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Effect of Intermediate Washing on Ozonolysis Delignification and Enzymatic Hydrolysis of Wheat Straw

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EFFECT OF INTERMEDIATE WASHING ON OZONOLYSIS DELIGNIFICATION AND ENZYMATIC HYDROLYSIS OF WHEAT STRAW

by

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AUTHOR'S DECLARATION

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ABSTRACT

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Wheat straw was pretreated with ozone to increase the enzymatic hydrolysis yield. Ozonolysis pretreatment was performed in two stages with an intermediate washing step with water in between. Part of the delignification products (lignin fragments) were removed by a washing step so ozone was used to oxidize more lignin rather than oxidizing lignin fragments.

Three parameters, i.e. Initial Water Content (IWC) in wheat straw, Washing Starting Time (WST), and Washing Contact Time (WCT), were optimized to minimize Acid Insoluble Lignin (AIL) content of ozonated wheat straw. Performing an experiment using optimal parameters' values, i.e. IWC equal to 45 wt. %, WST equal to 20 minutes and WCT equal to 80 seconds, showed a drop in AIL content to 9.35 % with an increase in enzymatic hydrolysis yield to 80 % of the theoretical. Readjusting water content of wheat straw to 45 % before performing the 2nd ozonolysis stage further reduced AIL content to 7.36 % and increased the hydrolysis yield to 90 %. Increasing wheat straw fiber size from < 2 mm to < 6 mm decreased the hydrolysis yield to 72 %. All results were considered significant improvements when compared to untreated wheat straw with AIL content of 20.5 % and hydrolysis yield of 23 %.

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NOMENCLATURE

A	Absorbance (-)
a	Absorptivity (l/g. cm)
AIA	Acid insoluble ash (wt. %)
AIL	Acid Insoluble Lignin (wt. %)
ASL	Acid Soluble Lignin (wt. %)
b	Cell path length (cm)
CF	Confidence function
CI	Confidence interval
d	Dilution factor (ml/ml)
DNS	Dinitosalicylic acid
FPU	Filter paper unit
IWC	Initial Water Content (wt. %)
MC	Moisture Content (wt. %)
min.	Minute
OD	Oven dry
PTFE	Polytetrafluoroethylene
RPD	Relative percent difference
RPM	Revolutions per minute
S_b	Standard deviation of the intercept
S_c	Standard deviation for result
sec.	Second

S_m	Standard deviation of slope
S_r	Standard deviation about regression
STDEV	Standard deviation
TS	Total Solids (wt. %)
V	Volume (liter)
WCT	Washing Contact Time (min)
WST	Washing Starting Time (min)

Greek Symbols

σ	Standard deviation of the population
σ^2	Variance of the population

Subscript

calc.	Calculated
exp.	Experimental

CHAPTER 1

INTRODUCTION

Conversion of abundant lignocellulosic biomass to bioethanol as transportation fuel presents a viable option for improving energy security and reducing greenhouse gas emissions. Unlike fossil fuels, which come from plants that grew millions of years ago, bioethanol is produced from plants grown today. It has been reported that cellulosic ethanol and ethanol produced from other biomass resources have the potential to cut greenhouse gas emissions by 86% (Wang and Huo, 2007). There are different sources of lignocellulosic biomasses such as agricultural residues (e.g., wheat straw, sugarcane bagasse, corn stover), forest products (hardwood and softwood), and dedicated crops (switchgrass, salix). Approximately 90% of the dry weight of most lignocellulosic biomasses is stored in the form of cellulose, hemicellulose, lignin, and pectin. For the conversion of lignocellulosic biomass to fuel, the cellulose and hemicellulose must be broken down or hydrolyzed into their corresponding monomers (sugars), so that microorganisms can ferment them to ethanol. Hemicellulose can be readily hydrolyzed by dilute acids under moderate conditions, but much more extreme conditions are needed for cellulose hydrolysis. Three major hydrolysis processes are typically used to produce a variety of sugars suitable for ethanol production: dilute or concentrated acid hydrolysis, and enzymatic hydrolysis which is more widely used.

Many physicochemical, structural and compositional factors hinder the hydrolysis of cellulose present in lignocellulosic biomass to sugars that can later be converted to ethanol. The presence of lignin in lignocellulosic biomass leads to a protective barrier that prevents plant cell attack by fungi and bacteria. The goal of a pretreatment process is

to break down the lignin structure and disrupt the crystalline structure of cellulose, so that the acids or enzymes can easily access and hydrolyze the cellulose. Pretreatment uses various techniques, including chemical, physical, and biological treatments, to alter the structure of lignocellulosic biomass cell wall and to make cellulose more accessible for hydrolysis.

In recent decades, ozonolysis pretreatment has shown its efficacy essentially degrading the lignin polymer content of lignocellulosic biomass. The main advantages linked to this process are the lack of any degradation products which might interfere with the subsequent steps of hydrolysis or fermentation, and the reactions occurring at ambient temperature. Ozone is highly reactive towards compounds incorporating conjugated double bonds and functional groups with high electron densities. Therefore, ozone preferably attack lignin rather than cellulose or hemicellulose due to its high content of C=C bonds. Ozonolysis of lignin releases soluble compounds of lower molecular weight, mainly organic acids such as formic and acetic acid. The range of ozonolysis products is influenced by the structure of the lignocellulosic biomass as well as its moisture content (Mamleeva et al., 2009).

Research objective

The objective of this research was to improve the ozonolysis delignification of wheat straw in order to enhance its subsequent enzymatic hydrolysis. This improvement was achieved by removing some of the delignification products (lignin fragments), such as carboxylic acids, enabling more ozone gas to oxidize even more lignin in the cell wall of wheat straw rather than oxidizing lignin fragments. Lignin fragments were removed by

performing the ozonolysis process in two stages with an intermediate washing step with distilled water followed by filtration before performing the second ozonolysis stage.

The effect of three parameters on the delignification of wheat straw was studied. The first parameter was the Initial Water Content (IWC) in wheat straw. The second parameter was the Washing Starting Time (WST) or reaction time during the first ozonolysis stage. The third parameter was the Washing Contact Time (WCT), or time of immersing wheat straw in distilled water, in between stages.

In summary, this work tried to answer the following questions:

1) What should the total ozonolysis time be?
2) How much water should be used in the intermediate washing step?
3) What are the optimum values of IWC, WST, and WCT to maximize delignification of wheat straw?
4) Will this delignification substantially enhance the enzymatic hydrolysis step?

CHAPTER 2

LITERATURE SURVEY

The increasing problem of CO_2 emissions, besides energy security concerns, has strengthened the interest in alternative nonpetroleum-based sources of energy. Biomass is the only suitable and renewable primary energy resource that can provide alternative transportation fuels such as bioethanol or biodiesel in the short-term.

So far, most bioethanol is made, from what is called first generation biofuel, by yeast fermentation based on soluble sugars that come from sugarcane and corn kernels. Ethanol derived from grain starch has many limitations. Most debates focus on the soaring price of food, which has a great impact on the whole chain of agricultural products and further leads to food security crisis. As a result, widely available lignocellulosic biomass for the production of a second generation biofuels is receiving more attention.

Lignocellulosic biomass includes: agricultural residues (e.g., wheat straw, corn stover, rice straw, cotton gin trash, etc.), forestry wastes (e.g., wood chips, and sawdust) bioenergy crops (sweet sorghum, switchgrass and common reeds), industrial wastes (e.g., paper sludge, recycled newspaper), and municipal solid wastes. Unlike food-based (starch-derived) biomasses, lignocellulosic biomasses offer a series of advantages, such as low cost, abundant supplies, non-competition with grain as food.

Biofuel can contribute to the Canadian energy sector by improving energy security, reducing greenhouse gas emissions and the economical contribution of renewable energy industry to the Canadian economy. In 2011, bioethanol production reached about 2

billion liters per year in Canada (Figure 2.1) and it will continue to grow because of the implementation of new federal renewable standard, i.e. imposing minimum 5 vol. % ethanol content requirements in gasoline in 2010, and because the transportation industries is expanding (Canadian Environmental Protection Act, 2010). Although Canada is self-sufficient in energy sources, the largest province (Ontario) is depending on importing transportation fuel from other provinces. Federal and provincial governments in Canada are trying to support biofuel industries by providing capital cost for building new biofuel plants, by offering tax incentives to producers, and implementing a renewable fuel standards. As a result of these efforts, biofuel industry is growing in Canada.

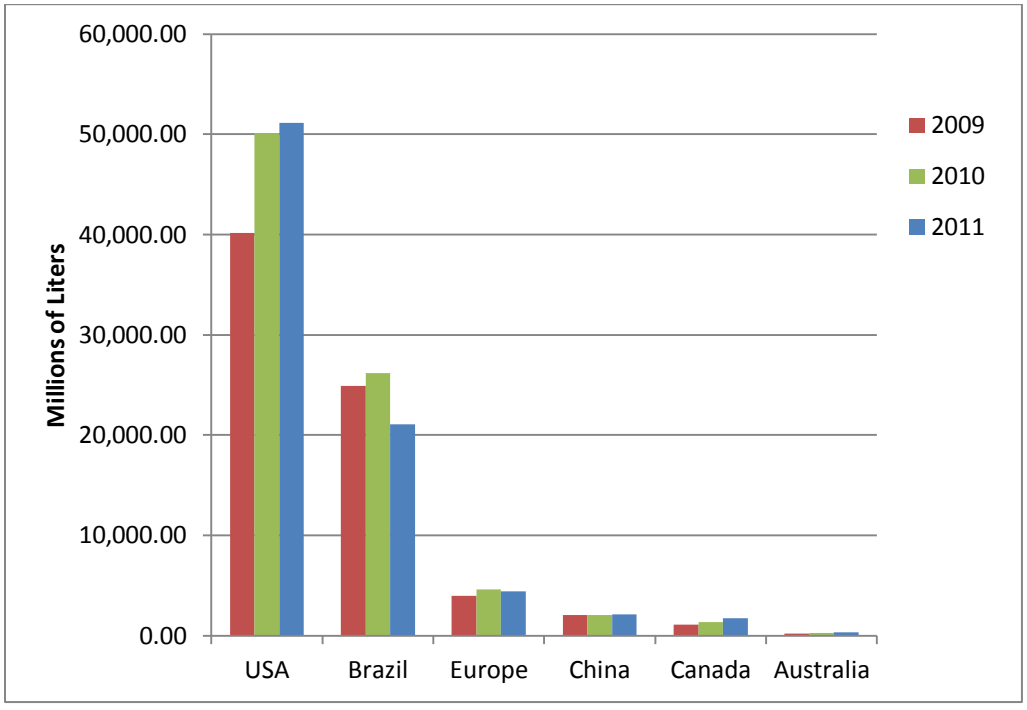

Figure 2-1 World fuel ethanol production (Renewable Fuels Association, 2012).

In 1980, a first bio-energy plant was built in Mimmedosa, Manitoba, followed by a second one in Lanigan, Saskatchewan, in 1990. Both of these plants used wheat starch as a feedstock. Corn starch ethanol production in Canada began in 1997 at a plant near Tiverton, Ontario. In 2009, an estimated 69 per cent of ethanol produced in Canada was made from corn starch, 30 per cent from wheat starch, and 1 per cent from wood waste and wheat straw (Dessureault, 2009).

2.1 Lignocellulosic ethanol

There is a strong interest in the use of lignocellulosic biomass as a feedstock for bio-ethanol production due to their widespread abundance and environmental benefits. However, lignocellulosic ethanol needs new generation of production technologies that have not yet reached the commercial stage of development. In this production technology, cellulose and hemicellulose are hydrolyzed to sugars such as glucose and xylose using either enzymes or (less commonly) diluted acid. These sugars are then converted to ethanol in a fermentation process.

Plant cells in lignocellulosic biomass are tightly packed, cross-linked networks of polysaccharides and contain physical barriers such as lignin and hemicellulose which limit enzyme access to cellulose. Cellulose crystallinity, which occurs due to the hydrogen binding of cellulose fiber sheets to each other, is another barrier against enzyme access to cellulose. Therefore, a pre-treatment process is necessary to enhance accessibility of enzymes to cell wall cellulose and to decrease cell wall crystallinity. Figure 2.2 shows the pathway for the production of bioethanol from lignocellulosic

biomass. The pretreatment process is followed by the enzymatic hydrolysis of cellulose and hemicellulose to their mono-sugars, followed by the fermentation of these sugars to ethanol. It is obvious that bioethanol production and burning cycle are not adding net CO_2 gas to the environment like the fossil fuels.

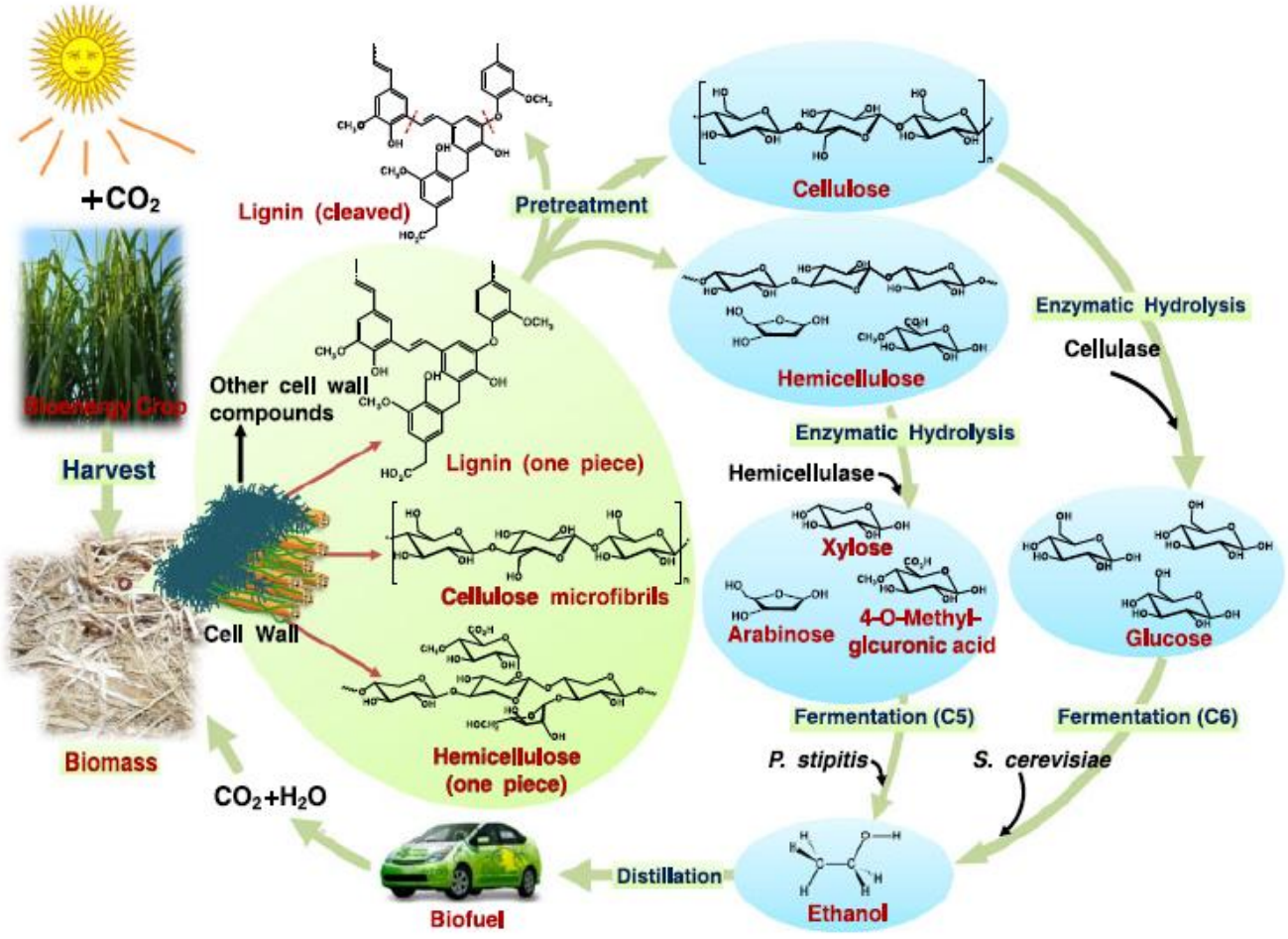

Figure 2-2 Bioethanol production pathway from lignocellulosic feedstock (Sivakumar et al., 2010).

Enzymatic hydrolysis is the process by which several enzymes, such as cellulases, and hemicellulases are used to decompose or hydrolyze polysaccharides (cellulose and hemicellulose) into their component monosaccharides. Saccharification of cellulose releases glucose which is a hexose (six carbons) sugar, while saccharification of hemicellulose releases pentose (five carbons) sugars, such as xylose and arabinose, and six carbon sugars such as glucose and mannose. Pentose sugars cannot be metabolized as efficiently as hexoses by the fermenting microbes and in some cases they can even inhibit fermentation. With the identification or engineering of more efficient microbes, fermentation of pentose sugars may be enhanced (Taherzadeh and Karimi, 2007).

There are several demonstration plants in Canada that produce small amounts of lignocellulosic ethanol. Iogen Corporation has opened its first commercial demonstration plant (Pileci, 2009) for lignocellulosic ethanol in Ottawa, Ontario in 2009. Iogen uses biochemical conversion to produce ethanol from agricultural residues, i.e. wheat, oat, and barley straws, using its own proprietary enzymes. Iogen Corporation has also partnered with Shell Oil to create a joint venture in lignocellulosic ethanol research and development (Iogen Corporation, 2012).

Choosing the right pretreatment method depends mainly on the composition of lignocellulosic biomass, which can vary from one plant species to another. In addition, the ratios between various constituents within a single plant vary with age, stage of growth, and other conditions. Furthermore, the choice of pretreatment has a large impact on all subsequent steps in the overall conversion scheme in terms of cellulose

digestibility and generation of toxic compounds potentially inhibitory for microorganism in the fermentation step.

Knowledge of the chemical structure of the major organic components in lignocellulosic biomass is quite valuable in the development of new processes and improving existing processes in biofuel industry.

2.2 Structure of lignocellulosic biomass

Figure 2.3 shows a basic cell wall structure of lignocellulosic biomass. Cellulose and hemicellulose are tangled together and wrapped by lignin. Cellulose polymer chains or sheets are binding to each other to make the crystalline form or cellulose fiber. Figure 2.3 also shows that hemicellulose connects cellulose to lignin. Lignin is surrounding both cellulose and hemicellulose to protect them against the attack of microorganisms. Depending on lignocellulosic biomass types, the dry weight typically makes up of around 35 to 50 wt. % of cellulose, 20 to 35 wt. % of hemicellulose and 10 to 25 wt. % of lignin (Demirbas et al., 2005).

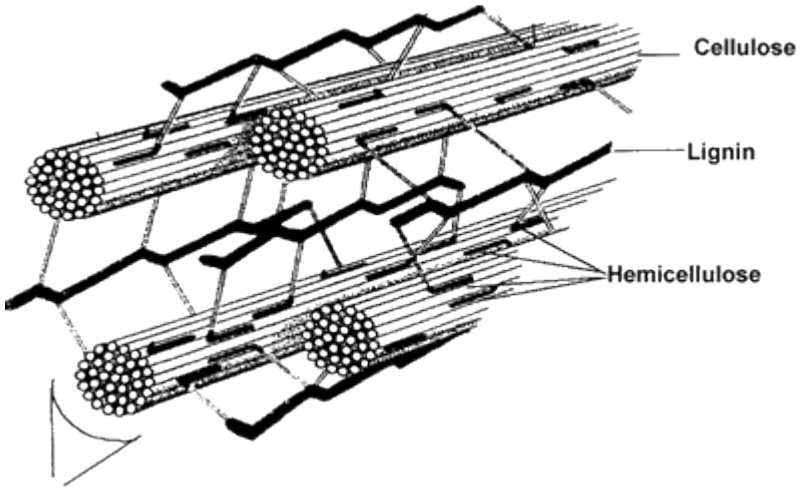

Figure 2-3 Basic structure of lignocellulosic biomass cell wall (Thostrup, 2006).

2.2.1 Cellulose structure

The primary structure of cellulose is a linear unbranched polymer of ß-glucose, connected with 1→4 β-glycosidic bonds. As shown in Figure 2.4 every second glucose residue is "turned upside down" compared to the previous one, i.e., the residues are rotated 180º towards each other. Thus, the repeated unit in cellulose is a cellobiose residue rather than a glucose residue. Cellobiose plays an important role in the enzymatic

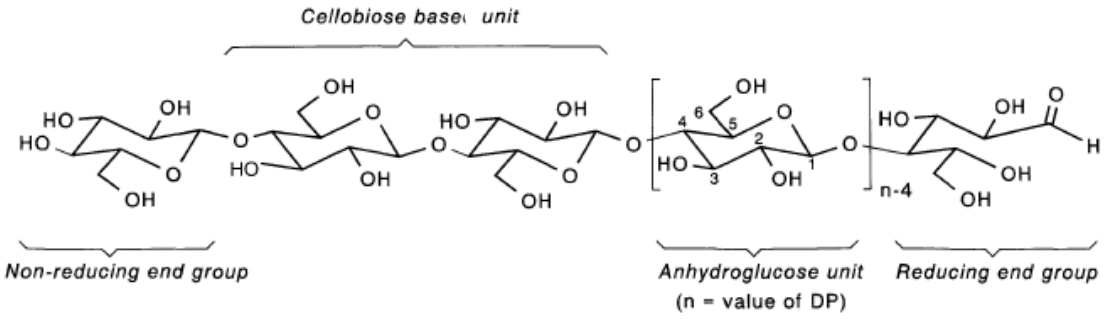

Figure 2-4 Primary structure of cellulose (Klemm et al., 1998).

hydrolysis of cellulose. It is one of the intermediate products in the enzymatic hydrolysis of cellulose which is then hydrolyzed to glucose.

Cellulose crystallinity, which has a great impact on the enzymatic hydrolysis process, occurs due to the binding of cellulose polymer chains (sheets) to each other by hydrogen bonds. When the cellulose sheets bind to each other, they can form two different crystal forms, cellulose I_α and I_β. This comes from the fact that the glucose residues of the first and the second sheets do not stack directly over each others, but there is a displacement in the position of the chains in the cellulose sheets (Figure 2.5). The third layer can be displaced in the same direction as the second, forming cellulose I_α, or in the opposed direction, forming cellulose I_β. The two crystalline forms are thought to co-exist in the cellulose. One of the main duties of the pretreatment of lignocellulosic biomass is to interrupt the crystallinity structure of cellulose to make it more accessible in the enzymatic hydrolysis.

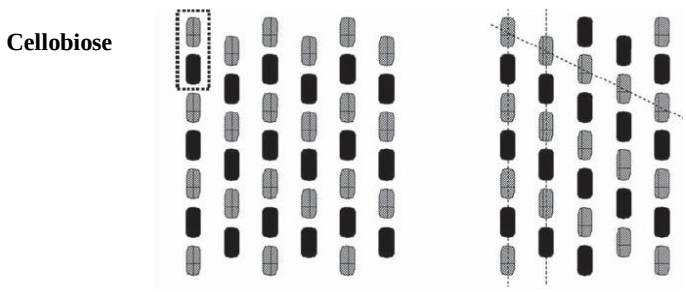

Figure 2-5 Cellulose I_α (right) and I_β (left) (Klemm et al., 1998).

2.2.2 Hemicellulose structure

Hemicellulose is a complex polysaccharide that occurs in association with cellulose in the cell walls of lignocellulosic biomass. Unlike cellulose, hemicellulose consists of branched structures, which vary significantly among different lignocellulosic biomass. Hemicellulose usually represents four general groups of structurally different polysaccharide types: xyloglycans (xylans), mannoglycans (mannans), xyloglucans, and mixed-linkage β-glucans (Ebringerova, 2006). Xylans and mannans are considered the most abundant hemicullulose type in plant kingdom. Figure 2.6 shows the structure of three types of xylan. Each type has a different linkage between its monomers. Figure 2.7 shows D-Galacto-D-mannans (GaM).

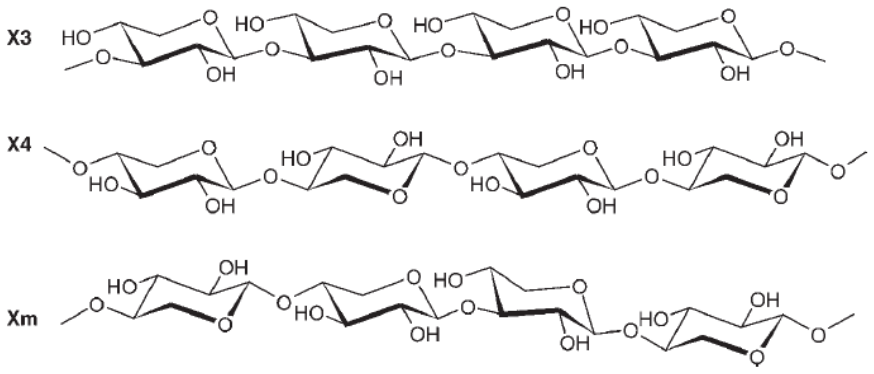

Figure 2-6 Structure of homoxylan chains with β (1→3) linkages (X3), β (1→4) linkages (X4), and mixed β (1→3, 1→4) linkages (Xm) (Ebringerova, 2006).

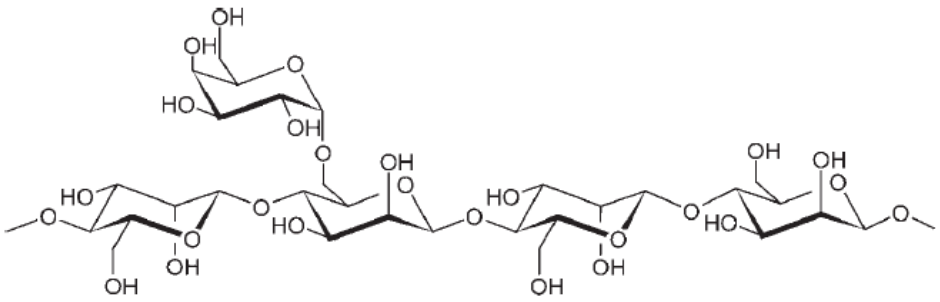

Figure 2-7 Primary structure of D-galacto-D-mannan (GaM) (Ebringerova, 2006).

The hemicellulose, mainly xylan or mannan, accounts for up to a third of the total carbohydrate in most lignocellulosic biomass. Thus, hemicellulose recovery can have a highly positive effect on the economics of ethanol production from lignocellulosic biomass. The observation of the hemicellulose hydrogen bonds to cellulose in lignocellulosic biomass suggests that they are cross-linked. One hemicellulose molecule could bind to more than one cellulose microfibril, thus both connecting and separating neighboring cellulose microfibrils. In this manner, cross-linking hemicellulosic molecules could both keep the cellulose microfibrils apart from each other and potentially influence the ability of the microfibrils to slip past one another. As a result of these cross-linked bonds between hemicellulose and cellulose, lignocellulosic biomass in its original form is relatively resistant to microbial attack, but pretreatment causes extensive changes in the structure of the cell wall which makes cellulose and hemicellulose more accessible to hydrolysis enzymes. Therefore, the enzymatic hydrolysis yield of lignocellulosic biomass is higher after the pretreatment process.

2.2.3 Lignin structure

The term lignin represents a large group of amorphous, highly complex, mainly aromatic polymers of phenylpropane units. Lignin structure could be represented mainly as polymerized hydroxycinnamyl alcohols, typically *p*-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol (Figure 2.8). Lignin derived from these hydroxycinnamyl alcohols are commonly referred to as hydroxyphenyl (H), guaiacyl (G) and syringyl (S) lignin, respectively (Mark and Kroschwitz, 2003).

The complex phenylpropanic structure of lignin provides the lignocellulosic plants cell wall with the physical rigidity to stand upright. The nature and randomness of the linkages in lignin also make lignin one of the hardest biopolymers to degrade, an ideal characteristic for a defensive barrier against the pathogens and herbivores.

Pan et al. (2005) suggested that the presence of lignin reduces the cellulose enzymatic hydrolysis by two distinct mechanisms: by forming a physical barrier that impedes or prevents enzyme from accessing to the cellulose, and by non-productively binding with cellulase enzymes. In fact, lignin irreversibly adsorbs the cellulase enzymes, preventing their action on cellulose (Mansfield et al., 1999). Consequently, for high lignin content biomass, enzymes are blocked by or adsorbed onto lignin allowing only a little to be adsorbed on the cellulose and hemicellulose surfaces. When lignin content in biomass is low, many enzymes can be adsorbed onto the cellulose and hemicellulose leading to a very effective and rapidly digestion of the biomass. The above facts show the importance of adding a pretreatment step before the enzymatic hydrolysis of lignocellulosic biomass in order to remove part of the lignin from the cell wall. In some pretreatment processes,

lignin is broken down to produce different types of lignin fragments. For example, in ozonolysis pretreatment, ozone gas either attacks the aromatic rings or the side chains of the hydroxycinnamyl alcohols (Figure 2.8) in lignin which results in a wide range of aldehydes and carboxylic acids as the delignification products.

