

# Optimization of Bio-based Succinic Acid Production from Hardwood Using the Two Stage pretreatments\*<sup>1</sup>

Ji Young Jung\*<sup>2</sup>, Jong Soo Jo\*<sup>3</sup>, Young Wun Kim\*<sup>4</sup>, Byeng Tae Yoon\*<sup>4</sup>,  
Choon Gil Kim\*<sup>5</sup>, and Jae Kyung Yang\*<sup>2†</sup>

## ABSTRACT

The steam explosion-chemical pretreatment is a more effective wood pretreatment technique than the conventional physical pretreatment by accelerating reactions during the pretreatment process. In this paper, two-stage pretreatment processes of hardwood were investigated for its enzymatic hydrolysis and the succinic acid yield from the pretreated solid. The first stage pretreatment was performed under conditions of low severity to optimize the amount of solid recovery. In the second stage pretreatment washed solid material from the first stage pretreatment step was impregnated again with chemical (alkaline or chlorine-based chemicals) to remove a portion of the lignin, and to make the cellulose more accessible to enzymatic attack. The effects of pretreatment were assessed by enzymatic hydrolysis and fermentation, after the two stage pretreatments. Maximum succinic acid yield (16.1 g L<sup>-1</sup> and 77.5%) was obtained when the two stage pretreatments were performed at steam explosion -3% KOH.

*Keywords* : hardwood, two stage pretreatments, steam explosion pretreatment, succinic acid

## 1. INTRODUCTION

Succinic acid, derived from fermentation of agricultural carbohydrates, has a specialty chemical market in industries producing food and pharmaceutical products, surfactants and detergents, green solvents and biodegradable plastics, and ingredients to stimulate animal and plant

growth (*Zeikus et al., 1999*). Lignocellulosic materials represent renewable resources for production of fuel ethanol from sugars. In particular, lignocellulosic material such as woody biomass is regarded as a promising energy source because it is renewable and consists of abundant carbohydrates.

\* 1 Received on November 23, 2012; accepted on March 7, 2013

\* 2 Division of Environmental Forest Science, Gyeongsang National University, Institute of Agriculture & Life Sciences, Jinju 660-701, Korea

\* 3 Gyeongnam National University of Science and Technology, Department of Interior Materials Engineering, Jinju 660-758, Korea

\* 4 Korea Research Institute of Chemical Technology, Daejeon 305-600, Korea

\* 5 SK Energy Institute of Technology, Daejeon 305-712, Korea

† Corresponding author : Jae-Kyung Yang (e-mail: jkyang@gnu.ac.kr)

Among the nutrient sources available, cellulosic and hemicellulosic materials such as wood and municipal solid wastes are renewable and most abundant (Kim *et al.*, 2004). Enzymatic hydrolysate of wood has been regarded as one of the most inexpensive carbon source for the fermentative production of biochemicals. Even though wood hydrolysate is a good source for fermentable glucose and xylose, it contains some unknown toxic or inhibitory compounds for cells, resulting in low efficiency of bioconversion (Lee *et al.*, 1999; Converti *et al.*, 1998; Palmqvist *et al.*, 2000).

The main drawbacks in hydrolysing lignocellulosic biomass are to do with the low specific activity of the enzymes and on the substrate structure itself. The utilization of lignocellulosic materials in a bioconversion process requires a pre-treatment step in order to break down the various physical and chemical barriers that greatly inhibit their susceptibility to bioprocess such as enzymatic hydrolysis and fermentation (Mosier *et al.*, 2005). Generally, the pretreatment process is crucial and costly, which has a significant impact on the efficiency of subsequent enzymatic hydrolysis. The enzymatic hydrolysis of lignocellulose is mainly limited by several factors including crystallinity of cellulose, degree of polymerization (DP), moisture content, available surface area and lignin content (Chang *et al.*, 2000; Hendriks and Zeeman, 2009).

To overcome these obstacles, many pretreatment processes, including mechanical pretreatment, thermal pretreatment, acid pretreatment, alkaline pretreatment, oxidative pretreatment, and some combinations, have been investigated extensively (Hendriks and Zeeman, 2009). Among the different existing pre-treatment methods, steam explosion is one of the most commonly used for fractionation of biomass components. High-pressure steaming followed by rapid decompression is called steam-explosion. The steam ex-

plosion process has been tried and found useful in the low cost biomass conversion of wood and crop residues (Taylor and Yu 1995). It has been found as an effective pretreatment of wood biomass for biotechnological conversion for production of food, feed and chemicals (Jackobsons *et al.*, 1995; Kallavus and Cravitis, 1995).

The treatment results in substantial breakdown of the lignocellulosic structure, hydrolysis of the hemicellulosic fraction, depolymerization of the lignin components and defibrillation. Therefore, the accessibility of the cellulose components to degradation by enzymes is greatly increased (Moniruzzaman *et al.*, 1996). Compared with alternative pre-treatment methods, the advantages of steam explosion include a significantly lower environmental impact, lower capital investment and less hazardous process chemicals (Li *et al.*, 2001). On the other hand, the presence of degradation products from sugar and lignin is unavoidable and must be taken into account in order to minimize the potential negative effect on subsequent steps (Tengborg *et al.*, 2001).

