

FERMENTATION OF GLUCOSE AND XYLOSE IN CATTAIL PROCESSED BY DIFFERENT PRETREATMENT TECHNOLOGIES

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The effects of different pretreatment technologies, including sulfuric acid, hot-water, NaOH, and MgCl₂ pretreatments, on the fermentation of xylose and glucose to ethanol by *Saccharomyces cerevisiae* ATCC 24858 and *Escherichia coli* KO11 were investigated. In this study, cattail was used as the feedstock. The use of aquatic plant cattails to produce biofuel will add value to land and reduce emissions of greenhouse gases by replacing petroleum products. The pretreated biomass first was enzymatically hydrolyzed for 2 days, followed by a 2-day Simultaneous Saccharification and Fermentation (SSF) using *S. cerevisiae*. The glucose to ethanol yields were approximately 85 to 91% of the theoretical yield for this *S. cerevisiae* strain. Glucose and xylose released from cattail cellulose and hemicellulose could be fermented to ethanol using *E. coli* KO11, resulting in approximately 85% of the theoretical ethanol yield using either a Separate Hydrolysis and Fermentation (SHF) process or a SSF process. In order to improve the sugars to ethanol yields, a detoxification process is necessary to remove the inhibitory compounds produced during the acid pretreatment process. Among the four pretreatment methods, the dilute acid pretreatment was found to be superior, and additional research is required to optimize the economics of the overall biorefinery process.

Keywords: Biomass; Cattail; Fermentation; Pretreatment; *E. coli* KO11

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INTRODUCTION

For a number of reasons, there has recently been an increasing interest in converting biomass to liquid fuels. Some of those reasons include limited availability and increasing demand for fossil fuels, especially in developing countries, increasing price, the need for national energy independence and safety, and the need for reduction in greenhouse gas (GHG) emissions (Huang *et al.* 2008). To this end, the U.S. federal government has been calling for research into ethanol production from a number of cellulosic sources (Zhang *et al.* 2008). Ethanol is considered the most potential next generation transportation fuel, and significant quantities of ethanol are currently being produced from corn and sugar cane via a fermentation process. Utilizing lignocellulosic biomass as a feedstock is seen as the next step towards significantly expanding the ethanol production capacity. However, technological barriers including pretreatment, enzyme hydrolysis, saccharification of cellulose and hemicellulose matrix, and

simultaneous fermentation of hexoses and pentoses, need to be addressed to efficiently convert lignocellulosic biomass into bioethanol (Zhang and Shahbazi 2011).

Saccharomyces cerevisiae (baker's yeast) has been used for ethanol production from hexoses (C6 sugars) for at least a thousand years. However, a significant amount of pentoses (C5 sugars) derived from the hemicellulose portion of the lignocellulosic biomass is present in the hydrolysate from the pretreatment process. Modern biotechnologies enable the fermenting microorganisms to use both C5 and C6 sugars available from the hydrolysate. This further increased the economic competitiveness of ethanol production and other bio-products from cellulosic biomass. Recently, microorganisms for cellulosic ethanol production, such as *Saccharomyces cerevisiae*, *Zymomonas mobilis*, and *Escherichia coli* have been genetically engineered using metabolic engineering approaches (Lau *et al.* 2010).

In this work, cattail (*Typha* species) was used as the feedstock. Cattails have been identified as a particularly suitable biomass crop for wetlands because of their superiority in productivity (40+ metric ton/ha standing crops), pest resistance, adaptability, and chemical composition (Apfelbaum 1985). Cattails have been used for phytoremediation in constructed wetlands (Suda *et al.* 2009). Recently, reed (*Typha*) was catalytically converted to liquid products by using organic solvents (methanol, ethanol, and acetone) with catalysts of 10% concentration (NaOH or ZnCl₂) (Küçük *et al.* 2005). The use of aquatic plant cattails to produce biofuel will add value to land and reduce emissions of greenhouse gases by replacing petroleum products. For this study, the effects of different pretreatment technologies, including dilute sulfuric acid, hot-water, NaOH, and MgCl₂ pretreatments on the fermentation of xylose and glucose to ethanol by the baker's yeast *Saccharomyces cerevisiae* ATCC 24858 and *Escherichia coli* KO11 were investigated.

