

Saccharification of a Potential Bioenergy Crop, *Phragmites australis* (Common Reed), by Lignocellulose Fractionation Followed by Enzymatic Hydrolysis at Decreased Cellulase Loadings

Noppadon Sathitsuksanoh,^{†,‡} Zhiguang Zhu,[†] Neil Templeton,[†] Joseph A. Rollin,[†] Steven P. Harvey,[§] and Y-H. Percival Zhang^{*,†,‡,||}

Biological Systems Engineering Department, Virginia Polytechnic Institute and State University, 210-A Seitz Hall, Blacksburg, Virginia 24061, Institute for Critical Technology and Applied Science (ICTAS), Virginia Polytechnic Institute and State University, Blacksburg, Virginia 24061, US Army ECBC, Research & Technology Directorate, 5183 Blackhawk, Road, Aberdeen Proving Ground Maryland 21010, and DOE BioEnergy Science Center (BESC), Oak Ridge, Tennessee 37831

Cost-effective biological saccharification of nonfood lignocellulosic biomass is vital to the establishment of a carbohydrate economy. *Phragmites australis* (common reed) is regarded as an invasive perennial weed with a productivity of up to 18–28 tons of dry weight per acre per year. We applied the cellulose solvent- and organic solvent-based lignocellulose fractionation (COSLIF) to the stems and leaves of *Phragmites* and optimized the pretreatment conditions (e.g., temperature, reaction time, and biomass moisture content) through response surface methodology (RSM). The optimal pretreatment conditions were 85% (w/v) H₃PO₄, 50 °C, and 60 min, regardless of the biomass moisture contents from 5–15% (w/w). Glucan digestibility of the COSLIF-pretreated common reed was 90% at hour 24 at a low cellulase loading (5 filter paper units and 10 β-glucosidase units per gram of glucan). Under these conditions, the overall sugar yields were 88% for glucose and 71% for xylose, respectively. Cellulose accessibility to cellulase (CAC) was increased 93.6-fold from 0.14 ± 0.035 to 13.1 ± 1.1 m² per gram of biomass with the COSLIF pretreatment. Results showed that cellulase concentrations could be reduced by 3-fold with only a slight reduction in sugar yield. This study suggested that *Phragmites* could be used as a carbon-neutral bioenergy feedstock, while its harvesting could help control its invasive growth and decrease nutrient pollution in adjacent waterways.

Introduction

The production of biofuels and biobased products from renewable lignocellulosic biomass will promote rural economy, decrease greenhouse gas emissions, and enhance energy security.^{1–3} Biomass saccharification usually involves two sequential steps: lignocellulose pretreatment/fractionation and enzymatic hydrolysis of cellulose. The largest technological and economical challenge for biomass biorefineries is the efficient release of fermentable soluble sugars from low-cost lignocellulosic biomass at competitive costs.^{4–7} Currently, the production of second generation biofuels, that is, cellulosic ethanol, cannot compete with that made from corn grain and sugar cane, because of its high processing costs (ca. \$1–3 per gallon of cellulosic ethanol), huge capital investment (\$2–10 per annual gallon ethanol capacity), and relatively low revenues from ethanol (\$2–3 per gallon of cellulosic ethanol).^{4,7,8}

Recently, a new technology called cellulose solvent- and organic solvent-based lignocellulose fractionation (COSLIF) has been developed to separate lignocellulose components (cellulose, hemicellulose, lignin, and acetic acid) by using a cellulose solvent, an organic solvent, and water.⁴ Different from other lignocellulose pretreatments, this technology can be conducted at modest reaction conditions (e.g., 50 °C and atmospheric

pressure) for minimizing sugar degradation. The key ideas of COSLIF are (1) partial removal of lignin and hemicellulose from cellulose, allowing more cellulose exposure to cellulase, (2) decrystallization of cellulose fibers (allowing cellulase to work more efficiently), and (3) modest reaction conditions (i.e., a decrease in sugar degradation, less inhibitor formation, lower energy requirement, and less capital investment). Higher glucan digestibility of the COSLIF-pretreated biomass was attributed to greater cellulose accessibility and more lignin removal, as compared to the dilute acid pretreatment.⁹ In addition, COSLIF can separate lignocellulose components on the basis of their different solubilities in solvents and exhibit coutilization of lignocellulose components such as lignin.^{4,7,10}

The DOE cellulosic ethanol workshop has summarized three distinct goals associated with potential bioenergy feedstocks: (1) maximizing the total amount of biomass produced per acre per year, (2) producing sustainable biomass with minimal inputs (e.g., pesticides, fertilizers, seeds, and harvesting), and (3) maximizing the amount of biofuels that can be produced per unit of biomass.¹¹ A yield of 20 dry tons per acre per year may be considered as a reasonable target in an area with adequate rainfall and good soil.¹¹

Phragmites australis (common reed) is a widespread perennial grass that grows in wetlands or near inland waterways throughout the world. Although it is harvested for thatched roofs, ropes, baskets, pulping feedstock, etc., in some areas of the world, common reed is typically regarded as an invasive weed, due to its vigorous growth and difficulty of eradication. *Phragmites*, a C4 photosynthesis plant, can grow as high as 18 feet, with enormously high productivities of 18–28 tons of dry biomass per acre per year.¹² This productivity is approximately

* To whom correspondence should be addressed. E-mail: ypzhang@vt.edu. Tel.: (540) 231-7414. Fax: (540) 231-3199.