Recently, steam-explosion has been considered as an effective pretreatment (Duff and Murray, 1996) of waste cellulosic materials for further processing, including olive stones (Fernández *et al.*, 1999). The resultant material is finely divided; and the main components-cellulose, hemicellulose, and lignin- are separated (Felizón *et al.*, 2000). Furthermore, the enzymatic hydrolysis of cellulose is enhanced, which could create alternative uses for the hardwood.

Some bases can also be used for pretreatment of lignocellulosic materials and the effect of alkaline pretreatment depends on the lignin content of the materials (Fan *et al.*, 1987). The mechanism of alkaline hydrolysis is believed to be saponification of intermolecular ester bonds cross-linking xylan hemicelluloses and other components, for example, lignin and other hemicellulose. The porosity of the lignocellulosic materials

increases with the removal of the crosslinks (Tarkow and Feist, 1969).

The aim of this work is to evaluate the fractionation of hard wood by the process sequence based on controlled steam explosion at high temperature followed by alkaline and chlorine pretreatment. Also, we reported, the succinic acid production from pretreated hard wood hydrolysate by *Actinobacillus succinogenes* CGMCC 1593.

## 2. MATERIALS and METHODS

### 2.1. Raw Material

The raw material used in the pretreatment experiments was wood chips, 2~10 mm in size, derived from four year old stems of a hardwood, which were chopped and gathered at Korea Forest Research Institute in the city of Seoul, Korea.

### 2.2. Pretreatment of Hardwood

#### 2.2.1. First Stage Pretreatment

Steam explosion pretreatment, which was used as a physicochemical pretreatment, was carried out in a batch pilot unit equipped with a 1 L reaction vessel. The “severity parameter” ( $Ro$ ) was used to map the destruction, desegregation, and depolymerization of *Quercus variabilis*.  $Ro$  was calculated using the following relationship :

$$Ro = \{t \cdot \exp[(T - 100)/14.5]\} \quad (1)$$

where  $T$  is the temperature (°C) and  $t$  the time (min). A steam temperature of 225°C and pretreatment time of 5 min were applied to realize a severity parameter value of  $\log Ro = 4.37$ , which ensured a significant reduction of DP with the lowest degradation and produced a material more suitable for hydrolysis (De Bari *et al.*,

2002). After the saturated steam exposure, a ball valve at the bottom of the reactor was opened suddenly to bring the reactor rapidly to atmospheric pressure. This yielded the product, containing liquid and solid materials (Take *et al.*, 2006). The exploded material was recovered in a cyclone and after cooling to about 40°C filtered for solid recovery. The solid fraction was water-washed and used for chemical pretreatment.

#### 2.2.2. Second Stage Pretreatment

We added 100 ml of 1%, 3% (w/v) of sodium hydroxide (NaOH), potassium hydroxide (KOH), Sodium hypochlorite (NaClO) and Sodium chlorite (NaClO<sub>2</sub>) to 200 ml Erlenmeyer flasks at a steam exploded sample loading of 1% (w/v) in an shaking incubator (IS-97IR from Jeio-Tech Co., Korea) at the room temperature and 100 rpm for 3 hours. After the pretreatment, the sample was washed with water several times, and stored at 4°C before enzymatic hydrolysis (Chang and Holtzapfel, 2000).

### 2.3. Enzymatic Hydrolysis of Hardwood Pretreated with Different Methods

The effects of the pretreatment conditions on the digestibility were assessed by enzymatic hydrolysis of pretreated material from the pretreatment step. Enzymatic hydrolysis was performed in 1 g batches with 2% (w/w) pretreated material in 0.1 M sodium citrate buffer (pH 4.8) at a temperature of 40°C for 96 h. A commercial cellulase mixture, kindly supplied by Novozymes A/S (Bagsværd, Denmark), consisting of Celluclast 1.5 l (65 FPU g<sup>-1</sup>), supplemented with the  $\beta$ -glucosidase preparation Viscozyme l (24 CBU g<sup>-1</sup>) was used in enzymatic hydrolysis. Samples were withdrawn after 0, 24, 48, 72 and 96 h to monitor the progress of hydrolysis. Duplicate experiments were run for each sample.

## 2.4. Succinic Acid Fermentation

*Actinobacillus succinogenes* CGMCC 1593 was used for succinic acid fermentations. Cells were anaerobically grown in sealed anaerobic bottles (100 ml) under CO<sub>2</sub> atmosphere. The medium for seed culture contained per liter : 2.5 g dextrose, 2.5 g dipotassium phosphate, 5 g sodium chloride, 17 g enzymatic digest of casein, 3 g enzymatic digest of soybean meal. The medium for fermentation followed as per liter : 30 g total sugar from wood hydrolysate, 10 g yeast extract, 6 g urea, 1.09 g Na<sub>2</sub>HPO<sub>4</sub>, 0.95 g NaH<sub>2</sub>PO<sub>4</sub>, 0.2 g NaCl, 0.2 g MgCl<sub>2</sub>, 0.2 g CaCl<sub>2</sub>, 40 g MgCO<sub>3</sub>, 20 μg Vitamin B12, 50 μg itamin B6, 50 μg Riboflavin, 50 μg Lipoic acid, 50 μg Niacin, 50 μg Thiamine hydrochloride, 50 μg Folic acid, 50 μg Vitamin B5, 50 μg ethyl p-aminobenzoate, 100 μg Biotin, to seed or fermentation medium, concentrated 2 M NaOH was added to adjust the pH to 6.5. The medium was heat sterilized (15 min at 121°C) in an anaerobic bottle sealed with a butyl rubber stopper with a nitrogen headspace. *A. succinogenes* CGMCC 1593 was incubated in sealed 150 ml anaerobic bottles containing 50 ml of the seed medium with CO<sub>2</sub> as the gas phase at 37°C for 24 h. For the fermentation experiments, the medium was inoculated with 5% (v/v) seed culture and incubated at 37°C for 48 h.