EXPERIMENTAL

Bacterial Strains and Media

Saccharomyces cerevisiae ATCC 24858 was the yeast organism used to ferment the enzymatically released glucose. Stock cultures were maintained on YM medium.

Escherichia coli KO11 was used to ferment the enzymatically released glucose and xylose. Chloramphenicol acyl transferase (*cat*) and the *Z. mobilis* genes for ethanol production (*pdc*, *adhB*) are integrated into the chromosome of this strain. Stock cultures were maintained on modified Luria-Bertani (LB) medium containing (per liter): 5 g NaCl, 5 g yeast extract, 10 g tryptone, 20 g xylose, 15 g agar, and 600 mg chloramphenicol (Moniruzzaman *et al.* 1998).

Feedstock and Pretreatment Processes

The aerial portions of cattails, *Typha latifolia*, were chopped with pruning shears, dried at 70°C for 5 days, and ground in a Wiley mill to 1 mm mesh size.

The pretreatment conditions were as follows: 4% NaOH at room temperature for 24 hours, 180°C hot-water for 15 minutes, MgCl₂ pretreatment with 0.4 M concentration at 180°C for 15 minutes, and dilute sulfuric acid pretreatment with 0.5% concentration at

180°C for 5 minutes. The detailed descriptions of NaOH, hot water, and H₂SO₄ pretreatment processes are presented elsewhere (Zhang *et al.* 2010, 2011a,b).

MgCl₂ pretreatment was done using a Dionex ASE 350 Accelerated Solvent Extractor (Dionex Corporation, Sunnyvale, CA). Approximately 2 to 3 g of ground biomass (composed of cattails) was placed into a tared 66 mL Dionex extraction cell containing a glass fiber filter. Then the appropriate number of 150-mL collection vials were weighed and placed onto the ASE system. The extractor passed 60 mL of magnesium chloride solution into the cell containing biomass. Then the cell was heated to the desired temperature (140 to 180°C) at a heating rate of 25°C/min, and the desired temperatures were maintained for 5 to 15 minutes. After treatment, 40 mL of the solution was passed into the cell to rinse the biomass. The resulting extractive and the rinsing solution (total about 100 mL) were collected in the collection vials. The extraction cell was cooled down to 25°C by sitting at room temperature for 30 min. The magnesium chloride treated biomass was filtered using a 12.5 cm diameter Whatman No. 1 filter paper in a Buchner funnel. Clean deionized water (300 mL) was washed through the filter cake. The yield percentage of each fraction from treatment is defined as:

$$\begin{aligned} \text{Treated biomass (\%)} &= (\text{Weight of treated biomass} / \text{Weight of starting biomass}) \times 100 \\ \text{Dissolved solids yield (\%)} &= (1 - \text{Weight of treated biomass} / \text{Weight of starting biomass}) \times 100 \end{aligned}$$

All experiments and analysis were performed in triplicate. Dry matter recoveries and compositional analyses of solids and liquids after the pretreatment step were used to develop a component balance for the pretreatment processes. The remaining soluble mass in the hydrolysate liquid was determined by difference.

Biomass Analytical Procedures

Compositional analysis of biomass was carried out using the laboratory analytical procedures (LAPs) developed by the National Renewable Energy Laboratory. The moisture content of the biomass was determined by the LAP #001 method, and the ash content of the biomass was determined by the LAP #005 method. Structural analyses of the samples were carried out according to the LAP #002 method. The composition of cattails and pretreated cattails is listed in Table 1.