[†] Biological Systems Engineering Department, Virginia Polytechnic Institute and State University.

[‡] Institute for Critical Technology and Applied Science (ICTAS), Virginia Polytechnic Institute and State University.

[§] US Army ECBC, Research & Technology Directorate.

^{||} DOE BioEnergy Science Center (BESC).

Table 1. The Features of *Phragmites* and Its Potential Advantages as a Bioenergy Crop

features	advantages
perennial grass	harvested yearly long canopy duration
C4 photosynthesis	high photosynthesis efficiency, e.g., 18–28 tons of dry biomass per acre per year
having seeds	easy large-scale planting at the beginning
growing from rhizomes	no yearly replanting
removing pollutant nutrients	no fertilizers waste water treatment
growing in wetland	no irrigation
few pests	low pesticide needed
invasive	harvesting = weed control
winter standing	long harvesting time (several months) low moisture content feedstock for winter harvesting
having rhizomes	recycling nutrients to rhizomes in winter
temperate and tropical regions	worldwide
marginal lands	no competition for arable land, preferring neutral and even alkaline wetlands

three to five times higher than a dedicated bioenergy crop—switchgrass. Since it produces seeds in addition to its growth from rhizomes, large-scale planting would be easier as compared to another potential bioenergy plant *Miscanthus*. Judging from annual inputs, the use of common reed as a bioenergy plant would have several advantages: growth from rhizomes after initial establishment from seeds or rhizomes, no or low fertilizer requirement, no irrigation (growing in wetlands), and low pesticides needed. Since common reed is regarded as an invasive weed by the U.S. Environmental Protection Agency (EPA), annual harvesting of common reed as a bioenergy feedstock can be regarded as weed control. In addition, existing strands of *Phragmites* are huge in the USA, and its further planting as a bioenergy crop seems promising. In fact, growth features of common reed are very good for biomass harvesting. Its winter standing allows a much longer harvesting time, as compared to corn stover. Also, harvesting of standing naturally dried strands with decreased moisture contents of ~5–15% would save drying costs and biomass transportation costs. Before winter, it can recycle its nutrients to rhizomes for the growth in next year. Common reed usually grows in neutral pH or alkaline tropical and temperate water lands or wetlands, which are not suitable for most crops. Because it can take up nutrients efficiently, harvesting of existing strands will effectively remove phosphorus and nitrogen from inland waterways, and prevent algal blooms and other microbial pollution.^{13–16} The features and associated advantages of common reed are presented in Table 1.

In this study, we investigated the feasibility of applying the COSLIF technology to common reed. We also sought to further improve the COSLIF technology by replacing the organic solvent (acetone) with ethanol for reductions in processing costs and capital investment for recycling of organic solvent. We optimized key pretreatment conditions by using response surface methodology (RSM), studied the release of soluble sugars from this potential bioenergy plant at decreased enzyme loadings, and analyzed potential economic benefits associated with low use of costly enzyme.

Materials and Methods

Chemicals and Materials. All chemicals were reagent grade and purchased from Sigma-Aldrich (St. Louis, MO), unless otherwise noted. Phosphoric acid (85%) and 95% ethanol were purchased from Fisher Scientific (Houston, TX). The *Trichoderma* cellulase (Novozyme 50013) and β -glucosidase (Novozyme 50010) were gifts from Novozymes North American

(Franklinton, NC). They had activities of 84 filter paper units (FPU) per mL and 270 β -glucosidase units per mL, respectively.

Common reed was obtained from the U.S. Army Edgewood Chemical Biological Center (Aberdeen, MD) in the early winter of 2007. The naturally dried common reed was milled into small particles by using the Pallmann counter-rotating knife ring flaker (Clifton, NJ). The resulting particulates were screened to the sizes of less than 40 mesh (i.e., smaller than 0.420 mm) and greater than 60 mesh (i.e., larger than 0.250 mm). The milled materials were slowly dried to a moisture content of ~5% at room temperature, whose moisture contents were determined by complete drying in a convection oven, at 105 ± 3 °C for 4 h or longer, until a constant weight was achieved. The different moisture contents of biomass samples were prepared by mixing ~5% moisture content biomass with water, and then equilibrating in a closed container at room temperature overnight.