## 2.5. Analytical Methods

The raw material composition was determined according to the National Renewable Energy Laboratory (NREL, Golden, CO) analytical methods for biomass. Extractives are determined as the solubilized material after a Soxhlet extraction with ethanol for 24 h. Ashes result from raw material calcination at 550°C. Cellulose and hemicellulose content was determined based on monomer content measured after a two-step acid hydrolysis procedure to fractionate the fiber. A first

step with 72% H<sub>2</sub>SO<sub>4</sub> at 30°C for 60 min was used. In a second step, the reaction mixture was diluted to 4% H<sub>2</sub>SO<sub>4</sub> and autoclaved at 121°C for 1 h. This hydrolysis liquid was then analysed for sugar content by high performance liquid chromatography (HPLC) in a Waters 2695 liquid chromatography with refractive index detector. An Aminex HPX-87P carbohydrate analysis column (Bio-Rad, Hercules, CA) operating at 85°C with deionised water as mobile-phase (0.6 ml/min) was used. The cellulose and hemicellulose contents were calculated using Eqs. (2) and (3), where 0.9 is the correction coefficient for hydration (Iyer and Lee, 1999):

$$\begin{aligned} & \% \text{ Cellulose} \\ & = \frac{\text{glucose released (g)} \times 0.9}{\text{sample dry weight (g)}} \times 100 \end{aligned} \quad (2)$$

$$\begin{aligned} & \% \text{ Hemicellulose} \\ & = \frac{(\text{xylose} + \text{galactose} + \text{arabinose released}) (\text{g}) \times 0.9}{\text{sample dry weight (g)}} \times 100 \end{aligned} \quad (3)$$

The solid residue remaining after this acid hydrolysis is considered as acid insoluble lignin. The acid-soluble lignin content in the hydrolysates is also quantified. The acid soluble lignin is determined based on UV spectrophotometry at 205 nm. Pre-treated solids were analyzed for sugar and acid insoluble lignin content as described above for raw material composition.

Glucose concentration from enzymatic hydrolysis samples was measured by high performance liquid chromatography (HPLC). Succinic acid was analyzed by HPLC (high-performance liquid chromatograph) system (Waters, Milford, MA, USA) equipped with a cation-exclusion column (Aminex HPX-87H; Bio-Rad Chemical Division, Richmond, CA) and a refractive index detector (Waters) as described previously (Liu *et al.*, 2008). The mobile phase was 0.005 N

H<sub>2</sub>SO<sub>4</sub> solution at a flow rate of 0.5 ml/min, and the column was operated at 55°C. Succinic acid yield was defined as the amount of succinic acid produced from 1 g sugar consumed, and was expressed as a percentage. The sugar utilization was calculated as sugar consumed initial sugar, and was expressed in percentage (Zheng *et al.*, 2009). All analytical determinations were performed in duplicate and average results are shown.

### 3. RESULTS and DISCUSSION

#### 3.1. Chemical Composition of Hardwood

The chemical composition of the untreated hardwood used in this study is presented in Table 1. Based on the HPLC carbohydrate analysis, the sugar fraction was 58.6% of the dry biomass. Glucose, which is derived from both the hardwood fiber and plant cell wall, was the major component at 44.6%. Xylose, which is the major hemicellulose constituent, was 10.8%. Arabinose, galactose and mannose accounted for only a small portion of the biomass. Both glucan and xylan content were lower than the reported values of 40~55% glucan and 15~40% xylan for other woody plant and agricultural residues (Milne *et al.*, 1992; Sun *et al.*, 2002; Zhu and Pan, 2010; J. Söderström *et al.*, 2003; Jorgensen *et al.*, 2007). The discrepancy between the holo-cellulose content and total sugars is probably due to sugar degradation during the intense hydrolysis with sulfuric acid (Badger, 2002) used for the carbohydrate analysis procedure. Since the carbohydrate content of hardwood (based on monomeric sugars) determined by HPLC analysis was more likely to represent the actual sugars available after the treatments, subsequent calculations and analysis of data in this study were performed on the basis of HPLC measurements. The acid insoluble and soluble material from woody biomass is normally classified as lignin. The acid-

Table 1. Chemical composition of untreated hardwood<sup>a</sup>

Composition	Dry weight (%) <sup>a</sup>
Extractives	5.7
Cellulose as glucose	44.6
Hemicellulose <sup>b</sup> as	14.0
Xylose	10.8
Galactose	2.4
Arabinose	0.5
Mannose	0.3
Acid insoluble lignin	24.2
Acid soluble lignin	3.3
Ash	2.9
Other	5.3

<sup>a</sup>Composition percentages are on a dry-weight basis.

insoluble content of the hardwood was 24.2% and lignin was higher than the reported values of 18~25% other hardwoods (Sun *et al.*, 2002; Ucar,1990; Mosier *et al.*, 2005).