Fermentation Using *Saccharomyces cerevisiae*

For ethanol production, 4 mL of *S. cerevisiae* seed culture were used to inoculate 40 mL YM medium in a 250-mL Erlenmeyer flask. The cultures were incubated in a shaker at 30°C and 200 rpm, and grown aerobically overnight. The yeast was harvested at room temperature by centrifugation at 2600 RCF for 15 minutes. The supernatant was discarded, and the cells were transferred to 250-mL screw-capped Erlenmeyer flasks containing 100 mL of hydrolysate. The initial cell mass concentration prior to fermentation in each experiment was 4 to 6 g dry weight/L. The flasks were then tightly capped to allow fermentation to occur under largely anaerobic conditions. The cultures were placed in a shaker and incubated at 30°C. Fermentation samples were filtered through 0.2 µm nylon membranes and analyzed by HPLC to determine the presence of ethanol and sugars.

Table 1. Biomass composition^{a,b} of pretreated cattails (% by weight)

Pretreatment Method ^d	Cellulose	Xylan	Other sugars ^c	Klason lignin
Unpretreated	34.5	11.7	3.4	26.4
NaOH	45.0	24.6	3.2	13.9
Hot-water	58.8	5.2	0.5	28.5
MgCl ₂	56.3	14.2	0.3	25.3
H ₂ SO ₄	57.0	-	-	42.1

a: Moisture-free basis

b: Biomass also contains acid-soluble lignin, extractives, acetyl acid groups, ash, and uronic acid groups.

c: Other sugars represent galactan, arabinan, and mannan.

d: The pretreatment conditions were as follows: 4% NaOH at room temperature for 24 hours, 180°C hot-water for 15 minutes, MgCl₂ pretreatment with 0.4 M concentration at 180°C for 15 minutes, and dilute sulfuric acid pretreatment with 0.5% concentration at 180°C for 5 minutes;

-: not detectable

Fermentation Using *Escherichia coli* KO11

For ethanol production, 4 mL of *E. coli* KO11 seed culture were used to inoculate 40 mL LB medium containing 2% glucose in a 250-mL Erlenmeyer flask (Moniruzzaman *et al.* 1998). The cultures were incubated in a shaker at 37°C and 200 rpm, and grown aerobically overnight. *E. coli* was harvested at room temperature by centrifugation at 2600 RCF for 15 minutes. The supernatant was discarded, and the cells were transferred to 250-mL screw-capped Erlenmeyer flasks containing 100 mL of hydrolysate. The initial cell mass concentration prior to fermentation in each experiment was 4 to 6 g dry weight/L. The cultures were placed in a shaker and incubated at 37°C. Fermentation samples were filtered through 0.2 µm nylon membranes and analyzed by HPLC to determine the presence of ethanol and sugars.

Detoxifying Hydrolysates from an Acid Pretreatment Process

Treatment of biomass hydrolysate with Ca(OH)₂ (overliming) is an effective method for detoxification (Martinez *et al.* 2001). The extracts (*i.e.*, hydrolytes) from a sulfuric acid pretreatment process were over-neutralized by adding slaked lime (*i.e.* Ca(OH)₂) until the pH value was 10. Then the pH was adjusted back to 7 by adding HCl solution (Lee *et al.* 1999). Before the enzyme hydrolysis step, the pH of detoxified hydrolysates was further adjusted to 5.0 by adding sodium citrate buffer.

RESULTS AND DISCUSSION

Fermentation of Pretreated Cattails with *Saccharomyces cerevisiae*

Cattails were pretreated with 4% NaOH for 24 h, with hot water at 180°C for 15 minutes, with 0.4 M MgCl₂ at 180°C for 15 minutes, or with 0.5% H₂SO₄ at 180°C for 5 minutes. All pretreated cattails were washed with deionized water. The pretreated biomass first was enzymatically hydrolyzed for 2 days using a cellulase loading of 15 FPU/g glucan, followed by a 2-day Simultaneous Saccharification and Fermentation (SSF) using *Saccharomyces cerevisiae* (ATCC 24858).