COSLIF Procedure. The COSLIF pretreatment for common reed was conducted as described elsewhere,^{4,10} with some modifications. Acetone was replaced with 95% (v/v) ethanol. One gram of dry common reed with a moisture content, varying from 5%, 10% to 15%, was mixed with 8 mL of 85% phosphoric acid at different temperatures (40, 50, and 60 °C) for different lengths of time (30, 60, and 90 min) in 50-mL plastic centrifuge tubes. The biomass dissolution and weak hydrolysis reactions were stopped by adding 20 mL ethanol. After mixing well, solid/liquid separation was conducted in a swinging bucket centrifuge at 4500 rpm at room temperature for 15 min. After the supernatant was decanted, an additional 40 mL of ethanol was mixed with the slurry containing cellulose and hemicellulose. The solid/liquid separation was again conducted by centrifugation. After the supernatant was decanted, the pellets were resuspended and washed twice with 40 mL of water. The residual amorphous solid pellet was neutralized to pH 5–7 with a small amount of 2 M sodium carbonate.

Carbohydrate and Lignin Assays. The structural carbohydrate composition of the biomass was determined with a modified quantitative saccharification (QS) procedure.¹⁷ In the modified QS, the secondary hydrolysis was conducted in the presence of 1% (w/w) sulfuric acid, rather than 4% sulfuric acid at 121 °C, for 1 h for more accurate determination of acid-labile carbohydrates (e.g., xylan and arabinan).¹⁷ Monomeric sugars were measured by a Shimadzu HPLC, with a Bio-Rad Aminex HPX-87P column (Richmond, CA), at 65 °C with a distilled water as a mobile phase at a rate of 0.6 mL per min.¹⁷ Lignin and ash were measured according to the standard NREL biomass protocol.¹⁸ The concentrations of glucose and xylose in the enzymatic hydrolysate were measured by a Shimadzu HPLC with a Bio-Rad Aminex HPX-87H chromatography column by using 0.1% (v/v) sulfuric acid as a mobile phase at a flow rate of 0.6 mL per minute and a column temperature of 65 °C.⁴

Enzymatic Hydrolysis. The pretreated common reed samples were diluted to 10 g glucan per liter in a 50 mM sodium citrate buffer (pH 4.8) with supplementary addition of 0.1% (w/v) NaN₃, which prevented the growth of microorganisms.⁴ All hydrolysis experiments were carried out in a rotary shaker at 250 rpm and 50 °C. Four enzyme loadings were tested: (1) 5 FPU cellulase and 30 units of β -glucosidase per gram of glucan; (2) 10 FPU cellulase and 30 units of β -glucosidase per gram of glucan; (3) 15 FPU cellulase and 30 units of β -glucosidase per gram of glucan; (4) 5 FPU cellulase and 10 units of β -glucosidase per gram of glucan. Eight hundred microliters of well-mixed hydrolysate were removed, followed by immediate centrifugation at 13 000 rpm for 5 min. Then exactly 500 μ L

of the supernatant was transferred to another microcentrifuge tube and incubated at room temperature for 30 min, enabling the conversion of (nearly) all cellobiose to glucose, by β -glucosidase in the supernatant. The supernatant was acidified by adding 50 μ L of 10% (w/w) sulfuric acid, and then was frozen overnight. The thawed liquid samples were mixed well and then centrifuged at 13 000 rpm for 5 min, to remove any solid sediment. The clear supernatants were used for determination of the released glucose by HPLC. After 72-h hydrolysis, the remaining hydrolysate was transferred to a 50 mL centrifuge tube, and centrifuged at 4500 rpm for 15 min. After decanting, the pellet was resuspended in 20 mL of water and centrifuged to remove soluble sugars. Following centrifugation, the remaining sugars and lignin in the lyophilized pellets were measured by QS. The soluble glucose and xylose (including galactose and mannose) in the enzymatic hydrolysate were measured by HPLC using a Bio-Rad HPX-87H column, as described above.

The enzymatic glucan digestibility (X)¹⁹ can be calculated in percent as

$$X = \frac{G_f}{(180/162)G_i} 100 \quad (1)$$

where G_f is the amount of soluble glucose plus celldextrins in the liquid phase after hydrolysis (g glucose equivalent, GE) and G_i is the initially added glucan in solid cellulosic samples before hydrolysis (g).

For biomass pretreatment and subsequent enzymatic hydrolysis, the biomass input (stream 1) generated two streams (pretreatment hydrolysate—stream 2 and pretreated biomass—stream 3) and then enzymatic hydrolysis (stream 3) produced the solid residue (stream 4) and the enzymatic hydrolysate (stream 5).²⁰ The overall glucose yield (Y_{Glu}), during the COSLIF pretreatment and enzymatic cellulose hydrolysis, is calculated in percent as

$$Y_{\text{Glu}} = \frac{\text{Glu}_2 + \text{Glu}_5}{(180/162)\text{Glu}_1} 100 \quad (2)$$

where Glu_2 and Glu_5 are mass amounts of glucose equivalent in streams 2 (the pretreatment liquid hydrolysate) and 5 (the enzymatic hydrolysate), respectively. Glu_1 is the initial glucan content before pretreatment. It is worth noting that commercial cellulase and β -glucosidase solutions contain very high concentrations of sugars (~20–100 g glucose per liter of enzyme solution).²¹ Therefore the glucose concentration in stream 5 needs to be reduced by the amount of sugars already present in the enzyme solutions.²¹