#### 3.2. Effect of Pretreatment on the Chemical Composition

The chemical composition change of hardwood is important indices for the effectiveness of its pretreatment. Table 2 shows the two stage pretreatment of this study, as well as yields of both the steam explosion pretreatment solid recovered (%) and the insoluble residue recovered by filtration after steam explosion-chemical pretreatment. It is found that the solid recovery is significantly decreased by the steam explosion treatment from 100% of raw material to 85% of the steam exploded material. Fiber losses occur during steam explosion because of the deposition of fibers on the walls of the cyclone as well as in the connecting piping between the reactor vessel and the cyclone. Losses also occurred through the escape of volatiles with the steam and through the degradation of sugars

Table 2. Solids recovery of hardwood after steam explosion-alkaline, chlorine pretreatment processes

Pretreatment	Pretreatment conditions			Solid recovered after pretreatment (%) <sup>a</sup>
	Conc. (%) <sup>b</sup>	Reaction temperature (°C)	Reaction time (min)	
First stage				
Steam explosion (log <i>Ro</i> 4.37)		225	5	85.0
Second stage				
Steam explosion-KOH	1	30	180	51.7
	3			50.7
Steam explosion-NaOH	1	30	180	51.0
	3			50.2
Steam explosion-NaClO	1	30	180	76.5
	3			67.4
Steam explosion-NaClO <sub>2</sub>	1	30	180	74.8
	3			50.2

<sup>a</sup>Percentages calculated from value on a dry-weight basis.

<sup>b</sup>Conc. : chemical concentration.

Table 3. Chemical composition of hardwood after steam explosion-alkaline, chlorine pretreatment processes

Pretreatment	Composition (%) <sup>a</sup>				
	Conc. (%) <sup>b</sup>	Cellulose	Hemicellulose	Lignin <sup>c</sup>	Ash
First stage					
Steam explosion (log <i>Ro</i> 4.37)		52.4 (44.5) <sup>d</sup>	4.0 (3.4)	31.7 (26.9)	2.4 (2.0)
Second stage					
Steam explosion-KOH	1	75.1 (38.8)	1.0 (0.5)	14.8 (7.7)	2.5 (1.3)
	3	75.2 (38.1)	- <sup>c</sup>	14.6 (7.4)	2.4 (1.2)
Steam explosion-NaOH	1	72.0 (36.7)	0.5 (0.3)	16.5 (8.4)	3.6 (1.8)
	3	69.9 (35.1)	0.2 (0.1)	17.4 (8.7)	3.0 (1.5)
Steam explosion-NaClO	1	50.2 (38.4)	0.4 (0.3)	33.2 (25.4)	3.0 (2.3)
	3	50.1 (33.8)	0.5 (0.3)	36.9 (24.9)	3.1 (2.1)
Steam explosion-NaClO <sub>2</sub>	1	53.4 (39.9)	0.6 (0.4)	33.1 (24.8)	2.8 (2.1)
	3	70.6 (35.4)	0.2 (0.1)	47.4 (23.8)	2.6 (1.3)

<sup>a</sup>Percentages calculated from values on a dry-weight basis.

<sup>b</sup>Conc. : chemical concentration.

<sup>c</sup>Amount of hemicellulose was too low to quantify.

<sup>d</sup>Data are expressed in parentheses as a percentage based on dry weight of raw material.

<sup>e</sup>Acid insoluble lignin and acid soluble lignin.

into furfural and 5-hydroxymethyl furfural, both of which are volatile compounds (Jeoh *et al.*, 1998). Steam explosion conditions (log *Ro* 4.37)

were optimized to obtain maximum solid recovery (data not shown). Alkaline and chlorine consisted of two stages : KOH, NaOH, NaClO and

Table 4. Weight loss of hardwood after steam explosion-alkaline, chlorine pretreatment processes

Pretreatment	Weight loss (%) <sup>a</sup>			
	Conc. (%) <sup>b</sup>	Cellulose	Hemicellulose	Lignin <sup>c</sup>
First stage				
Steam explosion (log <i>Ro</i> 4.37)		0.2	75.7	2.0
Second stage				
Steam explosion-KOH	1	13.0	96.3	72.2
	3	14.6	100.0	73.1
Steam explosion-NaOH	1	17.7	98.2	69.4
	3	21.4	99.3	68.2
Steam explosion-NaClO	1	14.0	97.8	7.6
	3	24.4	97.6	9.6
Steam explosion-NaClO <sub>2</sub>	1	10.5	96.8	10.0
	3	20.6	99.3	13.5

<sup>a</sup>Percentages calculated from values on a dry-weight basis.

<sup>b</sup>Conc. : chemical concentration.

<sup>c</sup>Acid insoluble lignin and acid soluble lignin.

