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Effects of ethanol organosolv pretreatment conditions on fermentable sugar production and lignin structure from yellow poplar and pitch pine

유기용매 전처리 조건이 백합나무와 리기다소나무의 발효 가능 당 생산과 리그닌 구조에 미치는 영향

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이 논문을 농학석사학위 논문으로 제출함

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Abstract

Effects of ethanol organosolv pretreatment conditions on fermentable sugar production and lignin structure from yellow poplar and pitch pine

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In this study, ethanol organosolv pretreatment was conducted under a variety of similar pretreatment conditions by controlling severity factors. The conditions were designed for evaluating the effectiveness of process parameters, such as reaction temperature, time and concentration of sulfuric acid, which was used as an acidic catalyst. A combined severity factor was therefore applied to compare the pretreatment parameters. The factors were applied at the same level that showed an optimal condition for glucose conversion in previous studies. Ethanol organosolv pretreatment was performed under 5 different conditions for yellow poplar (*Liriodendron tulipifera*) and pitch pine (*Pinus rigida*), respectively.

As the reaction temperature and pH were elevated (130 to 170°C and pH 0.79 to 1.90), lignin or xylan, mannan, and galactan (XMG) removals in the solid residue were increased until the ratio reached 79% and 94%. When the reaction time was extended, and the pH was increased (10 to 50 min and pH 1.31 to 2.01), degradation of XMG was mitigated. However lignin removal

increased slightly in *L. tulipifera*. The degradation tendency of *P. rigida* was similar to that of *L. tulipifera*, except for low lignin removal efficiency (below 45%). According to the results of the solid residues, the reaction temperature was considered to be as major factor that could influence pretreatment strongly. Enzymatic digestibility ranged from 84 to 98% in *L. tulipifera*, indicating that pretreatment efficiency was affected by individual process parameters. In the case of *P. rigida*, results of enzymatic digestibility were observed below 30% under all experimental conditions, except for the condition of 190°C at pH 1.90.

The ethanol organosolv lignin (EOL) was recovered from the liquid hydrolysate, and the yields ranged from 11 to 13% in *L. tulipifera* and approximately 6% in *P. rigida*. High reaction temperatures led to the reduction of the ether bonds in the EOL structure and the enhancement of uniformity, supported by the amount of nitrobenzene oxidation products and the resulting molecular weight distribution. To evaluate the productivity of monomeric compounds, the EOL of *L. tulipifera* was performed as a depolymerization using supercritical ethanol treatment. The lignin-derived monomeric compounds determined by GC/MS ranged from 1.7 to 2.8% based on 100 g of EOL.

Reaction temperature was therefore the most effective process parameter compared to reaction time and concentration of acidic catalyst. The conditions of 170°C, 10 min reaction time, and pH 1.90 might be optimal pretreatment condition, as indicated by the highest enzymatic digestibility (98%) and amount of phenolic monomer (2.8%).

Key words: organosolv pretreatment, ethanol organosolv lignin, combined severity factor, Liriodendron tulipifera, Pinus rigida

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1 Introduction

1.1 Lignocellulosic biomass and pretreatment

Lignocellulosic biomass is a carbon-rich material that could be converted to biofuel for transportation (Cherubini, 2010; Hamelinck et al., 2005; Sun & Cheng, 2002). If biomass is converted into biofuels, carbon emission by fossil fuel combustion will decrease (Naik et al., 2010). 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).

Bioethanol (one of the biofuels) can be produced in two steps: enzymatic hydrolysis from cellulose to fermentable monosaccharides by cellulases, and fermentation for production of the bioethanol from fermentable sugars (Demain et al., 2005; Percival Zhang et al., 2006; Ragauskas et al., 2006). However the natural resistance of lignocellulosic materials, known as biomass recalcitrance, has made it difficult to deconstruct these materials through enzymatic and microbial reactions (Himmel et al., 2007; Singh-Nee Nigam et al., 2004). For example, cellulose crystallinity, cellulase accessibility to cellulose and content of lignin are representative properties of recalcitrance (Agbor et al., 2011). The biomass recalcitrance can be overcome by the pretreatment process for bioethanol production.

Pretreatment is one of the most important steps in lignocellulosic biomass utilization. The pretreatment process incurs a high cost for the degradation of recalcitrance, and the pretreatment process also has a strong influence on the products of subsequent processes (Wooley et al., 2008; Wyman et al., 2005b). The objective of pretreatment is to mitigate the

complex structure of the biomass and to facilitate cellulosic proportions that are more accessible to enzymes for hydrolysis (Mosier et al., 2005).

1.2 Pretreatment methods and organosolv pretreatment

A number of different pretreatment strategies have been studied during the past decades for facilitating the use of the lignocellulosic biomass (Galbe & Zacchi, 2007). Table 1 summarized the pretreatment methods, which have been studied up to these days.

Table 1. Advantages and disadvantages of representative pretreatment strategies (Pedersen & Meyer, 2010)

	Advantages	Disadvantages
Dilute acid	Elimination of hemicellulose concomitantly some lignin removal	Corrosion of reactor, degradation products produced
Steam explosion	High performance of glucose recovery and hemicellulose degradation	Generation of inhibitors by temperature and pressure
Wet oxidation	Reduces formation of inhibitors, exothermic process	Need to pressure, temperature and equipment, high price of oxygen
AFEX	Low reaction temperature and inhibitor formation, reduces lignin and hemicellulose fraction	Not suitable for high lignin content such as softwood, high price of ammonia
Biological	Unnecessary corrosive resistant equipment, low energy consumption	Long time for digest the glucose by microorganism

Among the variety of pretreatment technologies, organosolv pretreatment has merits and advantages due to: (1) high enzymatic accessibility and low carbohydrate loss; (2) low requirements for chemicals, energy, and capital

investment; and (3) good lignin recovery for valuable applications (Holtzapple & Humphrey, 1984).

1.3 Evaluation of pretreatment using the severity factor

As mentioned above, diverse pretreatment methods have been developed and studied for all types of lignocellulosic biomass. However, comparison of these pretreatment strategies with one another is difficult because the ultimate results could be changed by a variety of factors. These factors consist of operating parameters, such as reaction temperatures, reaction times and pH. These different factors make it difficult to compare between the pretreatment processes (Pedersen & Meyer, 2010). The severity factor has been developed and improved to evaluate pretreatment conditions and parameters.

However, this equation requires a validation of the outputs of the pretreatment. Several studies have therefore been conducted on various sets of pretreatment conditions for investigation of the severity factor (Chum et al., 1990; Larsson et al., 1999; Tengborg et al., 1998). However, this research did not investigate the individual pretreatment parameters comprehensively. The results of this research do not clearly reveal that the lignocellulosic biomass can be damaged by a certain factor during pretreatment. Therefore, to understand the effect of each pretreatment parameter, it is necessary to operate under similar pretreatment conditions.

In this study, organosolv pretreatment was conducted under various conditions which have difference of reaction temperature, time and catalyst concentration for evaluating process parameters.

1.4 Conception of a biorefinery

The ethanol production from lignocellulosic biomass has encountered two major problems: (1) high cost of initial investment and (2) little price difference between biomass and product (Eggeman & Elander, 2005; Percival Zhang et al., 2006; Wyman et al., 2005a). Therefore, the conception of a biorefinery is required for the effective use of the lignocellulosic biomass (Fernando et al., 2006; Huber & Corma, 2007; Ragauskas et al., 2006). In this vision, plant biomass is used not only for production of transportation fuels but also as a means of producing value-added products. Hemicellulose and lignin have been used as an energy resource by combustion in a conventional bioethanol production. However, they are no longer burning materials in a biorefinery concept (Kleinert & Barth, 2008).

Efficiency of lignin application has been limited than hemicellulose application. Hemicellulose can be applied to the enzymatic hydrolysis process by a pentose recovery and converted to fermentable sugars. This process contributes to improve the total bioethanol production. However, nearly 98% of lignin, in spite of great amounts of lignin production in the paper industries, is directly combusted for a boiler fuels (Thielemans et al., 2001). Only 2% of lignin from sulfite pulping is converted to lignosulfonates, which are used in emulsifiers, binders and pesticides (Browning, 1955; Mathiasson & Kubat, 1994). However, lignosulfonates have been recognized as low-efficiency reactants because the elimination of sulfur is accompanied by high energy consumption (Calvo-Flores & Dobado, 2010). However, the ethanol organosolv lignin has good properties, such as low molecular weight, high purity and narrow distribution (Pan et al., 2005a; Pan et al., 2006b; Pan et al., 2005b). Therefore, in terms of lignin utility, the ethanol organosolv pretreatment can produce a high quality of lignin for biorefinery in a cost-competitive manner.

In terms of producing multiple-products, a biorefinery with organosolv pretreatment is similar to an oil refinery. The suspension resulting from organosolv pretreatment is fractionated and separated into two phases, a solid and a liquid fraction. Cellulose in the solid fraction and hemicellulose in the liquid fraction are recovered and later, produce bioethanol by passing the enzymatic hydrolysis and fermentation steps. Lignin in the liquid fraction is converted into chemicals and building blocks for many industries. Figure 1 shows an example of a process of a multi-product biorefinery with organosolv lignin (de Wild et al., 2011).

If a biorefinery for hemicellulose and lignin application will be cost competitive, its products can partially substitute for petrochemicals from crude oil because of a similarity between biorefinery and oil refinery (Fernando et al., 2006; Zhang, 2008).

