**A DISSERTATION FOR THE DEGREE OF DOCTOR OF PHILOSOPHY**

# Yield enhancement of glucose and xylooligosaccharide by controlling biomass constituents of *Eucalyptus pellita*

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# Abstract

## Yield enhancement of glucose and xylooligosaccharide by controlling biomass constituents of *Eucalyptus pellita*

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In this study, to understand effects of biomass constituents on enzymatic hydrolysis, highly recalcitrant lignocellulosic biomass (*Eucalyptus pellita*) was used for reactions of hemicellulose removal (liquid hot water treatment), delignification (sodium chlorite treatment), and cellulose crystallinity modification (sodium hydroxide treatment), respectively. Additionally xylooligosaccharides as a value-added byproduct were produced under an optimal condition of liquid hot water treatment. To elucidate the effect of the constituents of the lignocellulosic biomass, correlation and regression assessments were performed to develop a guideline model for determining which strategy to address unknown feedstock.

Xylooligosaccharides were suitably produced up to 8.3% (67.2% conversion rate) at 170°C for 50 min; however, the total amount decreased steadily to 0.8% with an increase in treatment severity. The maximum content of xylobiose, which was the most produced among xylooligosaccharide, was 3.0% under the aforementioned conditions. Meanwhile, the maximum

amounts of xylotetraose (1.5%), xylopentaose (1.0%), and xylohexaose (0.8%) also occurred under these conditions. After enzymatic hydrolysis using solid residue, the enzymatic digestibility and glucose yield increased to 21.3% and 28.2%, respectively, at 190°C for 50 min, even though the hemicellulose removal rate was 96.2% under this condition. A correlation analysis between biomass constituents and glucose yield was conducted using the statistical program SAS. Adjusted R-squared values of the hemicellulose and lignin removal rates for linear regression models were determined as 0.6768 and 0.0390, respectively. Meanwhile, a linear regression model for a double factor (hemicellulose and lignin) had a slightly higher adjusted R-squared value (0.7177) compared to that of the single factor.

The amount of total lignin in *E. pellita* (34.8%) was obviously reduced up to 9.0% under 4 g of sodium chlorite and 0.8 mL of acetic acid with 3 times input (total reaction time: 180 min). In this case, Klason lignin (2.4%) was entirely eliminated compared to that of *E. pellita* (29.0%), whereas acid-soluble lignin (6.6%) was preferably increased compared to that of *E. pellita* (2.3%). Meanwhile, the total lignin was almost removed up to 0.3% under 4 g of sodium chlorite and 0.8 mL of acetic acid with 2 times input (total reaction time: 120 min) using 75% hemicellulose removed solid residue. In addition, the maximum glucose yield (87.5%) after the sodium chlorite treatment with liquid hot water treatment was dramatically improved compared to that of liquid hot water alone (28.2%). However, a high glucose yield (83.9%) could also be obtained by treatment with sodium chlorite alone. According to the correlation analysis between the lignin removal rate and the glucose yield, the adjusted R-squared value was determined at 0.9063, which is much higher than the results obtained for liquid hot water treatment. Furthermore, the adjusted R-squared value of the double factor after linear regression analysis was increased to 0.9285, which indicates that lignin removal has a significant

influence on glucose production.

The crystallinity index of *E. pellita* was slightly increased from 59.7% to 68.9% after liquid hot water treatment. However, crystallinity index could not be determined after sodium hydroxide treatment because the peak of $I_{002}$, which indicates the crystalline region in cellulose structure, disappeared regardless of the preceding treatment. The sodium hydroxide treatment was beneficial for enhancing the glucose yield to 36.9% from 0.5% of untreated raw material. Meanwhile, in the case of liquid hot water and sodium chlorite treated solid residues, the glucose yield after sodium hydroxide treatment was similar or slightly decreased.

Consequently, treatments using liquid hot water, sodium chlorite (with acetic acid), and sodium hydroxide were induced for controlling a specific biomass constituent (hemicellulose, lignin, and cellulose crystallinity). According to the correlation analysis, existence of lignin in biomass revealed as the strongest factor to reduce cellulase activity and glucose production. Therefore, xylooligosaccharide and glucose can be produced suitably by application of appropriate technology for controlling hemicellulose or lignin content even though a high recalcitrant biomass was utilized. However, purification of xylooligosaccharide and recovery of lignin fraction should be investigated for ensuring feasibility of lignocellulosic biomass application in future studies.

**Keywords: liquid hot water treatment, xylooligosaccharide, delignification, glucose production, *Eucalyptus pellita***

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# List of Abbreviations

| | |
|---|---|
| HPLC | high performance liquid chromatography |
| WIS | water-insoluble solid residue |
| 5-HMF | 5-hydroxymethylfurfural |
| NMR | nuclear magnetic resonance |
| SAS | statistical analysis system |
| KL | Klason lignin |
| ASL | acid-soluble lignin |
| LHW | liquid hot water |
| AFEX | ammonia fiber explosion |
| DP | degree of polymerization |
| HRR | hemicellulose removal rate |
| LRR | lignin removal rate |
| CrI | crystallinity index |
| DF | degree of freedom |
| GY | glucose yield |
| ANOVA | analysis of variance |
| R-square | coefficient of determination |

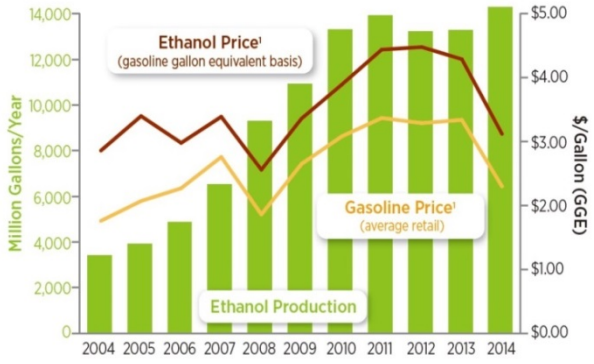
# *Chapter 1*

## Introduction

# 1. Background

## 1.1. Utilization of biomass as sustainable resource

Today fossil fuels are dominant energy source that supports everything in the human life through combustion of fossil fuels providing more than 80% of the total global energy demands (Escobar et al., 2009). Paradoxically, the utilization of fossil fuels encounters a crisis of human-being by three major problems (Zabed et al., 2016). First of all, every type of fossil fuel is rapidly depleted through fast industrialization and motorization in the developed and developing countries, because 58% of the application of fossil fuel is simply consumed for transport demands (Agarwal, 2007). In this trend, it is expected that the deposit of fossil fuel will be completely exhausted within approximately 50 years (Vohra et al., 2014). Another problem of fossil fuel utilization is significantly concerned to emissions for greenhouse gas, especially carbon dioxide that contributes climate change, sea level rising, and biodiversity limitation by the global warming (Singh et al., 2010). Finally, the international situation is worsening by frequent fluctuation of market price of fossil fuels that cause economic uneasiness and business depression in the world (Ogbonna et al., 2001). Therefore, development of energy source, which can alter the fossil fuels, is necessary for alleviating the global warming and greenhouse gas emission and the feedstock as an alternative energy source needs to inherent renewable, sustainable, efficient and cost-effective properties (Zabed et al., 2014).




| | Gasoline Price (average retail, $/gallon) | Ethanol Price (gasoline gallon equivalent basis, $/gallon) | Ethanol Production (million gallons/ year) |
|---|---|---|---|
| 2000 | $1.56 | $2.16 | 1,622 |
| 2001 | $1.50 | $2.38 | 1,765 |
| 2002 | $1.38 | $1.97 | 2,140 |
| 2003 | $1.54 | $2.27 | 2,810 |
| 2004 | $1.76 | $2.86 | 3,404 |
| 2005 | $2.07 | $3.40 | 3,904 |
| 2006 | $2.27 | $2.98 | 4,884 |
| 2007 | $2.76 | $3.39 | 6,521 |
| 2008 | $1.86 | $2.56 | 9,309 |
| 2009 | $2.65 | $3.36 | 10,938 |
| 2010 | $3.08 | $3.89 | 13,298 |
| 2011 | $3.37 | $4.44 | 13,929 |
| 2012 | $3.29 | $4.48 | 13,218 |
| 2013 | $3.34 | $4.29 | 13,293 |
| 2014 | $2.30 | $3.12 | 14,300 |

Figure 1-1. US corn ethanol production and prices (NREL, 2014).

Among the alternative energy source, lignocellulosic biomass is one of the most interested and researched resource, it also raises expectation because of its high amount of carbon deposit (Varanasi et al., 2012). Lignocellulosic biomass produces carbohydrates by conversion of light energy to chemical energy, named photosynthesis that enables to fix atmospheric carbons and stores in the body (Zhang, 2008). Thus, the amount of carbons by burning biofuels would be equal to the requirement for photosynthesis and additional $CO_2$ does not accumulate in the atmosphere (Naik et al., 2010). And, it is estimated that 5% to 8% of annual consumption of fossil fuel can cover the energy production from lignocellulosic biomass (Stark, 2011). Furthermore, 442 billion liters of bioethanol is annually produced using lignocellulosic biomass (Bohlmann, 2006). Additionally, 14,300 million gallons of bioethanol in USA is annually produced using biomass at 2014, as shown in Figure 1-1 (NREL, 2014). Another research reported that woody biomass afford to convert 120 to 300 liters of bioethanol based on one ton of dry raw material (Mabee & Saddler, 2010). The lignocellulosic biomass has a great possibility of development in term of alternative energy source for overcoming the problems of fossil fuels, but research necessity about lignocellulosic biomass is steadily required due to an inherent difficulty of conversion to bioenergy that causes by biomass recalcitrance (Balat, 2011).

## 1.2. Biomass recalcitrance

Lignocellulosic biomass is a promising material for altering all of carbon based resources because it comprised up to 75% carbohydrate (Gomez et al., 2008). This carbohydrate can be converted to useful material such as liquid biofuels, industrial chemicals, and etc. (Jørgensen et al., 2007). However biomass has constructed by cell wall structure through biosynthetic pathways, especially thorough and rigid form of cell wall existing in lignocellulosic biomass (Himmel et al., 2007). The cell wall structure has exhibited unique functions like as a shield against fungi, bacteria, insect, and animal that play a role as a pathogen or preyer for plant biomass (Gorshkova et al., 2005). This intrinsic resistance of biomass over external harmful factors is called biomass recalcitrance (Himmel et al., 2007).

The biomass recalcitrance is ordinarily considered the product of the plant evolution that leads to complex characteristics and structures of lignocellulosic biomass. Several researchers referred and classified to the factors of biomass recalcitrance that have defined to seven categories by Himmel at al: (1) cuticle and waxes in the epidermal tissue of plant, (2) density or arrangement of vascular bundles, (3) ratio of sclerenchymatous tissue in cell wall, (4) degree of lignification, (5) structural complexity and non-uniformity of microfibrils and macrofibrils in cell wall, (6) embodiment of insoluble substrate for microbial and animals, and (7) inhibitors containing in cell wall or deriving from conversion reactions (Himmel et al., 2005; Himmel et al., 2007). Among them, recalcitrance comes from epidermal and sclerenchymatous tissue and density of vascular structure can be classified anatomical factors, while lignification, microfibril complex, and existence of

insoluble substrate for pest and pathogen are contributed by chemical cause of cell wall structure (Zhu, 2006).

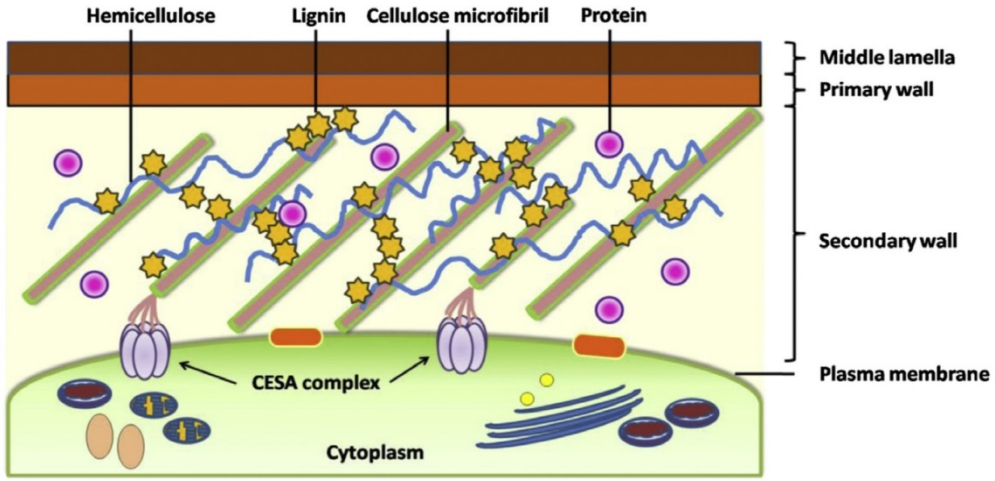



Figure 1-2. Simplified diagram showing complex structures of plant cell walls (Sticklen, 2008).

The biomass recalcitrance deriving from chemical structure in plant cell wall is caused by a matrix of cross-linked polysaccharide networks and lignin, as shown in Figure 1-2 (Sticklen, 2008; Zhao et al., 2012). The plant cell wall is generally constituted by three major components which are cellulose, hemicellulose and lignin that combine each other and form networks (Mood et al., 2013). Cellulose, the major structural constituent in lignocellulosic biomass, is a homogeneous-linked polymer of β-cellobiose that combines two D-glucose by β-(1,4)-glycosidic bonds forming a linear glycan chain (Harris & DeBolt, 2010). And the chain structure grants strong linkage with intra- and intermolecular hydrogen bonds (Barakat et al., 2015). Hemicellulose is a heterogeneous-linked polymer and consist of various groups that branch short-chain of substituted sugars (Scheller & Ulvskov, 2010). Hexoses (D-mannose, D-glucose, D-galactose), pentose (D-xylose, D-arabinose) and sugar-derived acids has known as major constituent of hemicellulose, and the backbones are primarily formed by polymers of xylopyranose and glucose with β-1,4-linkage (Scheller & Ulvskov, 2010). Lignin is synthesized by three types of phenyl propane monomers, p-coumaryl, coniferyl and sinapyl alcohols, that employ as a precursor to link through radical reaction for lignin macromolecule (Gomez et al., 2008).

## 1.3. Relationship between glucose production and biomass constituents

Enzymatic hydrolysis typically conducts for glucose production using cellulase because of high selectivity and productivity for cellulose degradation and glycosidic bond cleavage (Wojtusik et al., 2016). Once cellulases acting on the cellulose fibrils of lignocellulosic biomass, chemical and physical features of biomass affect to enzymatic digestibility such as cellulose crystallinity, amount of hemicellulose, lignin and acetyl group, pore volume, accessible surface area, degree of polymerization, cell wall thickness (Chang & Holtzapple, 2000a; McMillan, 1994; Yejun & Hongzhang, 2007). Among them, three major components of cell wall structure, which are cellulose, hemicellulose and lignin, have considerable hindrance for enzyme activity onto plant cell wall (Zhu, 2006).

Cellulose consists of two types of glucan chain, crystalline and amorphous regions. In case of crystalline region, cellulose is united by shape of microfibrils that combine paracrystalline state of several dozen β-D-1,4-glucan chains. The chains form hydrogen bond with adjacent microfibrils along their length (Laureano-Perez et al., 2005). The hydrogen bond is primary cause of rigid and stiff characteristic in the crystalline region, therefore, cellulose, which contains high crystalline region, more resist against fungal and microbial assault than that of amorphous region (Fan et al., 1981). In addition, cellulose crystallinity makes reduce the initial hydrolysis rate during enzymatic hydrolysis that reported by some researches (Fan et al., 1980; Koullas et al., 1990). Meanwhile, amorphous regions, less recalcitrant against enzymes, are typically hydrolyzed first, and the high amorphous cellulose is hydrolyzed from 3 to 30 times faster than cellulose having a lot of

high crystalline region (Hall et al., 2010; Zhang & Lynd, 2004). However most of researches utilize relatively pure celluloses as substrate followed by foundation for the correlation between cellulose crystallinity and enzymatic hydrolysis efficiency that is not enough to apply heterogeneous substrate like as lignocellulosic biomass (Chandra et al., 2007). Furthermore, some studies reported that cellulose crystallinity has no correlation with enzymatic activity (Puri, 1984; Yu et al., 2011). Another factor making a difficulty to understand effect of crystallinity is an interference of other factors, because solid fraction as subtract utilized for researching crystallinity effect from various pretreatment strategies. As a result, particle size or distribution of cell wall can change after pretreatment reaction that makes interrupt to comprehend the effect of crystallinity (Zhao et al., 2012).

Hemicellulose is known as a contributor to block the cellulose accessibility as physical barriers (Mussatto et al., 2008). Thus, hemicellulose removal process such as steam explosion or dilute acid pretreatment can induce an increase of glucose production using cellulase (Liao et al., 2005; Yang & Wyman, 2004). Some studies verified existence of xylan that allows limiting the access of enzyme to cellulose by addition of xylanase (Mansfield et al., 1997; Yoshida et al., 2008). According to results of these researches, improvement of enzymatic digestion can achieve by supplement of xylanase with cellulase (Edgar et al., 1998; Tabka et al., 2006). And influence of hemicellulose removal is not only advancement of cellulose accessibility but also decomposition of low molecular weight lignin fragments (Mansfield et al., 1997). However hemicellulose is considered less important for a factor of reducing enzymatic digestibility due to characteristic of hemicellulose that easily decomposes in relatively mild condition during pretreatment process (Zhu, 2011).

The mechanism of lignin impacting on cellulase activity through protection of carbohydrate do not clearly reveal, however, it is typically known as disturbing the enzymatic activity on the lignocellulosic biomass by some mechanisms (Laureano-Perez et al., 2005). First evidence is that lignin employs to limit access to cellulose for cellulase like as a physical barrier (Meunier-Goddik & Penner, 1999; Mooney et al., 1998). Because, lignin fills a form of covalent connection with hemicellulose in space of cell wall structure (Chabannes et al., 2001). Another reason is high degree of lignification observing in a matured woody biomass that makes limit pretreatment or degradation efficiency than that of grass biomass (Zhu, 2016). And various lignin structure as types and degree of cross-linkage with cellulose and hemicellulose can affect to glucose production (Laureano-Perez et al., 2005). Also phenolic polymers originated from lignin macromolecular that distribute in cell wall and hinder enzymatic hydrolysis (Chandra et al., 2007). According to a lot of researches, lignin removal becomes widely-accepted theory for improvement of glucose production using enzymes (Xue-bing et al., 2007). Removal of lignin can induce an increase of cellulase accessibility due to the expansion of specific area in the cell wall structure (Mansfield et al., 1999). Meanwhile, some studies reveal that elimination of lignin is not essential process for enzymatic hydrolysis even though delignification significantly contributes to increase of glucose production (Jang et al., 2016; Koo et al., 2012). The acidic hydrolysis is well known that it can decompose little amount of lignin in lignocellulosic biomass. However enzymatic digestibility improved through lignin structure change, and can achieve a little of lignin redistribution as well as hemicellulose removal after acidic hydrolysis (Yang & Wyman, 2008). A comparison between aqueous ammonia soaking and cellulose fractionation by solvent was conducted for evaluating enzymatic digestibility by Rollin at al. According to result of this

study, the later process achieves better glucose production but present lower delignification (Rollin et al., 2011). Another property of lignin also notably decreases enzymatic activity through irreversible adsorption of cellulase (Alvira et al., 2010; Pan, 2008). And the adsorption phenomenon for cellulose enzyme is typically reported in results of steam explosion or dilutes acid pretreatment (Converse et al., 1990; Palonen et al., 2004).

In general, plant cell wall has multilayered structure as linkage aspect with component for three layers; middle lamella, primary wall, and secondary wall (Chylińska et al., 2016). The middle lamella has fundamentally formed cellulose backbone by glycan cross-linkage that can be classified two types with plant species. Type I middle lamella is consist of glucan and xyloglucan with same amount that encapsulated in a pectin matrix, and this type is observed in dicotyledonous plant. Type II middle lamella is built by glucuronoarabinoxylan with cross linking glucans, while the pectin matrix absent unlike Type I middle lamella, and usually found in herbal plant and grasses (Sticklen, 2008).

In juvenile lignocellulosic biomass, the cell wall structure is commonly observed flexible and thin, this characteristic of young cell enables expansion of organs during the cell growing and dividing. Meanwhile, as growth of plant, the cell structure is significantly rigid and harder than young cell through grown-up of old tissues and secondary cell wall (Harris & DeBolt, 2010).

The secondary wall is organized with three types of layer, namely S1, S2, and S3 and these layers are located outer, middle, and inner space, respectively (Chundawat et al., 2011). A distinguishing feature of secondary wall is presence of lignin that surrounds the cellulose microfibrils and hemicellulose, and this property allows hardness and rigidity of cell wall in lignocellulosic biomass (Sticklen, 2008). S2 and S3 layer have the largest amount of cellulose in whole cell wall, and generally, secondary wall contains

more cellulose than middle lamella or primary wall (Agarwal, 2006). Also, hemicellulose mainly deposits in secondary wall, and its concentration is increased from middle lamellar to secondary wall (McMillan, 1994). Meanwhile most of lignin distributes in middle lamella, and lignin amount is decrease as closer to the secondary wall. For instance, lignin concentration of primary wall and S1 layer are much higher than those of S2 and S3 layers (Agarwal, 2006).

## 1.4. Xylan and xylooligosaccharides

Xylan backbone primarily consists of hemicellulose structure as a form of glucuronoxylan in hardwood that combined by xylose units through β-1,4-glycosidic bonds and branched with α-1,2-glycosidic bonds occasionally linked by 4-O-methlglucuroinc acid groups (Otieno & Ahring, 2012a). The O-acetyl group can be substituted on C2 and C3 position. In case of softwood, the acetyl group seemed to be arranged relatively small amount than hardwood in xylan backbone. Because hemicellulose structure in softwood is dominantly built by galactoglucomannan, which has rarely contained acetyl group even though units arabinofuranose units as additional branches linked with α-1,3-glycosidic bonds in arabinoglucuronoxylan (Demirbas, 2009). In the cell-wall structure, xylan deposits on xylan-lignin complex through covalent bonds, and the complex linked by other polysaccharide structure like as pectin (Das et al., 1984; Selvendran, 1985). Generally, four type of bond formation entangled with different molecules in hemicellulose; β-bonds, α-bonds, ether bonds and hydrogen bonds (Otieno & Ahring, 2012a). Among them, the β-1,4 bonds combining each xylose molecular considered relatively strong bonds that allows rigidity in biomass (York & O'Neill, 2008). And the

β-bonds arranged the xylose unit as a linear formation and horizontal configuration (Zhang et al., 2015). Meanwhile, the α-bonds contribute to branched structure in the xylan, and the branch structure has different formation on xylan backbone such as α-1,6, α-1,2, and α-1,3 linkage (Otieno & Ahring, 2012a).

Xylooligosaccharide is carbohydrate oligomer that consists of xylose units, and it commonly contained in fruits, vegetables, honey, and milk (Vazquez et al., 2000). However, how much xylooligosaccharide constitutes in these sources is not clearly verified, but it can define by degree of polymerization, monomeric units, and linkage formation (Ayyappan & Prapulla, 2011). Xylooligosaccharide is typically a mixture of xylose oligomer that combined by β-1,4-linkages (Aachary & Prapulla, 2008). The numbers of xylose units employ for xylooligosaccharide formation generally from 2 to 6 but other studies suggest from 2 to 10 xylose unit. Furthermore xylan typically branched with side groups such as acetyl groups, arabinofuranosyl unit, α-D-glucopyranosyl uronic acid or its 4-O-methyl derivatives. These side groups on xylan backbone allow inducing a variety of biological characteristics of xylooligosaccharide (Ayyappan & Prapulla, 2011).

Xylooligosaccharide cannot be digested for human-being due to an absence of enzymes which able to hydrolyze the β-linkage of carbohydrate in human body (Delgado et al., 2011). And xylooligosaccharide is not decomposed at pass the stomach until arrive in the large intestine (Nabarlatz et al., 2007). Therefore xylooligosaccharide is considered as non-digestible oligosaccharide and recognized prebiotics (Rivero-Urgell & Santamaria-Orleans, 2001), this characteristic of xylooligosaccharide also grants to use for low-calorie diet foods (Vazquez et al., 2000). These beneficent effects of xylooligosaccharide primarily came from its molecular weight distribution, because below than four monomeric units considerably influence for prebiotic

activity (Hughes et al., 2007). It is revealed that xylooligosaccharide containing this degree of polymerization facilitate the proliferation of advantageous microorganisms (bifidobacteria) for good intestine circumstance through prevent of growth of putrefactive and pathogenic bacteria (Gullón et al., 2008; Mussatto & Mancilha, 2007). The xylooligosaccharide usually takes as a functional food for getting dietetic benefits through active ingredients, that is reason to add for food or food industrial products (Nabarlatz et al., 2007). If xylooligosaccharide intakes into the human body as a form of food, it provides beneficent effect on biological properties such as betterment of bowel function, lipid metabolism and calcium absorption, dental-decaying protection, cardiovascular disease prevention and alleviation in attack rate of colon cancer through short-chain fatty acid formation (Grootaert et al., 2007; Wang et al., 2009). Furthermore, xylooligosaccharide gives a merit for health in human body such as blood, skin, anti-oxidant, anti-inflammatory effect, anti-allergic activities and immunological system (Aachary & Prapulla, 2009). Chung et al. provided four gram of xylooligosaccharide per day until three weeks for above sixty-five years old that observed beneficent effect for intestinal microiota (Chung et al., 2007). The xylooligosaccharide is attractive material for chemical and pharmaceutical industries due to the its technological characteristics, for instance, non-toxicity, low calorie content, manifestation of biological effect with small amount, heat resistance, and stability in acidic pH condition (Vázquez et al., 2002). As beneficent effect on human health and biological properties, xylooligosaccharide is dealing with expensive market price approximately from 22 to 50 dollars per kilogram in comparison with price of xylose approximately less than 5 dollars per kilogram (Taniguchi, 2004). And market price of xylooligosaccharide expected to steadily grow over time (Otieno & Ahring, 2012a).

# 2. Objectives

As mentioned before, to overcome the problem of climate change, fossil fuels depletion, and instability of its market price, the utilization of lignocellulosic biomass as an alternative energy source is very important. However, development of conversion technology from lignocellulosic biomass to renewable energy is necessary for facilitating application due to the biomass recalcitrance, which is similar with a shield against external harmful factors that derived from spontaneous generation in a long period. For utilization of lignocellulosic biomass, pretreatment process must be conducted for alleviating the recalcitrance and effective conversion to bioethanol or biochemical. However, prior to the operation of pretreatment process, how much existence of lignin and hemicellulose, cellulose crystallinity, lignin-carbohydrate covalent bond, and pore size in the cell-wall structure affect to reduce the pretreatment efficiency and enzymatic digestibility should be determining. In addition, xylooligosaccharide, which is attractive material as prebiotics, can produce as a high value-added product during the hemicellulose removal process.

In this study, to understand effect of the biomass constituents for enzymatic hydrolysis, highly recalcitrant lignocellulosic biomass, Eucalyptus, will be employed for hemicellulose elimination by liquid hot water treatment, lignin decomposition by sodium chlorite treatment, and cellulose crystallinity reduction by sodium hydroxide treatment. The reactions will be conducted with appropriate catalysts for each method, and will be carried out at various conditions (temperature, time and catalyst concentration) to evaluate the effect of reaction conditions on biomass degradation. And, xylooligosaccharide as a value-added byproduct will be produced through liquid hot water treatment.

Then, enzymatic hydrolysis will be carried out for determining the effect of recalcitrance factors on glucose production. For understanding the effect of recalcitrance factors of the lignocellulosic biomass, correlation and regression analysis will be progressed to develop approach strategy for handling the high recalcitrant feedstock.

In addition, a variety of structural and analytical methods (XRD, HPLC and Bio-LC) will be applied. The information obtained from various methods and tools will be analyzed by using the statistical analysis system software (SAS).

Therefore, the objectives of this study are:

(1) To evaluate effects of biomass recalcitrance factors through removal reactions for improving efficiency of enzymatic hydrolysis to produce glucose.
(2) To investigate production conditions for value-added product derived from lignocellulosic biomass such as xylooligosaccharide.
(3) To suggest a guideline model for glucose production based on highly recalcitrant biomass or unknown species.

# 3. Literature review

## 3.1. Hemicellulose removal and xylooligosaccharide production

### 3.1.1. Effect of hemicellulose concerning glucose production

Researches for evaluating the effect of hemicellulose content has been less frequent conducted because of easy decomposition by biological or chemical reaction that is one of the hemicellulose characteristic (Tanaka et al., 1988). Nevertheless, hemicellulose plays a role as physical barriers like as lignin in the cell wall structure, leading to reduce enzyme accessibility (Grethlein & Converse, 1991). The negative effect of hemicellulose for enzymatic hydrolysis has been reported by some studies that used pretreated solid fraction by steam explosion as a substrate (Boussaid et al., 2000; Fernandez-Bolanos et al., 2001; Vlasenko et al., 1997). Because the steam-pretreated substrates contained low amount of hemicellulose, and easily hydrolyzed by cellulase than that of rich hemicellulose raw materials. Additionally, hemicellulose removal contributes cellulase activity and glucose production due to expand the specific pore size in substrates (Chandra et al., 2007). Meanwhile, substitution rate of acetyl group is considered one of important factor because acetyl group and lignin easily adhere to hemicellulose structure, leading to reduce the cellulase accessibility (Chang & Holtzapple, 2000b).

### 3.1.2. Strategies of xylooligosaccharide production

Xylooligosaccharide can produce from biomass especially high xylan-containing sources through chemical and biological (enzymatic) methods (Izumi et al., 2004). The feedstocks through chemical methods for xylooligosaccharide manufacture have been reported various biomass such as bamboo (Ando et al., 2003), wheat straw (Sun et al., 2005), flax shive (Jacobs et al., 2003), rice hulls (Vila et al., 2002), corn fiber and stover (Kim et al., 2005), almond shells (Nabarlatz et al., 2005), barley hulls (Vegas et al., 2005), corn cob (Nabarlatz et al., 2004), sugarcane bagasse (Jacobsen & Wyman, 2002), crop residue (Endo & Kuroda, 2000), and hardwood (Garrote & Parajó, 2002; Parajó et al., 2004). A variety of biomass for xylooligosaccharide production is described in Table 1-1 (Moure et al., 2006).

In case of chemical method, autohydrolysis (liquid hot water treatment) or hydrothermal process is typical strategy for production of xylooligosaccharide that operates using water and heat with mineral acid or not (Izumi & Azumi, 2001). Numerous researches seek for suitable production and application of xylooligosaccharide, and most of that employ aqueous media such as heated or compressed steam or water (Vazquez et al., 2000). Because, the aqueous treatment has been accepted a proper method for hemicellulose degradation and xylan solubilization in the previous studies (Ikemizu & Azumi, 2002). During the autohydrolysis or hydrothermal process, hemicellulose structure is gradually cleaved due to hydrolytic action by hydronium ions that produced from organic acid and water autoionization (Moure et al., 2006). Therefore, as this mechanism, oligosaccharide dissolved into aqueous media from biomass with little chemical hindrance than that of cellulose and lignin (Yuan et al., 2004).

Table 1-1. Hydrothermal processing for production of xylooligosaccharide with various biomass (Moure et al., 2006)

| Raw material | Processing | Objectives of the work |
|---|---|---|
| Bamboo | Hydrothermal processing in a percolation reactor at temperatures in the range 175–180˚C | Optimization of XO production |
| Wheat straw | Steam explosion at temperatures in the range 200–220˚C | Characterization of degraded hemicellulosic polymers |
| Flax shive | Aqueous treatments in microwave oven for partial depolymerization and solubilization of hemicelluloses | Structural characterization of hemicellulose-derived products |
| Rice hulls | Two-step aqueous treatment (120˚C and 198˚C, respectively) for hydrolysis of hemicellulose and susceptible lignin | Production of high-DP xylan oligosaccharides and water-soluble lignins |
| Corn fiber | Hydrothermal processing at 160˚C for hemicellulose solubilization | Manufacture of oligosaccharide solutions for further conversion into monosaccharides |
| Corn stover | Hydrothermal treatments for fractionation (optimal temperature, 190˚C) | Optimization of the enzymatic digestibility of spent solids from autohydrolysis |
| Almond shells | Hydrothermal treatments at temperatures in the range 150–190˚C | Kinetics of hemicellulose decomposition into oligomers and monomers |
| Barley hulls | Non-isothermal hydrothermal treatments up to 220˚C | Kinetics of hemicellulose decomposition and byproduct characterization |
| Corn cob | Hydrothermal treatments at temperatures in the range 150–190˚C | Kinetics of hemicellulose decomposition into oligomers and monomers |
| Sugarcane bagasse | Hydrothermal processing at 200˚C | Assessment of the effects of the solid to liquor ratio |
| Crop residue | Two-step aqueous treatment (100–140˚C and below 200˚C, respectively) | Manufacture of XO by hydrothermal processing and further refining |
| Aspen wood | Aqueous treatments in microwave oven at 180˚C | Product purification and structural characterization |
| Eucalyptus globulus wood | Non-isothermal hydrothermal treatments up to 224˚C | Kinetics of hemicellulose decomposition into oligomers and monomers |
| Eucalyptus globulus wood | Hydrothermal treatment under optimal conditions for XO production | Comparative evaluation of substrates for XO production and product characterization |

Alkali and acidic catalyst also adopted to produce xylooligosaccharide, but it need to careful reaction for preventing excess decomposition to xylose as a form of monomeric unit due to high reactivity of these catalysts (Carvalho et al., 2013). Akpinar et al. compared with alkaline and acidic pretreatment using corn cob, and as results of this study, alkaline pretreatment suggested the best efficiency for xylooligosaccharide production with supplement of endoxylanases (Akpinar et al., 2010). Meanwhile, alkaline hydrolysis cannot maintain the acetyl groups in xylan structure that might cause to reduce the water miscibility of xylooligosaccharide (Nabarlatz et al., 2007). Nevertheless the alkaline pretreatment with hydrogen peroxide has a great performance to lignin removal and hemicellulose solubilization with conserving the cellulose fraction in sugarcane bagasse (Brienzo et al., 2009). Wheat stalk, sunflower stalk, cotton stalk, and tobacco stalk are utilized for evaluating production of xylooligosaccharide depending on various sulfuric acid concentrations from 1.22% to 4.90% (Akpinar et al., 2009). As a result of this research, reducing sugars derived from biomass more dissolved as increase the reaction time and sulfuric acid concentration. It is observed that monomeric sugar is actively produced than oligosaccharide in 4.9% of sulfuric acid at 100˚C reaction temperature condition. And a condition, which is 2.45% of sulfuric acid concentration for 30 min at 100˚C, is revealed the best circumstance for xylooligosaccharide production. However, conversion efficiency of xylooligosaccharide is significantly varied depending on biomass types; for example, production rate of xylooligosaccharide is evaluated to 13% from tobacco, 12.6% from sunflower stalk, 10.2% from wheat straw, and 7.5% from cotton stalk in the best condition above presented. In a recent study, high yield of xylooligosaccharide achieved through rapid thermos-acid process (Otieno & Ahring, 2012b). As result of this study, a condition, 0.1% of sulfuric acid concentration at 145˚C for 60 min of reaction

time, is the best condition this method, and the production ratio of xylooligosaccharide is differently presented depending on raw material; 92.2% of sugarcane bagasse, 84.1% of switchgrass, and 64.9% of morning light. The reaction condition of much diluted inorganic acid with low reaction temperature and long reaction time, namely rapid thermo-acid treatment mentioned in previous study, is reported to effective strategy for xylooligosaccharide production, and it suggested in other studies (Brienzo et al., 2010; Carvalho et al., 2013; Samanta et al., 2012). However, the acidic pretreatment has a drawback the production of toxic compounds that derived from carbohydrate such as furfural and 5-HMF. And these components induce an increase of operation cost through requirement of sensitive purification process (Carvalho et al., 2013).

## 3.2. Pretreatment strategies for lignin removal

### 3.2.1. Lignin removal methods and its efficiency

Alkaline hydrolysis is well known as a critical method for delignification of lignocellulosic biomass, especially, for an increase of utilization efficiency the remaining carbohydrate (Harmsen et al., 2010). During the alkaline pretreatment, alkali reagent or catalyst makes microfibril and cell-wall structure swelling followed by extracting lignin macromolecular and dissolving hemicellulose structure (Hendriks & Zeeman, 2009). In addition, saponification processes in xylan and lignin that crosslink through intermolecular ester bonds (Sun & Cheng, 2002). Sodium hydroxide or calcium hydroxide is typical reagent for alkaline pretreatment, and reaction with this catalyst can operate on relatively mild condition but prolonging the reaction time (Imadi & Kazi, 2015). This mild condition of alkaline pretreatment allows preventing carbohydrate conversion reaction that makes sugar derivatives such as 5-HMF, furfural, and organic acid (Chang & Holtzapple, 2000b). Delignification of corn stover conducted with 1% and 2% sodium hydroxide for 1 h that reported decrease of lignin content and disruption of crystalline region (He et al., 2010). High concentration of sodium hydroxide with long reaction time was applied on various reaction temperature, then amount of lignin decrease as 85.8%, 77.8%, and 62,9% at 121˚C, 50˚C, 21˚C at reaction temperature, respectively (Xu et al., 2010). Another typical catalyst for alkaline pretreatment is aqueous ammonia with heating for lignocellulosic biomass. The method using ammonia is usually defined to three processes, aqueous ammonia soaking, ammonia recycle percolation (ARP) and ammonia fibre explosion-method (AFEX) (Harmsen et

al., 2010). During the ARP, aqueous ammonia flows in column-shape reactor at high reaction temperature, and seals with biomass preventing evaporation steam (Kim et al., 2003). After the reaction, ammonia can recover from liquid hydrolysate and collect remaining lignin fraction (Kim & Lee, 2005). Aqueous ammonia soaking process runs at relative low reaction temperature than that of ARP for avoiding the damage to hemicellulose during lignin removal (Harmsen et al., 2010). This process applied for destarched barley hull using 15% and 30% aqueous ammonia at 30 to 75˚C for 12 h to 77 days (Kim et al., 2008). As a result of this study, optimal condition for enzymatic hydrolysis is 15% aqueous ammonia at 75˚C for 48 h, achieved 66% of lignin solubilization and obtained 83% of glucan and 63% of xylan saccharification efficiency, respectively. However, ammonia treatment processes are relatively expensive because of ammonia price and addition of ammonia recovery system (Holtzapple et al., 1994).

Organosolv pretreatment using organic solvent commonly employs a form of aqueous solution of organic solvent for delignification followed by improving cellulase activity using lignocellulosic biomass (Mou & Wu, 2016). Ethanol is typically utilized; also, methanol, acetone, and ethylene glycol are ordinary solvent for this process (Guo et al., 2015). During the organosolv pretreatment, hemicellulose can extract with removal of lignin, especially addition of inorganic acid-catalyst into aqueous solution of organic solvent. These acid catalysts (e.g. hydrochloric, nitric and sulfuric acid) have been primarily used as a helper of organosolv pretreatment (Jang et al., 2016). Especially, sulfuric acid is typically used that has superior properties for pretreatment; high efficiency and strong reactivity (Araque et al., 2008; Pan et al., 2007). Addition of acid catalysts can be made to accelerate delignification process and reduce the reaction temperature, thus required higher temperature as of 185~210˚C without a catalyst than as below 180˚C with a catalyst. Because, inorganic acid performs depolymerize the lignin macromolecular through cleavage of the ether linkages (α-O-4 or β-O-4 bond) during ethanol organosolv pretreatment (Sun et al., 2001). As this reaction progressed, the lignin fragments became smaller until enabling to dissolve in the ethanol solvent (Sannigrahi et al., 2009; Sun et al., 2001). The organosolv pretreatment methods with different feedstock and conditions are described in Table 1-2 (Harmsen et al., 2010).

Table 1-2. Organosolv pretreatment conditions with various biomass (Harmsen et al., 2010).

| Biomass | Remarks | Solvent | Catalyst | S/L ratio (%) | Temp. (˚C) | Time (h) | Lignin removal (%) | Cellulose recovery (%) | Hemi-cellulose removal (%) |
|---|---|---|---|---|---|---|---|---|---|
| Corn stover | Pretreated with dilute $H_2SO_4$ | Methanol, butanol, aromatic alcohols | $H_2SO_4$ | 5 | 160 | 1 | >90 | | |
| Corn stover | Chopped, pre-soaked | Ethanol | $H_2SO_4$ | 6 | 170 | 0.5 | 85 | 92 | 91 |
| Oak (red) | After steam pretreatment | Acetic acid | | 11 | 60 | 1 | ~60 | | |
| Poplar | Chips | Ethanol /MIBK | $H_2SO_4$ | 9 | 140 | 1 | 64 | | |
| Poplar | Chopped | Ethanol | $H_2SO_4$ | | 180 | 1 | 74 | 88 | |
| Rice straw | Chopped <1 cm | Butanol | Organic catalysts | | 120 | 2 | 54 | 83 | |
| Spruce (red) | Chipped <7 mm, flow-through reactor | Acetic acid | | | 180 | 3 | 51 | 93 | |
| Wheat straw | Pretreated with acid hydrolysis | Ethanol | $H_2SO_4$ | | 81 | 1.5 | >70 | >98 | 50 |
| Wheat straw | | Glycerol | | 15 | 240 | 4 | >70 | 95 | >90 |
| Woody biomass | | Ethanol | | 9-20 | 180-195 | 0.5-1.5 | | | |

The sodium chlorite treatment (acid-chlorite delignification) has been originally developed in pulping industry for biomass delignification (Hubbell & Ragauskas, 2010). Comparing to the sodium chlorite treatment, Kraft pulping is a commonly used technique for lignin decomposition of lignocellulosic biomass in the industrial scale. But, the Kraft pulping is considered as an inadequate process for investigating delignification due to a limitation for lignin removal and a significant degradation of structural sugar (Singh, 1982). Therefore, the sodium chlorite treatment, which uses sodium chlorite and acetic acid as a reagent, is proper for research in laboratory scale. Furthermore, it has several properties such as moderate reaction temperature (70-80°C), barely damaging for carbohydrate fraction, and selective delignification (Ahlgren & Goring, 1971). However, cellulose chain length was significantly reduced after the sodium chlorite treatment that reported in previous study (Kumar et al., 2009b).