NaClO<sub>2</sub> followed by steam explosion. The percentage of solid recovery ranged between 50.2 and 76.5%. As expected, a decrease of total gravimetric recovery was detected as the pretreatment chemical concentration increased. This is mainly attributed to the decrease of hemicellulosic fraction and lignin fraction; as can be seen in Table 3, the hemicellulosic-derived sugars and lignin recovery yield in the insoluble fiber (expressed as a percentage of the initial component in the raw material) decreased, with values ranging from 4 to 0.2%, 31.7~14.6%, respectively. Removal of hemicelluloses from the microfibrils is believed to expose the cellulose surface and increase enzyme accessibility to the cellulose microfibrils. Lignin is removed only to a limited extent during the steam explosion but is re-distributed on the fiber surfaces as a result of melting and depolymerization/repolymerization reactions (Kabel *et al.*, 2007; Li *et al.*, 2007).

The chemical composition change and weight loss of hardwood are important indices for the effectiveness of its Pretreatment. Table 3 and

Table 4 list the chemical composition and weight loss of hardwood after each treatment stage for steam explosion-alkaline, steam explosion-chlorine pretreatment processes, respectively. Results show that when compared with the untreated materials, both alkaline and chlorine pretreatment decrease lignin and hemicellulose and produce higher cellulose, ranging from 50.1% to 75.2%. The maximum cellulose content (75.2%) was attained at log *Ro* 4.37~3% KOH. Hemicellulose was completely removed at log *Ro* 4.37~3% KOH while the increase in chemical concentration resulted, in general, in increased solubilisation of hemicellulose. The lignin of the steam exploded material, referred to raw material, showed a slight decrease (31.7%). On the other hand, the lignin of the chemical pretreated residue, showed a rapidly decrease (maximum decrease of 14.6% at log *Ro* 4.37~3% KOH). The recovered hardwood of alkali pretreatment have significantly reduced lignin content compared to the untreated hardwood. The weight loss of hardwood after pretreatment is presented in Table 4.

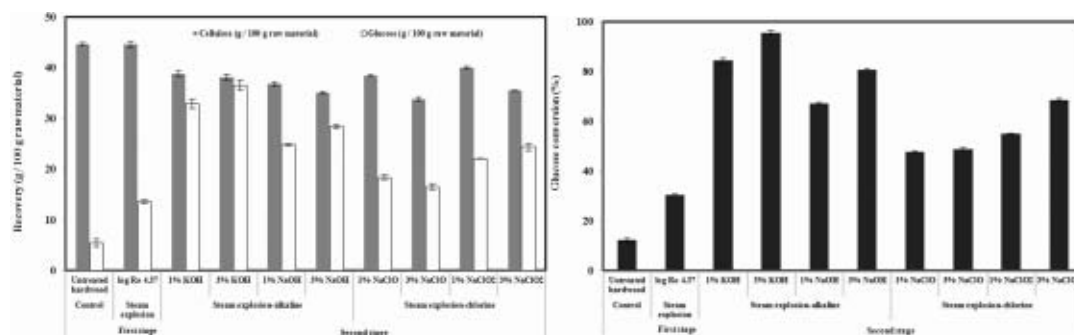


Fig. 1. Effect of steam explosion-alkaline, chlorine pretreatment processes on the production of glucose sugars by enzymatic hydrolysis: Symbols are cellulose recovery (■), glucose recovery (□) and glucose conversion (■).

A regular increase in degradation of hardwood was observed with the increase in chemical concentration from 1% to 3% (w/v). This behaviour, leading to a considerable loss of cellulose increasing with chemical concentration, is shown on hemicellulose and lignin. The steam explosion, steam explosion-alkaline and steam explosion-chlorine pretreatment removed most of the hemicellulose.

The lowest lignin and hemicelluloses content indicated that the log Ro 4.37~3% KOH pretreatment efficiently removed these components in hardwood. All these results show that the alkaline pretreatment is an efficient hardwood pretreatment technique. The best procedure for efficiently removing lignin from the steam exploded hard wood was the combination with alkaline treatment. The alkali treatment was originally suggested by Feist *et al.* (1970) as a way to partially solubilize lignin and improve the digestibility of hardwood substrate. Subsequently, it was also used to efficiently remove lignin from steam-exploded hardwood residues (Ramos *et al.*, 1992).

### 3.3. Effect of Pretreatment on Glucose Conversion

The solid material obtained after the second

pretreatment step was hydrolysed enzymatically to assess the effects of pretreatment. The solid material pretreated residue was submitted to enzymatic hydrolysis by a cellulase complex (Celluclast 1.5 ℓ) supplemented with β-glucosidase (Viscozyme ℓ). The concentration of glucose was determined every 24 h during a 96 h-period of enzymatic action. The cellulose recovery, glucose recovery and glucose conversion during hydrolysis is presented in Fig. 1. The result indicated that pretreatment of substrate enhanced the digestibility of lignocellulosic material. The glucose conversion of steam explosion-alkaline (67.4%~95.7%) was higher than that of steam explosion-chlorine (47.8%~68.7%). The highest conversion of cellulose into glucose is found when hardwood is pretreated at log Ro 4.37~3% KOH (95.7%). At high enzymatic concentrations, a maximum of 33 g and 36 g of the glucose recovery present in the insoluble residue (cellulose) of pretreated material at log Ro 4.37~1% KOH and log Ro 4.37~3% KOH, respectively, were solubilized after 96 h. The increase in the extent of saccharification was proportional to the slight alkaline delignification (Table 4), which occurred when the concentration of the alkaline treatment was increased. The cellulose fraction that was insoluble in the alkaline treatment was a more suitable substrate