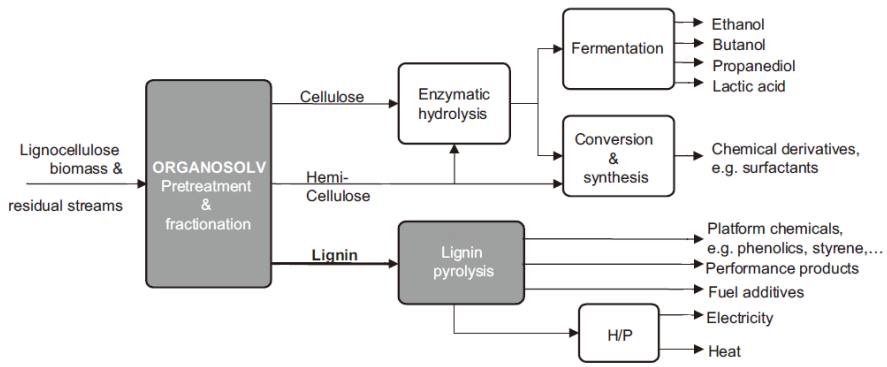

Figure 1. A process of lignocellulosic biomass biorefinery with organosolv pretreatment (de Wild et al., 2011)

1.5 Objectives

The potential demands for transportation fuels due to petroleum shortage, and concerns on climate change by greenhouse gas emissions from fossil fuels have induced an interest for an alternative sustainable biofuels and biochemicals from renewable resources such as lignocellulosic biomass. Lignocellulosic biomass, an abundant, sustainable, renewable, and environment friendly resource, can be converted to fermentable sugars for biotransformation into liquid transportation fuels as well as numerous commodity chemicals and biodegradable materials. However, the pretreatment process absolutely required to produce bioethanol from lignocellulosic biomass. In addition, this process is still considered as a cost expensive and energy consuming process. Therefore, the application of lignin, as a byproduct of pretreatment, has to encourage improving the price competitiveness of bioethanol production from lignocellulosic biomass. Furthermore, the technology of lignin depolymerization and its evaluation for conversion to valuable products are required in the future.

In this study, organosolv lignin will be produced under various organosolv pretreatment conditions, using lignocellulosic biomasses (*Liriodendron tulipifera* and *Pinus rigida*). The characteristics of the ethanol organosolv lignin will be evaluated by nitrobenzene oxidation and gel permeation chromatography. The ethanol organosolv lignins will be selected for low molecularization under supercritical ethanol.

Specifically, this study aims :

1. To evaluate such process parameters as reaction temperature, time and concentration of catalyst through organosolv pretreatment with theoretically the same pretreatment conditions.
2. To understand the characteristics of ethanol organosolv lignin,

which is produced under the optimal condition of saccharification with specific combined severity factors.

3. To determine an optimal organosolv pretreatment condition for production of sugars and lignin, which was determined by evaluation of degradation products and estimation of its yield.

2 Literature reviews

2.1 Organosolv pretreatment for lignocellulosic biomass

Organosolv pretreatment, one of the pretreatment methods, such as dilute-acid, steam explosion pretreatment and AFEX, is a suitable process for a biorefinery. Organosolv pretreatment can easily recover organic solvents and can obtain the high quality of lignin as a byproduct than other pretreatment methods (Pan et al., 2005a; Zhao et al., 2009). For instance, the lignin from other pretreatment methods, such as dilute-acid pretreatment and steam explosion, is only used as a boiler fuel (Pan et al., 2008).

A various types of the organic solvent or the aqueous mixture, such as methanol, ethanol, acetone and ethylene glycol, are utilized in organosolv pretreatment. The solvents enhance efficiency of the enzymatic hydrolysis (Dorrestijn et al., 2000; Mesa et al., 2011; Obama et al., 2012; Sannigrahi et al., 2010) and decompose the linkage of lignin structure (Curreli et al., 1997; Itoh et al., 2003; Pan et al., 2006a; Rolz et al., 1986). Ethanol and methanol have a low boiling point that is a good property for the pretreatment process. A low boiling point can enable to recover the solvent easily with low energy consumption (Mesa et al., 2011). Ethanol pretreatment is favored than methanol pretreatment because methanol is more toxic than ethanol (Zhao et al., 2009).

The alkaline catalysts, such as ammonia, sodium hydroxide and lime, can be used for organosolv pretreatment (Fan et al., 1987; McMillan, 1994). The alkaline catalysts not only expand the cellulose surface area by alkaline swelling or defiberization but also are effective for lignin removal. This effect of alkaline catalysts improves the enzyme accessibility (Zhang et al., 2007; Zhao et al., 2008). A study of organosolv pretreatment was reported using alkaline catalyst condition with *L. tulipifera*. In this study, ethanol solvent

(50%) and sodium hydroxide (1%, (w/w)) were used as a solvent. Organosolv pretreatment was conducted with reaction time of 10 min and the reaction temperature at 140, 150, and 160°C. As a result, the most enzymatic conversion (67%) was obtained at 160°C (Koo et al., 2011). However, the pretreatment using the alkaline catalysts is required for more reaction time than using acidic catalysts (Kumar et al., 2009).

The acidic catalysts, such as hydrochloric, nitric and sulfuric acid, have been mainly used for reagent under organosolv pretreatment conditions (Sarkanen & Tillman, 1980). Especially, sulfuric acid is the most-used catalyst that has superior properties for pretreatment; high efficiency and strong reactivity (Araque et al., 2008; Pan et al., 2007). An addition of acid catalysts can be made to accelerate delignification process and reduce the reaction temperature. Organosolv pretreatment required the high reaction temperature at 185 to 210°C without the acidic catalyst. However an utilization of the acidic catalyst enable to down the reaction temperature below 180°C (Duff & Murray, 1996). Organosolv pretreatment of pitch pine (*Pinus rigida*) was conducted using the acidic catalysts for estimating the enzymatic hydrolysis. The sulfuric acid (1% w/v) is used as the acidic catalyst and the pretreatment conducted under the various conditions that change the reaction temperature and reaction times. As a result, the maximum digestibility and glucose yield (56% and 75%) were observed at 170°C for 10 mins (Park et al., 2010). Organosolv pretreatment of *L. tulipifera* was studied under the various reaction temperatures (120 to 180°C). The cellulose to glucose conversion yield (98%) was obtained at the reaction temperature of 150°C, indicating that almost the enzymatic hydrolysis was finished (Gwak, 2012).

2.2 Development of the combined severity factor

For estimation of pretreatment severity and comparison with pretreatment methods, Overend and Chornet used the P factor of Brasch and developed the severity of different pretreatments (Brasch & Free, 1965; Overend et al., 1987). The severity factor (R_0) was invented for a benchmark in order to confirm efficiency of the various pretreatment methods. The R_0 enables to use for comparing of different pretreatment because the reaction temperature and reaction time were integrated in the severity factor. The severity factor could be utilized to estimate the xylan solubilization and the lignin reduction (Silverstein et al., 2007).

$$R_0 = \int_a^b e^{\left(\frac{T(t)-100}{14.75}\right)} dt = t \times e^{\left(\frac{T(t)-100}{14.75}\right)}$$

T(t) = reaction temperature (°C), t = reaction time (min)

Where 100 is the reference temperature and 14.75 is the arbitrary constant which was based on the activation energy from pseudo first order kinetics (Carvalheiro et al., 2009). The constant has been adjusted by some studies, but it made a little difference.

However, this equation doesn't explain the efficiency of the various catalysts that affected to biomass during pretreatment process. Abatzoglou et al. developed combined severity factor for estimating the effect of an acidic catalyst by adding the proton concentration in the equation (Abatzoglou et al., 1992).

$$R'_0 = R_0 \times [H^+]$$
$$\log(R'_0) = \log(R_0 \times [H^+]) = \log(R_0) - pH$$

The combined severity factor (CSF) which is $\log(R'_0)$ explained the effect of acidic catalysts especially in the pretreatment. The CSF was originated from pretreatment using acidic catalyst of aspen for estimating the lignin reduction and the xylan solubilization (Chum et al., 1990; Yang & Wyman, 2004).

2.3 Pretreatment studies with the combined severity factor

The combined severity factor (CSF) could be used for determining severity of the pretreatment methods and estimating the pretreatment results. A various pretreatment studies have been conducted in accord with CSF and improved the CSF equation (Chen et al., 2007).

The spruce wood pretreatment using sulfuric acid was conducted under the optimal condition for obtaining the fermentable sugars at the CS value 3.0 however the fermentation efficiency was decreased under this condition (Tengborg et al., 1998). The study of pretreatment using dilute acid was performed with spruce wood that combined severity factor ranged from 1.4 to 5.4 under 76 different conditions. As a result, the optimal condition for the fermentable sugar production was founded at 2.0 and 3.4 of the CS values (Larsson et al., 1999). The dilute acid pretreatment at the same CS value of 2.5 using sulfuric acid was operated with mixed hardwoods. This study reported that the reaction temperature was more affected than reaction time for producing the fermentable sugars (Lim & Lee, 2012).

Ethanol organosolv pretreatment was investigated based on the combined severity factor. A research of empty palm fruit bunch was conducted under conditions with a large variation of the severity values ($1.27<CS<2.45$). This study was reported the combined severity factor was trustworthy parameters

because CSF exhibits a good prediction for the xylan and lignin extraction (Goh et al., 2011). The experiment of ethanol organosolv lignin extraction with *Miscanthus×giganteus* was conducted under the range of combined severity factors ($1.75<CS<2.8$). The results revealed as an increase in the severity factor facilitated the dehydration reaction on the side chain and the condensation of lignin, declined the molecular weight of lignin fragment and augmented the concentration of phenolic monomers (El Hage et al., 2010).