Since a significant amount of xylooligosaccharides that cannot be measured in the presence of celldextrins, by the regular HPLC columns, could exist in stream 5, the overall xylose yield Y_{Xyl} can be calculated in an alternative way as

$$Y_{\text{Xyl}} = \frac{\text{Xyl}_2 + (150/132)(\text{Xyl}_3 - \text{Xyl}_4)}{(150/132)\text{Xyl}_1} 100 \quad (3)$$

where Xyl_3 and Xyl_4 are mass amounts of xylan in streams 3 and 4, respectively. Xylan contents in streams 1, 3, and 4 were measured by the HPLC HPX-87P column after quantitative saccharification.

Scanning Electron Microscopy (SEM). The biomass materials were imaged with a Zeiss-DSM940 (Carl Zeiss, Okerkochen, Germany). All samples were sputter-coated with gold and imaged by SEM, as described elsewhere.²²

Substrate Accessibility Assays. The total substrate accessibility to cellulase (TSAC) was determined on the basis of the

maximum adsorption capacity of the TGC protein.⁹ The TGC protein is a nonhydrolytic fusion protein, containing a green fluorescence protein and cellulose-binding module.²³ The recombinant TGC fusion protein was produced in *Escherichia coli* BL21 (pNT02),²³ and purified by affinity adsorption on regenerated amorphous cellulose,²² followed by modest desorption using ethylene glycol (EG).²⁴ EG was removed by membrane dialysis in a 50 mM sodium citrate buffer (pH 6.0). The TGC protein solution was reconcentrated using a 10000 Da molecular weight cutoff centrifugal ultrafilter column (Millipore, Billerica, MA). Mass concentration of the nonadsorbed TGC protein was measured on the basis of a fluorescent reading using a BioTek multidetection microplate reader, as described elsewhere.²³ Cellulose accessibility to cellulase (CAC, m^2/g biomass) can be measured on the basis of the maximum TGC adsorption capacity after the blocking by a large amount of BSA (e.g., 5 g/L). Noncellulose accessibility to cellulase (NCAC, m^2/g biomass) was calculated as $\text{NCAC} = \text{TSAC} - \text{CAC}$.⁹

Results

The common reed sample was harvested at the Aberdeen Proving Ground of Maryland. After complete drying, it contains $32.7 \pm 2.5\%$ glucan, $18.1 \pm 2.2\%$ xylan, $1.2 \pm 0.2\%$ galactan, $2.5 \pm 0.3\%$ arabinan, $22 \pm 2.0\%$ lignin, as well as $20 \pm 3.6\%$ mass weight for extractives, ashes, proteins, and so on.

Modified COSLIF Technology. The original version of COSLIF used a highly volatile organic solvent (acetone) between a cellulose solvent (concentrated phosphoric acid) and water. The functions of this organic solvent are (1) to partially remove lignin by dissolving it, (2) to decrease cellulose solvent recycling costs, and (3) to separate water-soluble depolymerized hemicellulose fragments and water-insoluble amorphous cellulose.^{4,10} Low boiling-point acetone can be recycled easily by simple flashing, but it must be recycled with very high yields (e.g., > 99.99%). Any loss in acetone would negatively impact the economics of COSLIF implementation.

Here we replaced acetone by using ethanol for the modified COSLIF. This modification brought several benefits such that (1) a much lower recycling efficiency of ethanol is acceptable because the remaining ethanol in the hydrolysate and cellulose phase can be recycled after ethanol fermentation, (2) ethanol is more chemically stable than acetone, and (3) ethanol is less corrosive to the following membrane-based separations. Furthermore, we decreased organic solvent use nearly 2-fold, from 100 volumes to 60 volumes.

Optimization of COSLIF Pretreatment Conditions. The yield of fermentable sugars from the lignocellulosic biomass is a critical factor for evaluating the overall performance of the saccharification process, because sugar yields correlate closely with revenue.^{10,19,20} Biomass saccharification usually involves two sequential steps: pretreatment and enzymatic hydrolysis. The COSLIF pretreatment conditions (temperature, time, and biomass moisture content) were optimized by using RSM.²⁵ The pretreatment temperature (T , 40, 50, and 60 °C), reaction time (t , 30, 60, and 90 min), and biomass moisture content (MC, 5, 10, and 15%) were chosen as independent variables (Table 2). The experimental design consisted of a 3-factor 2-level pattern with 20 experiments—14 combinations with 6 replications of the central point. The statistical software Design-Expert 6.0 (Stat-Ease Inc., Minneapolis, MN) was used to analyze the experimental results. The glucan retention after the COSLIF pretreatment, glucan digestibility, and glucan yield are presented in Table 2. The quadratic equation was obtained for the

