Two-Stage Flow-Through Pretreatment of *Helianthus tuberosus* Residue for Enzymatic Production of Fermentable Sugar by Alkaline and Acidic Solutions

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A response surface methodology (RSM) tool with the Box-Behnken design was used to determine the optimum pretreatment conditions of *Helianthus tuberosus* residue for the enzymatic production of fermentable sugar with aqueous ammonia and sulfuric acid solutions, for various parameters such as pretreatment solution concentration, temperature, and reaction time. The pretreatment of biomass was performed using these optimized parameters in aqueous ammonia and sulfuric acid solution, followed by hot water, under the same conditions. The process was then performed by changing the sequence.

**Keywords:** Biomass; Pretreatment; Response surface method; *Helianthus tuberosus* residue; Aqueous ammonia; Sulfuric acid

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**INTRODUCTION**

Cellulosic materials are the most economic and highly renewable natural resources in the world (Zhu *et al.* 2006). Cellulosic materials contain sugars that are polymerized to cellulose and hemicellulose; these can be liberated by hydrolysis and subsequently fermented by microorganisms to form different chemicals (Beak and Kwon 2007). Cellulose, hemicellulose, and lignin in plant biomass are useful resources that are convertible to not only pulp and foodstuff, but also energy resources (*e.g.*, alcohol and methane) and chemical raw materials (*e.g.*, furfural and organic acids). Because cellulose and hemicellulose in the cell wall become intimately associated with lignin during the growth of plants, an efficient pretreatment for separating cellulose and hemicellulose from plant biomass with ease at low cost is a very important goal (Sawada and Nakamura 2001).

Pretreatment using aqueous ammonia has been studied extensively. The major purpose of aqueous ammonia pretreatment is to remove lignin. Aqueous ammonia pretreatment can remove 60% of lignin from cellulosic biomass while achieving 70% enzymatic digestibility. In addition, ammonia can selectively react with lignin, ester, and especially ether bonds, causing the selective removal of lignin in biomass (Gao *et al.* 2012; Jurado *et al.* 2013; Wanga *et al.* 2016). Ammonia pretreatment material is a suitable substrate for enzymatic hydrolysis and ethanol production. The residual ammonia by pretreatment process is a potential nitrogen source for fermentation. The ammonia-treated biomass can be used without any extraction. Ammonia is characterized by non-toxicity and
provides assistance to subsequent processes (Mes-Hartree et al. 1988). Acid hydrolysis is one of the most promising pretreatment methods. Dilute sulfuric acid pretreatment has been studied for many types of cellulosic biomass; it results in a high recovery of hemicellulose in the pretreatment liquid and solid cellulose fraction (Hu and Ragauskas 2012; Jönsson and Martin 2016). Both concentrated and diluted acids have been used to pretreat cellulosic biomass. Acid pretreatment can be applied to solubilize partial hemicelluloses from cellulosic biomass. However, the analysis of this propensity is fundamentally a result of a batch reaction. The batch reaction process should be used for analysis after removing the reaction conditions (temperature, pressure, etc.). After the reaction is completed, because the separated material in the reaction are in the same space, the material dissolved in the biomass may be condensed again in the cellulose and hemicellulose; therefore, a flow-through process is required. However, the concentrated acid pretreatments likely lead to severe cellulose degradation, high inhibitor concentrations, and serious equipment corrosion (Alvira et al. 2010; Sun et al. 2016). To avoid degrading cellulose/hemicellulose, thus forming inhibitors, a flow-through reactor for pretreatment of cellulosic biomass at high temperatures and pressure (300 psig) is required. A flow-through column reactor performs reasonably well in this regard because it is packed with biomass, which allows for operations with a high solid/liquid ratio, attains the working temperature quickly, and may enable a clear interpretation of the effect of fractionation in a desired time (Martins et al. 2015; Reddy et al. 2015; Terán-Hilares et al. 2016).

The pretreatment of biomass is a process that can include fractionation of the inhibitor component affecting enzymatic hydrolysis and microorganism fermentation. The two-stage process increases the efficiency of pretreatment and fractionates specific components in the liquid phase. The liquid phase is fractionated in a solid phase by the action of a particular solvent, and the biomass swells from solid removal. This widens the surface area of the cellulose that reacts with the enzyme. For example, the first step of the two-stage pretreatment may consist of lignin removal by a basic solution, and the second step can involve fractionation to hemicellulose by an acidic solution. In addition, the percolation of the solution in the reverse sequence induces an efficient reaction in the respective solution. The two-stage pretreatment has an advantage; it elicits an appropriate pH value for the enzymatic hydrolysis of biomass.