3 Materials and Methods

3.1 Materials

Stems of twenty-year old *L. tulipifera* and *P. rigida* were dried, and milled to a particle size of 0.5 mm. The milled woods were air-dried, and the moisture content was less than 10%. The milled materials were stored at 4˚C until utilization.

3.2 Conditions of organosolv pretreatment

Ethanol organosolv pretreatment was conducted in a reactor composed of a vessel, an electric band heater, a magnetic drive with a paddle type impeller and a control box (HR-8300, Hanwoul Engineering Inc., Republic of Korea). The vessel was manufactured from stainless steel (SUS 316) and had a capacity of 1,000 mL. A Teflon gasket was applied to maintain the inner pressure. The thermocouple and pressure gauge were located inside the reactor to measure internal temperature and pressure during pretreatment. A Teflon impeller was located inside the reactor for regular stirring. The control box controlled the temperature of the vessel and heater, and the stirring rate of the impeller.

The ratio of the standard material to solvent was 1:10 (50 g : 500 mL). Ethanol (50% (v/v)) was used as an organic solvent. Sulfuric acid was added as a catalyst. The pretreatment was performed with consistent output of the electric band heater until the internal temperature reached the target (130, 150, 170°C in *L. tulipifera* and 150, 170, 190°C in *P. rigida*). When the internal temperature in the vessel reached the target temperature, the heating temperature were stopped, and reaction time of 10, 30 and 50 min was

imposed. After pretreatment, the vessel was quenched into an ice chamber and cooled to room temperature. The detailed conditions of ethanol organosolv pretreatment were shown in Table 2.

Table 2. The operation conditions of ethanol organosolv pretreatment using the acidic catalyst.

No.	Reaction time (min)	Reaction temp. (°C)	pH (±0.05)	CSF value
		Liriodendron tulipifera		
1	10	130	0.72	1.16
2	10	150	1.31	1.16
3	10	170	1.90	1.16
4	30	150	1.79	1.16
5	50	150	2.01	1.16
		Pinus rigida		
6	10	150	0.72	1.75
7	10	170	1.31	1.75
8	10	190	1.90	1.75
9	30	170	1.79	1.75
10	50	170	2.01	1.75

The product suspension was filtered through a Hyundai no. 20 filter paper. The solid residue was retained in the filter paper, while the liquid fraction was collected by filteration and poured into a plastic bottle. The ethanol organosolv lignin (EOL) was precipitated from the liquid fraction using distilled water (water : liquid 2 : 1, v/v) in conical tubes. The tubes were centrifuged, and the supernatant liquid was subsequently collected into a plastic bottle. After lyophilization, EOL was used for chemical analysis and supercritical treatment. The schemes of overall processes in this study are shown in Figure 2.

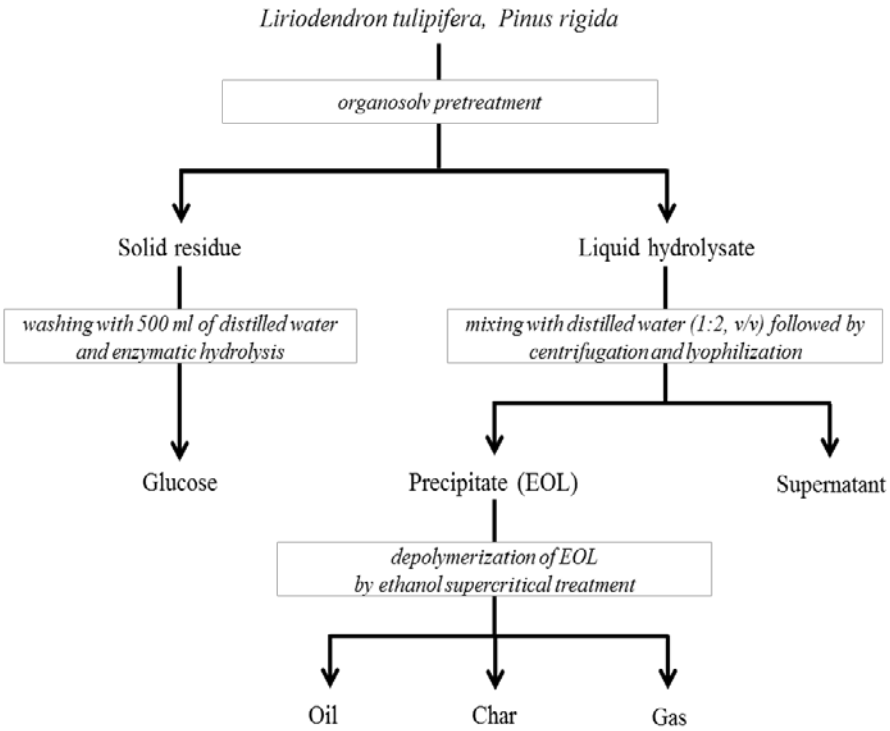

Figure 2. The procedure of ethanol organosolv pretreatment producing ethanol organosolv lignin (EOL) and the degradation products

3.3 Analysis of solid residues

3.3.1 Recovery rate and lignin content

The raw materials and filtered solid residues were dried in 105°C, 24h for measuring water-insoluble solid (WIS) recovery rates. Structural sugars and lignin were analyzed according to the NREL Laboratory Analytical Procedure (Sluiter et al., 2006).

The samples of oven-dried solid residue (0.3 g) were swelled in 3 mL of

72% sulfuric acid at 30°C for 1 h and later added to 84 mL distilled water for dilution (4% sulfuric acid). These samples were reacted in an autoclave at 121°C for 1 h and were filtered using distilled water through glass filters (1G4, Iwaki, Japan). The residue on the filters was oven-dried and weighed for measuring Klason lignin.

Acid-soluble lignin was analyzed by the absorbance of the filtrate which was determined using a UV-visible spectrophotometer (UV-1601 PC, Shimadzu, Japan). The filtrates were diluted with distilled water (1:14, v/v), and the absorbance was measured at 205 nm with a quartz cuvette. The acid-soluble lignin was calculated according to the following equation:

$$\text{Acid-soluble lignin (\%)} = \frac{Absorbance \times Sample\ volume \times Dilution}{\varepsilon \times Weight\ of\ Initial\ sample} \times 100$$

(ε = absorptivity, equal to 110 L/g·cm)

3.3.2 Carbohydrate composition

After Klason lignin analysis, the filtrates of raw material and solid residues were measured by high performance liquid chromatography (Ultimate 3000 series, Dionex, USA) for analysis of structural sugar content. Before the HPLC analysis, the filtrates were filtered through 0.45 μm hydrophilic PTFE syringe filters. The HPLC using an Aminex HPX-87H column (300 mm × 7.8 mm × 9 μm, Bio-Rad Laboratories, USA) was operated with a 0.5 mL/min flow of 0.01 N sulfuric acid as the mobile phase and a column temperature of 40°C.

3.4 Enzymatic hydrolysis for glucose conversion

Enzymatic hydrolysis was performed with 1 g of dry weight of solid residues in 50 mL of 50 mM sodium acetate buffer (pH 5.0). Cellulase complex NS 22086 (Novozymes, Denmark) with an activity of 1,000 BHU/g and β-glucosidase NS 22118 (Novozymes, Denmark) with an activity of 250 CBU/g were used to perform enzymatic hydrolysis and mixed with the sodium acetate buffer. The mixtures were incubated at 50°C for 72 h in a shaking incubator at 150 rpm.

For determination of enzymatic digestibility, the hydrolysates were filtered through glass filters (1G2, Iwaki, Japan), and the weight of the residues was measured. The filtrate was recovered and filtered using a 0.45 μm hydrophilic PTFE syringe filter. The glucose contents in the filtrate were determined by HPLC using a Sugar-pak column (300 mm × 6.5 mm× 5 μm, Waters, USA) at a column temperature of 80°C. Cellulose to glucose conversion yield and enzymatic digestibility were calculated by the following equations.

$$\text{Cellulose to glucose conversion yield (\%)} = \frac{Glucose\ content\ after\ enzymatic\ hydrolysis\ (g)}{Glucan\ content\ of\ soild\ residues\ (g)} \times 100$$

$$\text{Enzymatic digestibility (\%)} = \frac{Enzymatically\ hydrolyzed\ biomass\ (g)}{Amount\ of\ solid\ residues\ for\ enzymatic\ hydrolysis\ (g)} \times 100$$

3.5 Analysis of liquid hydrolysate

The sugar contents (glucose, xylose) and degradation products (furfural, HMF) of the liquid hydrolysate that was separated from the solid residues after organosolv pretreatment were analyzed using HPLC as previously suggested in Section 3.3.2.

3.6 Characterization of ethanol organosolv lignin

3.6.1 Lignin contents

Lignin content was determined according to the method previously described in Section 3.3.1.