Table 2. Pretreatment Conditions and Experimental Results for Glucan Retention after the COSLIF Pretreatment, Glucan Digestibility after Enzymatic Hydrolysis, And Glucan Yield That Equals Glucan Retention × Glucan Digestibility^a

run	<i>T</i>	MC	<i>t</i>	glucan retention (%)	glucan digestibility (%)	glucan yield (%)
1	33.2	10	60	98.0	82.5	80.9
2	40	5	30	93.9	87.8	82.4
3	40	5	90	95.1	92.6	88.1
4	40	15	30	95.1	87.3	83.0
5	40	15	90	91.1	91.9	83.8
6	50	1.6	60	88.6	93.1	82.5
7	50	10	9.6	92.4	73.1	67.5
8	50	10	60	92.7	93.8	86.9
9	50	10	60	91.9	93.9	86.3
10	50	10	60	94.3	92.3	87.0
11	50	10	60	94.5	93.6	88.4
12	50	10	60	91.6	93.5	85.6
13	50	10	60	91.4	93.8	85.8
14	50	10	110.5	85.9	93.2	80.1
15	50	18	60	92.8	88.9	82.5
16	60	5	30	84.4	93.2	78.6
17	60	5	90	68.7	89.9	61.8
18	60	15	30	79.3	92.4	73.3
19	60	15	90	59.0	89.9	53.0
20	66.8	10	60	47.5	85.3	40.5

^a All hydrolysis experiments were carried out at the same enzyme loading of 15 FPU of cellulase and 30 units of β -glucosidase per gram of glucan for 24 h.

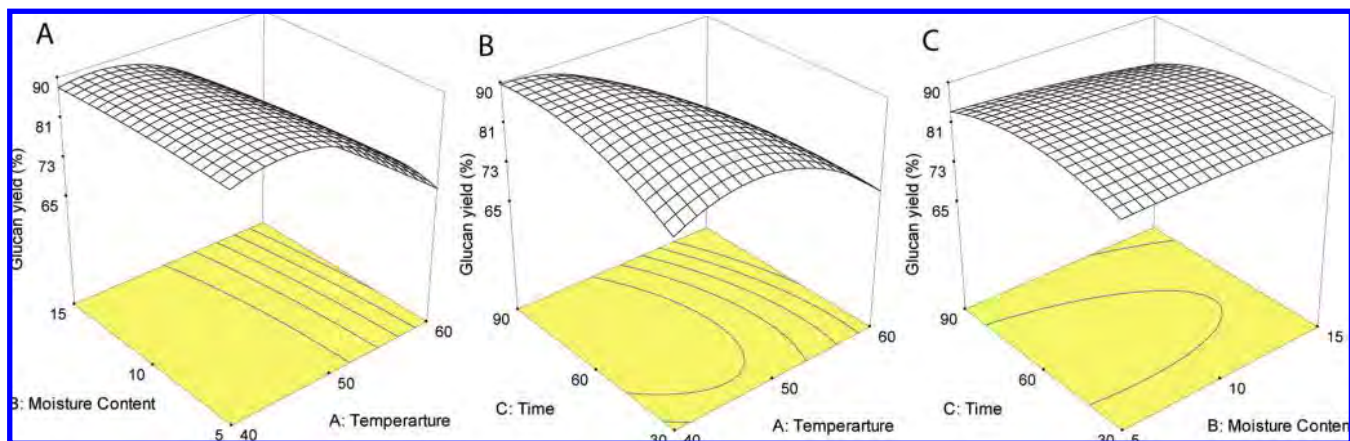


Figure 1. Response surface for the glucose yield from common reed pretreated by COSLIF at (a) temperature and moisture content, (b) temperature and reaction time, (c) moisture content and reaction time, followed by enzymatic cellulose hydrolysis (15 FPU of cellulase per gram of glucan).

maximum glucose release, from pretreatment and enzymatic hydrolysis as

$$\text{glucan yield (\%)} = -155.68 + 8.66T + 1.94MC + 1.44t - 0.083T^2 - 0.025(MC)^2 - 4.09 \times 10^{-3}t^2 - 0.026T(MC) - 0.018Tt - 6.69 \times 10^{-3}(MC)t \quad (4)$$

Figure 1A shows the effects of reaction temperature and biomass moisture content on glucan yield that equals glucan retention multiplied by glucan digestibility. Regardless of temperature, the moisture content alone, between 5 and 15% had little effect on glucan yield. Reaction temperatures, between 40 and 50 °C, did not differentially impact glucose yields, while the higher reaction temperature (60 °C) resulted in a much lower sugar yield. As shown in Figure 1B, at a long reaction time (90 min), increasing reaction temperature significantly decreased the sugar yield, mainly due to overhydrolysis of polysaccharides. There was a maximum glucan yield at approximately 50 °C for a short reaction time (30 min). Low reaction temperatures prevented overhydrolysis of the glucan, resulting in high glucan retention. However, when pretreatment conditions were not sufficient, the enzymatic digestibilities were much lower than those of well-pretreated samples (90%) (Table 2). Therefore, a

trade-off between pretreatment and hydrolysis was identified (i.e., maximum glucan yield) at two points: 40 °C for 90 min and 50 °C for 60 min. Figure 1C suggests that the maximum sugar yields were obtained when the reaction time was approximately 60 min, regardless of biomass moisture contents. All data suggested that biomass with a moisture content ranging from 5–15% did not affect pretreatment efficiency. The optimal pretreatment conditions for common reed were found to be 50 °C and 60 min, regardless of moisture content between 5 and 15%. After COSLIF treatment, ~93% of the glucan was retained, while 65% of the xylan and 28% of the lignin were removed.