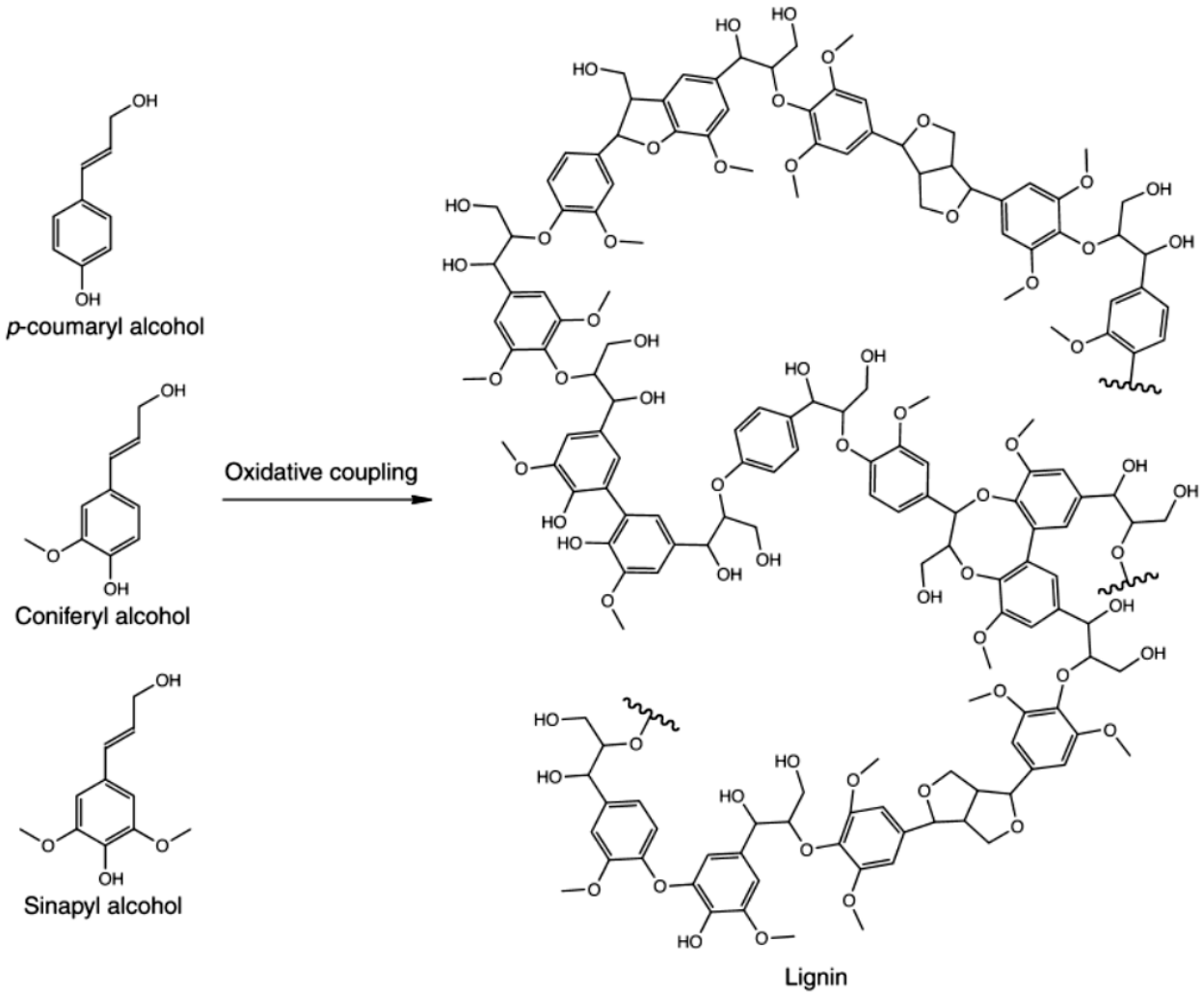

Figure 2-8 Simplified structure of lignin polymer in lignocellulosic biomass cell wall (Weng and Chapple, 2010).

2.3 Pretreatment of lignocellulosic biomass

Many physicochemical, structural and compositional factors hinder the hydrolysis of cellulose and hemicellulose present in lignocellulosic biomass to sugars that can later be converted to fuels. Various pretreatment techniques change the physical and chemical structure of the lignocellulosic biomass and improve hydrolysis rates.

The goal of a pretreatment process is to break down the lignin structure and disrupt the crystalline structure of cellulose, so that the acids or enzymes can easily access and hydrolyze the cellulose and hemicellulose. Pretreatment is considered an expensive process in biomass-to-fuels conversion. It is estimated that 33% of the total cost is due to the pretreatment (Tomas-Pejo et al., 2008). However, improvements in pretreatment efficiency and lowering of its costs merit further detailed research and development. Pretreatment processes must meet the following requirements:

1) Improve the formation of sugars or the ability to subsequently form sugars by hydrolysis,
2) Avoid the degradation or loss of sugars or carbohydrates,
3) Avoid the formation of byproducts that are inhibitory to the subsequent hydrolysis and fermentation processes, and
4) Be cost-effective.

Pretreatment processes can be roughly divided into six categories: physical (such as milling and grinding), physicochemical (such as steam pretreatment and Ammonia Fiber Explosion (AFEX), chemical (such as treatment with alkali, dilute acids, oxidizing agents, or organic solvents), biological, electrical, or a combination of these. After

pretreatment, acids or enzymes can be used to break down the cellulose into its constituent sugars.

There are two major barriers affecting the pretreatment process and the subsequent enzymatic hydrolysis: lignin content and cellulose crystallinity. Delignification is sufficient to obtain a high enzymatic hydrolysis yield regardless of cellulose crystallinity, while crystallinity significantly affects the initial hydrolysis rates but has less of an effect on ultimate hydrolysis yields (Chang and Holtzapple, 2000).

Various pretreatment processes for lignocellulosic biomass are summarized in Table 2.1. It can be seen from Table 2.1 that every pretreatment process has its own advantages and disadvantages but none of them has reached the commercial scale. The choice of the pretreatment technology used for a particular biomass depends on its composition and the byproducts produced as a result of pretreatment. These factors significantly affect the costs associated with a pretreatment method.

Table 2-1 Evaluation of some pretreatment processes in the conversion of lignocellulosic biomass (Kumar, 2009).

Pretreatment process	Advantages	Limitations and disadvantages
Biological	degrades lignin and hemicellulose; low energy requirements	rate of hydrolysis is very low
Pulsed electrical field	ambient conditions; disrupts plant cells; simple equipment	new technology: process needs more research
Mechanical comminution	reduces fiber size; cellulose crystallinity; increases accessible surface area of the substrate to enzymatic attack	power consumption usually higher than inherent biomass energy
Steam explosion	causes hemicellulose degradation and lignin redistribution (depolymerization /repolymerization reactions); increases accessible surface area of the substrate to enzymatic attack; cost-effective	partial hemicellulose degradation; generation of compounds inhibitory to microorganisms
AFEX	increases accessible surface area, removes lignin and hemicellulose to an extent; does not produce inhibitors for downstream processes	not efficient for biomass with high lignin content
CO_2 explosion	increases accessible surface area of the substrate to enzymatic attack; cost-effective; does not cause formation of inhibitory compounds	very high pressure requirements
Acid hydrolysis	hydrolyzes hemicellulose to xylose and other sugars; alters lignin structure	high cost; equipment corrosion; formation of toxic substances
Alkaline hydrolysis	removes part of hemicelluloses and lignin; increases accessible surface area of the biomass to enzymatic attack	long residence times required; irrecoverable salts formed and incorporated into biomass
Organosolv	hydrolyzes lignin and hemicellulose	solvents need to be drained from the reactor, evaporated, condensed, and recycled; high cost
Ozonolysis	reduces lignin content; does not produce toxic residues	large amount of ozone required

It must be emphasized that it is not always possible to transfer the results of pretreatment from one type of material to another. Furthermore, one technology that is efficient for a particular type of biomass material might not work for another material.

Previous studies have shown that ozonolysis pretreatment is an effective delignification process with very slight solubilization effect on both cellulose and hemicellulose in lignocellulosic biomass (Sun and Cheng, 2002). Ozone is highly reactive towards high electron density compounds and functional groups. Therefore, ozone most likely attacks lignin in lignocellulosic biomass due to its high content of C=C bounds and high electron density functional groups. Ozone attacks lignin in several stages releasing compounds of less molecular weight, mainly organic acids such as formic and acetic acids which can result in a drop in pH from 6.5 to 2. The main advantages linked to this process are the lack of any degradation products which might interfere with subsequent hydrolysis or fermentation processes, and the reactions occurring at room temperature and normal pressure. Furthermore, the fact that ozone can be easily decomposed by using a catalytic bed or increasing the temperature means that processes can be designed to minimize environmental pollution. A drawback of ozonolysis is that a large amount of ozone is required, which can make the process expensive. Therefore, the process needs further investigation to improve it. The high consumption of ozone could be coming from the use of ozone in the oxidation of lignin fragments. Therefore, it is worth to investigate the effect of removing some of these lignin fragments on the reduction of total ozone consumption during the ozonolysis process while keeping the desired delignification effect at its highest level.

2.4 Ozonolysis of lignocellulosic biomass

Ozone has been used to de-lignify a variety of lignocellulosic biomass such as wheat straw (Ben-Ghedalia and Miron, 1981), bagasse, green hay, peanut, pine (Neely, 1984), cotton straw (Ben-Ghedalia. and Shefet, 1983) and poplar sawdust (Vidal and Molinier, 1988).

The rate of enzymatic hydrolysis was increased by a factor of 5 following 60% removal of the lignin from wheat straw using ozone pretreatment (Ben-Ghedalia and Miron, 1981). Enzymatic hydrolysis yield increased from 0% to 57% as the percentage of lignin decreased from 29% to 8% after ozonolysis pretreatment of poplar sawdust (Vidal and Molinier, 1988).

Reactions of ozone with lignin and its derivative, lignin-sulfonate, have been studied for several years, and the kinetics of lignin oxidation by ozone in acidic and basic solutions has received attention. Lignin oxidation by ozone in acid media is accompanied by destruction of aromatic rings and formation of a number of carboxylic acids.

Most ozonolysis experiments were conducted in hydrated fixed bed reactors which led to more effective oxidation than in aqueous suspension batch reactors. Neely (1984) reported that the number of ozone moles consumed to oxidize one lignin mole was 3 in the solid state and 7 in the slurry. This result substantiated Neely's conclusions in which he identified excessive dilutions are the reason for excessive consumption of ozone. Euphrosine-Moy et al. (1991) ozonated hydrated poplar sawdust (45 wt. % moisture content) and identified oxalic and formic acids as the major products in the aqueous extract of the treated material, along with glycolic, glycoxylic, succinic, glyceric,

malonic, *p*-hydroxybenzoic, fumaric, and propanoic acids. Morrison and Akin (1990), on the other hand, used ozone to oxidize herbaceous species moistened to 50% and identified caproic, levulinic, *p*-hydroxybenzoic, vanillic, azelaic, and malonic acids and aldehydes such as *p*-hydroxybenzaldehyde, vanillin, and hydroquinone in the aqueous extract. It was concluded that delignification process is occurring in sequence reactions with a lot of intermediate products.

Ozone is one of the strongest oxidizing agents, exceeded in electronegative oxidation potential only by Fluorine, Fluorine monoxide, and the oxygen atom. Ozone is formed from oxygen in a strongly endothermic reaction and decomposes easily into molecular and atomic oxygen. Most commonly, ozone is obtained by passing air or oxygen through a corona discharge. Some of the oxygen molecules are dissociated into oxygen atoms, which in turn combine with oxygen molecules to form ozone.

The ozone molecule is generally classified as a 1, 3 dipole (Figure 2.9). In one resonance form, the central atom and one terminal atom possess full octets of electrons, while the other terminal oxygen atom has only a sextet of electrons. This electron deficiency in the terminal oxygen gives ozone an electrophilic character and is responsible for its electrophilic attack on electron rich substrates.

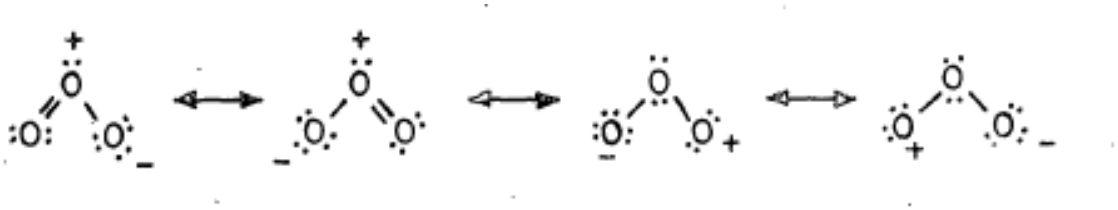

Figure 2-9 Resonance forms of ozone (Singh and Eckert, 1975).

The reaction of ozone with carbon-carbon double bonds has been thoroughly studied and a great deal of experimental data has been accumulated for this particular reaction. Since lignin contains olefinic and aromatic bonds, a brief discussion of ozone reaction with olefins is relevant. The ozonolysis reaction proceeds by a three-step mechanism proposed by Criegee (1975). The Criegee mechanism of ozonolysis is outlined in Figure 2.10. The olefins and ozone form an intermediate (initial ozonide). Next, the initial ozonide reverts to its corresponding carbonyl oxide (also called the Criegee zwitterion). The zwitterion serves as a reactive intermediate and can follow several pathways to produce reaction products. The most important pathway, from the standpoint of ozonolysis, is recombination of the zwitterion with the carbonyl fragment to form the final ozonide as shown in Figure 2.10.

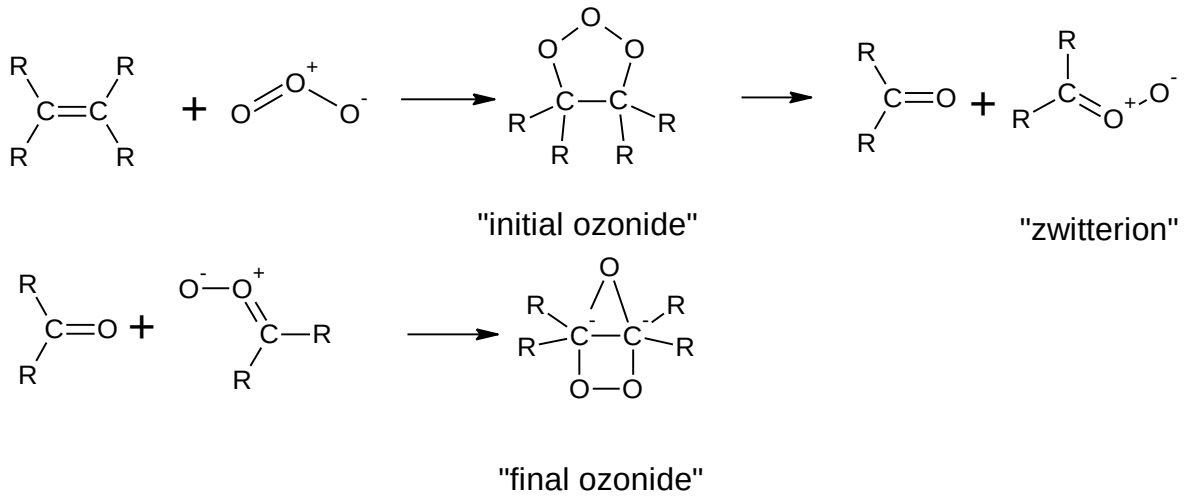

Figure 2-10 Criegee mechanism for ozonolysis of olefines (Criegee, 1975).

The second pathway involves reaction of the zwitterion with participating solvents. Figure 2.11 illustrates a zwitterion reaction with water to form hydroxyhydroperoxide, which can in turn hydrolyze to formaldehyde and hydrogen peroxide.

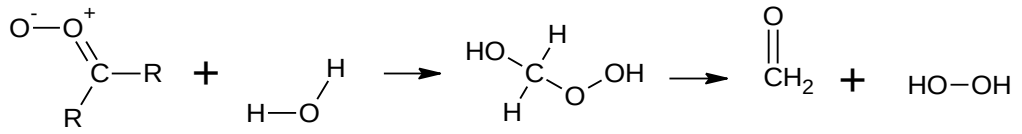

Figure 2-11 Reaction of zwitterion with water.

The reaction of ozone with aromatic compounds is more complicated than ozone reaction with olefins. For example, phenol was ozonated in water at 30 ºC and it was found that approximately 17 mole-equivalents of ozone were needed to consume one mole of phenol and much more to use up the intermediates (Yamamoto et al., 1979). The major products

were formic acid and carbon dioxide, several intermediates were detected such as catechol, hydroquinone, muconic acid, maleic aldehyde acid, glyoxalic acid, glyoxal, oxalic acid, and hydrogen peroxide (see Figure 2.11). In another study by Gould and Webber (1967), it was found that in an aqueous solution 4-6 moles of ozone per mole of phenol were sufficient to destroy phenol aromatic ring but that many more (about 150 moles or more) were required to destroy all organic materials completely to CO_2. These results show the excess amount of ozone which is consumed by the intermediate compounds to reach the final products.

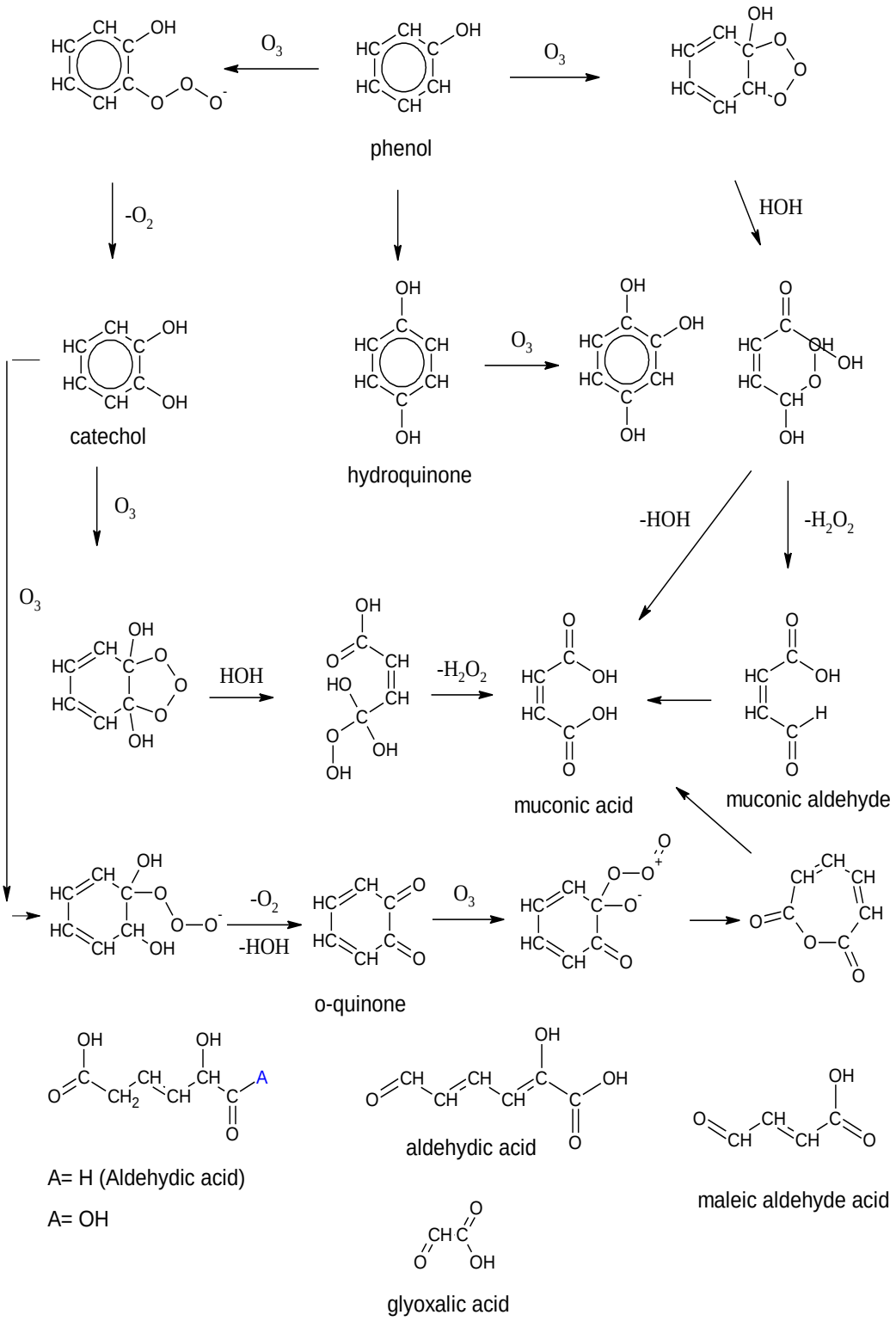

Figure 2-12 Ozonolysis of phenol in water (Yamamoto et al., 1979).

Many researchers have studied the reaction of ozone with lignin model compounds. Lignin model compounds have ranged from monomeric and dimeric lignin model compounds to more complicated structures including HCl, methanol and sulfite lignins. All these studies were conducted in order to understand the mechanisms of lignin ozonolysis.

In several studies, derivatives of guaiacyl or veratryl nuclei were used as a lignin model compounds. A selective ring cleavage was observed as a result of ozonolysis process. This resulted in the formation of muconic acid derivatives which underwent rearrangement, and were usually isolated as muconate lactones (Kojima et al., 1978). An example of the selective ring cleavage reaction is shown in Figure 2.13.

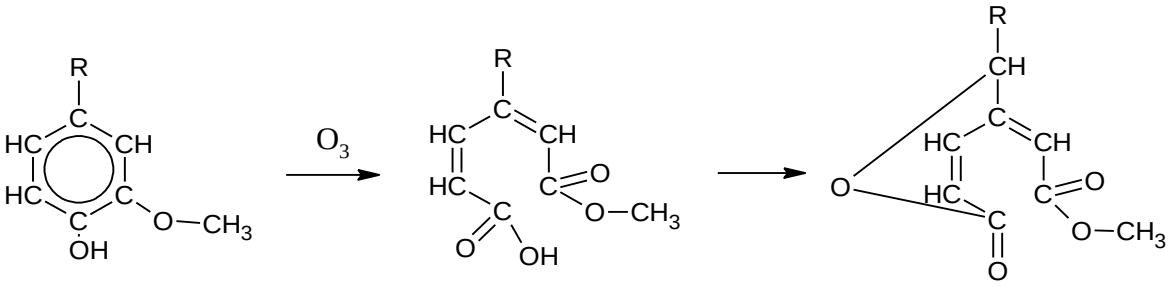

Figure 2-13 Selective ring-cleavage reaction for ozone-oxidation of lignin model compound (guaiacyl) (Kojima et al, 1978).

Ultimate degradation of the aromatic ring in lignin models compound (guaiacyl) was found to produce primarily acidic reaction products as shown in Figure 2.14.

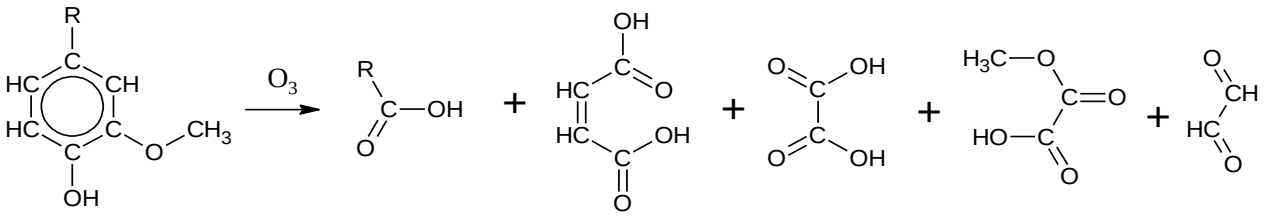

Figure 2-14 Ozone-oxidation of lignin model compound (guaiacyl) to form acidic reaction products (Kojima et al., 1978).

Side-chain oxidation was also observed in the ozonolysis of lignin model compounds (Kojima et al., 1978). Aldehydic or carboxylic functional groups were produced from this reaction as shown in Figure 2.15.

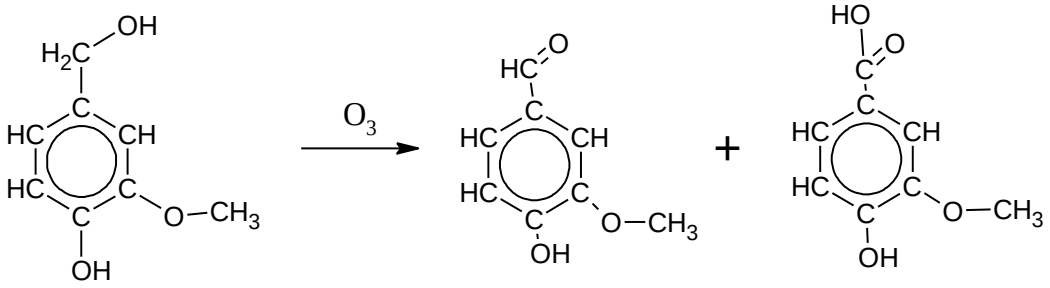

Figure 2-15 Ozonolysis of side-chain in lignin model compound (Kojima et al., 1978).

In the case of dimeric lignin model compounds, which consist of two aromatic monomers, it was found that ozone was capable of cleaving the bond connecting the lignin monomers and several types of oxidation products were observed as shown in Figure 2.16.

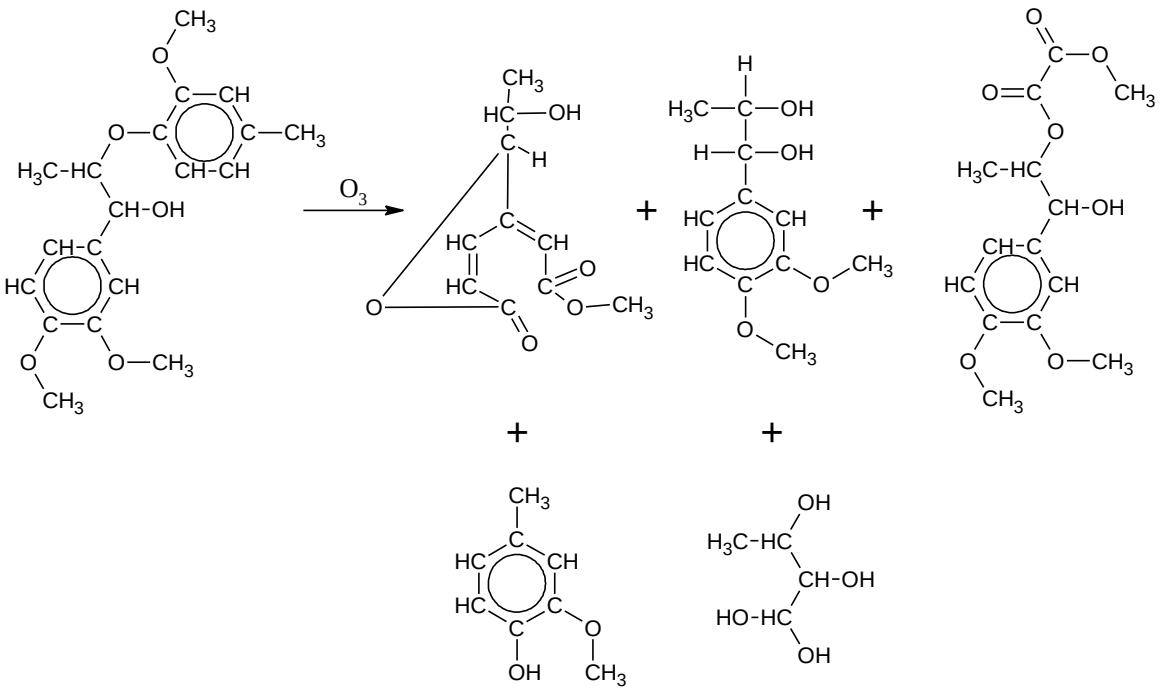

Figure 2-16 Ozonolysis of β-aryl ether lignin model according to Balousek (1979).

Kojima et al. (1978) also identified a poly-condensation reaction between ozone and lignin model compound (guaiacyl) in addition to the aromatic ring and side chain oxidation reactions. It was suggested that a radical coupling reactions between phenoxy radicals were also taking place (Figure 2.17). This reaction is mostly observed in the reaction of ozone with dry lignocellulosic biomass where lignin reacts with gaseous ozone.

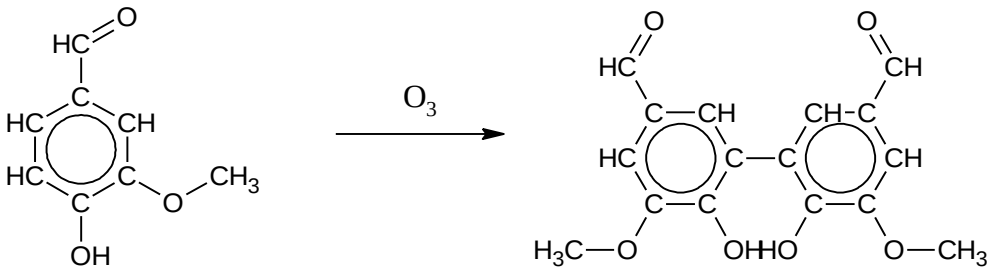

Figure 2-17 Formation of poly-condensed reaction products through ozonolysis of lignin model compound (Kojima et al., 1978).

The above mechanisms for the ozonolysis of lignin, either by the selective ring cleavage or by the side chain reaction, show that the excess amount of ozone is consumed by the intermediate products (lignin fragments). This consumption of ozone could be reduced significantly if part of these fragments were removed from the reaction surrounding. One way of achieving this is to wash partially ozonated lignocellulosic biomass with water to remove the soluble portion of lignin fragments. In this case the ozonolysis process will be divided in two stages with an intermediate washing step between them.

Since the substrate in this study was lignocellulosic biomass (wheat straw) rather than an isolated lignin, possible reaction of ozone with the cellulose must also be considered. Glucose was identified as the primary reaction product from the ozonolysis of methyl glucoside. A sharp drop in degree of polymerization for ozonated methyl cellulose and kraft pulp was also observed. Volatile materials such as methanol, formaldehyde, and formic acid were detected from the reaction of ozone with methyl glucoside and methyl cellulose (Katai and Schuerch, 1966).

Katai (1966) proposed a mechanism for the ozonolysis of cellulosic materials with direct ozone attack (ozone initiated hydrolysis) as the most important reaction (Figure 2.18).