3.6.2 Nitrobenzene oxidation

The phenolic acid and aldehyde composition was determined by nitrobenzene oxidation. Approximately 30 mg of oven-dried EOL was swelled with 4 mL of 2 M NaOH and 250 μL of nitrobenzene. The mixture was loaded into glass bombs and reacted at 170°C for 2 h with stirring every 20 min. Internal standard (20 μl) was injected into the oxidized sample, and the sample was subsequently extracted twice using 20 mL of dichloromethane and allowed to rest. HCl (4 M) was infused into extracted the sample until the pH of the aqueous phase was in the range of 1 to 2. After pH adjustment, the sample was extracted with dichloromethane twice (as in the previous process) but collected after this step. The extract was evaporated and dissolved in 1 mL of diethyl ether. For GC/MS analysis, silylation of the sample was conducted at 105°C for 2 h with stirring every 20 min using 200 μL of pyridine and N,O-

bis(trimethylsilyl)trifluoroacetamide (1:1, v/v). The silylated sample was filtered using a 0.45 μm PTFE hydrophobic syringe filter and analyzed using GC/MS. The GC was (Agilent 7890A) equipped with a 5974C mass selective detector and an FID. The GC was operated with a DB-5 capillary column (60 m × 0.25 mm × 0.25 μm) at a split ratio of 1:20. The temperatures of the injector and detector were set at 200°C and 250°C, respectively. The temperature of the oven started at 120°C for 10 min with a heating rate of 10°C/min to 280°C, and the temperature was maintained for 20 min (Kim et al., 2012).

3.6.3 Gel permeation chromatography

Molecular weight distributions of EOL were measured by gel permeation chromatography (GPC). For acetylation, 100 mg of EOL and milled wood lignin were reacted with 2 mL of a mixture of pyridine and acetic anhydride (1:1, v/v) at 105°C for 2 h with stirring and then dissolved in tetrahydrofuran. Samples were injected into the GPC (GPCmax, Vixcotek, USA) using a PLgel 3 μm MIXED-D column (300 mm × 7.5 mm, Varian Inc., USA), PLgel 3 μm MIXED-E column (300 mm × 7.5 mm, VARIAN, Inc) and PLgel 5 μm guard column (50 mm × 7.5 mm, VARIAN, Inc). The GPC was operated at a 1 mL/min flow rate and eluted with tetrahydrofuran. The calibration curves were set using polystyrene standards with various molecular weight ranges (580 ~ 3,250,000 Da).

3.7 Total phenol content

Total phenol content of the supernatant was determined by the Folin-Ciocalteu reagent. Vanillin (0.2 g) as a standard was dissolved in 100 mL of distilled water. To create a standard curve, the standard vanillin was diluted in different concentration ranges (1:10~1:1,000, v/v). The Folin-Ciocalteu reagent was prepared by 10-fold dilution, and 1 mL of this reagent was put into a vial. Next, 200 μL of the standard or the supernatant which had been diluted 50 times, and 800 μL of 17.5% sodium carbonate solution were injected into the vial sequentially. The mixture was agitated strongly and reacted in a dark-room for 2 h. The total phenol content was analyzed by absorbance of the sample of reacted supernatant using a UV-visible spectrophotometer (UV-1601 PC, Shimadzu, Japan). The absorbance was measured at 760 nm and quantified by standard curve.

3.8 Supercritical ethanol treatment of EOL

EOL was depolymerized using supercritical ethanol, in a hydrothermal reactor. The reactor was a stainless steel (SUS316) reactor with a volume of 50 mL, including a heater. The EOL sample (0.3 g) and 20 mL of ethanol were placed the reactor, and the reactor was purged for 2 min with nitrogen gas (10L/min) to remove any reactive air before reaction. The reaction temperature was later fixed at 350°C, and the reaction time was set to 40 min. After pyrolysis, the reactor was cooled in an ice chamber. The samples of oil were dissolved in 3 mL of acetone, and the suspension was mixed with 100 μL of fluoranthene (ca. 9.9 mg/10 mL in acetone) as an internal standard. Analysis of the oil fractions was conducted by GC/MS. The GC (Agilent HP7890A) was equipped with a DB-5 capillary column (30 m × 0.25 mm ID

× 0.25 μm) and an Agilent HP5975A mass selective detector (MSD). The oven temperatures began at 50°C for 5 min with a heating rate of 3°C/min to 140°C for 10 min, and an increase of 2 °C/min to 280°C for 10 min. The temperature of the injector and detector was set at 220°C and 300°C, respectively, and the purity of the He carrier gas was 99.99%. The mass selective detector (Agilent Technologies 5975A) was operated to observe the ratio of mass to charge (m/z) values for each compound at 70 eV (Kim et al., 2012).

4 Results and Discussions

4.1 Composition of water-insoluble solid residue

4.1.1 Degradation and delignification

The chemical composition of raw materials and solid residues after organosolv pretreatment is shown in Table 3. All of the pretreatments were carried out at CSF value in *L. tulipifera* (1.16) and *P. rigida* (1.75), respectively.

Under pretreatment condition 1, 2 and 3 (130, 150, and 170°C with pH 0.79, 1.31 and 1.90) in Table 3, water-insoluble solid (WIS) recovery rates and Klason lignin decreased, when the reaction temperature increased at the same reaction time (10 min). Although the concentration of acidic catalyst declined, the solid residues were more hydrolyzed and converted to degradation products, indicating that the increase of reaction temperature was a moer effective pretreatment factor than that of catalyst concentration.

As the reaction time increased with the increase in pH, WIS recovery rates and Klason lignin increased slightly (10, 30, and 50 min reaction time with pH 1.31, 1.79 and 2.01, reaction temperature was fixed at 150°C). An extension of reaction time was less effective for degradation of solid residues than an increment in catalyst concentration.

The variation of WIS recovery rates and Klason lignin under conditions with the reaction temperature fixed was broader than conditions when reaction time was fixed. As a result, the reaction temperature had more impact than the reaction time or pH. The strong effect of reaction temperature has been reported in many studies (Kabel et al., 2007; Wildschut et al., 2012).

A relatively high level of lignin removal was observed with an extension of the reaction time (30 and 50 min), indicating that long reaction time brought about delignification in the solid residue.

The trend in WIS recovery rates in *P. rigida* was similar to that in *L. tulipifera*. However, lignin removal was only slightly changed in all experiments. A higher value of CSF was therefore required to remove lignins from *P. rigida* because of a high recalcitrance of the gymnosperm lignin, a unique quality of softwoods (Palonen et al., 2004).

4.1.2 Structural sugars

Xylan was easily removed from biomass by pretreatment because the hemicellulose is the most reactive component among the major biomass components (Alvira et al., 2010; Huijgen et al., 2012; Zhao et al., 2009). In this study, most of the experimental conditions revealed that over 80% of the hemicellulosic sugars, such as xylan, mannan, and galactan (XMG), were decomposed and converted into the liquid hydrolysates (Table 3).

Under conditions of 30 and 50 min reaction time, XMG was removed relatively less than under other pretreatment conditions in *L. tulipifera* (80.6% and 74.1%). Low concentrations of sulfuric acid (above pH 1.79) with an extension of the reaction time (over 30 min) prevented the decomposition of XMG. Conditions of 30 and 50 min reaction times were therefore efficient for utilizing both hexose and pentose in the solid residue.

For *P. rigida*, as reaction temperature and pH increased (10 to 50 min with pH 1.31 to 2.01), the elimination of XMG increased and the range of variation (92.9~97.7%) was broader than under the fixed temperature conditions (96.5~92.5%). Reaction temperature was therefore a major factor

for lignin and XMG removal for ethanol organosolv pretreatment with sulfuric acid.

Generally, most of the glucan remained in the solid residues after organosolv pretreatment. An increase in the reaction temperature with dilution of the acidic catalyst could lead to a slight increase in glucose decomposition.

Table 3. WIS recovery rates, Klason lignin (KL), acid-soluble lignin (ASL), structural sugars and lignin removal rates of *L. tulipifera* and *P. rigida* after ethanol organosolv pretreatment

No.	WIS (g)	Lignins (g)			Structural sugars (g)			Total (g)	*Removal rates (%)	
		KL	ASL	Total	Glucan	XMG	Total		Lignin	XMG
Con. *L. tulipifera*		22.9 ±1.4	3.7 ±0.1	26.6 ±1.5	36.4 ±0.2	17.1 ±0.0	53.5 ±0.2	80.0 ±1.7		
1	52.6 ±0.6	9.5 ±0.7	0.9 ±0.0	10.4 ±0.7	31.0±0.1	2.0 ±0.1	33.0±0.2	43.4 ±1.0	58.3	88.1
2	45.2 ±0.7	6.3 ±0.5	0.7 ±0.0	7.0 ±0.5	29.6±1.4	1.3±0.0	30.9±1.4	37.9 ±2.0	72.5	92.6
3	42.4 ±0.1	4.9 ±0.3	0.6 ±0.0	5.5 ±0.3	29.6±0.2	1.0±0.0	30.5±0.2	36.0 ±0.5	78.7	94.3
4	47.4 ±0.7	4.5 ±0.9	0.5 ±0.0	5.0 ±0.9	35.7±0.3	3.3±0.0	39.0±0.3	44.0 ±1.2	80.2	80.6
5	49.5 ±1.1	4.8 ±0.9	0.6 ±0.1	5.4 ±0.9	36.0 ±0.6	4.4±0.2	40.4±0.7	45.7 ±1.7	79.1	74.1
Con. *P. rigida*		33.4 ±2.2	0.8 ±0.1	34.1 ±2.2	37.5±0.6	17.5±0.3	55.0±0.9	89.1 ±3.1		
6	58.0 ±1.7	19.0 ±0.5	0.2 ±0.0	19.2 ±0.5	33.5±0.4	1.2±0.2	34.7±0.5	53.9 ±1.0	43.1	92.9
7	52.7 ±0.7	18.5 ±0.6	0.2 ±0.0	18.8 ±0.6	32.0±0.2	0.7±0.0	32.7±0.2	51.5 ±0.8	44.4	96.0
8	47.3 ±0.7	18.3 ±0.8	0.3 ±0.0	18.6 ±0.9	28.2±0.1	0.4±0.0	28.6±0.1	47.2 ±0.9	45.1	97.7
9	56.8 ±2.3	21.0 ±2.2	0.3 ±0.0	21.2 ±2.2	33.1±1.3	1.0±0.0	34.1±1.3	54.5 ±2.4	39.6	94.5
10	57.6 ±1.1	18.4 ±0.4	0.3 ±0.0	18.6 ±0.5	34.4±1.2	1.3±0.0	35.8±1.2	54.4 ±1.7	44.9	92.5