When pretreatment is performed using a batch process, both the pretreatment solution and pretreated biomass remain together on the reactor, structurally. Therefore, it is disadvantageous when the fractionated component (liquid phase) and pretreated biomass are recombined. However, because the pretreatment solution can be stored in the reservoir tank without leaving the reactor, by controlling the reaction time, the flow-through process can be reduced to structurally combine both the fractionated component and pretreated biomass. Therefore, it is possible to clearly analyze the effect of removing inhibitors of enzymatic hydrolysis, according to the pretreatment conditions. Response surface methodology (RSM) allows for construction of a proper experimental design so that multivariate equations may be determined and simultaneously solved. The main advantage of RSM is that it reduces the number of experimental trials needed to evaluate multiple parameters and their interactions (Kim and Han 2012).

This study utilized a two-stage pretreatment process to assess the effects of these important parameters. The optimal treatment conditions were determined by an RSM statistical approach. The two-stage pretreatment process was then performed with the optimal conditions.
EXPERIMENTAL

Materials
Helianthus tuberosus residue was provided by the Korea Research Institute of Bioscience and Biotechnology (KRIBB) and milled to below 30 to 50 mesh. Aqueous ammonia (10 to 20 wt.%; Duksan, CAS No. 1336-21-6, Ansan, Republic of Korea) and 0.5 to 2 wt.% sulfuric acid (Duksan, CAS No. 7664-93-9) were used. For enzymatic hydrolysis Celluclast 1.5L (CAS No. 9012-54-8, Novozymes, Bagsvard, Denmark) and Novozyme-188 (CAS No. 9001-22-3, Novozymes, Bagsvard, Denmark), were used.

Response Surface Methodology (RSM)
The experimental design and statistical analysis optimization of process conditions are two of the most critical stages in the development of an efficient and economic bioprocess. Classical and statistical methodologies are available for optimizing process conditions, such as RSM. RSM is a powerful mathematical model with a collection of statistical techniques, wherein interactions between multiple process variables can be identified with fewer experimental trials. The RSM used in the present study was a Box-Behnken design involving three different factors. Experiments were conducted under specified conditions. The independent variables selected were pretreatment solution (wt.%), temperature (°C), and reaction time (min) (Sasikumar and Viruthagiri 2008).

The statistical software package, Design-Expert (Stat-Ease, Inc., Minneapolis, MN, USA), was used for regression analysis of experimental data and plotting the response surface. Analysis of variance (ANOVA) was used to estimate the statistical parameters (Jeya et al. 2009).

Flow-Through Pretreatment
The pretreatment of H. tuberosus residue was carried out using a flow-through column reactor. The pretreatment reaction was performed at a specified temperature and reaction time. The solid/liquid ratio of the reaction solution was 1/10. The system consisted of a stock solution reservoir, pump, temperature-programmable oven, SS-316 column reactor (3 cm internal diameter × 19.4 cm length, internal volume of 137 cm³), and liquid-holding tank. The reactor was operated in flow-through mode, where the liquid flowed through the reactor column packed with biomass. The reactor system was pressurized with nitrogen at 2.3 MPa to prevent flash evaporation. In a typical flow-through experiment, 35 g of biomass were packed into the reactor. The reaction was initiated by raising the reactor temperature in a forced-air convection oven. Approximately 15 min of preheating were required to reach the desired temperature. The reaction time was counted after the desired temperature was attained, and all flow-through experiments were run in duplicate (Kim et al. 2003; Kim and Kim 2010; Park and Kim 2012). The flow-through reactor was also used to perform a two-stage pretreatment to apply to different solutions according to the specified pretreatment conditions. The two-stage pretreatment process was sequentially applied to the basic solution, acidic solution, and hot water using a flow-through reactor.

Enzymatic Digestion
The pretreated H. tuberosus residue was hydrolyzed in Erlenmeyer flasks. The enzymatic digestions were performed in a 0.1 M citrate buffer solution (pH 4.8) shaken at 150 rpm for 72 h. The conditions of enzymatic digestion were a substrate concentration of 5 wt.%, temperature of 50 °C, enzyme loading of 65 FPU/mL (FPU: filter paper unit) of
substrate, and 32 CBU/mL (CBU: cellobiose unit) of substrate (Kim and Kim 2010; Park et al. 2011; Park and Kim 2011; Park and Kim 2012). The digestibility was calculated as:

$$\text{Enzymatic digestibility(\%) } = \left( \frac{\text{Amount of glucose released(g) \times 0.9}}{\text{Total initial glucan (g)}} \right) \times 100$$