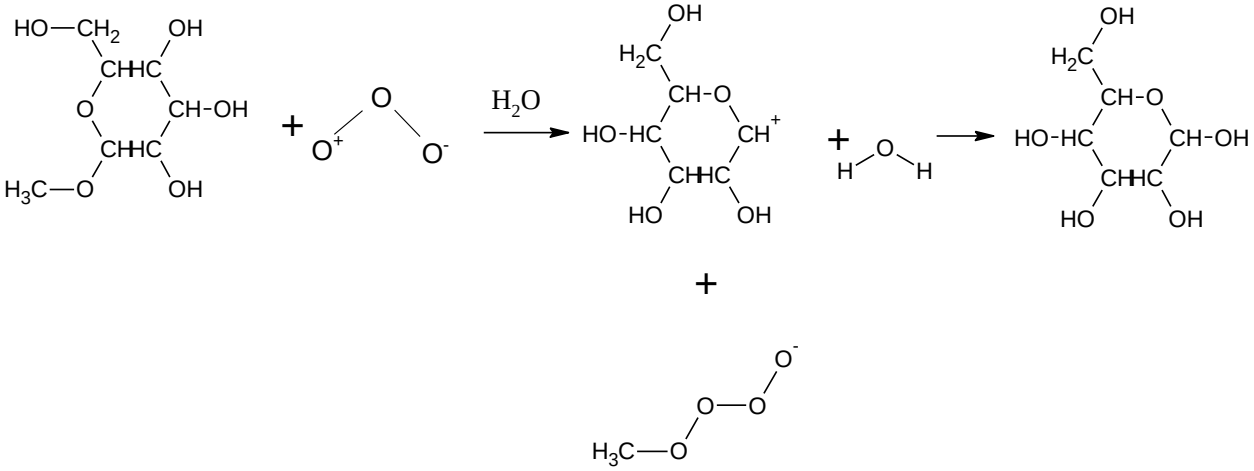

Figure 2-18 Mechanism for ozone-initiated hydrolysis of methyl cellulose (Katai, 1966).

According to the mechanism shown in Figure 2.18, although the reaction of cellulose with ozone is possible, García-Cubero et al. (2009) reported that this reaction is negligible and there was no loss of cellulose after the ozonolysis of wheat straw. Binder et al. (1980) also observed that a loss in cellulose due to ozonolysis pretreatment was negligible when working at low pH values.

2.4.1 Effect of water on ozonolysis process

It is well known that lignocellulosic materials swell in the presence of water or water vapor. The hydrogen bonds between water molecules and the hydroxyl groups of the carbohydrate fibers in the lignocellulosic biomass increase the gap between the carbohydrate fibers, resulting in swelling of the biomass. The distance between

carbohydrate fibers increase as a result of that and these gaps or cavities become filled with water. The saturation condition is achieved at equilibrium moisture content (EMC). Each lignocellulosic biomass has a specific EMC. For example, the value of EMC for wheat straw at 25 °C and relative humidity of 90 % is about 28 wt. % (Duggal and Mui, 1981). When the moisture content (MC) of the lignocellulosic biomass is above EMC, the cell cavities become filled with water. This type of water is called "free water". Water which is chemically bounded to the cell wall carbohydrate fibers by hydrogen bound is called "bound water" (Walker, 2006).

When ozone gas passes through wet biomass it will be absorbed and it will penetrate, due to diffusion phenomenon, through several layers of water until it reaches the cell wall surface. Reactions between dissolved ozone and lignin will occur at this stage. Complex polymer composites containing lignin fragments will be formed and remain inside the cell wall cavities. Therefore, lignin fragments will block the cavities and prevent dissolved ozone from reaching and further reacting with lignin. At this stage, ozone will mostly be used to oxidize these fragments breaking them down to smaller fragments. Dissolution of these fragments in water is limited because of the decreasing of bulk water content in the lignocellulosic biomass during the ozonolysis process. The decrease in water content is due to evaporation effect caused by ozone/ oxygen gas flow through the biomass. As MC becomes less than EMC, ozone consumption in the ozonolysis process will decrease until it reaches a point where most of lignin will react with gaseous ozone. At this stage, ozone reaction will become negligible and lignin will mostly react with ozone according to Poly-condensation mechanism which has been described in Figure 2.17 (Mamleeva et al., 2009).

It can be concluded that water has a major role in the ozonolysis of lignocellulosic biomass. Water induces cell wall swelling, acts as absorption solvent to dissolve ozone, transfers dissolved ozone to react with deep internal lignin inside cell wall, and dissolves some of the ozonolysis products (lignin fragments). It has to be emphasized that the dissolution of lignin fragments is limited due to the decreasing of water content during the ozonolysis process. Using an excess amount of water content in lignocellulosic biomass will increase the non productive consumption of ozone during the ozonolysis process. On the other hand, using water content less than EMC will almost eliminate the delignification process. Therefore, there is an optimal range of the water content that should be used during the ozonolysis process to get the desired delignification effect. Working with oak sawdust, Neely (1984) stated that the optimum range of water content should be 25–35 wt. %, while Vidal and Molinier (1988) working with poplar sawdust obtained an optimum water content of 70 wt. %.

The substantial goal of the pretreatment is to enhance the enzymatic hydrolysis of lignocellulosic biomass. Therefore, a brief description of enzymatic hydrolysis of lignocellulosic biomass is necessary.

2.5 Enzymatic hydrolysis of lignocellulosic biomass

Enzymatic hydrolysis of cellulose and hemicellulose in lignocellulosic biomass can be carried out using cellulase and hemicellulase enzymes. Cellulose hydrolysis is the cleavage of glycosidic bonds between two anhydroglucose units in polymer chains. This can be understood as the addition of one molecule of water during cleavage of the glycosidic bond which is the reversal of macromolecule formation by poly-condensation

process. Since the middle of the last century, enzyme-catalyzed cleavage of glycosidic bonds by cellulase enzyme has received considerable attention, promoted by the anticipation of an eco-compatible alternative choice for degrading cellulose.

Enzymatic hydrolysis of cellulose can be achieved by using cellulase enzymes. Cellulase enzymes consist of at least three major types of enzymes: endo-glucanase, exo-glucanase, and ß-glucosidase. These enzymes are working in a synergistic action to achieve the hydrolysis. Low corrosion problem, energy consumption, and toxicity are the main advantages of enzymatic hydrolysis process.

Enzymatic hydrolysis of cellulose is usually performed under mild conditions, e.g. temperature in the range of 40-50 °C and pH in the range of 4.5-5.5. The mechanism of enzymatic hydrolysis can be summarized as follows (Figure 2.19):

1) The adsorption of cellulase enzyme onto the surface of cellulose
2) Endo-glucanase enzyme attacks the low-crystallinity regions of the cellulose fiber and creates free chain-ends
3) Exo-glucanase (exocellobiohydrolase) further degrades the sugar chain by removing cellobiose units (dimers of glucose) from the free chain-ends
4) Produced cellobiose is then cleaved to glucose by β-glucosidase
5) Desorption of cellulase enzymes.

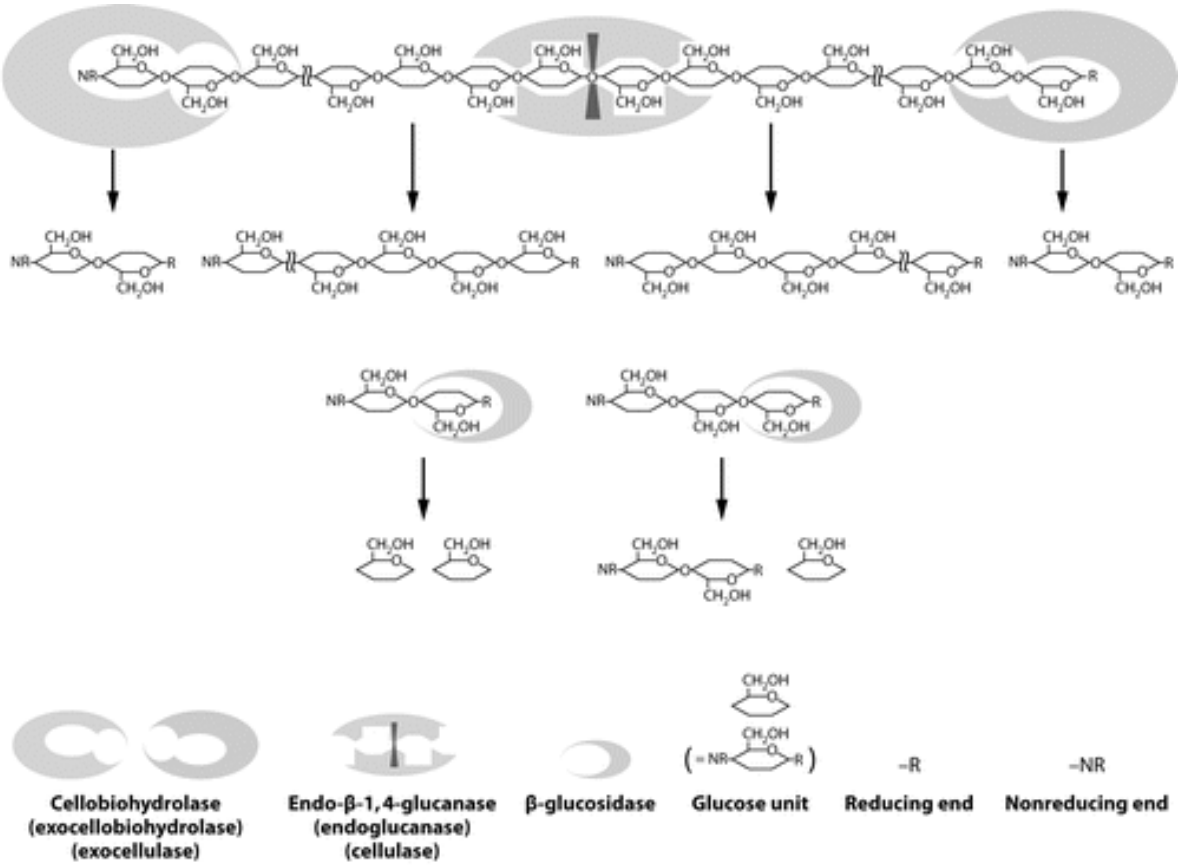

Figure 2-19 Scheme of enzymatic hydrolysis of cellulose (Watanabe and Tokuda, 2010).

Since hemicellulose contains many types of sugar units, the hemicellulase enzymes are more complex and involve at least endo-1, 4-β-D-xylanases, exo-1, 4-β-D-xylosidases, endo- 1, 4-β-D-mannanases, β-mannosidases, acetyl xylan esterases, α-glucuronidases, α-L-arabinofuranosidases, and α-galactosidases. There are several micro-organisms able to produce cellulase and hemicellulase enzymes, including bacteria, such as *Cellulomonas*, *Thermomonospora*, and *Clostridium*, and fungi such as *Phanerochaete*, *Penicillium*, and *Trichoderma*. Cellulases of *Trichoderma reesei* or *T. viride* have been the most broadly

studied and best characterized. Stability under enzymatic hydrolysis conditions and resistivity to chemical inhibitors are the main advantages of using cellulases produced by *Trichoderma*. The main disadvantage of *Trichoderma* cellulase is the low activity of ß-glucosidase. This disadvantage is usually overcome by supplementary addition with extra ß-glucosidase.

There are several factors affecting the enzymatic hydrolysis process: pre-treatment method (if any), biomass quality, and enzyme activities. On the other hand, the operating conditions such as pH, temperature, and mixing, have a significant effect on the hydrolysis yield. The optimum temperature and pH of cellulases are usually reported to be in the range of 40-50 °C and pH 4-5.5. High solid load (substrate concentration in the hydrolysis solution) can cause inhibition of the enzymes resulting in a reduction of the yield of the hydrolysis. Novozyme Bioenergy (2012) has recommended a solid load in the range of 1-5 wt. % (Total solids).

One of the significant technical barriers in bioethanol production technology is the cost of enzymes. Joint collaboration and investment has been made with the aim of increasing the effectiveness of enzymes, developing of novel technology for high solid handling and reducing the enzyme cost by several folds. Novozymes AS unveiled its second generation of lignocellulosic enzymes which is claimed to be a key step toward delivering commercially viable enzymes for lignocellulosic ethanol production. These enzymes require one-third the dose of its first generation enzyme to achieve 80 % of conversion, and work with a wide range of lignocellulosic biomass and pretreatment methods. By further decrease in the cost of enzymes for hydrolysis, and modern technology,

lignocellulosic ethanol will gain the potential to compete on a large scale with gasoline in near future

It can be concluded that pretreatment of lignocellulosic biomass is necessary to obtain high sugar yields through enzymatic hydrolysis. It is in the best interest of the biomass industry to understand biomass structure and the effects of pretreatments on structure in order to minimize the cost of both pretreatment and enzymes. As the prices of current starch ethanol feedstocks such as corn are estimated to increase, lignocellulosic biomass remains the only viable candidate to serve as a renewable feedstock for ethanol production. There are huge amounts of wheat straw that are currently burned on the field or otherwise wasted which could be used as low value raw material for ethanol production. Despite extensive technological advances in ethanol production from lignocellulosic biomass over last few decades, the price of the lignocellulosic ethanol is still high and remains around US$ 0.7 per liter (Kumar et al., 2009). Development of more effective pretreatment methods will substantially decrease the total cost of lignocellulosic ethanol. Ozonolysis is an efficient pretreatment for lignocellulosic biomass. The advantages of ozonolysis pretreatment: it is carried out at room temperature and normal pressures; the degradation is mainly limited to lignin; ozone is the only needed chemical which can be generated on-site; freedom from environmental problems involving toxic wastes or by-products. The only disadvantage is the large amount of ozone needed. Ozone degrades lignin in several stages either by selective ring cleavage, side chain reaction or poly-condensation reaction. Lignin fragments are believed to be responsible for the most consumption of ozone gas. The amount of ozone gas consumed could be reduced significantly if these lignin fragments were removed from the

lignocellulosic biomass cell wall. For example, this can be done by performing the ozonolysis process in two stages with an intermediate washing step with water. This study was an effort to examine this idea and to see if it will enhance the delignification and enzymatic hydrolysis of wheat straw.

CHAPTER 3

MATERIAL AND METHODS

3.1 Material

Wheat straw

Wheat (*Triticum sativum* Soft White Superior) was received as straw in October 2010. It was generously provided by Mr. John Morrisson, Cookston, Ontario. The wheat straw was received dry and packed in bags with different fiber sizes. Received wheat straw was milled using Retsch Cutting Miller type SM 100 and passed through one of two sieves (2 mm or 6 mm). Milled wheat straw was stored in sealed plastic bags at room temperature until being used for ozonolysis pretreatment, enzymatic hydrolysis and/or composition analysis.

Enzymes

A cellulase complex (NS22086) consisting mainly of endo-glucanase, exo-glucanase and β-glucosidase enzymes, and β-glucosidase (NS22118) were kindly donated by Novozymes A/S (Bagsvaerd, Denmark). Table 3.1 shows recommended ranges of dosage amounts and operating conditions (Novozymes Bioenergy, 2012).

Table 3-1 Recommended ranges of enzyme dosages, pH, and temperature (Novozymes Bioenergy, 2012).

Enzymes	Density	pH	Temperature (°C)	Dosage (% wt./wt. (TS*))
Cellulase complex NS22086	1.15	5.0-5.5	45-50	1-5
β -glucosidase NS22118	1.2	2.5-6.5	45-70	0.2-0.6

*TS= Total solids.

Total solids (TS) content was determined following NREL laboratory analytical procedures LAP 001 (Appendix A).

3.2 Design of experiments

Table 3.2 shows the parameters that were fixed during this study. These parameters were fixed in order to reduce the number of experiments.

Table 3-2 Operating parameters fixed during ozonolysis pretreatment of wheat straw in this study.

Fixed Parameters	Value	Unit
O_3/O_2 flow rate	1	liter/min
Ozone concentration	3	wt.%
Wheat straw (OD)* weight	5	g
Wheat straw fiber size	< 2 or < 6	mm

*OD= Oven dry

The first set of experiments was designed to study the effect of ozonolysis time on the acid insoluble lignin (AIL) content in ozonated wheat straw in order to select the total ozonolysis time for the subsequent experiments. The total ozonolysis time should be enough to cause significant delignification effect without using an excessive amount of ozone gas. Five grams (OD) of wheat straw with Initial Water Content (IWC) of 50 wt. %

and fiber size < 2 mm was used in these set of experiments. Six separate experiments were run in a single ozonolysis process using different ozonolysis times, i.e. 5, 15, 30, 60, 120 and 180 minutes. The AIL content of ozonated wheat straw was determined after each experiment following NREL laboratory analytical procedures LAP 003 (Appendix B). Total ozonolysis time was chosen from plotting ozonolysis time against AIL content in ozonated wheat straw.

The second set of experiments was designed to choose an appropriate volume of washing water to be used in the intermediate washing step between the two ozonolysis stages. The volume of washing water should be enough to remove part of the lignin fragments produced from the 1st ozonolysis stage in order to improve the delignification of the final ozonated wheat straw without using an excessive amount of washing water. In the beginning, four experiments were run with 5 g OD straw ozonated for 30, 60, 120 or 180 minutes in a single ozonolysis stage. After each experiment, ozonated wheat straw was washed with seven 200-ml portions of distilled water. Each portion was recovered by filtration and the pH of each filtrate was measured. The increase in the filtrate pH was used as an indication of the removal of carboxylic acids produced from the ozonolysis reaction. A curve of this pH against the washing water volume was plotted, and an arbitrary washing water volume was chosen from this curve (500 ml). This arbitrary washing water volume was then compared with the minimum volume (100 ml) of washing water that could totally immerse the 5 g of wheat straw used in this study. In this comparison, two sets of experiments were run using ozonolysis in two stages with an intermediate washing step between them. The AIL content in the ozonated wheat straw was determined after each experiment. The means for both sets of experiments were

calculated and statistical compared to ascertain whether there was a statistically-significant difference between them. STATGRAPHICS® Centurion XV and Minitab 16 software programs were used to perform the statistical comparison of the means using t-test (Appendix F). No significant difference between the means would indicate that 100 ml could be used as the intermediate washing water volume instead of 500 ml. In the two sets of experiments, three parameters were included in the design of experiment to have more comprehensive comparison between the two washing water volumes. The first parameter was the initial water content (IWC) of wheat straw, which ranged from 30 to 70 wt. %. The second parameter was the Washing Starting Time (WST), or the duration of the first ozonolysis stage, studied in the range of 1/3 and 2/3 of the total ozonolysis time. The third parameter was the Washing Contact Time (WCT), or time during which the wheat straw and the washing water were in contact (not including filtration time), at a range of 1 to 5 minutes. A mixed level factorial design of experiment (3×2^2) was used to perform these two sets of experiments (Table B3, Appendix B). The term (3) in the mixed level factorial design represented the three levels for each parameter in the design (Low, medium and high). For example, the IWC had three levels (30, 50 and 70 wt. %) as shown in Table B3 (Appendix B). On the other hand, the term (2^2) in the mixed level factorial design represented 2^{p-1}, where p is the number of parameters, which are three (IWC, WST and WCT) in this case. The design of experiment was generated in random sequence using STATGRAPHICS® Centurion XV software with two center points (run number 4 and 13) to increase the accuracy of the design. The first four columns of Table B3 (Appendix B) show the design of experiment.

A final set of experiments was performed with the intermediate washing water volume found above to optimize the effect of the above three parameters, i.e. IWC, WST and WCT, with the same above ranges, in order to have maximum delignification effect (minimum AIL content in ozonated wheat straw). The same mixed level factorial design as described above was used to perform this set of experiments. The first four columns of Table B4 (Appendix B) shows the design of experiment for this set of experiments.

3.3 Experimental procedures

3.3.1 Ozonolysis pretreatment

Figure 3.1 shows a schematic diagram of the ozonolysis reactor set-up used in this project. Compressed oxygen gas flowed from the gas cylinder to the ozone gas generator (WEDECO, model GL-1). The amount of generated ozone gas was controlled by two methods. The first method was to control the oxygen gas flow rate supplied to the ozone gas generator using the rotameter (RO-1). Increasing the oxygen flow rate through the ozone gas generator would decrease the ozone gas concentration in the outlet stream due to a dilution effect. The second method was to control the voltage supplied to the ozone gas generator. Increasing the voltage or the power supplied to the generator would increase the ozone gas generation or the ozone gas concentration in the oxygen gas stream. The ozone concentration in the gas phase was measured with an OZOCAN analyzer (OZOCAN Corporation).

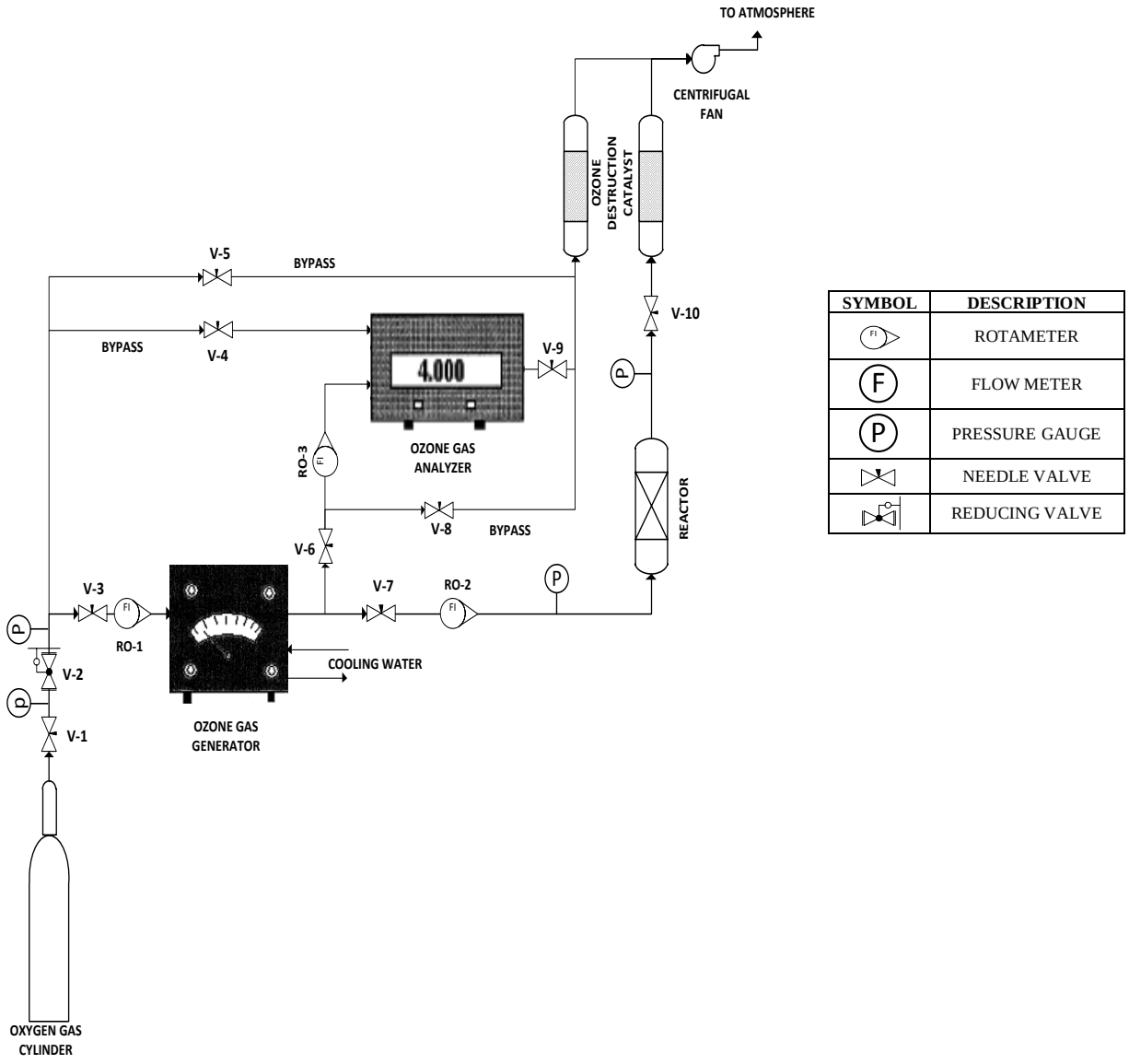

Figure 3-1 Schematic illustration of the ozone generation and of the reactor system used in this study. Operating conditions: temperature = 25 ºC, pressure = 1 atm, reactor volume = 192 cm^3, ozone/oxygen flow rate = 1 l/min and ozone concentration = 3 wt. %.

Figure 3.2 shows the ozonolysis reactor assembly. The ozonolysis reactor consisted of a polytetrafluoroethylene (PTFE) cylindrical tube of 3.5 cm diameter and 20 cm height. The lower part of the reactor contained a stainless steel mesh (sieve number 80) and a mesh holder. The mesh acted as a holder of the wheat straw and as distributor of the O_3/ O_2 gas to the straw. Four bolts with their nuts attached the bottom flanges with a gasket coated with silicone to prevent gas leakage from the reactor. The top part of the reactor consisted of a PolyTetraFluoroEthylene (PTFE) union with its gasket coated with silicone and a top mesh (sieve number 80) to prevent the fibers from moving out of the reactor and into the ozone destruction zone. Bypass streams were provided to isolate the ozone generator, ozone analyzer or the reactor from the gas stream when it was necessary. Ozone gas in the outlet gas stream form the reactor and in the bypass streams was destructed, for safety and as protection to the environment, by passing it through packed columns containing a Carus catalyst (Carus Chemical Company, Peru, IL).

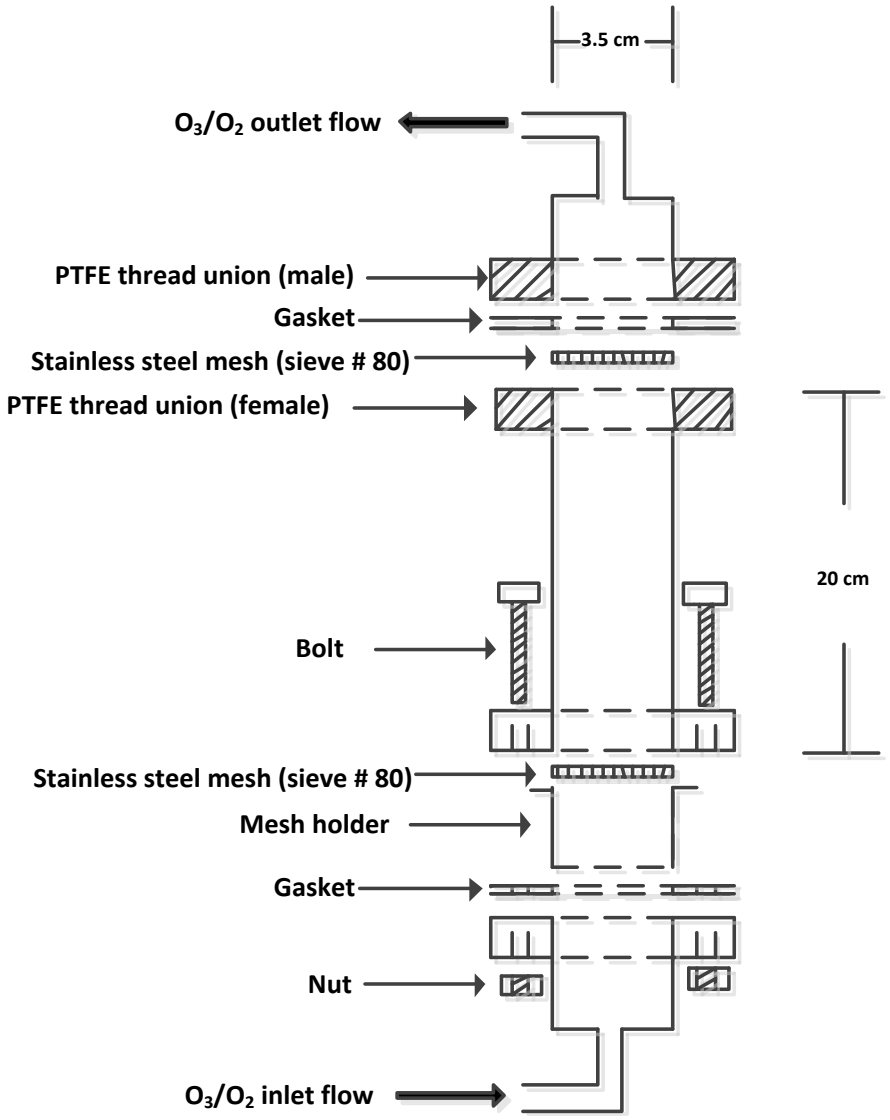

Figure 3-2 Ozonolysis reactor assembly.

The ozonolysis process used the following procedure:

1) Turn on the ozone analyzer and allow it to warm up for about 10 minutes. The ozone analyzer should read 0.000 wt. % after warming up.
2) Turn on the light and the ventilation in the hood. The cooling water should also be turned on to cool the ozone generator at this time.

3) After placing the lower stainless steel mesh in the mesh holder, assemble the lower part of the reactor by tightening the four bolts with their nuts.
4) Load wheat straw to the reactor and assemble the upper part of the reactor after putting the gasket and the stainless steel mesh. The normal load of 5 grams of wheat straw (oven dry weight) with fiber size < 2 mm and initial water content of 70 wt. % should occupy less than 50 % of the reactor volume.
5) Connect the inlet of the ozonolysis reactor to the outlet of the ozone gas generator. Connect the outlet of the ozonolysis reactor to the inlet of the ozone destruction catalyst. PTFE tubes are used in these connections.
6) Turn valves (V-3, 6, 7, 9 and 10) on. These valves are connecting the ozone generator to the reactor and the ozone analyzer. Bypass valves (V-4, 5 and 8) have to be turned off.
7) Turn valve (V-1) on. Make sure there is enough oxygen gas in the cylinder to perform the experiment by watching the pressure gauge connected to the gas cylinder.
8) Turn the reducing valve (V-2) on to give a pressure in the range of 20-25 psig in the pressure gauge connected to the gas stream entering the ozone generator. Be sure there is no pressure build up in the system caused by a closed valve by mistake.
9) Adjust the rotameter (RO-1) in the ozone gas generator inlet to read in the range of 20-30 cubic feet per hour (CFH). Adjust the rotameter (RO-2) connected to the reactor inlet to read 1 liter per minute. Adjust the rotameter (RO-3) which is connected to the ozone analyzer to read 1 l/min.