During the sodium chlorite treatment, chloride and chlorine anion generated by dissociating the sodium chlorite in aqueous solution (Abdel-Halim, 2014). The ions react with lignin macromolecular of biomass, and then lignin fraction decomposed into smaller compound by oxidative reaction (Rabetafika et al., 2014). Moreover, the acetic acid donates hydrogen ions for facilitating the activity of chloride and chlorine anion following as lignin decomposition and removal (Hubbell & Ragauskas, 2010). In previous study, approximately above 90% of Klason lignin removal rate after the sodium chlorite treatment was observed with a tiny degradation of glucan and xylan using switchgrass, poplar, corn stover, and pine sawdust (Kumar et al., 2013). In another research, lignin removal through the sodium chlorite treatment using softwood and hardwood pulp was conducted with extensive dose of sodium chlorite upward 6 times the sample weight (Yu et al., 2011). As the result of the study, about 78% of total lignin was eliminated and a significant

glucose production was achieved in case of softwood pulp after the treatment.

### 3.2.2. Lignin effect concerning glucose production

Reduction of cellulase activity was observed approximately 60% for a filter paper as a substrate through supplement of 1.5 mg/ml lignin, and the research reported that the enzyme loading from 5 to 50 FPU/g can appropriately the mitigate effect of lignin addition and hindrance (Sewalt et al., 1997). Meanwhile, approximately 90% of delignification from sugarcane bagasse was observed using alkali reagent and peractic acid (Zhao et al., 2009b). Then pretreated slurry applied to enzymatic hydrolysis that revealed remarkable improvement of cellulase activity than that of pretreated slurry derived from dilute acid pretreatment (Zhao et al., 2011). Furthermore, sources and types of lignin significantly affect to adsorption aspect of cellulase against lignin fraction (Sutcliffe & Saddler, 1986). And lignin structure can diversely change depending on pretreatment strategies or conditions during delignification reaction (Pan, 2008). In another study, AFEX treated lignin presented relatively low cellulose adsorption than that of dilute acid or $SO_2$ pretreatment (Kumar & Wyman, 2009). Meanwhile, high adsorption of cellulase was observed in organosolv pretreated lignin than that of steam explosion treated lignin due to a large proportion of phenolic hydroxyl group in organosolv lignin (Sewalt et al., 1997). Meanwhile, another research reported that aliphatic hydroxyl group has concerned about characteristics of hydrogen bonding between lignin and cellulase as well as phenolic hydroxyl group (Nakagame, 2010). However, one of study suggested no obvious interaction between adsorption ability of lignin model compounds and methoxyl groups (Pan, 2008). Carboxylic acid might enhance to overcoming lignin adsorption against the cellulase during enzymatic

hydrolysis (Berlin et al., 2006). And cellulase-lignin linkage can affect lignin adsorption aspect through electrostatic and hydrophobic interaction (Nakagame, 2010). These interactions between lignin and enzyme take an important role for irreversible binding of enzymes and determination of cellulase doses for facilitating the glucose production (Nakagame et al., 2011a), and the inhibition of lignin to cellulose is illustrated as three mechanisms in Figure 1-2 (Saini et al., 2016). However which factors of lignin structure strongly affect the reduction of cellulase activities that hard to clearly comprehend because of complexity of lignin and cell-wall structure in the lignocellulosic biomass (Nakagame, 2010).

Several strategies have been improved to reduce the negative impact on enzyme activities by lignin. Among them, lignin removal has two advantages for glucose production; i) destruction of physical barriers formed by lignin, ii) get rid of a change for interaction between lignin and cellulase followed by non-productive adsorption (Zhao et al., 2012). In this respect, alkaline and organosolv pretreatment commonly refer to effective delignification method for improving enzymatic digestibility (Sun & Cheng, 2002; Zhao et al., 2009a). The alkaline and organosolv pretreatment are simply similar with the conventional pulping process, but some of critical differences appear between these pretreatment method and the pulping process. Generally, the pulping process seeks for optimizing to holding the fiber integrity in the pulp, so it does not pursue improvement of cellulose accessibility. Furthermore the pulping process is usually known as expensive method that is not inadequate for lignocellulosic biomass pretreatment (Zhao et al., 2009a).

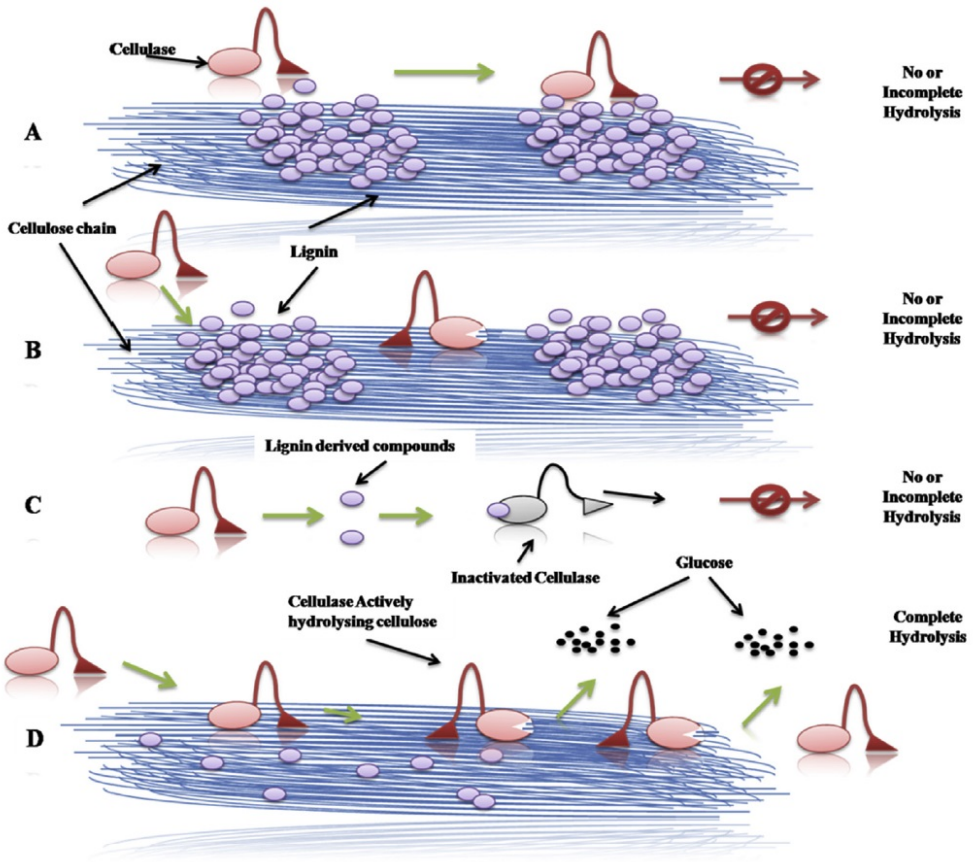



Figure 1-3. Inhibition of cellulase by lignin. a) Non-productive adsorption of cellulase onto lignin, b) Physical blockage of cellulase progress on lignocellulose chain, c) enzyme inhibition due to soluble lignin-derived compounds, and d) normal functioning of cellulase on cellulose chain to release glucose in presence of no or very low amount of lignin (Saini et al., 2016)

## 3.3. Glucose production according to cellulose properties

### 3.3.1. Crystallinity concerning glucose production

Crystallinity of cellulose chain has been believed one of important factor for cellulase activity and enzymatic hydrolysis (Chang & Holtzapple, 2000b). However, effect of cellulose properties for enzymatic digestibility in terms of biomass recalcitrance do not simply explain due to a discrepancy in several data from results of independent investigations in previous researches (Puri, 1984). In addition, amorphous region more easily eliminates than crystalline region through pretreatment methods that made an increase of crystalline territory (Mansfield et al., 1999).

Nevertheless, cellulose crystallinity has a significant impact on initial step during enzymatic hydrolysis (Fan et al., 1980; Zhang & Lynd, 2004). As results of this study, cellulose substrate mixed with crystalline and amorphous region is hydrolyzed first on amorphous domain, thus crystallinity index increases due to the remaining crystallinity region (Ooshima et al., 1983). Meanwhile, various analytical methods (e.g. X-ray diffraction and solid state $^{13}$C-NMR) conducted for clearly prove the effect of cellulose crystallinity using pure cellulose and lignocellulose biomass (Zhang et al., 2006b). Despite all these efforts, whether cellulose crystallinity is a key factor for enzymatic hydrolysis or not is difficult to conclusions, because some studies reported no correlation between cellulase activity and crystallinity (Lynd et al., 2002; Mansfield et al., 1999). Avicel, filter paper, bacterial cellulose, cotton, and cotton linter are applied for evaluating effect of cellulose crystallinity that had different types of cellulose due to different degrees of crystallinity (McLean et al., 2002; Väljamäe et al., 1999). In these studies, a direct relationship

between crystallinity index and degradation ratio of these samples found during enzymatic hydrolysis (Hoshino et al., 1997; Puri, 1984).

Meanwhile, adsorption capacity of enzymes onto the cellulose chain is considered to significant criterion for explanation of crystallinity effect (Steiner et al., 1988). If amount of adsorbing cellulase increases to cellulose structure, the rate of enzymatic hydrolysis generally increases (Medve et al., 1994; Sattler et al., 1989). However, it is reported that the adsorption capacity of endoglucanase differently presented depending on difference of reactivity between the amorphous and crystalline cellulose (Klyosov et al., 1986). In addition, the highest adsorption rate is observed in low crystallinity index despite of given enzyme dose strongly affecting the cellulase adsorption rate (Lee et al., 1982). The amorphous cellulose has been typically researched for evaluation of cellulase activity that avoids interference by crystalline region (Stone et al., 1969; Szijártó et al., 2008). 85% of phosphoric acid was employed to generate the phosphoric acid swollen cellulose that appeared complete dissolution of sample, but it reported that cellulase activity had little relationship with reducing-end concentration by degree of polymerization of samples in this experience (Zhang et al., 2006a).

### 3.3.2. Strategy for cellulose structure and crystallinity change

Most of pretreatment methods for lignocellulosic biomass commonly drive an increase of crystallinity index because of easily decomposing amorphous region in cellulose and hemicellulose. Additionally, chemically pretreated solid fraction tends to contain a large of crystalline region that might reduce the cellulose accessibility. Therefore, modification of rigid and strict property of crystalline structure is necessary for facilitating the enzymatic hydrolysis process.

Mercerization is a typical fiber treatment that developed to improve hydrophobic characteristic of natural fiber, interfacial linkage between fiber and matrix, surface wettability and roughness, and reduction of moisture absorption (George et al., 2001; Ku et al., 2011). As these benefits of mercerization, this process is commonly adopted in textile industry (Wang et al., 2003). Mercerization is one of the alkaline treatment process, and concentrated aqueous solution of strong base catalyst, leading to high degree of swelling of fiber that makes relaxation of crystalline structure in cellulose chain (Bledzki & Gassan, 1999). A monoclinic crystalline lattice of cellulose-I, which is native cellulose occurring in nature, can be modified to different forms of polymorphous such as cellulose-II and alkali-cellulose through mercerization (John & Anandjiwala, 2008). It is reported that types and concentration of alkali reagent affect to swelling efficiency (Hashim et al., 2012). During mercerization process, $Na^+$ ion appears a suitable diameter for expanding the smallest pores in the lattice planes through penetrating into this structure, therefore, utilization of sodium hydroxide reported the best degree of swelling (Mwaikambo & Ansell, 2002). Generally, concentration of NaOH ranges from 1% to 25% and the reaction runs for approximately 60 min for mercerization (Symington et al., 2009).

# *Chapter 2*

## Hemicellulose degradation and xylooligosaccharide production with correlation analysis between biomass constituents and glucose yield

# 1. Introduction

Oligosaccharides such as fructooligosaccharide, galactooligosacchride, and xylooligosaccharide have recently been attracting attention due to their properties as prebiotics (Barreteau et al., 2006). Among them, xylooligosaccharide is one of the most notable in food or its additives, and its beneficial influence on the human body has been reported in several previous studies (Aachary & Prapulla, 2011; Vázquez et al., 2002). It contributes primarily to the proliferation of bifidobacteria, which improves intestinal condition and inhibits pathogenic and putrefactive microorganisms (Gullón et al., 2008). And, as a non-digestible oligosaccharide, xylooligosaccharide, has been supplied as a low-caloric food for weight loss, and it has also advantageous effects such as reduction of cardiovascular symptoms, alleviation of dental decay, activation of calcium absorption, and improvement of bowel function (Grootaert et al., 2007). Moreover, xylooligosaccharide is traded typically at a higher price than that of xylose even though both products originated from xylan commonly (Taniguchi, 2004). Therefore, a value-added utilization of lignocellulosic biomass, which contains a large amount of xylan and is abundant worldwide, can be achieved as an application for a source of xylooligosaccharide.

Generally, conventional production of xylooligosaccharide follows a particular process: xylan extraction from biomass, enzymatic hydrolysis using xylanase, and purification (Chapla et al., 2012). However, this process has several drawbacks such as the high cost of purchasing xylanase, the long time needed for hydrolysis, and only xylobiose (DP=2) and xylotriose (DP=3) are produced (Brienzo et al., 2010). To overcome these limitations, non-biological approaches have been developed to produce xylooligosaccharide using

hydrothermal methods (Otieno & Ahring, 2012b). However, hemicellulose structure can easily decompose into monomeric sugars due to thermal energy and acidic conditions (Akpinar et al., 2009). Therefore, the investigation of appropriate conditions is important for suitable xylooligosaccharide production using hydrothermal treatments.

*Eucalyptus* is native to Australia; however, it now widely grows in subtropical and tropical regions owing to artificial planting (Florence, 2004). And, it has numerous diversities over 700 species with several groups that are classified by genetic and geographical relationship. Among the species, *Eucalyptus globulus* or *Eucalyptus grandis* has been employed as a feedstock in many studies regarding pretreatment or enzymatic hydrolysis because it is easily supplied for chemical treatment in the United States (Romaní et al., 2011; Yu et al., 2010). Because this species was introduced to California by Australians in the early 1900s, it is typically used for construction, furniture making and railroad ties as a form of timber (Santos, 1997). Meanwhile, *Eucalyptus pellita*, which is a feedstock for this study, has been rarely applied for pretreatment research, but it has recently been widely used for artificial planting in South and Southeast Asia. Furthermore, foreign species such as *Eucalyptus pellita* can be imported for chemical conversion, and then the products are utilized for industrial, pharmaceutical, and food markets using the tropical species.

At present, some research has been conducted to evaluate the influence of lignocellulosic biomass constituents on glucose production after pretreatment and enzymatic hydrolysis. Silverstein et al. investigated xylan solubilization and delignification from cotton stalk using sodium hydroxide, hydrogen peroxide, and ozone pretreatment (Silverstein et al., 2007). In the previous study, the results of pretreatment were analyzed using the statistical program SAS, which estimated the correlation between xylan solubilization

(or lignin reduction) and modified severity parameters. However, studies of the correlation between biomass constituents and glucose production through enzymatic hydrolysis have been rarely carried out using statistical approaches.

In this study, liquid hot water treatment, which is among the hydrothermal treatments without a catalyst, was performed using *E. pellita* to produce xylooligosaccharide. Moreover, a trend of xylan decomposition was investigated depending on the conditions of the liquid hot water treatment, and xylooligosaccharide content was evaluated by the degree of polymerization. In addition, the control of biomass constituents such as hemicellulose and lignin removal rate for glucose production was evaluated by the regression analysis using SAS. The variation in the biomass constituents was employed for correlation analysis given empirical data which are presented in this chapter. Finally, single or multiple regression analysis was performed using experimental data of glucose yield and the change in biomass constituents, and a regression model was established analyzing statistical significance and the squared of correlation coefficients (R-squared values).

# 2. Materials and methods

## 2.1. Materials

*Eucalyptus pellita*, which is the feedstock of this study, was generously supplied by the Korea Research Institute of Chemical Technology (Daejeon, Republic of Korea). Although Australia and Papua New Guinea are native habitat of *Eucalyptus pellita*, this biomass is widely inhabited all over the world such as Brazil, Congo, Indonesia and etc. Before proceeding treatments, *Eucalyptus pellita* was milled by twin-extruder for reduction of dust generation and extractives removal. The particle size of feedstock was approximately 0.5 mm and sealed in the zipper bag.

## 2.2. Xylooligosaccharide production

For producing xylooligosaccharide, liquid hot water treatment was induced for twin-extruder treated *Eucalyptus pellita*, the feedstock of this study. 122.9 g of wet feedstock, which is same with 50 g of oven-dried weight, and 500 mL of distilled water (solid:liquid ratio = 1:10 (w/v), considering moisture content in the wet feedstock) were loaded into a 1000-mL capable batch type reactor (HR-8300, Hanwoul Engineering Inc., Gunpo, Republic of Korea). The reactor was heated by metal jacket until the sample reached the target temperature (130, 150, 170, 180, 190, 210°C) within heating time (50 min). And then the target temperature (reaction temperature) was maintained for 10, 20, 30, 50, or 70 min. After the reaction is completed, the reactor was quenched to 50°C using ice chamber and liquid hydrolysate was separated from treated solid residue by filter paper (No. 52, Hyundai micro CO., Seoul,

Republic of Korea). For analysis of sugar and its derivatives contents, the liquid hydrolysate was filtered using a 0.45 μm membrane filter (Advantec, Tokyo, Japan). The solid residue was washed with distilled water, and kept at 4°C for enzymatic hydrolysis.


Figure 2-1. Reaction system for liquid hot water treatment.

Table 2-1. Reaction temperature and time of liquid hot water treatment.

| Conditions | |
|---|---|
| Reaction temperature (°C) | Reaction time (min) |
| 130 | 10 |
| 150 | 10 |
| 170 | 10 |
| 170 | 20 |
| 170 | 30 |
| 170 | 50 |
| 170 | 70 |
| 180 | 10 |
| 180 | 30 |
| 180 | 50 |
| 190 | 10 |
| 190 | 30 |
| 190 | 50 |
| 210 | 10 |

## 2.3. Analysis of solid residues

### 2.3.1. Water-insoluble solid recovery rate

The water-insoluble solid (WIS) recovery rate of solid residue after liquid hot water treatment was calculated by weighing of the wet solid residue and estimating its moisture content (Eq. 2-2).

$$\text{WIS recovery rate (\%)} = \frac{\text{weight of solid residue after treatment}}{\text{weight of initial biomass}} \times 100 \qquad \text{(Eq. 2-2)}$$

### 2.3.2. Chemical composition analysis

The chemical composition (extractives, lignin, and sugars) of feedstock and solid residues obtained through liquid hot water treatment were determined using the procedures described by Laboratory Analytical Procedure (LAP) of the National Renewable Energy Laboratory (NREL) and the Association of Official Agricultural Chemists (AOAC) (Latimer, 2012; Sluiter et al., 2008a; Sluiter et al., 2008b). Detailed conditions for determining extractives, lignin, and sugars content were described in section 2.3.3, section 2.3.4, and section 2.3.5 of Chapter 2.

### 2.3.3. Determination of extractives

Content of extractives in the feedstock (twin-extruder treated *Eucalyptus pellita*) were measured according to NREL and AOAC method (Latimer, 2012; Sluiter et al., 2008b). Before measurement of extractives, the feedstock was air-dried until its moisture content was below 10%. 2 g of the air-dried

feedstock were put in a thimble filter (grade 84, Advantec, Tokyo, Japan) and 150 mL of alcohol-benzene (1:2 (v/v)) solution (Samchun Chemicals, Pyeongtaek, Korea) were used to extract in Soxhlet extractor for 6 h at 80°C. And then the extractives in the round-bottom flask were incrassated by a rotary evaporator (N-1110, Tokyo Rikakikai Co., Ltd., Tokyo, Japan) and they were dried in an oven (HB-501M, Hanbaek Scientific Technology, Bucheon, Republic of Korea) at 105°C for 24 h. The content of extractive based on the weight of oven-dried feedstock was determined as follow equation (Eq. 2-3):

$$\text{Extractive content (\%)} = \frac{\text{weight of incrassated extractives}}{\text{weight of initial biomass}} \times 100 \qquad \text{(Eq. 2-3)}$$

### 2.3.4. Determination of Klason lignin and acid-soluble lignin

Amount of Klason lignin and acid-soluble lignin were determined by NREL method (Sluiter et al., 2008a). 0.3 g of air-dried feedstock and solid residues after liquid hot water treatment were swelled in 3 mL of 72% (w/w) sulfuric acid in an Erlenmeyer flask at 30°C for 1 h. Then, 84 mL of distilled water were added to dilute for acid concentration to 4% and the samples were reacted using an autoclave at 121°C for 1 h. After hydrolysis, the samples were filtered through glass filter (1G4, Iwaki, Tokyo, Japan) using distilled water. The residues on the filters were oven-dried at 105°C and weighed for measuring Klason lignin. Klason lignin content based on the weight of oven-dried feedstock was calculated by following formula (Eq. 2-4):

$$\text{Klason lignin (\%)} = \frac{\text{weight of residue on the glass filter}}{\text{weight of initial biomass}} \times 100 \qquad \text{(Eq. 2-4)}$$

Acid-soluble lignin was determined through the absorbance of the filtrate that was measured at 205 um using a UV-visible spectrophotometer (UV-1601 PC, Shimadzu, Kyoto, Japan) with a quartz cuvette. Acid-insoluble lignin content based on the weight of oven-dried feedstock was calculated as follow (Eq. 2-5):

$$\text{Acid-soluble lignin (\%)} = \frac{\text{UV}_{\text{absorbance}} \times \text{V}_{\text{filtrate}} \times \text{Dilution}}{\varepsilon \times \text{W}_{\text{initial biomass}}(\text{g}) \times \text{P (cm)}} \times 100 \qquad \text{(Eq. 2-5)}$$

### 2.3.5. Determination of monomeric sugars

The monomeric sugar (glucose, xylose, arabinose, galactose, and mannose) content in the feedstock and solid residues after liquid hot water treatment was determined using ion chromatography (ICS2500, Thermo Dionex, Palo Alto, CA, USA). It equipped with a CarboPac PA-1 column (250 × 4 mm, Dionex, Palo Alto, CA, USA) and a pulsed amperometric detector (ED40, Dionex, Palo Alto, CA, USA). The monomeric sugar analysis was conducted at 40°C using potassium hydroxide as an eluent (2 mM for 1–36 min, 2 to 100 mM for 35–36 min, 100 mM for 36–56 min, 100 to 2 mM: 56–57 min, and 2 mM for 58–63 min) at a flow rate of 1 mL/min with injection volume of sample was 10 μL. The standard solutions of glucose, xylose, arabinose, galactose, and mannose were prepared using reagents, which were purchased by Sigma-Aldrich Korea CO. (Yongin, Republic of Korea) to make calibration curve for quantitative analysis. All peaks by ion chromatography were identified via comparison of peak retention times and measured each concentration of sugars corresponding to the different peaks (Jang et al., 2017a).

## 2.4. Analysis of liquid hydrolysates

### 2.4.1. Xylose and xylooligosaccharide analysis

The xylose and xylooligosaccharide (xylobiose, xylotriose, xylotetraose, xylopentaose, and xylohexaose) content in the liquid hydrolysate after liquid hot water treatment was determined using ion chromatography (ICS5000, Thermo Dionex, Palo Alto, CA, USA). It equipped with a CarboPac SA-10 column (250 × 4 mm, Dionex, Palo Alto, CA, USA) and a pulsed amperometric detector (ED40, Dionex, Palo Alto, CA, USA) with a gold electrode. The xylose and xylooligosaccharide analysis was conducted at 32°C using 100 mM and 200 mM of sodium hydroxide with 100 mM and 1 mM of sodium acetate at a flow rate of 0.85 mL/min with injection volume of sample was 10 μL. The standard solutions of xylose was purchased by Sigma-Aldrich Korea CO. (Yongin, Republic of Korea), while the standard solutions of xylobiose, xylotriose, xylotetraose, xylopentaose, and xylohexaose were purchased by Megazyme (Wicklow, Ireland) to make calibration curve for quantitative analysis. All peaks by ion chromatography were identified via comparison of peak retention times and measured each concentration of xylose and xylooligosaccharides corresponding to the different peaks .

### 2.4.2. Monomeric sugar analysis

The monomeric sugar (glucose, xylose, arabinose, galactose, and mannose) content in the liquid hydrolysate after liquid hot water treatment and glucose content after enzymatic hydrolysis were determined as described in section 2.3.5 of Chapter 2.

### 2.4.3. Sugar derivatives analysis

The amount of sugar derivatives (furfural, 5-hydroxymethylfurfural (5-HMF), levulinic acid, acetic acid, and formic acid) in the liquid hydrolysate after liquid hot water treatment were determined using a high performance liquid chromatography (Ultimate 3000, Thermo Dionex, Palo Alto, CA, USA) outfitted with a Aminex 87H column. The sugar derivatives analysis was conducted at 40°C using 0.1 N of sulfuric acid as an eluent at a flow rate of 0.5 mL/min with injection volume of sample was 10 μL. The standard solutions of furfural, 5-HMF, levulinic acid, acetic acid, and formic acid was purchased by Sigma-Aldrich Korea CO. (Yongin, Republic of Korea) to make calibration curve for quantitative analysis. All peaks by high performance liquid chromatography were identified via comparison of peak retention times and measured each concentration of sugar derivatives corresponding to the different peaks (Jang et al., 2017b).

## 2.5. Enzymatic hydrolysis

Enzymatic hydrolysis was performed using 1 g of oven-dried weight of the feedstock and solid fractions obtained by liquid hot water treatments. The substrates were mixed with 50 mM sodium acetate buffer (pH 5.0). And then, Cellulase complex Cellic CTec2 (Novozymes, Bagsværd, Denmark) was employed with an activity of 15 FPU/g to add the substrate and buffer mixture. The mixture was incubated at 50°C for 72 h in a hybridization incubator (combi-D24, FINEPCR, Gunpo, Republic of Korea). After the process, the mixture was filtered by filter paper (No. 52, Hyundai micro CO., Seoul,

Republic of Korea). Residues on the filter paper were dried in an oven (HB-501M, Hanbaek Scientific Technology, Bucheon, Republic of Korea) at 65°C for 24 h, and it was weighed for estimation of enzymatic digestibility. Enzymatic digestibility based on the weight of oven-dried substrate was calculated as follow (Eq. 2-6):

$$\text{Enzymatic digestibility (\%)} = \frac{\text{1- (weight of residues (g))}}{\text{weight of substrate (g) (equal to 1)}} \times 100 \qquad \text{(Eq. 2-6)}$$

Meanwhile, the filtrate was filtered once more using a 0.45 μm membrane filter (Advantec, Tokyo, Japan) was collected to determine glucose yield. The glucose yield based on the glucose content in the feedstock was determined as follow equation (Eq. 2-7) (Jang et al., 2017a):

$$\text{Glucose yield (\%)} = \frac{\text{weight of glucose in filtrate by chromatography(g)}}{\text{weight of glucose in the initial biomss(g)}} \times 100 \qquad \text{(Eq. 2-7)}$$

## 2.6. Correlation analysis

The material of correlation analysis was empirical data sets which were obtained from the results of the liquid hot water treatment and enzymatic hydrolysis in this chapter. In other words, the experimental data which consisted of hemicellulose and lignin removal rate was corresponded with glucose yield. Thus, the arranged empirical results were employed for statistical analysis using computer program. The statistical analysis program, SAS (SAS Institute, Cary, NC, USA), was used for investigating the correlation between the biomass constituents and the glucose yield. It was fit using PROC REG for evaluating the effect of single or multiple factor, and the

model of linear regression analysis was established with checking the significance and correlation coefficient. In addition, the single or multiple regression model was also confirmed the signification and the squared of correlation coefficient (R-squared value).

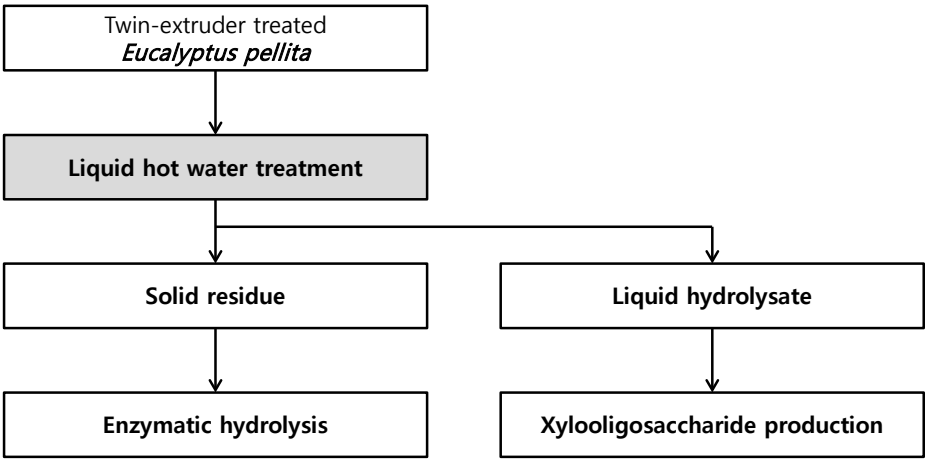



Figure 2-2. Schematic experimental flowchart of xylooligosaccharide production process by liquid hot water treatment of *Eucalyptus pellita*.

# 3. Results and discussion

## 3.1. Conversion characteristics of *Eucalyptus pellita* under a liquid hot water treatment

### 3.1.1. Physicochemical characteristics of solid residues

During a hydrothermal treatment such as liquid hot water treatment, chemical components of lignocellulosic biomass degrade into smaller molecule (Pérez et al., 2008). For example, glycosidic bonds of cellulose and hemicellulose in cell-wall structure are easily cleaved and dissolved into a solvent as a form of monomeric or oligomer sugar (Yu et al., 2010). Thus, the weight of lignocellulosic biomass typically decreases under the hydrothermal treatment, and it is related closely with reaction conditions such as reaction temperature, reaction time, catalyst concentration, and type of solvent and reactor (Kumar et al., 2009a).

A change in water insoluble solid (WIS) recovery rate from twin-extruder-treated *E. pellita* after liquid hot water treatment is shown in Figure 2-3. The WIS recovery rates under two conditions, 130°C for 10 min and 150°C for 10 min, were 97.9% and 97.9%, respectively, which is similar to that of initial biomass (100%). Meanwhile, the WIS recovery rate decreased to 76.6% at 210°C for 10 min which was the highest reaction temperature among the conditions of the liquid hot water treatment. The WIS recovery rate typically decreased with the extension of the reaction time among similar reaction temperature conditions, for example, the 90.6% WIS recovery rate at 170°C for 10 min decreased to 80.8% at 170°C for 70 min. The severity of the hydrothermal treatment is usually determined by investing the thermal energy

which is controlled by reaction temperature or time. Therefore, harsh conditions such as 210°C for 10 min during this study resulted in significant damage to the chemical structure of *E. pellita*. The severity of the hydrothermal treatment is also affected by the dose of the catalyst. Although no catalyst was employed for liquid hot water treatment, acetic acid can be released into the solvent originating from the lignocellulosic biomass (Zhuang et al., 2016). In the case of hardwood, hydroxyl groups are substituted by acetyl groups at C2 and/or C3 carbon of the xylose unit in the hemicellulose chain (Laine, 2005). The acetyl groups are released more with an increase in the severity of the hydrothermal treatment, which enhances the acidity of the solvent (Cara et al., 2007). Consequently, a low WIS recovery rate of solid residues at high reaction temperatures might be caused by an increase in acetyl group decomposition as well as thermal energy.

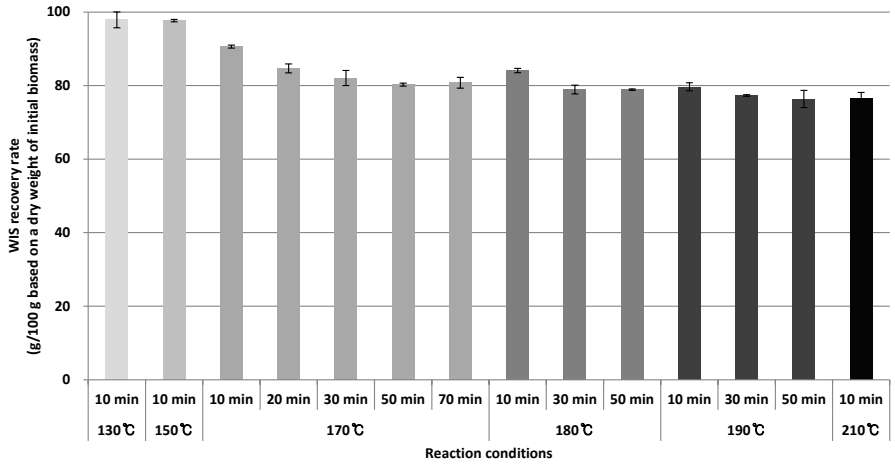



Figure 2-3. Water insoluble solid (WIS) recovery rate (%, based on a dry weight of biomass) of solid residues after liquid hot water treatment of *Eucalyptus pellita* depending on reaction temperature and time.

Table 2-2 summarizes the structural sugar (glucan, xylan, galactan, mannan, and arabinan) content of *E. pellita* and the solid residues after liquid hot water treatment. The twin-extruder-treated *E. pellita* used as a raw material in this study was composed of the following carbohydrates: 50.9% glucan, 10.8% xylan, 1.6% galactan, 0.6% mannan, and 0.3% arabinan. The sub-/tropical species, *E. pellita*, has a large amount of xylan (10.8%) compared to that of mannan (0.6%). Generally, hemicellulose in hardwood is primarily composed of glucuronoxylan (15-30, % of the wood), while that of softwood mainly consists of galactoglucomannan (10-15, % of the wood) (Sjostrom, 2013). This difference regarding the type of the dominant backbone in hemicellulose structure is revealed by the amount of xylan or mannan in the biomass. Thus, *E. pellita* might have a similar hemicellulose structure compared to that of hardwood because of the high amount of xylan.

Glucan (50.9%) comprised the largest portion of structural sugar in *E. pellita*, and glucan content was approximately constant even though there were changes in reaction temperature and time during the liquid hot water treatment. Cellulose has different properties compared to those of hemicellulose, as it is only composed of glucose, has a linear polymer topology, there is no substitution at the side group, a crystalline region exists, it has low reactivity, and it is poorly soluble in water (Hendriks & Zeeman, 2009). These features of cellulose provide resistance and it is not easily decomposed by hydrothermal treatment. Meanwhile, the amount of glucan in solid residues after liquid hot water treatment decreased slightly less than that in *E. pellita*. It is assumed that the small amount of glucose that was dissolved into liquid hydrolysate originated from side chains of hemicellulose, not cellulose.

The amount of xylan in the solid residues was maintained under the conditions of 130°C (10.5%) and 150°C (10.5%) for 10 min when compared

to that of *E. pellita* (10.8%). As the reaction temperature increased to 180°C for 10 min, xylan content dramatically decreased to 3.4%, and a minimal amount of xylan (0.5%) remained in the solid residue under conditions of 210°C for 10 min. This decomposition trend was quite different from the case of cellulose previously mentioned. It showed that hemicellulose is more reactive and susceptible under conditions of hydrothermal reaction (Kumar et al., 2008). On the other hand, mannan and arabinan were fully decomposed at 130°C, and galactan dissipated at 170°C in the solid residue.

Table 2-2. Structural sugar content of the twin-extruder treated *Eucalyptus pellita* and the solid residues obtained by liquid hot water treatment as a function of reaction temperature and time

| Conditions | | Structural sugar (%)[1] | | | | | Total[2] |
|---|---|---|---|---|---|---|---|
| Reaction temp. (°C) | Reaction time (min) | Glucan | Xylan | Galactan | Mannan | Arabinan | |
| *E. pellita* | | 50.9±1.5 | 10.8±0.4 | 1.6±0.1 | 0.6±0.0 | 0.3±0.0 | 64.2 |
| 130 | 10 | 47.3±0.7 (48.3)[3] | 10.5±0.3 (10.7) | 1.2±0.0 (1.2) | 0.0±0.0 (0.0) | 0.0±0.0 (0.0) | 59.0 |
| 150 | 10 | 47.3±0.7 (48.4) | 10.5±0.3 (10.7) | 1.2±0.0 (1.2) | 0.0±0.0 (0.0) | 0.0±0.0 (0.0) | 59.0 |
| 170 | 10 | 47.5±0.5 (52.4) | 7.7±0.5 (8.5) | 0.6±0.1 (0.7) | 0.0±0.0 (0.0) | 0.0±0.0 (0.0) | 55.8 |
| | 20 | 46.5±0.7 (54.9) | 3.1±0.2 (3.7) | 0.3±0.1 (0.3) | 0.0±0.0 (0.0) | 0.0±0.0 (0.0) | 49.9 |
| | 30 | 46.3±2.7 (56.4) | 2.6±0.3 (3.1) | 0.0±0.0 (0.0) | 0.0±0.0 (0.0) | 0.0±0.0 (0.0) | 48.9 |
| | 50 | 47.4±2.3 (59.1) | 1.8±0.3 (2.3) | 0.0±0.0 (0.0) | 0.0±0.0 (0.0) | 0.0±0.0 (0.0) | 49.2 |
| | 70 | 46.7±0.6 (57.8) | 1.6±0.2 (2.0) | 0.0±0.0 (0.0) | 0.0±0.0 (0.0) | 0.0±0.0 (0.0) | 48.3 |
| 180 | 10 | 46.3±1.5 (55.1) | 3.4±0.3 (4.1) | 0.0±0.0 (0.0) | 0.0±0.0 (0.0) | 0.0±0.0 (0.0) | 49.7 |
| | 30 | 45.4±0.0 (57.5) | 1.5±0.1 (1.9) | 0.0±0.0 (0.0) | 0.0±0.0 (0.0) | 0.0±0.0 (0.0) | 46.9 |
| | 50 | 43.4±0.3 (55.0) | 1.1±0.3 (1.5) | 0.0±0.0 (0.0) | 0.0±0.0 (0.0) | 0.0±0.0 (0.0) | 44.5 |
| 190 | 10 | 46.4±0.9 (58.2) | 1.8±0.4 (2.2) | 0.0±0.0 (0.0) | 0.0±0.0 (0.0) | 0.0±0.0 (0.0) | 48.2 |
| | 30 | 42.7±2.3 (55.2) | 0.7±0.0 (0.9) | 0.0±0.0 (0.0) | 0.0±0.0 (0.0) | 0.0±0.0 (0.0) | 43.4 |
| | 50 | 45.2±2.0 (59.2) | 0.5±0.1 (0.7) | 0.0±0.0 (0.0) | 0.0±0.0 (0.0) | 0.0±0.0 (0.0) | 45.7 |
| 210 | 10 | 44.6±1.3 (58.2) | 0.5±0.1 (0.7) | 0.0±0.0 (0.0) | 0.0±0.0 (0.0) | 0.0±0.0 (0.0) | 45.1 |

Values are the mean ± standard deviation

[1] %, based on a dry weight of initial biomass

[2] Sum of structural sugar content

[3] %, based on a dry weight of the solid residue

Lignin (Klason lignin and acid-soluble lignin) and extractive content of twin-extruder-treated *E. pellita* and the solid residues after liquid hot water treatment are shown in Table 2-3. 2.0% of the extractive content was observed in the raw material, but it was not found in the solid residues. The amount of lignin typically reported is 18-25% in hardwoods and 25-30% in softwoods (Harkin & Rowe, 1971). Thus, *E. pellita* had significant lignin content (34.8% of total lignin) compared to that of other lignocellulosic biomass.

The amount of Klason lignin in *E. pellita* (32.5%) was generally maintained during liquid hot water treatment. For example, Klason lignin was 32.6% at 130℃ for 10 min and 32.7% at 210℃ for 10 min. Therefore, it is considered that Klason lignin was barely affected by the changes in the reaction conditions. Acid hydrolysis occurs under liquid hot water treatment that is critical for hemicellulose decomposition with an increase in reaction temperature and time as previously mentioned (Esteghlalian et al., 1997). This is because acetic acid, which is derived from acetyl groups in biomass, is effective in cleaving the ether bond in a hemicellulose chain (Kim et al., 2009). Cellulose chains can be broken into glucose that was not observed in this study when the acidity increases due to acidic catalysts such as sulfuric acid or hydrochloric acid during hydrothermal treatment (Hsu et al., 2010). However, these acidic catalysts do not work on aryl-ether or aryl-aryl bonds in the lignin structure (Jang et al., 2017a). Meanwhile, a control variable is well established over hemicellulose for investigation of the relationship with glucose production after enzymatic hydrolysis.

On the other hand, acid-soluble lignin content in *E. pellita* (2.3%) slightly decreased after the liquid hot water treatment, but it was generally constant among the reaction conditions.