Effect of Enzyme Loading and Mass Balance. Since cellulase is still a relatively costly biocatalyst accounting for a significant fraction of the processing costs for cellulosic ethanol production (approximately 30–100 cents per gallon of ethanol),² we studied the effects of an enzyme decrease from 15 to 5 FPU per gram on glucan digestibility. Figure 2 shows the glucan digestibility profiles of the common reed pretreated under the optimum condition (50 °C, 1 atm, and 60 min) at different enzyme loadings. Since enzymatic cellulose hydrolysis involves a rate-limiting primary cellulose hydrolysis (soluble cellodextrin

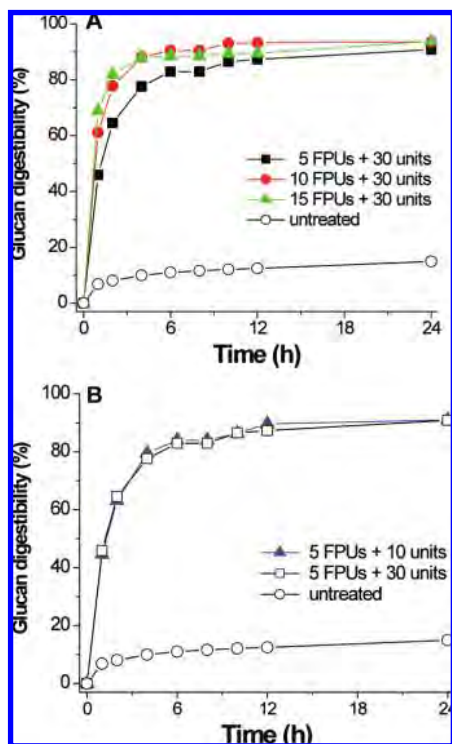


Figure 2. Enzymatic cellulose hydrolysis profiles for the COSLIF-pretreated common reed at different enzyme loadings (A, various cellulase and 30 units of beta-glucosidase; and B, 5 FPU of cellulase and 10 or 30 units of beta-glucosidase).

release from solid cellulose), and a fast secondary cellulose hydrolysis (glucose generation from cellooligosaccharides mainly mediated by β -glucosidase),²⁶ we first tested the effects of decreased cellulase loadings from 15 to 10 to 5 FPU per gram of glucan, with a fixed high β -glucosidase loading (30 units per gram of glucan). High β -glucosidase loading can prevent any possible cellobiose inhibition. At a high enzyme loading (15 FPU per gram of glucan), glucan digestibility reached 94% at hour 24 before leveling off (Figure 2A). When cellulase loading was decreased, glucan digestibility decreased slightly. At a low cellulase loading (5 FPU per gram of glucan), glucan digestibilities were 87% at hour 12, 90% at hour 24, and nearly leveled off after 24 h.

Furthermore, we investigated the effect of 3-fold reduction in β -glucosidase from 30 to 10 units of per gram of glucan on glucan digestibility at 5 FPU per gram of glucan. As shown in Figure 2C, a 3-fold reduction in total cellulase loading resulted in only 1–2% decrease in final glucan digestibility, and decreased hydrolysis rate only during the first 12 h. There was no significant difference in hydrolysis rates and final glucan digestibilities.

Figure 3 presents the mass balance of common reed pretreated by the COSLIF process and hydrolysis with 5 FPU of cellulase as well as 10 units of β -glucosidase per gram of glucan. The enzymatic digestibilities at a low enzyme loading were 90% for glucan and 46% for xylan, respectively. The overall glucose and xylose yields, including enzymatic hydrolysis and pretreatment (water stream), were 88% and 71%, respectively.