10) Turn on the main switch of the ozone generator. Increase the voltage supplied to the generator by turning the voltage knob in the ozone gas generator clockwise slowly while watching the digital reading in the ozone analyzer until the analyzer start reading a value equal to 3.000 wt. %. At this point, start the stop watch as the beginning of the ozonolysis experiment.
11) After reaching the required ozonolysis time of wheat straw, decrease the voltage supply to the ozone generator by turning the voltage knob counter clockwise until the scale read zero voltage. Turn off the main switch of the ozone generator. Keep the flow of the oxygen gas for 15 seconds after reading zero wt. % in the ozone analyzer to be sure no ozone gas remains in the system.
12) Turn the oxygen valve (V-1) off. Turn off the valve (V-10) which is connecting the reactor to the ozone destruction catalyst to protect the catalyst.
13) Turn off the ozone analyzer, cooling water, and the hood ventilation.
14) Disassemble the reactor by disassembling the top union first and remove the top gasket and mesh. Flip the reactor upside down to transfer the ozonated wheat straw into a 600 ml beaker. Disassemble the lower part of the reactor by loosening the four bolts, and transfer the remaining of wheat straw which was stuck to the reactor inside wall to the beaker.
15) Wash the reactor cylinder and meshes, dry them with air then assemble the lower part of the rector as mentioned in step 3 to prepare the reactor for another experiment.

Figure 3.3 is a block diagram summarizing the ozonolysis pretreatment and enzymatic hydrolysis of wheat straw used in this study.

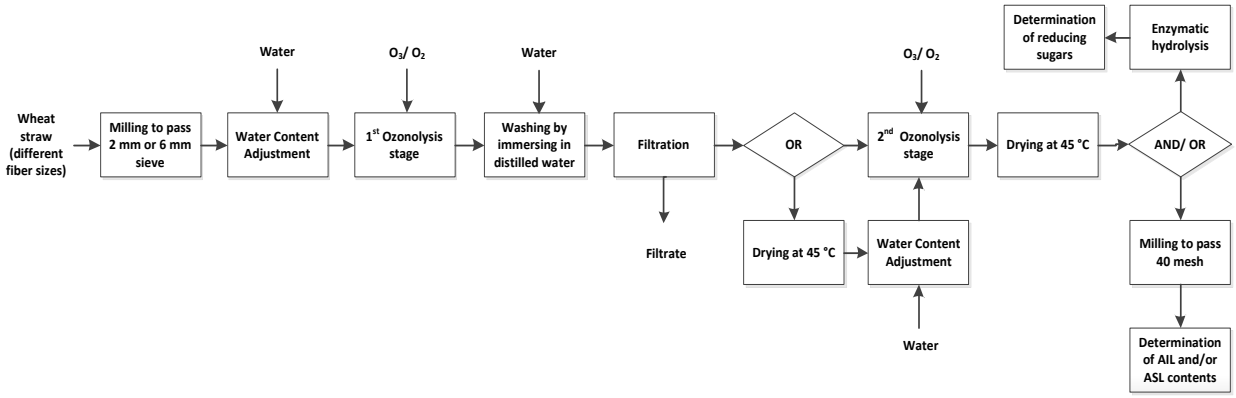

Figure 3-3 Block diagram for ozone pretreatment and enzymatic hydrolysis of wheat straw used in this study.

As shown in Figure 3.3, ozonated wheat straw was either:

1) Dried in an oven at 45 ºC to reach constant weight, stored in a freezer at -20 ºC, and used for Acid Insoluble Lignin (AIL) Acid Soluble Lignin (ASL) content analyses following NREL laboratory analytical procedures LAP 003 and 004 (Appendices B and C respectively) and/or enzymatic hydrolysis following NREL laboratory analytical procedures LAP 009 (Appendix D) or;
2) Washed with distilled water, filtered (see section 3.3.1.1) then ozonated in a 2nd stage. In some cases, the biomass was dried and its water content was readjusted (see section 3.3.1.2) before ozonolysis in the 2nd stage.

3) The biomass from the 2nd ozonolysis stage was dried at 45 ºC to reach constant weight, stored in a freezer at -20 ºC, and used for enzymatic hydrolysis and/or AIL and/or ASL content analyses.

3.3.1.1 Intermediate washing

In the case of two stage ozonolysis process, an intermediate washing and filtration process was performed between the two ozonolysis stages. The intermediate washing step was performed as follows:

1) After finishing the 1st ozonolysis stage, wheat straw was transferred from the ozonolysis reactor to a 600 ml beaker. Caution was taken to be sure all wheat straw fibers were transferred from the reactor to the beaker.
2) A specific volume of distilled water (either 500 or 100 ml) was added to the beaker containing wheat straw for a specific period of time (between 1- 5 min) while mixing with spatula for the first 30 seconds to be sure that all wheat straw was immersed in water.
3) The aqueous suspension of wheat straw was poured and filtered in a vacuum filter system. The filtration media was a glass microfibre filter, 691 (VWR Cat. # 28297-289). Caution was taken to be sure all wheat straw fibers were transferred from the beaker to the filtration system.
4) In some cases, the washing and filtration of wheat straw were performed in steps of 200 ml of distilled washing water in each step to reach a total of 1400 ml of washing water. The acidity (pH) of the filtrate was measured after each washing step as an indication of the removing of carboxylic acids from ozonated wheat straw.

5) The filtered wheat straw was transferred to the ozonolysis reactor to perform the 2nd ozonolysis stage. Caution was taken to be sure all wheat straw fibers were transferred from the filtration system to the ozonolysis reactor.
6) In the case of water content readjustment before performing the 2nd ozonolysis stage, the filtered wheat straw was dried at 45 ºC to a constant weight (usually over night). Dried wheat straw was transferred to a desiccator until it cooled down. Then a portion of the dried wheat straw was analyzed to determine moisture (water) content. The required amount of distilled water was calculated using Eq. 3.4. The IWC was readjusted following steps 2-4 in section 3.3.1.2.

3.3.1.2 Adjusting the initial water content (IWC) of wheat straw

The following procedure was followed whenever there was need to adjust the water content in wheat straw:

1) The Initial Water Content (IWC) in wheat straw was calculated based on the following equation:

$$IWC = \frac{weight\ of\ water\ in\ wheat\ straw\ (g)}{weight\ of\ (water + wheat\ straw)\ (g)} \times 100 \qquad (3.1)$$

It was assumed that the wheat straw originally contains x_1 (g) of water before water content adjustment. Therefore, the weight of water in wheat straw after readjustment:

$$Amount\ of\ water\ in\ wheat\ straw\ (g) = (x_1 + x_2) \qquad (3.2)$$

x_2 = amount of water needs to be added for water content adjustment.

Substitute Eq. 3.2 in Eq. 3.1:

$$IWC = \frac{(x_1 + x_2)}{(x_1 + x_2 + x_3)} \times 100 \quad (3.3)$$

x_3 = weight of wheat straw (g).

Rearranging Eq. 3.3:

$$x_2(g) = \frac{(x_1 + x_3) \times \frac{IWC}{100} - x_1}{\left(1 - \frac{IWC}{100}\right)} \quad (3.4)$$

2) The required amount of distilled water was added plus 10 % extra water to the beaker containing the wheat straw with mixing for 5 minutes using a clean, dry spatula.
3) The weight of the beaker, containing wheat straw, was monitored with continuous mixing until the extra amount of water added was evaporated.
4) Wheat straw (with adjusted IWC) was loaded to the reactor to start the ozonolysis process.

3.3.1.3 Enzymatic hydrolysis

Enzymatic hydrolysis was performed on untreated and some of the best ozonated wheat straw as a standard test to verify the improvement in delignification of wheat straw caused by the ozonolysis pretreatment. In the first and second hydrolysis experiments, untreated wheat straw was used with fiber sizes of < 2 mm and < 6 mm respectively. The first experiment was called "2-control" and the second experiment was called "6-control". Delignified wheat straw with fiber size < 2 mm, which was ozonated in two stages using

optimum operating parameter of IWC, WST and WCT, was used in the third hydrolysis experiment. The third experiment was called "2-optimum". The fourth experiment was similar to the third experiment except that water content in wheat straw was readjusted to be equal to the optimal value of IWC before performing the second ozonolysis stage. The fourth experiment was called "2-enhance". The fifth and last hydrolysis experiment was similar to the third experiment except using wheat straw with fiber size < 6 mm. The fifth experiment was called "6-optimum". Table 3.3 shows a summary description of wheat straw used in each of these enzymatic hydrolysis experiments. Enzymatic hydrolysis was performed using cellulase complex (NS22086) and β-glucosidase (NS22118) enzymes.

Table 3-3 Untreated and delignified wheat straw used in enzymatic hydrolysis experiments.

Experiment name	Fiber size (mm)	Process description
2-control	< 2	Untreated
2-optimum	< 2	Using optimum IWC, WST and WCT: adjusting IWC to 45 wt. %, ozone treated for 20 min, immersed in 100 ml distilled water for 80 sec, filtered, ozone treated for 10 min, dried at 45 ºC.
2-enhance	< 2	Using optimum IWC, WST and WCT with enhancement by readjusting WC before the 2nd ozonolysis stage: adjusting IWC to 45 wt. %, ozone treated for 20 min, immersed in 100 ml distilled water for 80 sec, filtered, dried at 45 ºC, adjusting water content to 45 wt. %, ozone treated for 10 min and dried at 45 ºC.
6-control	< 6	Untreated
6-optimum	< 6	Using optimum IWC, WST and WCT: adjusting water content to 45 wt. %, ozone treated for 20 min, immersed in 100 ml distilled water for 80 sec, filtered, ozone treated for 10 min and dried at 45 ºC.

Enzymatic hydrolysis process was performed using modified NREL laboratory analytical procedures LAP 009 (Appendix D). The modification in LAP 009 procedure was done to take the recommended conditions (including enzymes dosages, total solid loading, temperature and pH) of the new generation of Novozymes enzymes in consideration (Table 3.1). Enzymatic hydrolysis was performed with 2.0 g of either dry untreated wheat straw or (dried and frozen) ozonated wheat straw. The wheat straw was suspended in 50 ml acetate buffer 0.1 M (pH 5.25) and 1 ml of sodium azide (2 wt. %). Water was added to bring the total volume to 100 ml in a 250 ml Erlenmeyer flask. The hydrolysis temperature was set at 47.5 ºC. Each of the five experiments, shown in Table 3.3, was accompanied with its buffer blank (same composition as the experiment except there were no enzymes added). The five experiments were accompanied with one enzyme

blank (same composition as the five experiments except there is no wheat straw added). Buffer blanks was used as the reference while enzyme blank was used to correct the measurement of reducing sugars concentration. The eleven test flasks were sealed by rubber stoppers during the hydrolysis process. Flasks were placed in a rotary incubator (New Brunswick, model INNOVA 40), shaken at speed of 68 rpm and air heated to 47.5 ºC (Appendix D). After reaching the set temperature of 47.5 ºC, the rubber stoppers were removed from the five hydrolysis experiments and the enzyme blank flasks and volumes of 87 µl NS22086 cellulase enzyme and 10 µl NS22118 β-glucosidase were added to the six flasks to initiate the enzymatic hydrolysis process. Enzymes volumes were equivalent to the recommended dosages of 5 % wt. /wt. (TS) for NS22086 and 0.6 % wt. /wt. (TS) for NS22118 (Appendix D). All flasks were sealed tightly again with their rubber stoppers. The rubber stoppers were only removed temporarily to take samples during the hydrolysis process. Samples of 1.5 ml were taken from each flask at specific times after starting the hydrolysis process (2, 4, 16, 40, 64, 88, 112, 136 and 160 hours). The samples were centrifuged and 1.0 ml of the supernatant were tested for total reducing sugars (a glucose equivalent) using DNS method (Appendix D).

The hydrolysis yield compared the amount of reducing sugars experimentally released by the enzymatic hydrolysis of the cellulose and hemicellulose in wheat straw to the theoretical amount of reducing sugars expected to be released from the complete degradation of cellulose and hemicellulose (Appendix D).

The reducing sugars yields can be written as follow:

$$\text{Reducing sugars yields}(\%\ theoretical) = \frac{[Experimental\ reducing\ sugars\ (^{mg}/_{ml})]}{[Theoretical\ reducing\ sugars\ (^{mg}/_{ml})]} \times 100 \qquad (3.5)$$

3.4 Analytical methods

Total solids and moisture content, acid insoluble lignin content, and acid soluble lignin content in raw material and ozonated wheat straw were determined following NREL laboratory analytical procedures LAP 001, 003, and 004 respectively (see Appendices A, B, and C). Enzymatic hydrolysis of wheat straw was performed following NREL laboratory analytical procedures LAP 009 (Appendix D). The concentration of reducing sugars released from the enzymatic hydrolysis of cellulose and hemicellulose in wheat straw was determined by a Dinitrosalicylic Acid (DNS) method (Appendix D). Cellulase enzyme activity was measured using modified NREL laboratory analytical procedure LAP 006 (see Appendix E).

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Total Solids (TS), Moisture Content (MC), Acid Insoluble Lignin (AIL) and Acid Soluble Lignin (ASL) in untreated wheat straw

Total solids (TS), moisture content (MC), acid insoluble lignin (AIL), and acid soluble lignin (ASL) in the untreated wheat straw are summarized in Table 4.1.

Table 4-1 Total solids, moisture content, acid insoluble lignin, and acid soluble lignin for untreated wheat straw.

Content	Equivalent to	Mean ± CF[1]
TS	$\frac{dry\ wheat\ straw\ (g)}{original\ wheat\ straw\ (g)} \times 100$	92.31 ± 0.12 wt. %
MC	$100 - \%\ TS$	7.69 ± 0.12 wt. %
AIL	$\frac{AIL\ (g)}{dry\ wheat\ straw\ (g)} \times 100$	20.50 ± 0.15 wt. %
ASL	$\frac{ASL\ (g)}{dry\ wheat\ straw\ (g)} \times 100$	2.30 ± 0.06 wt. %

[1]CF = Confidence function at 95 % probability.

Details for the calculation of TS and MC can be found in Table A1 (Appendix A). The high value of TS (92.31 wt. %) and the low value of MC (7.69 wt. %) were expected because the original wheat straw (as received) was very dry. Section G1 (Appendix G) shows an example for the detailed calculation of TS and MC.

The high value of AIL (20.50 wt. %) in the untreated wheat straw – see raw data in Table B1 (Appendix B) – indicated the importance of a delignification process to make the

enzymatic hydrolysis of the straw more efficient. Jacobs et al. (2000) reported that lignin content varied within and along the length of the wheat straw. Their study showed that the AIL content changed from 13.8 wt. % in the leaves to 22.3 wt. % in the internodes. The values of AIL for wheat straw in Table 4.1 agree with the ranges reported by Jacobs et al. (2000). Section G2 (Appendix G) shows an example for the calculation of AIL content.

As can be seen in Table 4.1, the ASL content (2.30 wt. %) was found to be small compared to the AIL content. Therefore, AIL was considered the main dependent variable in subsequent experiments. The value of ASL was taken from Table C1 (Appendix C). Section G3 (Appendix G) shows an example for the calculation of ASL.

Determination of TS for untreated wheat straw was essential for the determination of AIL and ASL. The values of TS and MC were also used as the bases for the calculation of water needed to be added to readjust the IWC of wheat straw in the subsequent experiments. Section G4 (Appendix G) shows an example for the calculation of the amount of water needed to be added to readjust IWC. On the other hand, the AIL content for untreated wheat straw was used as a reference in the subsequent experiments to determine the effectiveness of the delignification process.

4.2 Total ozonolysis time

Figure 4.1 shows the AIL content of ozonated wheat straw as a function of ozonolysis time. Table B2 (Appendix B) contains the raw data which were used to establish Figure

4.1. The confidence interval bars are not shown in Figure 4.1 because of the low values of the confidence functions (CF, ≤ 0.32 wt. %) as can be seen in Table B2 (Appendix B).

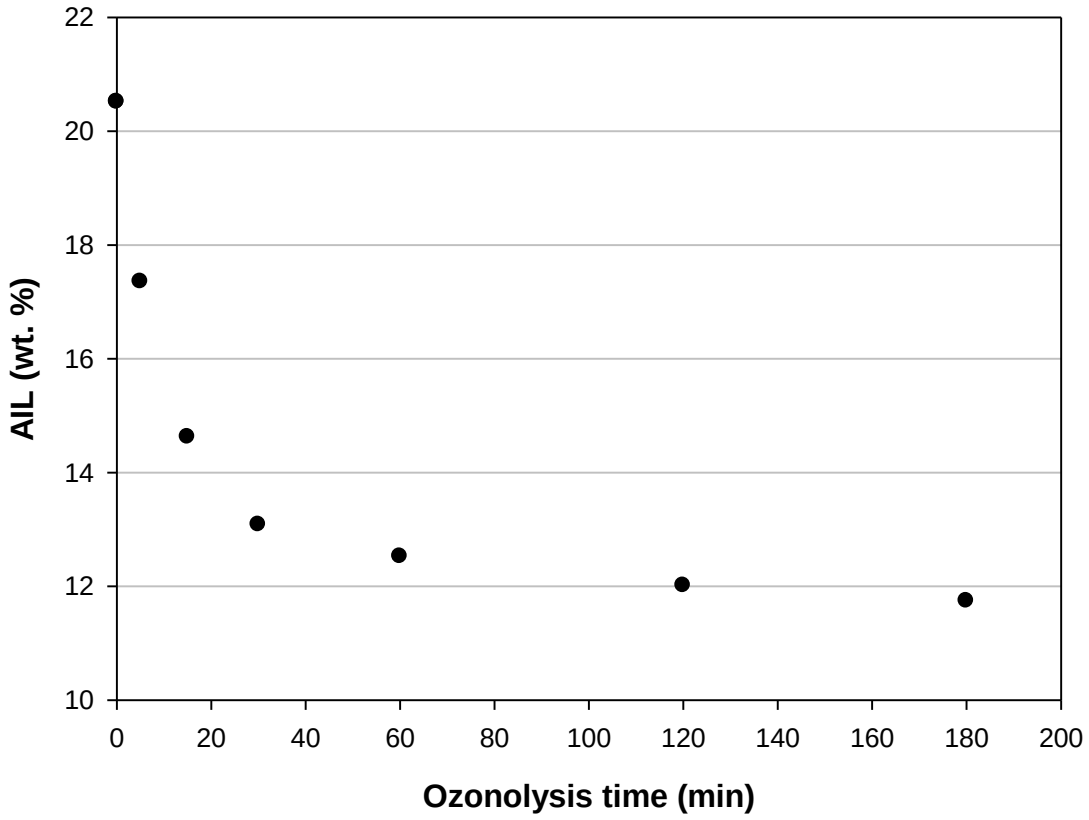

Figure 4-1 Acid Insoluble Lignin (AIL) content of ozonated wheat straw as a function of ozonolysis time. Five grams of wheat straw, with fiber size < 2 mm and Initial Water Content (IWC) = 50 wt. %, ozonated in a single stage at O_3/O_2 flow rate = 1 l /min and ozone concentration = 3 wt. %.

It can be seen from Figure 4.1 that the AIL content dropped rapidly from 20.50 wt. % to 13 wt. % in the first 30 min of ozonolysis process, while remaining almost constant at 12 wt. % after 60 min of ozonolysis time. The high rate of delignification, which was markedly observed at the first 30 min of ozonolysis, seems to indicate that most of ozone gas was consumed by lignin at the surface of the wheat straw cell wall. As the ozonolysis

process continued, ozone gas might have been consumed by lignin decomposition fragments, and it might have become harder for ozone gas to reach lignin in the deep cavities of the wheat straw cell wall. The delignification process could also have been slowed down due to the effect of water evaporating from the wheat straw cell wall because of the O_3/O_2 gas stream flow. Another important factor which might affect the delignification process is the ozone concentration which was fixed at 3 wt. % during these experiments. The delignification process could be improved further by optimizing ozone concentration and O_3/O_2 flow rate. The delignification rate continued to decrease until it reached a very small rate where the AIL content became almost constant. In the end (> ~ 100 minutes), most of ozone can be assumed to be consumed by lignin fragments.

An ozonolysis time of 30 min was chosen for the remainder of experiments since it results in a significant delignification of wheat straw without using an excessive amount of ozone gas.

The next step was to choose an appropriate volume of washing water to remove as much lignin fragments as possible.

4.3 Intermediate washing water volume

Figure 4.2 shows that the variation of filtrate pH with washing water volume is almost similar for the three experiments with various ozonolysis time, i.e. 30, 60, 120 and 180 minutes. Figure 4.2 also shows that the filtrate pH varies sharply with the first 600 ml of washing water volume and the variation starts to decrease until it seems to become

negligible around 1400 ml of washing water. The difference between using 600 ml and 1400 ml of washing water resulted in an increase of the filtrate pH from around 3.4 to almost 4.0. Therefore, it was decided to choose 500 ml arbitrarily as the washing water volume.

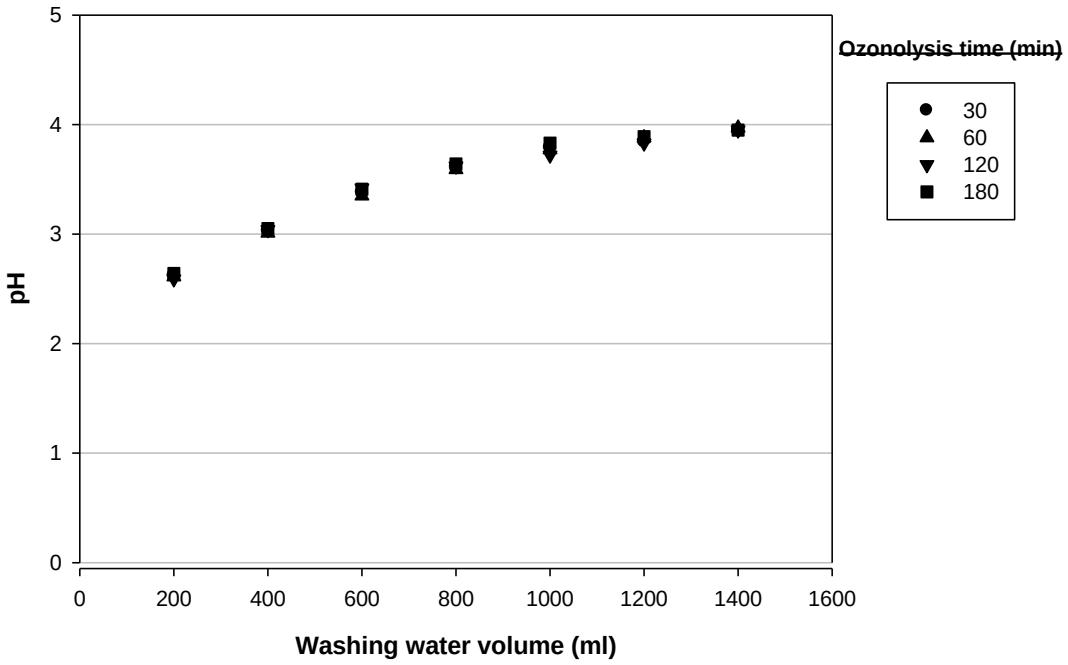

Figure 4-2 Effect of volume of washing water on the removal of carboxylic acids from wheat straw subjected to various contact times with ozone. Five grams of wheat straw, with fiber size < 2 mm and Initial Water Content (IWC) = 50 wt. %, ozonated in a single stage at O_3/O_2 flow rate = 1 l /min and ozone concentration = 3 wt. %.

A set of 14 experiments with different IWC, WST and WCT, using 500 ml washing water were run. The AIL content of the ozonated wheat straw was determined after each experiment. Another set of 14 experiments were run using the same design of experiment as in the first set but with only 100 ml of washing water volume. The results of the two sets of experiments are shown in Table B3 (Appendix B). The comparison between the

two sets of experiments, including the check for normality, AIL variances differences and AIL means differences, is summarized in Table 4.2. Details of the calculations are shown in Appendix F. The normality test showed that the two sets of experiments came from normal distribution, which allowed the use of an F-test to compare the AIL variances of the two sets of experiments. The F-test showed that there were no significant differences between the AIL variances of the two sets of experiments. The results of F-test helped to use a simple t-test to compare the AIL means of the two sets of experiments. Finally, the t-test showed that there were no significant differences between the AIL means of the two sets of experiments. Therefore, a volume of 100 ml was chosen to be used as the intermediate washing water in the next set of ozonolysis experiments.

Table 4-2 Comparison between using 100 ml and 500 ml as an intermediate washing water volume in two stages ozonolysis process. Five grams of wheat straw, with fiber size < 2 mm ozonated in two stage at O_3/O_2 flow rate = 1 l /min and ozone concentration = 3 wt. %.

	Set # 1 (using 100 ml washing water)	Set # 2 (using 500 ml washing water)
Number of experiments (n)	14	14
AIL mean ($\bar{x}$)	13.8021	13.9864
AIL Standard deviation (s)	2.26407	2.43606
AIL Variance (s^2)	5.12602	5.93441
AIL Minimum	10.42	10.31
AIL Maximum	17.27	17.61
Degree of freedom (df)	13	13
P-value (normality test)	0.145	0.063
F-value (variances equality test)	1.58	
t-value (means equality test)	- 0.207	

4.4 Delignification of wheat straw

After choosing the total ozonolysis time (30 min) and the intermediate washing water volume (100 ml), the last set of experiment was performed to find the relation between the IWC, WST and WCT with the AIL content of ozonated wheat straw. Using mixed level factorial design helped to find interactions between these parameters and any non linear relation between these parameters and the AIL content of ozonated wheat straw. Assessing the impact of these parameters and their interactions on the AIL content of the final ozonated wheat straw was explored to find the parameter which shows the most effect on the AIL content. The results of this set of experiments are shown in Table B4 (Appendix B). An example for the calculation of AIL content is shown in section G.2 (Appendix G). The first look at the results of Table B4 did not give a clear idea for the relation between IWC, WST and WCT with the AIL content. Therefore, a mathematical model was generated using STATGRAPHICS® Centurion XV software to show the effect of these three parameters on the AIL content in the final ozonated wheat straw based on the raw results of Table B4 (Appendix B). The model is:

$$AIL = 29.677 - 0.755 \times IWC - 0.047 \times WST - 0.394 \times WCT + 0.009 \times IWC^2 - 0.002 \times IWC \times WST + 0.002 \times IWC \times WCT + 0.014 \times WST \times WCT \quad (4.1)$$

The accuracy of the AIL model (Eq. 4.1) was examined by comparing the experimental values of AIL content used to generate the model with the calculated AIL contents from Eq. 4.1. The experimental AIL content values (AIL_{exp}) were taken from column 15 of

Table B4 (appendix B). The comparisons are shown in Table 4.3. From this comparison, the model (Eq. 4.1) was found to represent the experimental values at a confidence or probability of 97.5 %. For example, the calculated AIL (AIL_{calc}) from Eq. 4.1 in Run # 4, which is equal to 18.94 wt. %, is included in the range of the confidence interval of the experimental AIL (AIL_{exp}), which is equal to 16.45 ± 2.78, when the probability is equal to 97.5 %.

Table 4-3 Comparison between experimental and calculated AIL content from the model (Eq. 4.1).

Run #	**IWC**	**WST**	**WCT**	**AIL_{exp} ± CF[1]**	**AIL_{calc}**	**STDEV[2]**
	wt. %	min	min	wt. %	wt. %	wt. %
1	50	15	3	10.53 ± 1.61	11.97	1.02
2	70	10	5	16.17 ± 2.60	18.49	1.64
3	50	15	3	10.66 ± 1.47	11.97	0.93
4	70	10	1	16.45 ± 2.78	18.94	1.76
5	70	20	5	15.47 ± 2.07	17.32	1.31
6	30	10	5	12.24 ± 0.95	13.09	0.60
7	70	20	1	15.49 ± 1.93	17.21	1.22
8	50	20	1	9.66 ± 2.03	11.47	1.28
9	50	10	1	11.97 ± 0.93	12.80	0.59
10	30	20	1	12.74 ± 0.22	12.93	0.14
11	50	20	5	9.59 ± 2.05	11.42	1.29
12	30	20	5	12.85 ± 0.15	12.72	0.09
13	30	10	1	13.43 ± 0.49	13.86	0.31
14	50	10	5	11.81 ± 0.42	12.19	0.27

[1]CF= Confidence Function at 97.5 % probability.
[2]STDEV = Standard deviation.

In order to understand the method which was used to construct Table 4.3, Run # 4 was taken as an example:

AIL_{exp} = 16.45 wt. % (from AIL mean in Run # 4, Table B4, Appendix B)

AIL_{calc} was calculated using Eq. 4.1:

AIL_{calc} = 29.677 - 0.755 * IWC - 0.047 * WST - 0.394 * WCT + 0.009 * (IWC) ^ 2 - 0.002 * IWC *WST + 0.002 * IWC *WCT + 0.014 *WST *WCT

AIL_{calc} = 29.677 - 0.755 * 70 - 0.047 * 10 - 0.394 * 1 + 0.009 * (70) ^ 2 - 0.002 * 70 *10 + 0.002 * 70 *1 + 0.014 *10 *1

AIL_{calc} = 18.94 wt. %

The mean (average):

$$\bar{x} = \frac{AIL_{exp} + AIL_{calc}}{2} = \frac{16.45 + 18.94}{2} = 17.70\ wt.\%$$

The standard deviation:

$$STDEV = \frac{\sqrt{\sum(x_i - \bar{x})^2}}{N - 1}$$

$$STDEV = \frac{\sqrt{(16.45 - 17.70)^2 + (18.94 - 17.70)^2}}{2 - 1} = 1.76\ wt.\%$$

The confidence function at 97.5 % probability:

$$CF = \pm 2.23 \times \frac{STDEV}{\sqrt{N}}$$

$$CF = \pm 2.23 \times \frac{1.76}{\sqrt{2}} = 2.78\ wt.\%$$

Figure 4.3 shows the effect of each parameter (IWC, WST, or WCT) on the AIL content of ozonated wheat straw. Each parameter varies from its lower value to its higher value while the other two parameters are fixed at their middle values. For example, for the IWC curve in Figure 4.3, the IWC varies from its lower value (30 wt. %) to its higher value (70 wt. %) while WST is fixed at its middle value (15 min) and WCT at its middle value (3 min).