Table 2-3. Lignin and extractive content of the twin-extruder treated *Eucalyptus pellita* and the solid residues obtained by liquid hot water treatment as a function of reaction temperature and time

| Conditions | | Lignin (%)[1] | | Total[2] | Extractive |
|---|---|---|---|---|---|
| Reaction temp. (°C) | Reaction time (min) | Klason lignin | Acid-soluble lignin | | |
| *E. pellita* | | 32.5±1.0 | 2.3±0.3 | 34.8 | 2.0±0.0 |
| 130 | 10 | 32.6±1.7 (33.3)[3] | 2.1±0.1 (2.1) | 34.7 | 0.0±0.0 (0.0) |
| 150 | 10 | 31.4±2.5 (32.2) | 1.8±0.2 (1.9) | 33.2 | 0.0±0.0 (0.0) |
| 170 | 10 | 32.3±0.5 (35.6) | 1.1±0.1 (1.3) | 33.4 | 0.0±0.0 (0.0) |
| | 20 | 30.8±0.3 (36.3) | 1.1±0.2 (1.3) | 31.9 | 0.0±0.0 (0.0) |
| | 30 | 30.5±1.3 (37.1) | 1.3±0.1 (1.6) | 31.8 | 0.0±0.0 (0.0) |
| | 50 | 30.9±0.2 (38.6) | 0.9±0.0 (1.2) | 31.8 | 0.0±0.0 (0.0) |
| | 70 | 29.8±0.2 (36.9) | 1.0±0.0 (1.2) | 30.8 | 0.0±0.0 (0.0) |
| 180 | 10 | 32.7±0.7 (38.9) | 0.9±0.0 (1.1) | 33.6 | 0.0±0.0 (0.0) |
| | 30 | 31.6±0.4 (40.0) | 0.9±0.1 (1.1) | 32.5 | 0.0±0.0 (0.0) |
| | 50 | 31.3±0.6 (39.7) | 1.4±0.1 (1.8) | 32.7 | 0.0±0.0 (0.0) |
| 190 | 10 | 30.5±0.9 (38.3) | 1.1±0.1 (1.4) | 31.6 | 0.0±0.0 (0.0) |
| | 30 | 32.2±0.1 (41.6) | 0.9±0.1 (1.2) | 33.1 | 0.0±0.0 (0.0) |
| | 50 | 33.1±0.2 (43.4) | 0.9±0.0 (1.1) | 34.0 | 0.0±0.0 (0.0) |
| 210 | 10 | 32.7±1.4 (42.7) | 1.1±0.2 (1.5) | 33.8 | 0.0±0.0 (0.0) |

Values are the mean ± standard deviation

[1] %, based on a dry weight of initial biomass

[2] Sum of Klason lignin and acid-soluble lignin content

[3] %, based on a dry weight of the solid residue

### 3.1.2. Chemical composition of liquid hydrolysates

Liquid hydrolysates were collected using filtering slurries obtained after liquid hot water treatment. The amount of monomeric sugars (glucose, xylose, galactose, mannose, and arabinose) was determined using bio-liquid chromatography and as is illustrated in Figure 2-4. The monomeric sugars were not detected at conditions of 130°C for 10 min and 150°C for 10 min. It is assumed that nearly all of the carbohydrates are not released into the liquid hydrolysate due to insufficient thermal energy under mild conditions during the liquid hot water treatment. This also corresponded to the results of structural sugar content in section 3.1.1 of Chapter 2.

The amount of glucose ranged from 0.0% (130°C for 10 min and 150°C for 10 min) to 1.2% (190°C for 50 min). Considering the glucan content in *E. pellita* (50.9%), a very small amount of glucose was released into liquid hydrolysate. At the same reaction temperature conditions of 170°C or 180°C or 190°C, glucose content increased as the reaction time was prolonged. Based on this result, decomposition of the cellulose chain might be enhanced by affording sufficient thermal energy.

Xylose was more released than glucose into the liquid hydrolysate, and the maximum content was 2.2% under the conditions of 180°C for 50 min and 190°C for 30 min. The trend of glucose content increased with an extension of reaction time under the same reaction temperature was also denoted in case of xylose. However, xylose content decreased slightly (2.1%) under high reaction temperatures and time such as 190°C for 50 min. It is known that the monomeric sugars derived from hemicellulose can be easily converted into various sugar derivatives during hydrothermal treatment (Lü et al., 2017). This conversion reaction might be enhanced by an increase in reaction

temperature, reaction time, and concentration of acidic catalysts (Alvira et al., 2010). This point was revealed obviously at conditions of 210°C for 10 min, as all the monomeric sugars diminished dramatically to less than under 0.3% in these conditions.

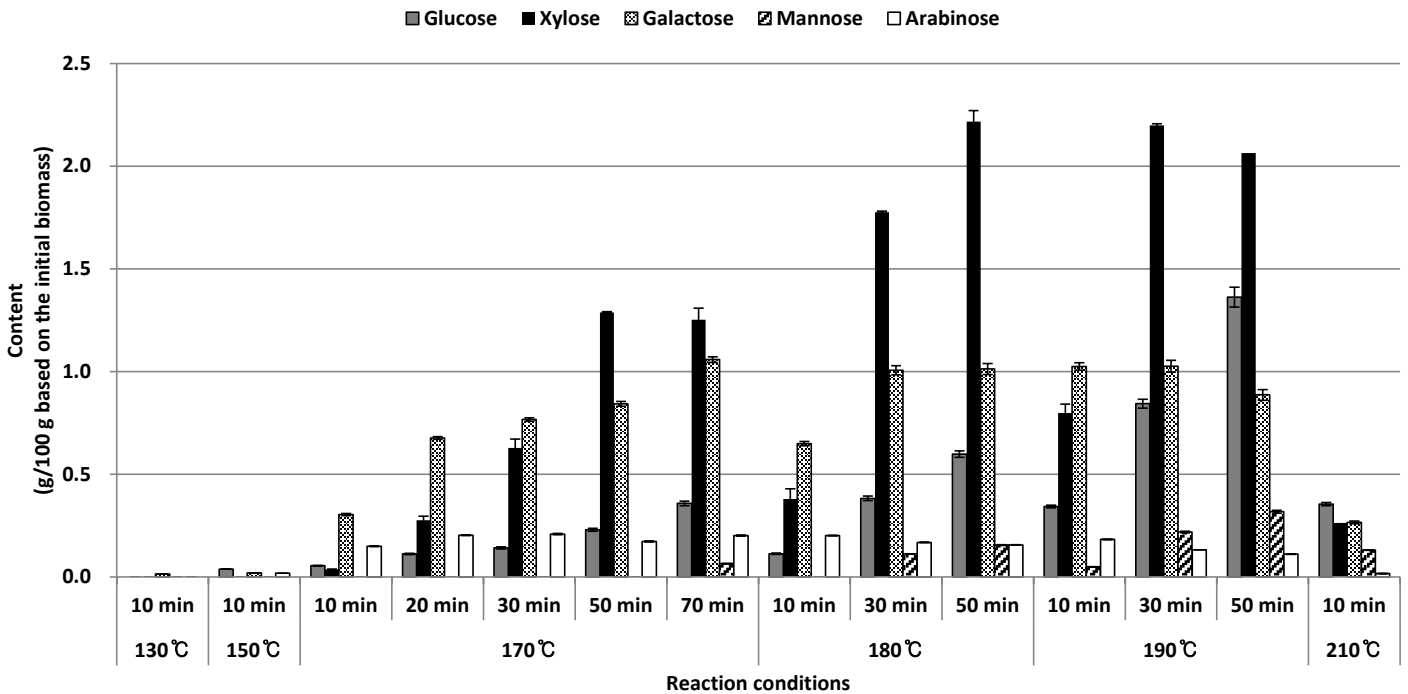



Figure 2-4. Monomeric sugar content (g/100 g initial biomass) of liquid hydrolysate after liquid hot water treatment of twin-extruder treated *Eucalyptus pellita* depending on reaction temperature and time.

The amount of sugar derivatives (formic acid, acetic acid, levulinic acid, 5-hydroxylmethylfurfural (5-HMF), and furfural) are shown in Figure 2-5. No sugar derivatives were detected under the conditions of 130°C for 10 min and 150°C for 10 min, because monomeric sugars serves as precursors for sugar derivatives and are rarely released into liquid hydrolysate under these conditions.

Acetic acid has been noted as a major factor in allowing acid hydrolysis during the liquid hot water treatment due to enhancement of the acidity. Among the sugar derivatives, acetic acid was the most active conversion product during the liquid hot water treatment in this study. The amount of acetic acid in the liquid hydrolysate increased steadily with an extension in reaction time at the same reaction temperature (170°C or 180°C or 190°C) or an increase in reaction temperature at the same reaction time (10 min or 30 min or 50 min) where more thermal energy was employed. The maximum content of acetic acid (2.8%) was observed at 190°C for 50 min, similar to acetic acid content at 210°C for 10 min (2.7%).

The amount of furfural was less than 1% below 170°C reaction temperature, but it increased sharply up to 3.0% at 190°C for 50 min. Furfural was converted typically from pentose through isomerization, enolization, and dehydration during acid hydrolysis (Feather et al., 1972). Thus, a sufficient amount of xylose or arabinose in the liquid hydrolysate needs to be secured to facilitate furfural production. A significant amount of acetic acid was released simultaneously under the severe reaction conditions (190°C for 50 min) that might have also accelerated furfural conversion from pentose. Meanwhile, the furfural content slightly decreased at 210°C for 10 min because of the further conversion reaction from furfural to formic acid or humin formation (Morone et al., 2015).

5-HMF and levulinic acid were rearely observed in the liquid hydrolysates under all conditions of liquid hot water treatment. These sugar derivatives are known as mainly being converted from hexose (Girisuta et al., 2006). However, glucose, galactose, and mannose as a source of 5-HMF or levulinic acid were released at less than 1.0% into the liquid hydrolysate due to the relatively mild conditions of the liquid hot water treatment which has no catalyst for the reaction.

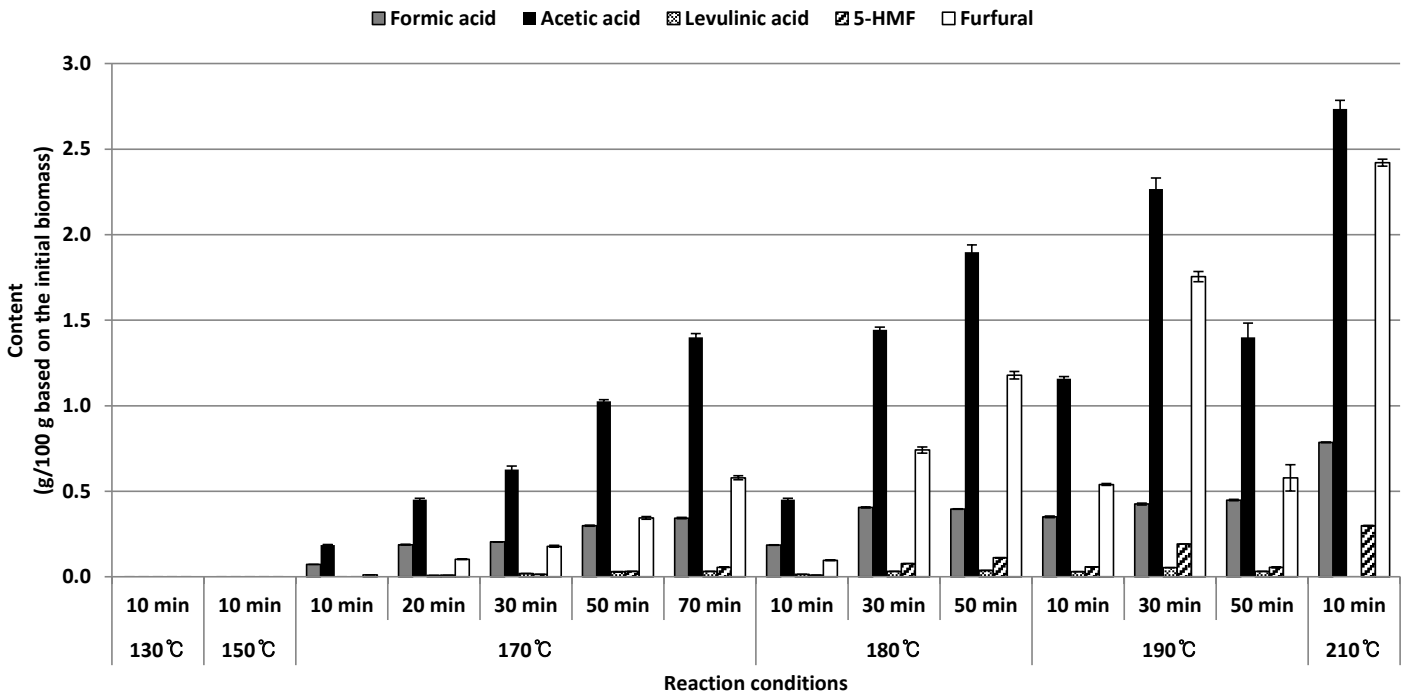



Figure 2-5. Sugar derivatives content (g/100 g initial biomass) of liquid hydrolysate after liquid hot water treatment of twin-extruder treated *Eucalyptus pellita* depending on reaction temperature and time.

## 3.2. Xylooligosaccharide production via liquid hot water treatment

In section 3.1 of this chapter, the conversion route of pentose from hemicellulose (xylan) to furfural was denoted by the amount of structural sugar in the solid residues and the amount of monomeric sugars and sugar derivatives in the liquid hydrolysate. However, a significant amount of xylan decomposed by thermal energy during the liquid hot water treatment could be not identified. The mechanism of pentose degradation originating from biomass or not has been studied by numerous researchers (Nimlos et al., 2006; Qian et al., 2005). According to Figure 2-6, xylooligosaccharide (xylobiose, xylotriose, etc.) is depicted as an intermediate of decomposition between hemicellulose (xylan) and xylose during acid hydrolysis (Enslow & Bell, 2012). Therefore, the amount of xylooligosaccharide that may be dissolved into liquid hydrolysates was investigated using bio-liquid chromatography as described in this section.

Xylose and xylooligosaccharide (xylobiose, xylotriose, xylotetraose, xylopentaose, and xylohexaose) content of liquid hydrolysate after liquid hot water treatment are illustrated in Figure 2-7. Total xylooligosaccharide content was 0.1% at 130°C and 150°C for 10 min because of the very mild condition for decomposition of *E. pellita* which was revealed by the results of monomeric sugar and its derivatives in the liquid hydrolysate.

The amount of total xylooligosaccharide increased from 0.8% to 8.3% at 50 min of reaction time at 170°C, but it decreased slightly (7.3%) as the reaction time extended to 70 min. According to the xylan content of solid residues (Table 2-2), the decomposition of xylan in *E. pellita* began in earnest at 170°C of reaction temperature as the reaction time was prolonged.

Therefore, the maximum content of total xylooligosaccharide (8.3%) in this study was observed after 50 min of reaction time. The conditions of short reaction time (10 min) had poor production of total xylooligosaccharide compared to that of conditions of long reaction time (30 min or 50 min), especially at 170°C. Thus, sufficient reaction time is required for reducing the degree of polymerization (DP) from high molecular weight xylooligosaccharide which is separated from solid residues to low molecular weight xylooligosaccharide. Meanwhile, a decrease in the total xylooligosaccharide content in the condition of 70 min might show that a long reaction time excessively reduces the DP or converts it into another product, although almost xylan was already removed from *E. pellita*.

This trend in changes of the total xylooligosaccharide content depending on reaction time at 170°C could be found at 180°C. As reaction time extended from 30 min to 50 min, total xylooligosaccharide content was decreased from 5.8% to 4.5%. A declining trend in total xylooligosaccharide content continued steadily at 190°C as an increase in reaction time, and total xylooligosaccharide content was 0.8% at 210°C for 10 min. Therefore, the extension of reaction time above 180°C conditions except for that of 10 min had a negative effect on the production of xylooligosaccharide. This is because, furfural, a derivative from xylose and xylooligosaccharide, was produced vigorously above 180°C (Figure 2-5).

The severity factor of the liquid hot water treatment condition in which the total xylooligosaccharide was produced the most was 3.76. In general, the conditions of a severity factor lower than 3.76 were not achieved for sufficient decomposition of xylan in *E. pellita*, while the conditions of a severity factor higher than 3.76 denoted an increase in xylose or furfural in the liquid hydrolysate. However, the total xylooligosaccharide content at 170°C for 70 min (7.3%) was higher than that of 180°C for 30 min (5.8%) even though the

former condition had a higher severity factor (3.91) than that of the latter (3.83). It is assumed that the supply of thermal energy through an extension of reaction time rather than an increase in reaction temperature is effective for xylooligosaccharide production using the liquid hot water treatment.

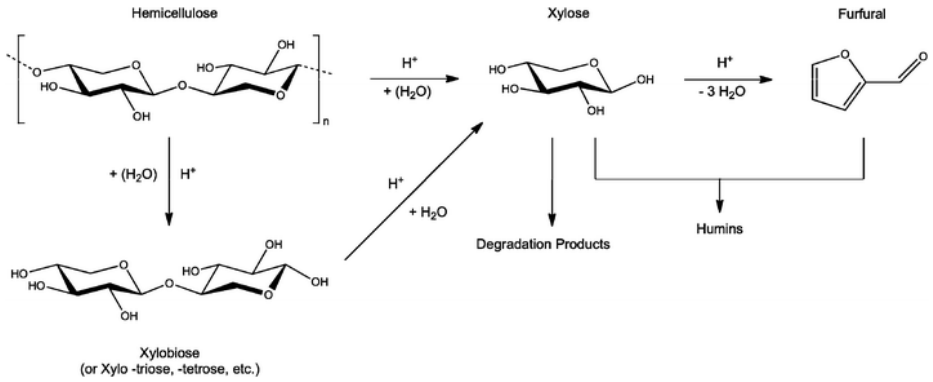



Figure 2-6. Conversion mechanism from xylose to furfural during an acidic catalyzed treatment condition (Enslow & Bell, 2012).

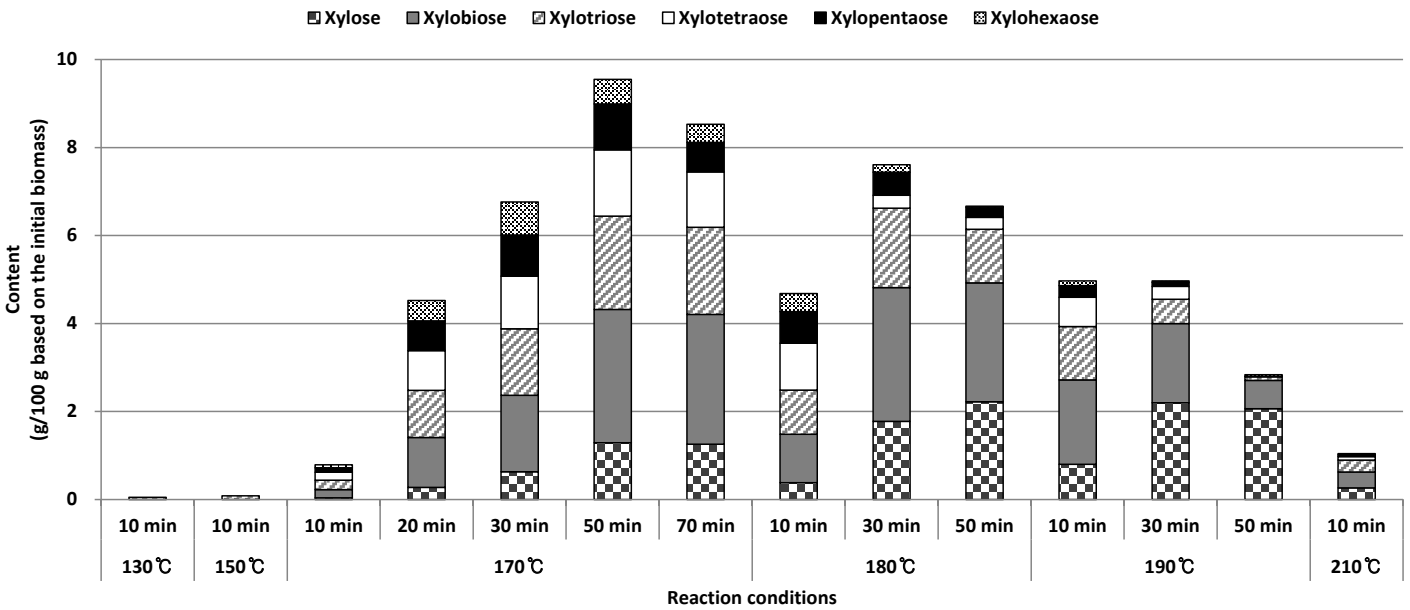



Figure 2-7. Xylose and xylooligosaccharide content (g/100 g initial biomass) of liquid hydrolysate after liquid hot water treatment of twin-extruder treated *Eucalyptus pellita* depending on reaction temperature and time.

The xylose and xylooligosaccharide content in the liquid hydrolysate depending on DP are presented in Figure 2-8. After the liquid hot water treatment, a relatively similar amount of xylose and xylooligosaccharide was observed and no experimental condition dominantly produced a single product of xylose or xylooligosaccharide. However, changes in xylose and xylooligosaccharide content as a difference in DP could be found among the various reactions condition of the liquid hot water treatment.

Xylobiose (DP=2) was the most produced among the xylooligosaccharide including xylose, and the maximum amount of xylobiose (3.0%) was obtained at 180°C for 30 min (Figure 2-8B). The reason for the high content of xylobiose compared to xylose under most of the conditions is the conversion of furfural from the xylose was facilitated on the high reaction temperatures with long reaction times (Garrote et al., 1999). Under the condition of 170°C for 50 min, xylotriose (2.1%, DP=3), xylotetraose (1.5%, DP=4), and xylopentaose (1.0%, DP=5) showed their maximum quantity, respectively (Figure 2-8B and C). Meanwhile, the maximum content of xylohexaose (0.8%, DP=6) was observed at 170°C for 30 min. Considering the maximum quantity of xylose content (2.2%) was at 180°C for 50 min, the reaction condition for the maximum content of xylose or xylooligosaccharides was milder as an increase in their DP.

Furthermore, the maximum content of xylooligosaccharides decreased with an increase in thier DP. Generally, the chain length of hemicellulose is known as barely hundreds units of monomeric sugars (Parajó et al., 2004). Thus, for the high molecular weight xylooligosaccharide it is difficult to maintain the chain length, even if hydrolysis of the liquid hot water treatment occurs under mild conditions.

Xylooligosaccharide over DP 4 cannot be produced during a conventional process for xylooligosaccharide production that uses xylanases.

Therefore, most of the xylooligosaccharide sold on the market is made up of only xylobiose and xylotriose, and their production was confirmed in this study using a hydrothermal treatment, liquid hot water treatment (Figure 2-8B). Furthermore, xylotetraose, xylopentaose, and xylohexaose could be obtained in this study (Figure 2-6C), overcoming the conventional enzymatic process, and the trend in xylooligosaccharide production as DP was investigated. If the beneficial physiological activity of xylooligosaccharide above DP 4 is revealed by other research, the results of this study may contribute high value-added utilization of lignocellulosic biomass. However, a separation and purification process of xylooligosaccharide having a specific DP was not developed.

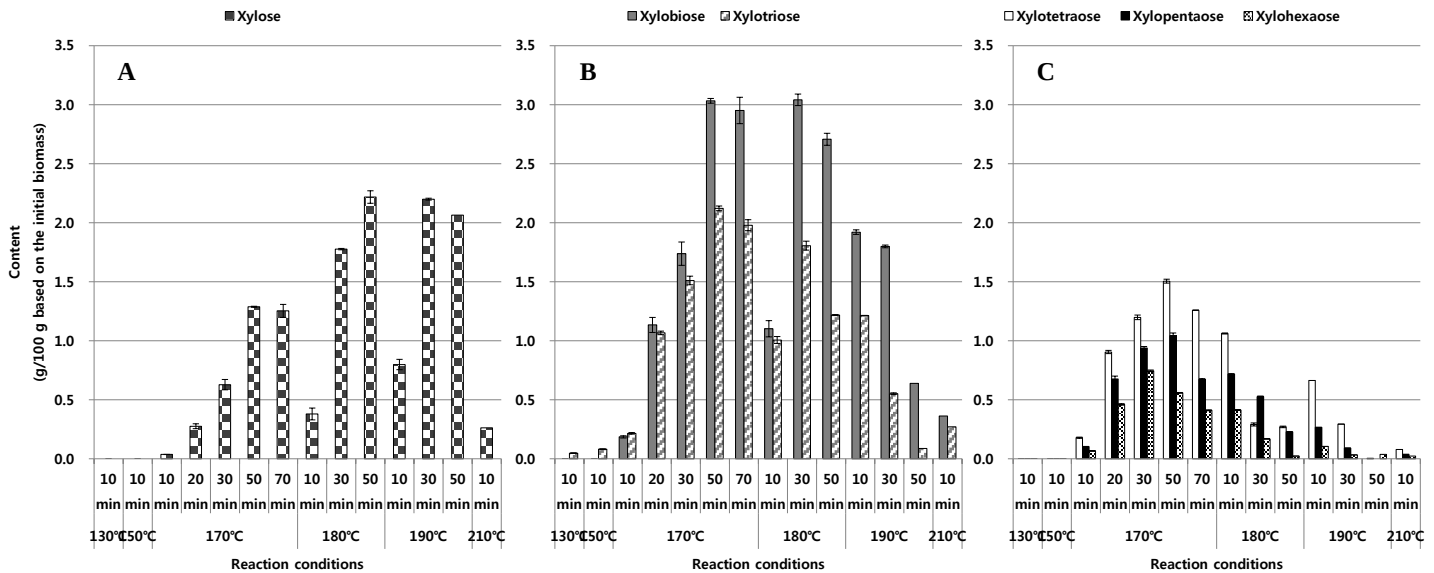



Figure 2-8. Xylose and xylooligosaccharide content (g/100 g initial biomass) of liquid hydrolysate after liquid hot water treatment of twin-extruder treated *Eucalyptus pellita* depending on reaction temperature and time as a degree of polymerization (DP) (A: xylose (DP=1), B: xylobiose and xylotriose (DP=2 and 3), C: xylotetraose, xylopentaose, and xylohexaose (DP=4, 5, and 6)).

The conversion rate of xylooligosaccharide from xylose in raw material is shown in Table 2-4. Generally, the total conversion rate of xylooligosaccharide increased/or decreased evenly for each DP of xylooligosaccharide, and it did not change as the variation in conversion rate of xylooligosaccharide which had a certain DP. The maximum conversion rate of xylooligosaccharide (67.2%) was obtained under the 170°C for 50 min conditions, and the maximum conversion rate of xylooligosaccharide from xylobiose to xylopentaose could be observed under this condition except for xylohexaose. In the case of 170°C conditions, the conversion rate of xylohexaose most sensitively responded to the extension of the reaction time. According to previous study, the higher DP of xylooligosaccharide had a larger rate constant in first-order reaction kinetics to convert the lower DP of xylooligosaccharide during the liquid hot water treatment for xylan decomposition (Akpinar et al., 2009). Consequently, a high DP xylooligosaccharide such as xylohexaose might have difficulty surviving even with a slight extension in reaction time.

Table 2-4. Conversion rate of xylooligosaccharide from xylan in twin-extruder treated *Eucalyptus pellita* after liquid hot water treatment depending on reaction temperature and time

| Conditions | | Conversion rate of xylooligosaccharide (%)[1] | | | | | Total[2] |
|---|---|---|---|---|---|---|---|
| Reaction temp. (℃) | Reaction time (min) | Xylobiose | Xylotriose | Xylo-tetraose | Xylo-pentaose | Xylo-hexaose | |
| 130 | 10 | 0.0 | 0.4 | 0.0 | 0.0 | 0.0 | 0.4 |
| 150 | 10 | 0.0 | 0.7 | 0.0 | 0.0 | 0.0 | 0.7 |
| | 10 | 1.5 | 1.8 | 1.5 | 0.8 | 0.5 | 6.1 |
| | 20 | 9.2 | 8.7 | 7.4 | 5.5 | 3.7 | 34.5 |
| 170 | 30 | 14.1 | 12.3 | 9.7 | 7.6 | 6.1 | 49.9 |
| | 50 | 24.7 | 17.2 | 12.2 | 8.5 | 4.5 | 67.2 |
| | 70 | 24.0 | 16.1 | 10.2 | 5.5 | 3.3 | 59.1 |
| | 10 | 9.0 | 8.2 | 8.6 | 5.8 | 3.4 | 35.0 |
| 180 | 30 | 24.7 | 14.7 | 2.4 | 4.3 | 1.4 | 47.4 |
| | 50 | 22.0 | 9.9 | 2.2 | 1.9 | 0.2 | 36.1 |
| | 10 | 15.6 | 9.9 | 5.4 | 2.2 | 0.8 | 33.9 |
| 190 | 30 | 14.6 | 4.5 | 2.4 | 0.7 | 0.3 | 22.5 |
| | 50 | 5.2 | 0.7 | 0.0 | 0.0 | 0.3 | 6.3 |
| 210 | 10 | 2.9 | 2.2 | 0.6 | 0.3 | 0.2 | 6.3 |

Values are the mean ± standard deviation

[1] %, based on the xylose content in the initial biomass

[2] %, Sum of conversion rate of xylooligosaccharide

According to the aforementioned sections in this chapter, xylan degradation from *E. pellita* during liquid hot water treatment (section 3.1.1), conversion of xylose and furfural in the liquid hydrolysate (section 3.1.2), and xylooligosaccharide production (section 3.2) were investigated on the route of xylan conversion mechanism under an acidic catalyst circumstance. The mass balance of these products (xylose in the solid residue and liquid hydrolysate, xylooligosaccharide, furfural, and the undetected portion) as a result of liquid hot water treatment from xylan is illustrated in Figure 2-9.

The unknown portion which could not be identified by HPLC or Bio-LC was appreciably over the 170°C of reaction temperature, and it might be understood as two cases depending on reaction conditions. First, the content of the undetected portion under conditions of 170°C for 10-30 min appeared from 2.4% to 4.1%, and is speculated as high weight molecular xylooligosaccharide over DP 6. The conditions of 170°C for 10-30 min could result in a partial degradation of xylan in *E. pellita*, but the xylan which was dissolved into the solvent was cleaved sparsely through acid hydrolysis. Indeed, the peaks of xylooligosaccharide had a higher DP than that of xylohexaose were found using a Bio-LC chromatogram at a long retention time (Figure 2-10).

In another case, the unknown portion of conditions that had a higher severity of reaction than that of 170°C for 50 min could be observed, notably ranging from 1.3% to 8.2%. As the reaction severity increased, xylooligosaccharide or xylose was readily converted to a sugar derivative such as furfural that was previously investigated. However, even excluding the furfural content on the mass balance, a significant portion remained as unknown. Formic acid has been known to be derived from furfural as a degradation product through an extreme reaction condition in acidic hydrolysis (Danon et al., 2014). Formic acid content increased obviously at

conditions of 210°C for 10 min (Figure 2-5). However, humin might be considered as a major part of the unknown portion because the maximum amount of formic acid (0.8%) was relatively small. Humin, an inhomogeneous dark brown solid, is typically produced by dehydration of sugars, and it forms larger molecular through the condensation of sugars with furfural and 5-HMF (Hu et al., 2011). Previous studies have reported that a significant amount of humin was converted from aldose, 5-HMF, and furfural in biomass after the acid hydrolysis process (Sumerskii et al., 2010). Humin formation is favored by thermodynamic changes, because it requires a lower activation energy than that of the dehydration of xylose for conversion to furfural (Eifert & Liauw, 2016). Furthermore, humin formation is known to be facilitated under conditions of high reaction temperature or long reaction time during hydrothermal treatment, corresponding to the results of this study (Chen et al., 2015).

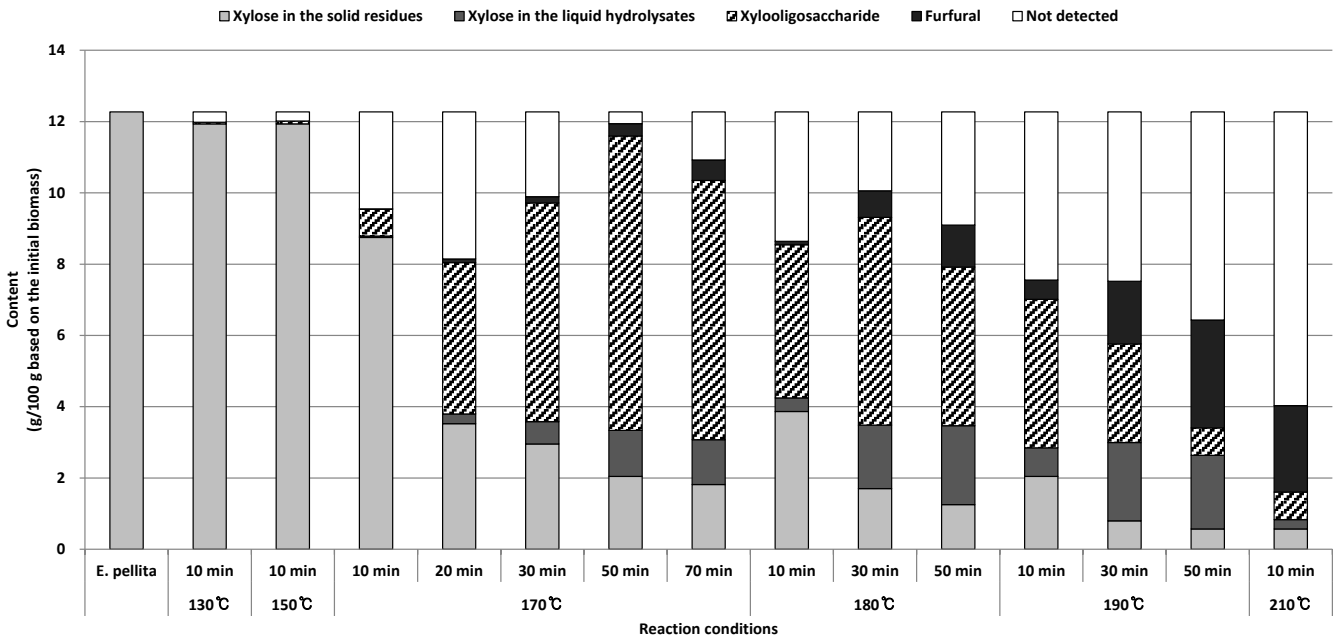



Figure 2-9. Mass balance for xylose in the solid residue and liquid hydrolysate, xylooligosaccharide, furfural, and not detected portion (g/100 g initial biomass) by liquid hot water treatment of twin-extruder treated *Eucalyptus pellita*.

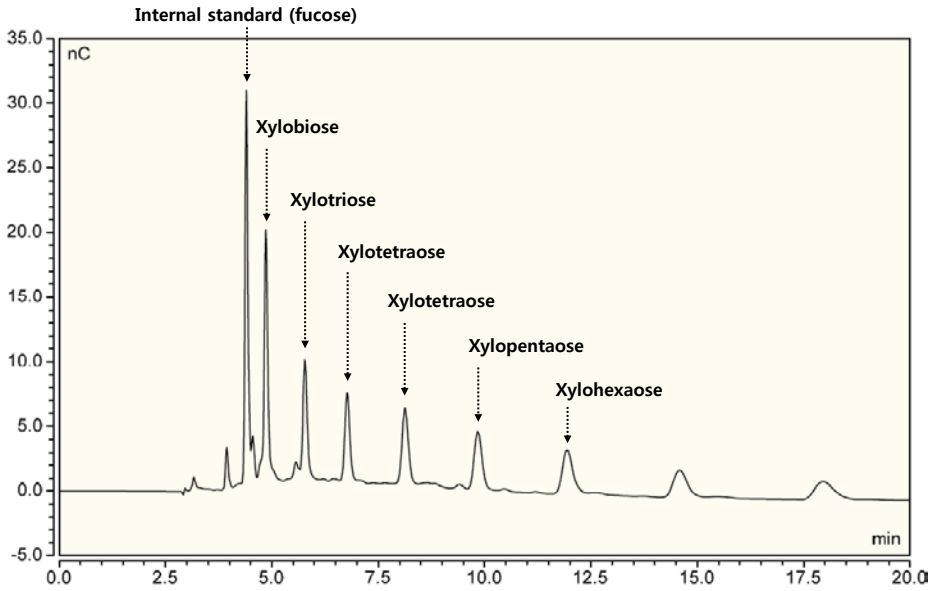



Figure 2-10. Bio-liquid chromatogram of liquid hydrolysate after liquid hot water treatment of twin-extruder treated *Eucalyptus pellita*.

## 3.3. Enzymatic hydrolysis of solid residues

The solid residues recovered by filtration after the liquid hot water treatment were employed in the enzymatic hydrolysis for glucose production. Then, the results of enzymatic hydrolysis were evaluated suing two parameters, enzymatic digestibility and glucose yield (Table 2-5). After the enzymatic hydrolysis using solid residues, enzymatic digestibility and glucose yield were different under the same reaction condition, and glucose yield was slightly higher than that of enzymatic digestibility under most conditions. However, the variation in enzymatic digestibility and glucose yield was similar in response to the change in liquid hot water treatment conditions. As the severity factor increased, the enzymatic digestibility and glucose yield increased gradually until 190°C for 10 min as 21.3% and 28.2%, respectively. However, the enzymatic digestibility and glucose yield decreased slightly at 210°C for 10 min as 18.3% and 25.9%, respectively, even though the glucan content in the solid residue did not decrease significantly compared to that of *E. pellita* (Table 2-2). It is assumed that a pore-size reduction in the cell wall occurred due to extreme dehydration during hydrothermal treatment under severe conditions (Ertas et al., 2014). Additionally, degradation products from hemicellulose such as humin might adhere to the lignin fraction of the liquid-hot-water-treated solid residues, and the complex of humin and lignin reduced cellulase accessibility on the surface of cellulose fibrils (Filiciotto et al., 2017).

The enzymatic digestibility and glucose yield were relatively low compared to that of previous studies adopting liquid hot water treatment (Mosier et al., 2005; Pérez et al., 2008). Thus, *E. pellita* could be considered a high recalcitrant species among lignocellulosic biomass. However, the poor results of enzymatic hydrolysis were expected improve through further processing to solid residue.

Table 2-5. Enzymatic digestibility (%, based on a dry weight of substrate) and glucose yield (%, based on the glucose content in the initial biomass) after enzymatic hydrolysis of twin-extruder treated *Eucalyptus pellita* and solid residues after liquid hot water treatment as a function of reaction temperature and time.

| Conditions | | Enzymatic digestibility (%)[1] | Glucose yield (%)[2] |
|---|---|---|---|
| Reaction temp. (°C) | Reaction time (min) | | |
| *E. pellita*[3] | | 0.0±0.0 | 0.5±0.0 |
| 130 | 10 | 0.0±0.0 | 6.0±0.1 |
| 150 | 10 | 2.4±0.5 | 6.2±0.3 |
| 170 | 10 | 5.4±0.2 | 6.9±0.0 |
| | 20 | 7.6±0.0 | 10.4±0.2 |
| | 30 | 7.1±0.4 | 11.8±0.5 |
| | 50 | 9.8±0.0 | 13.0±0.2 |
| | 70 | 9.6±0.4 | 14.0±0.2 |
| 180 | 10 | 9.4±0.3 | 10.5±0.2 |
| | 30 | 11.2±0.1 | 18.3±0.4 |
| | 50 | 14.8±0.2 | 21.0±0.2 |
| 190 | 10 | 12.0±0.1 | 22.9±0.0 |
| | 30 | 17.6±0.4 | 25.1±0.3 |
| | 50 | 21.3±0.7 | 28.2±0.0 |
| 210 | 10 | 18.3±0.2 | 25.9±2.8 |

Values are the mean ± standard deviation

[1] %, based on a dry weight of substrate (equal to 1 g)

[2] %, based on glucose content in the initial biomass

Hemicellulose removal rate and lignin removal rate as related to enzymatic digestibility and glucose yield are presented in Figure 2-10. The hemicellulose removal rate was estimated based on the amount of total hemicellulose which consists of xylose, galactose, mannose, and arabinose, while the lignin removal rate was determined based on the amount of total lignin which consists of Klason lignin and acid-soluble lignin.

The lignin removal rate generally maintained a constant value under all conditions of liquid hot water treatment, thus there was no obvious relationship with the variation in enzymatic digestibility and glucose yield.

Meanwhile, the hemicellulose removal rate might be considered to have a certain relationship with the results of enzymatic hydrolysis. Enzymatic digestibility and glucose yield increased with an increase in the hemicellulose removal rate, as revealed at 170°C for 20-70 min and 180°C for 10-50 min. Hemicellulose is believed to be an inhibitor serving as a barrier with respect to cellulase accessibility during enzymatic hydrolysis (Mussatto et al., 2008). Furthermore, hemicellulose removal through hydrothermal treatment can ensure room, where hemicellulose was previously, that improves enzyme activity in cell wall structure (Yoshida et al., 2008). In contrast, enzymatic digestibility and glucose yield increased slightly at 150°C for 10 min and 170°C for 10-20 min in spite of a sharp increase in the hemicellulose removal rate. In addition, enzymatic hydrolysis and glucose yield increased under 190°C condition compared to that of 190°C condition even though the both of the hemicellulose removal rates under 180°C and 190°C conditions were similar. During the liquid hot water treatment, an increase in reaction temperature could result in an enlargement of specific surface area as reported in a previous study (Thompson et al., 1992). Therefore, the hemicellulose removal rate cannot be considered as a major factor for enzymatic hydrolysis after liquid hot water treatment.