Figure 1 illustrates the concentrations of glucose and ethanol over the course of fermentation of cellulose from dilute-sulfuric acid pretreated cattails. In this case, the acid pretreated cattails of three extraction tubes (solids) were pooled, giving a glucan loading of approximately 2 g/100 mL liquid (i.e., 2% (w/v)). The maximum glucose yield and the theoretical ethanol yield from 2 g glucan are 2.22 g/100 mL and 1.14 g/100 mL, respectively. The hydrolysate from undiluted pretreated cattails (first 2-day hydrolysis) gave a fermentable glucose yield of 1.7% w/v, which is lower than the maximum glucose yield of 2.2%. An incomplete sugar conversion is usually observed in the enzymatic hydrolysis of lignocellulosic biomass. The factors such as the product inhibition, lignin of biomass, crystallinity, and accessible pore volume are the major concerns (Yu *et al.* 2011; Park *et al.* 2010; Yu *et al.* 2012; Xiao *et al.* 2004). During the SSF, more glucan was converted into glucose, and the glucose was rapidly converted to ethanol (<8 h). The final ethanol yield was 0.99% w/v, suggesting that glucose produced from cattails cellulose can be efficiently fermented to ethanol. The glucose to ethanol yields were approximately 90% of the theoretical yield for this *S. cerevisiae* strain.

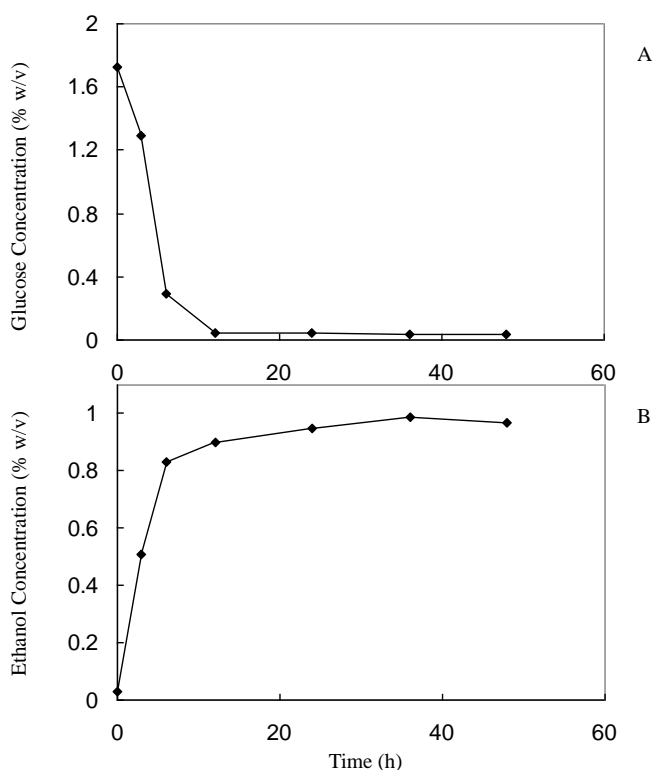


Fig. 1. Fermentation of cellulose from dilute-sulfuric acid pretreated cattails. Cattails were pretreated at 180°C for 5 minutes with 0.5% dilute-sulfuric acid. The pretreated biomass first was enzymatically hydrolyzed for 2 days using a cellulase loading of 15 FPU/g glucan, followed by a 2-day Simultaneous Saccharification and Fermentation (SSF) using *S. cerevisiae* (ATCC 24858). A) Glucose consumption, B) Ethanol Yield

Comparing to other pretreatment technologies, the result of dilute sulfuric acid pretreatment is similar to the results of the hot water, $MgCl_2$, or $NaOH$ pretreated cattails. When diluted pretreated cattails (1 to 2 g glucan/100 mL volume) were used, glucose to

ethanol yields were approximately 91%, 88.7%, and 87% of the theoretical yield for NaOH, hot water, and MgCl₂ pretreatments.