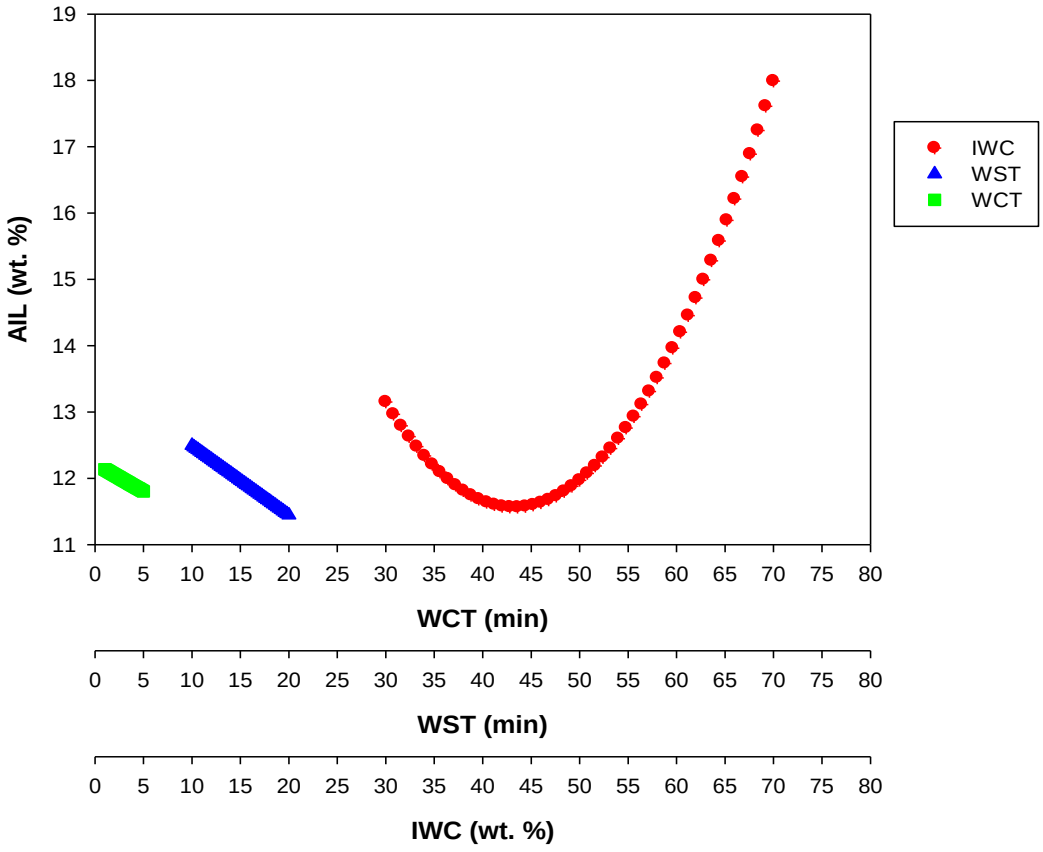

Figure 4-3 Variation of AIL content of the final ozonated wheat straw with each of the parameters: IWC, WST, and WCT. Five grams of wheat straw, with fiber size of < 2 mm was ozonated in two stages at O_3/O_2 flow rate = 1 l /min and ozone concentration = 3 wt. %. A 100 ml of washing water was used in the intermediate washing step.

Figure 4.3 shows that the greatest effect on AIL content came from IWC. The relation between IWC and AIL content is non linear. This non linear relation between IWC and the AIL content suggested that working with IWC value close to the optimal value will result in the highest delignification of wheat straw.

The effect of WST on AIL content, as can be seen from Figure 4.3, was also observed. This indicated that the WST has an effect on the delignification process although it is not as significant as that of IWC. Choosing the right washing starting time would allow removing the highest portion of lignin fragments and allowing ozone to oxidize more lignin rather than oxidizing lignin fragments.

Finally, the effect of WCT had the least effect on the delignification process comparing to the effect of IWC and WST. This can be interpreted as there is a minimum WCT for the lignin fragments to diffuse from the wheat straw cell wall to the bulk of the washing water. Passing this minimum time, the WCT will have no effect on the delignification process.

Knowing that the main effects on the AIL content came from IWC and WST, a response surface was plotted using Eq. 4.1 with the WCT fixed at its middle value (3.0 min). The response surface plot provides a better visualization for the dependence of AIL content of the ozonated wheat straw on both the IWC and WST.

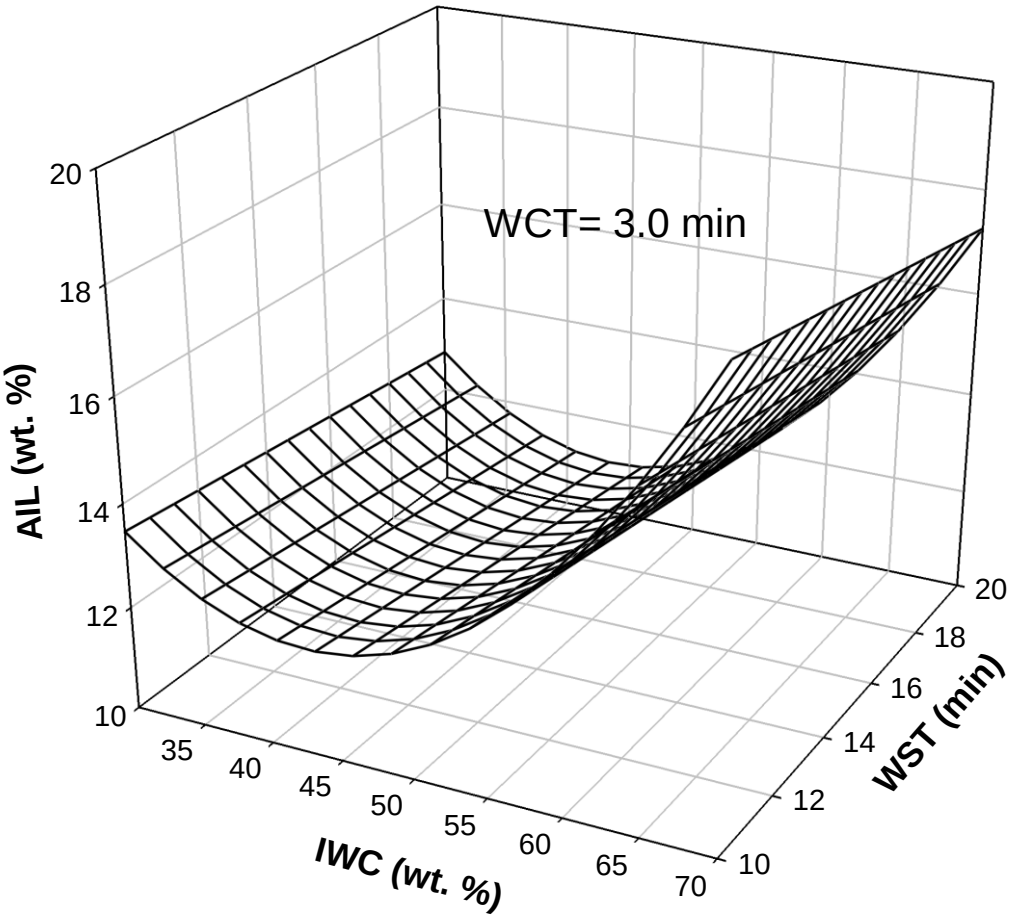

Figure 4-4 Predicted response surface plot for the variation of AIL content of ozonated wheat straw with the IWC and WST while WCT is fixed at 3.0 min. Five grams of wheat straw with fiber size < 2 mm was ozonated in two stages at O_3/O_2 flow rate = 1 l /min and ozone concentration = 3 wt. %. A 100 ml of washing water was used in the intermediate washing step.

Figure 4.4 shows that the minimum values of AIL content were obtained in the range of 40- 50 wt. % of IWC. The predicted optimum parameters values of IWC (45 wt. %), WST (20 min), and WCT (80 seconds), at which a minimum AIL content of ozonated wheat straw could be obtained, were used in an experiment. The result showed that the

AIL content dropped from 20.5 wt. % for untreated wheat straw to 9.34 wt. % (Table B5, Appendix B). Although this drop in AIL content was considered a significant improvement in the delignification of wheat straw, there was a belief that the delignification process could be improved further.

It was noticed that the water content of the wheat straw entering the 2nd ozonolysis stage was higher than the IWC of wheat straw entering the 1st ozonolysis process (45 wt. %) due to the intermediate washing and filtration step. The water content of wheat straw entering the 2nd ozonolysis stage was found to be equal to 72 wt. % (Table A2, Appendix A). Ozonated wheat straw, which was washed and filtered after the 1st ozonolysis stage, was dried in the oven at 45 ^{o}C to reach constant weight, then its water content was readjusted to 45 wt. %. The AIL content of wheat straw after the 2nd ozonolysis stage dropped to 7.36 wt. % (Table B5, Appendix B).

The above results for AIL content (9.34 wt. % and 7.36 wt. %) were considered a significant improvement in delignification of wheat straw in two stages when they were compared with the work of García-Cubero et al. (2009), where it was reported that an AIL content of 11.2 wt. % was reached after 2.5 hours of a single ozonolysis stage with very close operating conditions, i.e., water content equal to 40 wt.%, O_3/air flow rate equal to 1.5 l/min, ozone concentration equal to 3 wt.% and wheat straw fiber size equal to 3-5 mm.

The improvement in the delignification process of wheat straw due to the use of two ozonolysis stages with an intermediate washing step was needed to be verified by examining its effect on the enzymatic hydrolysis yield of ozonated wheat straw.

4.5 Enzymatic hydrolysis of wheat straw

Figure 4.5 shows the change in reducing sugar yields with time for the five enzymatic hydrolysis experiments which were performed using two types of untreated wheat straw (2-control and 6-control) and three types of ozonated wheat straw (2-optimum, 2-enhance and 6-optimum). Descriptions of the five types of wheat straw are shown in Table 3.3. The data for the standardized enzymatic hydrolysis experiments are shown in Tables D3, D4 and D5 respectively (Appendix D). Section G.5 (Appendix G) shows an example for the calculation of reducing sugars yield.

Figure 4.5 shows the low reducing sugars yield (around 23 % theoretical) for the two untreated wheat straw experiments (2-control and 6-control). The results of the two experiments were almost identical, which seems to indicate that the fiber size (< 2 mm versus < 6 mm) has no significant effect on the productivity of the enzymes. The low reducing sugars yields for the two untreated wheat straw experiments was expected due to the high lignin content in untreated wheat straw. This also shows the importance of the pretreatment process to alter the structure of wheat straw cell wall and remove part of the lignin. It was noticed from Figure 4.5 that the rate of hydrolysis yield accelerated in the first 16 hours of hydrolysis. This acceleration in hydrolysis yield could be caused by the relatively easy hydrolysis of exposed cellulose and hemicellulose on the surface of the wheat straw cell wall. As the time passing by, it becomes harder for the enzymes to reach the internal cellulose and hemicellulose inside the cell wall of wheat straw. Therefore, the rate of enzymatic hydrolysis yield starts to decrease.

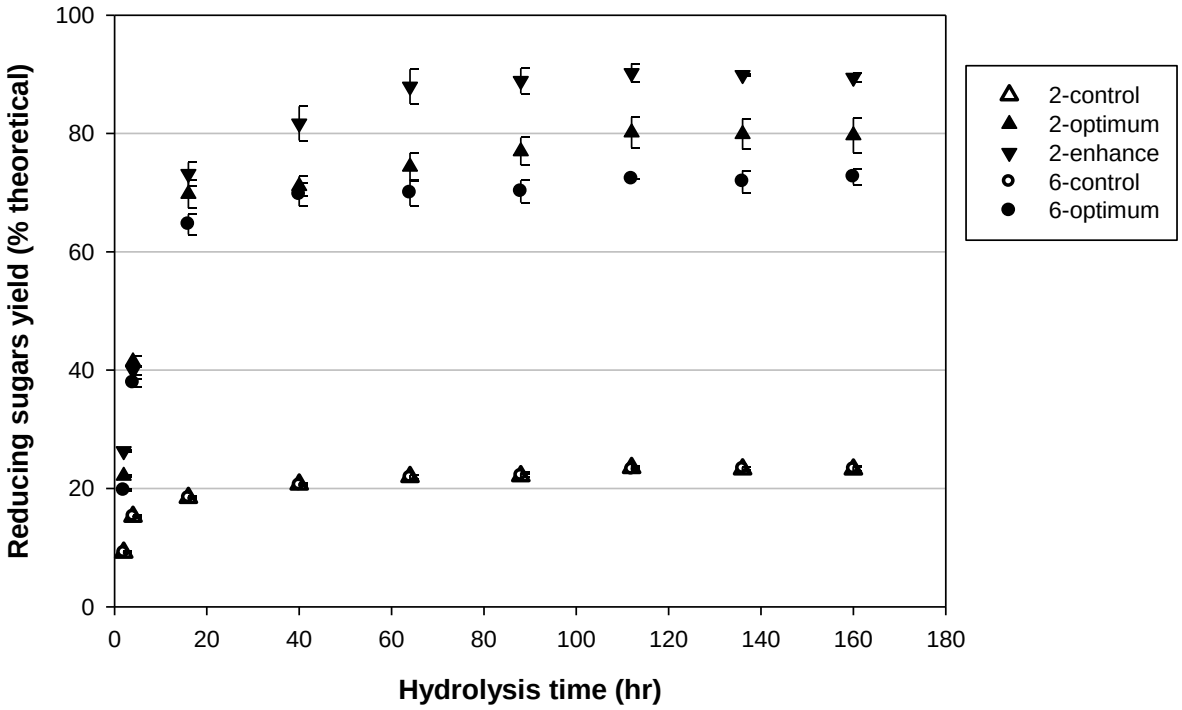

Figure 4-5 Reducing sugars yield from enzymatic hydrolysis of untreated and ozonated wheat straws. Vertical bars are confidence intervals for each duplicate runs at 95% probability.

The reducing sugars yield reached around 80 % theoretical for the "2-optimum" experiment after 112 hours of hydrolysis time. This was a significant improvement in reducing sugars yield for ozonated wheat straw, compared to untreated wheat straw of 23 % theoretical. The improvement in sugars yield was the result of the effective delignification of wheat straw using the optimum parameters values of IWC, WST and WCT in an ozonolysis process of two stages with washing step between them. The highest reducing sugars yield was noticed for "2-enhance" experiment where the reducing sugars yield had reached around 90 % theoretical after 112 hours of hydrolysis time. It can be seen that slight drop in AIL content from 9.35 wt. % (for "2-optimum"

experiment) to 7.36 wt. % (for "2-enhance" experiment) caused an increase in reducing sugars yield by around 10 % theoretical.

Although fiber size differences for untreated wheat straw in experiments "2-control " and "6-control " did not show significant effect on the hydrolysis yield because they had almost the same hydrolysis yield, the same thing cannot be said for ozonated wheat straw in experiments "2-optimum" and "6-optimum". The difference between the reducing sugars yield for the "2-optimum" and "6-optimum" experiments was around 8 % theoretical after 112 hours of hydrolysis time. This difference in reducing sugars yield gave an indication that fiber size has noticeable effect on the ozonolysis process even if the fiber sizes are too close (< 2 mm and < 6 mm).

There are several factors which might cause to obtain high hydrolysis yields in this research. One of these factors is the error in the measurement of reducing sugars, especially at high sugars concentrations, using glucose standard curve as shown in section D2 (Appendix D). The other factor is the assumption that all reducing sugars have an absorbance equivalent to the absorbance of glucose sugar at a wavelength equal to 540 nm which is not true. This assumption was used to simplify the measurement of reducing sugars concentration using glucose standard curve in the DNS method as shown in section D2 (Appendix D). The second assumption, which was also used to simplify the calculation of hydrolysis yield, is that the wheat straw, which was used in this study, has the same compositions of wheat straw analyzed by Mckean and Jacobs (1997). This assumption might help to obtain these high hydrolysis yields. The activity of Cellulase enzyme (NS22086), which was used in the enzymatic hydrolysis experiments, might have another important role in having high hydrolysis yield. The activity of NS22086

enzyme was determined and it was found to be equal to 106 FPU/ ml. The activity of NS22086 was compared with the activity of the old generation of cellulase enzyme NS50013 and it was found that the activity of the new generation of cellulase has almost double the activity of the old generation as shown in Table E2 (Appendix E). The high activity of NS22086 enzyme which was used in the enzymatic hydrolysis experiments during this research supported the high enzymatic hydrolysis yields which were obtained in this research. A detail of the calculation of cellulase enzyme activity is shown in section G.6 (Appendix G).

CHAPTER 5

CONCLUSION

The results of this study showed that:

1) Ozonolysis pretreatment of wheat straw in two stages with an in-between washing of the straw showed a significant improvement on the delignification of the straw and on its subsequent enzymatic hydrolysis.
2) Maximum delignification of wheat straw was achieved with optimal Initial Water Content (IWC) of 45 wt. %, Washing Starting Time (WST) of 20 min and Washing Contact Time (WCT) of 80 sec. AIL content was reduced from 20.5 wt. % for untreated wheat straw to 9.34 wt. % for ozonated wheat straw using optimal conditions of IWC, WST and WCT and the reducing sugars yield was increased from 23 % theoretical for untreated wheat straw to 80 % theoretical for ozonated wheat straw. The high delignification effect might be related to the washing out of lignin fragments from the cell wall of wheat straw so ozone gas could oxidize more lignin rather than oxidizing lignin fragments.
3) IWC in wheat straw had the most significant effect on the ozonolysis delignification process among the other two parameters, i.e., WST and WCT. Readjusting water content to the optimal value of 45 wt. % in ozonated wheat straw before performing the second ozonolysis stage further improved the delignification process and enhanced enzymatic hydrolysis. AIL content was reduced to 7.36 wt. % for ozonated wheat straw. The reducing sugars yield was increased to 90 % theoretical for ozonated wheat straw.

4) Increasing untreated wheat straw fiber size from < 2 mm to < 6 mm did not show significant effect on the enzymatic hydrolysis yield of untreated wheat straw but it showed a negative effect on the hydrolysis yield of ozonated wheat straw using optimal conditions of IWC, WST and WCT. The reducing sugars yield decreased from 80 % theoretical to 72 % theoretical.

5) The improvement in the ozonolysis pretreatment of wheat straw, which has been achieved in this study, is another step toward decreasing the operating cost of the ozonolysis pretreatment process by making ozone consumption more efficient. Investigating alternative lignocellulosic biomass sources, optimizing pretreatment and enzymatic hydrolysis processes, and reducing byproduct wastes will make lignocellulosic bioethanol competitive to fossil fuel in the near future.

RECOMMENDATIONS

This research, as part of the effort to improve bio-ethanol production from lignocellulosic biomass, could be extended to include the following:

1) Optimize other operating parameters of ozonolysis pretreatment such as, O_3/O_2 flow rate, ozone concentration, and a wider range of biomass fiber sizes. The suggested ranges are: O_3/O_2 flow rate (1 - 4 l/min), ozone concentration (0.5 - 4 wt. %) and different fiber sizes including as received wheat straw in order to reduce the cost of pretreatment milling.
2) Study the composition of the organic compounds in the filtrate from the intermediate washing process and try to recover these chemicals and reuse washing water. Recovering these chemicals will decrease the cost of wastewater treatment. For example, the recovery can be investigated using extraction with organic solvents such as amine derivatives.
3) Study the economic feasibility of performing the ozonolysis delignification in two stages with an intermediate washing step. The expected reduction in ozone generation and consumption costs, caused by this modification, can be investigated. Then, the saving in ozone generation can be compared to the cost of adding the intermediate washing step.
4) Optimize the enzymatic hydrolysis operating conditions in order to reduce operating cost. The optimization can include: enzyme dosages, total solids (TS) loading, temperature and pH used in the enzymatic hydrolysis process based on the recommended ranges which were supplied by the enzymes production company (Novozymes).

5) Study the effect of using the intermediate washing step on the ozonolysis delignification and enzymatic hydrolysis yield of other types of biomass such as barley and oat straws. Improving the delignification of other straws will increase the potential of bioethanol production in Canada.

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APPENDICES

Appendix A: Determination of Total Solids (TS) in wheat straw

Laboratory Analytical Procedure # 001 (NREL, 1995)

This method involves drying a sample at 105 ºC ± 3 ºC in a convection oven. Each sample was run in duplicates.

1) Put a pre-dried aluminum foil weighing dish on an analytical balance and tare the balance.
2) Thoroughly mix wheat straw and then weigh out a sample of 2 grams, to the nearest 0.1 mg, into the weighing dish. Record the weight of the sample.
3) Place the sample into a convection oven at 105 ºC ± 3 ºC and dry to constant weight (± 0.1% change in the amount of moisture present upon one hour of reheating).
4) Remove the sample from the oven and place in a desiccator; cool to room temperature.
5) Weigh the oven-dried sample to the nearest 0.1 mg and record this weight.

Calculations

Calculate the percent total solids on a 105 ºC dry weight basis as follows:

$$\%\, Total\ Solids\ (TS) = \frac{weight\ of\ dried\ \ straw}{weight\ of\ original\ \ straw} \times 100 \qquad (A1)$$

The percent moisture can also be calculated:

$$\%\, Moisture\ Content\ (MC) = \ 100 - \%\, TS \qquad (A2)$$

Table A1 shows the calculated means, confidence functions, standard deviations and the relative percent differences for % TS and % MC of untreated wheat straw. An example of the calculation of % TS and % MC is shown in section G.1 (Appendix G).

The standard deviation (STDEV) was calculated using the following equation:

$$STDEV = \sqrt{\frac{\sum(x - \bar{x})^2}{n - 1}} \qquad (A3)$$

The confidence function (CF) was calculated using the following equation:

$$CF = 1.96 \times \left(\frac{STDEV}{\sqrt{n}}\right) \qquad (A4)$$

$n = number\ of\ replicates (2\ in\ this\ case)$

Laboratory Analytical Procedure LAP# 001 by NREL stated that if Relative Percent Difference (% RPD) for duplicate runs to determine % TS is higher than 1.1 % then the experiment must be rerun. The %RPD can be defined as follow:

$$\%\ RPD = \frac{|x_1 - x_2|}{\frac{(x_1 + x_2)}{2}} \times 100 \qquad (A5)$$

where x_1 and x_2 are the values of % TS determined in replicate # 1 and 2 respectively.

Table A1. Percent total solids and moisture content for untreated wheat straw (fiber size < 2 mm).

	Replicate # 1	Replicate # 2	Mean ± CF[1]	STDEV[2]	% RPD[3]
Weight of dry straw (g)	1.845	1.847			
Weight of original straw (g)	2.000	2.000			
Total Solids (wt. %)	92.25	92.37	92.31 ± 0.12	0.08	0.13
Moisture Content (wt. %)	7.75	7.63	7.69 ± 0.12	0.08	1.56

[1]CF = Confidence Function at 95 % probability.
[2]STDEV = Standard Deviation.
[3]RPD = Relative percent difference.

Table A2 shows the % TS and % MC for ozonated wheat straw produced from washing and filtration step performed after the 1st ozonolysis stage.

Table A2. Percent total solids and moisture content for ozone treated, washed, and filtered, wheat straw produced from the 1st ozonolysis stage (fiber size < 2 mm).

	Replicate # 1	Replicate # 2	Mean ± CF[1]	STDEV[2]	% RPD[3]
Weight of dry straw (g)	4.163	4.169			
Weight of original straw (g)	15.095	15.243			
Total solids (wt. %)	27.58	27.35	27.47 ± 0.23	0.16	0.84
Moisture Content (wt. %)	72.42	72.65	72.54 ± 0.23	0.16	0.32

[1]CF = Confidence Function at 95 % probability.
[2]STDEV = Standard Deviation.
[3]RPD = Relative percent difference.

Appendix B: Determination of Acid Insoluble Lignin (AIL) in wheat straw (Klason Lignin procedure)

Laboratory Analytical Procedure LAP# 003 (NREL, 1995)

1) Individually label the crucibles needed for analysis, and ignite them with an individual glass fiber filter at 575 ± 25 ºC to achieve a constant weight of ± 0.3 mg. Store the ignited crucibles and their glass fiber filter in a desiccator until needed.
2) Fiber size wheat straw used in this procedure has to be less than 1 mm. if the fiber size is bigger than 1 mm then grind and sieve the fiber to pass sieve number 40.
3) Weigh 1.0 ± 0.05 g wheat straw sample to the nearest 0.1 mg and place in a 20 x 150 mm test tube. Record the weight as the initial sample weight (W_1). Each sample must be run in duplicate.

   Note: Samples for total solids determination (Appendix B) must be weighed out at the same time as the samples for the acid-insoluble lignin determination. If this is done later, it can introduce an error in the calculation because ground biomass can rapidly gain or lose moisture when exposed to the atmosphere. Determine and record the average total solids value as TS. This value is used to correct the weight of the wheat straw used in the lignin analysis, as described in the calculations section. Wheat straw with total solids content less than 85 wt. %, on a 105 ºC dry weight basis, was dried in oven at 45 ºC.
4) Add 15.00 ± 0.02 ml of 72% wt. /wt. H_2SO_4. Use a glass stirring rod to mix for 1 minute, or until the sample is thoroughly wetted. Some of the wheat straw fiber will stick to the mixing rod, so keep the mixing rod immersed in the acid inside the test tube.

5) Hydrolyze the sample for 2 hours at room temperature (approximately 20 ºC), stirring every 15 minutes to assure complete mixing and wetting.

6) Transfer the hydrolyzate, which looks like a black slurry, to a 1000 ml Erlenmeyer flask and dilute to a 3 wt. % acid concentration with 560 ml of distilled water. Caution must be taken to transfer all the residual solids along with the hydrolysis liquid. Use hot water to wash any particles clinging on the test tube or the mixing rod walls.

7) Place the flask on a hot plate heater and attach to the reflux condenser. Heat the liquid to a gentle boil. Start timing at the onset of boiling, and reflux for 4 hours ± 5 minutes.

8) At the end of 4 hours, rinse the condenser with a small amount of distilled water before disassembling the reflux apparatus.

9) Vacuum filter the hydrolysis solution through one of the previously ignited glass fiber filter.

   Note: If an Acid Soluble Lignin (ASL) determination (Appendix C) is to be done, record the volume of the collected filtrate. Decant 15-25 ml of filtrate into a container to be used for ASL analysis. If this aliquot is not used immediately for ASL analysis, it should be store in refrigerator at 4 ºC. Acid-soluble lignin should be analyzed within 24 hours, preferably within 6 hours of hydrolysis.

10) Use hot distilled water to wash any particles clinging to the hydrolyzing flask to the crucible. Wash the filtered residue with a small amount of hot distilled water to make it free of acid using at the end of the vacuum filtration.

11) Put the glass fiber filter with the filtered residue in its pre-ignited crucible.

12) Dry the crucible and contents at 105 ± 3 ºC for 2 hours or until constant weight is achieved (±0.3 mg upon reheating for 20 min).
13) Cool in desiccator and record the weight as W_2, (weight of the crucible + glass fiber filter + acid-insoluble lignin + acid-insoluble ash) to the nearest 0.1 mg. the residue (acid insoluble lignin + acid insoluble ash) will look like a black spot on the filter.
14) Place the crucible and contents in the muffle furnace (Thermo Scientific, Model: BF 51828C-1, USA) and ignite at 575 ± 25 ºC for a minimum of 3 hours, or until all the carbon (black spot) turns into ash. Heat at a rate of 10 ºC/min to avoid flaming. If the sample tends to flare up, the container should be partially covered with ceramic cover during this step. Avoid heating above the maximum stated temperature.
15) Cool in desiccator and record the weight, W_3 (weight of the crucible + glass fiber filter + and acid insoluble ash) to the nearest 0.1 mg.

Calculations

Calculate % acid-insoluble lignin on an as received 105 ºC dry weight basis as follows:

$$\%\ Acid\ Insoluble\ Lignin\ (AIL) = \frac{W_2 - W_3}{W_1 \times \frac{TS}{100}\%} \times 100 \qquad (B1)$$

Where:

W_1 = initial sample weight (1.000 g).

W_2 = weight of crucible + glass fiber filter + Acid-Insoluble Lignin + Acid Iinsoluble Ash.

W_3 = weight of crucible + glass fiber filter + Acid Insoluble Ash.

TS = % total solids content of the prepared sample used in this lignin analysis, on a 105 ^{o}C dry weight basis, as determined by the LAP-001 (Appendix A).

Table B1 shows the calculated means, standard deviations and the relative percent differences for AIL of untreated wheat straw.

The standard deviation (STDEV) was calculated using the following equation:

$$STDEV = \sqrt{\frac{\sum(x - \bar{x})^2}{n - 1}} \qquad (B2)$$

The confidence function (CF) was calculated using the following equation:

$$CF = \pm 1.96 \times \left(\frac{STDEV}{\sqrt{n}}\right) \qquad (B3)$$

$n = number\ of\ replicates (2\ in\ this\ case)$

Laboratory Analytical Procedure LAP# 003 by NREL stated that if the Relative percent difference (RPD) higher than 3.4%, the sample must be rerun. The %RPD is a criterion to measure the difference between two samples and it can be defined as follow:

$$\%\ RPD = \frac{|x_1 - x_2|}{\frac{(x_1 + x_2)}{2}} \times 100 \qquad (B4)$$

x_1 and x_2 in this case are the two % AIL determined in a duplicate runs.