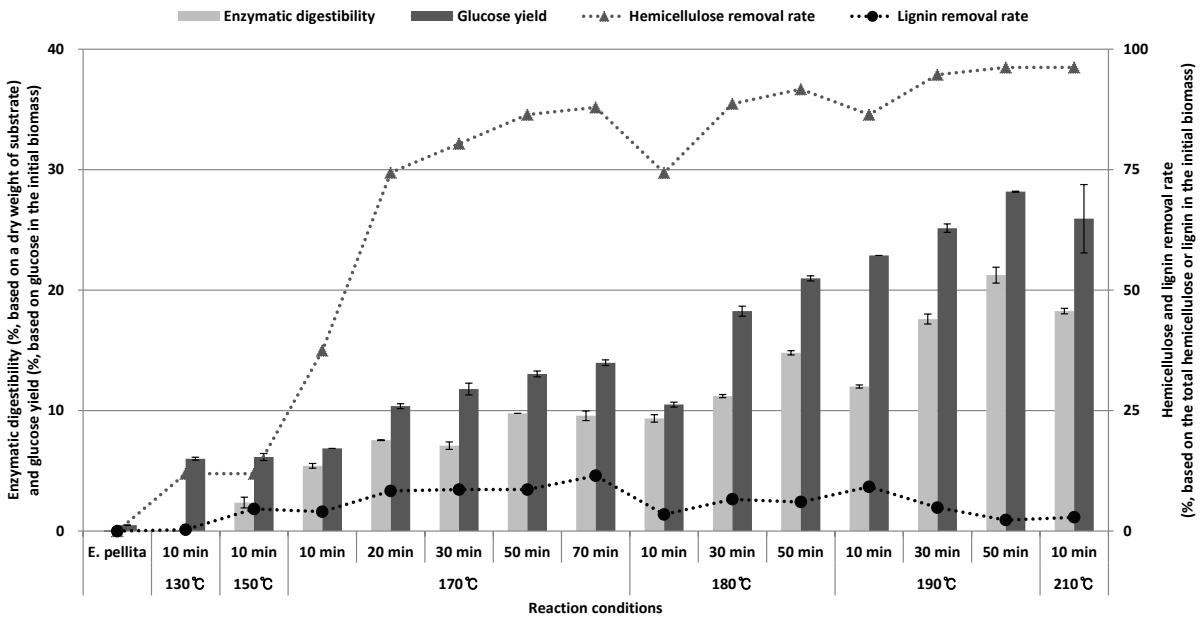



Figure 2-11. Enzymatic digestibility (%, based on a dry weight of substrate) and glucose yield (%, based on the glucose content in the initial biomass) after enzymatic hydrolysis of twin-extruder treated *Eucalyptus pellita* and solid residues after liquid hot water treatment with hemicellulose removal rate and lignin removal rate (%, based on the content in the initial biomass).

## 3.4. Effects of biomass constituents on enzymatic hydrolysis

### 3.4.1. A relationship between hemicellulose removal and glucose production

The relationship between the hemicellulose removal rate and glucose yield was analyzed using a statistical method with regression analysis and is summarized in Table 2-6. Fourteen empirical data after liquid hot water treatment and enzymatic hydrolysis were employed for this regression analysis. According to the results of statistical analysis, less than 0.0001 of p-value was obtained, and it was considered significant. Because, the p-value is less than 0.05 the correlation has a significance relation. Thus, the correlation between the hemicellulose removal rate and glucose yield may form a strong relationship after liquid hot water treatment. As the shown in the results of the ANOVA table, the linear regression formula of hemicellulose removal rate for glucose yield can be determined as follows:

Glucose yield = 0.72693 + 0.20583 × HRR (hemicellulose removal rate)

In addition, an adjusted R-squared value was determined to be 0.6768, indicating that approximately 68% of the results of all the experiments conducted in this study can be denoted using this linear regression formula. In other words, an influence of hemicellulose decomposition was revealed for glucose production even though the glucose yield did not reach more than 30% using the liquid hot water treatment.

The correlation plot of the regression analysis between the hemicellulose removal rate and glucose yield is shown in Figure 2-12. Some of the dots in the plots are placed within the 95% confidence limits, but others deviated. This means that other factors except for hemicellulose removal of lignocellulosic biomass might affect glucose yield.

Table 2-6. Analysis of variance (ANOVA) and parameter estimates for glucose yields corresponded hemicellulose removal rate (HRR) after liquid hot water treatment from *Eucalyptus pellita* (R-Square=0.6999, Adjust R-Square=-0.6768)

| Analysis of Variance | | | | | |
|---|---|---|---|---|---|
| Source | DF[1] | Sum of Squares | Mean Square | F Value | Pr > F |
| Model | 1 | 697.15459 | 697.15459 | 30.32 | 0.0001 |
| Error | 13 | 298.89606 | 22.99200 | - | - |
| Corrected Total | 14 | 996.05065 | - | - | - |
| **Parameter Estimates** | | | | | |
| Variable | DF | Parameter Estimate | Standard Error | t Value | Pr > \|t\| |
| Intercept | 1 | 0.72693 | 2.82437 | 0.26 | 0.8009 |
| HRR[2] | 1 | 0.20583 | 0.03738 | 5.51 | 0.0001 |

[1] DF: degree of freedom
[2] HRR: hemicellulose removal rate

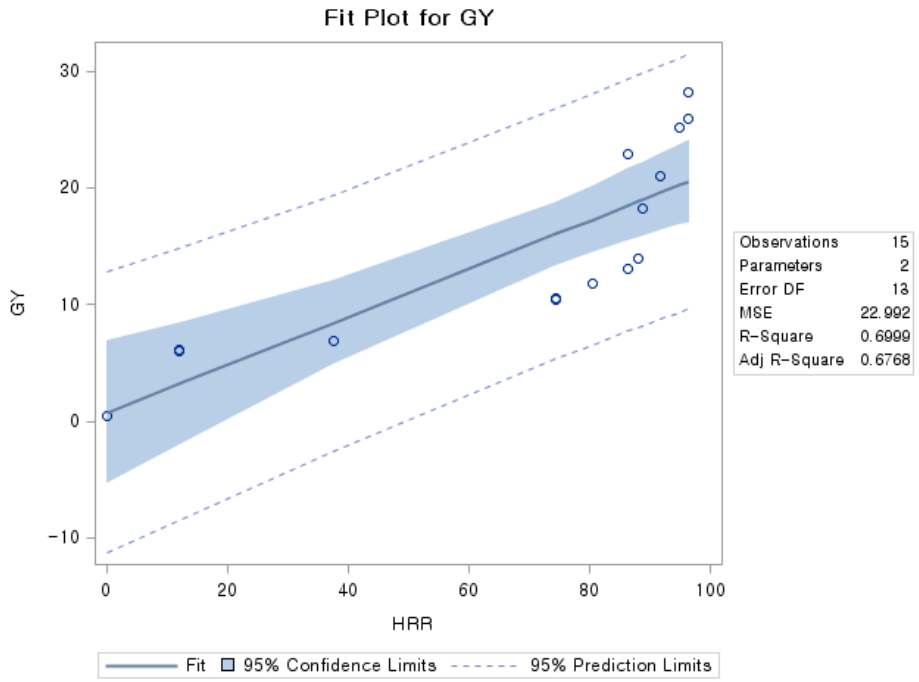



Figure 2-12. Correlation plot for glucose yields (GY) vs. hemicellulose removal rate (HRR) after liquid hot water treatment from *Eucalyptus pellita.*

### 3.4.2. A relationship between lignin removal and glucose production

A correlation analysis between the lignin removal rate and glucose yield was conducted using the empirical results (Table 2-7). For the result, the p-value of the lignin removal rate was 0.4805 and thus could not ensure the significance of the independent variable. Considering the empirical data originated from various reaction conditions, lignin removal rate and glucose yield do not have a significant relationship regardless of treatment conditions.

In addition, the square of the correlation coefficient (R-squared value) for the lignin removal rate model was 0.0390. This R-squared value, approximately 0, means that the correlation model cannot adequately explain the results in this study. However, the lignin removal rate did not increase notably under the liquid hot water treatment. Thus, this empirical data set might be comprehended for evaluating the correlation between lignin removal and glucose yield as there is no significant variance in lignin content as an independent variable.

The results of the correlation analysis between the lignin removal rate and the glucose yield are plotted in Figure 2-13. A trend line, which was determined by regression analysis, with an area of 95% confidence limits, is indicated in the plot. According to the dots of the plot, glucose yield (GY) was dispersed regardless of lignin removal rate. In addition, the range of lignin removal rate was relatively narrow compared to that of hemicellulose removal rate (Figure 2-12). Therefore, an appropriate delignification process from *E. pellita* might be required for investigating the precise effect of lignin content on glucose yield.

Table 2-7. Analysis of variance (ANOVA) and parameter estimates for glucose yields corresponded lignin removal rate (LRR) after liquid hot water treatment from *Eucalyptus pellita* (R-Square=0.0390, Adjust R-Square=-0.0349)

| Analysis of Variance | | | | | |
|---|---|---|---|---|---|
| Source | DF[1] | Sum of Squares | Mean Square | F Value | Pr > F |
| Model | 1 | 38.85174 | 38.85174 | 0.53 | 0.4805 |
| Error | 13 | 957.19891 | 73.63069 | - | - |
| Corrected Total | 14 | 996.05065 | - | - | - |
| **Parameter Estimates** | | | | | |
| Variable | DF | Parameter Estimate | Standard Error | t Value | Pr > \|t\| |
| Intercept | 1 | 12.03953 | 4.28703 | 2.81 | 0.0148 |
| LRR[2] | 1 | 0.49175 | 0.67697 | 0.73 | 0.4805 |

[1] DF: degree of freedom
[2] LRR: lignin removal rate

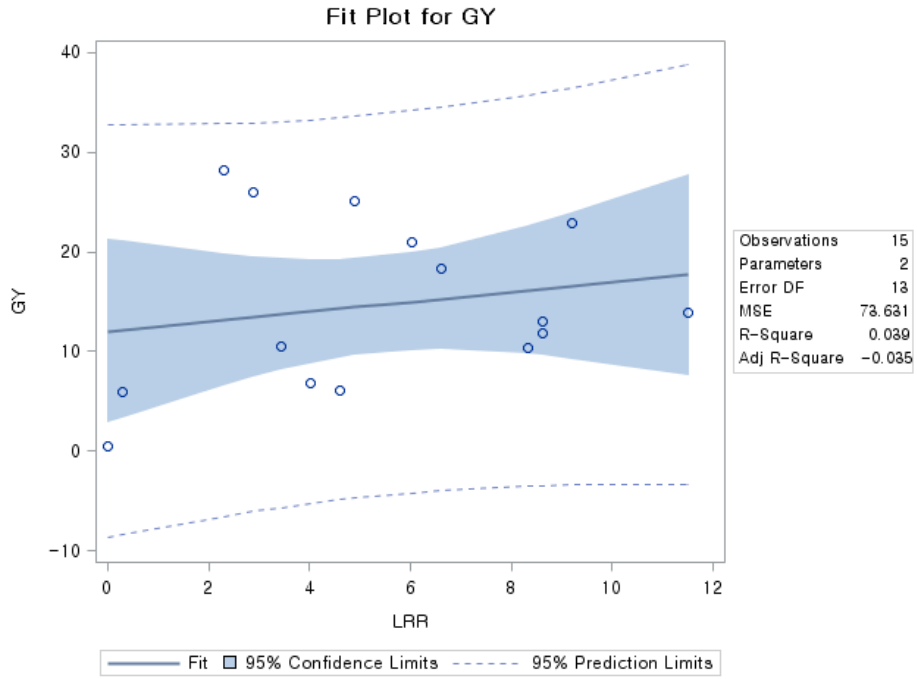



Figure 2-13. Correlation plot for glucose yields (GY) vs. lignin removal rate (LRR) after liquid hot water treatment from *Eucalyptus pellita.*

### 3.4.3. A relationship between double factors and glucose production

The correlation between the glucose yield and double factor (hemicellulose and lignin removal rate), which varied at the same time, was evaluated using the REG procedure in SAS (Table 2-8). According to the p-values in the parameter estimates, both the hemicellulose (less than 0.0001) and lignin removal rate (0.0264) had an impactful significance on the change in glucose yield under the liquid hot water treatment. The results of the correlation analysis by double factors were in contrast to previous results which analyzed lignin removal rate (single factor) and glucose yield, The p-value was 0.4805. This may be understood as the influence of hemicellulose removal was dominantly determined for improving glucose yield, while the lignin removal rate remained a constant value at same time. Using the results of correlation analysis for hemicellulose and lignin removal rate with glucose yield, a regression model was deducted as follows:

Glucose yield = 2.35853 + 0.25911 × HRR – 0.96839 × LRR (lignin removal rate)

The adjusted R-squared value of this model was 0.7717, a slightly higher value than that of the single factor hemicellulose removal rate (0.6768). It is thought that the lignin removal rate as a single recalcitrant factor for enzymatic hydrolysis was insufficient for improving glucose production during the liquid hot water treatment, but the lignin removal rate as a double recalcitrant factors with hemicellulose removal rate can concurrently contribute to glucose yield.

Table 2-8. Analysis of variance (ANOVA) and parameter estimates for glucose yields corresponded hemicellulose removal rate (HRR) and lignin removal rate (LRR) after liquid hot water treatment from *Eucalyptus pellita* (R-Square=0.8043, Adjust R-Square=0.7717)

Analysis of Variance

| Source | DF[1] | Sum of Squares | Mean Square | F Value | Pr > F |
|---|---|---|---|---|---|
| Model | 2 | 801.10919 | 400.55460 | 24.66 | <.0001 |
| Error | 12 | 194.94146 | 16.24512 | - | - |
| Corrected Total | 14 | 996.05065 | - | - | - |

Parameter Estimates

| Variable | DF | Parameter Estimate | Standard Error | t Value | Pr > \|t\| |
|---|---|---|---|---|---|
| Intercept | 1 | 2.35853 | 2.46014 | 0.96 | 0.3566 |
| HRR[2] | 1 | 0.25911 | 0.03783 | 6.85 | <.0001 |
| LRR[3] | 1 | -0.96839 | 0.38282 | -2.53 | 0.0264 |

[1] DF: degree of freedom
[2] HRR: hemicellulose removal rate
[3] LRR: lignin removal rate

# 4. Conclusions

In this chapter, a liquid hot water treatment was conducted using twin-extruder treated *Eucalyptus pellita* for investigating the degradation trend of chemical components and conversion mechanisms of xylooligosaccharide. Enzymatic hydrolysis was performed using solid residues after the liquid hot water treatment for glucose production.

After the liquid hot water treatment, xylan content in the solid residues decreased reduced appreciably with an increase in reaction temperature and time. The xylan content was 10.5%, similar to the xylan content of *E. pellita* (10.8%) at 130°C and 150°C for 10 min conditions, while the xylan content in the solid residue decreased dramatically to 0.5% at 210°C for 10 min. However, glucan and total lignin in the solid residues had no significant decomposition into liquid hydrolysates under all conditions of liquid hot water treatment, and their content was generally maintained with similar results of *E. pellita*, 50.9% and 34.8%, respectively.

Xylose, which showed the greatest decrease in solid residue during the liquid hot water treatment, was significantly observed in the liquid hydrolysate that separated from the solid residues, and its maximum content was 2.2% under 180°C for 50 min and 190°C for 30 min. Meanwhile, a trace amount (< 1.0%) of sugar derivatives was detected in the liquid hydrolysates under all conditions of liquid hot water treatment except for acetic acid.

The dominant constituent in the liquid hydrolysate was xylooligosaccharide, which is considered as a partially cleaved molecule by acidic hydrolysis from xylan. The maximum content of total xylooligosaccharide (8.3%) was obtained at 170°C for 50 min. The maximum content of each xylooligosaccharide was found under slightly different

conditions; 3.0% of xylobiose (DP=2) at 180°C for 30 min, 2.1% of xylotriose (DP=3) at 170°C for 50 min, 1.5% of xylotetraose (DP=4) at 170°C for 50 min, 1.0% of xylopentaose (DP=5) at 170°C for 50 min, and 0.8% of xylohexaose (DP=6) at 170°C for 30 min. Thus, it was revealed that the larger DP of xylooligosaccharide was produced more under the mild conditions after liquid hot water treatment.

In terms of mass balance, a mismatch among the xylose, xylooligosaccharide, and furfural content in liquid hydrolysate and the xylan content in solid residue was found, upward of 8.2% at 210°C for 10 min. The unknown portion might be considered as humin, a condensed material combining sugars and sugar derivatives.

Meanwhile, the maximum value of enzymatic digestibility and glucose yield was observed as 21.3% and 28.2%, respectively, after enzymatic hydrolysis using solid residues after liquid hot water treatment. The liquid hot water treatment might be considered as an inadequate strategy for glucose production from *E. pellita*, even the xylooligosaccharide was produced suitably.

Correlation analysis between the changes in biomass constituents (hemicellulose and lignin) and the glucose yield was performed through regression modeling using SAS for statistical analysis. The empirical data set was supplied by the experimental results of liquid hot water treatment and enzymatic hydrolysis in this chapter. According to the results of single factor analysis, the hemicellulose removal rate was denoted a significant parameter for glucose yield compared to that of lignin removal through the liquid hot water treatment. Adjusted R-squared value was observed as 0.6768 through a linear regression model between the hemicellulose removal rate and glucose yield. However, a considerable effect of lignin removal rate on glucose yield could not be observed. Its adjusted R-squared value was 0.0390. The

hemicellulose removal rate on glucose yield had a significant influence in the double factor correlation analysis (R-squared value=0.7717), which was slightly higher than that of the single factor (hemicellulose removal rate) analysis. However, the linear regression model may insufficiently explain the improvement of glucose yield because the maximum glucose yield cannot exceed approximately 40% through the model.

Consequently, additional treatments that control recalcitrance factors such as lignin and cellulose crystallinity of lignocellulosic biomass may be required to improve glucose production.

# *Chapter 3*

## Selective lignin decomposition with correlation analysis between biomass constituents and glucose yield

# 1. Introduction

According to Chapter 2, poor glucose production by enzymatic hydrolysis with liquid hot water treatment was observed using *Eucalyptus pellita*. However, most hardwood was thoroughly degraded for suitable enzymatic hydrolysis through liquid hot water treatment in a previous study (Lehto et al., 2017). Thus, *E. pellita* is considered as highly recalcitrant biomass, and the liquid hot water treatment may not be sufficient for weakening the complexity of its cell wall structure and removing the recalcitrant factors, which reduce the glucose production (Yu et al., 2013).

Lignin in the lignocellulosic biomass has been recognized as a critical recalcitrant factor in several studies, which has been applied to various delignification methods for improving glucose production (Yu et al., 2011). According to previous studies, lignin is known as three mechanisms to inhibit glucose production from cellulose: i) irreversible adsorption between cellulose and the lignin fraction, ii) physical hindrance of cellulase activity on the cellulose structure, and iii) reducing enzymatic activity by small molecular weight products including lignin compounds (Saini et al., 2016).

Generally, a covalent bond is formed between lignin and hemicellulose in the cell wall of the biomass (Chabannes et al., 2001). In addition, lignin removal approaches commonly accompany hemicellulose decomposition due to low resistance against chemical reactions (Palamae et al., 2014). Thus, selective lignin decomposition is required to investigate the effect of the lignin content in a biomass for enzymatic hydrolysis. Sodium chlorite treatment (acid-chlorite delignification) was developed for determining the amount of holocellulose in lignocellulosic biomass (Wise, 1946). Therefore, a precise evaluation of the influence of lignin content can be expected because the method is designed based on very low damage to the carbohydrate fraction

in the biomass (Jang et al., 2017b).

In this study, the sodium chlorite treatment was conducted using *E. pellita* for investigating lignin decomposition. The glucose production was evaluated depending on the lignin content in the solid residues. Furthermore, the effect of the hemicellulose and lignin removal rate was investigated on the glucose production after sodium chlorite treatment with the liquid hot water treatment under various conditions.

# 2. Materials and methods

## 2.1. Materials

The raw material of this study, twin-extruder treated *Eucalyptus pellita*, was described circumstantially in section 2.1 of Chapter 2, and the chemical composition of *E. pellita* was listed in Table 2-2 and Table 2-3.

Meanwhile, the solid residues which were collected after liquid hot water treatment were employed lignin decomposition process for investigating an impact of hemicellulose and lignin in terms of enzymatic hydrolysis. And the chemical composition of solid residues was presented in Table 3-4.

## 2.2. Sodium chlorite treatment

For lignin decomposition of biomass, sodium chlorite ($NaClO_2$) treatment was conducted using twin-extruder treated *E. pellita* and the solid residues which were treated by liquid hot water process. 15 g of *E. pellita* or the solid residues were loaded into a 1000 mL-volume of Erlenmeyer flask, and it mixed with 150 mL of distilled water. The sodium chlorite treatment was reacted in water bath (LEB-106D, DAIHAN LABTECH Co., LTD, Namyangju, Republic of Korea), and reaction temperature and reaction time were set as 80°C and 60 min, respectively. During the treatment, the samples in Erlenmeyer flask were shaken every 15 min. When *E. pellita* was a feedstock, the sodium chlorite was used as 1 g, 2 g, 3 g, and 4 g with 0.2 mL, 0.4 mL, 0.6 mL, and 0.8 mL of acetic acid, respectively. And the reagents were inputted for 1, 2, and 3 times before each reaction time (60 min). Thus,

as the number of reagents (sodium chlorite and acetic acid) dose was increased once, the reaction time was also extended to add 60 min.

When the liquid hot water treated solid residues were used as feedstock, the sodium chlorite was added as 1.5 g, 2.5 g, 4 g, and 4 g (2 times) with 0.3 mL, 0.5 mL, 0.8 mL, and 0.8 mL (2 times) of acetic acid, respectively. The conditions of sodium chlorite treatment using liquid hot water treated solid residues were designed and modified to reflect the results using *E. pellita* for controlling the lignin content appropriately. After the reaction was finished, the flasks were cooled to room temperature, and then the samples were washed by 300 mL of distilled water with filtration by filter paper (No. 52, Hyundai micro CO., Seoul, Republic of Korea). For analysis of sugar and its derivatives contents, the liquid hydrolysate was filtered using a 0.45 μm membrane filter (Advantec, Tokyo, Japan). The solid residue was washed with distilled water, and kept at 4°C for liquid hot water treatment and enzymatic hydrolysis. The sodium chlorite and acetic acid that used in this study were purchased from Samchun pure chemical (Pyeongtaek, Republic of Korea) and Sigma-Aldrich Korea CO. (Yongin, Republic of Korea), respectively.

## 2.3. Analysis of solid residues

### 2.3.1. Water-insoluble solid recovery rate

The water-insoluble solid (WIS) recovery rate of solid residue after sodium chlorite treatment was determined as described in section 2.3.1 of Chapter 2.

### 2.3.2. Determination of Klason lignin and acid-soluble lignin

The Klason lignin and acid-soluble lignin content of solid residue after sodium chlorite treatment were determined as described in section 2.3.4 of Chapter 2.

### 2.3.3. Determination of structural sugar

The structural sugar (glucose, xylose, mannose, galactose, and arabinose) of solid residue after sodium chlorite treatment were determined as described in section 2.3.5 of Chapter 2.

## 2.4. Enzymatic hydrolysis

The enzymatic digestibility and glucose yield of solid residue after sodium chlorite treatment were determined as described in section 2.5 of Chapter 2.

## 2.5. Correlation analysis

The material of correlation analysis was empirical data sets which were obtained from the results of the sodium chlorite treatment and enzymatic hydrolysis in this chapter. In other words, the experimental data which consisted of hemicellulose and lignin removal rate was corresponded with glucose yield. And, the linear regression analysis between single or double factors and the glucose yield was corresponded as described in section 2.6 of Chapter 3.

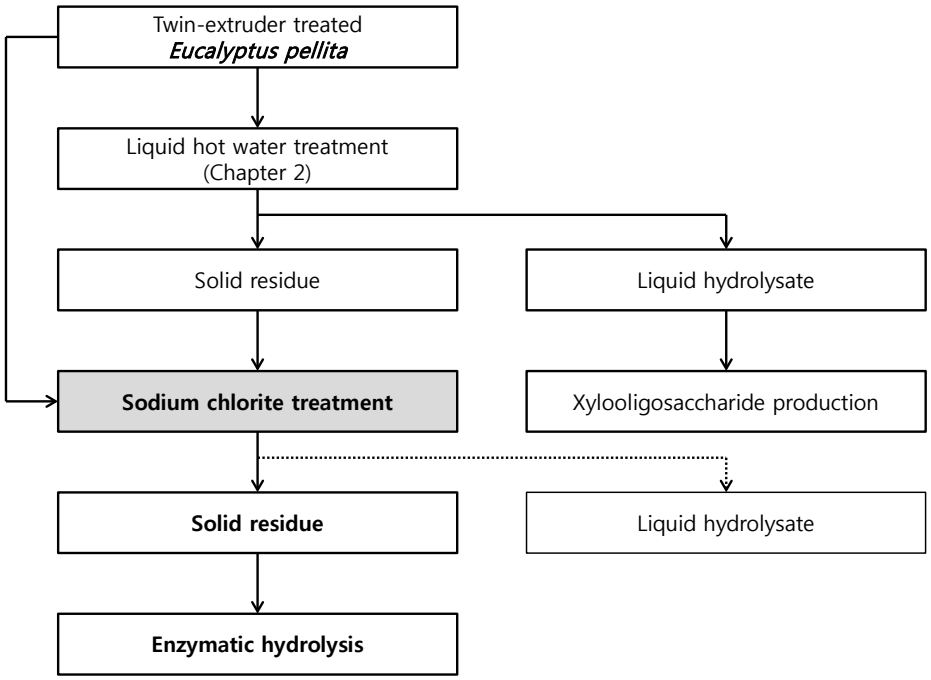



Figure 3-1. Schematic experimental flowchart of lignin decomposition by sodium chlorite treatment of *Eucalyptus pellita* and liquid hot water treated *Eucalyptus pellita*.

# 3. Results and discussion

## 3.1. Lignin decomposition of *Eucalyptus pellita* by sodium chlorite treatment

### 3.1.1. Physicochemical characteristics of solid residues

In this study, the sodium chlorite dose designed for achieving differential lignin decomposition in the solid residues during sodium chlorite treatment was used to assess the independent influence of lignin content for enzymatic hydrolysis. The dose of acetic acid was calculated based on the sodium chlorite dose according to a previous study (Wise, 1946).

The WIS recovery rate of solid residues after sodium chlorite treatment as a function of reagent dose is presented in Figure 3-2. As the sodium chlorite and acetic acid dose increased from 1 g and 0.2 mL to 4 g and 0.8 mL, the WIS recovery rate declined steadily from 98.8% to 89.0% for 60 min of reaction time. This trend in WIS recovery rate as with an increasing reagent dose could also be observed for the 120 min and 180 min conditions, which indicates that a significant degradation of the chemical component in cell wall structure occurs through the sodium chlorite treatment.

The 95.3% WIS recovery rate under the 1 g of sodium chlorite and 0.2 mL of acetic acid condition was decreased dramatically to 83.8% under 2 g of sodium chlorite and 0.4 mL of acetic acid condition for 120 min of reaction time. However, the WIS recovery rate of solid residues was maintained from 3 to 4 g of sodium chlorite and from 0.6 to 0.8 mL acetic acid conditions for 120 and 180 min of reaction time. Therefore, a specific component, which is assumed to be lignin in *E. pellita*, was well decomposed by sodium chlorite

and acetic acid, but a critical point of reagent dose for lignin decomposition was observed under the same dose conditions.

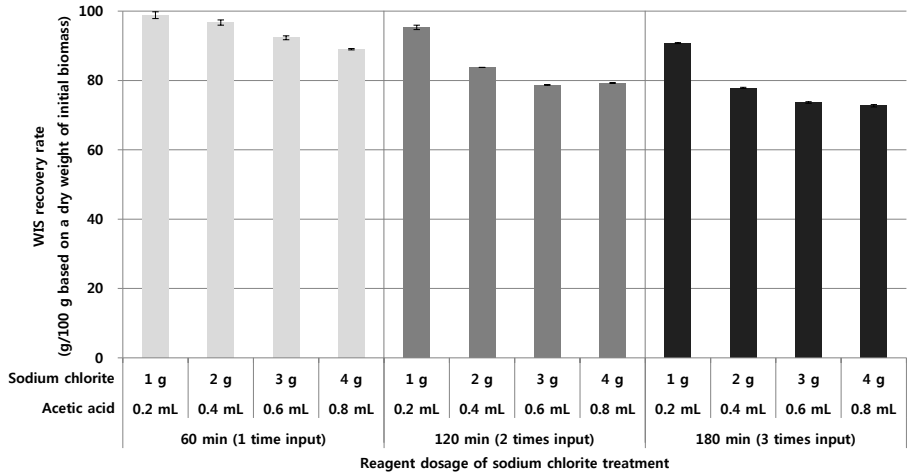



Figure 3-2. Water insoluble solid (WIS) recovery rate (%, based on a dry weight of biomass) of solid residues after sodium chlorite treatment of *Eucalyptus pellita* depending on reagent (sodium chlorite and acetic acid) dose and reaction time.

The structural sugar content of *E. pellita* and the solid residues after sodium chlorite treatment are summarized in Table 3-1. After sodium chlorite treatment, the amount of glucan in the solid residues had no significant change in spite of the high dose of reagents (4 g of sodium chlorite and 0.8 mL of acetic acid) employed for the 180 min condition. Meanwhile, galactan, mannan, and arabinan contents were declined with an increase in the reagent dose, even mannan and arabinan were eliminated in the solid residues for the 180 min condition.

Xylose, which is a dominantly composed hemicellulose chain, was significantly degraded through the liquid hot water treatment that did not use any acid or alkali catalyst in Chapter 2. Moreover, acetic acid originating from substituted acetyl groups in hemicellulose was considered to be a catalyst for facilitating the acid hydrolysis. However, the hemicellulose fraction did not receive any damage during sodium chlorite treatment even though acetic acid was added for an appropriate reaction due to prohibition of oxidative degradation under high pH conditions (Lewin & Epstein, 1962). It is speculated that the reaction temperature (80°C) was insufficient for hemicellulose decomposition despite of the long reaction time (180 min) with reagents. Meanwhile, a previous study reported that the chain length of cellulose was reduced through acid-chlorite delignification (sodium chlorite treatment), but no significant decomposition of cellulose in the solid residues was observed in this study (Millett et al., 1954). Consequently, the structural sugar were mostly preserved through oxidative degradation and acid hydrolysis caused by sodium chlorite and acetic acid during the sodium chlorite treatment.

Table 3-1. Structural sugar content of the solid residues after sodium chlorite treatment from twin-extruder treated *Eucalyptus pellita* as a function of sodium chlorite and acetic acid dose, and reaction time

| Conditions | | | Structural sugar (%)[1] | | | | | Total[2] |
|---|---|---|---|---|---|---|---|---|
| Time (min)[3] | Sodium chlorite (g) | Acetic acid (mL) | Glucan | Xylan | Galactan | Mannan | Arabinan | |
| *E. pellita* | | | 50.9±1.5 | 10.8±0.4 | 1.6±0.1 | 0.6±0.0 | 0.3±0.0 | 64.2 |
| 60 | 1 | 0.2 | 49.1±1.7 (49.6)[4] | 10.1±0.2 (10.2) | 1.4±0.1 (1.4) | 0.4±0.1 (0.7) | 0.2±0.0 (0.4) | 61.2 |
| | 2 | 0.4 | 49.7±2.2 (51.3) | 10.8±0.4 (11.1) | 1.2±0.3 (1.3) | 0.5±0.0 (1.0) | 0.2±0.0 (0.5) | 62.3 |
| | 3 | 0.6 | 49.0±1.5 (53.1) | 10.6±0.6 (11.5) | 1.0±0.1 (1.1) | 0.4±0.1 (1.0) | 0.3±0.0 (0.6) | 61.4 |
| | 4 | 0.8 | 49.4±0.0 (55.4) | 10.7±0.2 (12.1) | 1.4±0.0 (1.5) | 0.5±0.0 (1.1) | 0.2±0.0 (0.6) | 62.2 |
| 120 | 1×2 | 0.2×2 | 50.3±1.0 (52.8) | 11.0±0.4 (11.5) | 1.0±0.1 (1.0) | 0.4±0.1 (0.4) | 0.0±0.0 (0.0) | 62.6 |
| | 2×2 | 0.4×2 | 47.7±2.0 (56.9) | 10.0±0.1 (11.9) | 1.1±0.3 (1.3) | 0.3±0.1 (0.4) | 0.0±0.0 (0.0) | 58.9 |
| | 3×2 | 0.6×2 | 47.7±0.1 (60.6) | 9.5±0.2 (12.1) | 1.4±0.2 (1.8) | 0.2±0.0 (0.4) | 0.0±0.0 (0.0) | 59.3 |
| | 4×2 | 0.8×2 | 49.1±0.4 (61.9) | 9.5±0.1 (12.2) | 1.0±0.0 (1.4) | 0.4±0.1 (0.6) | 0.0±0.0 (0.0) | 59.6 |
| 180 | 1×3 | 0.2×3 | 49.2±0.9 (54.2) | 10.6±0.5 (11.6) | 0.8±0.0 (0.9) | 0.0±0.0 (0.0) | 0.0±0.0 (0.0) | 60.6 |
| | 2×3 | 0.4×3 | 49.4±3.5 (63.5) | 10.7±0.8 (13.8) | 0.8±0.2 (1.0) | 0.0±0.0 (0.0) | 0.0±0.0 (0.0) | 60.9 |
| | 3×3 | 0.6×3 | 49.1±0.9 (67.7) | 10.8±0.3 (14.7) | 0.9±0.0 (1.2) | 0.0±0.0 (0.0) | 0.0±0.0 (0.0) | 60.7 |
| | 4×3 | 0.8×3 | 49.2±1.0 (67.6) | 10.7±0.2 (14.7) | 0.9±0.0 (1.2) | 0.0±0.0 (0.0) | 0.0±0.0 (0.0) | 60.8 |

Values are the mean ± standard deviation

[1] %, based on a dry weight of initial biomass

[2] Sum of structural sugar content

[3] Reaction time

[4] %, based on a dry weight of the solid residue

The amount of lignin (Klason lignin and acid-soluble lignin) of *E. pellita* and the solid residues after sodium chlorite treatment are shown in Table 3-2. Under the 1 g of sodium chlorite and 0.2 mL acetic acid condition, 33.3% of the total lignin content was observed, and the amount of total lignin decreased steadily to 19.2% under the 4 g of sodium chlorite and 0.8 mL acetic acid condition for 60 min of reaction time. This declining trend of total lignin content as an increase of reagent dose could also be found for 120 and 180 min of reaction time. However, a sharp decrease of total lignin content, from 27.5% to 17.5% for the 120 min condition and from 22.0% to 11.8% for the 180 min condition, was observed when the sodium chlorite and acetic acid dose was increased from 1 to 2 g and 0.2 to 0.4 mL, respectively. The WIS recovery rate after sodium chlorite treatment also significantly decreased in the given conditions. Thus, the change of WIS recovery rate seems to deeply involve the total lignin content, not structural sugar content that was maintained after sodium chlorite treatment.

Sodium chlorite typically dissociates into a chlorine anion ($ClO_2^-$) and chloride anion ($Cl^-$) that are highly reactive in aqueous circumstances in the presence of acetic acid (Abdel-Halim, 2014). The two molecules that come from sodium chlorite have been known as primarily attack to lignin macromolecules until smaller phenolic compounds (Rabetafika et al., 2014). Moreover, the phenolic compounds typically convert into some kind of intermediates such as quinone, muconic acid ester, and other reactive lignin through aromatic ring opening with oxidative degradation (Figure 3-3) (Tarvo et al., 2010). Meanwhile, the reaction products commonly have permanent whiteness by washing process (Abdel-Halim, 2014). Furthermore, the sodium chlorite treatment provides selective decomposition for lignin with a low degree of carbohydrate degradation compared with alkaline peroxide which

excessively degrades cellulose by peroxide radicals (Hubbell & Ragauskas, 2010).

The amount of Klason lignin in the solid residues changed as a similar variation with that of total lignin; i.e., a sharp decrease in the Klason lignin content under the 2 g of sodium chlorite and 0.4 mL of acetic acid condition for 120 and 180 min condition corresponded to total lignin content. This steady reduction of Klason lignin content could be comprehended by the acidified chlorite reaction that was mentioned above.

Meanwhile, the amount of acid-soluble lignin showed two different variations compared with that of the Klason lignin. First, the acid-soluble lignin content in the solid residues was higher than that of *E. pellita* under all conditions of sodium chlorite treatment unlike the Klason lignin. It has been speculated that the lignin structure in *E. pellita* that does not readily dissolve in sulfuric acid during Klason lignin measurement is converted into a suitable structure for dissolving in acidic circumstances through sodium chlorite treatment. Finally, the amount of acid-soluble lignin increased from 4.3% to 9.6% in response to increase in the sodium chlorite and acetic acid dose for 60 min of reaction time. However, the acid-soluble lignin content had a constant value from 7.2% to 8.1% for the 120 min condition, and it decreased slightly from 8.3% to 6.6% with an increase in the reagents dose for the 180 min condition. A similar variation of acid-soluble lignin could be found in a previous study using black spruce that presented an increase of acid-soluble lignin content until 50% of delignification, and then the content decreased until 92.5% of delignification after sodium chlorite treatment (Ahlgren & Goring, 1971). The reason for this result is unclear, but the lignin structure in the solid residue was converted into one with a higher affinity for acid or aqueous solvent through sodium chlorite treatment. Meanwhile, in the case of 3 times input condition (180 min of reaction time), the lignin macromolecular

undergoes significant damage by a high dose of reagents; then, it might be converted into small molecular weight degradation products that have less absorbance at 205 nm by UV-vis spectroscopy.

Table 3-2. Lignin content of the solid residues after sodium chlorite treatment from twin-extruder treated *Eucalyptus pellita* as a function of sodium chlorite and acetic acid dose, and reaction time

| Conditions | | | Lignin (%)[1] | | Total[2] |
|---|---|---|---|---|---|
| Time (min)[3] | Sodium chlorite (g) | Acetic acid (mL) | Klason lignin | Acid-soluble lignin | |
| *E. pellita* | | | 32.5±1.0 | 2.3±0.3 | 34.8 |
| 60 | 1 | 0.2 | 29.0±1.1 (29.3)[4] | 4.3±0.3 (4.4) | 33.3 |
| | 2 | 0.4 | 21.7±1.3 (22.4) | 6.7±0.2 (6.9) | 28.4 |
| | 3 | 0.6 | 14.8±0.9 (16.0) | 8.4±0.1 (9.0) | 23.1 |
| | 4 | 0.8 | 9.6±0.2 (10.8) | 9.6±0.1 (10.8) | 19.2 |
| 120 | 1×2 | 0.2×2 | 20.3±0.0 (21.3) | 7.2±0.2 (7.6) | 27.5 |
| | 2×2 | 0.4×2 | 9.3±0.1 (11.1) | 8.1±0.2 (9.7) | 17.5 |
| | 3×2 | 0.6×2 | 3.9±0.1 (4.9) | 7.8±0.1 (9.9) | 11.7 |
| | 4×2 | 0.8×2 | 2.6±0.2 (3.3) | 7.4±0.0 (9.4) | 10.1 |
| 180 | 1×3 | 0.2×3 | 14.3±0.8 (15.7) | 7.7±0.1 (8.5) | 22.0 |
| | 2×3 | 0.4×3 | 3.5±0.1 (4.5) | 8.3±0.6 (10.7) | 11.8 |
| | 3×3 | 0.6×3 | 2.2±0.5 (3.0) | 7.3±0.4 (10.0) | 9.6 |
| | 4×3 | 0.8×3 | 2.4±0.2 (3.3) | 6.6±0.1 (9.0) | 9.0 |

Values are the mean ± standard deviation

[1] %, based on a dry weight of initial biomass

[2] Sum of Klason lignin and acid-soluble lignin content

[3] Reaction time

[4] %, based on a dry weight of the solid residue

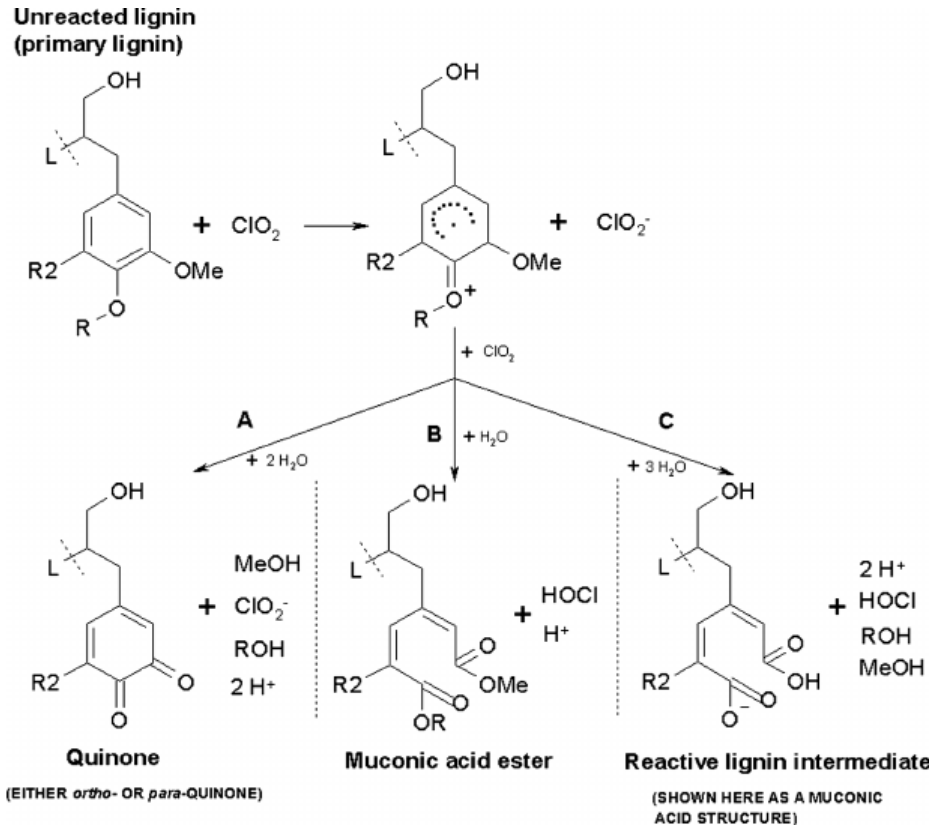



Figure 3-3. The oxidation of primary lignin by chlorine dioxide. (R2=H for guaiacyl lignin, R2=OMe for syringyl lignin; R=H for phenolic lignin, R=Lignin for non-phenolic lignin) (Tarvo et al., 2010).