Xylose Only Fermentation

The ability of utilizing xylose by *E. coli* KO11 was tested via xylose-only fermentation. *E. coli* KO11 was cultured in LB medium containing 4% w/v xylose under either aerobic or anaerobic conditions. After 2-day fermentation, the ethanol concentrations were 1.98% and 2.14% w/v for aerobic and anaerobic conditions, respectively. The xylose-to-ethanol yields were 97±1.0% and 98.1±0.9% of the theoretical yield for aerobic and anaerobic conditions, respectively. The differences in results between aerobic and anaerobic conditions were not statistically significant at a 95% confidence level.

Comparison of SHF and SSF Processes for Ethanol Production by *E. coli* KO11

The effects of the Separate Hydrolysis and Fermentation (SHF) process and Simultaneous Saccharification and Fermentation (SSF) on ethanol production were compared using cattails pretreated by hot water at 180°C for 15 minutes. For the separate hydrolysis and fermentation (SHF) process, the pretreated cattails were hydrolyzed for two days, and then the resulting hydrolysate was fermented at 37°C for 48 h by *E. coli* KO11. During the simultaneous saccharification and fermentation (SSF) process, the pretreated cattails were loaded in a 250-mL Erlenmeyer flask along with LB medium, cellulase, citric acid buffer, and *E. coli* KO11 cells. The initial cell mass concentration prior to fermentation in each experiment was 4 to 6 g dry weight/L. The cultures were placed in a shaker and incubated at 37°C for 48 h. Cellulase loadings for both SHF and SSF were 15 FPU/glucan.

The hot-water pretreated cattails contained approximately 58.8% cellulose and 5.2% xylan. During both SHF and SSF processes, the amount of xylose present in the hydrolysate was fermented within 6 hours by *E. coli* (Fig. 2A, B). For SHF, the glucose also was rapidly converted to ethanol. The ethanol reached the highest concentration within 6 hours. During the SSF process, the glucose was released gradually over the course of SSF, reaching minimum fermentable glucose concentration after 48 hours of fermentation. The sugars to ethanol yields were 82.6% and 85.8% of the theoretical yield for SHF and SSF processes, respectively. The results showed that SSF process is more favorable for bioethanol production from cattails. The principal advantages of performing the SSF process are the reduced end-product inhibition of the enzymatic hydrolysis, and the reduced investment costs. However, the major challenge is to find favorable conditions (*e.g.* temperature and pH) for both the enzymatic hydrolysis and the fermentation and the difficulty to recycle the fermenting organism and the enzymes (Olofsson *et al.* 2008). The actual choice of fermentation technology should be based on the pretreatment approach and the fermentation strain selection.

The SHF process was also used to ferment cattails pretreated with 4% NaOH and 0.4 M MgCl₂. The sugars to ethanol yields were approximately 85.9% and 87.5% of the theoretical yield for NaOH and MgCl₂ pretreatments, respectively.

Fermentation of Cattail Hydrolysates from Pretreatment Processes

The extracts (*i.e.*, hydrolytes) of the hot-water and the sulfuric acid pretreatment processes were directly used for the SHF fermentation processes.