* Following formula were used for calculating removal rate of lignin and XMG.

$$\text{Lignin removal rate} = \left(1 - \frac{A\ total\ of\ lignins\ in\ solid\ residues}{A\ total\ of\ lignins\ in\ raw\ material}\right) \times 100$$

$$\text{XMG removal rate} = \left(1 - \frac{XMG\ content\ in\ solid\ residues}{XMG\ content\ in\ raw\ material}\right) \times 100$$

4.2 Composition of liquid hydrolysate

4.2.1 Sugar composition

The glucose and xylose content of the liquid hydrolysate from *L. tulipifera* was elevated by an increase in reaction temperature and pH because reaction temperature had a major influence on decomposition of glucan and XMG in the solid residues (conditions 1, 2 and 3 in Table 4). The effect of pH was more remarkable than that of reaction time under the conditions with fixed reaction temperature at 150°C (conditions 2, 4 and 5 in Table 4) similar to the result reported in Section 4.1.2. Under conditions of 170°C in *L. tulipifera*, the highest content of glucose and xylose was observed, indicating that severity of this condition was harder than other conditions.

When the temperature was augmented from 150 to 190°C, the content of xylose declined from 9.1 to 7.5% in *P. rigida*. The decrease in xylose content could be explained by converting xylose to degradation products, and then dissolving the degradation products into the liquid hydrolysate. Content of glucose increased from 3.1 to 5.5% with an increase in reaction temperature, indicating that further degradation of glucose had not yet occurred.

Overall, the glucose decomposition in *P. rigida* was higher than the glucose decomposition in *L. tulipifera* because different CSF values (1.16 and 1.75) were obtained by increases in reaction temperature.

4.2.2 Carbohydrate derivatives

In *L. tulipifera*, the trend in the furfural content was similar to that in the xylose content at fixed reaction temperature and time, indicating that xylose was not completely converted to furfural or other degradation products. The

highest contents of xylose and furfural (10.1% and 0.8%) was observed under the same conditions.

The content of acetic acid in *L. tulipifera* was higher than that of acetic acid in *P. rigida* because structural sugars in the solid residues from *L. tulipifera* were more degraded than those from in *P. rigida*. The degradation of the structural sugars could be explained by the glucan and XMG, which remained in the solid residues (Table 3).

As the reaction temperature was augmented, the content of furfural was increased remarkably in *P. rigida*, closely related to decreases in xylose. The content of furfural was increased by a drop in the xylose content. A CSF value of 1.75 thus led to the conversion of xylose into degradation products in *P. rigida*, and the sum of degradation products in *P. rigida* was higher than those in *L. tulipifera*.

Table 4. Structural sugars and degradation products in the liquid hydrolysate of *L. tulipifera* and *P. rigida* after ethanol organosolv pretreatment

No.	WIS (%)	Structural sugars (%)				Organic acid (%)	Degradation products (%)			
		Glucose	Xylose	Arabinose	Total	Acetic acid	Levulinic acid	HMF	Furfural	Total
1	52.6 ±0.6	0.7 ±0.0	7.5 ±0.0	0.5 ±0.0	8.7 ±0.1	2.2 ±0.0	0.0 ±0.0	0.1 ±0.0	0.2 ±0.0	0.2±0.0
2	45.2 ±0.7	1.2 ±0.1	9.2 ±0.4	0.8 ±0.1	11.2 ±0.6	2.3 ±0.0	0.0 ±0.0	0.1 ±0.0	0.6 ±0.1	0.7±0.1
3	42.4 ±0.1	1.5 ±0.0	10.1 ±0.0	0.9 ±0.0	12.5 ±0.1	2.3 ±0.1	0.0 ±0.0	0.1 ±0.0	0.8 ±0.0	0.9±0.0
4	47.4 ±0.7	1.0 ±0.0	8.9 ±0.2	0.7 ±0.0	10.7 ±0.3	2.2 ±0.0	0.0 ±0.0	0.1 ±0.0	0.4 ±0.0	0.4±0.0
5	49.5 ±1.1	0.9 ±0.1	7.8 ±0.5	0.6 ±0.0	9.3 ±0.6	1.9 ±0.1	0.0 ±0.0	0.0 ±0.0	0.2 ±0.0	0.2±0.1
6	58.0 ±1.7	3.1 ±0.2	9.1 ±0.1	2.6 ±0.1	14.9 ±0.4	0.8 ±0.1	0.1 ±0.1	0.4 ±0.0	1.1 ±0.2	1.6±0.3
7	52.7 ±0.7	4.3 ±0.1	8.6 ±0.0	3.1 ±0.1	16.0 ±0.2	0.7 ±0.0	0.3 ±0.0	0.7 ±0.0	1.6 ±0.1	2.6±0.1
8	47.3 ±0.7	5.5 ±0.1	7.5 ±0.2	3.3 ±0.0	16.3 ±0.2	0.6 ±0.1	0.4 ±0.0	1.3 ±0.0	2.2 ±0.1	3.9±0.1
9	56.8 ±2.3	3.1 ±0.0	9.2 ±0.1	2.6 ±0.0	14.9 ±0.1	0.8 ±0.1	0.1 ±0.0	0.6 ±0.0	1.1 ±0.0	1.7±0.0
10	57.6 ±1.1	2.8 ±0.0	9.2 ±0.1	2.5 ±0.0	14.4 ±0.2	0.7 ±0.1	0.0 ±0.0	0.6 ±0.0	0.9 ±0.0	1.5±0.1

4.3 Enzymatic hydrolysis

The cellulose to glucose conversion yields and enzymatic digestibility were shown to be over 80% under all experimental conditions in *L. tulipifera*, while those parameters were below 45% in *P. rigida* except for the 190°C condition (Figure 3). Condition 2 (150°C for 10 min with pH 1.31) from *L. tulipifera* was designated as a standard condition for optimal enzymatic hydrolysis based on previous studies (Gwak, 2012). The result of enzymatic hydrolysis under other experimental conditions in *L. tulipifera* was similar to condition 2, indicating that CSF values affected the result of enzymatic hydrolysis.

The highest level was observed under condition 3 (170ºC for 10 min and pH 1.90). Lignin in solid residues was recognized as an inhibitor for enzymatic hydrolysis (Huijgen et al., 2011). However, compared with conditions 4 and 5 (150 ºC for 30, and 50 min), condition 3 did not exhibit the lowest Klason lignin content. The efficiency of enzymatic hydrolysis was determined not only by Klason lignin content in the solid residue but also by degradation of cellulose such as the decline in crystallinity and an expansion of the surface of microfibrils.

The highest cellulose to glucose conversion yield and enzymatic digestibility were observed at condition 8 (190ºC for 10 min, and pH 1.90) in all experiments with softwoods (69.4% and 54.2%). These values were significantly higher than the values observed at condition 7 (170ºC for 10 min, and pH 1.31 in *P. rigida*). Condition 7 was designated as an optimal condition for enzymatic hydrolysis in *P. rigida* (Park et al., 2010). The conformation of the cell walls was dramatically disassembled when the reaction temperature reached 190°C.