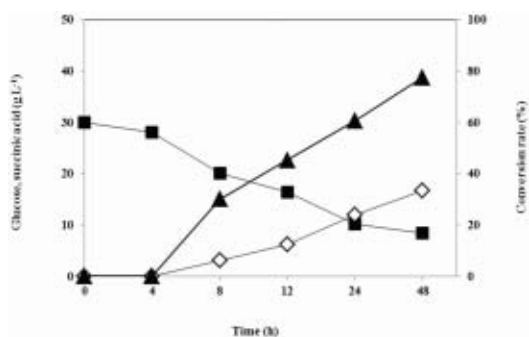


Fig. 2. Time course of glucose consumption, production of succinic acid and conversion rate in fermentation from wood hydrolysates of steam explosion -3% KOH pretreated hardwood by *A. succinogenes* CGMCC 1593. Symbols are glucose (■), succinic acid (◇) and conversion rate (▲).

for the cellulases than the original cellulose. This could be due to the greater susceptibility of cellulose crystalline form II hydrolysis by both endo- and exoglucanases. Furthermore, the alkaline treatment apparently altered the fibre structure, leading to greater accessibility of the cellulose to enzymatic attack (Chang and Holtzapfel, 2000).

### 3.4. Succinic Acid Production from the Wood Hydrolysate Based Medium

Batch fermentation was carried out with the treated wood hydrolysate based medium to investigate the time profile of glucose consump-

Table 5. Comparison of succinic acid yield produced from various lignocellulosic materials and pretreatments

Lignocellulosic material	Pretreatment	Strain	Succinic acid (%)	Reference
Oak wood	Steam explosion 6 min, 215°C	<i>M. succiniciproducens</i> MBEL55E	55.0	Kim <i>et al.</i> (2004)
Cane molasses	Sulfuric acid (5H <sub>2</sub> SO <sub>4</sub> , 60°C, 2 h)	<i>A. succinogenes</i> CGMCC1593	80.5	Liu <i>et al.</i> (2008)
	Potassium ferrocyanide (100 ppm, room temperature, 24 h)		78.6	
	Resin (cation exchange resin, 250 rpm, 6 h, 30°C)		80.0	
	Activated carbon (50°C, 15 min)		80.2	
Corn straw	Dilute-alkali	<i>A. succinogenes</i> CGMCC1593	81.0	Zheng <i>et al.</i> (2009)
Rice straw	89.1			
Corn core	62.8			
Wheat straw	74.1			
Corn stover	0.75% H <sub>2</sub> SO <sub>4</sub> , 121°C, 1 h	<i>A. succinogenes</i> CGMCC1593	23.8	Zheng <i>et al.</i> (2010)
	1% NaOH, 121°C, 1 h		98.8	
	10 % ammonia, 30°C, 24h		25.9	
	2% H <sub>2</sub> O <sub>2</sub> /4M NaOH, 30°C, 16 h		23.2	
	Steam explosion 5 min, 187°C		38.4	
Hard wood	Two stage (Steam explosion 5 min, 225°C - 3% (w/v) KOH/room temperature, 3 h, 100 rpm)	<i>A. succinogenes</i> CGMCC1593	77.5	This work

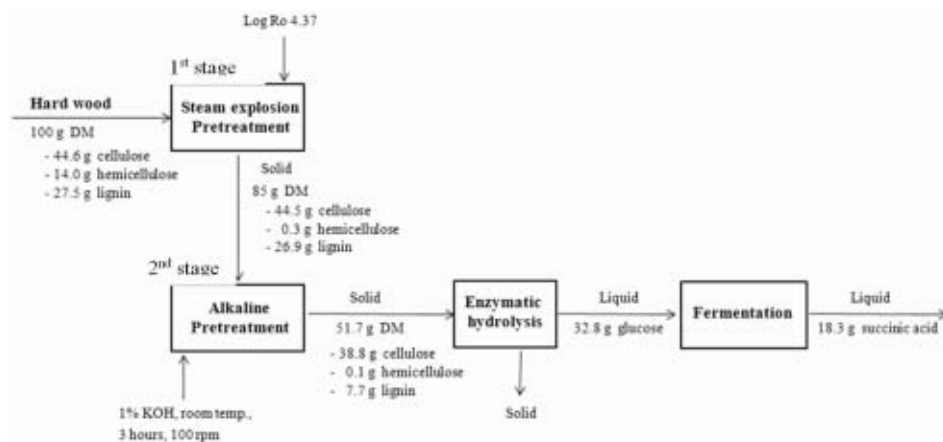


Fig. 3. Mass balance diagram of steam explosion - 3% KOH pretreatment followed by fermentation.

tion and succinic acid formation. The time courses of fermentation processes are shown in Fig. 2. When the log  $Ro$  4.37~3% KOH treated wood hydrolysate based medium containing 30.0 g  $\ell^{-1}$  of glucose was used, 21.6 g  $\ell^{-1}$  of glucose was consumed. Similarly to glucose fermentations, succinic acid was a major product. The succinic acid concentration and conversion rate of succinic acid obtained at the end fermentation were 16.7 g  $\ell^{-1}$ , 77.5%, respectively. When compared with succinic acid production by microorganism from pretreatment and renewable material as shown in Table 5, Among all the pretreatment cases, succinic acid concentration produced from diluted alkaline pretreated lignocellulosic material was the highest for fermentation. Compared with the steam explosion pretreatment (Kim *et al.*, 2004; Zheng *et al.*, 2009), two stage (steam explosion-alkaline) pretreatment has the advantages over the former in the aspects of high reducing sugar production ability and less inhibitory substances formation. The hardwood hydrolysate was successfully utilized for succinic acid production.