(1)

**Analytical Methods**

The compositions of sugars and acid-insoluble lignin (AIL) were determined according to the National Renewable Energy Laboratory (NREL) standard biomass analytical procedures. The compositions of the hydrolysates from the enzymatic digestion were determined using high-performance liquid chromatography (HPLC). The HPLC system consisted of a Bio-Rad Aminex HPX-87H column (Hercules, USA) and a refractive index detector. The mobile phase was 5 mM sulfuric acid at a flow rate of 0.6 mL/min at 60 °C. Prior to injection into the HPLC apparatus, all samples were centrifuged at 15,000 rpm for 10 min and filtered through 0.2 µm syringe filters (Kim and Kim 2010; Park and Kim 2011, 2012; Park et al. 2011).

**RESULTS AND DISCUSSION**

**Compositions of Cellulosic Biomass**

The compositions of biomass were analyzed before pretreatment. *Helianthus tuberosus* residue was used as the cellulosic biomass, and raw *H. tuberosus* residue was composed of 42.4% cellulose, 17.1% hemicellulose, and 21.9% acid-insoluble lignin (AIL).

**Pretreatment by Aqueous Ammonia for RSM**

These experiments were designed using the RSM tool and Box-Behnken design to optimize the conditions for pretreatment by aqueous ammonia. The independent variables in the experiment were aqueous ammonia concentration, temperature, and reaction time, as depicted in Table 1. The experimental conditions consisted of 10 to 20 wt.% aqueous ammonia, a temperature of 130 to 210 °C, and reaction time of 20 to 40 min.

| Table 1. RSM Design Conditions for Aqueous Ammonia |
|---------------------------------|---------|--------|--------|
| Independent Variable           | Symbol  | Levels |
| Aqueous ammonia concentration (wt.%) | X1      | 10     | 15     | 20     |
| Temperature (°C)               | X2      | 130    | 170    | 210    |
| Reaction time (min)            | X3      | 20     | 30     | 40     |

The pretreated *H. tuberosus* residue underwent enzymatic hydrolysis. The response categories under the RSM conditions included solid remaining, glucose recovery yield, delignification yield, and enzymatic digestibility, as depicted in Table 2; the response result is illustrated in Fig. 1. The RSM tool predicted the optimum conditions for pretreatment by aqueous ammonia. The flow-through reaction with biomass was based on the experimental results, and a predictive model was proposed for enzymatic hydrolysis. The coefficient of determination ($R^2$) for the predictive model displayed a high reliability of 0.9826. The optimum conditions were predicted as 19 wt.% aqueous ammonia, 163 °C,
and a reaction time of 38 min. This result was expressed by Eq. 2.

\[
\text{Predicted enzymatic digestibility(\%) } = -136.72031 + 0.58175X_1 + 0.95287X_2 + 4.8415X_3 - 0.007875X_1X_2 - 0.0285X_1X_3 - 0.012875X_2X_3 + 0.0804X_1^2 + 0.000241X_2^2 - 0.03315X_3^2
\] (2)

The pretreatment of the \textit{H. tuberosus} residue was performed by a flow-through process using predicted optimum pretreatment conditions from the RSM model. Following pretreatment, the solid remaining, glucan recovery yield, delignification yield, and enzymatic digestibility were 60.1\%, 95.6\%, 63.0\%, and 70.9\%, respectively. Pretreatment by hot water, for comparison, was performed at the same temperature and reaction time as the ammonia pretreatment.

Table 2. RSM Results for Aqueous Ammonia

<table>
<thead>
<tr>
<th>No.</th>
<th>X1</th>
<th>X2</th>
<th>X3</th>
<th>Solid Remaining (%)</th>
<th>Glucan Recovery Yield (%)</th>
<th>Delignification Yield (%)</th>
<th>Enzymatic Digestibility (% Theoretical Max. Glucose)</th>
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<tr>
<td>1</td>
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<td>40</td>
<td>75.2</td>
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<td>56.6</td>
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<td>2</td>
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<td>210</td>
<td>30</td>
<td>55.4</td>
<td>81.5</td>
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<td>20</td>
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<td>20</td>
<td>56.6</td>
<td>88.1</td>
<td>33.0</td>
<td>99.6</td>
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<tr>
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<td>30</td>
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<td>86.3</td>
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<td>38.2</td>
<td>72.6</td>
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</table>

The effects of two-stage pretreatment by aqueous ammonia and hot water were investigated. Pretreatment under the same conditions resulted in a level of solids remaining, glucan recovery yield, delignification yield, and enzymatic digestibility of 59.2\%, 91.4\%, 57.0\%, and 77.6\%, respectively.