### 3.1.2. Enzymatic hydrolysis of solid residues

The solid residues after sodium chlorite treatment were washed for reagent removal, and then subjected to the enzymatic hydrolysis. The enzymatic digestibility and glucose yield were increased obviously from 6.3% to 53.6% and from 11.5% to 51.3%, respectively, as there was an increase of sodium chlorite and acetic acid dose for 60 min of reaction time (Table 3-3). This trend of the enzymatic digestibility and glucose yield continued for 120 and 180 min conditions, but the maximum values were much higher than that of the 60 min condition. The maximum enzymatic digestibility was 93.9% (2 times input) and 94.7% (3 times input) under 4 g of sodium chlorite and 0.8 mL of acetic acid, while the maximum glucose yield was 83.9% (2 times input, 120 min reaction time) and 81.7% (3 times input, 180 min reaction time) under the given condition. According to the results in Chapter 2, the maximum enzymatic digestibility and glucose yield were observed to be 21.3% and 28.8%, respectively, after liquid hot water treatment and enzymatic hydrolysis. Therefore, the sodium chlorite treatment turned out to be much more effective than liquid hot water treatment for glucose production from *E. pellita* under all experimental conditions in this study.

The hemicellulose and lignin removal rate corresponded with the enzymatic digestibility and glucose yield, which are illustrated in Figure 3-4. The hemicellulose removal rate ranged from 3.1% to 15.6% under all conditions of sodium chlorite treatment and might be considered as a relatively good variable control. Meanwhile, the lignin removal rate, an independent variable, changed dramatically from 4.4% to 74.2%, as intended for investigating the effect of delignification on enzymatic hydrolysis. Compared with the effect of the hemicellulose removal rate, the effect of the

lignin removal rate on the results of enzymatic hydrolysis are shown in Figure 3-4.

Generally, lignin macromolecules are distributed to cover the cellulose chain as a covalent bond with hemicellulose in cell wall structures (Mood et al., 2013). Thus, lignin has been considered to preform a similar role with hemicellulose, serving as a barrier against the access of enzyme to cellulose (Laureano-Perez et al., 2005). However, unlike hemicellulose, the lignin fraction can adsorb an active site of cellulase by electrostatic and hydrophobic interactions, and this cellulase-lignin linkage is irreversible and induces a permanent loss of enzyme activity (Nakagame et al., 2011b). Therefore, the maximum enzymatic digestibility (94.7%) could be obtained with the highest lignin removal rate (72.5%) condition through the sodium chlorite treatment.

Table 3-3. Enzymatic digestibility (%, based on a dry weight of substrate) and glucose yield (%, based on the glucose content in the initial biomass) after enzymatic hydrolysis of the solid residues after sodium chlorite treatment from twin-extruder treated *Eucalyptus pellita* as a function of sodium chlorite and acetic acid dose, and reaction time

| Conditions | | | Enzymatic digestibility (%)[1] | Glucose yield (%)[2] |
|---|---|---|---|---|
| Time (min)[3] | Sodium chlorite (M) | Acetic acid (M) | | |
| *E. pellita* | | | 0.0±0.0 | 0.5±0.0 |
| 60 | 1 | 0.2 | 6.3±0.6 | 11.5±0.1 |
| | 2 | 0.4 | 17.4±0.4 | 20.7±0.5 |
| | 3 | 0.6 | 35.3±0.8 | 35.8±0.1 |
| | 4 | 0.8 | 53.6±1.0 | 51.3±0.4 |
| 120 | 1×2 | 0.2×2 | 17.8±1.6 | 19.6±0.0 |
| | 2×2 | 0.4×2 | 57.5±0.1 | 56.3±0.8 |
| | 3×2 | 0.6×2 | 88.4±0.8 | 83.1±0.3 |
| | 4×2 | 0.8×2 | 93.9±0.4 | 83.9±3.1 |
| 180 | 1×3 | 0.2×3 | 35.8±2.8 | 34.9±1.6 |
| | 2×3 | 0.4×3 | 86.7±0.2 | 76.8±1.2 |
| | 3×3 | 0.6×3 | 94.7±0.0 | 76.9±0.0 |
| | 4×3 | 0.8×3 | 94.3±0.3 | 81.7±1.8 |

Values are the mean ± standard deviation

[1] %, based on a dry weight of substrate (equal to 1 g)

[2] %, based on glucose content in the initial biomass

[3] Reaction time

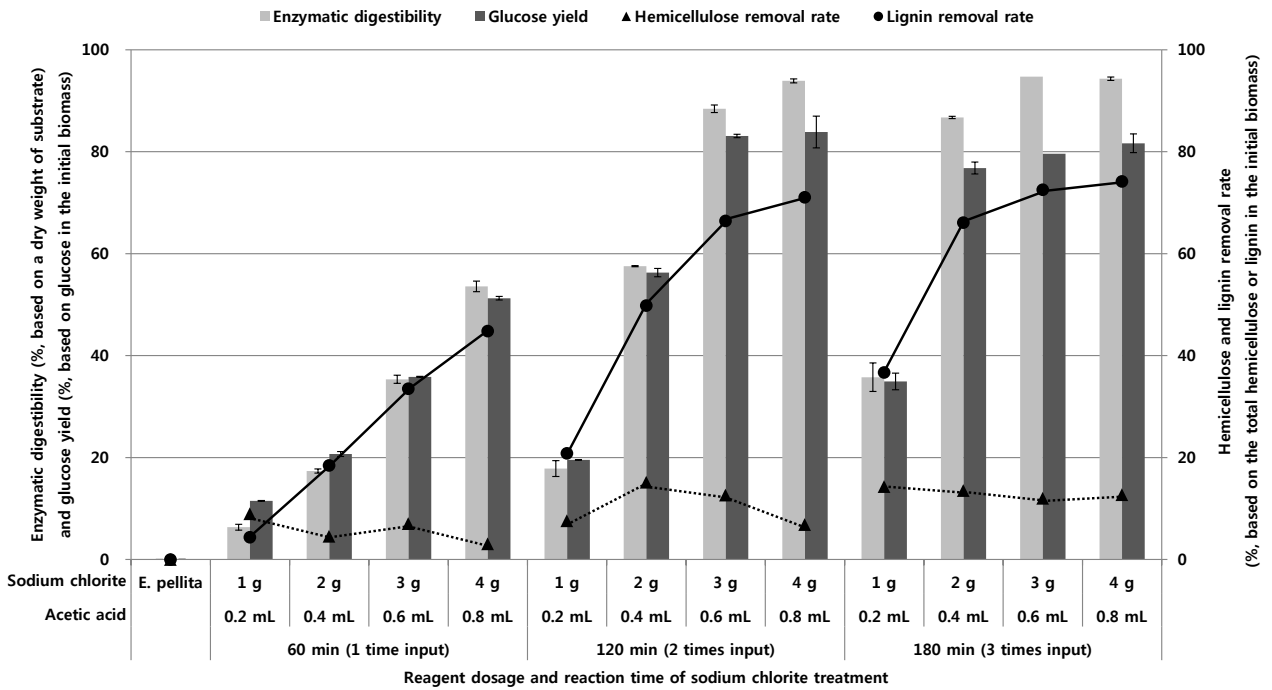



Figure 3-4. Enzymatic digestibility (%, based on a dry weight of substrate) and glucose yield (%, based on the glucose content in the initial biomass) after enzymatic hydrolysis of the solid residues after sodium chlorite treatment of *Eucalyptus pellita* with hemicellulose removal rate and lignin removal rate.

## 3.2. Lignin decomposition of liquid hot water treated *Eucalyptus pellita* by sodium chlorite treatment

In the section 3.1 of this chapter, the change of lignin content depending on the conditions of sodium chlorite treatment was investigated and the effect of the lignin removal rate was evaluated in terms of the enzymatic digestibility and glucose yield after enzymatic hydrolysis. Meanwhile, the relationship between the hemicellulose removal rate and enzymatic hydrolysis was investigated with little delignification after liquid hot water treatment in Chapter 2. Thus, these experimental conditions were induced based on a single independent variable (hemicellulose or lignin) with controlling other chemical constituents. In this section, samples whose hemicellulose and lignin removal rates changed at the same time were prepared through sodium chlorite treatment with liquid hot water treatment. Then, the effect of lignin and hemicellulose content with a variety of cases were analyzed over the results of enzymatic hydrolysis.

For achieving the above purpose, hemicellulose in *E. pellita* was removed appropriately by liquid hot water treatment that causes less damage to the lignin fraction and the structural sugar content of the solid residues are summarized in Table 3-4 and Table 3-5. The 0% hemicellulose removal rate was designated with twin-extruder treated *E. pellita* (raw material), while a 75% of lignin removal rate was set with the solid residue at 170°C for 50 min that resulted in the maximum content of xylooligosaccharide in Chapter 2. Furthermore, 160°C for 10 and 50 min conditions for liquid hot water treatment were conducted using *E. pellita* for investigating the effect of hemicellulose removal with sodium chlorite treatment. In the previous chapter, xylan content was dramatically reduced from the 150°C to the 170°C

condition; thus, the additional liquid hot water treatment at 160°C for giving a variation of hemicellulose removal rate.

According to the results of liquid hot water treatment, the amount of glucan and total lignin of the prepared solid residues for sodium chlorite treatment were maintained after liquid hot water treatment. Meanwhile, xylan content was decreased from 8.4% to 1.8% with an increase of reaction severity under the given reaction conditions.

Table 3-4. Structural sugar content of the solid residues after liquid hot water treatment under modified conditions as a function of reaction temperature and time

| Conditions | | Structural sugar (%)[1] | | | | | Total[2] |
|---|---|---|---|---|---|---|---|
| Reaction temp. (°C) | Reaction time (min) | Glucan | Xylan | Galactan | Mannan | Arabinan | |
| *E. pellita* | | 50.9±1.5 | 10.8±0.4 | 1.6±0.1 | 0.6±0.0 | 0.3±0.0 | 64.2 |
| 160 | 10 | 47.5±0.3 (49.4)[4] | 8.4±0.1 (8.7) | 0.9±0.1 (1.0) | 0.0±0.0 (0.0) | 0.0±0.0 (0.0) | 56.8 |
| 160 | 50 | 48.6±0.3 (55.9) | 5.6±0.5 (6.4) | 0.0±0.0 (0.0) | 0.0±0.0 (0.0) | 0.0±0.0 (0.0) | 54.2 |
| 170 | 50 | 47.4±2.3 (59.1) | 1.8±0.3 (2.3) | 0.0±0.0 (0.0) | 0.0±0.0 (0.0) | 0.0±0.0 (0.0) | 49.2 |

Values are the mean ± standard deviation

[1] %, based on a dry weight of initial biomass

[2] Sum of structural sugar content

[3] %, based on a dry weight of the solid residue

Table 3-5. Lignin content of the solid residues after liquid hot water treatment under modified conditions as a function of reaction temperature and time

| Conditions | | Lignin (%)[1] | | Total[2] |
|---|---|---|---|---|
| Reaction temp. (°C) | Reaction time (min) | Klason lignin | Acid-soluble lignin | |
| *E. pellita* | | 32.5±1.0 | 2.3±0.3 | 34.8 |
| 160 | 10 | 30.8±0.3 (31.6)[4] | 2.1±0.0 (2.2) | 32.9 |
| 160 | 50 | 30.8±0.7 (35.8) | 2.0±0.0 (2.3) | 32.8 |
| 170 | 50 | 30.9±0.2 (38.6) | 0.9±0.0 (1.2) | 31.8 |

Values are the mean ± standard deviation

[1] %, based on a dry weight of initial biomass

[2] Sum of Klason lignin and acid-soluble lignin content

[3] %, based on a dry weight of the solid residue

### 3.2.1. Physicochemical characteristics of solid residues

The sodium chlorite and acetic acid doses for sodium chlorite treatment using hemicellulose removed solid residues were designated 1.5 g, 2.5 g, 4 g, and 4 g and given 2 times (sodium chlorite) with 0.3 mL, 0.5 mL, 0.8 mL, and 0.8 mL given 2 times (acetic acid). These conditions of sodium chlorite treatment were modified through reflecting on the results of section 3.1 in this Chapter. Because 2 (reaction time: 120 min) and 3 times (reaction time: 180 min) reagents input conditions presented an excessive decomposition of Klason lignin after sodium chlorite treatment. Thus, the reagent dose was modified as above to give apparent variation of total lignin content among the solid residues.

After the sodium chlorite treatment using the solid residues recovered after liquid hot water treatment, the WIS recovery rate is shown in Figure 3-5. As the dose of sodium chlorite and acetic acid increased, the WIS recovery rate decreased steadily could be observed in each hemicellulose removal rate condition. Meanwhile, a significant drop of WIS recovery rate presented under the conditions that used liquid hot water treatment (25%, 50%, and 75% of hemicellulose removal rate), and it decreased until 48.6% under 4 g for 2 times input of sodium chlorite and 0.8 mL for 2 times input of acetic acid condition (reaction time: 120 min) using 75% of hemicellulose removed solid residue. The inordinate decrease of WIS recovery rate might be caused by the liquid hot water treatment and sodium chlorite treatment run in sequence, and the hemicellulose and lignin fraction of the biomass seems to be damaged during the two-step treatment.

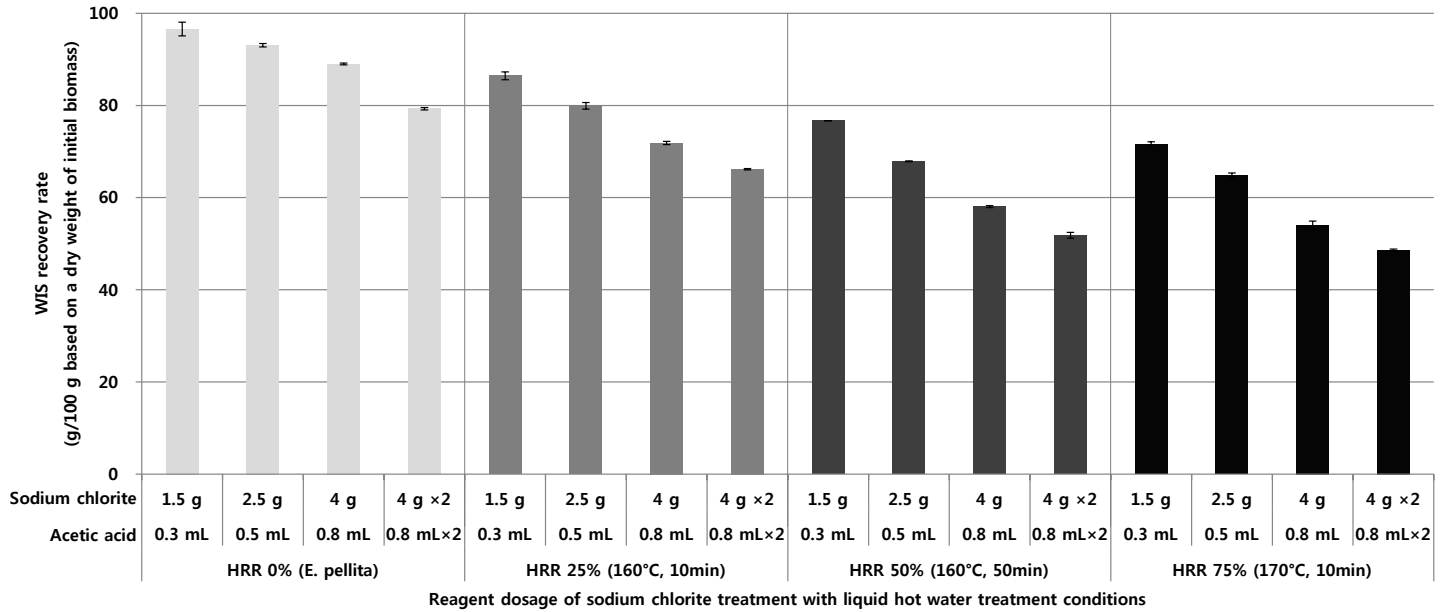



Figure 3-5. Water insoluble solid (WIS) recovery rate (%, based on a dry weight of biomass) of solid residues after sodium chlorite treatment of *Eucalyptus pellita* and liquid hot water treated *Eucalyptus pellita* depending on reagents (sodium chlorite and acetic acid) dose and reaction time (HRR: hemicellulose removal rate).

After the sodium chlorite treatment, the structural sugar content of the solid residues that had removed hemicellulose are presented in Table 3-6 (0% and 25% of hemicellulose removal rate) and Table 3-7 (50% and 75% of hemicellulose removal rate). All of the structural sugar content had no significant changes after sodium chlorite treatment that might be comprehended as an oxidative reaction for the lignin fraction selectively as can be observed in Table 3-1. The glucan content of the solid residues at 25%, 50%, and 75% of hemicellulose removal rate was slightly decreased compared to that of the 0% hemicellulose removal rate because the glucan content had already declined after the liquid hot water treatment.

As the hemicellulose removal rate increased to 50%, the xylan content of the solid residue was decreased to range from 4.0% to 2.6% (Table 3-7). Compared with cases the 0% and 25% hemicellulose removal rates, the xylan content suffered little damage, and it decreased compared to that of the solid residue (5.6%) that was employed as a feedstock for sodium chlorite treatment. This result might be understood as that the tight complexity of the cell wall structure began to loosen in response to the liquid hot water treatment under condition of 160°C for 50 min, and the hemicellulose fraction was hydrolyzed by acetic acid during the sodium chlorite treatment. The xylan decomposition after the sodium chlorite treatment could be expected in the case of the 75% hemicellulose removal rate condition, but the remaining xylan content was too tiny (1.8%) to confirm the effect of liquid hot water treatment properly.

Table 3-6. Sugar contents of the solid residues after sodium chlorite treatment with liquid hot water treatment as a function of sodium chlorite and acetic acid dose, and hemicellulose removal rate (HRR, 0% or 25%)

| Conditions | | | Structural sugar (%)[1] | | | | | Total[2] |
|---|---|---|---|---|---|---|---|---|
| HRR[3] | Sodium chlorite (g) | Acetic acid (mL) | Glucan | Xylan | Galactan | Mannan | Arabinan | |
| 0% | 1.5 | 0.3 | 50.2±2.2 (51.0)[4] | 9.8±0.3 (10.1) | 1.0±0.1 (1.1) | 0.5±0.1 (0.6) | 0.0±0.0 (0.0) | 61.5 |
| | 2.5 | 0.5 | 50.6±0.0 (54.5) | 10.5±0.0 (11.2) | 1.1±0.0 (1.2) | 0.5±0.0 (1.1) | 0.0±0.0 (0.0) | 62.7 |
| | 4 | 0.8 | 49.4±0.0 (55.4) | 10.7±0.2 (12.1) | 1.4±0.0 (1.5) | 0.5±0.0 (1.1) | 0.2±0.0 (0.6) | 62.2 |
| | 4×2 | 0.8×2 | 49.1±0.4 (61.9) | 9.5±0.1 (12.2) | 1.0±0.0 (1.4) | 0.4±0.1 (0.6) | 0.0±0.0 (0.0) | 59.6 |
| 25% | 1.5 | 0.3 | 46.4±0.1 (51.7) | 8.5±0.0 (9.4) | 0.8±0.1 (0.9) | 0.0±0.0 (0.0) | 0.0±0.0 (0.0) | 56.0 |
| | 2.5 | 0.5 | 46.6±0.2 (56.2) | 8.5±0.2 (10.2) | 0.5±0.0 (0.7) | 0.0±0.0 (0.0) | 0.0±0.0 (0.0) | 56.5 |
| | 4 | 0.8 | 47.1±0.4 (63.1) | 8.3±0.0 (11.2) | 0.7±0.0 (1.0) | 0.0±0.0 (0.0) | 0.0±0.0 (0.0) | 55.6 |
| | 4×2 | 0.8×2 | 46.8±0.4 (68.1) | 7.7±0.1 (11.1) | 0.6±0.0 (0.9) | 0.0±0.0 (0.0) | 0.0±0.0 (0.0) | 52.2 |

Values are the mean ± standard deviation

[1] %, based on a dry weight of initial biomass

[2] Sum of structural sugar content

[3] HRR: hemicellulose removal rate (from solid residues obtained by liquid hot water treatment)

[4] %, based on a dry weight of the solid residue

Table 3-7. Sugar contents of the solid residues after sodium chlorite treatment with liquid hot water treatment as a function of sodium chlorite and acetic acid dose, and hemicellulose removal rate (HRR, 50% or 75%)

| Conditions | | | Structural sugar (%)[1] | | | | | Total[2] |
|---|---|---|---|---|---|---|---|---|
| HRR[3] | Sodium chlorite (g) | Acetic acid (mL) | Glucan | Xylan | Galactan | Mannan | Arabinan | |
| 50% | 1.5 | 0.3 | 47.8±0.0 (61.5)[4] | 3.9±0.0 (5.1) | 0.4±0.0 (0.6) | 0.0±0.0 (0.0) | 0.0±0.0 (0.0) | 52.2 |
| | 2.5 | 0.5 | 48.4±0.8 (73.0) | 4.0±0.1 (5.8) | 0.5±0.1 (0.7) | 0.0±0.0 (0.0) | 0.0±0.0 (0.0) | 52.9 |
| | 4 | 0.8 | 47.6±2.0 (80.9) | 3.2±0.1 (5.5) | 0.5±0.2 (0.8) | 0.0±0.0 (0.0) | 0.0±0.0 (0.0) | 51.3 |
| | 4×2 | 0.8×2 | 46.2±1.8 (88.0) | 2.6±0.1 (4.9) | 0.3±0.1 (0.6) | 0.0±0.0 (0.0) | 0.0±0.0 (0.0) | 49.1 |
| 75% | 1.5 | 0.3 | 47.8±0.0 (67.3) | 1.4±0.2 (2.0) | 0.0±0.0 (0.0) | 0.0±0.0 (0.0) | 0.0±0.0 (0.0) | 49.3 |
| | 2.5 | 0.5 | 48.1±0.1 (74.3) | 1.4±0.7 (2.2) | 0.0±0.0 (0.0) | 0.0±0.0 (0.0) | 0.0±0.0 (0.0) | 49.6 |
| | 4 | 0.8 | 47.6±0.5 (88.0) | 1.5±0.2 (2.7) | 0.0±0.0 (0.0) | 0.0±0.0 (0.0) | 0.0±0.0 (0.0) | 49.0 |
| | 4×2 | 0.8×2 | 46.5±1.2 (95.7) | 1.1±0.1 (2.3) | 0.0±0.0 (0.0) | 0.0±0.0 (0.0) | 0.0±0.0 (0.0) | 47.6 |

Values are the mean ± standard deviation

[1] %, based on a dry weight of initial biomass

[2] Sum of structural sugar content

[3] HRR: hemicellulose removal rate (from solid residues obtained by liquid hot water treatment)

[4] %, based on a dry weight of the solid residue

The lignin content of the solid residue as a function of the hemicellulose removal rate and the dose of reagent for the sodium chlorite treatment are summarized in Table 3-8. In all cases of hemicellulose removal rate, total lignin content declined gradually as an increase of sodium chlorite and acetic acid dose. Meanwhile, the amount of total lignin was decreased in response to same dose of sodium chlorite and acetic acid as the hemicellulose removal rate increased. For instance, 28.5% of total lignin content in the 0% of hemicellulose removal rate condition declined steadily from 21.7% at the 75% hemicellulose removal rate condition under 1.5 g of sodium chlorite and 0.3 mL of acetic acid. According to this trend, the minimum content of total lignin (0.3%) was observed under 4 g of sodium chlorite and 0.8 mL of acetic acid at the condition of 75% hemicellulose removal rate. As hemicellulose was removed by the hydrothermal reaction, the lignin fraction might be more easily disintegrated without maintaining a strong linkage with the cell wall structure (Zhao et al., 2012). Consequently, hemicellulose degradation through the liquid hot water treatment was considered to contribute to suitable lignin decomposition after the sodium chlorite treatment.

According to the section 3.1.1 in this chapter, the amount of acid-soluble lignin of the solid residues varied as the dose of reagents changed (Table 3-2). With an increase in the sodium chlorite and acetic acid dose, the acid-soluble lignin of the solid residues was more highly produced, but its content declined when the dose of reagent was higher than a certain amount. This trend of acid-soluble lignin content could be found under variety of hemicellulose removal rate conditions (Table 3-8). It was thought that the sodium chlorite treatment might decompose it and convert it into the lignin fraction, which has a high affinity for acidic solvent.

However, the maximum amount of acid-soluble lignin was decreased from 9.9% to 1.7% as an increase of hemicellulose removal rate from 0% to

75%. This phenomenon could be interpreted to mean that the lignin macromolecules may be strongly decomposed into small fragments that are not detected by UV-vis spectroscopy during sodium chlorite treatment as a response to enhancement of hemicellulose removal through liquid hot water treatment. Furthermore, the hemicellulose-lignin bond may prevent the oxidative decomposition of the lignin structure by covering reaction sites. Finally, both the amounts of Klason lignin and acid-soluble lignin were extremely decreased under high hemicellulose removal rate conditions with a high dose of sodium chlorite and acetic acid.

Table 3-8. Lignin content of the solid residues after sodium chlorite treatment with liquid hot water treatment as a function of sodium chlorite and acetic acid dose, and hemicellulose removal rate (%, based on the total hemicellulose content in the initial biomass)

| Conditions | | | Lignin (%)[1] | | Total[2] |
|---|---|---|---|---|---|
| HRR[3] | Sodium chlorite (M) | Acetic acid (M) | Klason lignin | Acid-soluble lignin | |
| 0% | 1.5 | 0.3 | 23.2±0.2 (24.1)[4] | 5.2±0.0 (5.4) | 28.5 |
| | 2.5 | 0.5 | 16.9±0.1 (18.1) | 7.7±0.0 (8.2) | 24.6 |
| | 4 | 0.8 | 9.6±0.2 (10.8) | 9.6±0.1 (10.8) | 19.2 |
| | 4×2 | 0.8×2 | 2.6±0.2 (3.3) | 7.4±0.0 (9.4) | 10.1 |
| 25% | 1.5 | 0.3 | 19.0±0.2 (22.0) | 5.2±0.2 (6.0) | 24.2 |
| | 2.5 | 0.5 | 11.7±0.4 (14.6) | 6.7±0.0 (8.3) | 18.3 |
| | 4 | 0.8 | 5.1±0.7 (7.1) | 6.1±0.3 (8.5) | 11.2 |
| | 4×2 | 0.8×2 | 2.6±0.4 (3.9) | 4.4±0.2 (6.6) | 6.9 |
| 50% | 1.5 | 0.3 | 18.8±0.4 (24.2) | 3.4±0.3 (4.4) | 22.2 |
| | 2.5 | 0.5 | 10.4±0.3 (15.2) | 3.5±0.1 (5.0) | 13.9 |
| | 4 | 0.8 | 3.2±0.3 (5.5) | 2.4±0.1 (4.0) | 5.6 |
| | 4×2 | 0.8×2 | 1.0±0.3 (1.9) | 0.6±0.1 (1.1) | 1.6 |
| 75% | 1.5 | 0.3 | 20.0±0.2 (28.0) | 1.7±0.5 (2.4) | 21.7 |
| | 2.5 | 0.5 | 13.3±1.3 (20.5) | 1.4±0.3 (2.1) | 14.6 |
| | 4 | 0.8 | 2.8±0.2 (5.2) | 1.1±0.0 (2.1) | 4.0 |
| | 4×2 | 0.8×2 | 0.3±0.2 (0.6) | 0.0±0.0 (0.0) | 0.3 |

Values are the mean ± standard deviation

[1] %, based on a dry weight of initial biomass

[2] Sum of Klason lignin and acid-soluble lignin content

[3] HRR: hemicellulose removal rate (from solid residues obtained by liquid hot water treatment)

[4] %, based on a dry weight of the solid residue

### 3.2.2. Enzymatic hydrolysis of solid residues

The enzymatic digestibility and glucose yield after enzymatic hydrolysis using the solid residue after sodium chlorite treatment and liquid hot water treatment are stated in Table 3-9. The solid residues were decomposed entirely after enzymatic hydrolysis, and 100% of enzymatic digestibility was obtained under 4 g of sodium chlorite and 0.8 mL of acetic acid conditions with liquid hot water treatment even though a small amount of lignin remained in the solid residues. This result could not be understood completely, but it is assumed that the lignin fraction of the solid residue which has a very small molecular weight, passed through the filter paper during the washing process after enzymatic hydrolysis.

The maximum glucose yield (87.5%, 86.2%, and 82.9%) was observed at the condition of 4 g of sodium chlorite and 0.8 mL of acetic acid at 25%, 50%, and 75% of hemicellulose removal rate conditions, respectively, except for the 0% of hemicellulose removal rate. A disagreement between the enzymatic digestibility and glucose yield may be caused by glucan loss after liquid hot water treatment. Meanwhile, the glucose yield was decreased slightly with a 2 times reagents dose that can be explained as damage to the cellulose as mentioned above.

Therefore, a high glucose yield (87.5%) was achieved from *E. pellita*, a highly recalcitrant species, through sodium chlorite treatment with liquid hot water treatment, and the combined treatment can reduce the reagents dose and reaction time.

Table 3-9. Enzymatic digestibility (%, based on a dry weight of substrate) and glucose yield (%, based on the glucose content in the initial biomass) after enzymatic hydrolysis of the solid residues after sodium chlorite treatment with liquid hot water treatment as a function of sodium chlorite and acetic acid dose, and hemicellulose removal rate

| Conditions | | | Enzymatic digestibility (%)[1] | Glucose yield (%)[2] |
|---|---|---|---|---|
| HRR[3] | Sodium chlorite (M) | Acetic acid (M) | | |
| 0% | 1.5 | 0.3 | 11.0±2.0 | 14.7±0.2 |
| | 2.5 | 0.5 | 26.0±0.7 | 26.6±0.2 |
| | 4 | 0.8 | 53.6±1.0 | 51.3±0.4 |
| | 4×2 | 0.8×2 | 93.9±0.4 | 83.9±3.1 |
| 25% | 1.5 | 0.3 | 32.3±1.4 | 34.5±0.0 |
| | 2.5 | 0.5 | 73.9±0.9 | 65.1±3.9 |
| | 4 | 0.8 | 96.7±0.9 | 87.5±3.0 |
| | 4×2 | 0.8×2 | 100.0±0.6 | 84.4±0.4 |
| 50% | 1.5 | 0.3 | 37.2±0.2 | 38.3±0.1 |
| | 2.5 | 0.5 | 71.9±0.2 | 66.0±0.1 |
| | 4 | 0.8 | 99.3±0.4 | 86.2±0.1 |
| | 4×2 | 0.8×2 | 100.0±0.2 | 82.7±0.3 |
| 75% | 1.5 | 0.3 | 37.0±1.2 | 38.6±0.8 |
| | 2.5 | 0.5 | 59.2±0.2 | 57.3±0.1 |
| | 4 | 0.8 | 99.6±0.0 | 82.9±1.0 |
| | 4×2 | 0.8×2 | 100.0±0.0 | 81.3±1.5 |

Values are the mean ± standard deviation

[1] %, based on a dry weight of substrate (equal to 1 g)

[2] %, based on glucose content in the initial biomass

[3] HRR: hemicellulose removal rate (from solid residues obtained by liquid hot water treatment)

The enzymatic digestibility and glucose yield after enzymatic hydrolysis with hemicellulose and lignin removal rate based on initial content in *E. pellita* are illustrated in Figure 3-6. As shown in the graph, the effect of the hemicellulose removal rate for enzymatic hydrolysis was remarkable, and it is considered to have a strong relationship with glucose production. The proportional relation was observed in all groups that had a similar hemicellulose removal rate. However, the glucose yield was decreased slightly from 87.5% to 84.4% even though the lignin removal rate was increased from 67.8% to 80.2% as the reagents' dose doubled (4 g of sodium chlorite and 0.8 mL of acetic acid) at 25% of the hemicellulose removal rate condition. Meanwhile, the hemicellulose removal rate after sodium chlorite treatment did not change significantly within the group that had the same hemicellulose removal rate by liquid hot water treatment and the relationship between the enzymatic digestibility and glucose yield was not clear.

As mentioned in the paragraph, the effect of hemicellulose removal with sodium chlorite treatment was indicated by the results of enzymatic hydrolysis. In addition, high enzymatic digestibility was achieved through a low dose of the reagents with liquid hot water treatment. Therefore, it is clear that the sequential removal of hemicellulose and lignin of the solid residues resulted in synergy. However, a statistical analysis approach seems to be required to evaluate the influence of the interaction of recalcitrance factors removal for improving glucose production that will proceed in section 3.3 of this chapter.

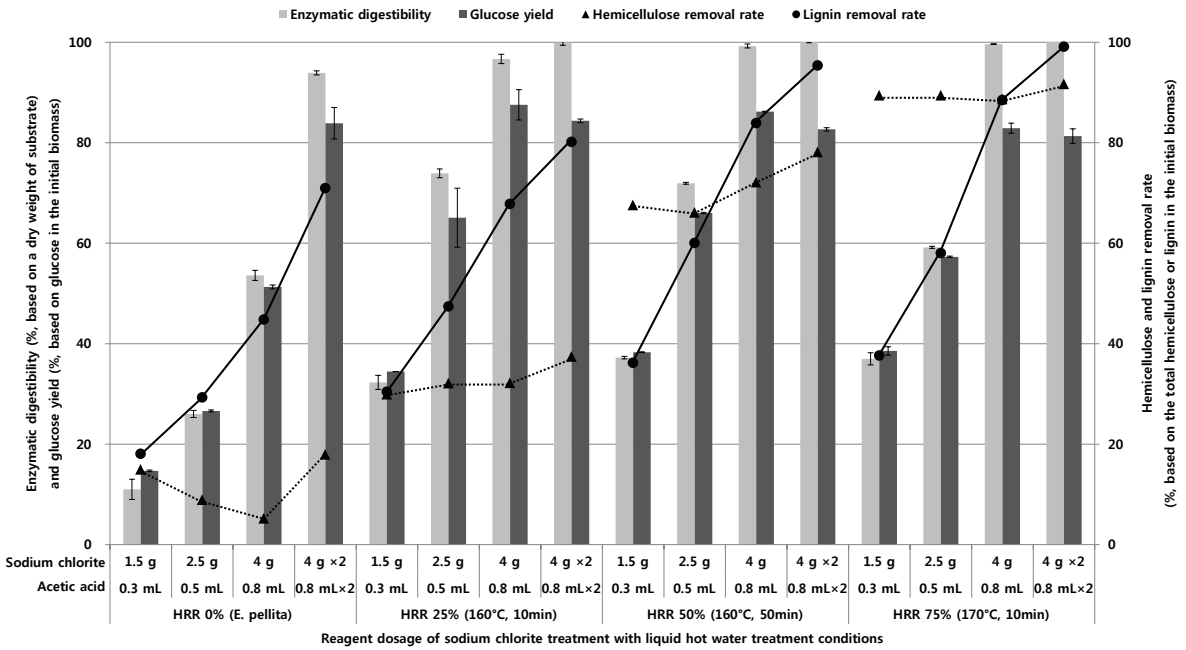



Figure 3-6. Enzymatic digestibility (%, based on a dry weight of substrate) and glucose yield (%, based on the glucose content in the initial biomass) after enzymatic hydrolysis of the solid residues after sodium chlorite treatment of *Eucalyptus pellita* and liquid hot water treated *Eucalyptus pellita* with hemicellulose removal rate and lignin removal rate.

## 3.3. Effects of biomass constituents on enzymatic hydrolysis

### 3.3.1. A relationship between hemicellulose removal and glucose production

Correlation analysis was conducted between biomass constituents and glucose yield as same procedure that described in section 3.4 of Chapter 2. And, a linear regression model was established for evaluating the effect of hemicellulose removal rate on glucose production after sodium chlorite treatment (Table 3-10). However, the regression model cannot explain the significant relationship between the hemicellulose removal rate and glucose yield due to the very low value of the adjusted square of the correlation coefficient (0.0936). Furthermore, the p-value of the hemicellulose removal rate against glucose yield was determined as 0.0588, which cannot have significance for this model. Thus, hemicellulose removal may be comprehended as a negligible factor for improving the glucose yield.

This result of correlation analysis between hemicellulose removal rate and glucose yield can be easily understood through a plot of linear regression model (Figure 3-7). According to the plot, dots, which were placed in the low value of hemicellulose removal rate, were dispersed approximately from 0% to 80% of the glucose yield. Therefore, other factors except for hemicellulose removal rate seem to strongly interfere for change of glucose yield after the sodium chlorite treatment regardless of liquid hot water treatment.

Table 3-10. Analysis of variance (ANOVA) and parameter estimates for glucose yields corresponded hemicellulose removal rate (HRR) after liquid hot water or/and sodium chlorite treatment (R-Square=0.1260, Adjust R-Square=-0.0936)

Analysis of Variance

| Source | DF[1] | Sum of Squares | Mean Square | F Value | Pr > F |
|---|---|---|---|---|---|
| Model | 1 | 2663.46935 | 2663.46935 | 3.89 | 0.0588 |
| Error | 27 | 18475 | 684.25117 | - | - |
| Corrected Total | 28 | 21138 | - | - | - |

Parameter Estimates

| Variable | DF | Parameter Estimate | Standard Error | t Value | Pr > \|t\| |
|---|---|---|---|---|---|
| Intercept | 1 | 45.80256 | 6.99585 | 6.55 | <.0001 |
| HRR[2] | 1 | 0.30625 | 0.15523 | 1.97 | 0.0588 |

[1] DF: degree of freedom
[2] HRR: hemicellulose removal rate

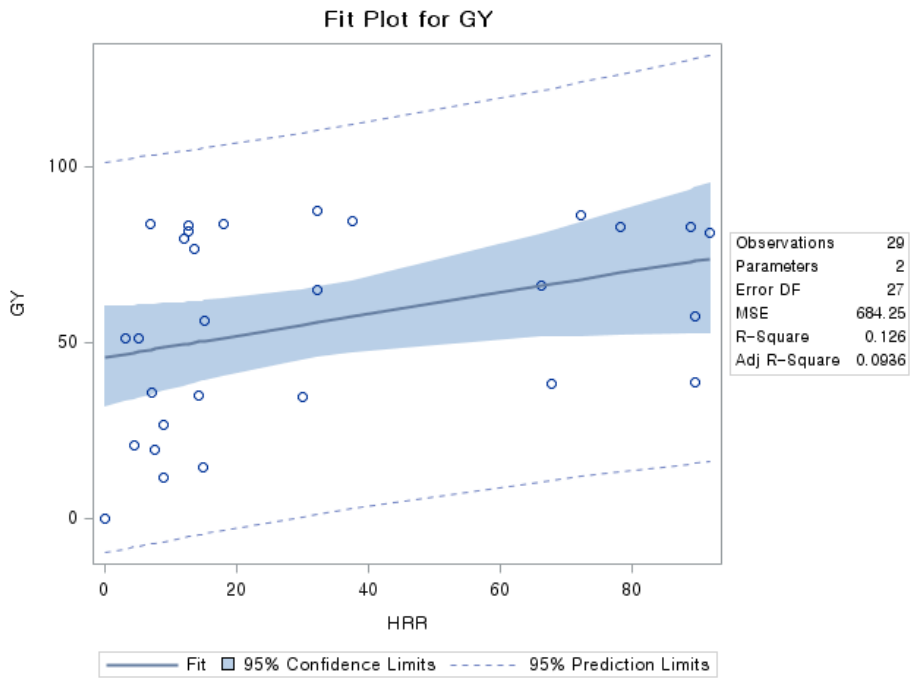



Figure 3-7. Correlation plot for glucose yields (GY) vs. hemicellulose removal rate (HRR) after liquid hot water or/and sodium chlorite treatment.

### 3.3.2. A relationship between lignin removal and glucose production

According to the single regression analysis between lignin removal rate and glucose yield, a p-value of less than 0.0001 was observed, which revealed a strong influence of this independent variable. The result was the opposite of the previous result for section 3.4.2 of Chapter 2, which conducted liquid hot water treatment only, and the p-value of the lignin removal rate was 0.4805. Thus, the effect of lignin removal on glucose production cannot be adequately considered due to insufficient delignification through liquid hot water treatment. Meanwhile, the correlation plots from regression analysis indicated the dominant influence of the lignin removal rate after sodium chlorite treatment was related to the glucose yield (Figure 3-11).

$$\text{Glucose yield} = 4.79449 + 0.98044 \times \text{LRR}$$

The adjusted R-squared value of the above linear regression model was estimated at 0.9063, and it relatively higher that the result of the correlation analysis for the hemicellulose removal rate after liquid hot water treatment (0.6768) in section 3.4.2 of Chapter 2. Therefore, the lignin removal rate could be considered as an more impactful factor for improving glucose yield than the hemicellulose removal rate among the biomass constituents. The correlation plot also presented an obvious proportional relationship between lignin removal and glucose yield (Figure 3-8).