A mass balance diagram is shown in Fig. 3 for better understanding (assumption a thumb rule is 50% of the glucose will be converted into ethanol with an efficiency of 90%).

## 4. CONCLUSION

This study showed a promising combined method for hardwood pretreatment using steam explosion, alkaline and chlorine. Pretreatment of hardwood with log  $Ro$  4.37- 3% KOH was the most effective method for succinic acid production by *A. succinogenes* CGMCC1593. The solid recovery and chemical composition showed that the two stage pretreatment resulted in partial removal of hemicelluloses and lignin. The hardwood was subjected to two stage pretreatment, yielding cellulose fractions of 50.1~75.2%. Among the two stage pretreatments examined, the steam explosion-KOH pretreatment was the most effective one in improving enzymatic hydrolysis of the untreated hardwood, with glucose recovery of 36 g per 100 g raw material. The concentration and yield of succinic acid in SHF process at 48 h could reach 16.7 g/ $\ell$  and 77.5%, respectively. This result suggested a potential of succinic acid industrial production by two stage pretreatment and using lignocellulosic biomass materials.

## ACKNOWLEDGEMENT

This study was carried out with the support of “Research Center for Ecological Restoration in Damaged land (project No. S211212L0504)” provide by Korea Forest Service, Republic of Korea and partially supported by “Development of Application Technology using Succinic Acid Derivatives (project No. 10033530; Ministry of Knowledge Economy)”.

## REFERENCES

1. Badger, P. C. 2002. Ethanol from cellulose: a general review. In: Janick, J., Whipkey, A. (Eds.), Trends in New Crops and New Uses. ASHS Press, Alexandria, VA, 17~21.
2. Chang, V. S. and M. T. Holtzapfle. 2000. Fundamental factors affecting enzymatic reactivity. Applied Biochemistry and Biotechnology 84 ~ 86: 5~37.
3. Converti, A. and M. D. Borghi. 1998. Inhibition of the fermentation of oak hemicellulose acid hydrolysate by minor sugars. Journal of Biotechnology 64: 211~218.
4. De Bari, I., Viola, E., Barisano, D., Cardinale, M., Nanna, F., Zimbardi, F., Cardinale, G., and G. Braccio. 2002. Ethanol production at flask and pilot scale from concentrated slurries of steamexploded aspen. Industrial & Engineering Chemistry Research 41: 1745~1753.
5. Duff, S. J. B. and W. D. Murray. 1996. Bio-conversion of forest products industry waste cellulosic to fuel ethanol: A Review. Bioresource Technology 55: 1~33.
6. Fan, L. T., Gharpuray, M. M., and Y. H. Lee. 1987. In: Cellulose hydrolysis biotechnology monographs. Springer, Berlin, p. 57.
7. Feist, W. C., baker A. J., and H. Tarkow. 1970. Alkali requirements for improving digestibility of hardwoods by rumen micro-organisms. Journal of Animal Science 30: 832~836.
8. Felizón, B., Fernández-Bolaños, J., Heredia, A., and R. Guillén. 2000. Steam explosion pretreatment of olive cake. Journal of the American Oil Chemists' Society 77: 15~22.
9. Fernández-Bolaños, J., Felizón, B., Heredia, A., Guillén, R., and A. Jiménez. 1999. Characterization of the lignin obtained by alkaline delignification and of the cellulose residue from steam exploded olive stones. Bioresource Technology 68: 121~132.
10. Hendriks, A. T. W. M. and G. Zeeman. 2009. Pretreatments to enhance the digestibility of ligno-cellulosic biomass. Bioresource Technology 100: 10~18.
11. Iyer P. V. and Y. Y. Lee.1999. Product inhibition in simultaneous saccharification and fermentation of cellulose into lactic acid. Biotechnology Letters 21: 371~373.
12. Jacobsons, J., Hortling, B., Erins, P., and J. Sundquist. 1995. Characterization of alkali soluble fraction of steam exploded birch wood. Holzforschung 49(1): 51~59.
13. Jeoh, T. Steam explosion pretreatment of cotton gin waste for fuel ethanol production. Master's thesis, Virginia Tech. University, VA, 1998.
14. Jorgensen, H., Kristensen, J. B., and C. Felby. 2007. Enzymatic conversion of lignocellulose into fermentable sugars: Challenges and opportunities. Biofuels, Bioproducts and Biorefining 1: 119~134.
15. Kabel, M. A., Bos, G., Zeevalking, J., Voragen, A. G., and H. A. Schols. 2007. Effect of pretreatment severity on xylan solubility and enzymatic breakdown of the remaining cellulose from wheat straw. Bioresource Technology 98: 2034~2042.
16. Kallavus, U. and Gravitis. J. 1995. A comparative investigation of the ultrastructure of steam exploded wood with light, scanning and transmission electron microscopy. Holzforschung 49(2): 182~188.
17. Kim, D. Y., Yim, S. C., Lee, P. C., Lee, W. G., Lee, S. Y., and H. N. Chang. 2004. Batch and continuous fermentation of succinic acid from wood hydrolysate by *Mannheimia succiniciproducens* MBEL55E. Enzyme and Microbial Technology 35: 648~653.
18. Lee, W. G., Lee, J. S., Shin, C. S., Park, S. C.,

