC by allowed cellulose degradation

% Cellulose Degradation	Optimal % Lignin Degradation	Time (min)	Temperature (°C)	Final Solid Composition		
				Cellulose %	Lignin %	Hemicellulose %
50.0	71.0	12.2	190	70.8	28.2	1.0
45.0	71.0	12.2	190	70.8	28.2	1.0
41.0	71.0	12.2	190	70.8	28.2	1.0
40.0	70.1	11.7	190	64.7	33.1	2.2
35.0	61.4	7.6	190	60.3	36.8	2.9
30.0	51.8	4.5	190	56.2	40.2	3.6
25.0	45.0	2.8	190	53.6	42.1	4.2

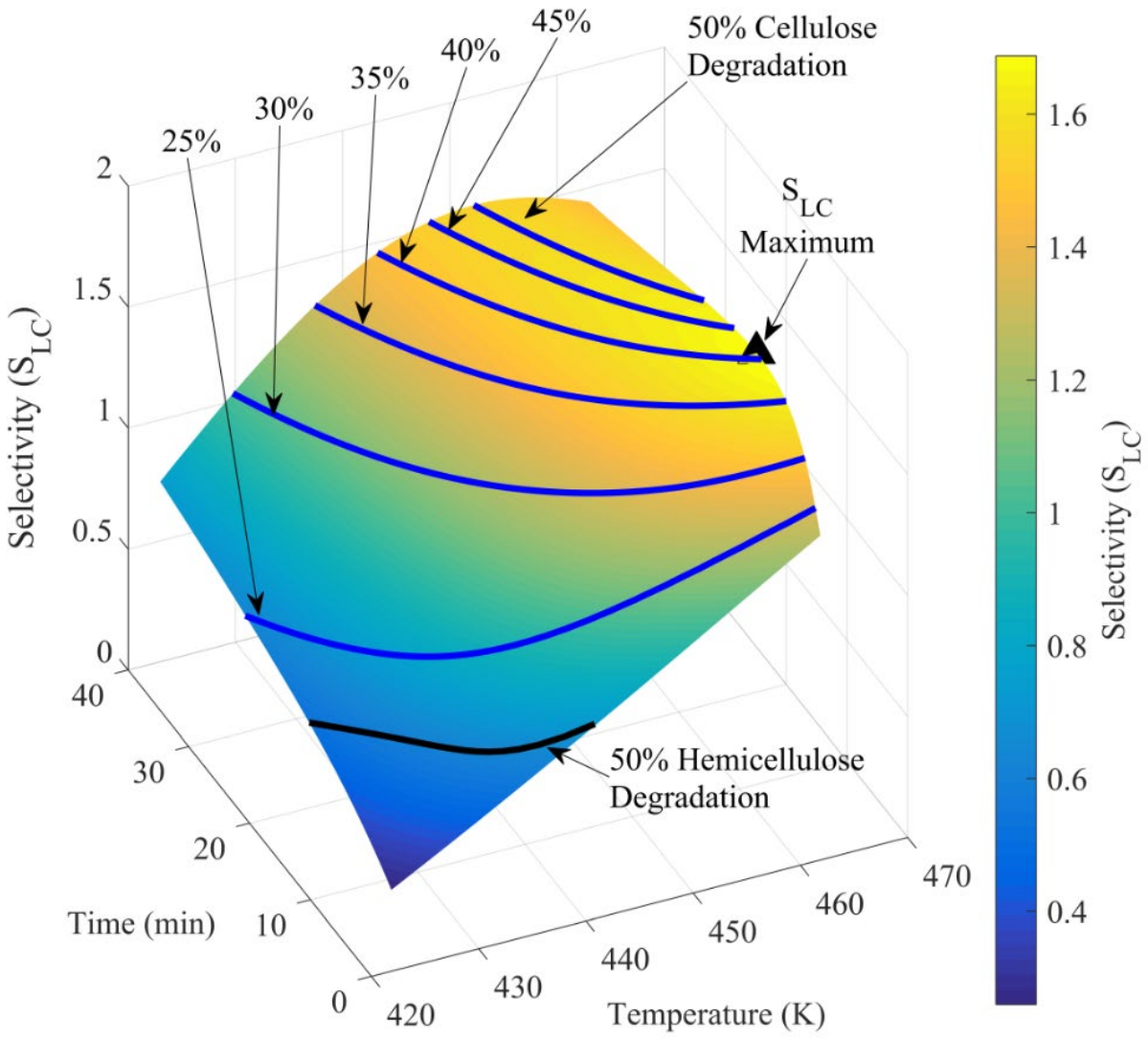

Figure 6.6.8 Surface plot of SLC (degradation of lignin/ degradation of cellulose) versus time (min) and temperature (K), black curve denotes 50 % hemicellulose degradation, black triangle denotes maximum S_{LC}, blue curves denote cellulose degradation

lower cellulose degradation, shorter times were found to be more optimal. Below 25 % cellulose degradation, the optimisation was not valid due to the t > 2 min restriction. For a cellulose degradation of 25 %, the maximum found was at 190 °C and 2.8 min, with lignin degradation of 45.0 %.

6.10 Conclusions

The Levenberg-Marquardt method used was to determine Arrhenius parameters for the fitting of the experimental data to two parallel pseudo-first-order reactions derived from the simplified WO model proposed by Li, Chen [71]. The model showed an excellent fit, with almost all prediction values falling within the 95 % prediction intervals. Activation energies found agree with previous kinetic studies of oxidative pre-treatments on lignocellulosic biomass. Through selectivity analysis, optimal operating conditions were found in the temperatures and times studied.

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