Table 3-11. Analysis of variance (ANOVA) and parameter estimates for glucose yields corresponded lignin removal rate (LRR) after liquid hot water or/and sodium chlorite treatment (R-Square=0.9096, Adjust R-Square=0.9063)

| Analysis of Variance | | | | | |
|---|---|---|---|---|---|
| Source | DF[1] | Sum of Squares | Mean Square | F Value | Pr > F |
| Model | 1 | 19228 | 19228 | 271.75 | <.0001 |
| Error | 27 | 1910.38278 | 70.75492 | - | - |
| Corrected Total | 288 | 21138 | - | - | - |
| **Parameter Estimates** | | | | | |
| Variable | DF | Parameter Estimate | Standard Error | t Value | Pr > \|t\| |
| Intercept | 1 | 4.79449 | 3.46250 | 1.38 | 0.1775 |
| LRR[2] | 1 | 0.98044 | 0.05948 | 16.48 | <.0001 |

[1] DF: degree of freedom
[2] LRR: lignin removal rate

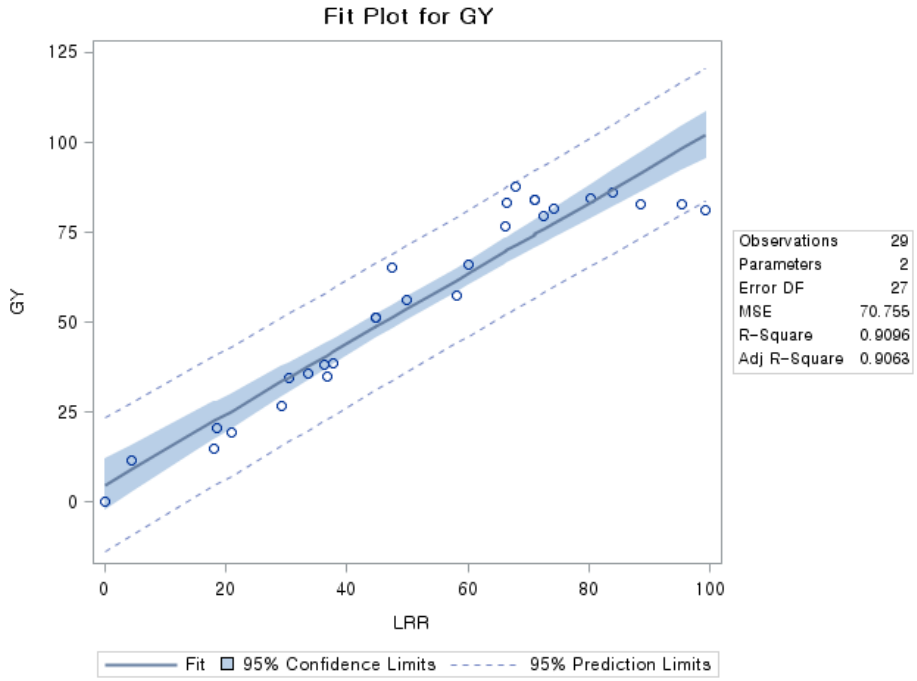



Figure 3-8. Correlation plot for glucose yields (GY) vs. lignin removal rate (LRR) after liquid hot water or/and sodium chlorite treatment.

### 3.3.3. A relationship between double factors and glucose production

The double factors of biomass constituents (hemicellulose and lignin removal rate), which were controlled simultaneously through liquid hot water and sodium chlorite treatment, were employed for a linear regression analysis corresponding to the glucose yield, and the result of the correlation analysis is shown in Table 2-12. After investigating the parameter significance, the case of lignin removal rate had the highest significance (p-value < 0.0001), and the significance of the hemicellulose removal rate had a low p-value (0.0050). The linear regression formula was established as follows:

$$\text{Glucose yield} = 4.91249 - 0.15545 \times \text{HRR} + 1.07521 \times \text{LRR}$$

According to the regression model that was calculated by double biomass constituents, the adjusted squared of correlation coefficient was observed at 0.9285, and it was relatively higher than that of single factor analysis for the lignin removal rate (0.9063). This result could be explained as a synergetic effect by hemicellulose removal and lignin removal for improving glucose production through liquid hot water and sodium chlorite treatment. Indeed, a slightly higher glucose yield could be observed after combined treatment of *E. pellita* than that of sodium chlorite treatment only.

Consequently, the lignin removal rate revealed a dominant factor for improving glucose production using the high recalcitrant species, *E. pellita* according to the delignification and correlation analysis. In contrast, several studies reported a high glucose yield without lignin decomposition through mild conditions of reactions such as dilute acid pretreatment using hardwood (Lim & Lee, 2013). However, the result of high glucose production after enzymatic hydrolysis can be achieved after only a minimal removal of the

lignin fraction when low recalcitrant species such as hardwood used as a feedstock. Therefore, the results of correlation analysis suggest that an effort of controlling the lignin content in lignocellulosic biomass should be considered as a priority for improving glucose production.

On the other hand, considering the linear regression model, 99.6% of glucose yield could be reached when the lignin removal rate was 88%. However, the glucose yield was not increased above 90% when there was a more than 80% lignin removal rate among experimental results (Figure 3-8). Therefore, it is clear that the lignin content in biomass is a most important factor in determining glucose production, but a limitation for entire glucose conversion appeared at high lignin removal conditions. As the condition of sodium chlorite treatment becomes more severe, the cell wall structure of *E. pellita* may be changed in way that is not beneficial for enzymatic hydrolysis such as an increase of cellulose crystallinity.

Table 3-12. Analysis of variance (ANOVA) and parameter estimates for glucose yields corresponded hemicellulose removal rate (HRR) and lignin removal rate (LRR) after liquid hot water or/and sodium chlorite treatment (R-Square=0.9336, Adjust R-Square=0.9285)

Analysis of Variance

| Source | DF[1] | Sum of Squares | Mean Square | F Value | Pr > F |
|---|---|---|---|---|---|
| Model | 2 | 19734 | 9867.22005 | 182.75 | <.0001 |
| Error | 26 | 1403.81096 | 53.99273 | - | - |
| Corrected Total | 28 | 21138 | - | - | - |

Parameter Estimates

| Variable | DF | Parameter Estimate | Standard Error | t Value | Pr > \|t\| |
|---|---|---|---|---|---|
| Intercept | 1 | 4.91249 | 3.02492 | 1.62 | 0.1164 |
| HRR[2] | 1 | -0.15545 | 0.05075 | -3.06 | 0.0050 |
| LRR[3] | 1 | 1.07521 | 0.06047 | 17.78 | <.0001 |

[1] DF: degree of freedom
[2] HRR: hemicellulose removal rate
[3] LRR: lignin removal rate

# 4. Conclusions

In this chapter, sodium chlorite treatment was performed using twin-extruder treated *Eucalyptus pellita* to evaluate the delignification trend with changes of conditions and the effect of lignin decomposition for enzymatic hydrolysis. Moreover, feedstocks that controlled the amount of hemicellulose were prepared by liquid hot water treatment, and were employed for sodium chlorite treatment to investigate the changes in recalcitrant factors (hemicellulose and lignin) in terms of glucose production.

The amount of glucan and xylan remained relatively constant (approximately 49% and 10%, respectively) with that of *E. pellita* (50.9% and 10.8%) after sodium chlorite treatment even though acetic acid was employed for the reaction. Meanwhile, total lignin content of the solid residues was decreased obviously as an increase of sodium chlorite and acetic acid dose regardless of the number of inputs. In addition, the lowest amount of total lignin (9.0%) was observed under the 4 g of sodium chlorite and 0.8 mL of acetic acid condition for 180 min of reaction time (3 times input). The variation of Klason lignin content typically corresponded to that of total lignin, while the acid-soluble lignin content was increased until 9.6% after sodium chlorite treatment. After enzymatic hydrolysis using sodium chlorite treated solid residues, the enzymatic digestibility and glucose yield were increased dramatically to 94.7% and 83.9%, respectively, with high doses of reagents.

To evaluate the effect of hemicellulose content changes, solid residues that were treated by liquid hot water treatment under a variety of conditions were applied for sodium chlorite treatment with modified conditions. As a result, glucan was hardly decomposed and its content was maintained at approximately 47%, and xylan mostly remained at a similar amount within

each feedstock and the liquid hot water treated solid residue. Meanwhile, the amount of total lignin declined sharply in response to an increase in reagents dose and hemicellulose removal rate, and its content was decreased to 0.3% under the harshest condition in this chapter. The variation of Klason lignin and acid-soluble lignin content was corresponded with the results of sodium chlorite treatment from *E. pellita*, but the maximum amount of acid-soluble lignin dropped from 9.9% to 1.7% with an increase in hemicellulose removal rate. Thus, the enzymatic digestibility and glucose yield were increased intensively through the elimination both of the recalcitrant factors, hemicellulose and lignin, and their maximum values were achieved as 100% and 87.5%, respectively.

According to the correlation analysis and linear regression modelling between single factor (hemicellulose or lignin) and glucose yield, the hemicellulose removal rate had not significant effect on enzymatic hydrolysis because the low adjusted square of the correlation coefficient (0.0936) after sodium chlorite treatment in compared with the result of liquid hot water treatment in Chapter 2 (R-squared value=0.6768). Meanwhile, a strong influence of lignin removal rate on the glucose yield by a high adjusted R-squared value (0.9063) was observed through delignification and correlation analysis in contrast to the result of liquid hot water treatment. In addition, the lignin removal rate had a high adjusted R-squared value (0.9285) for glucose production that was determined by regression analysis for double factors (hemicellulose and lignin) with the glucose yield.

Consequently, a dominant influence of lignin decomposition in solid residues for enzymatic hydrolysis was revealed through the sodium chlorite treatment. Furthermore, the combined (liquid hot water and sodium chlorite) treatment, which sequentially removed hemicellulose and lignin from *E. pellita*, can contribute to improving the glucose yield in comparison with

sodium chlorite treatment, as observed by correlation analysis and linear regression modelling.

# *Chapter 4*

## Change of cellulose crystalline structure and glucose production

# 1. Introduction

Crystallinity is a unique characteristic of cellulose, which has a long chain length of approximately 5 μm with no substitution in side groups (Mazeau, 2011). Compared to amorphous regions, crystalline regions typically formed by inter-/intramolecular hydrogen bond induce strong hydrophobicity, low reactive properties, and difficulty in hydrolysis by chemical reaction (Chundawat et al., 2011). These characteristics of crystalline regions hinder a suitable mechanism of cellulase for a cellulose chain compared to an amorphous region (Fan et al., 1980). Therefore, the crystallinity of lignocellulosic biomass has been recognized as a recalcitrant factor and an inhibitor for enzymatic hydrolysis (Hoshino et al., 1997).

In a previous study, a variety of feedstocks such as Avicel, filter paper, cotton, and bacterial cellulose were employed for evaluating the effect of cellulose crystallinity (McLean et al., 2002). As a result, an efficiency of enzymatic hydrolysis was increased along with a decrease in crystallinity index. Furthermore, adsorption capacity of endoglucanase toward cellulose was enlarged when the substrate had low crystallinity index (Lee et al., 1982).

However, the correlation between the crystallinity of cellulose and the recalcitrant factor against enzyme activity is still debated unlike hemicellulose and lignin (Puri, 1984). There has been inconclusive findings on the effect of crystallinity index, and an obvious relationship has not been found in some studies (Lynd et al., 2002).

The evaluation method of crystallinity index has been researched for several decades, given that the crystallinity of cellulose was recognized as an important property for determining the reactivity of a sample (Zhao et al., 2006). To date, typically four measurement methods for crystallinity index

have been developed; i) X-ray diffraction (XRD) peak height method (Segal method), ii) XRD deconvolution method, iii) XRD amorphous subtraction method (Ruland-Vonk method), and iv) Nuclear magnetic resonance (NMR) C4 peak separation method (Park et al., 2010). Among them, the Segal method was proposed as an empirical measurement, and it supplied simple and fast analysis for comparison of cellulosic feedstocks (Segal et al., 1959). Additionally, this method is useful in discriminating the characteristic differences of samples (Park et al., 2010).

In this study, concentrated sodium hydroxide treatment was performed for mitigating the crystallinity of both cellulose from *E. pellita* as well as solid residues treated by liquid hot water and/or sodium chlorite. After the reaction, the crystallinity index of the solid residue was determined by Segal method. In addition, the crystallinity index was compared with the results of liquid hot water treatment or sodium chlorite treatment before and after enzymatic hydrolysis. Finally, the glucose yield after enzymatic hydrolysis was evaluated to investigate the relationship with crystallinity index.

# 2. Materials and methods

## 2.1. Materials

The raw material of this study, twin-extruder treated *Eucalyptus pellita*, was stated in section 2.1 of Chapter 2 in detail.

Meanwhile, the solid residue, which was controlled the hemicellulose content through liquid hot water treatment in Chapter 3, was used for reducing the crystallinity index. Moreover, the solid residue, which was controlled the hemicellulose and lignin content by liquid hot water treatment with sodium chlorite treatment in Chapter 3, was employed experiments in this chapter for reducing the crystallinity.

## 2.2. Treatment for crystallinity change

To reduce the crystallinity, concentrated sodium hydroxide (NaOH) solution was employed for (1) *E. pellita*, (2) liquid hot water treated solid residue, and (3) sodium chlorite with liquid hot water treated solid residue. 4 g of the samples was put into a conical tube, and then mixed with 8% or 12% (w/w) of sodium hydroxide solution. The sodium hydroxide treatment was conducted in ice-water chamber (0°C), and the slurry was reacted until 60 min or 180 min. During the reaction, the slurry was thoroughly stirred by glass stick. When the sodium hydroxide treatment was finished, the conical tube was centrifuged (mega 17R, HANIL SME Co., LTD, Anyang, Republic of Korea) at 12,000 rpm for 10 min that separated the treated samples by

supernatant (sodium hydroxide solution). The treated samples were lyophilized using freeze-dryer (FD8508, IlShinBioBase, Seoul, Republic of Korea) for 72 h, and then the dried samples were grounded by pulverizer (RT-02SF, Tsong, Taichung, Taiwan). Finally, the sodium hydroxide treated sample was collected and employed for x-ray diffraction analysis and enzymatic hydrolysis.

## 2.3. X-ray diffraction (XRD) analysis of solid residues

The twin-extruder treated *E. pellita* and the solid residue, which originated the liquid hot water or/and sodium chlorite treatment, after the sodium hydroxide treatment were analyzed by X-ray diffractometer (D8 ADVANCE with DAVINCI, Bruker, Germany). And it equipped with a LYNXEYE XE detector and a sealed tube Cu Ka source with wavelength 1.5418 Å (40 kV voltage and 40 mA current by a generator). Scans were selected from 2θ = 3° to 50° with step size of 0.02 at 0.5 s/step (Jang et al., 2017a).

Crystallinity index was determined by Segal method (peak height ration method). It was calculated from the ration of the height of the 002 peak ($I_{002}$) and the height of the minimum ($I_{AM}$) between the 002 and the 110 peaks as following formula (Eq. 4-1).

$$\text{Crystallinity index (\%)} = \frac{I_{002} - I_{AM}}{I_{002}} \times 100 \qquad \text{(Eq. 4-1)}$$

$I_{002}$ = max intensity in 002 plane peak

$I_{AM}$ = min intensity between the 002 plane and 110 plane peaks

## 2.4. Enzymatic hydrolysis

The process of enzymatic hydrolysis and the calculation of glucose yield of solid residues after the sodium hydroxide treatment were described in section 2.5 of Chapter 2.

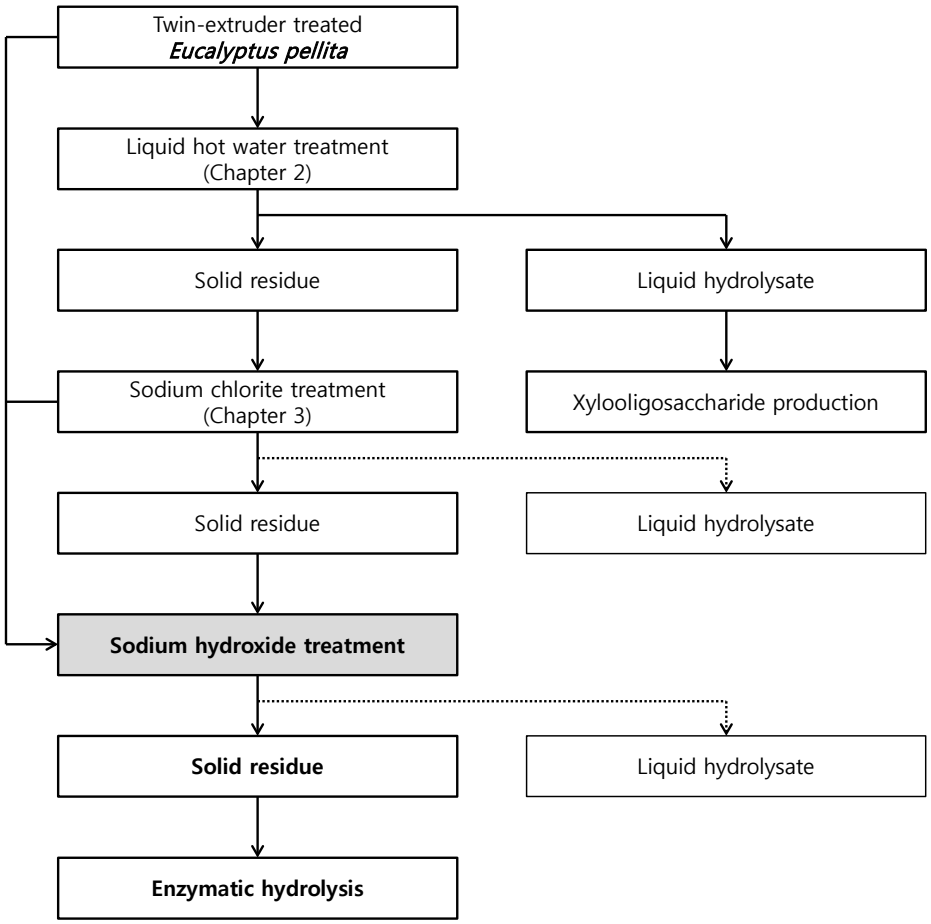



Figure 4-1. Schematic experimental flowchart of crystallinity change by sodium hydroxide treatment of liquid hot water and sodium chlorite treated *Eucalyptus pellita*.

# 3. Results and discussion

## 3.1. Crystallinity index of solid residues after liquid hot water treatment

The concentrated sodium hydroxide treatment was applied to three categories of feedstocks in this study; i) the raw material, *E. pellita*, which had no reaction, ii) the solid residue, which was treated by liquid hot water but had no reaction with sodium chlorite, and iii) the solid residue, which was treated by liquid hot water and sodium chlorite. The crystallinity index of *E. pellita* as well as only liquid hot water treated solid residues, which had same reaction condition in Chapter 3, before and after the sodium hydroxide treatment are summarized in Table 4-1.

After the liquid hot water treatment, the crystallinity index of solid residues was slightly increased under three conditions until 69.6% compared to that of *E. pellita* (59.7%) (Table 4-1). Hemicellulose fraction in the solid residues typically dissolved into liquid hydrolysate, which could be observed in the result of this study (Table 3-4). Therefore, the increase of crystallinity index might be explained by the result of hemicellulose removal because it consists of only amorphous regions (Sun et al., 2016). However, an additional increase of crystallinity index was not found as the reaction temperature rose from 160°C to 170°C.

Table 4-1. Crystallinity index of *Eucalyptus pellita* and the solid residues before sodium hydroxide treatment as a function of liquid hot water treatment condition

| Conditions | | Crystallinity index (%)[1] |
|---|---|---|
| Reaction temp. (°C) | Reaction time (min) | |
| *E. pellita* | | 59.7±0.2 |
| 160 | 10 | 65.6±0.1 |
| 160 | 50 | 69.6±0.5 |
| 170 | 50 | 68.9±0.3 |

Values are the mean ± standard deviation

[1] calculated by Segal method :crystallinity index (%)=$(I_{002}-I_{AM})/I_{002} \times 100$

After the sodium hydroxide treatment using 8% and 12% (w/w) of sodium hydroxide, the crystallinity index could not be calculated by Segal method. Since the X-ray diffractograms were obviously changed after the sodium hydroxide treatment, they were compared with the result of *E. pellita* (Figure 4-2). According to Figure 4-3A, the highest peak ($I_{002}$) was at around 22° and the minimum peak ($I_{AM}$) was at 18°. The highest peak was determined as the crystalline region of the sample. The diffractogram shape of *E. pellita* can be typically found in untreated lignocellulosic biomass.

However, the major peaks, which determine the crystallinity index ($I_{002}$ and $I_{AM}$), are obscured around from 18° to 22° under both (8% and 12%) conditions of sodium hydroxide treatment (Figure 4-2B and C). In addition, a general form of diffractogram had a significant difference before and after sodium hydroxide treatment using *E. pellita*. Therefore, a precise value of crystallinity index could not be deduced through the results of XRD after sodium hydroxide treatment. It is assumed that the cellulose structure of *E. pellita* changed, as the distinction between the crystalline and amorphous regions was unclear through sodium hydroxide treatment (Hashim et al., 2012).

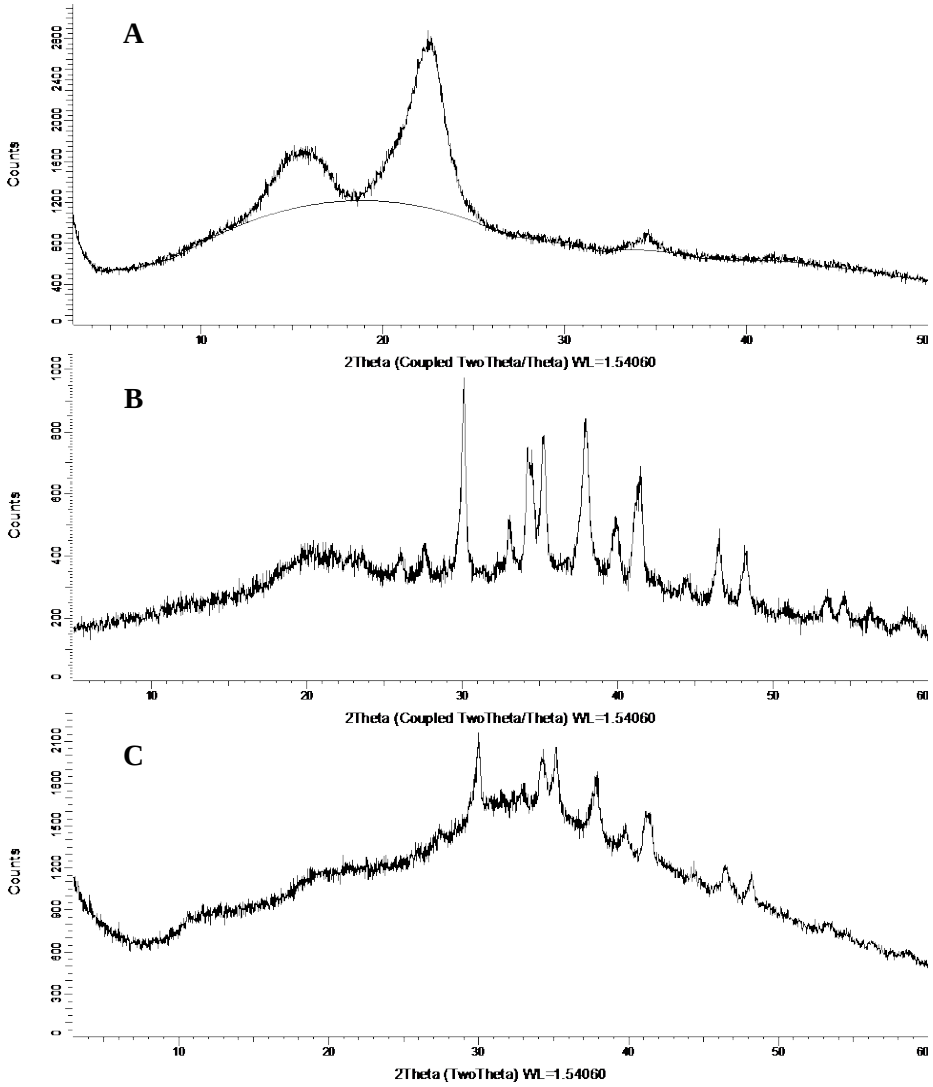



Figure 4-2. X-ray diffractograms of twin-extruder treated *Eucalyptus pellita* before (A) and after the sodium hydroxide treatment (B: 8% (w/w) of sodium hydroxide at 0°C for 60 min, C: 12% (w/w) of sodium hydroxide at 0°C for 180 min).

Meanwhile, the shape of X-ray diffractograms of the liquid hot water treated solid residue was similar with that of *E. pellita*, and both $I_{002}$ and $I_{AM}$ are presented in Figure 4-3A. Thus, hemicellulose removal was rarely affected in the crystalline region of the cellulose structure. However, the shape of X-ray diffractograms was considerably changed and the main peaks, such as $I_{002}$ and $I_{AM}$, at 18° to 22° were indistinct (Figure 4-3B and C). As demonstrated by results of XRD analysis, accurate determination of crystallinity index was impossible, while the crystalline region of cellulose structure definitely decomposed after treatment by 8% or 12% sodium hydroxide solution. It is thought that the cellulose lattice structure might significantly expand and swell with immersion in a concentrated alkaline solution.

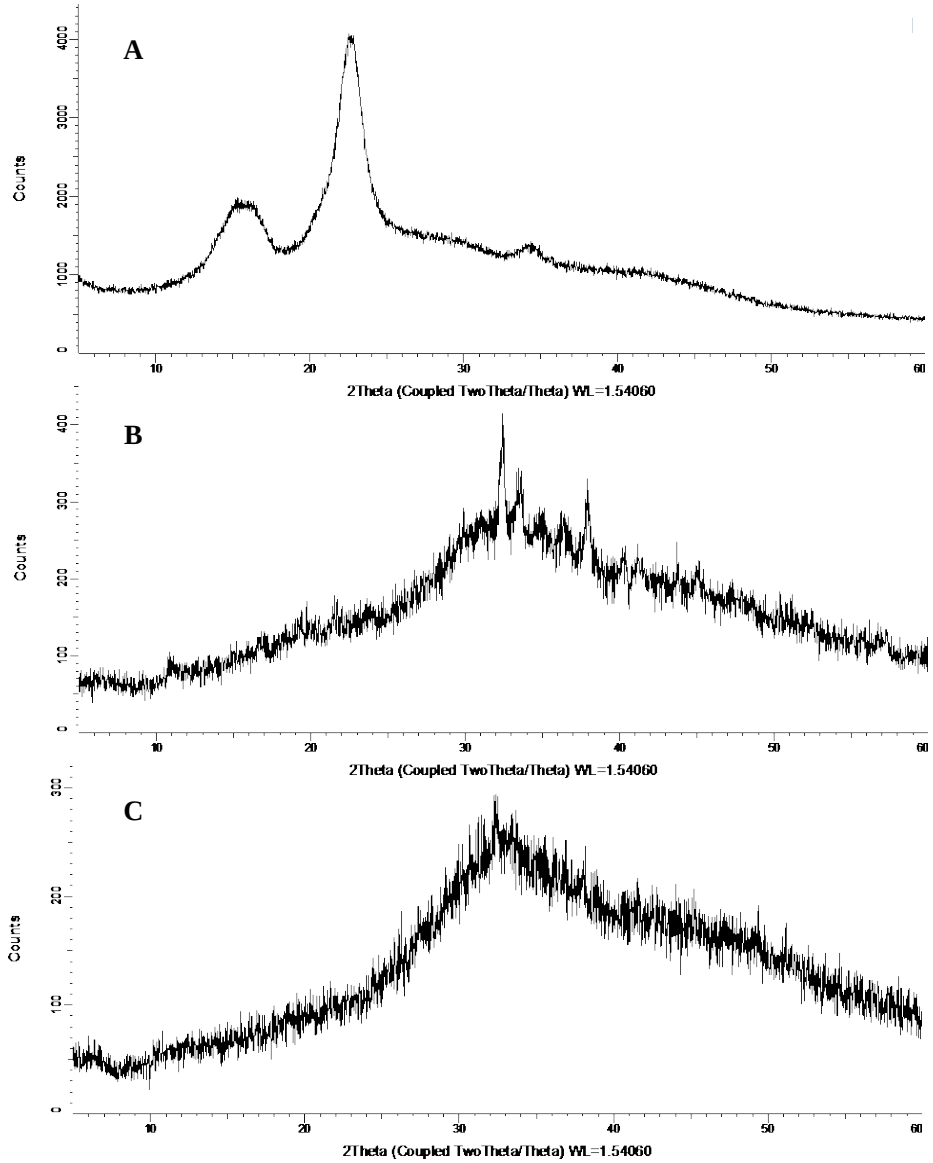



Figure 4-3. X-ray diffractograms of the liquid hot water treated solid residue before (A) and after the sodium hydroxide treatment (B: 8% (w/w) of sodium hydroxide at 0°C for 60 min, C: 12% (w/w) of sodium hydroxide at 0°C for 180 min).

The cellulose structure of lignocellulosic biomass typically has a monoclinic crystalline lattice of cellulose type I, and its structure can be modified to a different conformation by chemical treatment (John & Anandjiwala, 2008). When alkali treatment is conducted using lignocellulosic biomass (cellulose I), it commonly induced swelling of cellulose structure and lattice transformation into cellulose II (Hashim et al., 2012). In particular, sodium hydroxide treatment leads to extensive swelling. Because sodium ion ($Na^+$) has a suitable diameter for penetrating into the lattice plane, the reaction in cellulose expands the smallest pores among the distance of the lattice (Li et al., 2007). As a result of the above reaction, a new cellulose lattice structure, Na-cellulose I lattice, is formed by changing the hydroxyl groups in cellulose to O-Na groups, leading to enlargement of molecular dimension (Mwaikambo & Ansell, 2002). Thus, the Na-cellulose I lattice has a relatively large space among the cellulose molecules, and this space is sometimes filled with water (Sreenivasan et al., 1996). The lattice structure of cellulose I, II, and Na-Cellulose I are shown in Figure 4-4.

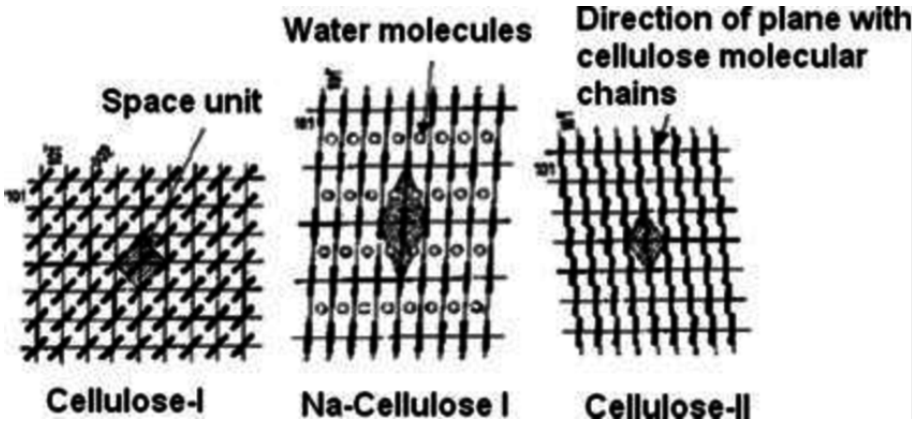



Figure 4-4. Lattice structure of cellulose I and cellulose II with sodium hydroxide treatment (Hashim et al., 2012).

## 3.2. Crystallinity index of solid residues after liquid hot water treatment with sodium chlorite treatment

The crystallinity index of the solid residues before concentrated sodium hydroxide treatment is stated in Table 4-2. These solid residues were treated by liquid hot water and sodium chlorite under a variety of conditions. As a result, the crystallinity index was gradually increased with increasing reagent (sodium chlorite and acetic acid) dose for the sodium chlorite treatment. For instance, 66.0% of crystallinity index (1.5 g and 0.3 mL of sodium chlorite and acetic acid respectively, under 25% of hemicellulose removal rate) was increased to 75.1% (4 g and 0.8 mL for 120 min (2 times input) of sodium chlorite and acetic acid, respectively). Meanwhile, the maximum crystallinity index was increased by an increase in hemicellulose removal rate. For instance, 66.7% of crystallinity index was the maximum value among 0% of hemicellulose removal rate conditions, but the maximum crystallinity index was determined as 81.5% under 75% of hemicellulose removal rate condition. Thus, the former result demonstrated that the delignification promotes an increase of crystallinity index, while hte latter result showed that the hemicellulose removal increases the crystallinity index, too. It is speculated that a space forms in the cell wall structure through the liquid hot water and sodium chlorite treatment, resulting in deformation including both shrinkage of size and development of new hydrogen bonds rather. These new hydrogen bonds are presumed to form during the washing process in the liquid hot water treatment and sodium chlorite treatment, since it was observed that the washing process during the sodium hydroxide treatment led to an increase in crystallinity index.

Table 4-2. Crystallinity index of the solid residues before sodium hydroxide treatment as a function of hemicellulose removal rate and sodium chlorite treatment condition

| Conditions | | | Crystallinity index (%)[2] |
|---|---|---|---|
| HRR[1] | Sodium chlorite (g) | Acetic acid (mL) | |
| 0% | 1.5 | 0.3 | 60.1±0.6 |
| | 2.5 | 0.5 | 62.4±1.1 |
| | 4 | 0.8 | 63.9±2.1 |
| | 4×2 | 0.8×2 | 66.7±1.2 |
| 25% | 1.5 | 0.3 | 66.0±1.6 |
| | 2.5 | 0.5 | 71.6±1.4 |
| | 4 | 0.8 | 72.7±1.7 |
| | 4×2 | 0.8×2 | 75.1±2.9 |
| 50% | 1.5 | 0.3 | 72.0±2.7 |
| | 2.5 | 0.5 | 75.1±0.9 |
| | 4 | 0.8 | 77.2±2.0 |
| | 4×2 | 0.8×2 | 79.2±3.0 |
| 75% | 1.5 | 0.3 | 71.6±1.9 |
| | 2.5 | 0.5 | 74.3±0.8 |
| | 4 | 0.8 | 79.7±1.1 |
| | 4×2 | 0.8×2 | 81.5±0.7 |

Values are the mean ± standard deviation

[1] HRR: hemicellulose removal rate (from solid residues obtained by liquid hot water treatment)

[2] calculated by Segal method :crystallinity index (%)=$(I_{002}-I_{AM})/I_{002} \times 100$

The X-ray diffractograms of the solid residue after only sodium chlorite treatment are presented in Figure 4-5. There was no significant change among the diffractograms as the reagent dose for sodium chlorite treatment increased from 1.5 g to 4 g with 2 times input. Additionally, the shape of the diffractograms was similar with that of *E. pellita* or liquid hot water treated solid residues. Therefore, sodium chlorite treatment as well as liquid hot water treatment might not affect the crystalline region in cellulose structure.

When the liquid hot water treatment and sodium chlorite treatment were sequentially performed, X-ray diffractograms of the solid residue were similar with those of only sodium chlorite treatment (Figure 4-6). However, the highest peak at 22°, which typically represented the crystalline region, was intensively detected compared to the case of only sodium chlorite treated solid residues. Thus, delignification through sodium chlorite with high hemicellulose removal might increase the crystallinity index by changing the cell wall structure from rigid to brittle.

Even though the crystallinity index was clearly increased after liquid hot water and sodium chlorite treatment, the glucose yield was increased dramatically with increasing reagent doses for sodium chlorite treatment (Table 3-9). This phenomenon could be understood as a characteristic of the cellulase (Cellic CTec2) used in this study. Cellic CTec2 is known as a cocktail enzyme for glucose production, consisting of endoglucanase, cellobiohydrolase, and β-glucosidase. In addition, the cocktail cellulase is designed for appropriate decomposition of amorphous and crystalline regions in cellulose fibril, and is superior to the previous version of commercial cellulase by Novozyme. Therefore, the increase of crystallinity index did not prevent glucose production after delignification using Cellic CTec2 in this study.

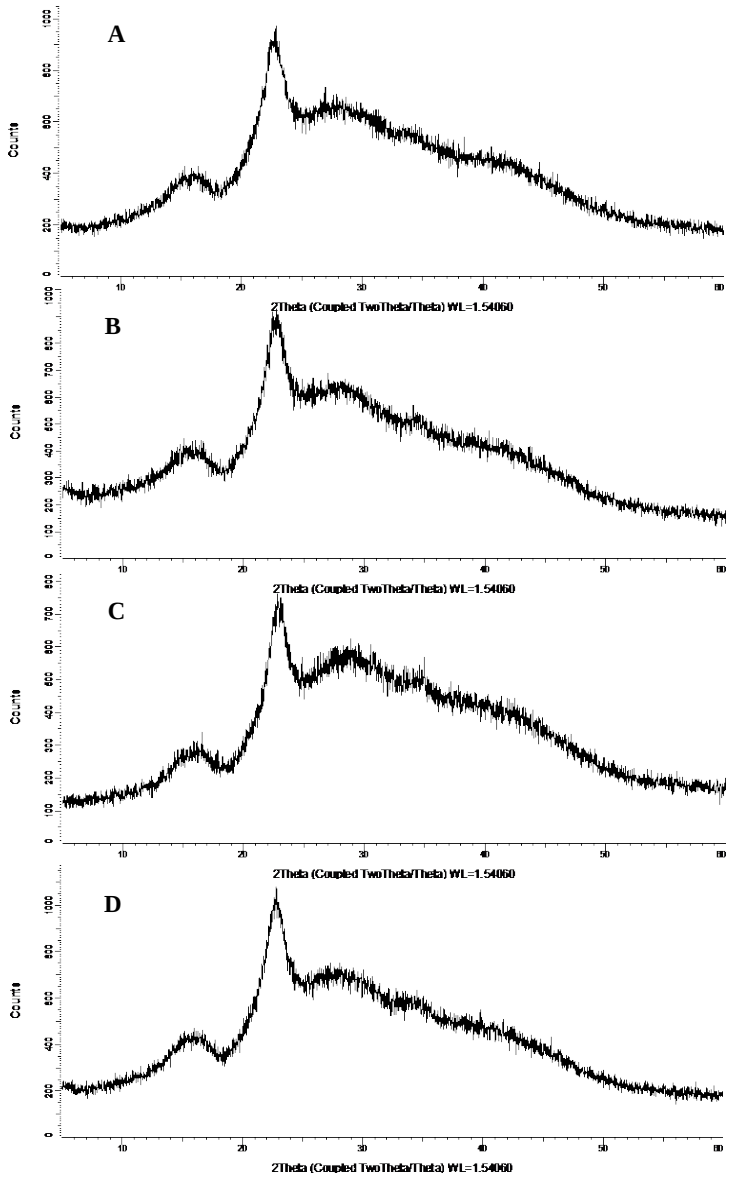



Figure 4-5. X-ray diffractograms of the sodium chlorite treated solid residues before sodium hydroxide treatment as a function of sodium chlorite and acetic acid dose (A: 1.5 g and 0.3 mL, B: 2.5 g and 0.5 mL, C: 4 g and 0.8 mL, D: 4 g and 0.8 mL with 2 times input)

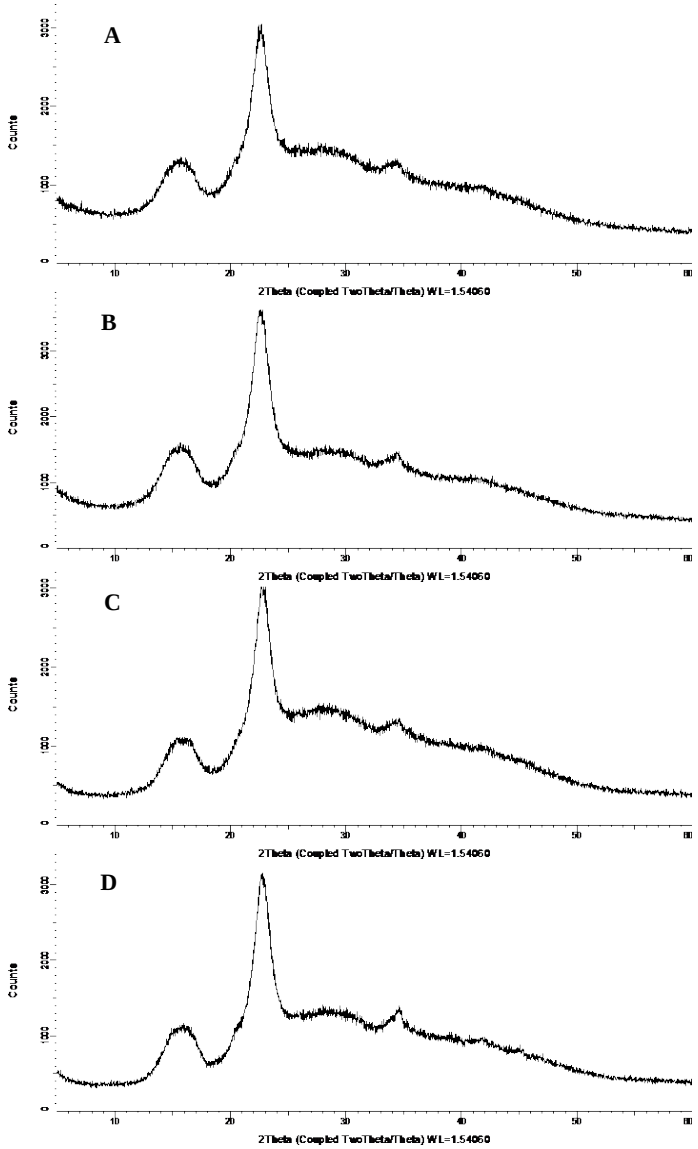



Figure 4-6. X-ray diffractograms of the liquid hot water (HRR: 75%) and sodium chlorite treated solid residues before sodium hydroxide treatment as function of sodium chlorite and acetic acid dose (A: 1.5 g and 0.3 mL, B: 2.5 g and 0.5 mL, C: 4 g and 0.8 mL, D: 4 g and 0.8 mL with 2 times input)

The liquid hot water and sodium chlorite treated solid residues were employed for sodium hydroxide treatment, and then X-ray diffraction analysis was conducted for determination of crystallinity index. The X-ray diffractograms of 8% (w/w) sodium hydroxide treatment are illustrated in Figure 4-7 and 4-8. The shape of X-ray diffractogram was quite different to that before sodium hydroxide treatment, but was similar that of *E. pellita* or liquid hot water treatment with sodium hydroxide treatment. Thus, the crystallinity index could not calculated by Segal method by disappearance of main peaks, which indicated cellulose crystallinity. In the case of low lignin removal rate, the highest peak remained at around 20° after three treatments (Figure 4-7B, C, and D). However, no peak was evident from 18° to 22° in the case of highly lignin-removed solid residue (Figure 4-8).