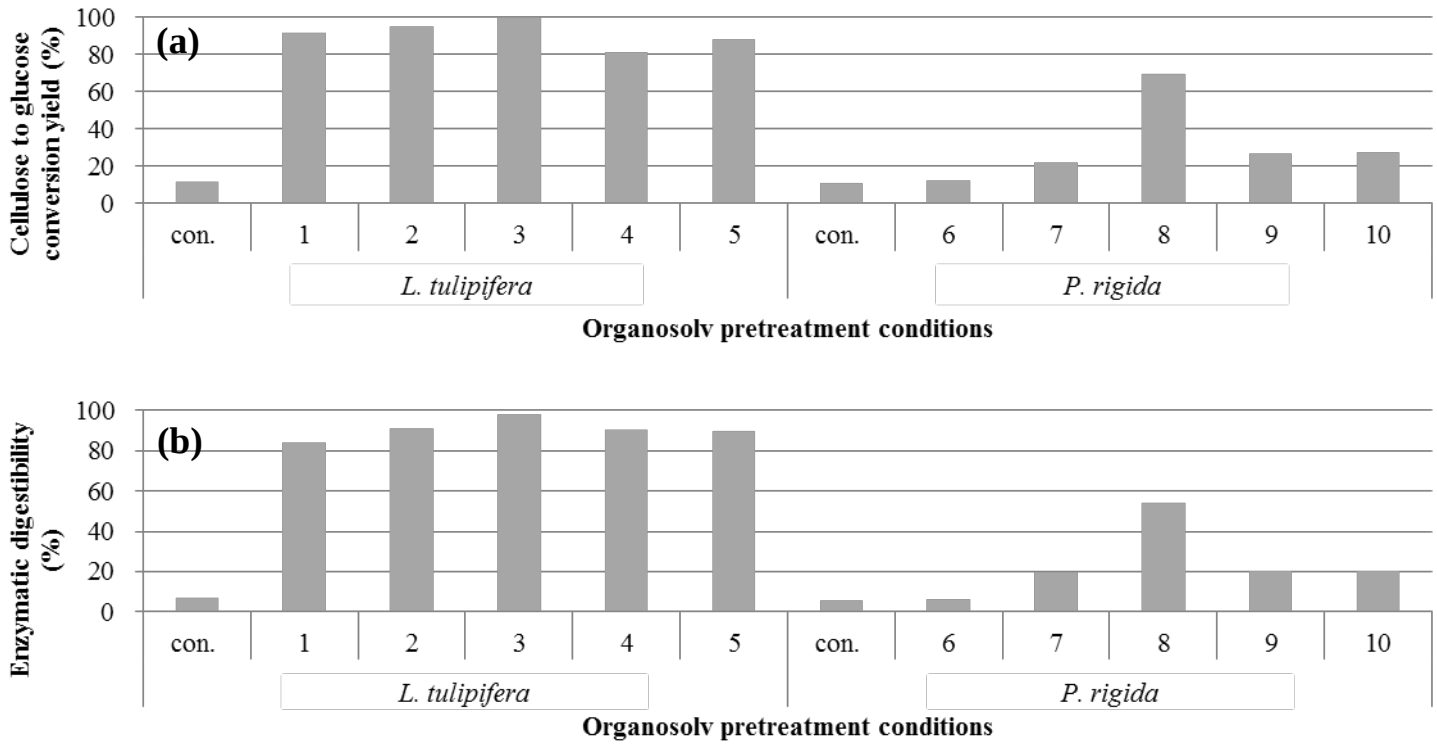

Figure 3. Cellulose to glucose conversion yield (a) and enzymatic digestibility (b) of *L. tulipifera* and *P. rigida* after ethanol organosolv pretreatment

4.4 Chemical structures of ethanol organosolv lignin

4.4.1 Yields of ethanol organosolv lignin

The EOL yields ranged from 11.2 to 12.9% in *L. tulipifera* and from 6.0 to 6.5 in *P. rigida* (Figure 4).

When the reaction temperature and pH increased, the EOL yields were declined. The largest EOL yield (12.9%) was observed under condition 1 (130ºC for 10 min and pH 0.79). This result is opposed to a previous report that an increase in reaction temperature led to the growth of the EOL yields (Wildschut et al., 2012). Decrease in concentration of the acidic catalyst strongly affected EOL yield, even though the reaction temperature was the major factor for organosolv pretreatment under conditions 1 to 3 (pH 0.79 to 1.90). During ethanol organosolv pretreatment, sulfuric acid depolymerized macromolecular lignin by cleaving the ether linkages (α-O-4 or β-O-4 bonds). As this reaction progressed, the lignin fragments became smaller until the lignin fragments dissolved in the ethanol solvent (El Hage et al., 2010; Sannigrahi et al., 2009; Sun et al., 2001). The organosolv pretreatment at a low concentration of acidic catalyst, such as condition 3, might cause a low production of EOL.

Among the conditions with fixed reaction temperature, the highest EOL yield (12.4%) was obtained from condition 4 (30 min and pH 1.79), indicating that an extension of reaction time could contribute to the growth of the EOL yields. When reaction time exceeded 30 min, the EOL yield declined (11.5%) because a negative effect might work on the EOL yields caused by a low concentration of catalyst (< pH 2.01).

The EOL yields were observed at approximately 6% under all experimental conditions in *P. rigida*. There was no significant difference in the EOL yields. The EOL yields were assumed to be related to the Klason

lignin in the solid residues. Further degradation of lignins did not occur below the 20% of Klason lignin obtained in previous results (Table 3 and 4.1.1). A CSF value of 1.75 might be insufficient for lignin removal in softwoods. As a result, the amount of lignins was maintained at a certain level.

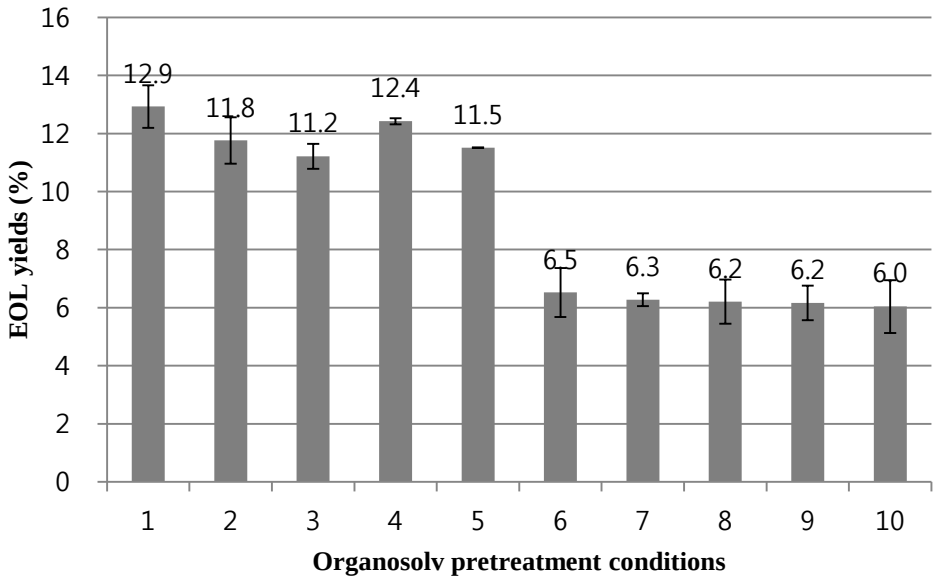

Figure 4. EOL yield (%) of *L. tulipifera* and *P. rigida* after ethanol organosolv pretreatment

4.4.2 Composition of phenolic acid and aldehydes

Nitrobenzene oxidation (NBO) has been recognized as a good analytical method for measuring ether linkages in the lignin structure. A high amount of NBO products indicated few condensed linkages, such as carbon-carbon bonds (Ehara et al., 2002). The results of EOL analysis by NBO are shown in Table 5. The H-units, such as benzoic acid, benzaldehyde and acetophenone, were hardly detected in *L. tulipifera*.

As reaction time and pH increased, the total amounts of G-units and S-units were observed to rise under conditions of fixed reaction temperature (conditions 2, 4 and 5 in Table 5). This result was assumed to be closely related to a change in concentration of the acidic catalyst. The condition 2 observed the lowest amount of NBO products because the EOL structure of condition 2 was more suffered by sulfuric acid.

Total amounts of G-units and S-units were slightly decreased as reaction time and pH increased (conditions 1, 2, and 3 in Table 5). EOL under condition 3 was composed of carbon-carbon linkages on the lignin structure. Ether bonds of the lignin macromolecule were assumed to be cleaved, and lignin fragments were then condensed with other fragments with carbon linkages. This change was accelerated as reaction temperature rose during organosolv pretreatment. This result was interesting because formation of the EOL, which was related to EOL yield, was strongly affected by the acidic catalyst, while reaction temperature might affect the structure of EOL.

Total amounts of G-units in *P. rigida* did not exhibit any trend that was related to EOL yield (Table 6). EOL yields in *P. rigida* were shown to have analogous values under all experimental conditions, indicating that degree of EOL condensation in both softwoods was similar.

Table 5. The amount of G-units, S-units and S/G ratio of ethanol organosolv lignin in *L. tulipifera* after nitrobenzene oxidation

No.	Amount of G-units (%)					Amount of S-units (%)				S/G ratio
	Guaiacol	Vanillin	Vanillic acid	Acetovanilone	Total	Syringol	Syringaldehyde	Syringic acid	Total	
1	0.1 ±0.0	4.4 ±0.2	0.8 ±0.1	0.2 ±0.0	5.4 ±0.4	0.0 ±0.0	12.9 ±0.6	2.8 ±0.4	15.7 ±1.1	2.9
2	0.1 ±0.0	3.9 ±0.0	0.7 ±0.1	0.2 ±0.0	4.8 ±0.1	0.1 ±0.0	9.3 ±0.1	2.3 ±0.0	11.7 ±0.1	2.4
3	0.1 ±0.0	3.6 ±0.1	0.7 ±0.0	0.2 ±0.0	4.5 ±0.2	0.1 ±0.0	8.7 ±0.2	2.1 ±0.0	10.9 ±0.3	2.4
4	0.0 ±0.0	4.7 ±0.3	0.7 ±0.0	0.2 ±0.0	5.6 ±0.3	0.1 ±0.0	11.0 ±0.6	2.2 ±0.2	13.3 ±0.8	2.4
5	0.1 ±0.0	5.2 ±0.3	0.6 ±0.1	0.2 ±0.1	6.0 ±0.5	0.1 ±0.0	12.4 ±0.5	2.1 ±0.2	14.6 ±0.7	2.4

Table 6. The amounts of H-units and G-units of ethanol organosolv lignin in *P. rigida* after nitrobenzene oxidation

No.	Amount of H-units (%)				Amount of G-units (%)				
	Benzoic acid	Benz-aldehyde	Acetophenone	Total	Guaiacol	Vanillin	Vanillic acid	Acetovanilone	Total
6	0.1 ±0.0	0.1 ±0.0	0.0 ±0.0	0.2 ±0.0	0.1 ±0.0	4.1 ±0.4	1.2 ±0.1	0.3 ±0.0	5.7 ±0.5
7	0.1 ±0.0	0.1 ±0.0	0.0 ±0.0	0.2 ±0.1	0.1 ±0.0	5.8 ±1.6	1.7 ±0.4	0.4 ±0.0	8.0 ±2.3
8	0.1 ±0.0	0.1 ±0.0	0.0 ±0.0	0.2 ±0.0	0.1 ±0.0	5.0 ±0.0	1.5 ±0.1	0.3 ±0.0	6.9 ±0.1
9	0.2 ±0.0	0.1 ±0.0	0.0 ±0.0	0.3 ±0.0	0.2 ±0.0	7.8 ±0.9	1.8 ±0.4	0.2 ±0.3	9.9 ±1.6
10	0.1 ±0.0	0.1 ±0.0	0.0 ±0.0	0.2 ±0.1	0.1 ±0.0	4.8 ±1.5	1.0 ±0.2	0.3 ±0.0	6.1 ±1.7

4.4.3 Examination of average molecular weight and polydispersity

The average molecular weight (Daltons) and polydispersities (M_w/M_n) of milled wood lignin (MWL) and EOL were analyzed by gel permeation chromatography (GPC). The results are shown in Table 7.