Surface Morphology and Substrate Accessibility. Figure 4 shows the surface morphology changes in intact and COSLIF-treated common reed samples. The intact plant cell wall structures of common reed presents its plant cell vascular bundles and its fibril structure (Figure 4A). Concentrated H_3PO_4 can overcome biomass recalcitrance by dissolving crystalline cellulose fibers,

accompanied with increasing cellulose accessibility.^{4,9,22} The sequential washing by the organic solvent can partially remove lignin.⁹ A well-treated lignocellulose sample (85% H_3PO_4 , 50 °C and 60 min) shows no fibrous structure (Figure 4C), suggesting that all fibrous structures of the lignocellulose were completely disrupted. However, this disruption required sufficient reaction time at the set temperature.⁹ Figure 4B shows that 20 min reaction time at 50 °C looks to break large fibrils of common reed but is not as efficient as that in Figure 4C. We further measured the substrate accessibility before and after the COSLIF pretreatment. This measurement was based on adsorption of a nonhydrolytic fusion protein TGC containing green fluorescent protein and a cellulose-binding module.²³ Through the COSLIF pretreatment, the total substrate accessibility to cellulase (TSAC) increased from 0.35 ± 0.056 to 16.1 ± 1.3 m² per gram of biomass (Table 3). To eliminate interference from the remaining lignin and other noncellulose components, cellulose accessibility to cellulase (CAC) was measured on the basis of the adsorption of TGC after blocking with BSA. The CAC values of the intact common reed and pretreated common reed were 0.14 ± 0.035 and 13.1 ± 1.1 m² per gram of biomass, respectively. This result suggested that COSLIF can increase substrate accessibility 93.6-fold and yield a cellulosic product with high substrate digestibility mediated by cellulase and fast enzymatic hydrolysis rate even at a low enzyme loading. A 14.4-fold increase in noncellulose accessibility (NCAC), from 0.21 to 3.03 m² per gram of biomass, was much lower than a 93.6-fold increase in CAC, suggesting the importance of increasing cellulose accessibility through biomass pretreatment.

Discussion

This study showed that very high overall yields (88% for glucose and 71% for xylose) were achieved for the COSLIF-pretreated common reed at a low cellulase loading (5 FPU of cellulase and 10 units of β -glucosidase per gram of glucan) within 24 h hydrolysis. The optimal pretreatment conditions through surface response methodology were 50 °C, 1 atm, and 60 min in the presence of 85% H_3PO_4 , regardless of the moisture contents of the feedstock, from 5 to 15% (w/w). Glucan digestibility (94%) of the pretreated common reed at a high enzyme loading was slightly lower than the previous results (i.e., 96–97%) for corn stover, switchgrass, poplar, and hemp hurds. This small difference was attributed to less efficient lignin removal (28% of overall lignin) in the modified COSLIF as compared to those achieved (40–50% of overall lignin)^{4,10} by decreasing the use of organic solvent.

Water in ~5–15% moisture content biomass did not dilute concentrated phosphoric acid significantly below the critical values (e.g., 80–83%) as a cellulose solvent.^{10,22} The moisture contents of harvested biomass range widely from ~5 to 40% w/w, depending on the harvesting season and biomass type.²⁷ Winter harvesting of the standing bioenergy plants after natural drying to 5–15% moisture contents, such as common reed, would save feedstock transportation costs as compared to that of freshly cut wet biomass feedstock. This study suggested the technological feasibility of efficient sugar release from a perennial grass, the common reed.

Cost analysis associated with enzyme costs and sugar-to-ethanol revenues suggests that decreasing cellulase use would compensate for the slight revenue loss, resulting from a slightly low overall sugar yields at a decreased enzyme loading (Figure 5). It was estimated that approximately 79.4 and 75.4 gallons of cellulosic ethanol per ton of common reed could be produced at high (15 FPU per gram of glucan) and low (5 FPU per

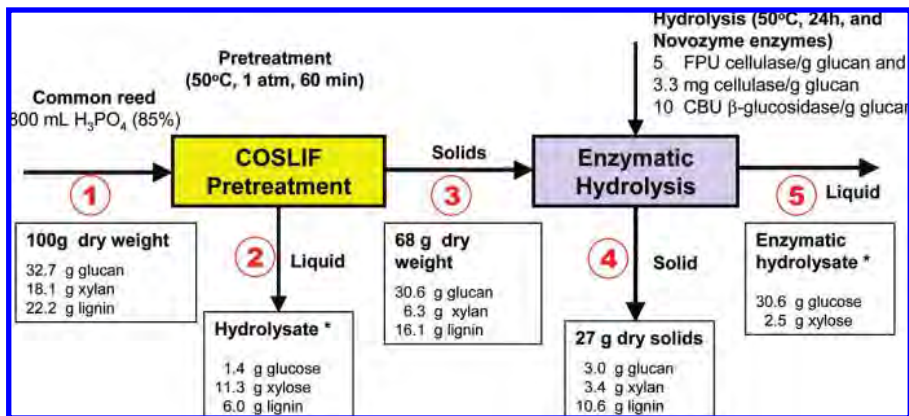


Figure 3. Mass balance for common reed pretreated by COSLIF followed by enzymatic hydrolysis at a low enzyme loading (5 FPU of cellulase and 10 units of β -glucosidase per gram of glucan).