As the concentration of sodium hydroxide solution increased to 12%, the crystalline region of cellulose obviously swelled and decomposed, as shown in Figure 4-9. In addition, overall peak intensity was reduced in the X-ray diffractogram regardless of hemicellulose removal rate.

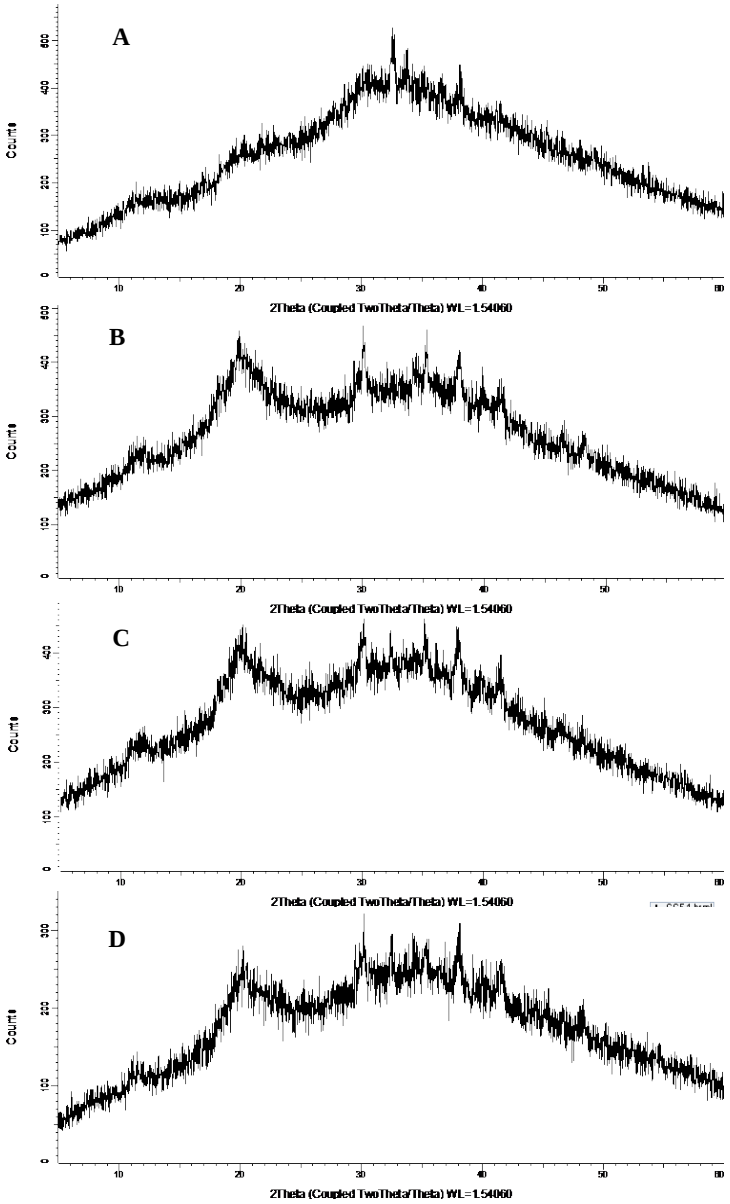



Figure 4-7. X-ray diffractograms of the liquid hot water and sodium chlorite treated (1.5 g of sodium chlorite and 0.3 mL of acetic acid) solid residues after sodium hydroxide treatment (8%) as a function of hemicellulose removal rate (A: 0%, B: 25%, C: 50%, D: 75%)

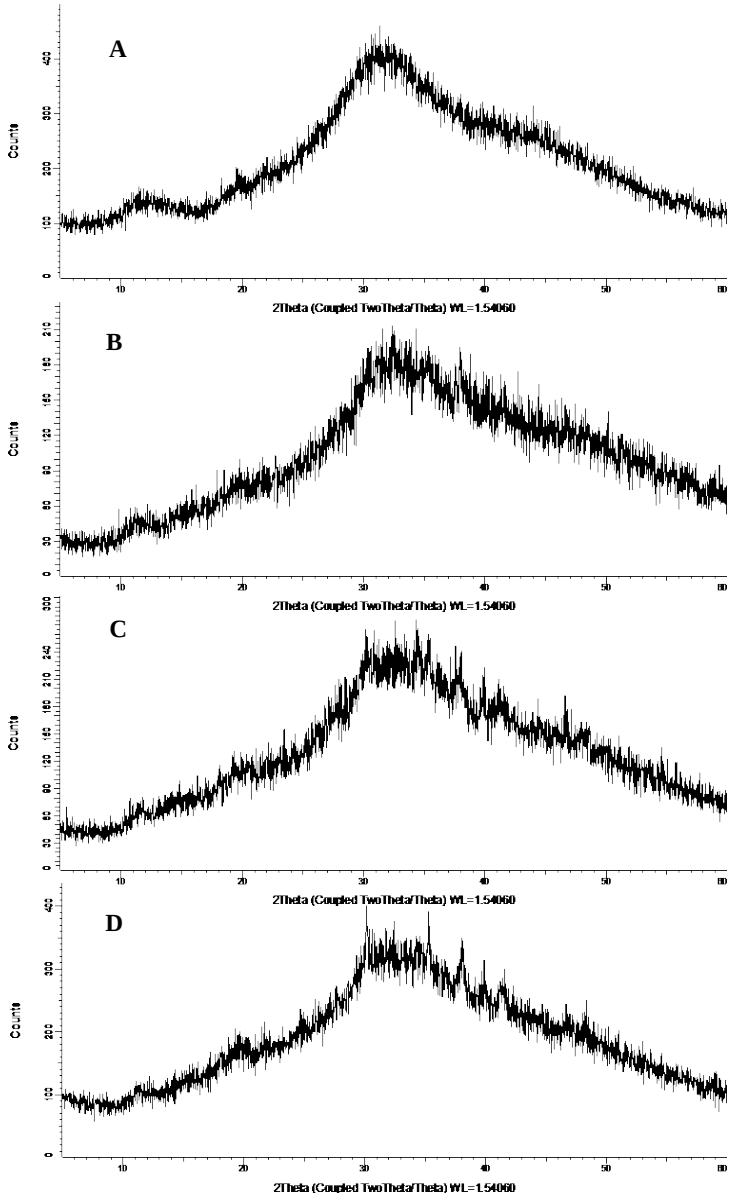



Figure 4-8. X-ray diffractograms of the liquid hot water and sodium chlorite treated (4 g of sodium chlorite and 0.8 mL of acetic acid) solid residues after sodium hydroxide treatment (8%) as a function of hemicellulose removal rate (A: 0%, B: 25%, C: 50%, D: 75%)

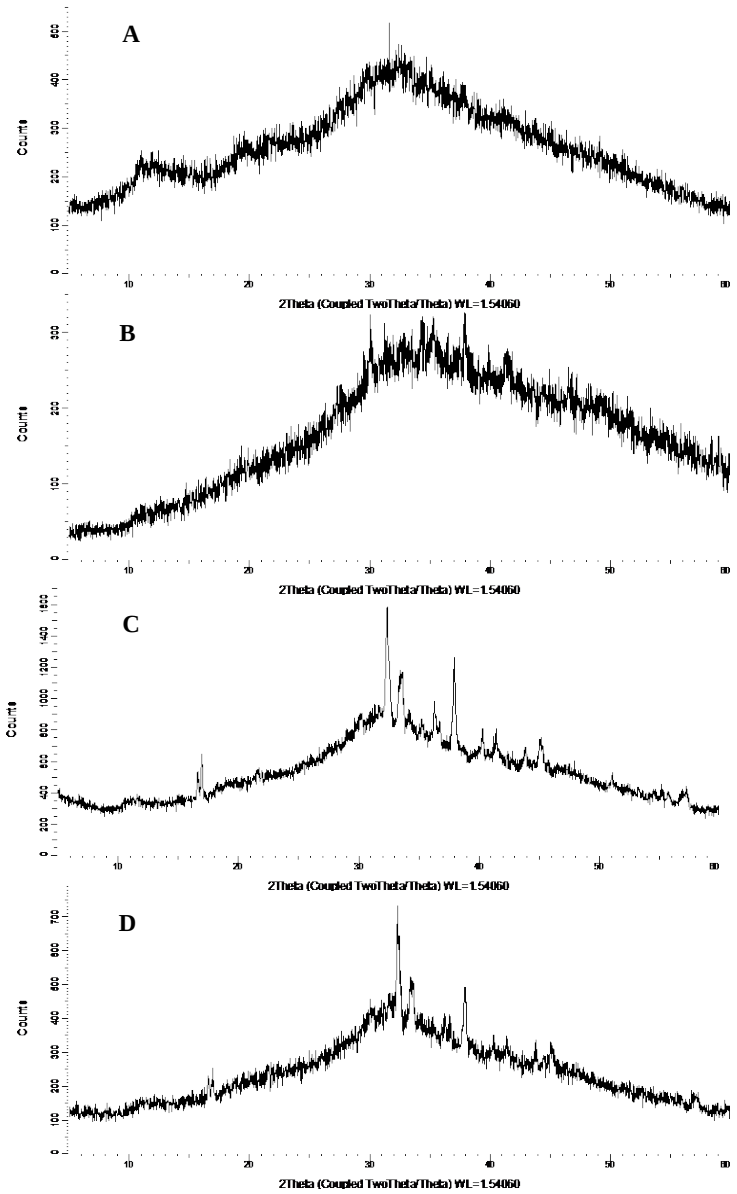



Figure 4-9. X-ray diffractograms of the liquid hot water and sodium chlorite treated (4 g of sodium chlorite and 0.8 mL of acetic acid) solid residues after sodium hydroxide treatment (12%) as a function of hemicellulose removal rate (A: 0%, B: 25%, C: 50%, D: 75%)

## 3.3. Enzymatic hydrolysis of solid residue

The glucose yield from *E. pellita* and the solid residues obtained by liquid hot water treatment after the sodium hydroxide treatment is listed in Table 4-5. The glucose yield was obviously increased from 0.5% (untreated *E. pellita*) to 36.9% (12% of sodium hydroxide treated *E. pellita*). Thus, the reduction of crystallinity index contributed to improvement of glucose production, and the result was better than that of the maximum glucose yield (28.2%) after liquid hot water treatment (Table 2-5). In the case of liquid hot water treatment before the sodium hydroxide treatment, the glucose yield was approximately 35%, which was maintained regardless of liquid hot water treatment conditions.

Meanwhile, the glucose yield of *E. pellita* and the liquid hot water treated solid residues was slightly increased with an increase of sodium hydroxide concentration from 8% and 12% as well as an extension of reaction time from 60 min to 180 min. A relaxation of crystalline structure is mitigated through a strong base catalyst that may enhance improvement of the glucose yield (Bledzki & Gassan, 1999). However, the glucose production mentioned above was not remarkable compared to that of the results using sodium chlorite treated solid residues.

Table 4-3. Glucose yield (%, based on the glucose content in the initial biomass) of the *Eucalyptus pellita* and the solid residues after sodium hydroxide treatment as a function of liquid hot water treatment condition

| Conditions | | Glucose yield (%) | |
|---|---|---|---|
| Reaction temp. (°C) | Reaction time (min) | 8% (w/w) NaOH at 0°C for 60 min | 12% (w/w) NaOH at 0°C for 180 min |
| *E. pellita* | | 30.5±2.2 | 36.9±1.3 |
| 160 | 10 | 34.7±1.5 | 39.1±0.7 |
| 160 | 50 | 34.1±0.2 | 38.9±2.1 |
| 170 | 50 | 35.8±1.1 | 39.9±1.3 |

Values are the mean ± standard deviation

The solid residues, which were treated by liquid hot water, sodium chlorite, and sodium hydroxide, were employed as substrates for enzymatic hydrolysis, and the results of glucose yield, are summarized in Table 4-6 (8% of sodium hydroxide at 0°C for 60 min) and Table 4-7 (12% of sodium hydroxide at 0°C for 180 min). Compared to the results of sodium chlorite treatment (Table 3-9), glucose yield was similar or slightly decreased after sodium hydroxide treatment (Table 4-6). For instance, the maximum glucose yield (74.6%) was observed after the sodium hydroxide (8%) treatment, while 87.5% of maximum glucose yield was obtained without the sodium hydroxide treatment. Moreover, a similar trend of the glucose yield could be found using 12% of sodium hydroxide treatment (Table 4-7), even though the glucose yield was generally higher than that of the results using 8% of sodium hydroxide treatment. It is speculated that the concentrated sodium hydroxide solution induced excessive degradation of cellulose followed by reduction of glucose yield after enzymatic hydrolysis.

Consequently, the effect of sodium hydroxide treatment was investigated in changing the crystallinity index and the glucose yield; however, the results were difficult to discriminate unlike the results of *E. pellita* and the solid residues, which were only treated by liquid hot water, since the reduction of crystallinity index was hard to control after the sodium chlorite treatment. As sodium chlorite and acetic acid dose increased, the crystallinity index was increased regardless of conditions of the sodium hydroxide treatment previously mentioned. In this situation, the glucose yields were strongly affected by the effect of lignin decomposition, and this trend questions whether crystallinity index is an important factor for enzymatic hydrolysis.

Table 4-4. Glucose yield (%, based on the glucose content in the initial biomass) of the solid residues after sodium hydroxide treatment (8%, w/w) at 0°C for 60 min as a function of liquid hot water and sodium chlorite treatment condition

| Conditions | | | Glucose yield (%) |
|---|---|---|---|
| HRR[1] | Sodium chlorite (g) | Acetic acid (mL) | |
| 0% | 1.5 | 0.3 | 17.5±1.2 |
| | 2.5 | 0.5 | 30.7±0.9 |
| | 4 | 0.8 | 48.9±1.1 |
| | 4×2 | 0.8×2 | 77.2±2.1 |
| 25% | 1.5 | 0.3 | 31.3±0.9 |
| | 2.5 | 0.5 | 57.2±3.2 |
| | 4 | 0.8 | 73.4±0.1 |
| | 4×2 | 0.8×2 | 74.6±1.8 |
| 50% | 1.5 | 0.3 | 30.9±1.6 |
| | 2.5 | 0.5 | 55.6±0.2 |
| | 4 | 0.8 | 71.1±0.8 |
| | 4×2 | 0.8×2 | 70.6±0.4 |
| 75% | 1.5 | 0.3 | 33.2±0.4 |
| | 2.5 | 0.5 | 56.5±0.5 |
| | 4 | 0.8 | 73.4±2.5 |
| | 4×2 | 0.8×2 | 73.1±0.5 |

Values are the mean ± standard deviation

[1] HRR: hemicellulose removal rate (from solid residues obtained by liquid hot water treatment)

Table 4-5. Glucose yield (%, based on the glucose content in the initial biomass) of the solid residues after sodium hydroxide treatment (12%, w/w) at 0°C for 180 min as a function of liquid hot water and sodium chlorite treatment condition

| Conditions | | | Glucose yield (%) |
|---|---|---|---|
| HRR[1] | Sodium chlorite (g) | Acetic acid (mL) | |
| 0% | 1.5 | 0.3 | 21.8±1.2 |
| | 2.5 | 0.5 | 36.2±2.8 |
| | 4 | 0.8 | 53.9±0.1 |
| | 4×2 | 0.8×2 | 76.3±3.1 |
| 25% | 1.5 | 0.3 | 36.8±0.2 |
| | 2.5 | 0.5 | 59.3±0.6 |
| | 4 | 0.8 | 74.1±1.7 |
| | 4×2 | 0.8×2 | 75.7±0.3 |
| 50% | 1.5 | 0.3 | 33.5±1.3 |
| | 2.5 | 0.5 | 60.0±1.2 |
| | 4 | 0.8 | 73.8±0.9 |
| | 4×2 | 0.8×2 | 71.3±2.4 |
| 75% | 1.5 | 0.3 | 35.6±1.4 |
| | 2.5 | 0.5 | 57.5±2.5 |
| | 4 | 0.8 | 76.2±0.7 |
| | 4×2 | 0.8×2 | 77.1±1.6 |

Values are the mean ± standard deviation

[1] HRR: hemicellulose removal rate (from solid residues obtained by liquid hot water treatment)

# 4. Conclusions

In this chapter, concentrated sodium hydroxide treatment was used to control the crystallinity index using a raw material (*Eucalyptus pellita*) and two pretreated solid residues (only liquid hot water treated solid residue, and liquid hot water and sodium chlorite treated solid residue). In addition, the glucose production (glucose yield) was evaluated depending on conditions of sodium hydroxide treatment and pretreatments.

The crystallinity index of *E. pellita* and liquid hot water treated solid residue (160°C for 50 min) was observed as 59.7% and 69.6%, respectively, before sodium hydroxide treatment. However, the crystallinity index after 8% or 12% of sodium hydroxide treatment could not be determined due to disappearance of main peaks, which represented the crystalline region of cellulose fibril, in X-ray diffractograms. Meanwhile, the glucose yield was increased form 0.5% to 36.9% after 12% of sodium hydroxide treatment using *E. pellita*. In the case of liquid hot water treated solid residue, the maximum glucose yield (39.9%) was obtained after 12% of sodium hydroxide treatment. Therefore, the concentrated sodium hydroxide treatment seemed to be effective for improving the glucose production without delignification.

The crystallinity index was remarkably increased up to 81.5% through the sodium chlorite treatment by increasing the dose of reagent (sodium chlorite and acetic acid). However, regarding the sodium hydroxide treatment, a significant change of X-ray diffractogram of the sodium chlorite treated solid residue that was similar with the results of *E. pellita* and liquid hot water treatment was found. Meanwhile, the sodium hydroxide treatment had a weak influence on the improvement of glucose production after the sodium chlorite treatment. The variation of glucose yield might be more seriously influenced by the lignin decomposition than crystallinity index, while overall glucose

yield was similar or slightly decreased after the sodium hydroxide treatment. Consequently, the modification of crystalline structure in cellulose through the sodium hydroxide treatment had a small impact on glucose production when the sodium chlorite treatment was applied for delignification using *E. pellita*.

# *Chapter 5*

## Concluding remarks

The recalcitrance, which is inherent characteristic of lignocellulosic biomass, has been considered as a major obstacle for the suitable utilization of lignocellulosic biomass. Because, biomass fractionation through damaging to cell wall structure using various pretreatment methods must be preceded for stable obtaining or conversion of the desired substance in lignocellulosic biomass. Generally, the cell wall structure is mainly consists of cellulose, hemicellulose, and lignin that are complex and tightly coupled together. Among the major component in cell wall, cellulose provides a necessity for active utilization and research of lignocellulosic biomass. Since, glucose can be produced through hydrolysis process using cellulose, and it can be converted to ethanol for transportation fuel or various material for applying industry, pharmaceutical, and food market. Therefore, the improvement of glucose production was investigated through controlling the recalcitrant factors such as an existence of hemicellulose and lignin, and crystalline region in cellulose fibril that recognize as recalcitrant factors in previous studies. Meanwhile, xylooligosaccharide, which is attractive product as prebiotics, conversion was evaluated after hemicellulose removal process. Finally, the effect of recalcitrant factors for glucose production was determined by correlation and regression analysis.

For investigating the xylooligosaccharide (xylobiose, xylotriose, xylotetraose, xylopentaose, and xylohexaose) conversion and the effect of hemicellulose removal for glucose production, liquid hot water treatment was performed under various conditions using *Eucalyptus pellita*, highly recalcitrant lignocellulosic biomass from tropical region. The maximum amount of total xylooligosaccharide (8.3%) in the liquid hydrolysate was obtained at 170°C for 50 min condition, and it was corresponded to 67.2% of initial amount of xylan in *E. pellita*. On the other hand, xylobiose was greatly produced than other xylooligosaccharide even xylose, and its maximum

content was observed as 3.0% at 180°C for 30 min condition. In addition, xylotetraose, xylopentaose, and xylohexaose, which are difficult to produce in conventional process using xylanase, were converted properly during liquid hot water treatment under 170°C for 50 min condition. Furthermore, the xylooligosaccharide has more high DP that hardly maintained its chain length, then easily decomposed into low DP xylooligosaccharide or xylose. Meanwhile, xylan elimination through liquid hot water treatment was successfully preceded, and most of xylan in the solid residue was removed at over 180°C conditions. After the enzymatic hydrolysis, the maximum value of glucose yield was 28.2% from the solid residue, which is treated by liquid hot water under 190°C for 50 min condition. Therefore, significant conversion of xylooligosaccharide was observed after liquid hot water treatment in spite of requirement of separation or purification process. However, the glucose production was relatively low even though entire hemicellulose fraction in *E. pellita* dissolved into the liquid hydrolysate. The correlation analysis and regression modeling between the biomass constituents (hemicellulose and lignin) and glucose yield corresponding in same reaction condition was conducted using computer program (SAS). According to correlation analysis of single factor, adjust R-squared value was presented through linear regression model as 0.6768 (hemicellulose removal rate) and 0.0349 (lignin removal rate), respectively. Meanwhile, 0.7717 of adjust R-squared value was observed through correlation analysis of double factor. Therefore, hemicellulose removal rate, which controlled by liquid hot water treatment, has influence for glucose production, but over 40% of glucose yield cannot be expected as increase of hemicellulose removal rate according to the linear regression model.

Sodium chlorite with acetic acid treatment (acid-chlorite delignification) was conducted for evaluating the effect of lignin decomposition on glucose

production. And, the solid residues with or without liquid hot water treatment was employed the sodium chlorite treatment for investigating single or combined effect of lignin removal. In case of untreated feedstock, the glucan and xylan in the solid residues did not significantly decompose and its content maintained constant value (approximately 49% and 10%) that similar with *E. pellita* under all conditions of sodium chlorite treatment. On the other hand, the amount of total lignin was gradually decreased from 33.3% to 9.0% as increase of reagent (sodium chlorite and acetic acid) dose and number of reagent input even though acid-soluble lignin was increased than that of *E. pellita*. Meanwhile, the glucose yield using sodium chlorite treated solid residue was steadily increased from 11.5% to 83.9% as larger reagent dose.

Moreover, solid residue, which prepared to control the hemicellulose content by liquid hot water treatment, was adapted to the sodium chlorite treatment for evaluating the combined effect of lignin and hemicellulose on glucose production. The amount of glucan was slightly decreased at about 47% after liquid hot water treatment, but the content was roughly kept under all conditions of the sodium chlorite treatment. Meanwhile, the total lignin content was remarkably decreased from 28.5% to 0.3% due to the intensive reduction of amount of Klason and acid-soluble lignin, simultaneously. After enzymatic hydrolysis using the solid residue obtained through the both reaction, the glucose yield was increased sharply from 14.7% to 86.2% that similar results with using the untreated solid residue. Consequently, the selective decomposition of lignin in biomass was obtained through the sodium chlorite that is appropriate for evaluating the effect of lignin content on glucose production. And, high glucose yield over about 80% could be observed at the solid residues, which had below 10% of total lignin content. After the correlation analysis between single factor and glucose yield, adjust R-squared value of hemicellulose removal rate was 0.0936, while that of

lignin removal rate was 0.9063 that were determined using empirical data obtained Chapter 3. The result of regression analysis through the sodium chlorite treatment was opposite with the results of liquid hot water treatment in Chapter 2. According to the correlation analysis with double factor, high adjust R-squared value (0.9285) for glucose production was determined that validates a strong influence of lignin removal regardless of hemicellulose content of *E. pellita*.

For reducing the crystallinity index, the concentrated sodium hydroxide treatment was performed using the solid residue controlled the amount of hemicellulose and lignin. In case of *E. pellita*, the crystallinity index was slightly increased from 59.7% to 69.6% after liquid hot water treatment, and typical shape of X-ray diffractograms for biomass were appeared. However, change of crystallinity index through the sodium hydroxide treatment could be not investigated due to excessive swelling in crystalline region of cellulose fibril. Meanwhile, the glucose yield was improved as 30.5% and 36.9% using *E. pellita* after 8% and 12% sodium hydroxide treatment, respectively. In addition, up to 39.9% of glucose yield was obtained after the sodium hydroxide treatment using liquid hot water treated solid residue. But, in case of sodium chlorite treated solid residues, the glucose yield was increased as an increase of reagent input for the sodium chlorite treatment regardless of changing the crystallinity index.

In this study, the high value added utilization of lignocellulosic biomass, *E. pellita*, was confirmed by investigation of suitable xylooligosaccharide production. Furthermore, three method of treatment such as liquid hot water treatment, sodium chlorite treatment, and sodium hydroxide was employed for controlling the hemicellulose, lignin, and crystallinity index, respectively. According to result of correlation and regression analysis, lignin in biomass is a critical factor that statistically proved for appropriate glucose production

regardless of hemicellulose content or crystallinity index using high recalcitrant species. Although the sodium chlorite treatment is very effective for selective lignin decomposition, recovery and utilization of lignin technology has not been considered very much after the treatment. However, relatively high yield of xylooligosaccharide and glucose could be achieved within the sequential treatment processes. And, the result of this study expected to be applicable to other high recalcitrant lignocellulosic biomass as well.

# *References*

Aachary, A.A., Prapulla, S.G. 2008. Corncob-induced endo-1, 4-β-D-xylanase of Aspergillus oryzae MTCC 5154: production and characterization of xylobiose from glucuronoxylan. *Journal of agricultural and food chemistry*, **56**(11), 3981-3988.

Aachary, A.A., Prapulla, S.G. 2009. Value addition to corncob: production and characterization of xylooligosaccharides from alkali pretreated lignin-saccharide complex using Aspergillus oryzae MTCC 5154. *Bioresource technology*, **100**(2), 991-995.

Aachary, A.A., Prapulla, S.G. 2011. Xylooligosaccharides (XOS) as an emerging prebiotic: microbial synthesis, utilization, structural characterization, bioactive properties, and applications. *Comprehensive Reviews in Food Science and Food Safety*, **10**(1), 2-16.

Abdel-Halim, E. 2014. Chemical modification of cellulose extracted from sugarcane bagasse: Preparation of hydroxyethyl cellulose. *Arabian Journal of Chemistry*, **7**(3), 362-371.

Agarwal, A.K. 2007. Biofuels (alcohols and biodiesel) applications as fuels for internal combustion engines. *Progress in energy and combustion science*, **33**(3), 233-271.

Agarwal, U.P. 2006. Raman imaging to investigate ultrastructure and composition of plant cell walls: distribution of lignin and cellulose in black spruce wood (Picea mariana). *Planta*, **224**(5), 1141.

Ahlgren, P., Goring, D. 1971. Removal of wood components during chlorite delignification of black spruce. *Canadian Journal of Chemistry*, **49**(8), 1272-1275.

Akpinar, O., Erdogan, K., Bakir, U., Yilmaz, L. 2010. Comparison of acid and enzymatic hydrolysis of tobacco stalk xylan for preparation of xylooligosaccharides. *LWT-Food Science and Technology*, **43**(1), 119-125.

Akpinar, O., Erdogan, K., Bostanci, S. 2009. Production of xylooligosaccharides by controlled acid hydrolysis of lignocellulosic materials. *Carbohydrate Research*, **344**(5), 660-666.

Alvira, P., Tomás-Pejó, E., Ballesteros, M., Negro, M. 2010. Pretreatment technologies for an efficient bioethanol production process based on enzymatic hydrolysis: a review. *Bioresource technology*, **101**(13), 4851-4861.

Ando, H., Morita, S.-i., Furukawa, I., Kamino, Y., Sasaki, T., Hirosue, H. 2003. Generation of xylooligosaccharides from moso bamboo (Phyllostachys pubescens) using hot compressed water. *Journal of the Japan Wood Research Society (Japan)*.

Araque, E., Parra, C., Freer, J., Contreras, D., Rodríguez, J., Mendonça, R., Baeza, J. 2008. Evaluation of organosolv pretreatment for the conversion of Pinus radiata D. Don to ethanol. *Enzyme and Microbial Technology*, **43**(2), 214-219.

Ayyappan, A.A., Prapulla, S. 2011. Xylooligosaccharides (XOS) as an Emerging Prebiotic: Microbial Synthesis, Utilization, Structural Characterization, Bioactive Properties, and Applications. *Comprehensive Reviews in Food Science and Food Safety*, **10**, 2-16.

Balat, M. 2011. Production of bioethanol from lignocellulosic materials via the biochemical pathway: a review. *Energy conversion and management*, **52**(2), 858-875.

Barakat, A., Monlau, F., Solhy, A., Carrere, H. 2015. Mechanical dissociation and fragmentation of lignocellulosic biomass: Effect of initial moisture, biochemical and structural proprieties on energy requirement. *Applied Energy*, **142**, 240-246.

Barreteau, H., Delattre, C., Michaud, P. 2006. Production of Oligosaccharides as Promising New Food Additive Generation. *Food Technology & Biotechnology*, **44**(3).

Berlin, A., Balakshin, M., Gilkes, N., Kadla, J., Maximenko, V., Kubo, S., Saddler, J. 2006. Inhibition of cellulase, xylanase and β-glucosidase activities by softwood lignin preparations. *Journal of Biotechnology*, **125**(2), 198-209.

Bledzki, A., Gassan, J. 1999. Composites reinforced with cellulose based fibres. *Progress in polymer science*, **24**(2), 221-274.

Bohlmann, G.M. 2006. Process economic considerations for production of ethanol from biomass feedstocks. *Industrial Biotechnology*, **2**(1), 14-20.

Boussaid, A.-L., Esteghlalian, A.R., Gregg, D.J., Lee, K.H., Saddler, J.N. 2000. Steam pretreatment of Douglas-fir wood chips. *Twenty-First Symposium on Biotechnology for Fuels and Chemicals*. Springer. pp. 693-705.

Brienzo, M., Carvalho, W., Milagres, A.M. 2010. Xylooligosaccharides production from alkali-pretreated sugarcane bagasse using xylanases from Thermoascus aurantiacus. *Applied biochemistry and biotechnology*, **162**(4), 1195-1205.

Brienzo, M., Siqueira, A., Milagres, A.M.F. 2009. Search for optimum conditions of sugarcane bagasse hemicellulose extraction. *Biochemical Engineering Journal*, **46**(2), 199-204.

Cara, C., Romero, I., Oliva, J.M., Sáez, F., Castro, E. 2007. Liquid hot water pretreatment of olive tree pruning residues. in: *Applied Biochemistry and Biotecnology*, Springer, pp. 379-394.

Carvalho, A.F.A., de Oliva Neto, P., Da Silva, D.F., Pastore, G.M. 2013. Xylo-oligosaccharides from lignocellulosic materials: chemical structure, health benefits and production by chemical and enzymatic hydrolysis. *Food Research International*, **51**(1), 75-85.

Chabannes, M., Ruel, K., Yoshinaga, A., Chabbert, B., Jauneau, A., Joseleau, J.P., Boudet, A.M. 2001. In situ analysis of lignins in transgenic tobacco reveals a differential impact of individual transformations on the spatial patterns of lignin deposition at the cellular and subcellular levels. *The Plant Journal*, **28**(3), 271-282.

Chandra, R.P., Bura, R., Mabee, W., Berlin, d.A., Pan, X., Saddler, J. 2007. Substrate pretreatment: The key to effective enzymatic hydrolysis of lignocellulosics? in: *Biofuels*, Springer, pp. 67-93.

Chang, V.S., Holtzapple, M.T. 2000a. Fundamental factors affecting biomass enzymatic reactivity. *Applied biochemistry and biotechnology*, **84**(1), 5-37.

Chang, V.S., Holtzapple, M.T. 2000b. Fundamental factors affecting biomass enzymatic reactivity. *Twenty-first symposium on biotechnology for fuels and chemicals*. Springer. pp. 5-37.

Chapla, D., Pandit, P., Shah, A. 2012. Production of xylooligosaccharides from corncob xylan by fungal xylanase and their utilization by probiotics. *Bioresource Technology*, **115**, 215-221.

Chen, Z., Zhang, W., Xu, J., Li, P. 2015. Kinetics of xylose dehydration into furfural in acetic acid. *Chinese Journal of Chemical Engineering*, **23**(4), 659-666.

Chundawat, S.P., Bellesia, G., Uppugundla, N., da Costa Sousa, L., Gao, D., Cheh, A.M., Agarwal, U.P., Bianchetti, C.M., Phillips Jr, G.N., Langan, P. 2011. Restructuring the crystalline cellulose hydrogen bond network enhances its depolymerization rate. *Journal of the American Chemical Society*, **133**(29), 11163-11174.

Chung, Y.-C., Hsu, C.-K., Ko, C.-Y., Chan, Y.-C. 2007. Dietary intake of xylooligosaccharides improves the intestinal microbiota, fecal moisture, and pH value in the elderly. *Nutrition Research*, **27**(12), 756-761.

Chylińska, M., Szymańska-Chargot, M., Zdunek, A. 2016. FT-IR and FT-Raman characterization of non-cellulosic polysaccharides fractions isolated from plant cell wall. *Carbohydrate polymers*, **154**, 48-54.

Converse, A., Ooshima, H., Burns, D. 1990. Kinetics of enzymatic hydrolysis of lignocellulosic materials based on surface area of cellulose accessible to enzyme and enzyme adsorption on lignin and cellulose. *Applied Biochemistry and Biotechnology*, **24**(1), 67-73.

Danon, B., Marcotullio, G., de Jong, W. 2014. Mechanistic and kinetic aspects of pentose dehydration towards furfural in aqueous media employing homogeneous catalysis. *Green Chemistry*, **16**(1), 39-54.

Das, N.N., Das, S.C., Sarkar, A.K., Mukherjee, A.K. 1984. Lignin-xylan ester linkage in mesta fiber (Hibiscus cannabinus). *Carbohydrate research*, **129**, 197-207.

Delgado, G.T.C., Tamashiro, W.M.d.S.C., Junior, M.R.M., Moreno, Y.M.F.,

Pastore, G.M. 2011. The putative effects of prebiotics as immunomodulatory agents. *Food Research International*, **44**(10), 3167-3173.

Demirbas, A. 2009. Progress and recent trends in biodiesel fuels. *Energy conversion and management*, **50**(1), 14-34.

Edgar, C.D., Mansfield, S.D., Gübitz, G.M., Saddler, J.N. 1998. The Synergistic effects of endoglucanase and xylanase in modifying Douglas fir kraft pulp, ACS Publications.

Eifert, T., Liauw, M. 2016. Process analytical technology (PAT) applied to biomass valorisation: a kinetic study on the multiphase dehydration of xylose to furfural. *Reaction Chemistry & Engineering*, **1**(5), 521-532.

Endo, M., Kuroda, Y. 2000. Production method of xylose and xylooligosaccharides from natural compounds containing xylan by hot water pretreatment. *JP Patent*, **2000236899**.

Enslow, K.R., Bell, A.T. 2012. The kinetics of Brønsted acid-catalyzed hydrolysis of hemicellulose dissolved in 1-ethyl-3-methylimidazolium chloride. *RSC Advances*, **2**(26), 10028-10036.

Ertas, M., Han, Q., Jameel, H., Chang, H.-m. 2014. Enzymatic hydrolysis of autohydrolyzed wheat straw followed by refining to produce fermentable sugars. *Bioresource technology*, **152**, 259-266.

Escobar, J.C., Lora, E.S., Venturini, O.J., Yáñez, E.E., Castillo, E.F., Almazan, O. 2009. Biofuels: environment, technology and food security. *Renewable and sustainable energy reviews*, **13**(6), 1275-1287.

Esteghlalian, A., Hashimoto, A.G., Fenske, J.J., Penner, M.H. 1997. Modeling and optimization of the dilute-sulfuric-acid pretreatment of corn stover, poplar and switchgrass. *Bioresource Technology*, **59**(2-3), 129-136.

Fan, L., Lee, Y.H., Beardmore, D. 1981. The influence of major structural features of cellulose on rate of enzymatic hydrolysis. *Biotechnology and Bioengineering*, **23**(2), 419-424.

Fan, L., Lee, Y.H., Beardmore, D.H. 1980. Mechanism of the enzymatic

hydrolysis of cellulose: effects of major structural features of cellulose on enzymatic hydrolysis. *Biotechnology and Bioengineering*, **22**(1), 177-199.

Feather, M.S., Harris, D.W., Nichols, S.B. 1972. Routes of conversion of D-xylose, hexuronic acids, and L-ascorbic acid to 2-furaldehyde. *The Journal of Organic Chemistry*, **37**(10), 1606-1608.

Fernandez-Bolanos, J., Felizon, B., Heredia, A., Rodrıguez, R., Guillen, R., Jimenez, A. 2001. Steam-explosion of olive stones: hemicellulose solubilization and enhancement of enzymatic hydrolysis of cellulose. *Bioresource Technology*, **79**(1), 53-61.

Filiciotto, L., Balu, A.M., Van der Waal, J.C., Luque, R. 2017. Catalytic insights into the production of biomass-derived side products methyl levulinate, furfural and humins. *Catalysis Today*.

Florence, R.G. 2004. *Ecology and silviculture of eucalypt forests*. Csiro Publishing.

Garrote, G., Dominguez, H., Parajó, J.C. 1999. Mild autohydrolysis: an environmentally friendly technology for xylooligosaccharide production from wood. *Journal of Chemical technology and Biotechnology*, **74**(11), 1101-1109.

Garrote, G., Parajó, J. 2002. Non-isothermal autohydrolysis of Eucalyptus wood. *Wood Science and Technology*, **36**(2), 111-123.

George, J., Sreekala, M., Thomas, S. 2001. A review on interface modification and characterization of natural fiber reinforced plastic composites. *Polymer Engineering & Science*, **41**(9), 1471-1485.

Girisuta, B., Janssen, L., Heeres, H. 2006. A kinetic study on the decomposition of 5-hydroxymethylfurfural into levulinic acid. *Green Chemistry*, **8**(8), 701-709.

Gomez, L.D., Steele-King, C.G., McQueen-Mason, S.J. 2008. Sustainable liquid biofuels from biomass: the writing's on the walls. *New Phytologist*, **178**(3), 473-485.

Gorshkova, T., Nikolovski, N., Finaev, D. 2005. Plant cell wall is a stumbling

stone for molecular biologists. *Russian Journal of Plant Physiology*, **52**(3), 392-409.

Grethlein, H.E., Converse, A.O. 1991. Common aspects of acid prehydrolysis and steam explosion for pretreating wood. *Bioresource technology*, **36**(1), 77-82.

Grootaert, C., Delcour, J.A., Courtin, C.M., Broekaert, W.F., Verstraete, W., Van de Wiele, T. 2007. Microbial metabolism and prebiotic potency of arabinoxylan oligosaccharides in the human intestine. *Trends in Food Science & Technology*, **18**(2), 64-71.

Gullón, P., Moura, P., Esteves, M.a.P., Girio, F.M., Domínguez, H., Parajó, J.C. 2008. Assessment on the fermentability of xylooligosaccharides from rice husks by probiotic bacteria. *Journal of agricultural and food chemistry*, **56**(16), 7482-7487.

Guo, Y., Zhou, J., Wen, J., Sun, G., Sun, Y. 2015. Structural transformations of triploid of Populus tomentosa Carr. lignin during auto-catalyzed ethanol organosolv pretreatment. *Industrial Crops and Products*, **76**, 522-529.

Hall, M., Bansal, P., Lee, J.H., Realff, M.J., Bommarius, A.S. 2010. Cellulose crystallinity–a key predictor of the enzymatic hydrolysis rate. *The FEBS journal*, **277**(6), 1571-1582.

Harkin, J.M., Rowe, J.W. 1971. Bark and its possible uses.

Harmsen, P., Huijgen, W., Bermudez, L., Bakker, R. 2010. Literature review of physical and chemical pretreatment processes for lignocellulosic biomass. *Energy Research Centre of the Netherlands*, 10-13.

Harris, D., DeBolt, S. 2010. Synthesis, regulation and utilization of lignocellulosic biomass. *Plant biotechnology journal*, **8**(3), 244-262.

Hashim, M.Y., Roslan, M.N., Amin, A.M., Zaidi, A.M.A., Ariffin, S. 2012. Mercerization treatment parameter effect on natural fiber reinforced polymer matrix composite: A brief review. *World academy of science, engineering and technology*, **68**, 1638-1644.