The average molecular weight (M_w) of the EOL in *L. tulipifera* and *P. rigida* ranged from 2153 to 4276 Daltons and from 1706 to 3078 Daltons. If this result compared with the M_w value of the MWL (9159 and 8483 Da), the lignin macromolecule had become disorganized during organosolv pretreatment.

The average values of the M_w of the EOL in *L. tulipifera* and *P. rigida* were 3182 Daltons and 2066 Daltons, respectively. The EOL was comprised of approximately 15 and 10 monomeric units, if the weight of the monomeric lignin precursors (coniferyl and sinapyl alcohols) were estimated to be approximately 200 Daltons.

With an increase in reaction temperature and pH, the M_w and polydispersities decreased in both *L. tulipifera* and *P. rigida* (conditions 1, 2, 3 and 6, 7, 8). In particular, the M_w in condition 1 (130ºC for 10 min and pH 0.79 in *L. tulipifera*) and 6 (130ºC for 10 min and pH 0.79 in *P. rigida*) was 4276 Daltons and 3078 Daltons, the highest values in each species. As mentioned in Section 4.1.2, the reaction temperature strongly affected the decomposition of lignin. The lignin fragments that were insufficiently cleaved at the low reaction temperature were solubilized into the ethanol solvent. The highest polydispersity under these conditions could thus be understood because of the relatively high portion of the lignin fragments that were not sufficiently decomposed.

The M_w under the conditions with fixed reaction temperature was raised with an increase in the reaction time (conditions 2, 4 and 5), indicating the effect of the acidic catalyst. However, conditions 7, 9 and 10 did not exhibit

any difference in the M_w and polydispersity. Conditions 8, 9 and 10 showed the same polydispersity (1.7), indicating that the EOLs were uniformly composed of G-units.

At condition 1, the highest EOL yield was detected in *L. tulipifera* (Figure 4), but the EOL was organized with the lignin which was less depolymerized with low uniformity. However, condition 3 might be considered an optimal condition for ethanol organosolv lignin application due to its polydispersity (1.9).

Table 7. The average molecular weights and polydispersities of milled wood lignin (MWL), *L. tulipifera* and *P. rigida* after ethanol organosolv pretreatment

No.	**M_n (Dalton)**	**M_w (Dalton)**	**Polydispersity (M_w/M_n)**
MWL (*L. tulipifera*)	3946	9159	2.3
1	1366	4276	3.1
2	1252	2921	2.3
3	1155	2153	1.9
4	1367	3087	2.3
5	1444	3473	2.4
MWL (*P. rigida*)*	2666	8483	3.2
6	1521	3078	2.0
7	1160	1942	1.7
8	1082	1731	1.6
9	1170	1871	1.6
10	1074	1706	1.6

* Referred by Chemical structural characterization for lignin extracted from pitch pine with ionic liquid. Kim, 2012

4.5 Total phenol in the supernatant

The sum of lignins in the solid residue (Klason lignin and acid-soluble lignin) and EOL were measured to estimate the mass balance of lignin in *L. tulipifera* and *P. rigida*. The total lignin including solid and EOL was consisted of a small amount of lignin in each raw material, indicating that the ethanol solubilized lignin still remained in the supernatant after precipitation and centrifugation. To determine the undetectable lignin, analysis of total phenol was performed using supernatants. Figure 5 illustrates the mass balance of total lignins in *L. tulipifera* and *P. rigida*, where the total phenol ranged from 4.7 to 8.0% under all experimental conditions.

The highest total phenol was observed under condition 3 (8.0%). The large production of small lignin fragments caused by the high temperature might cause an increase of the portion of unprecipitated lignins. This assumption was supported by the analysis of GPC reported in Section 4.4.3.

In particular, under condition 1, total lignin exceeded the sum of Klason lignin and acid-soluble lignin in unpretreated *L. tulipifera*. The Folin-Ciocalteu reagent that was used for detection of phenolic compounds could react with any reducing constituents, such as sugars (Singleton et al., 1999). Certain pentoses in the liquid hydrolysate and supernatant after EOL recovery might react with the reagent.

Total phenols in the supernatant from *P. rigida* did not exhibit any trend, which was not expected.

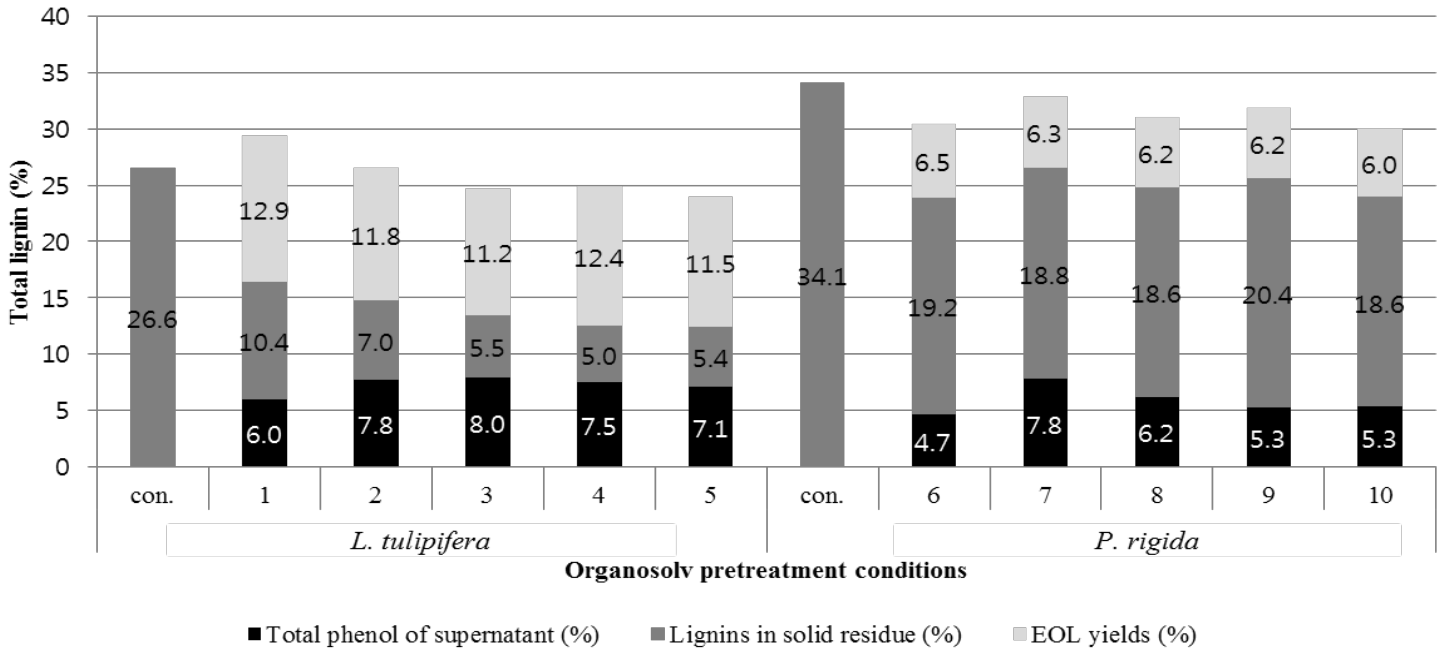

Figure 5. Mass balance (%) of the total lignin in *L. tulipifera* and *P. rigida* after ethanol organosolv pretreatment

*Lignins in solid residue : the sum of Klason lignin and ASL were shown in Table 3.

4.6 Production of bio-oil by supercritical ethanol treatment

The recoverable products (oil and char) from supercritical ethanol treatment were evaluated by the yield of depolymerized products. Figure 6 illustrates the yields of oil, char and gas under conditions 1 to 3. The yields of low molecular weight EOL were observed with 20.8~28.4% oil, 26.9~39.4% char and 39.5~44.7% gas according to the organosolv pretreatment conditions for EOL. A decreasing tendency for the yield in the oil fraction appeared under conditions 1 to 3.