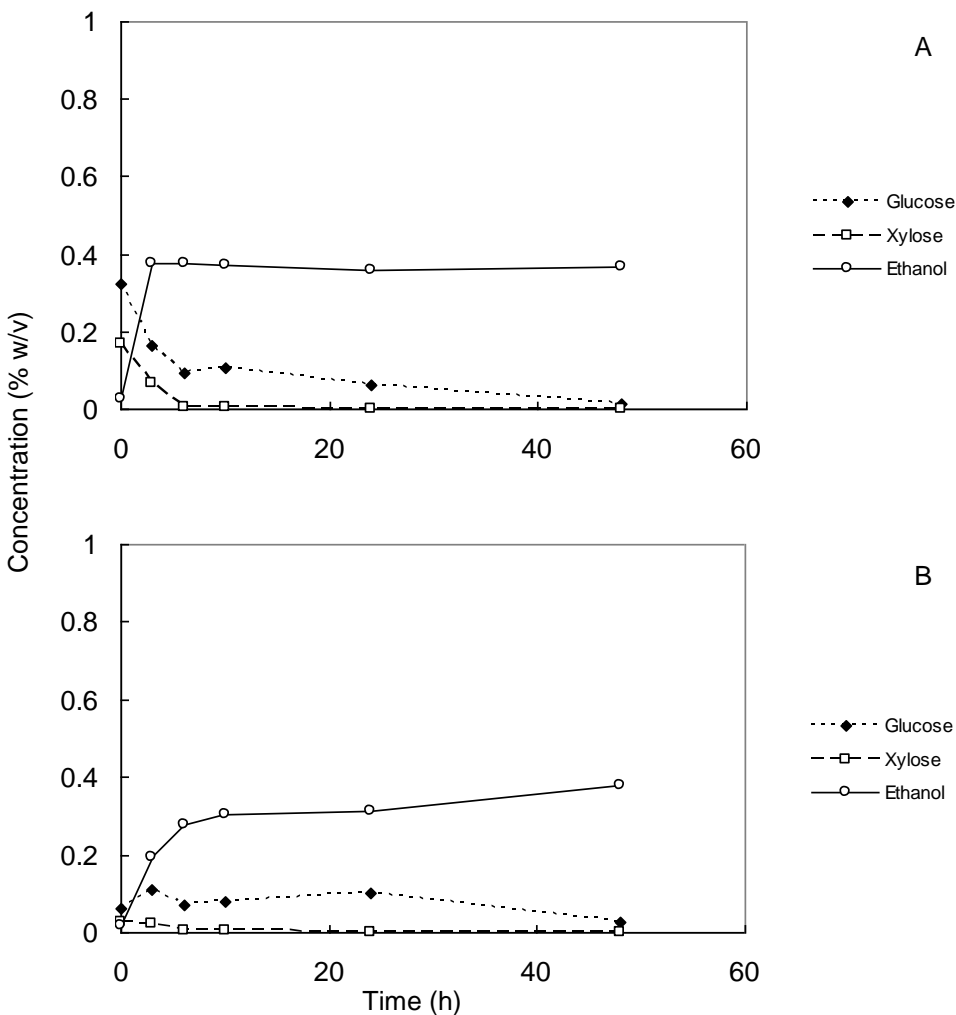


Fig. 2. Glucose, xylose, and ethanol profiles during the fermentation process of hot water pretreated cattail. A.) Separate Hydrolysis and Fermentation (SHF); B.) Simultaneous Saccharification and Fermentation (SSF)

The pH of the hydrolysates from a hot-water pretreatment process was adjusted to 5.0 by the addition of sodium citrate buffer. Then the extracts were further hydrolyzed using cellulase and hemicellulase for 2 days. *E. coli* was pre-cultured in 40 mL LB medium containing 2% xylose, then *E. coli* cells were transferred into the hydrolysates. *E. coli* can utilize the glucose and xylose in the extracts and produce ethanol (Fig. 3A), resulting in an ethanol yield of 73.5% of the theoretical yield.

The extracts from a sulfuric acid pretreatment process were over-neutralized by adding lime (*i.e.* Ca(OH)₂) until the pH value was 10. Then the pH was adjusted back to 7 by the addition of HCl solution. Before the enzyme hydrolysis step, the pH of detoxified

hydrolysates was further adjusted to 5.0 by the addition of sodium citrate buffer. The extracts after detoxification were hydrolyzed using cellulase and hemicellulase, resulting in a slightly increased sugar yield. *E. coli* was pre-cultured in 40 mL LB medium containing 2% xylose, then *E. coli* cells were transferred into the hydrolysates.