Table B2 shows the variation in AIL content with the ozonolysis time. In each experiment, five grams (oven dry basis) of wheat straw, with fiber size < 2 mm and Initial Water Content (IWC) equal to 50 wt. %, was ozonated at O_3/O_2 flow rate equal to 1 l/min and ozone concentration equal to 3 wt. %.

Table B3 shows the AIL content for ozonated wheat straw after ozonolysis in two stages with an intermediate washing step in between. A mixed level factorial design of experiment was used to study the effect of IWC, WST and WCT on the AIL content of the final ozonated wheat straw. A 100 ml of washing water volume was used in the first set of experiments and 500 ml of washing water was used in the second set. The comparison between the AIL content means for the two sets of experiments are shown in Appendix F.

Table B4 shows the AIL content results (two replicate runs) for ozonated wheat straw after ozonolysis in two stages with an intermediate washing step in between. A mixed level factorial design of experiment was used to study the effect of IWC, WST and WCT on the AIL content of the final ozonated wheat straw. An example in section G.2 (Appendix G) shows the details for the calculation of AIL content which was used in Tables B3 and B4.

Table B5 shows the AIL content for two types of ozonated wheat straw. The first type (2-optimum) was ozonated in two stages with an intermediate washing step with 100 ml of distilled water using optimal parameter values, i.e. IWC equal to 45 wt. %, WST equal to 20 min and WCT equal to 80 sec. The second ozonated wheat straw (2-enhance) was ozonated using the same optimal values of IWC, WST and WCT as in the 2-optimum

experiment with water content readjusted to 45 wt. % before performing the second ozonolysis stage.

Table B1. Acid Insoluble Lignin (AIL) content of untreated wheat straw calculated using Eq. B1 (Appendix B).

Replicate # 1					Replicate # 2					AIL Mean ± CF[2]	STDEV[3]	RPD[4]
initial sample weight (W_1)	weight of crucible + filter + AIL + AIA[1] (W_2)	weight of crucible + filter + AIA[1] (W_3)	Total solids (TS)	AIL	initial sample weight (W_1)	weight of crucible + filter + AIL + AIA[1] (W_2)	weight of crucible + filter + AIA[1] (W_3)	Total solids (TS)	AIL			
g	g	g	wt. %	wt. %	g	g	g	wt. %	wt. %	wt. %	wt. %	wt. %
1.000	113.040	112.850	92.30	20.58	1.000	116.326	116.137	92.30	20.43	20.51 ± 0.15	0.11	0.73

[1]AIA = Acid Insoluble Ash.
[2]CF = Confidence Function at 95 % probability.
[3]STDEV = Standard Deviation.
[4]RPD = Relative Percent Difference.

Table B2. Acid Insoluble Lignin (AIL) content, determined using Eq. B1 (Appendix B), of ozonated wheat straw at various ozonolysis times. In each experiment, 5 grams (oven dry basis) of wheat straw, with fiber size < 2 mm and Initial Water Content (IWC) = 50 wt. % was ozonated at O_3/O_2 flow rate= 1 l/min and ozone concentration= 3 wt. %.

Ozonolysis time	Replicate # 1					Replicate # 2					AIL Mean ± CF[2]	STDEV[3]	RPD[4]
	Initial sample weight (W_1)	weight of crucible + filter + AIL + AIA[1] (W_2)	weight of crucible + filter + AIA[1] (W_3)	Total solids (TS)	AIL	Initial sample weight (W_1)	weight of crucible + filter + AIL + AIA[1] (W_2)	weight of crucible + filter + AIA[1] (W_3)	Total solids (TS)	AIL			
min	g	g	g	wt. %	wt. %	g	g	g	wt. %	wt. %	wt. %	wt. %	wt. %
0	1.000	113.038	112.848	92.30	20.59	1.000	113.039	112.850	92.50	20.43	20.51 ± 0.15	0.11	0.75
5	1.000	112.979	112.817	92.50	17.51	1.000	112.669	112.510	92.50	17.19	17.35 ± 0.32	0.23	1.83
15	1.000	112.023	111.887	92.30	14.73	1.000	116.238	116.104	92.40	14.51	14.62 ± 0.22	0.16	1.51
30	1.000	116.239	116.117	92.20	13.23	1.000	112.923	112.804	92.10	12.92	13.08 ± 0.30	0.22	2.37
60	1.000	112.014	111.898	92.00	12.61	1.000	112.320	112.205	92.50	12.43	12.52 ± 0.18	0.13	1.45
120	1.000	116.225	116.115	92.40	11.90	1.000	116.244	116.132	92.50	12.11	12.01 ± 0.21	0.15	1.75
180	1.000	112.931	112.823	92.60	11.66	1.000	112.927	112.818	92.30	11.82	11.74 ± 0.15	0.11	1.32

[1]AIA = Acid Insoluble Ash.
[2]CF = Confidence Function at 95 % probability.
[3]STDEV = Standard Deviation.
[4]RPD = Relative Percent Difference.

Table B3. Acid Insoluble Lignin (AIL %) content, determined using Eq. B1 (Appendix B), of ozonated wheat straw. In each run, 5 grams (oven dry basis) of wheat straw with fiber size < 2 mm was ozonated in two stages at O_3/O_2 flow rate= 1 l/min and ozone concentration= 3 wt. %. Two washing water volumes (100 ml and 500 ml) were used.

Run #	IWC	WST	WCT	Set # 1 (using 100 ml washing water)					Set # 2 (using 500 ml washing water)				
				Initial sample weight (W_1)	weight of crucible + filter + AIL + AIA[1] (W_2)	weight of crucible + filter + AIA[1] (W_3)	Total solids (TS)	AIL	Initial sample weight (W_1)	weight of crucible + filter + AIL + AIA[1] (W_2)	weight of crucible + filter + AIA[1] (W_3)	Total solids (TS)	AIL
	wt. %	min	min	g	g	g	wt %	wt. %	g	g	g	wt %	wt. %
1	70	10	1	1.000	112.050	111.890	97.60	16.40	1.000	116.282	116.120	95.80	16.90
2	30	20	5	1.000	112.010	111.891	92.90	12.81	1.000	112.941	112.819	96.40	12.65
3	70	15	5	1.000	116.252	116.084	97.30	17.27	1.000	112.985	112.813	97.70	17.61
4	50	15	3	1.000	112.906	112.804	97.90	10.42	1.000	116.225	116.126	96.00	10.31
5	70	10	5	1.000	116.276	116.119	97.40	16.12	1.000	112.044	111.889	94.90	16.34
6	30	15	5	1.000	112.014	111.897	97.10	12.05	1.000	112.928	112.815	92.40	12.23
7	30	15	1	1.000	112.933	112.815	97.10	12.15	1.000	112.008	111.888	96.60	12.43
8	30	10	1	1.000	116.237	116.108	96.30	13.39	1.000	116.250	116.124	96.30	13.08
9	30	10	5	1.000	112.966	112.848	96.80	12.19	1.000	112.930	112.810	95.80	12.53
10	70	20	1	1.000	116.273	116.124	96.40	15.46	1.000	116.269	116.115	96.80	15.91
11	70	15	1	1.000	112.975	112.820	96.10	16.13	1.000	112.048	111.887	97.10	16.58
12	70	20	5	1.000	112.966	112.819	95.30	15.42	1.000	116.272	116.119	96.80	15.80
13	50	15	3	1.000	116.232	116.129	96.10	10.72	1.000	112.928	112.824	97.60	10.66
14	30	20	1	1.000	112.017	111.894	96.80	12.70	1.000	112.013	111.889	97.10	12.78

[1]AIA = Acid Insoluble Ash.

Table B4. Acid Insoluble Lignin (AIL %) content, determined using Eq. B1 (Appendix B), of ozonated wheat straw. In each run, 5 grams (oven dry basis) wheat straw with fiber size < 2 mm ozonated in two stages at O_3/O_2 flow rate= 1 l/min and ozone concentration= 3 wt. %. Washing water volume = 100 ml was used.

Run	IWC	WST	WCT	Replicate # 1					Replicate # 2					AIL Mean ± CF[2]	STDEV[3]	RPD[4]
				Initial sample weight (W_1)	weight of crucible + filter + AIL + AIA[1] (W_2)	weight of crucible + filter + AIA[1] (W_3)	Total solids (TS)	AIL	Initial sample weight (W_1)	weight of crucible + filter + AIL + AIA[1] (W_2)	weight of crucible + filter + AIA[1] (W_3)	Total solids (TS)	AIL			
	wt. %	min	min	g	g	g	wt %	wt. %	g	g	g	wt %	wt. %	wt. %	wt. %	wt.%
1	50	15	3	1.000	112.906	112.804	97.90	10.42	1.000	116.230	116.127	96.90	10.63	10.53 ± 0.21	0.15	1.99
2	70	10	5	1.000	116.276	116.119	97.40	16.12	1.000	112.967	112.813	95.00	16.22	16.17 ± 0.10	0.07	0.62
3	50	15	3	1.000	116.232	116.129	96.10	10.72	1.000	112.928	112.825	97.20	10.60	10.66 ± 0.11	0.08	1.18
4	70	10	1	1.000	112.050	111.890	97.60	16.40	1.000	112.052	111.889	98.70	16.51	16.45 ± 0.11	0.08	0.69
5	70	20	5	1.000	112.966	112.819	95.30	15.42	1.000	116.272	116.122	96.70	15.51	15.47 ± 0.08	0.06	0.55
6	30	10	5	1.000	112.966	112.848	96.80	12.19	1.000	112.007	111.889	96.00	12.29	12.24 ± 0.10	0.07	0.81
7	70	20	1	1.000	116.273	116.124	96.40	15.46	1.000	112.962	112.812	96.60	15.53	15.49 ± 0.07	0.05	0.44
8	50	20	1	1.000	111.983	111.890	97.10	9.58	1.000	111.984	111.890	96.50	9.74	9.66 ± 0.02	0.011	1.64
9	50	10	1	1.000	112.926	112.812	95.60	11.93	1.000	112.929	112.813	96.60	12.01	11.97 ± 0.08	0.06	0.71
10	30	20	1	1.000	112.017	111.894	96.80	12.70	1.000	112.013	111.889	97.10	12.77	12.74 ± 0.07	0.05	0.55
11	50	20	5	1.000	116.223	116.132	95.70	9.51	1.000	112.906	112.813	96.20	9.67	9.59 ± 0.15	0.11	1.63
12	30	20	5	1.000	112.010	111.891	92.90	12.81	1.000	112.018	111.891	98.50	12.89	12.85 ± 0.08	0.06	0.63
13	30	10	1	1.000	116.237	116.108	96.30	13.39	1.000	116.250	116.120	96.60	13.46	13.43 ± 0.07	0.05	0.48
14	50	10	5	1.000	112.927	112.813	97.00	11.75	1.000	112.926	112.812	96.00	11.87	11.81 ± 0.11	0.08	1.01

[1]AIA = Acid Insoluble Ash.
[2]CF = Confidence Function at 95 % probability.
[3]STDEV = Standard Deviation.
[4]RPD = Relative Percent Difference.

Table B5. Acid Insoluble Lignin (AIL %) content, determined using Eq. B1 (Appendix B), of ozonated wheat straw. In each run, 5 grams (oven dry basis) wheat straw with fiber size < 2 mm ozonated in two stages at IWC = 45 wt. %, WST = 20 min, WCT = 80 sec., O_3/O_2 flow rate= 1 l/min and ozone concentration= 3 wt. %. Washing water volume = 100 ml was used.

Experiment name[1]	Replicate # 1					Replicate # 2					AIL Mean ± CF[3]	STDEV[4]	RPD[5]
	initial sample weight (W_1)	weight of crucible + filter + AIL + AIA[2] (W_2)	weight of crucible + filter + AIA[2] (W_3)	Total solids (TS)	AIL	initial sample weight (W_1)	weight of crucible + filter + AIL + AIA[2] (W_2)	weight of crucible + filter + AIA[2] (W_3)	Total solids (TS)	AIL			
	g	g	g	wt. %	wt. %	g	g	g	wt. %	wt. %	wt. %	wt. %	wt. %
2-optimum	1.000	114.587	114.499	95.55	9.21	1.000	114.112	114.022	95.04	9.47	9.34 ± 0.25	0.18	2.78
2-enhance	1.000	115.148	115.079	94.91	7.27	1.000	113.877	113.807	93.96	7.45	7.36 ± 0.18	0.13	2.45

[1]See Table 3.3 for descriptions.
[2]AIA = Acid Insoluble Ash.
[3]CF = Confidence Function at 95 % probability.
[4]STDEV = Standard Deviation.
[5]RPD = Relative Percent Difference.

Appendix C: Determination of Acid Soluble Lignin (ASL) in wheat straw

Laboratory Analytical Procedure #004 (NREL, 1995)

1) Set up and calibrate the spectrophotometer following the protocols recommended in the instrument manual.
2) Put the required amount of the hydrolyzate (filtrate produced from step 11 in LAP # 0 03 in Appendix B) in the UV cuvette.
3) Measure the absorbance of the hydrolyzate at 205 nm, using the 1-cm light path cuvette. A 3 wt. % solution of H_2SO_4 should be used as a reference blank.

If the absorbance reading exceeds 0.7, the sample must be diluted. Dilute the sample so the resulting absorbance reading falls between 0.2 and 0.7. The 4% H_2SO_4 must be diluted in the same ratio as the sample and used as the reference blank for this repeat analysis.

Calculations

An absorptivity (extinction coefficient) value of 110 L/g-cm is used to calculate the amount of acid-soluble lignin present in the hydrolyzate. The 205 nm absorptivities reported for most woods fall in the range of 88 to 113 L/g-cm.

For a liquid process sample, an estimate can be made of the amount of acid-soluble lignin present as follows:

$$\%\, ASL = \frac{\frac{A}{b \times a} \times d \times V \times \frac{L}{1000\, mL}}{W_1 \times \frac{\%\, TS}{100}} \times 100 \qquad (C1)$$

Where:

A = absorbance at 205 nm of the filtrate obtained in step 9 of the LAP- 003- Klason lignin procedure (Appendix B).

d = dilution factor.

b = cell path length, 1 cm.

a = absorptivity, equal to 110 l/g. cm.

V = the volume of the filtrate obtained in step 9 of the LAP- 003- Klason lignin procedure (Appendix B) expressed in ml.

W_1 = initial wheat straw weight in grams from LAP-003 (Appendix B), 1.000 g.

TS = % total solids content of the prepared sample used in this lignin analysis, on a 105 ºC dry weight basis, as determined by the LAP-001 (appendix A).

Table C1 shows the means, confidence functions, standard deviations and relative percent differences of ASL content for untreated wheat straw. A sample for the details calculation of ASL content can be found in section G.3 (Appendix G).

The standard deviation (STDEV) was calculated using the following equation:

$$STDEV = \sqrt{\frac{\sum(x - \bar{x})^2}{n - 1}} \qquad (C2)$$

The confidence function (CF) was calculated using the following equation:

$$CF = \pm 1.96 \times \left(\frac{STDEV}{\sqrt{n}}\right) \qquad (C3)$$

$n = number\ of\ replicates (2\ in\ this\ case)$

Laboratory Analytical Procedure LAP# 004 by NREL stated that if the calculated Relative percent difference (RPD) of ASL is higher than 15.5 %, the sample must be rerun. The % RPD is a criterion to measure the difference between two samples and it can be defined as follow:

$$\% RPD = \frac{|x_1 - x_2|}{\frac{(x_1 + x_2)}{2}} \times 100 \qquad (C4)$$

x_1 and x_2 in this case are the two % ASL determined in a duplicate runs.

Table C1. Acid Soluble Lignin (ASL) content for untreated wheat straw.

Replicate # 1						Replicate # 2						ASL Mean ± CF[1]	ASL STDEV[2]	ASL % RPD[3]
absorbance at 205 nm (A)	dilution factor (d)	Filtrate volume (V)	Initial sample weight (W_1)	Total solids (TS)	ASL	absorbance at 205 nm (A)	dilution factor (d)	Filtrate volume (V)	Initial sample weight (W_1)	Total solids (TS)	ASL			
(-)		ml	g	wt.%	w.%	(-)		ml	g	wt. %	wt. %	wt. %	wt. %	wt. %
0.587	7	570	1.000	93.75	2.27	0.601	7	565	1.000	93.31	2.32	2.30 ± 0.06	0.04	2.18

[1]CF = Confidence Function at 95 % probability.
[2]STDEV = Standard Deviation.
[3]RPD = Relative Percent Difference.

Appendix D: Enzymatic hydrolysis of wheat straw

D.1 Enzymatic hydrolysis Procedure

Modified NREL Laboratory Analytical Procedure # 009 (NREL, 1995)

Reagents and Materials

Citrate Buffer

Enzymatic hydrolysis was carried out in 0.10 M citrate buffer (pH 5.25).

To prepare 1.0 M citrate buffer, dissolve 210 g of Citric acid monohydrate in 750 ml of distilled water. Adjust the pH of the solution to 4.3 by adding NaOH (50-60 g).

To prepare 0.10 M citrate buffer, dilute 100 ml of 1 M citrate buffer stock to 1 liter by adding 900 ml distilled water. Adjust the pH of the solution to 5.25 by adding NaOH.

Procedure

1) Perform LAP-001 (Appendix A) "Determination of Total Solids in Biomass" for all wheat straw to be hydrolyzed.

   Note: all lignocellulosic materials which have undergone some aqueous pretreatment must never be air-dried prior to enzyme digestibility, since irreversible pore collapse can occur in the micro-structure of the biomass leading to decreased enzymatic release of glucose from the cellulose.

2) Weigh out a sample equal to 2.0 g of oven-dried wheat straw to be hydrolyzed and add to a 250 ml Erlenmeyer flask. Same amount of each wheat straw should be weighted and put in a separate 250 ml Erlenmeyer flask to be used as a buffer blank accompanying each hydrolysis test. Label each hydrolysis flask and its accompanying buffer flask. Each blank will be used as a reference when measuring the reducing sugars concentration.

3) Label another 250 ml Erlenmeyer flask which will be used as enzyme buffer. Reducing sugars concentration measured for the enzyme buffer will be subtracted from reducing sugars concentration measurement of each hydrolysis test for accurate results.
4) To each flask, add 50 ml (0.1 M, and pH 5.25) sodium citrate buffer and 1 ml of a 2 wt. % sodium azide.
5) Add 47 ml of distilled water to each flask to bring the total volume to 100 ml.

   Note: the volume of enzymes added is so small that can be neglected in calculation of the total volume. To simplify the calculation, all solutions and the biomass are assumed to have specific gravity of 1.000 g/ml.
6) Close all the flasks with rubber stoppers then bring the contents of each flask to 47.5 ºC by warming in a rotary incubator set at 47.5 ± 1 ºC. Add the required volumes of the cellulase enzyme (NS22086) equivalent to 5 % wt. /wt. (TS) and β-glucosidase enzyme (NS22118) equivalent to 0.6 % wt. /wt. (TS) to each hydrolysis test flask and to the enzyme blank. Be careful not to add enzymes to the buffer blank flasks.

The densities of the enzymes (Table 3.1):

cellulase enzyme (NS22086) sp. gr. = 1.15 mg/ µl

β-glucosidase enzyme (NS22118) sp. gr. = 1.20 mg/ µl

Therefore, the volume of each enzyme added:

$$Cellulase\ (NS22086) = 0.05 \frac{mg\ enzyme}{mg\ Total\ solid} \times 2000\ mg \times \frac{\mu l}{1.15\ mg} = 87\ \mu l$$

$$\beta - \text{glucosidase } (NS22118) = 0.006 \frac{mg\ enzyme}{mg\ Total\ solid} \times 2000\ mg \times \frac{\mu l}{1.20\ mg} = 10\ \mu l$$

Note: The enzymes are always added last since the reaction is initiated by the addition of enzyme.

7) Close all the flasks tightly with the rubber stoppers and place them again in the rotary incubator set at 47.5 ºC. Incubate with gentle rotation (68 RPM) until the measured reducing sugars concentration become almost constant in subsequent samples.

9) One and a half ml aliquot is removed at each predetermined time intervals, i.e. 2, 4, 16, 40, 64, 88, 112, 136 and 160 hours. This is accomplished by using a 5.0 ml pipette with the tip of the plastic tip slightly cut off to prevent clogging of the pipette. The sample is expelled into a 1.5 ml micro-centrifuge tube and centrifuged for 1.5 minutes. A volume of 1.0 ml of the supernatant is subjected to reducing sugar analysis using DNS method.

D.2 Reducing sugar determination by Dinitrosalicylic Acid (DNS) Reagent (Ghose, 1987)

DNS Reagent

Dissolve 10.6 g of 3, 5 Dinitrosalicylic acid and 19.8 g of Sodium hydroxide then add 306 g of Rochelle salts (sodium potassium tartrate), 7.6 ml of Phenol and 8.3 g of Sodium metabisulfite.

Titrate 3 ml sample with 0.1 N HCl to the phenolphthalein endpoint. It should take 5-6 ml of HCl. Add NaOH if required.

Glucose standards

A working stock solution of anhydrous glucose (12 mg/ml) should be made up. Aliquots of this working stock should be tightly sealed and stored frozen. The standard should be vortexed after thawing to ensure adequate mixing.

Dilutions are made from the working stock in the following manner:

1.0 ml + 0.0 ml buffer = 1:1 (12.0 mg/ml)

1.0 ml + 0.33 ml buffer = 1:1.33 (9.0 mg/ml)

1.0 ml + 1.0 ml buffer = 1:2 (6.0 mg/ml)

1.0 ml + 3.0 ml buffer = 1:4 (3.0 mg/ml)

1.0 ml + 11.0 ml buffer = 1:12 (1.0 mg/ml)

1.0 ml + 23.0 ml buffer = 1:24 (0.5 mg/ml)

1.0 ml + 59.0 ml buffer = 1:60 (0.2 mg/ml)

Take 1.0 ml of each glucose dilution and put them in a 13 x 100 mm test tube.

Color development

1) Transfer the 1.0 ml of the supernatant for hydrolysis samples, buffer blanks and enzyme blank to 13 x 100 mm test tubes.
2) Add 3.0 ml of DNS reagent to test tubes containing hydrolysis samples, buffer blanks, enzyme blank and the glucose standards.
3) Boil all test tubes for exactly 5.0 minutes in a vigorously boiling water bath containing sufficient water to cover the portions of the tubes occupied by the reaction mixture plus reagent. All samples, controls, blanks, and glucose standards should be boiled together. After boiling, transfer to a cold ice-water bath.

4) Dilute all tubes (assays, blanks and standards) in water (0.200 ml of color-developed reaction mixture plus 2.5 ml of water)
5) Determine color formation by measuring absorbance for hydrolysis samples and glucose standards against their buffer blank at 540 nm.

Calculations

1) Construct a linear glucose standard curve using the absolute amounts of glucose concentration (mg/ml) plotted against Absorbance at 540 nm. The data for the standard curve should closely fit a calculated straight line.
2) Using this standard curve determine the amount of glucose released for each sample tube after subtraction of enzyme blank.

The absorbance values were measured using Biochrom UV spectrophotometer, model number: Ultraspec 50, England. Table D1 shows the data for the standard glucose curve. Figure D1 shows the plot of the glucose standard curve.

Table D1. Data for glucose standard curve (DNS method).

[Glucose] (x_i)	Absorbance replicate # 1	Absorbance replicate # 2	Absorbance Mean (y_i)	(x_i^2)	(y_i^2)	($x_i y_i$)
(mg/ml)	(-)	(-)	(-)	$(mg/ml)^2$	(-)	(mg/ml)
0.200	0.014	0.015	0.015	0.040	0.000	0.003
0.500	0.058	0.059	0.059	0.250	0.003	0.030
1.000	0.144	0.152	0.148	1.000	0.022	0.148
3.000	0.377	0.421	0.399	9.000	0.159	1.197
6.000	0.824	0.880	0.852	36.000	0.726	5.112
9.000	1.228	1.274	1.251	81.000	1.565	11.259
12.000	1.592	1.653	1.623	144.000	2.634	19.476
31.700			4.347	271.290	5.110	37.225

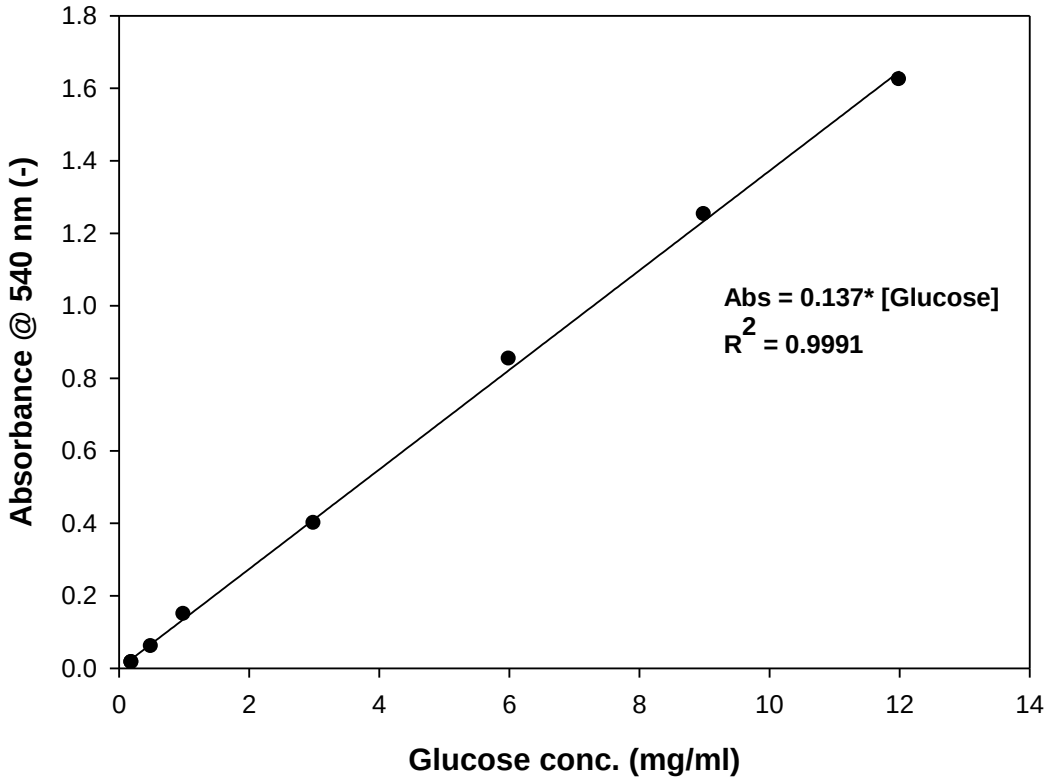

Figure D1. Glucose standard curve (DNS method) for reducing sugars concentration measurement. Vertical bars are the confidence intervals for duplicate runs at 95 % probability.

Columns 3, 4, and 5 of Table D1 contain computed values for x_i, y_i and $x_i y_i$, with their sums appearing as the last entry in each column which were used to analyze the reducing sugars measurements by the least square method (Skoog et al., 2007).

The calculation of the slope and intercept is simplified by defining three quantities S_{xx}, S_{yy} and S_{xy} as follows:

$$S_{xx} = \sum x_i^2 - \frac{(\sum x_i)^2}{N} = 271.290 - \frac{(31.700)^2}{7} = 127.734\ (\frac{mg}{ml})^2$$

$$S_{yy} = \sum y_i^2 - \frac{(\sum y_i)^2}{N} = 5.110 - \frac{(4.347)^2}{7} = 2.411\ (-)$$

$$S_{xy} = \sum x_i y_i - \frac{\sum x_i \sum y_i}{N} = 37.225 - \frac{31.700 \times 4.347}{7} = 17.539\ (\frac{mg}{ml})$$

The slope of the line, m:

$$m = \frac{S_{xy}}{S_{xx}} = \frac{17.539}{127.734} = 0.137\ (\frac{ml}{mg})$$

The means (averages) for x and y values:

$$\bar{x} = \frac{\sum x_i}{N} = \frac{31.700}{7} = 4.529\ (\frac{mg}{ml})$$

$$\bar{y} = \frac{\sum y_i}{N} = \frac{4.347}{7} = 0.621\ (-)$$

The intercept, b:

$$b = \bar{y} - m\bar{x} = 0.621 - 0.137 \times 4.529 = 0.0005\ \approx 0.000\ (-)$$

Thus, the equation for the lease square line of the standard curve is:

$$Abs. = 0.137 \times [Glucose] \qquad \text{(D1)}$$

The standard deviation about regression:

$$S_r = \sqrt{\frac{S_{yy} - m^2 S_{xx}}{N-2}} = \sqrt{\frac{2.411 - (0.137)^2 \times 127.734}{7-2}} = 0.020868\ (-)$$

The standard deviation of the slope:

$$S_m = \sqrt{\frac{S_r^2}{S_{xx}}} = \sqrt{\frac{(0.052)^2}{127.734}} = 0.001846\ (\frac{ml}{mg})$$

The standard deviation of the intercept:

$$S_b = S_r \sqrt{\frac{1}{N - (\sum x_i)^2 / \sum x_i^2}} = 0.052 \times \sqrt{\frac{1}{7 - (31.700)^2/271.290}} = 0.011494\ (-)$$

The standard deviation for results obtained from the standard curve:

$$S_c = \frac{S_r}{m} \sqrt{\frac{1}{M} + \frac{1}{N} + \frac{(\bar{y}_c - \bar{y})^2}{m^2 S_{xx}}}$$

$$S_c = \frac{0.020868}{0.137} \times \sqrt{\frac{1}{2} + \frac{1}{7} + \frac{(\bar{y}_c - 0.621)^2}{(0.137)^2 \times 127.734}} \left(\frac{mg}{ml}\right) \qquad \text{(D2)}$$

Where:

M = number of replicates.

N = number of points used in the standard curve.

$\bar{y}_c$ = mean of the Absorbance measured in replicates # 1 and # 2.