The measured enzymatic digestibility displayed 9.4\% increase in the two-stage reaction over the single reaction. These results confirmed a synergy between the aqueous ammonia and hot water pretreatment reactions.
Pretreatment by Sulfuric Acid Solution with RSM

Pretreatment experiments were performed in sulfuric acid solution. The concentration, temperature, and reaction time in sulfuric acid solution were the independent variables, as depicted in Table 3. The experimental conditions were 0.5 to 1.5 wt.% sulfuric acid solution, 130 to 210 °C, and a reaction time of 20 to 40 min. The response results under RSM conditions, such as solid remaining, glucan recovery yield, delignification yield, and enzymatic digestibility are displayed in Table 4 and Fig. 2.

**Table 3. RSM Design Conditions for Sulfuric Acid Solution**

<table>
<thead>
<tr>
<th>Independent Variable</th>
<th>Symbol</th>
<th>Levels</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sulfuric acid solution concentration (wt.%)</td>
<td>X1</td>
<td>0.5 1.0 1.5</td>
</tr>
<tr>
<td>Temperature (°C)</td>
<td>X2</td>
<td>130 170 210</td>
</tr>
<tr>
<td>Reaction time (min)</td>
<td>X3</td>
<td>20 30 40</td>
</tr>
</tbody>
</table>
Similarly to the use of ammonia, optimum conditions were predicted with the RSM tool, and a predictive model was proposed for enzymatic hydrolysis. The coefficient of determination ($R^2$) for the predictive model was 0.9504. The predicted optimum conditions were 0.6 wt.% sulfuric acid solution, 169 °C and a reaction time of 22 min. This result was expressed by Eq. 3:

$$\text{Predicted enzymatic digestibility(\%) = -425.47969 + 88.3775X_1 + 4.20637X_2 + 2.32925X_3 - 0.52375X_1X_2 - 0.165X_1X_3 - 0.014X_2X_3 + 1.48X_1^2 - 0.007566X_2^2 + 0.00095X_3^2}$$  (3)

The pretreatment of the *H. tuberosus* residue was performed using a flow-through process at predicted optimum conditions. The solid remaining, glucose recovery yield, delignification yield, and enzymatic digestibility were 54.9%, 89.2%, 36.9%, and 48.1%, respectively.

Pretreatment by hot water, in comparison, was performed under the same temperature and reaction time. In addition, a two-stage pretreatment was performed by sulfuric acid solution and hot water. These results indicated that the solids remaining, glucose recovery yield, delignification yield, and enzymatic digestibility were 48.2%, 80.6%, 40.9%, and 56.7%, respectively.
Mass Balance in Single Reaction for Biomass

The pretreatment of biomass was performed at predicted optimum conditions for aqueous ammonia and sulfuric acid solution by hot water. Two pretreated biomasses were compared, and their results are illustrated in Fig. 3. The solids remaining in the biomass pretreated by aqueous ammonia were lower than in the case of pretreatment by hot water. However, the loss of cellulose and hemicellulose components in the biomass pretreated by aqueous ammonia was lower than that pretreated by hot water. The biomass pretreated by aqueous ammonia exhibited a delignification of 64.8%, while that pretreated by hot water was 22%. The effect of delignification by hot water was lower than that by aqueous ammonia. The glucose recovery yield of biomass pretreated by aqueous ammonia was 67.7%, while that pretreated by hot water was 37.0%. The glucose recovery yield of biomass pretreated by aqueous ammonia was 83% higher than that pretreated by hot water, but the solid remaining was lower than that with hot water. The effect of delignification by aqueous ammonia was three fold higher than that of delignification by hot water. Therefore, the solid remaining decreased due to the removal of lignin. However, a positive effect on the removal of an inhibitor for enzymatic hydrolysis was observed; the glucose conversion yield increased by about 1.8 fold over biomass pretreated by hot water.
Fig. 3. Mass balance of pretreated biomass by single process (NH₄OH and hot-water)

The results of the sulfuric biomass by single process (NH₄OH and hot-water)