- Chang, H. N., and Y. K. Chang. 1999. Ethanol production using concentrated oak wood hydrolysates and methods to detoxify. *Applied Biochemistry and Biotechnology* 77~79: 547~559.
19. Li, J., Lennholm, H., Henriksson, G., and G. Gellerstedt. 2001. Bio-refinery of lignocellulosic materials for ethanol production. II. Fundamentals and strategic design of steam explosion. In: Kyritsis S, Beenackers AACM, Helm P, Grassi A, Chiaramonti D, editors. Proceedings of the first world conference on biomass for energy and industry 1: 767~770.
  20. Li, J., Henriksson, G., and G. Gellerstedt. 2007. Lignin depolymerization/repolymerization and its critical role for delignification of aspen wood by steam explosion. *Bioresource Technology* 98: 3061~3068.
  21. Liu, Y. P., Zheng, P., Sun, Z. H., Ni, Y., Dong, J. J., and L. L. Zhu. 2008. Economical succinic acid production from cane molasses by *Actinobacillus succinogenes*. *Bioresource Technology* 99: 1736~1742.
  22. Milne, T. A., Chum, H. L., Agblevor, F. A., and D. K. Johnson. 1992. Standardized analytical methods. *Biomass and Bioenergy* 2: 341~366.
  23. Mosier, N., Wyman, C., Dale, B., Elander, R. Lee, Y. Y., Holtzapfel, M., and M. Ladisch. 2005. Features of promising technologies for pretreatment of lignocellulosic biomass. *Bioresource Technology* 96: 673~686.
  24. Moniruzzaman, M. 1996. Effect of steam explosion on the physicochemical properties and enzymatic saccharification of rice straw. *Applied Biochemistry and Biotechnology* 59: 283~297.
  25. Palmqvist, E. and B. Hahn-Hägerdal. 2000. Fermentation of lignocellulosic hydrolysates. I: Inhibition and detoxification. *Bioresource Technology* 74: 17~24.
  26. Ramos, L. P., Breuil, C., Kushner, D. N., and J. N. Saddler. 1992. Steam pretreatment conditions for effective enzymatic hydrolysis and recovery yields of *Eucalyptus viminalis* wood chip. *Holzforchung* 46: 149~154.
  27. Söderström, J., Pilcher, L., Galbe, M., and G. Zacchi. 2003. Two-step pretreatment of softwood by dilute H<sub>2</sub>SO<sub>4</sub> impregnation for ethanol production. *Biomass and Bioenergy* 24: 475~486.
  28. Sun, Y. and J. Cheng. 2002. Hydrolysis of lignocellulosic materials for ethanol production: a review. *Bioresource Technology* 83: 1~11.
  29. Take, H., Andou, Y., Nakamura, Y., Kobayashi, F., Kurimoto, Y., and M. Kuwahara. 2006. Production of methane gas from Japanese cedar chips pretreated by various delignification methods. *Biochemical Engineering Journal* 28: 30~35.
  30. Tarkow, H. and W. C. Feist. 1969. In: A Mechanism for Improving the Digestibility of Lignocellulosic Materials with Dilute Alkali and Liquid NH<sub>3</sub> *Advance Chemistry Series* 95. American Chemical Society, Washington, DC, 197~218.
  31. Taylor, J. D. and E. K. C. Yu. 1995. Continuous Steam Explosion. *Chemtech-American Chemical Society* 2: 38~41.
  32. Tengborg, C., Galbe, M., and G. Zacchi. 2001. Reduced inhibition of enzymatic hydrolysis of steam-pretreated softwood. *Enzyme and Microbial Technology* 28: 835~844.
  33. Zeikus, J. G., Jain, M. K., and P. Elankovan. 1999. Biotechnology of succinic acid production and markets for derived industrial products. *Applied Microbiology and Biotechnology* 51: 545~552.
  34. Zheng, P., Dong, J. J., Sun, Z. H., Ni, Y., and L. Fang. 2009. Fermentative production of succinic acid from straw hydrolysate by *Actinobacillus succinogenes*. *Bioresource Technology* 100: 2425~2429.
  35. Zheng, P., Fang, L., Xu, Y., Dong, J. J., Ni, Y., and Z. H. Sun. 2010. Succinic acid production from corn stover by simultaneous saccharification and fermentation using *Actinobacillus succinogenes*. *Bioresource Technology* 101: 7889~7894.
  36. Zhu, J. Y. and X. J. Pan. 2010. Woody biomass pretreatment for cellulosic ethanol production: technology and energy consumption evaluation. *Bioresource Technology* 101: 4992~5002.
  37. Ucar, G. 1990. Pretreatment of poplar by acid and alkali for enzymatic hydrolysis. *Wood Science and Technology* 24: 171~180.