He, X., Miao, Y., Jiang, X., Xu, Z., Ouyang, P. 2010. Enhancing the enzymatic

hydrolysis of corn stover by an integrated wet-milling and alkali pretreatment. *Applied biochemistry and biotechnology*, **160**(8), 2449-2457.

Hendriks, A., Zeeman, G. 2009. Pretreatments to enhance the digestibility of lignocellulosic biomass. *Bioresource technology*, **100**(1), 10-18.

Himmel, M., Vinzant, T., Bower, S., Jechura, J. 2005. BSCL use plan: solving biomass recalcitrance. National Renewable Energy Laboratory (NREL), Golden, CO.

Himmel, M.E., Ding, S.-Y., Johnson, D.K., Adney, W.S., Nimlos, M.R., Brady, J.W., Foust, T.D. 2007. Biomass recalcitrance: engineering plants and enzymes for biofuels production. *science*, **315**(5813), 804-807.

Holtzapple, M.T., Ripley, E.P., Nikolaou, M. 1994. Saccharification, fermentation, and protein recovery from low-temperature AFEX-treated coastal bermudagrass. *Biotechnology and bioengineering*, **44**(9), 1122-1131.

Hoshino, E., Shiroishi, M., Amano, Y., Nomura, M., Kanda, T. 1997. Synergistic actions of exo-type cellulases in the hydrolysis of cellulose with different crystallinities. *Journal of Fermentation and Bioengineering*, **84**(4), 300-306.

Hsu, T.-C., Guo, G.-L., Chen, W.-H., Hwang, W.-S. 2010. Effect of dilute acid pretreatment of rice straw on structural properties and enzymatic hydrolysis. *Bioresource technology*, **101**(13), 4907-4913.

Hu, X., Lievens, C., Larcher, A., Li, C.-Z. 2011. Reaction pathways of glucose during esterification: Effects of reaction parameters on the formation of humin type polymers. *Bioresource technology*, **102**(21), 10104-10113.

Hubbell, C.A., Ragauskas, A.J. 2010. Effect of acid-chlorite delignification on cellulose degree of polymerization. *Bioresource Technology*, **101**(19), 7410-7415.

Hughes, S., Shewry, P., Li, L., Gibson, G., Sanz, M., Rastall, R. 2007. In vitro fermentation by human fecal microflora of wheat arabinoxylans. *Journal of agricultural and food chemistry*, **55**(11), 4589-4595.

Ikemizu, S., Azumi, N. 2002. Melanin formation inhibitors containing acidic xylooligosaccharides. *Japan Patent JP*, **200**.

Imadi, S.R., Kazi, A.G. 2015. Extraction of lignin from biomass for biofuel production. in: *Agricultural Biomass Based Potential Materials*, Springer, pp. 391-402.

Izumi, K., Azumi, N. 2001. Xylooligosaccharide compositions useful as food and feed additives. *Japan Patent JP*, **2**(001), 226,409.

Izumi, Y., Ikemizu, S., Shizuka, F. 2004. Intestinal environment improving agents containing acidic xylooligosaccharides. *Patent Japanese*, **2004182609**.

Jørgensen, H., Kristensen, J.B., Felby, C. 2007. Enzymatic conversion of lignocellulose into fermentable sugars: challenges and opportunities. *Biofuels, Bioproducts and Biorefining*, **1**(2), 119-134.

Jacobs, A., Palm, M., Zacchi, G., Dahlman, O. 2003. Isolation and characterization of water-soluble hemicelluloses from flax shive. *Carbohydrate research*, **338**(18), 1869-1876.

Jacobsen, S.E., Wyman, C.E. 2002. Xylose monomer and oligomer yields for uncatalyzed hydrolysis of sugarcane bagasse hemicellulose at varying solids concentration. *Industrial & engineering chemistry research*, **41**(6), 1454-1461.

Jang, S.-K., Jeong, H., Kim, H.-Y., Choi, J.-H., Kim, J.-H., Koo, B.-W., Choi, I.-G. 2017a. Evaluation of correlation between glucan conversion and degree of delignification depending on pretreatment strategies using Jabon Merah. *Bioresource Technology*, **236**, 111-118.

Jang, S.-K., Kim, H.-Y., Jeong, H.-S., Kim, J.-Y., Yeo, H., Choi, I.-G. 2016. Effect of ethanol organosolv pretreatment factors on enzymatic digestibility and ethanol organosolv lignin structure from Liriodendron tulipifera in specific combined severity factors. *Renewable Energy*, **87**, 599-606.

Jang, S.-K., Kim, J.-H., Jeong, H., Choi, J.-H., Lee, S.-M., Choi, I.-G. 2017b. Investigation of conditions for dilute acid pretreatment for improving xylose solubilization and glucose production by supercritical water

hydrolysis from Quercus mongolica. *Renewable Energy*.

John, M.J., Anandjiwala, R.D. 2008. Recent developments in chemical modification and characterization of natural fiber-reinforced composites. *Polymer composites*, **29**(2), 187-207.

Kim, T.H., Kim, J.S., Sunwoo, C., Lee, Y. 2003. Pretreatment of corn stover by aqueous ammonia. *Bioresource Technology*, **90**(1), 39-47.

Kim, T.H., Lee, Y. 2005. Pretreatment of corn stover by soaking in aqueous ammonia. *Twenty-Sixth Symposium on Biotechnology for Fuels and Chemicals*. Springer. pp. 1119-1131.

Kim, T.H., Taylor, F., Hicks, K.B. 2008. Bioethanol production from barley hull using SAA (soaking in aqueous ammonia) pretreatment. *Bioresource Technology*, **99**(13), 5694-5702.

Kim, Y., Hendrickson, R., Mosier, N., Ladisch, M.R. 2005. Plug-flow reactor for continuous hydrolysis of glucans and xylans from pretreated corn fiber. *Energy & fuels*, **19**(5), 2189-2200.

Kim, Y., Mosier, N.S., Ladisch, M.R. 2009. Enzymatic digestion of liquid hot water pretreated hybrid poplar. *Biotechnology Progress*, **25**(2), 340-348.

Klyosov, A.A., Mitkevich, O.V., Sinitsyn, A.P. 1986. Role of the activity and adsorption of cellulases in the efficiency of the enzymic hydrolysis of amorphous and crystalline cellulose. *Biochemistry*, **25**(3), 540-542.

Koo, B.-W., Min, B.-C., Gwak, K.-S., Lee, S.-M., Choi, J.-W., Yeo, H., Choi, I.-G. 2012. Structural changes in lignin during organosolv pretreatment of Liriodendron tulipifera and the effect on enzymatic hydrolysis. *Biomass and bioenergy*, **42**, 24-32.

Koullas, D., Christakopoulos, P., Kekos, D., Macris, B., Koukios, E. 1990. Effect of cellulose crystallinity on the enzymic hydrolysis of lignocellulosics by Fusarium oxysporum cellulases. *Cellulose chemistry and technology*, **24**, 469-474.

Ku, H., Wang, H., Pattarachaiyakoop, N., Trada, M. 2011. A review on the tensile properties of natural fiber reinforced polymer composites.

*Composites Part B: Engineering*, **42**(4), 856-873.

Kumar, P., Barrett, D.M., Delwiche, M.J., Stroeve, P. 2009a. Methods for pretreatment of lignocellulosic biomass for efficient hydrolysis and biofuel production. *Industrial & engineering chemistry research*, **48**(8), 3713-3729.

Kumar, R., Hu, F., Hubbell, C.A., Ragauskas, A.J., Wyman, C.E. 2013. Comparison of laboratory delignification methods, their selectivity, and impacts on physiochemical characteristics of cellulosic biomass. *Bioresource technology*, **130**, 372-381.

Kumar, R., Mago, G., Balan, V., Wyman, C.E. 2009b. Physical and chemical characterizations of corn stover and poplar solids resulting from leading pretreatment technologies. *Bioresource Technology*, **100**(17), 3948-3962.

Kumar, R., Singh, S., Singh, O.V. 2008. Bioconversion of lignocellulosic biomass: biochemical and molecular perspectives. *Journal of industrial microbiology & biotechnology*, **35**(5), 377-391.

Kumar, R., Wyman, C.E. 2009. Access of cellulase to cellulose and lignin for poplar solids produced by leading pretreatment technologies. *Biotechnology Progress*, **25**(3), 807-819.

Lü, H., Shi, X., Li, Y., Meng, F., Liu, S., Yan, L. 2017. Multi-objective regulation in autohydrolysis process of corn stover by liquid hot water pretreatment. *Chinese Journal of Chemical Engineering*, **25**(4), 499-506.

Laine, C. 2005. *Structures of hemicelluloses and pectins in wood and pulp*. Helsinki University of Technology.

Latimer, G.W. 2012. *Official methods of analysis of AOAC International*. AOAC international.

Laureano-Perez, L., Teymouri, F., Alizadeh, H., Dale, B.E. 2005. Understanding factors that limit enzymatic hydrolysis of biomass. *Twenty-sixth symposium on biotechnology for fuels and chemicals*. Springer. pp. 1081-1099.

Lee, S.B., Shin, H., Ryu, D.D., Mandels, M. 1982. Adsorption of cellulase on cellulose: effect of physicochemical properties of cellulose on adsorption and rate of hydrolysis. *Biotechnology and bioengineering*, **24**(10), 2137-2153.

Lehto, J., Louhelainen, J., Huttunen, M., Alén, R. 2017. Spectroscopic analysis of hot-water-and dilute-acid-extracted hardwood and softwood chips. *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy*, **184**, 184-190.

Lewin, M., Epstein, J.A. 1962. Functional groups and degradation of cotton oxidized by hypochlorite. *Journal of Polymer Science Part A: Polymer Chemistry*, **58**(166), 1023-1037.

Li, X., Tabil, L.G., Panigrahi, S. 2007. Chemical treatments of natural fiber for use in natural fiber-reinforced composites: a review. *Journal of Polymers and the Environment*, **15**(1), 25-33.

Liao, W., Wen, Z., Hurley, S., Liu, Y., Liu, C., Chen, S. 2005. Effects of hemicellulose and lignin on enzymatic hydrolysis of cellulose from dairy manure. *Twenty-Sixth Symposium on Biotechnology for Fuels and Chemicals*. Springer. pp. 1017-1030.

Lim, W.-S., Lee, J.-W. 2013. Effects of pretreatment factors on fermentable sugar production and enzymatic hydrolysis of mixed hardwood. *Bioresource technology*, **130**, 97-101.

Lynd, L.R., Weimer, P.J., Van Zyl, W.H., Pretorius, I.S. 2002. Microbial cellulose utilization: fundamentals and biotechnology. *Microbiology and molecular biology reviews*, **66**(3), 506-577.

Mabee, W., Saddler, J. 2010. Bioethanol from lignocellulosics: Status and perspectives in Canada. *Bioresource technology*, **101**(13), 4806-4813.

Mansfield, S.D., de Jong, E., Stephens, R.S., Saddler, J.N. 1997. Physical characterization of enzymatically modified kraft pulp fibers. *Journal of biotechnology*, **57**(1-3), 205-216.

Mansfield, S.D., Mooney, C., Saddler, J.N. 1999. Substrate and enzyme characteristics that limit cellulose hydrolysis. *Biotechnology progress*, **15**(5), 804-816.

Mazeau, K. 2011. On the external morphology of native cellulose microfibrils. *Carbohydrate polymers*, **84**(1), 524-532.

McLean, B.W., Boraston, A.B., Brouwer, D., Sanaie, N., Fyfe, C.A., Warren, R.A.J., Kilburn, D.G., Haynes, C.A. 2002. Carbohydrate-binding modules recognize fine substructures of cellulose. *Journal of Biological Chemistry*, **277**(52), 50245-50254.

McMillan, J.D. 1994. Pretreatment of lignocellulosic biomass, ACS Publications.

Medve, J., Ståhlberg, J., Tjerneld, F. 1994. Adsorption and synergism of cellobiohydrolase I and II of Trichoderma reesei during hydrolysis of microcrystalline cellulose. *Biotechnology and bioengineering*, **44**(9), 1064-1073.

Meunier-Goddik, L., Penner, M.H. 1999. Enzyme-catalyzed saccharification of model celluloses in the presence of lignacious residues. *Journal of Agricultural and Food Chemistry*, **47**(1), 346-351.

Millett, M., Moore, W., Saeman, J. 1954. Preparation and properties of hydrocelluloses. *INDUSTRIAL AND ENGINEERING CHEMISTRY*, **46**(7), 1493-1497.

Mood, S.H., Golfeshan, A.H., Tabatabaei, M., Jouzani, G.S., Najafi, G.H., Gholami, M., Ardjmand, M. 2013. Lignocellulosic biomass to bioethanol, a comprehensive review with a focus on pretreatment. *Renewable and Sustainable Energy Reviews*, **27**, 77-93.

Mooney, C.A., Mansfield, S.D., Touhy, M.G., Saddler, J.N. 1998. The effect of initial pore volume and lignin content on the enzymatic hydrolysis of softwoods. *Bioresource Technology*, **64**(2), 113-119.

Morone, A., Apte, M., Pandey, R. 2015. Levulinic acid production from renewable waste resources: Bottlenecks, potential remedies, advancements and applications. *Renewable and Sustainable Energy Reviews*, **51**, 548-565.

Mosier, N., Hendrickson, R., Ho, N., Sedlak, M., Ladisch, M.R. 2005. Optimization of pH controlled liquid hot water pretreatment of corn stover. *Bioresource technology*, **96**(18), 1986-1993.

Mou, H., Wu, S. 2016. Comparison of organosolv and hydrotropic pretreatments of eucalyptus for enhancing enzymatic saccharification. *Bioresource technology*, **220**, 637-640.

Moure, A., Gullón, P., Domínguez, H., Parajó, J.C. 2006. Advances in the manufacture, purification and applications of xylo-oligosaccharides as food additives and nutraceuticals. *Process Biochemistry*, **41**(9), 1913-1923.

Mussatto, S.I., Fernandes, M., Milagres, A.M., Roberto, I.C. 2008. Effect of hemicellulose and lignin on enzymatic hydrolysis of cellulose from brewer's spent grain. *Enzyme and Microbial Technology*, **43**(2), 124-129.

Mussatto, S.I., Mancilha, I.M. 2007. Non-digestible oligosaccharides: a review. *Carbohydrate polymers*, **68**(3), 587-597.

Mwaikambo, L.Y., Ansell, M.P. 2002. Chemical modification of hemp, sisal, jute, and kapok fibers by alkalization. *Journal of applied polymer science*, **84**(12), 2222-2234.

Nabarlatz, D., Ebringerová, A., Montané, D. 2007. Autohydrolysis of agricultural by-products for the production of xylo-oligosaccharides. *Carbohydrate Polymers*, **69**(1), 20-28.

Nabarlatz, D., Farriol, X., Montané, D. 2005. Autohydrolysis of almond shells for the production of xylo-oligosaccharides: product characteristics and reaction kinetics. *Industrial & engineering chemistry research*, **44**(20), 7746-7755.

Nabarlatz, D., Farriol, X., Montane, D. 2004. Kinetic modeling of the autohydrolysis of lignocellulosic biomass for the production of hemicellulose-derived oligosaccharides. *Industrial & engineering chemistry research*, **43**(15), 4124-4131.

Naik, S.N., Goud, V.V., Rout, P.K., Dalai, A.K. 2010. Production of first and second generation biofuels: a comprehensive review. *Renewable and sustainable energy reviews*, **14**(2), 578-597.

Nakagame, S. 2010. The influence of lignin on the enzymatic hydrolysis of pretreated biomass substrates, University of British Columbia.

Nakagame, S., Chandra, R.P., Kadla, J.F., Saddler, J.N. 2011a. Enhancing the enzymatic hydrolysis of lignocellulosic biomass by increasing the carboxylic acid content of the associated lignin. *Biotechnology and bioengineering*, **108**(3), 538-548.

Nakagame, S., Chandra, R.P., Saddler, J.N. 2011b. The influence of lignin on the enzymatic hydrolysis of pretreated biomass substrates. in: *Sustainable production of fuels, chemicals, and fibers from forest biomass*, ACS Publications, pp. 145-167.

Nimlos, M.R., Qian, X., Davis, M., Himmel, M.E., Johnson, D.K. 2006. Energetics of xylose decomposition as determined using quantum mechanics modeling. *The Journal of Physical Chemistry A*, **110**(42), 11824-11838.

NREL. 2014. 2014 Renewable Energy Data. *US Department of Energy*.

Ogbonna, J.C., Mashima, H., Tanaka, H. 2001. Scale up of fuel ethanol production from sugar beet juice using loofa sponge immobilized bioreactor. *Bioresource Technology*, **76**(1), 1-8.

Ooshima, H., Sakata, M., Harano, Y. 1983. Adsorption of cellulase from Trichoderma viride on cellulose. *Biotechnology and Bioengineering*, **25**(12), 3103-3114.

Otieno, D.O., Ahring, B.K. 2012a. The potential for oligosaccharide production from the hemicellulose fraction of biomasses through pretreatment processes: xylooligosaccharides (XOS), arabinooligosaccharides (AOS), and mannooligosaccharides (MOS). *Carbohydrate Research*, **360**, 84-92.

Otieno, D.O., Ahring, B.K. 2012b. A thermochemical pretreatment process to produce xylooligosaccharides (XOS), arabinooligosaccharides (AOS) and mannooligosaccharides (MOS) from lignocellulosic biomasses. *Bioresource technology*, **112**, 285-292.

Pérez, J., Ballesteros, I., Ballesteros, M., Sáez, F., Negro, M., Manzanares, P. 2008. Optimizing liquid hot water pretreatment conditions to enhance sugar recovery from wheat straw for fuel-ethanol production. *Fuel*, **87**(17), 3640-3647.

Palamae, S., Palachum, W., Chisti, Y., Choorit, W. 2014. Retention of hemicellulose during delignification of oil palm empty fruit bunch (EFB) fiber with peracetic acid and alkaline peroxide. *biomass and bioenergy*, **66**, 240-248.

Palonen, H., Tjerneld, F., Zacchi, G., Tenkanen, M. 2004. Adsorption of Trichoderma reesei CBH I and EG II and their catalytic domains on steam pretreated softwood and isolated lignin. *Journal of Biotechnology*, **107**(1), 65-72.

Pan, X. 2008. Role of functional groups in lignin inhibition of enzymatic hydrolysis of cellulose to glucose. *Journal of Biobased Materials and Bioenergy*, **2**(1), 25-32.

Pan, X., Xie, D., Yu, R.W., Lam, D., Saddler, J.N. 2007. Pretreatment of lodgepole pine killed by mountain pine beetle using the ethanol organosolv process: fractionation and process optimization. *Industrial & engineering chemistry research*, **46**(8), 2609-2617.

Parajó, J., Garrote, G., Cruz, J., Dominguez, H. 2004. Production of xylooligosaccharides by autohydrolysis of lignocellulosic materials. *Trends in Food Science & Technology*, **15**(3), 115-120.

Park, S., Baker, J.O., Himmel, M.E., Parilla, P.A., Johnson, D.K. 2010. Cellulose crystallinity index: measurement techniques and their impact on interpreting cellulase performance. *Biotechnology for biofuels*, **3**(1), 10.

Puri, V.P. 1984. Effect of crystallinity and degree of polymerization of cellulose on enzymatic saccharification. *Biotechnology and Bioengineering*, **26**(10), 1219-1222.

Qian, X., Nimlos, M.R., Davis, M., Johnson, D.K., Himmel, M.E. 2005. Ab initio molecular dynamics simulations of β-D-glucose and β-D-xylose degradation mechanisms in acidic aqueous solution. *Carbohydrate research*, **340**(14), 2319-2327.

Rabetafika, H.N., Bchir, B., Blecker, C., Paquot, M., Wathelet, B. 2014. Comparative study of alkaline extraction process of hemicelluloses from pear pomace. *Biomass and Bioenergy*, **61**, 254-264.

Rivero-Urgell, M., Santamaria-Orleans, A. 2001. Oligosaccharides: application in infant food. *Early Human Development*, **65**, S43-S52.

Rollin, J.A., Zhu, Z., Sathitsuksanoh, N., Zhang, Y.H.P. 2011. Increasing cellulose accessibility is more important than removing lignin: A comparison of cellulose solvent-based lignocellulose fractionation and soaking in aqueous ammonia. *Biotechnology and bioengineering*, **108**(1), 22-30.

Romaní, A., Garrote, G., López, F., Parajó, J.C. 2011. Eucalyptus globulus wood fractionation by autohydrolysis and organosolv delignification. *Bioresource Technology*, **102**(10), 5896-5904.

Saini, J.K., Patel, A.K., Adsul, M., Singhania, R.R. 2016. Cellulase adsorption on lignin: A roadblock for economic hydrolysis of biomass. *Renewable Energy*, **98**, 29-42.

Samanta, A., Jayapal, N., Kolte, A., Senani, S., Sridhar, M., Suresh, K., Sampath, K. 2012. Enzymatic production of xylooligosaccharides from alkali solubilized xylan of natural grass (Sehima nervosum). *Bioresource Technology*, **112**, 199-205.

Sannigrahi, P., Ragauskas, A.J., Miller, S.J. 2009. Lignin structural modifications resulting from ethanol organosolv treatment of loblolly pine. *Energy & Fuels*, **24**(1), 683-689.

Santos, R.L. 1997. The eucalyptus of California. *Alley-Cass Publications, California*.

Sattler, W., Esterbauer, H., Glatter, O., Steiner, W. 1989. The effect of enzyme concentration on the rate of the hydrolysis of cellulose. *Biotechnology and bioengineering*, **33**(10), 1221-1234.

Scheller, H.V., Ulvskov, P. 2010. Hemicelluloses. *Annual review of plant biology*, **61**.

Segal, L., Creely, J., Martin Jr, A., Conrad, C. 1959. An empirical method for estimating the degree of crystallinity of native cellulose using the X-ray diffractometer. *Textile Research Journal*, **29**(10), 786-794.

Selvendran, R. 1985. Developments in the chemistry and biochemistry of

pectic and hemicellulosic polymers. *J Cell Sci*, **1985**(Supplement 2), 51-88.

Sewalt, V., Glasser, W., Beauchemin, K. 1997. Lignin impact on fiber degradation. 3. Reversal of inhibition of enzymatic hydrolysis by chemical modification of lignin and by additives. *Journal of Agricultural and Food Chemistry*, **45**(5), 1823-1828.

Silverstein, R.A., Chen, Y., Sharma-Shivappa, R.R., Boyette, M.D., Osborne, J. 2007. A comparison of chemical pretreatment methods for improving saccharification of cotton stalks. *Bioresource technology*, **98**(16), 3000-3011.

Singh, A., Pant, D., Korres, N.E., Nizami, A.-S., Prasad, S., Murphy, J.D. 2010. Key issues in life cycle assessment of ethanol production from lignocellulosic biomass: challenges and perspectives. *Bioresource technology*, **101**(13), 5003-5012.

Singh, O. 1982. Kinetics and mechanism of hypochlorite oxidation of cellulose. *Textile Dryer & Printer*, 35-38.

Sjostrom, E. 2013. *Wood chemistry: fundamentals and applications*. Elsevier.

Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D. 2008a. Determination of sugars, byproducts, and degradation products in liquid fraction process samples. *National Renewable Energy Laboratory*.

Sluiter, A., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D. 2008b. Determination of extractive in biomass. *Laboratory Analytical Procedure (LAP)*.

Sreenivasan, S., Iyer, P.B., Iyer, K.K. 1996. Influence of delignification and alkali treatment on the fine structure of coir fibres (Cocos nucifera). *Journal of Materials Science*, **31**(3), 721-726.

Stark, A. 2011. Ionic liquids in the biorefinery: a critical assessment of their potential. *Energy & Environmental Science*, **4**(1), 19-32.

Steiner, W., Sattler, W., Esterbauer, H. 1988. Adsorption of Trichoderma reesei cellulase on cellulose: experimental data and their analysis by

different equations. *Biotechnology and bioengineering*, **32**(7), 853-865.

Sticklen, M.B. 2008. Plant genetic engineering for biofuel production: towards affordable cellulosic ethanol. *Nature Reviews Genetics*, **9**(6), 433-443.

Stone, J., Scallan, A., Donefer, E., Ahlgren, E. 1969. Digestibility as a simple function of a molecule of similar size to a cellulase enzyme.

Sumerskii, I., Krutov, S., Zarubin, M.Y. 2010. Humin-like substances formed under the conditions of industrial hydrolysis of wood. *Russian Journal of Applied Chemistry*, **83**(2), 320-327.

Sun, R., Lu, Q., Sun, X. 2001. Physico-chemical and thermal characterization of lignins from Caligonum monogoliacum and Tamarix spp. *Polymer degradation and stability*, **72**(2), 229-238.

Sun, S., Sun, S., Cao, X., Sun, R. 2016. The role of pretreatment in improving the enzymatic hydrolysis of lignocellulosic materials. *Bioresource technology*, **199**, 49-58.

Sun, X., Xu, F., Sun, R., Geng, Z., Fowler, P., Baird, M. 2005. Characteristics of degraded hemicellulosic polymers obtained from steam exploded wheat straw. *Carbohydrate Polymers*, **60**(1), 15-26.

Sun, Y., Cheng, J. 2002. Hydrolysis of lignocellulosic materials for ethanol production: a review. *Bioresource technology*, **83**(1), 1-11.

Sutcliffe, R., Saddler, J.N. 1986. The role of lignin in the adsorption of cellulases during enzymatic treatment of lignocellulosic material.

Symington, M.C., Banks, W.M., West, D., Pethrick, R. 2009. Tensile testing of cellulose based natural fibers for structural composite applications. *Journal of Composite Materials*.

Szijártó, N., Siika-aho, M., Tenkanen, M., Alapuranen, M., Vehmaanperä, J., Réczey, K., Viikari, L. 2008. Hydrolysis of amorphous and crystalline cellulose by heterologously produced cellulases of Melanocarpus albomyces. *Journal of biotechnology*, **136**(3), 140-147.

Tabka, M., Herpoël-Gimbert, I., Monod, F., Asther, M., Sigoillot, J. 2006. Enzymatic saccharification of wheat straw for bioethanol production by a combined cellulase xylanase and feruloyl esterase treatment. *Enzyme and Microbial Technology*, **39**(4), 897-902.

Tanaka, M., Ikesaka, M., Matsuno, R., Converse, A.O. 1988. Effect of pore size in substrate and diffusion of enzyme on hydrolysis of cellulosic materials with cellulases. *Biotechnology and bioengineering*, **32**(5), 698-706.

Taniguchi, H. 2004. Carbohydrate research and industry in Japan and the Japanese Society of Applied Glycoscience. *Starch-Stärke*, **56**(1), 1-5.

Tarvo, V., Lehtimaa, T., Kuitunen, S., Alopaeus, V., Vuorinen, T., Aittamaa, J. 2010. A model for chlorine dioxide delignification of chemical pulp. *Journal of Wood Chemistry and Technology*, **30**(3), 230-268.

Thompson, D.N., Chen, H.-C., Grethlein, H.E. 1992. Comparison of pretreatment methods on the basis of available surface area. *Bioresource Technology*, **39**(2), 155-163.

Väljamäe, P., Sild, V., Nutt, A., Pettersson, G., Johansson, G. 1999. Acid hydrolysis of bacterial cellulose reveals different modes of synergistic action between cellobiohydrolase I and endoglucanase I. *European Journal of Biochemistry*, **266**(2), 327-334.

Vázquez, M., Alonso, J., Domínguez, H., Parajó, J. 2002. Enzymatic processing of crude xylooligomer solutions obtained by autohydrolysis of eucalyptus wood. *Food Biotechnology*, **16**(2), 91-105.

Varanasi, P., Singh, P., Arora, R., Adams, P.D., Auer, M., Simmons, B.A., Singh, S. 2012. Understanding changes in lignin of Panicum virgatum and Eucalyptus globulus as a function of ionic liquid pretreatment. *Bioresource technology*, **126**, 156-161.

Vazquez, M., Alonso, J., Domınguez, H., Parajo, J. 2000. Xylooligosaccharides: manufacture and applications. *Trends in Food Science & Technology*, **11**(11), 387-393.

Vegas, R., Alonso, J.L., Domínguez, H., Parajó, J.C. 2005. Manufacture and

refining of oligosaccharides from industrial solid wastes. *Industrial & engineering chemistry research*, **44**(3), 614-620.

Vila, C., Garrote, G., Domínguez, H., Parajó, J.C. 2002. Hydrolytic processing of rice husks in aqueous media: a kinetic assessment. *Collection of Czechoslovak chemical communications*, **67**(4), 509-530.

Vlasenko, E.Y., Ding, H., Labavitch, J., Shoemaker, S. 1997. Enzymatic hydrolysis of pretreated rice straw. *Bioresource technology*, **59**(2), 109-119.

Vohra, M., Manwar, J., Manmode, R., Padgilwar, S., Patil, S. 2014. Bioethanol production: feedstock and current technologies. *Journal of environmental chemical engineering*, **2**(1), 573-584.

Wang, H., Postle, R., Kessler, R., Kessler, W. 2003. Removing pectin and lignin during chemical processing of hemp for textile applications. *Textile Research Journal*, **73**(8), 664-669.

Wang, J., Yuan, X., Sun, B., Cao, Y., Tian, Y., Wang, C. 2009. On-line separation and structural characterisation of feruloylated oligosaccharides from wheat bran using HPLC-ESI-MSn. *Food Chemistry*, **115**(4), 1529-1541.

Wise, L.E. 1946. Chlorite holocellulose, its fractionation and bearing on summative wood analysis and on studies on the hemicelluloses. *Paper Trade*, **122**, 35-43.

Wojtusik, M., Zurita, M., Villar, J.C., Ladero, M., Garcia-Ochoa, F. 2016. Influence of fluid dynamic conditions on enzymatic hydrolysis of lignocellulosic biomass: Effect of mass transfer rate. *Bioresource technology*, **216**, 28-35.

Xu, J., Cheng, J.J., Sharma-Shivappa, R.R., Burns, J.C. 2010. Sodium hydroxide pretreatment of switchgrass for ethanol production. *Energy & Fuels*, **24**(3), 2113-2119.

Xue-bing, Z., Lei, W., De-hua, L. 2007. Effect of several factors on peracetic acid pretreatment of sugarcane bagasse for enzymatic hydrolysis. *J Chem Technol Biotechnol*, **82**, 1115-21.

Yang, B., Wyman, C.E. 2004. Effect of xylan and lignin removal by batch and flowthrough pretreatment on the enzymatic digestibility of corn stover cellulose. *Biotechnology and bioengineering*, **86**(1), 88-98.

Yang, B., Wyman, C.E. 2008. Pretreatment: the key to unlocking low-cost cellulosic ethanol. *Biofuels, Bioproducts and Biorefining*, **2**(1), 26-40.

Yejun, H., Hongzhang, C. 2007. Plant cell wall proteins & enzymatic hydrolysis of lignocellulose.

York, W.S., O'Neill, M.A. 2008. Biochemical control of xylan biosynthesis—which end is up? *Current opinion in plant biology*, **11**(3), 258-265.

Yoshida, M., Liu, Y., Uchida, S., Kawarada, K., Ukagami, Y., Ichinose, H., Kaneko, S., Fukuda, K. 2008. Effects of cellulose crystallinity, hemicellulose, and lignin on the enzymatic hydrolysis of Miscanthus sinensis to monosaccharides. *Bioscience, biotechnology, and biochemistry*, **72**(3), 805-810.

Yu, Q., Zhuang, X., Lv, S., He, M., Zhang, Y., Yuan, Z., Qi, W., Wang, Q., Wang, W., Tan, X. 2013. Liquid hot water pretreatment of sugarcane bagasse and its comparison with chemical pretreatment methods for the sugar recovery and structural changes. *Bioresource technology*, **129**, 592-598.

Yu, Q., Zhuang, X., Yuan, Z., Wang, Q., Qi, W., Wang, W., Zhang, Y., Xu, J., Xu, H. 2010. Two-step liquid hot water pretreatment of Eucalyptus grandis to enhance sugar recovery and enzymatic digestibility of cellulose. *Bioresource Technology*, **101**(13), 4895-4899.

Yu, Z., Jameel, H., Chang, H.-m., Park, S. 2011. The effect of delignification of forest biomass on enzymatic hydrolysis. *Bioresource Technology*, **102**(19), 9083-9089.

Yuan, Q., Zhang, H., Qian, Z., Yang, X. 2004. Pilot-plant production of xylo-oligosaccharides from corncob by steaming, enzymatic hydrolysis and nanofiltration. *Journal of Chemical Technology and Biotechnology*, **79**(10), 1073-1079.

Zabed, H., Faruq, G., Sahu, J.N., Azirun, M.S., Hashim, R., Nasrulhaq Boyce, A. 2014. Bioethanol production from fermentable sugar juice. *The*

*Scientific World Journal*, **2014**.

Zabed, H., Sahu, J., Boyce, A., Faruq, G. 2016. Fuel ethanol production from lignocellulosic biomass: an overview on feedstocks and technological approaches. *Renewable and Sustainable Energy Reviews*, **66**, 751-774.

Zhang, T., Kumar, R., Tsai, Y.-D., Elander, R.T., Wyman, C.E. 2015. Xylose yields and relationship to combined severity for dilute acid post-hydrolysis of xylooligomers from hydrothermal pretreatment of corn stover. *Green Chemistry*, **17**(1), 394-403.

Zhang, Y.-H.P. 2008. Reviving the carbohydrate economy via multi-product lignocellulose biorefineries. *Journal of industrial microbiology & biotechnology*, **35**(5), 367-375.

Zhang, Y.-H.P., Cui, J., Lynd, L.R., Kuang, L.R. 2006a. A transition from cellulose swelling to cellulose dissolution by o-phosphoric acid: evidence from enzymatic hydrolysis and supramolecular structure. *Biomacromolecules*, **7**(2), 644-648.

Zhang, Y.-H.P., Himmel, M.E., Mielenz, J.R. 2006b. Outlook for cellulase improvement: screening and selection strategies. *Biotechnology advances*, **24**(5), 452-481.

Zhang, Y.H.P., Lynd, L.R. 2004. Toward an aggregated understanding of enzymatic hydrolysis of cellulose: noncomplexed cellulase systems. *Biotechnology and bioengineering*, **88**(7), 797-824.

Zhao, H., Kwak, J.H., Wang, Y., Franz, J.A., White, J.M., Holladay, J.E. 2006. Effects of crystallinity on dilute acid hydrolysis of cellulose by cellulose ball-milling study. *Energy & fuels*, **20**(2), 807-811.

Zhao, X., Cheng, K., Liu, D. 2009a. Organosolv pretreatment of lignocellulosic biomass for enzymatic hydrolysis. *Applied microbiology and biotechnology*, **82**(5), 815-827.

Zhao, X., Peng, F., Cheng, K., Liu, D. 2009b. Enhancement of the enzymatic digestibility of sugarcane bagasse by alkali–peracetic acid pretreatment. *Enzyme and Microbial technology*, **44**(1), 17-23.

Zhao, X., Song, Y., Liu, D. 2011. Enzymatic hydrolysis and simultaneous

saccharification and fermentation of alkali/peracetic acid-pretreated sugarcane bagasse for ethanol and 2, 3-butanediol production. *Enzyme and microbial technology*, **49**(4), 413-419.

Zhao, X., Zhang, L., Liu, D. 2012. Biomass recalcitrance. Part I: the chemical compositions and physical structures affecting the enzymatic hydrolysis of lignocellulose. *Biofuels, Bioproducts and Biorefining*, **6**(4), 465-482.

Zhu, J. 2011. Physical Pretreatment− Woody Biomass Size Reduction− for Forest Biorefinery. in: *Sustainable production of fuels, chemicals, and fibers from forest biomass*, ACS Publications, pp. 89-107.

Zhu, J. 2016. Pretreatment of Woody Biomass for Biofuel Production.

Zhu, L. 2006. Fundamental study of structural features affecting enzymatic hydrolysis of lignocellulosic biomass, Texas A&M University.

Zhuang, X., Wang, W., Yu, Q., Qi, W., Wang, Q., Tan, X., Zhou, G., Yuan, Z. 2016. Liquid hot water pretreatment of lignocellulosic biomass for bioethanol production accompanying with high valuable products. *Bioresource technology*, **199**, 68-75.

# 초 록

## 바이오매스 구성요소 조절에 의한 유칼립투스로부터 글루코오스와 자일로올리고당의 수율 향상 연구

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본 연구에서는 효소 당화에 미치는 바이오매스 구성요소의 영향을 이해하기 위해 난분해성 수종(유칼립투스)을 대상으로 헤미셀룰로오스 제거(열수 처리), 리그닌 제거(아염소산나트륨 처리), 셀룰로오스 결정화도 조절(수산화나트륨 처리)를 실시하였다. 그리고 열수 처리를 이용하여 고부가가치 산물인 자일로올리고당의 최적 생산 조건을 구명하였다. 또한, 바이오매스 구성요소 변화와 글루코오스 수율에 대해 상관관계분석 및 회귀분석을 실시하여 처리 방법이 알려지지 않은 수종에 대한 활용 방안과 가이드라인을 제시하고자 하였다.

열수 처리(170°C, 50분)를 통해 8.3%(전환율: 67.2%)의 자일로올리고당을 생산하였으나, 자일로올리고당의 함량은 열수처리 조건이 가혹해짐에 따라 0.8%까지 감소하였다. 자일로올리고당 중

함량이 가장 높은 것은 자일로비오스로써 상기 조건에서 3.0%까지 생산되었다. 또한, 자일로테트라오스(1.5%), 자일로펜타오스(1.0%), 자일로헥사오스(0.8%) 역시 상기 조건에서 생산되었다. 한편, 열수 처리(190°C, 50분) 후 고형분을 이용하여 효소 당화를 진행한 결과, 헤미셀룰로오스 제거율은 96.2%로 나타난 반면, 효소 소화율과 글루코오스 수율은 각각 21.3%와 28.2%으로 나타났다. 이를 통해 바이오매스의 구성요소와 글루코오스 수율 사이의 상관관계를 통계 분석 프로그램(SAS)를 이용하여 분석하였다. 그 결과, 헤미셀룰로오스와 리그닌 제거율에 따른 결정계수는 각각 0.6768과 0.0390으로 나타났다. 한편, 두 인자 (헤미셀룰로오스와 리그닌)에 대한 선형 회귀 모델의 결정계수는 0.7177로 단일 인자의 결과보다 다소 증가하였다.

34.8%였던 *E. pellita*의 리그닌 함량은 4 g의 아염소산나트륨과 0.8 mL의 아세트산을 3회(총 반응시간: 180분) 투입한 반응 조건에서 9.0%까지 감소하였다. 상기 반응 결과, 29.0%였던 *E. pellita*의 Klason 리그닌 함량은 2.4%까지 감소한 반면, acid-soluble 리그닌은 *E. pellita*(2.3%)보다 오히려 증가(6.6%)한 결과를 나타냈다. 한편, 헤미셀룰로오스 제거(75%)를 진행한 고형분에 대해 4 g의 아염소산나트륨과 0.8 mL의 아세트산을 2회 처리(총 반응시간: 120분)한 결과, 총 리그닌은 0.3%까지 크게 감소하였다. 28.2%가 최대 글루코오스 수율이였던 열수 처리 후 당화 결과와 비교하여, 열수 처리와 아염소산나트륨 처리를 순차적으로 진행한 경우 최대 글루코오스 수율은 87.5%까지 크게 증가하였다. 하지만, 열수 처리 없이 아염소산나트륨 처리만 진행한 결과에서도 83.9%의 높은 글루코오스 수율을 획득할 수 있었다.

리그닌 제거율과 글루코오스 수율 사이의 상관관계 분석 결과에 따르면, 결정계수는 0.9063으로 나타났고 이는 열수 처리 결과에 비해 크게 증가한 것이다. 게다가 두 인자의 선형 회귀 분석 결과 나타난 결정계수는 0.9285로 보다 증가하였고 이는 리그닌의 제거가 글루코오스 생산에 큰 영향력은 주는 인자임을 나타내는 결과이다.

59.7%였던 *E. pellita*의 결정화도는 열수 처리를 통해 68.9%까지 다소 증가하였다. 하지만 수산화나트륨 처리 후의 시료에 대한 결정화도는 계산할 수 없었는데, 선행 처리 결과에 상관없이 수산화나트륨 처리를 진행할 경우, 결정화 영역을 나타내는 $I_{002}$ 피크가 사라졌기 때문이다. 효소 당화 결과를 통해 수산화 나트륨 처리를 함으로써 원시료의 글루코오스 수율이 향상되는 것으로 나타났다. 한편, 열수 처리와 아염소산 나트륨 처리를 순차적으로 진행한 시료에 대해서는 수산화나트륨 처리를 하지 않은 시료와 비교할 경우 글루코오스 수율이 유사하거나 다소 감소하였다.

본 연구에서는 열수 처리, 아염소산나트륨 처리, 수산화나트륨 처리를 통해 바이오매스의 구성요소(헤미셀룰로오스, 리그닌, 셀룰로오스 결정화도)을 조절하였다. 상관관계 분석을 통해 바이오매스 내 리그닌의 존재는 효소 활성과 글루코오스 생산을 방해하는 강력한 인자로 밝혀졌다. 그러므로 높은 난분해성을 지닌 바이오매스라도 헤미셀룰로오스와 리그닌의 적절한 분해 기술 적용을 통해 자일로올리고당과 글루코오스를 원활하게 생산할 것으로 사료된다. 하지만, 추후 연구를 통해 목질계 바이오매스의 안정적인 활용을 위하여 생산된 자일로올리고당의 정제 및 분해된 리그닌의 회수가 수행되어야 할 것이다.

키워드 : 열수처리, 자일로올리고당, 탈리그닌, 글루코오스 생산,
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