The results of the sulfuric acid solution process are illustrated in Fig. 4. The solids remaining in the biomass pretreated by sulfuric acid were lower than what was obtained by pretreatment with hot water, but the loss of cellulose was similar between the two. However, the loss of hemicellulose components by the biomass pretreated by sulfuric acid was much higher than that pretreated by hot water. The biomass pretreated by a sulfuric acid solution exhibited delignification of 37.0%, while that pretreated by hot water was 25.1%. The glucose recovery yield compared to the initial biomass was 42.9%, while that from biomass pretreated by hot water was 28.8%. Although the glucose recovery yield of biomass pretreated by sulfuric acid solution was higher than that pretreated by hot water, the difference was small. The hemicellulose was fractionated mostly into the liquid phase, and the pretreated biomass retained its components, except for hemicellulose in the solid phase. The surface area available to contact the enzyme increased because considerable empty space was created in the structure while removing the cellulose and hemicellulose from the biomass.
**Fig. 4.** Mass balance of pretreated biomass by single process ($H_2SO_4$ and hot-water)

### Mass Balance in Two-Stage Reaction for Biomass

The pretreatments were performed via a two-stage method with aqueous ammonia and hot water; results are illustrated in Fig. 5. The first stage was pretreatment by aqueous ammonia, and the second stage was pretreatment by hot water under the same conditions. Conversely, the pretreatment was also performed by changing the sequence. The cellulose and hemicellulose components were hardly different after pretreatments by aqueous ammonia-hot water and hot water-aqueous ammonia; the delignification of both was almost similar. However, the glucose recovery yield after enzymatic hydrolysis by aqueous ammonia-hot water was 68.2%, while that of hot water-aqueous ammonia was 55.7%. The yield was higher in the case of aqueous ammonia-hot water sequence, with a difference of 22%. The fractionation effect of hemicellulose in the two-stage process by hot water was evaluated after lignin removal by aqueous ammonia. A slightly higher glucose conversion yield was observed when compared to the single reaction process. However, the hot water two-stage process exhibited a much lower glucose conversion yield than in a single reaction process. The delignification and hemicellulose fractionation of hot water in the two-stage process were higher than in the ammonia two-stage process, while the enzymatic hydrolysis was relatively lower. These results indicated that there was a structural space for the enzyme to function in during fractionation of hemicellulose after pulverizing the lignin.
**Fig. 5.** Mass balance of pretreated biomass by two-stage process (NH$_4$OH – Hot-water)

Pretreatments by sulfuric acid solution were performed using the two-stage method. First, pretreatment was performed by sulfuric acid, followed by pretreatment with hot water under the same conditions. When the sequence of the pretreatment was modified, the cellulose and hemicellulose components were similar following two-stage pretreatments by sulfuric acid solution-hot water and hot water-sulfuric acid solution; the delignification in both was also similar. The glucose recovery yield after enzymatic hydrolysis for biomass under a two-stage pretreatment by sulfuric acid solution-hot water was 45.8%, while that with hot water-sulfuric acid solution was 51.9%. The glucose recovery yield was no different for both sulfuric acid solution-hot water and hot water-sulfuric acid solution under the two-stage pretreatment process. However, a loss of hemicellulose was observed in sulfuric acid solution and hot water; most of the hemicellulose was fractionated through the liquid phase.
**CONCLUSIONS**

1. This study was designed using the response surface method (RSM) tool in the Box-Behnken design to determine the optimum pretreatment conditions with aqueous ammonia and sulfuric acid solutions. The RSM tool predicted the optimum conditions for pretreatment by aqueous ammonia and sulfuric acid solution. The optimum conditions with ammonia were: 19 wt.% aqueous ammonia, 163 °C, and a reaction time of 38 min. The optimum conditions with sulfuric acid were: 0.6 wt.% sulfuric acid, 169 °C, and a reaction time of 22 min.

2. The pretreatment was performed using a two-stage method. First, pretreatment by aqueous ammonia or sulfuric acid solution was performed, followed by hot water under the same conditions. Conversely, the pretreatment was also performed by changing the sequence. The glucan recovery yield in the two-stage process by aqueous ammonia-hot water was 22% lower than that of hot water-aqueous ammonia. The glucan recovery yield from biomass pretreated by sulfuric acid did not display any differences for both sulfuric acid solution-hot water and hot water-sulfuric acid solution used in a two-stage pretreatment process.

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*Fig. 6.* Mass balance of pretreated biomass by two-stage process (H₂SO₄ – Hot-water)
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REFERENCES CITED


Kim, I., and Han, J. (2012). “Optimization of alkaline pretreatment conditions for enhancing glucose yield of rice straw by response surface methodology,” Biomass and Bioenergy 46, 210-217. DOI: 10.1016/j.biombioe.2012.08.024


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