Analysis of the oil fraction was performed by GC/MS to identify the monomeric compounds, and the results are summarized and organized by molecular weight (MW) in Table 8. The monomeric compounds were observed from approximately 36.1 min to 72.6 min of the retention time (RT). The peak of fluoranthene that was used as an internal standard was detected at 96 min RT. As a result of the peak analysis, 17 monomeric units were detected by GC/MS using the NIST MS Search 2.0 (NIST/EPA/NIH Mass Spectral Library; NIST 02) and references (Faix et al., 1990).

At the EOL of condition 3, a total of 27.9 mg/g of phenolic compounds were obtained after the supercritical treatment. The products of the reaction mainly consisted of 9.2 mg/g of 4-methylsyringol, 6.6 mg/g of syringol, 1.9 mg/g of syringyl acetone and 1.8 mg/g of 3-methoxy catechol. The monomeric compounds in condition 3 were composed primarily of S-units.

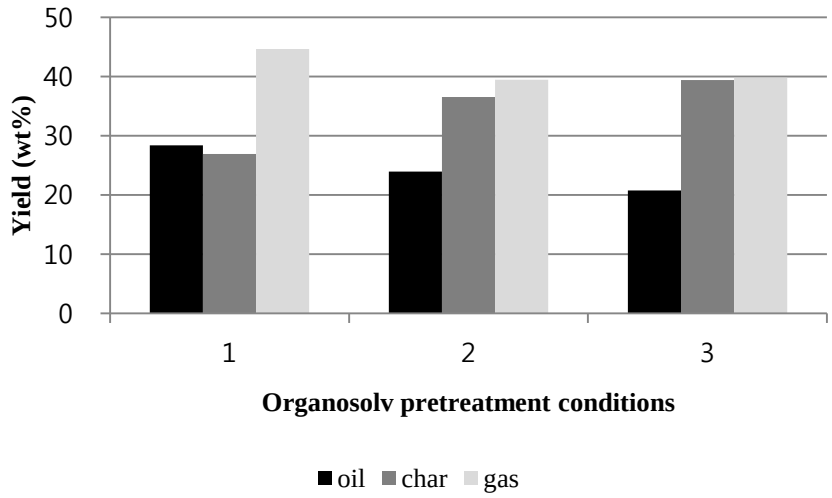

Figure 6. The product yield after supercritical ethanol treatment from EOL in *L. tulipifera*

The yields of the three main products (oil, gas, and char) were determined as followed equation:

$$\text{Oil yields (wt\%)} = \frac{the\ weight\ of\ oil\ fraction}{the\ weight\ of\ the\ EOL\ samples} \times 100$$

$$\text{Char yields (wt\%)} = \frac{the\ weight\ of\ solid\ fraction}{the\ weight\ of\ the\ EOL\ samples} \times 100$$

$$\text{Gas yields (wt\%)} = 100 - (Oil\ yield + Char\ yield)$$

Table 8. Phenolic monomers in oil determined using GC/MS after ethanol supercritical treatment

No.	Monomeric compounds	Type	RT	MW	Amount of monomeric compounds (mg/g)		
					1	2	3
1	Guaiacol	G	36.05	124	1.7	0.3	1.0
2	3-methyl guaiacol	G	41.16	138	0.2	0.4	0.3
3	4-methyl guaiacol	G	41.95	138	1.3	2.0	1.8
4	3-methoxy catechol	S	46.79	140	0.1	0.3	1.0
5	4-ethylguaiacol	G	48.05	152	0.6	0.2	0.8
6	Syringol	S	53.32	154	3.4	3.8	6.6
7	4-propylguaiacol	G	54.61	154	0.3	1.4	0.6
8	4-methylsyringol	S	60.03	168	2.5	4.4	9.2
9	acetoguaiacone	G	63.13	166	0.2	0.2	0.2
10	3-ethylsyringol	S	63.86	182	0.4	0.1	0.2
11	4-ethylsyringol	S	65.28	182	2.2	1.7	1.0
12	guaiacyl acetone	G	65.81	180	0.2	0.2	1.1
13	4-(2-propenyl)-syringol	S	70.16	194	0.3	0.6	0.6
14	4-propylsyringol	S	70.62	196	0.4	0.3	1.0
15	4-(2-propenyl)-syringol	S	68.40	194	0.1	0.4	0.1
16	Acetosyringone	S	70.70	196	0.1	0.3	0.6
17	Syringyl acetone	S	72.55	210	2.6	2.2	1.9
Total of monomeric compounds derived from G-units (mg/g)					4.6	4.7	5.7
Total of monomeric compounds derived from S-units (mg/g)					12.1	14.2	22.2
Total of monomeric compounds (mg/g)					16.7	18.9	27.9

* No. 9, 10, 11, 12 and 17 were quantified by assuming response factor as 1

**Identification was referred by (Faix et al., 1990).

5 Conclusions

The conversion characteristics of the solid and liquid fractions, ethanol organosolv lignin and supernatant after organosolv pretreatment of *L. tulipifera* and *P. rigida* were investigated under various conditions with theoretically identical pretreatment conditions.

The reaction temperature was considered the most influential process parameter for organosolv pretreatment in *L. tulipifera*, producing XMG and lignin removal from the solid residue, sugar decomposition and degradation products of the liquid hydrolysate, enzymatic hydrolysis and properties of EOL after organosolv pretreatment. Effective lignin removal and glucose conversion were obtained under condition 3. This condition was characterized by the highest degree of condensation and good distribution of EOL at the same time. The second influential factor was concentration of the acidic catalyst that slightly affected the degradation of the solid residues and the EOL yield. The structural sugars in the solid residue and the nitrobenzene oxidation products were affected by reaction time, while reaction time was the weakest parameter in organosolv pretreatment. Maintenance of reaction temperature was less important at a certain temperature. The trend in sugar composition of *P. rigida* was similar to that in *L. tulipifera*, but few differences were observed in the values related to the degradation of lignin, such as EOL yield and total phenols in the supernatant, under various pretreatment conditions.

The average EOL recovery in *L. tulipifera* was shown to be approximately twice than the average EOL recovery in *P. rigida*, and the efficiency of the glucose conversion in *L. tulipifera* was vastly superior. This result indicated that *L. tulipifera* was appropriate for sugar and EOL production, and this finding might be related to the high recalcitrance of softwoods. A further process was therefore conducted using the EOL of

hardwoods to evaluate the feasibility of a biorefinery, and approximately 2.5% of the conversion rates for phenolic compounds was detected after supercritical ethanol treatment.

The effect of reaction temperature was clearly revealed in the analysis of degradation products from solid and liquid fractions and also in the formation of EOL. According to the results of the process parameters evaluation, optimal organosolv pretreatment condition for the production of sugars and lignin might be determined to be condition 3 because high enzymatic digestibility, low polydispersity of EOL and the amount of phenolic monomer resulting from supercritical treatment were determined under in this condition, even if the lowest EOL yield was observed.

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초 록

본 연구에서는 combined severity factor를 참고하여 다양한 조건의 에탄올 유기용매 전처리가 진행되었다. 유기용매 전처리 조건은 반응 온도, 시간 그리고 산촉매의 농도와 같은 인자들의 영향력을 평가하기 위해 고안되었다. 수종별로 각각 5가지의 전처리 조건이 정해졌고, 이전에 연구되었던 최적 효소 당화 조건을 CSF 값의 기준점으로 삼았다.

백합나무를 이용한 유기용매 전처리 결과, 반응 온도와 pH가 상승 (130~170°C, pH 0.79~1.90)함에 따라 고형분 내의 리그닌과 자일란의 제거가 각각 79%와 94%가 될 때까지 증가하였다. 반면, 반응 시간이 늘어나고, pH가 증가할 때(10~50분, pH 1.31~2.01), 자일란의 분해는 완화되었으나 리그닌의 제거량은 조금 증가하였다. 리기다소나무의 분해 양상은 대체로 백합나무와 유사했으나 대신에 리그닌 제거 효율은 떨어졌다. 고형분에 대한 분석을 통해, 반응 온도는 유기용매 전처리 환경에 가장 큰 영향을 미치는 인자로 판단된다.

백합나무의 경우, 84~98%의 효소 소화율을 나타냈고, 이는 동일한 CSF 값이라 할지라도 각 변인들에 의해 효소 당화율이 달라질 수 있음을 보여주었다. 반면 리기다소나무의 경우, 190°C, pH 1.90의 조건을 제외하고 모든 실험 조건에서 30%이하의 효소 소화율 결과를 나타났다.

유기용매 리그닌의 수율은 각각 백합나무에서 약 11~13%, 리기다소나무에서 약 6% 정도를 보였다. 유기용매 리그닌의 특성을 분석하기 위해 nitrobenzene oxidation과 GPC 분석을 실시하였다. 그 결과, 높은 반응 온도는 유기용매 리그닌의 축합을 야기하고 구조의

균질성을 향상시키는 것으로 나타났다. 리그닌 단량체 수율을 평가하기 위해 초임계 에탄올 환경에서 단분자화를 실시하였다. 그 결과, 100 g의 유기용매 리그닌을 기준으로 1.7~2.8% 정도의 리그닌 유래 단량체를 생산하였다.

본 실험을 통해 유기용매 전처리에 영향을 미치는 각 변인들의 영향력을 알아보았고, 반응 온도가 전처리 공정에 있어서 주된 요인임을 파악하였다. 또한, 높은 효소 소화율 (98%)과 단량체 수율(2.8%) 결과를 통해, 백합나무의 170°C, 10분 그리고 pH 1.90 조건이 당과 리그닌을 동시에 생산하기에 적합한 조건으로 사료된다.