Table D2 shows the measured absorbance for the replicates of enzymatic hydrolysis experiments at different hydrolysis times. The mean of absorbance for the replicates ($\bar{y}_c$) was calculated and used to calculate the reducing sugars concentration by rearranging the lease square equation (Eq. D1) for the line. The standard deviation (S_c) for the absorbance was determined using to Eq. D2.

The maximum standard deviation for the reducing sugars concentration, which was determined from the standard curve as shown in Table D2, was for the 2-enhance experiments after 112 hrs of hydrolysis time and it is equal to 0.152 (mg/ml). The Confidence Interval (CI) was calculated at 95 % probability using the following equation:

$$CI = \bar{x} \ \pm 1.96 \times \left(\frac{S_c}{\sqrt{M}}\right)$$

$$CI = 11.322 \pm 1.96 \times \left(\frac{0.152}{\sqrt{2}}\right) = 11.322 \pm 0.21 = (11.112, 11.532)\frac{mg}{ml}$$

$\bar{x}$ is the mean (average) value of the reducing sugars concentration for the two replicates which were determined from the standard curve as shown in Table D2.

Figure D2 shows the change in reducing sugar concentration with time for the five enzymatic hydrolysis experiments which were started with two types of untreated wheat straw and three types of ozonated wheat straw. The vertical bar is the confidence interval for the maximum standard deviation which was calculated above.

Table D2. Summary of the enzymatic hydrolysis yield of the duplicate runs of enzymatic hydrolysis of untreated and ozonated wheat straw.

Hydrolysis time (hr)	2-control[1]					2-optimum[1]				
	Absorbance Replicate # 1	Absorbance Replicate # 2	Absorbance Mean ($\bar{y}_c$)	Reducing sugars concentration Mean ($\bar{x}$) ± CF[2]	Standard deviation (S_c)	Absorbance Replicate # 1	Absorbance Replicate # 2	Absorbance Mean ($\bar{y}_c$)	Reducing sugars concentration Mean ($\bar{x}$) ± CF[2]	Standard deviation (S_c)
	(-)	(-)	(-)	mg/ml	mg/ml	(-)	(-)	(-)	mg/ml	mg/ml
2	0.158	0.158	0.158	1.151 ± 0.18	0.129	0.384	0.387	0.385	2.811 ± 0.17	0.124
4	0.261	0.262	0.261	1.908 ± 0.17	0.126	0.713	0.731	0.722	5.271 ± 0.17	0.122
16	0.316	0.317	0.316	2.309 ± 0.17	0.125	1.192	1.237	1.215	8.866 ± 0.19	0.134
40	0.354	0.356	0.355	2.590 ± 0.17	0.124	1.222	1.254	1.238	9.035 ± 0.19	0.135
64	0.376	0.377	0.376	2.748 ± 0.17	0.124	1.273	1.317	1.295	9.450 ± 0.19	0.138
88	0.370	0.388	0.379	2.766 ± 0.17	0.124	1.318	1.362	1.340	9.779 ± 0.19	0.14
112	0.402	0.404	0.403	2.941 ± 0.17	0.123	1.371	1.419	1.395	10.185 ± 0.20	0.143
136	0.398	0.400	0.399	2.913 ± 0.17	0.123	1.366	1.415	1.390	10.150 ± 0.20	0.143
160	0.400	0.397	0.399	2.909 ± 0.17	0.123	1.360	1.414	1.387	10.122 ± 0.20	0.142
	2-enhance[1]									
2	0.453	0.457	0.455	3.320 ± 0.17	0.122					
4	0.682	0.700	0.691	5.042 ± 0.17	0.122					
16	1.244	1.285	1.265	9.231 ± 0.19	0.137					
40	1.384	1.434	1.409	10.285 ± 0.20	0.144					
64	1.491	1.549	1.520	11.095 ± 0.21	0.15					
88	1.514	1.559	1.536	11.215 ± 0.21	0.151					
112	1.525	1.577	1.551	11.322 ± 0.21	0.152					
136	1.551	1.556	1.554	11.340 ± 0.21	0.152					
160	1.549	1.543	1.546	11.285 ± 0.21	0.151					

[1]See Table 3.3 for descriptions.
[2]CF = Confidence Function at 95 % probability.

Table D2. Summary of the enzymatic hydrolysis yield of the duplicate runs of enzymatic hydrolysis of untreated and ozonated wheat straw.

Hydrolysis time (hr)	6-control[1]					6-optimum[1]				
	Absorbance Replicate # 1	Absorbance Replicate # 2	Absorbance Mean ($\bar{y}_c$)	Reducing sugars concentration Mean ($\bar{x}$) ± CF[2]	Standard deviation (S_c)	Absorbance Replicate # 1	Absorbance Replicate # 2	Absorbance Mean ($\bar{y}_c$)	Reducing sugars concentration Mean ($\bar{x}$) ± CF[2]	Standard deviation (S_c)
	(-)	(-)	(-)	mg/ml	mg/ml	(-)	(-)	(-)	mg/ml	mg/ml
2	0.159	0.152	0.156	1.136 ± 0.18	0.13	0.340	0.342	0.341	2.491 ± 0.17	0.124
4	0.260	0.260	0.260	1.898 ± 0.17	0.126	0.651	0.661	0.656	4.789 ± 0.17	0.121
16	0.313	0.312	0.313	2.284 ± 0.17	0.125	1.107	1.134	1.120	8.177 ± 0.18	0.131
40	0.351	0.348	0.350	2.552 ± 0.17	0.124	1.192	1.223	1.208	8.816 ± 0.19	0.134
64	0.375	0.369	0.372	2.714 ± 0.17	0.124	1.195	1.230	1.213	8.852 ± 0.19	0.134
88	0.373	0.381	0.377	2.752 ± 0.17	0.124	1.202	1.231	1.216	8.88 ± 0.19	0.135
112	0.396	0.394	0.395	2.882 ± 0.17	0.123	1.255	1.286	1.270	9.273 ± 0.19	0.137
136	0.399	0.396	0.397	2.900 ± 0.17	0.123	1.231	1.260	1.245	9.091 ± 0.19	0.136
160	0.398	0.394	0.396	2.891 ± 0.17	0.123	1.249	1.269	1.259	9.190 ± 0.19	0.136

[1]See Table 3.3 for descriptions.
[2]CF = Confidence Function at 95 % probability.

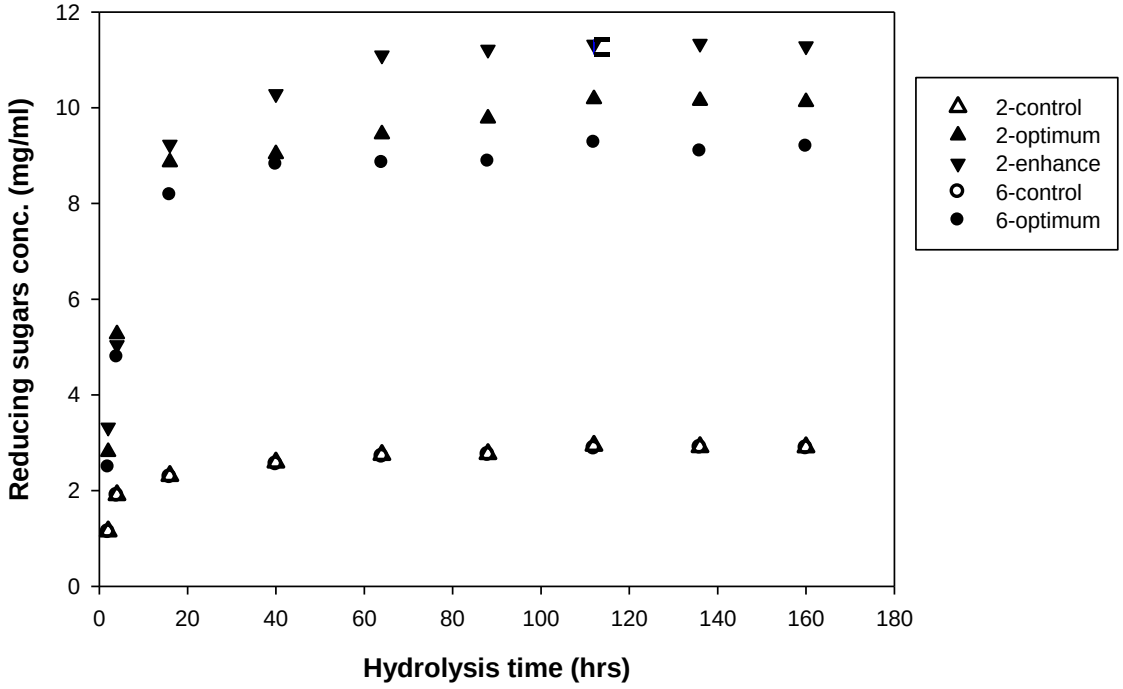

Figure D2 Reducing sugars concentration from enzymatic hydrolysis of untreated and ozonated wheat straws. Vertical bar is confidence interval for duplicate runs at 95% probability.

D.3 Enzymatic hydrolysis yield

The hydrolysis yield compared the amount of reducing sugars exp erimentally released by the enzymatic hydrolysis of the cellulose and hemicellulose in wheat straw to the theoretical amount of glucose expected from the complete degradation of cellulose and hemicellulose.

Hemicellulose and cellulose are the two main structural carbohydrates of wheat straw that can be hydrolyzed into simple sugars.

Cellulose, which is a homogeneous polymer (glucan), is converted into glucose, a six carbon monomer:

$$[\boldsymbol{C_6(H_2O)_5}]_n + \boldsymbol{nH_2O} \rightarrow \boldsymbol{nC_6H_{12}O_6} \qquad (D3)$$

cellulose n (glucose)

The hydrolysis of hemicellulose can be simplified as:

$$[\boldsymbol{C_5(H_2O)_4}]_n + \boldsymbol{nH_2O} \rightarrow \boldsymbol{nC_5H_{10}O_5} \qquad (D4)$$

hemicellulose n (xylose)

Mckean and Jacobs (1997) reported that wheat straw contains around 34 wt. % cellulose and 25 wt. % hemicellulose. To simplify the calculation, same compositions of cellulose and hemicellulose have been used in this study.

When using 2 g wheat straw (oven dry weight) in the enzymatic hydrolysis experiments to make a total volume of 100 ml solution (including citrate buffer, sodium azide, enzymes, and water), then:

cellulose = 2 g × 0.34 = 0.68 g

hemicellulose = 2 g × 0.25 = 0.5 g

According to Eq. D3, one mole of cellulose (equivalent to 162 g) will release one mole of glucose (equivalent to 180 g). Therefore:

0.68 g of cellulose will release = $0.68 \times \left(\frac{180}{162}\right) = 0.76$ g of glucose

According to Eq. D4, 132 g hemicellulose (equivalent to 1 mole) gives 150 g of xylose (equivalent to 1 mole). Therefore:

0.5 g of hemicellulose will release = $0.5 \times \left(\frac{150}{132}\right) = 0.57$ g of xylose

Consequently, 1.33 g of reducing sugars (glucose + xylose) are theoretically expected t o be released from 2 g of wheat straw,

Therefore, the theoretical reducing sugars concentration (dissolved in 100 ml solution) will be:

$$\text{theoretical reducing sugars concentration } = \frac{1.33\ g}{100\ ml} \times \frac{1000\ mg}{1\ g} = 13.3\ {}^{mg}/_{ml}$$

In this case, the hydrolysis yield (% conversion) can be reported as:

$$Reducing\ sugars\ yield\ (\%\ theoretical) = \frac{\left[Experimental\ reducing\ sugar\ ({}^{mg}/_{ml})\right]}{13.3\ ({}^{mg}/_{ml})} \times 100$$

In general, if there is y amount of total solids as in 2 g of wheat straw, then the amount of total reducing sugars can be calculated as follows:

$$\text{glucose theoretically to be released (g)} = 0.34 \times y \times \frac{180}{162} = 0.3778\ y$$

$$\text{xylose theoretically to be released (g)} = 0.25 \times y \times \frac{150}{132} = 0.2841\ y$$

In a solution of 100 ml, then the theoretical concentration of reducing sugars will be:

$$\text{theoretical reducing sugars concentration} = \frac{[0.3778\ y + 0.2841\ y]g}{100\ ml} \times \frac{1000\ mg}{g}$$

$$\text{theoretical reducing sugars concentration } \left({}^{mg}/_{ml}\right) = 6.618\, y$$

or:

$$Theoretical\ reducing\ sugars\ \left({}^{mg}/_{ml}\right) = 6.618 \times\ [Total\ solids\ (g)] \qquad (D5)$$

The yield will be:

$$Reducing\ sugars\ yield\ (\%\ theoretical) = \frac{\left[Experimental\ reducing\ sugars\ \left({}^{mg}/_{ml}\right)\right]}{\left[Theoretical\ reducing\ sugars\ \left({}^{mg}/_{ml}\right)\right]} \times 100 \qquad (D6)$$

Enzymatic hydrolysis experiments of untreated and ozonated wheat straw were performed in duplicate. Table D3 shows the results of replicate # 1 of the enzymatic hydrolysis of untreated and ozonated wheat straw while Table D4 shows replicate # 2.

The first column in Tables D3 and D4 shows the name of the experiment (see Table 3.3). The second column in Tables D3 and D4 shows the total solids of wheat straw used in each hydrolysis experiment which was determined by calculating the total solid content % TS (Appendix A) and multiply it by the wheat straw weight. The theoretical reducing sugar concentration in column three of Tables D3 and D4 was calculated using Eq. D5. Finally the reducing sugars yield in Tables D3 and D4 was calculated according to Eq. D6 after determining the experimental reducing sugars concentration in each sample using DNS method. Section G.5 (Appendix G) shows an example for the calculations of reducing sugars yield in Tables D3 and D4. Table D5 is a summary of the enzymatic hydrolysis yield of the duplicate experiments. The table shows the mean, standard deviation and the confidence function at 95 % probability level.

Table D3. Results for enzymatic hydrolysis of untreated and ozonated wheat straw (Replicate # 1).

Experiment name[1]	Total solids (OD weight)	Theoretical reducing sugar concentration	Hydrolysis time (hr)					
			2		4		16	
			Measured reducing sugars concentration	Reducing sugars yield	Measured reducing sugars concentration	Reducing sugars yield	Measured reducing sugars concentration	Reducing sugars yield
	g	mg/ml	mg/ml	% theoretical	mg/ml	% theoretical	mg/ml	% theoretical
2-control	1.874	12.402	1.152	9.29	1.904	15.35	2.303	18.57
2-optimum	1.918	12.693	2.800	22.06	5.208	41.03	8.702	68.56
2-enhance	1.902	12.587	3.304	26.25	4.977	39.54	9.080	72.14
6-control	1.867	12.356	1.162	9.40	1.895	15.34	2.287	18.51
6-optimum	1.914	12.667	2.485	19.62	4.753	37.52	8.079	63.78
			40		64		88	
2-control	1.874	12.402	2.583	20.83	2.744	22.13	2.702	21.79
2-optimum	1.918	12.693	8.920	70.27	9.290	73.19	9.619	75.78
2-enhance	1.902	12.587	10.100	80.24	10.880	86.44	11.05	87.79
6-control	1.867	12.356	2.563	20.74	2.734	22.13	2.721	22.02
6-optimum	1.914	12.667	8.702	68.70	8.723	68.86	8.772	69.25
			112		136		160	
2-control	1.874	12.402	2.933	23.65	2.905	23.42	2.919	23.54
2-optimum	1.918	12.693	10.01	78.86	9.974	78.58	9.924	78.18
2-enhance	1.902	12.587	11.13	88.42	11.32	89.96	11.31	89.85
6-control	1.867	12.356	2.89	23.42	2.91	23.55	2.906	23.52
6-optimum	1.914	12.667	9.157	72.29	8.985	70.93	9.12	72.00

[1]See Table 3.3 for descriptions.

Table D4. Results for enzymatic hydrolysis of untreated and ozonated wheat straw (Replicate # 2).

Experiment name[1]	Total solids (OD weight)	Theoretical reducing sugar concentration	Hydrolysis time (hr)					
			2		4		16	
			Measured reducing sugars concentration	Reducing sugars yield	Measured reducing sugars concentration	Reducing sugars yield	Measured reducing sugars concentration	Reducing sugars yield
	g	mg/ml	mg/ml	% theoretical	mg/ml	% theoretical	mg/ml	% theoretical
2-control	1.907	12.621	1.150	9.11	1.912	15.15	2.314	18.34
2-optimum	1.921	12.713	2.822	22.20	5.333	41.95	9.029	71.02
2-enhance	1.911	12.647	3.336	26.38	5.107	40.38	9.382	74.18
6-control	1.883	12.462	1.11	8.92	1.90	15.25	2.281	18.30
6-optimum	1.908	12.627	2.497	19.77	4.825	38.21	8.274	65.53
			40		64		88	
2-control	1.907	12.621	2.596	20.57	2.751	21.80	2.829	22.42
2-optimum	1.921	12.713	9.150	71.97	9.610	75.59	9.939	78.18
2-enhance	1.911	12.647	10.470	82.79	11.310	89.43	11.380	89.98
6-control	1.883	12.462	2.541	20.39	2.693	21.61	2.783	22.33
6-optimum	1.908	12.627	8.930	70.72	8.980	71.12	8.987	71.17
			112		136		160	
2-control	1.907	12.621	2.948	23.36	2.920	23.14	2.899	22.97
2-optimum	1.921	12.713	10.360	81.49	10.325	81.22	10.320	81.18
2-enhance	1.911	12.647	11.514	91.04	11.360	89.82	11.260	89.03
6-control	1.883	12.462	2.874	23.06	2.889	23.18	2.875	23.07
6-optimum	1.908	12.627	9.389	72.36	9.196	72.82	9.260	73.33

[1]See Table 3.3 for descriptions.

Table D5. Summary of the enzymatic hydrolysis yield of the duplicate runs of enzymatic hydrolysis of untreated and ozonated wheat straw.

Hydrolysis time (hr)	2-control[1]				2-optimum[1]			
	Reducing sugar yield Replicate # 1	Reducing sugar yield Replicate # 2	Reducing sugar yield Mean ± CF[2]	Reducing sugar yield STDEV[3]	Reducing sugar yield Replicate # 1	Reducing sugar yield Replicate # 2	Reducing sugar yield Mean ± CF[2]	Reducing sugar yield STDEV[3]
	% theoretical	% theoretical	% theoretical	% theoretical	% theoretical	% theoretical	% theoretical	% theoretical
2	9.29	9.11	9.20 ± 0.18	0.13	22.06	22.2	22.13 ± 0.14	0.10
4	15.35	15.15	15.25 ± 0.20	0.14	41.03	41.95	41.49 ± 0.90	0.65
16	18.57	18.34	18.45 ± 0.23	0.16	68.56	71.02	69.79 ± 2.41	1.74
40	20.83	20.57	20.70 ± 0.25	0.18	70.27	71.97	71.12 ± 1.67	1.20
64	22.13	21.8	21.96 ± 0.32	0.23	73.19	75.59	74.39 ± 2.35	1.70
88	21.79	22.42	22.10 ± 0.62	0.45	75.78	78.18	76.98 ± 2.35	1.70
112	23.65	23.36	23.50 ± 0.28	0.21	78.86	81.49	80.17 ± 2.58	1.86
136	23.42	23.14	23.28 ± 0.27	0.20	78.58	81.22	79.90 ± 2.59	1.87
160	23.54	22.97	23.25 ± 0.56	0.40	78.18	81.18	79.68 ± 2.94	2.12
	2-enhance[1]							
2	26.25	26.38	26.32 ± 0.13	0.09				
4	39.54	40.38	39.96 ± 0.82	0.59				
16	72.14	74.18	73.16 ± 2.00	1.44				
40	80.24	83.18	81.71 ± 2.88	2.08				
64	86.44	89.43	87.94 ± 2.93	2.11				
88	87.79	89.98	88.89 ± 2.15	1.55				
112	89.42	91.04	90.23 ± 1.59	1.15				
136	89.96	89.82	89.89 ± 0.14	0.10				
160	89.85	89.03	89.44 ± 0.80	0.58				

[1]See Table 3.3 for descriptions.
[2]CF = Confidence Function at 95 % probability.
[3]STDEV = Standard Deviation.

Table D5. Summary of the enzymatic hydrolysis yield of the duplicate runs of enzymatic hydrolysis of untreated and ozonated wheat.

Hydrolysis time (hr)	6-control[1]				6-optimum[1]			
	Reducing sugar yield Replicate # 1	Reducing sugar yield Replicate # 2	Reducing sugar yield Mean ± CF[2]	Reducing sugar yield STDEV[3]	Reducing sugar yield Replicate # 1	Reducing sugar yield Replicate # 2	Reducing sugar yield Mean ± CF[2]	Reducing sugar yield STDEV[3]
	% theoretical	% theoretical	% theoretical	% theoretical	% theoretical	% theoretical	% theoretical	% theoretical
2	9.4	8.92	9.16 ± 0.47	0.34	19.62	19.77	19.69 ± 0.15	0.11
4	15.34	15.25	15.29 ± 0.09	0.06	37.52	38.21	37.86 ± 0.68	0.49
16	18.51	18.3	18.40 ± 0.21	0.15	63.78	65.53	64.65 ± 1.71	1.24
40	20.74	20.39	20.56 ± 0.34	0.25	68.7	70.72	69.71 ± 1.98	1.43
64	22.13	21.61	21.87 ± 0.51	0.37	68.86	71.12	69.99 ± 2.21	1.60
88	22.02	22.33	22.17 ± 0.30	0.22	69.25	71.17	70.21 ± 1.88	1.36
112	23.42	23.06	23.24 ± 0.35	0.25	72.29	72.36	72.32 ± 0.07	0.05
136	23.55	23.18	23.36 ± 0.36	0.26	70.93	72.82	71.87 ± 1.85	1.34
160	23.52	23.07	23.29 ± 0.44	0.32	72	73.33	72.66 ± 1.30	0.94

[1]See Table 3.3 for descriptions.
[2]CF = Confidence Function at 95 % probability.
[3]STDEV = Standard Deviation.

Appendix E: Cellulase enzyme activity measurement

Modified Laboratory Analytical Procedure #006 (NREL, 1995)

Reagents and Materials

DNS Reagent

Dissolve 10.6 g of 3, 5 Dinitrosalicylic acid and 19.8 g of Sodium hydroxide then add 306 g of Rochelle salts (sodium potassium tartrate), 7.6 ml of Phenol and 8.3 g of Sodium metabisulfite.

Titrate 3 ml sample with 0.1 N HCl to the phenolphthalein endpoint. It should take 5-6 ml of HCl. Add NaOH if required.

Citrate Buffer

Novozymes Cellulase enzyme (NS 22086) assays are carried out in 0.05 M citrate buffer pH 5.0.

To prepare 1.0 M citrate buffer, dissolve 210 g of Citric acid monohydrate in 750 ml of distilled water. Adjust the pH of the solution to 4.3 by adding NaOH (50-60 g).

To prepare 0.05 M citrate buffer, dilute 50 ml of 1 M citrate buffer stock to 1 liter by adding 950 ml distilled water. Adjust the pH of the solution to 5.0 by adding NaOH.

Activity measurement procedure (Filter Paper Assay)

The detection of glycosidic bond cleavage by this method involves the parallel and identical treatment of three categories of experimental tubes (assay mixtures, blanks and controls, and glucose standards), prepared as detailed below. The substrate is a 50 mg Whatman No. 1 filter paper strip (1.0 x 6.0 cm).

Enzyme assay tubes

1) Place a rolled filter paper strip into each 13 x 100 test tube.
2) Add 1.0 ml 0.05 M citrate buffer, pH 5.0 to the tube; the buffer should saturate the filter paper strip.
3) Equilibrate tubes with buffer and substrate to 47.5 ºC.
4) Add 0.5 ml enzyme diluted appropriately in citrate buffer. At least two dilutions must be made of each enzyme sample, with one dilution releasing slightly more than 2.0 mg of glucose (absolute amount) and one slightly less than 2.0 mg of glucose. Target 2.1 and 1.9 mg glucose, respectively, for these two dilutions. Depending on the enzyme these targets may be hard to achieve and additional dilutions must be run.
5) Incubate at 47.5 ºC for exactly 60 min.
6) At the end of the incubation period, remove each assay tube from the 47.5 ºC bath and stop the enzyme reaction by immediately adding 3.0 ml DNS reagent and mixing.

Blank and controls

Reagent blank: 1.5 ml of 0.05 M citrate buffer.

Enzyme control: 1.0 ml of 0.05 M citrate buffer + 0.5 ml enzyme dilutions (prepare a separate control for each dilution tested).

Substrate control: 1.5 ml of 0.05 M citrate buffer + filter-paper strip.

Glucose standards

A working stock solution of anhydrous glucose (10 mg/ml) should be made up. Aliquots of this working stock should be tightly sealed and stored frozen. The standard should be vortexed after thawing to ensure adequate mixing.

Dilutions are made from the working stock in the following manner:

1.0 ml + 0.5 ml buffer = 1:1.5 (3.35 mg/0.5 ml).

1.0 ml + 1.0 ml buffer = 1:2 (2.5 mg/0.5 ml).

1.0 ml + 2.0 ml buffer = 1:3 (1.65 mg/0.5 ml).

1.0 ml + 4.0 ml buffer = 1:5 (1.0 mg/0.5 ml).

Glucose standard tubes should be prepared by adding 0.5 ml of each of the above glucose dilutions to 1.0 m of 0.05 M citrate buffer in a 13 x 100 mm test tube.

Blanks, controls and glucose standards should be incubated at 47.5 ºC along with the enzyme assay tubes, and then "stopped" at the end of 60 minutes by addition of 3.0 ml of DNS reagent.

Color development

1) Boil all tubes for exactly 5.0 minutes in a vigorously boiling water bath containing sufficient water to cover the portions of the tubes occupied by the reaction mixture plus reagent. All samples, controls, blanks, and glucose standards should be boiled together. After boiling, transfer to a cold ice-water bath.
2) Let the tubes sit until all the pulp has settled, or centrifuge briefly. Dilute all tubes (assays, blanks, standards and controls) in water (0.200 ml of color-developed reaction mixture plus 2.5 ml of water in a spectrophotometer cuvette works well, use the pipettor to mix by drawing the mixture into the pipettor tip repeatedly). Determine color formation by measuring absorbance against the reagent blank at 540 nm. With this dilution the glucose standards described above should give absorbance in the range of 0.1 to 1.0 A.

Calculations

1) Construct a linear glucose standard curve using the absolute amounts of glucose (mg/0.5 ml) plotted against A540. Verify the standard curve by running a calibration verification standard, an independently prepared solution containing a known amount of glucose which falls about midpoint on the standard curve.
2) Using this standard curve determine the amount of glucose released for each sample tube after subtraction of enzyme blank.
3) Estimate the concentration of enzyme which would have released exactly 2.0 mg of glucose by means of a plot of glucose standard curve. To find the required enzyme concentration take two data points that are very close to 2.0 mg and draw a straight line between them, use this line to interpolate between the two points to find the enzyme dilution that would produce exactly 2.0 mg glucose equivalents of reducing sugar.

   Note: In this plot, and in the equation below for calculating FPU, the term "enzyme" refers to the proportion of the original enzyme solution present in each enzyme dilution (i.e., the number of ml of the original solution present in each ml of the dilution).

$$Cellulase\ activity\left(FPU/_{ml}\right) = \frac{0.37}{[enzyme]\ releasing\ 2.0\ mg\ glucose} \qquad (E1)$$

[enzyme] represents the dilution factor or the proportion of original enzyme solution present in the directly tested enzyme dilution (that dilution of which 0.5 ml is added to the assay mixture).

Table E1 shows the raw data for the change of absorbance with glucose concentration variation. These data were used to plot the glucose standard curve as shown in Figure E1.

The absorbance values were measured using Biochrom UV spectrophotometer, model number: Ultraspec 50, England. Table E1 shows the data for the standard glucose curve. Figure E1 shows the plot of the glucose standard curve.

Table E1. Data for glucose standard curve (DNS method).

[Glucose] (x_i)	Absorbance replicate # 1	Absorbance replicate # 2	Absorbance Mean (y_i)	(x_i^2)	(y_i^2)	($x_i y_i$)
(mg/ 0.5 ml)	(-)	(-)	(-)	(mg/0.5 ml)^2	(-)	(mg/0.5 ml)
1.00	0.215	0.219	0.217	1	0.047089	0.217000
1.65	0.346	0.350	0.348	2.7225	0.121104	0.574200
2.50	0.519	0.531	0.525	6.2500	0.275625	1.312500
3.35	0.692	0.707	0.700	11.2225	0.490000	2.345000
8.50			1.790	21.1950	0.933818	4.448700

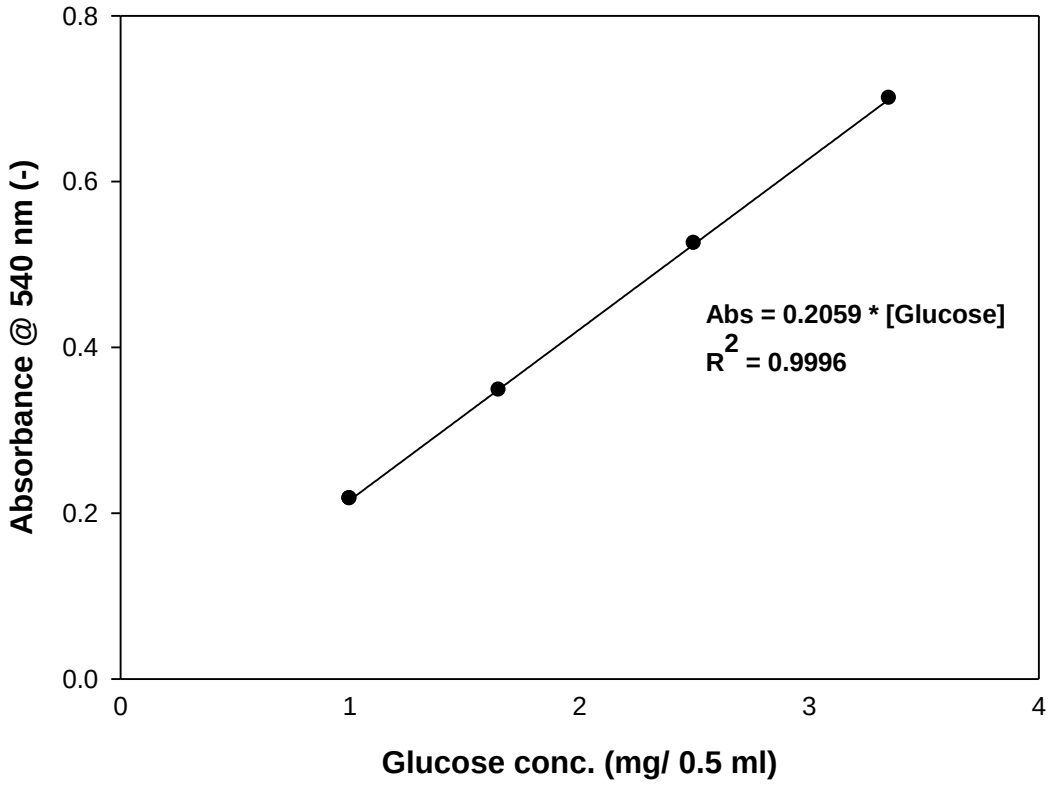

Figure E1. Glucose standard curve (DNS method) used for cellulase enzyme activity measurement.