Figure 3B shows the glucose, xylose, and ethanol profiles during the fermentation of cattail hydrolysates from the sulfuric acid pretreatment process. The glucose was rapidly converted to ethanol within 10 h of fermentation. The xylose was fermented much more slowly, reaching a minimum fermentable xylose concentration after 24 hours of fermentation. The ethanol yields from this fermentation were approximately 87% of the theoretical yield, which is higher than that of the hot-water pretreatment. The hot-water pretreatment may produce inhibitory compounds for enzyme hydrolysis and fermentation processes. So the detoxification is necessary for utilizing the hydrolytes from the acid or hot-water pretreatment process of cattails.

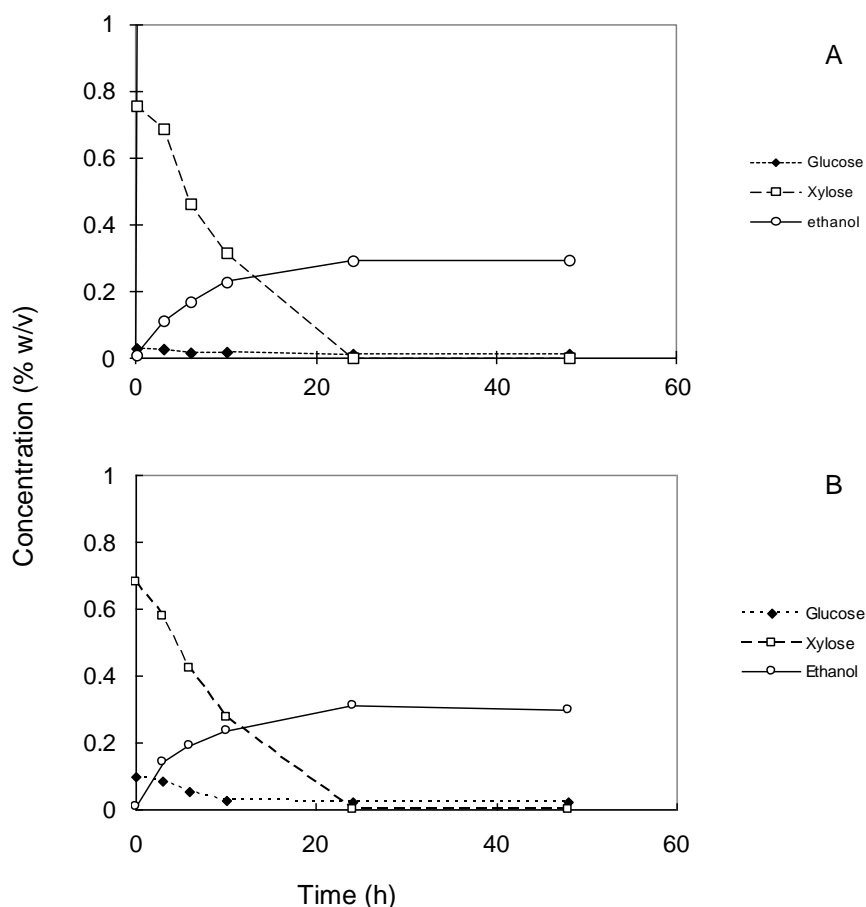


Fig. 3. Glucose, xylose, and ethanol profiles of SHF fermentation of cattail hydrolysates from A) the hot-water pretreatment process, and B) the sulfuric acid pretreatment process

Comparison of Four Biomass Pretreatment Methods

For each pretreatment technology, the cellulose to ethanol yields and hemicellulose to ethanol yields are summarized in Table 2. The selection of the fermentation strains needs to match the pretreatment technologies and the feedstock used,

as well as the process. For example, if a dilute acid pretreatment is used, most of the hemicellulose is degraded, the pretreated biomass can be fermented using baker's yeast *S. cerevisiae*, but pentose sugars-rich hydrolyates must be fermented by a microorganism that uses both hexose and pentose sugars. In our calculations, all streams containing both hexose and pentose sugars were assumed to be fermented using *E. coli* KO11.