Columns 3, 4, and 5 of Table E1 contain computed values for x_i, y_i and x_iy_i, with their sums appearing as the last entry in each column which were used to analyze the glucose measurements by the least square method (Skoog et al., 2007).

The calculation of the slope and intercept is simplified by defining three quantities S_{xx}, S_{yy} and S_{xy} as follows:

$$S_{xx} = \sum x_i^2 - \frac{(\sum x_i)^2}{N} = 21.195 - \frac{(8.500)^2}{4} = 3.1325\ (\frac{mg}{0.5\ ml})^2$$

$$S_{yy} = \sum y_i^2 - \frac{(\sum y_i)^2}{N} = 0.933818 - \frac{(1.790)^2}{4} = 0.132793\ (-)$$

$$S_{xy} = \sum x_i y_i - \frac{\sum x_i \sum y_i}{N} = 4.4487 - \frac{8.500 \times 1.790}{4} = 0.64495\ (\frac{mg}{0.5\ ml})$$

The slope of the line, m:

$$m = \frac{S_{xy}}{S_{xx}} = \frac{0.64495}{3.1325} = 0.20589\ (\frac{0.5\ ml}{mg})$$

The means (averages) for x and y values:

$$\bar{x} = \frac{\sum x_i}{N} = \frac{8.500}{4} = 2.125\ (\frac{mg}{0.5\ ml})$$

$$\bar{y} = \frac{\sum y_i}{N} = \frac{1.790}{4} = 0.4475\ (-)$$

The intercept, b:

$$b = \bar{y} - m\bar{x} = 0.4475 - 0.20589 \times 2.125 = 0.009\ \approx 0.000\ (-)$$

Thus, the equation for the lease square line of the standard curve is:

$$Abs. = 0.2059 \times [Glucose] \qquad \text{(E2)}$$

The standard deviation about regression:

$$S_r = \sqrt{\frac{S_{yy} - m^2 S_{xx}}{N-2}} = \sqrt{\frac{0.132793 - (0.20589)^2 \times 3.1325}{4-2}} = 0.00093\ (-)$$

The standard deviation of the slope:

$$S_m = \sqrt{\frac{S_r^2}{S_{xx}}} = \sqrt{\frac{(0.00093)^2}{3.1325}} = 0.000526\ (\frac{0.5\ ml}{mg})$$

The standard deviation of the intercept:

$$S_b = S_r \sqrt{\frac{1}{N - (\sum x_i)^2 / \sum x_i^2}} = 0.00093 \times \sqrt{\frac{1}{7 - (8.500)^2/21.195}} = 0.00049\ (-)$$

The standard deviation for results obtained from the standard curve:

$$S_c = \frac{S_r}{m} \sqrt{\frac{1}{M} + \frac{1}{N} + \frac{(\bar{y}_c - \bar{y})^2}{m^2 S_{xx}}}$$

$$S_c = \frac{0.00093}{0.20589} \times \sqrt{\frac{1}{2} + \frac{1}{4} + \frac{(\bar{y}_c - 0.4475\ )^2}{(0.20589)^2 \times 3.1325}}\ \left(\frac{mg}{0.5\ ml}\right) \qquad \text{(E3)}$$

Where:

M = number of replicates.

N = number of points used in the standard curve.

$\bar{y}_c$ = mean of the Absorbance measured in replicates # 1 and # 2.

The Confidence function (CF) was calculated at 95 % probability using the following equation:

$$CF = \pm 1.96 \times \left(\frac{S_c}{\sqrt{M}}\right) \qquad (E4)$$

Tables E2 and E3 show the glucose concentration measured using DNS method for five dilutions of cellulase enzymes NS22086 and NS50013 respectively. The glucose concentration data was used to find the dilution that will release 2.000 gm/ 0.5 ml of glucose sugar. Then this dilution was used to calculate the cellulase enzymes activity using Eq. E.1. A sample of the enzyme activity measurement is shown in section G6 (Appendix G).

Table E2. Dilution and glucose concentration of Cellulase enzyme (NS22086) stock that had been diluted in citrate buffer.

Run #	Dilution factor	Replicate # 1		Replicate # 2		Glucose Conc. Mean ± CF[1]	Standard deviation (S_c)
		Absorbance At 540 nm	Glucose Conc.	Absorbance At 540 nm	Glucose Conc.		
	(ml/ml)	(-)	(mg/ 0.5 ml)	(-)	(mg/ 0.5 ml)	(mg/ 0.5 ml)	(mg/ 0.5 ml)
1	0.0166	0.942	-	0.992	-	-	-
2	0.0100	0.912	-	0.912	-	-	-
3	0.0067	0.597	2.900	0.599	2.911	2.905 ± 0.04	0.03
4	0.0050	0.594	2.886	0.596	2.895	2.890 ± 0.04	0.03
5	0.0033	0.388	1.884	0.387	1.880	1.882 ± 0.03	0.02

[1]CF = Confidence Function at 95 % probability.

Table E3. Dilution and glucose concentration of Cellulase enzyme (NS50013) stock that had been diluted in citrate buffer.

Run #	Dilution factor	Replicate # 1		Replicate # 2		Glucose Conc. Mean ± CF[1]	Standard deviation (S_c)
		Absorbance At 540 nm	Glucose Conc.	Absorbance At 540 nm	Glucose Conc.		
	(ml/ml)	(-)	(mg/ 0.5 ml)	(-)	(mg/ 0.5 ml)	(mg/ 0.5 ml)	(mg/ 0.5 ml)
1	0.0250	0.740	-	0.789	-		
2	0.0150	0.658	3.185	0.675	3.277	3.231 ± 0.05	0.03
3	0.0100	0.509	2.473	0.517	2.509	2.491 ± 0.04	0.03
4	0.0075	0.438	2.128	0.416	2.018	2.073 ± 0.03	0.02
5	0.0050	0.317	1.540	0.327	1.590	1.565 ± 0.02	0.01

[1]CF = Confidence Function at 95 % probability.

Table E4 shows cellulase enzymes NS22086 and NS50013 activities measured by using the filter paper unit method. The absorbance for the duplicate sets with the means,

standard deviations and the confidence functions at 95 % probability level are shown in Table E4.

The standard deviation (STDEV) was calculated using the following equation:

$$STDEV = \sqrt{\frac{\sum(x - \bar{x})^2}{n - 1}} \qquad (E5)$$

The confidence function (CF) was calculated using the following equation:

$$CF = \pm 1.96 \times \left(\frac{STDEV}{\sqrt{n}}\right) \qquad (E6)$$

$n = number\ of\ replicates (2\ in\ this\ case)$

Enzyme NS50013 is the old generation of cellulase enzyme produced by Novozyme Company. The activity of the new cellulase enzyme NS22086 has almost double the activity of NS50013 enzymes as shown in Table E4.

Table E4. Cellulase enzymes activity measured by filter paper unit method.

Cellulase enzyme	Replicate # 1		Replicate # 2		Cellulase activity Mean ± CF[1]	STDEV[2]
	[Enzyme]	Cellulase activity	[Enzyme]	Cellulase activity		
	(ml/ml)	FPU/ ml	(ml/ml)	FPU/ ml	FPU/ ml	FPU/ ml
NS22086	0.00349	106	0.00350	106	106 ± 0.00	0
NS50013	0.00695	53	0.00739	50	52 ± 2.94	2.12

[1]CF = Confidence Function at 95 % probability.
[2]STDEV = Standard Deviation.

Appendix F: Comparison between the effect of using 100 ml and 500 ml washing water volume on the AIL content

A statistical analysis was used to compare the effect of using 100 ml and 500 ml of distilled water in the intermediate washing step between the two ozonolysis stages on the AIL content of the final ozonated wheat straw. As can be seen form Table B3 (appendix B), 14 experiments were run in each experiments. Table F1 shows summary statistics for the two sets of experiments.

Table F1 Summary statistics of the AIL content from Table B3 (Appendix B).

	Set # 1 (using 100 ml washing water)	Set # 2 (using 500 ml washing water)
Number of experiments (n)	14	14
AIL mean ($\bar{x}$)	13.8021	13.9864
AIL Standard deviation (s)	2.26407	2.43606
AIL Variance (s^2)	5.12602	5.93441
AIL Minimum	10.42	10.31
AIL Maximum	17.27	17.61
Degree of freedom (df)	13	13

The AIL means of the two sets of experiments were compared to determine if there is a statistical significant deference between the AIL of the two sets of experiments. If the statistical analysis shows that there is no significant difference between AIL content of the two sets of experiments, then a volume of 100 ml can be used in the intermediate washing step in order to minimize the volume of distilled water used.

In order to choose the right comparison method between the means, the results (AIL) of the two sets of experiments have been tested to see if they are coming from normal distribution or not. Anderson-Darling (Stephens, 1974) normality test was used.

In the Anderson-Darling test there are two hypotheses (H_0 and H_a)

H_0: The set of experiment follows normal distribution

H_a: The set of experiment do not follow normal distribution

And the Anderson-Darling test is defined as:

$$AD = -n - S \qquad (F1)$$

n is the number of experiments in each set (= 14)

$$S = \sum_{i=1}^{n} [\frac{(2i-1)}{n} \ln F(xi) + \ln(1 - F(x_{n+1-i}))] \qquad (F2)$$

x is AIL content detriment for each experiment.

F is the cumulative distribution function of the continuous distribution:

$$F(x) = \int_{-\infty}^{x} f(\mu) d\mu \qquad (F3)$$

The AD value calculated by using Eq. F1 is used to calculate p-value (probability). If the calculated p-value is greater than the critical value of 0.05, then the H_0 hypothesis can't be rejected and the distribution follows normal distribution. Minitab software was used to performed the normality test using the Anderson-Darling test to see if the results (AIL content) of the two sets of experiments are following normal distribution or not. Figure F1 shows the result of the Anderson-Darling test. Since p-value is greater than 0.05, it was concluded that the distribution of AIL content for the 1st set of experiments, where 100 ml of washing water was used, is coming from normal distribution. Same thing can

be said regarding the distribution of AIL content for the set of experiments, where 500 ml of washing water was used, as can be seen from Figure F2.

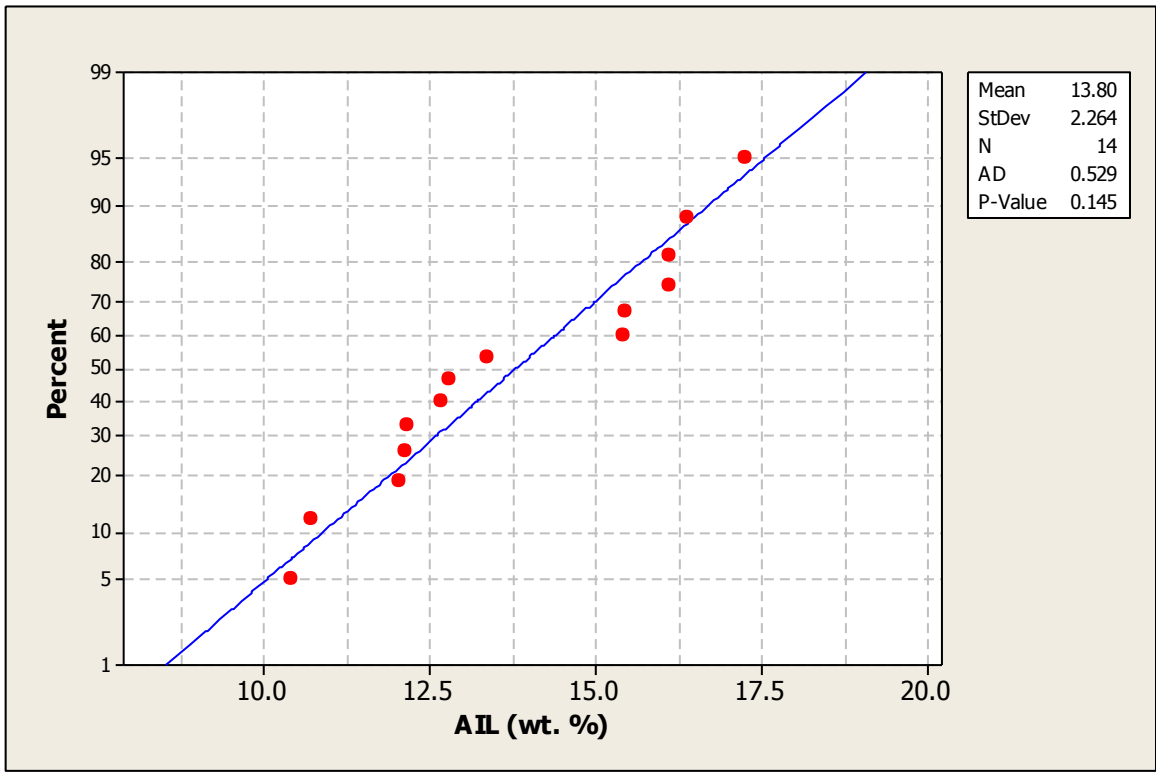

Figure F1 Probability plot Acid Insoluble Lignin (AIL %) content, determined using Eq. B1 (Appendix B), of ozonated wheat straw. In each run, 5 grams (oven dry basis) of wheat straw with fiber size < 2 mm was ozonated in two stages at O_3/O_2 flow rate= 1 l/min and ozone concentration= 3 wt. %. Washing water volume of 100 ml was used.

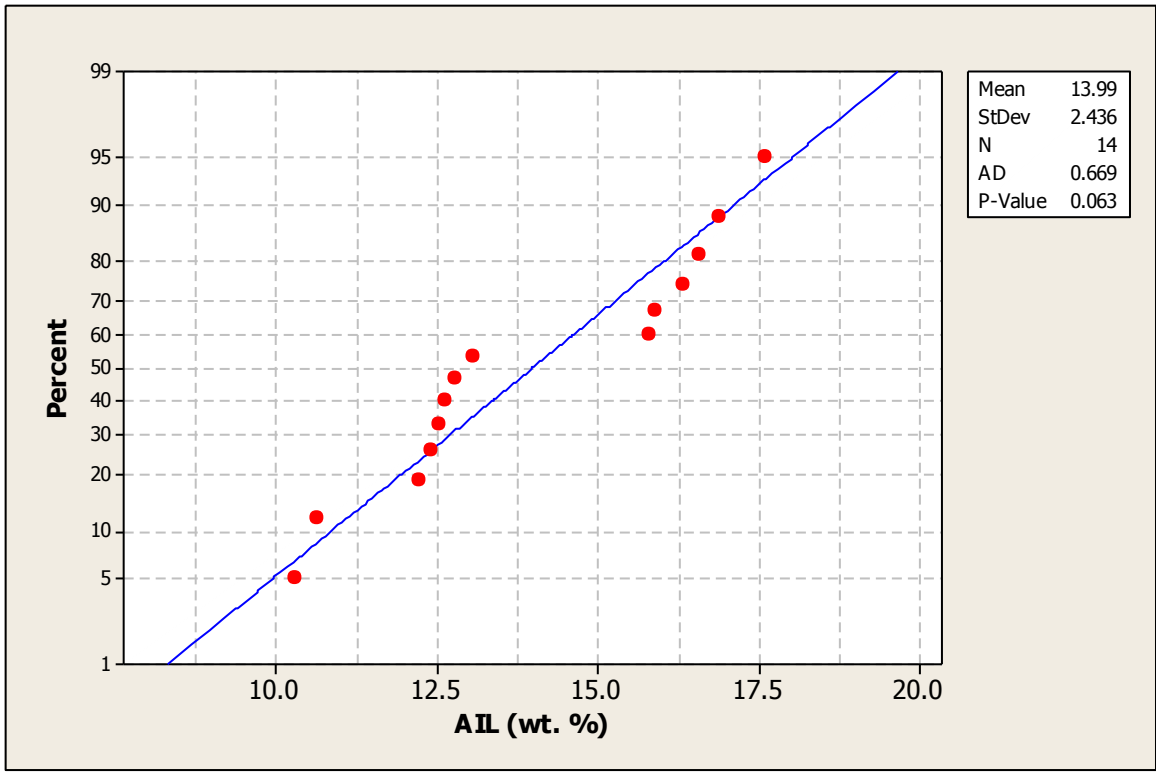

Figure F2 Probability plot Acid Insoluble Lignin (AIL %) content, determined using Eq. B1 (Appendix B), of ozonated wheat straw. In each run, 5 grams (oven dry basis) of wheat straw with fiber size < 2 mm was ozonated in two stages at O_3/O_2 flow rate= 1 l/min and ozone concentration= 3 wt. %. Washing water volume of 100 ml was used.

The next step, after determining that the two sets of experiments are coming from normal distribution, was to see if the two population AIL variances (σ^2), which were the two AIL variances (s^2) of the two sets of experiments come from, are equal or not. Knowing if the population variances are equal or not will help to determine the method to examine the hypothesis of equal AIL means for the two sets of experiments. F-test was used to compare the two population AIL variances. F value can be calculated by taking the ratio of the larger sample (set of experiments) AIL variance to the lesser AIL variance:

$$F = \frac{S_1^2}{S_2^2} = \frac{5.934}{5.126} = 1.58$$

In F-test there are two hypotheses, the first hypothesis (H_0) is the population AIL variances (σ^2) are equal. The alternative hypothesis (H_a) is that the population AIL variances are not equal.

H_0: $\sigma_1^2 = \sigma_2^2$

H_a: $\sigma_1^2 \neq \sigma_2^2$

F critical value can be found in F- tables. For a probability of 95 % and degree of freedom (df) = 13 (for the 1st and the 2nd sets of experiments), the F critical value is ± 2.58. This means if the calculated F value is within the range:

$$-2.58 < F < 2.58$$

Then, H_0 hypothesis can't be rejected and the AIL variances, were the two sets of experiments come from, are not significantly different. In this case the AIL variances of the two sets of experiments (using 100 ml and 500 ml of washing water) are equal or not significantly different.

The third and last step is to compare the two AIL means of the two sets of experiment. If the statistical analysis shows that the two AIL means are equal or not significantly different then 100 ml of washing water can be used in the washing water step and the washing water volume will be minimized. Because the previous step showed that the AIL variances of the two sets of experiments were equal, a simple t-test can be used to compare the two AIL means. The t value can be calculated using the following equation:

$$t = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{\frac{s_1^2}{n_1} + \frac{s_2^2}{n_2}}}$$

$$t = \frac{13.802 - 13.986}{\sqrt{\frac{5.126}{14} + \frac{5.934}{14}}} = -0.207$$

Calculated t value is compared to t critical value obtained from t-tables for 95 % probability and degree of freedom:

$$(df) = n_1 + n_2 - 2 = 14 + 14 - 2 = 26\ .$$

The critical value for probability of 95 % and df of 26 is ± 1.706. If the calculated t value is within the range of critical t:

$$-1.706 < t < 1.706$$

Then the two means are not significantly different. In this case the t value of -0.207 is within the range and it can be said that the two AIL means of the two sets of experiments (using 100 ml and 500 ml washing water) are not significantly difference. Therefore, a volume of 100 ml washing water was used in the intermediate washing step instead of 500 ml. In this case the amount of washing water used in the intermediate washing step was minimized.

Appendix G: Samples of calculations

G.1 Total Solids (TS) and Moisture Content (MC) of wheat straw

2.000 grams of original wheat straw was weighed and dried at 105 ºC in a convection oven (Thermolyne, Model: 9000, USA). The dry weight of Replicate # 1 (Table A1) wheat straw was 1.845. The % TS was calculated using Eq. A1:

$$\%\, TS = \frac{1.845}{2.000} \times 100 = 92.25\ wt.\%$$

The % MC was calculated using Eq. A2:

$$\%\, MC = 100 - 92.25 = 7.75\ wt.\%$$

The mean (average) of % TS for Replicate # 1 and 2 (Table A1) was calculated as follow:

$$\%\, TS\ mean = \frac{92.25 + 92.37}{2} = 92.31\ wt.\%$$

The standard deviation of % TS for Replicate # 1 and 2 (Table A1) was calculated using Eq. A3 as follow:

$$STDEV = \sqrt{\frac{(92.25 - 92.31)^2 + (92.37 - 92.31)^2}{2 - 1}} = 0.08\ wt.\%$$

The confidence function (CF) of % TS for Replicate # 1 and 2 (Table A1) at probability of 95 % was calculated using Eq. A4 as follow:

$$CF = 1.96 \times \frac{0.08}{\sqrt{2}} = 0.12\ wt.\%$$

The relative percent difference (RPD) of % TS for Replicate # 1 and 2 (Table A1) at probability of 95 % was calculated using Eq. A5 as follow:

$$\% \, RPD = \frac{|92.25 - 92.37|}{\frac{(92.25 + 92.37)}{2}} \times 100 = 0.13 \, wt.\%$$

G.2 Acid Insoluble Lignin (AIL)

The following showed the details for the calculation of AIL in Run # 8, Replicate # 1, Table B4 (Appendix B).

Wheat straw produced form the second ozonolysis stage was dried at 45 ºC then grounded using home miller to pass 40 mesh sieves. Two samples were taken at the same time. A first sample of 2.000 g was taken to determine the % Total Solids (TS):

W_1 = Original wheat straw weight = 2.000 g

The sample was dried in a convection oven, Thermolyne, Model: 9000, USA, at 105 ºC to constant weight (2-3 hours):

Dry wheat straw (oven dried at 105 ºC) weight = 1.942 g

The % TS was calculated using Eq. A1 (Appendix A):

$$\% \, TS = \frac{1.942}{2.000} \times 100 = 97.10 \, wt.\%$$

The second sample was 1.000 g and it was used to determine the % AIL. The crucible and the glass fiber filter were ignited at 575 ºC before they were used.

After filtration (Appendix B), the crucible with its contents of glass fiber filter and the remaining solids (AIL + Acid Insoluble Ash (AIA)) was dried in the convection oven, Thermolyne, Model: 9000, USA, at 105 ºC to constant weight:

W_2 = (crucible + filter + AIL + AIA) weight = 111.983 g

Then the crucible and its contents were ignited in a muffle furnace (Thermo Scientific, Model: BF 51828C-1, USA) at 575 ºC to constant weight (around 4 hours):

W_3 = (crucible + filter + AIA) weight = 111.890 g

The % AIL was calculated using Eq. B1 (Appendix B):

$$\%\, AIL = \frac{W_2 - W_3}{W_1 \times \frac{\%\, TS}{100}} \times 100 = \frac{(111.983 - 111.890)g}{1.000\ g \times \frac{97.10}{100}} = 9.58\ wt.\%$$

G.3 Acid Soluble Lignin (ASL)

The following shows the details for the calculation of ASL in Replicate # 1, Table C1 (Appendix C).

The volume (V) of the filtrate, which was collected from step 9 in the AIL determination procedure (Appendix C), was measured:

V = 570 ml

The absorbance of the filtrate was measured at 205 nm using cuvette with 1 cm path length (b) was used. The reference for the absorbance measurement was 3 wt. % H_2SO_4.

The filtrate and the reference were diluted (d) 1:7 until the absorbance (A) of the filtrate became within the allowed range of (0.2- 0.7):

A= 0.587

d = 7

The weight of the untreated wheat straw which was used in this experiment to determine both the AIL and the ASL was 1.000 g (W_1). The % TS was determined the same way as shown in G.1 (appendix G).

% TS = 93.75 wt. %

The % ASL was determined using Eq. C1 (Appendix C):

$$\% \, ASL = \frac{\frac{0.587}{110\frac{l}{g.cm} \times 1 \, cm} \times 7 \times 570 \, ml \times \frac{l}{1000 \, ml}}{1.000 \, g \times \frac{93.75}{100}} \times 100 = 2.27 \, wt.\%$$

G.4 Initial Water Content (IWC) adjustment

To have 5 g (oven dried at 105 ºC) of wheat straw with IWC of 50 wt. %:

Untreated wheat straw contained 92.31 wt. % TS (Appendix A). Therefore, required wheat straw was calculated by rearranging Eq. A1 (Appendix A);

$$original \, wheat \, straw = \frac{dry \, wheat \, straw}{(\% \, TS/100)} = \frac{5}{0.9231} = 5.416 \, g$$

$$and \, original \, moisture \, (water) \, content = 5.416 - 5 = 0.416 \, g$$

The required amount of water needs to adjust the water content to 50 wt. % was calculated using Eq. 3.4:

$$x = \frac{5.416 \times \left(\frac{50}{100}\right) - 0.416}{1 - (\frac{50}{100})} = 4.584 \, g$$

G.5 Enzymatic hydrolysis of wheat straw

The following are the details for the calculation of reducing sugars yield for experiment "2-optimum" at 112 hours of hydrolysis time in Table D4 (Appendix D).

Two samples of the ozonated wheat straw, which was dried and stored in the freezer, were taken at the same time. The first sample was 2.000 g and it was used to determine the % TS according to the procedure in Appendix A. The % TS was found to be 96.05 wt. %.

The second ozonated wheat straw sample (2.000 g) was used for the enzymatic hydrolysis. The TS was calculated using % TS calculated earlier and rearranging Eq. A1:

$$Total\ solids = 2.000 \times \frac{96.05}{100} = 1.921\ g$$

The theoretical reducing sugars that could be released were calculated using Eq. D5:

$$Theoretical\ reducing\ sugars = 6.618 \times 1.921\ g = 12.713\ g$$

After 112 hours of hydrolysis, samples were taken from the enzymatic hydrolysis, buffer blank, and enzyme blank flasks as described in the enzymatic hydrolysis procedure of Appendix D. The supernatants from the centrifugation of the three samples were subjected to DNS test as described in Appendix D.

The absorbance for the color developed from DNS test of the hydrolysis sample was measured at 540 nm using UV spectrophotometer (Biochrom Model: Ultra spec 50, England). The reference was the buffer blank. The absorbance of the enzyme blank, which should be subtracted from the hydrolysis sample absorbance, was negligible.

Glucose concentration for the hydrolysis sample was calculated from its absorbance (1.421) using the glucose standard curve of Figure D1.

$$Measured\ reducing\ sugars = 10.360\ g$$

Finally, the reducing sugars yield (% theoretical) using Eq. D6:

$$Reducing\ sugars\ yiels\ = \frac{10.360\ g}{12.713\ g} \times 100 = 81.49\ \%\ theoretical$$

G.6 Cellulase enzyme activity measurement

The following is the details for the calculation of cellulase (NS22086) enzyme activity in the replicate #1 of Table E2 (Appendix E).

All enzyme dilutions were made in citrate buffer, pH 5.0, as indicated in the following table from a working enzyme stock solution that had been diluted 1:15 in citrate buffer.

Table G1. Dilution of Cellulase enzyme NS22086 from enzyme stock that had been diluted 1:15 in citrate buffer.

Dilution #	Citrate buffer	1:15 Enzyme	Dilution factor*	Absorbance at 540 nm	[Glucose]
	(ml)	(ml)	(ml/ml)	(-)	(mg/ 0.5 ml)
1	1.65	0.35	0.0166	0.942	-
2	1.70	0.30	0.0100	0.912	-
3	1.80	0.20	0.0067	0.597	2.900
4	1.85	0.15	0.0050	0.594	2.886
5	1.90	0.10	0.0033	0.388	1.884

*The term "Dilution factor" is used to represent the proportion of the original enzyme solution present in the dilution added to the assay mixture. For example a 1:10 dilution (Dilution # 3) of the 1:15 working stock of enzyme will have:

$$Dilution\ factor = (\frac{\frac{0.2}{0.2+1.80}}{15}) = 0.0067.$$

In another word, the original enzyme was diluted two times. In the first dilution, it was diluted (1:15) and in the second dilution (1:10). Therefore:

$$Dilution\ factor = \left(\frac{1}{15}\right) \times \left(\frac{1}{10}\right) = 0.0067$$

After performing the hydrolysis and the DNS test described in Appendix E, glucose concentrations of the five cellulase enzyme assays were determined using glucose standard curve of Figure E1. The first two assays were above the measuring limits of the glucose standard curve of Figure E1. Therefore, Table G1 is not showing the glucose concentration for the first two assays.

It can be seen from Table G1 that the dilution factor of cellulase enzyme which released 2.0 mg/ 0.5 ml glucose was between 0.0050 and 0.0033. Linear interpolation between these two enzyme dilutions showed that the enzyme dilution which had released 2.0 mg/ 0.5 ml glucose was equal to 0.00349. Therefore, the cellulase enzyme activity (using Eq. E1):

$$Cellulase\ activity = \frac{0.37}{0.00349} = 106\ FPU/ml$$