Table 2. Comparison of Four Biomass Pretreatment Methods

Pretreatment Methods	Pretreatment/hydrolysis stage		Fermentation stage		Total sugars to ethanol yield (% of total sugars)
	Glucose yield (% of total cellulose)	Xylose yield (% of total xylan)	Glucose to ethanol yield (% of the theoretical yield)	Xylose to ethanol yield (% of the theoretical yield)	
NaOH	77.5	43.6	85.9	85.9	59.2
Hot-water	77.6	70	85.8	82.6	64.2
MgCl ₂	61.7	90	87.5	87.5	60.2
H ₂ SO ₄	97.0	90	90 ^a	87	85.1

a: The steam is fermented by *S. cerevisiae*. The rest streams were assumed to be fermented using *E. coli* KO11.

All four pretreatments were able to increase enzymatic digestibility of cattail. However, each pretreatment method has its own advantages and disadvantages (Zhang and Shahbazi 2011). Using alkaline chemicals to remove lignin has long been known to improve cellulose digestibility. But sodium hydroxide and other bases are expensive, and the recovery process is complex. Hot-water pretreatment enhances enzyme digestibility of the biomass by penetrating the cell structure, hydrating cellulose, and removing hemicellulose. The major advantages are less expense, lower corrosion to equipment, less xylose degradation, and thus fewer byproducts, including inhibitory compounds in the extracts. Application of Lewis acids (such as MgCl₂) as a novel biomass fractionation approach will require lower xylanase loading for hydrolysis, and have lower corrosion to equipment comparing to other acid pretreatment processes. MgCl₂ treatment enhanced xylan degradation and delignification, and increased enzymatic digestibility of cattail cellulose and xylan. While using three pretreatment technologies mentioned above, approximately 60% of original sugars were converted to ethanol. A possible explanation is that cattails are particularly recalcitrant. These three pretreatment methods may not be the most effective method for this biomass.

Dilute acid (0.5 to 1.0% sulfuric acid) pretreatment at moderate temperatures (140 to 190°C) can effectively remove and recover most of the hemicellulose as dissolved sugars, and lignin is disrupted and partially dissolved, increasing cellulose susceptibility to enzymes (Yang and Wyman 2008). When using a dilute sulfuric acid pretreatment, the highest total glucose and xylose yield for the pretreatment/hydrolysis stages was 97% and 90%, respectively, and about 85% of the original sugars in the cattails were converted to ethanol. Comparing to bioethanol yields of using other diluted sulfuric acid pretreated lignocellulosic feedstocks, the results from this study are slightly lower than that of corn stover (92.5%) and corn cob (94.5%), but similar to that of switchgrass (87.5%), sweetgum (86.5%) (Wyman *et al.* 1992), and *Populus* (86.5%) (Spindler *et al.* 1991).

Drying biomass is one of the important steps during biomass processing. It is especially important for biomass size reduction. Normally, the feedstock, such as corn stover, is field-dried until the feedstock has less than 15% moisture. According to the study by Ioelovich and Morag (2011), drying of the wet celluloses caused the decreasing of the hydrolysability. The severe drying condition used in this study may also cause the decreasing of the hydrolysability of the biomass. If non-dried cattails could be used in the study, the hydrolysability and fermentability may be further improved.

CONCLUSIONS

Glucose released from cattail cellulose could be fermented to ethanol using *S. cerevisiae*, resulting in approximately 85 to 91% of the theoretical ethanol yield. Glucose and xylose released from cattail cellulose and hemicellulose could be fermented to ethanol using *E. coli* KO11, resulting in approximately 85% of the theoretical ethanol yield. In order to improve the sugars to ethanol yields, a detoxification process is necessary to remove the inhibitory compounds produced during the pretreatment process. Among four pretreatment methods, the dilute acid pretreatment was found to be superior, and additional research is required to optimize the economics of the overall biorefinery process.

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