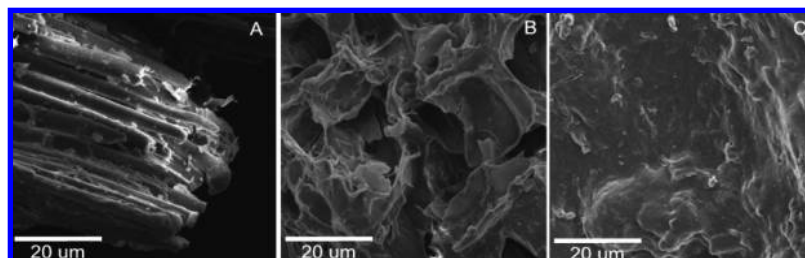


Figure 4. SEM images for the common reed samples before (A) and after the COSLIF pretreatment (B, 20-min dissolution; and C, 40-min dissolution).

Table 3. The Substrate Accessibilities (TSAC, Total Substrate Accessibility to Cellulase; CAC, Cellulose Accessibility to Cellulase; and NCAC, Noncellulose Accessibility to Cellulase) for Intact and COSLIF-Pretreated Biomass

sample	TSAC (m ² /g biomass)	CAC (m ² /g biomass)	NCAC (m ² /g biomass)
intact	0.35 ± 0.056	0.14 ± 0.035	0.21 ± 0.066
pretreated	16.1 ± 1.3	13.1 ± 1.1	3.0 ± 1.7

gram of glucan) cellulase loadings, respectively. If cellulase costs are \$0.30 per gallon based on an enzyme use of 15 FPU per gram of glucan, a 3-fold reduction in cellulase use can save \$0.20 per gallon and decrease the ethanol revenues of \$0.126 per gallon, resulting in a net savings of 7.4 cents per gallon of ethanol, \$5.55 per ton of common reed, or a \$3.7 million of annual cost savings for a biorefinery processing 2000 tons of biomass per day. The cost saving would increase drastically to \$0.27 and \$0.53 per gallon of ethanol, if the cellulase costs were

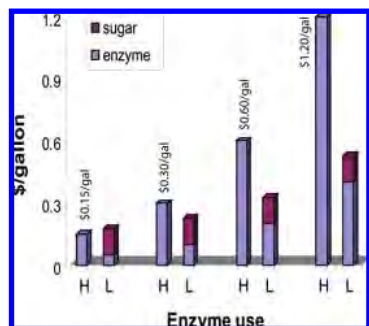


Figure 5. Enzyme cost analysis under the low (L, 5 FPU of cellulase per gram of glucan) and high (H, 15 FPU of cellulase per gram of glucan) cellulase loadings. Given an assumption of ethanol fermentation yield = 95% of theoretical yield for glucose and xylose, the overall ethanol yields were 79.4 and 75.4 gallons per ton of dry common reed, at the high and low enzyme loadings, respectively. The selling price of cellulosic ethanol was assumed to be \$2.50 per gallon.

\$0.60 and \$1.20 per gallon, respectively. On the other hand, if cellulase costs were decreased to \$0.15 per gallon of ethanol, a saving in enzyme cost could not be enough to compensate for the sugar loss.

The COSLIF technology may be regarded as a nearly generic pretreatment. It has previously been shown to efficiently increase the glucan digestibility of a relatively broad range of feedstocks, including corn stover, switchgrass, hemp hurds, and poplar.^{4,10} This study extended the range of feedstocks to the common reed and also made improvements in the COSLIF process. Different from widely studied dilute acid pretreatment,^{28–31} which substantially removes hemicelluloses thereby disrupting the linkages among cellulose, hemicellulose, and lignin, the COSLIF pretreatment not only partially removes lignin and hemicelluloses, but also substantially disrupts the fibrillar structure of biomass. The resulting fast hydrolysis rates and high glucan enzymatic digestibilities of the COSLIF-pretreated common reed are attributed to (i) more efficient biomass structure destruction, qualitatively shown by SEM images (Figure 4), and (ii) higher substrate accessibility to cellulase (Table 3).

Conclusion

The pretreatment conditions were optimized for the common reed through surface response methodology for the maximal release of soluble sugars. At a low enzyme loading (5 FPU of cellulase and 10 units of β -glucosidase), the overall glucose and xylose yields were 88% and 71%, respectively. Low use of costly cellulase would significantly improve the overall economics of cellulosic ethanol production. Since the COSLIF technology is still at a relatively early stage of development, more detailed economic analyses, based on rigorous Aspen-plus models are needed to understand its potential for practical applications. This study clearly suggests that currently growing *Phragmites*, an invasive weed, can be used for bioenergy

feedstock and would be planted as a bioenergy crop at marginal wetlands in the future.

Acknowledgment

This work was supported mainly by a DOD grant (W911SR-08-P-0021), and partially by the USDA-sponsored Bioprocessing and Biodesign Center, and the DOE BioEnergy Science Center (BESC). NS was partially supported by the ICTAS scholar program. NT was supported by the NSF REU program. The authors are grateful for the free cellulase samples provided from Novozymes North American.

Note Added after ASAP Publication: The version of this paper that was published on the ASAP website May 28, 2009 had errors in the presentation of data for run 20 in Table 2. The corrected version of this paper was reposted to the Web June 2, 2009.

Nomenclature

BSA = bovine serum albumin
 CAC = cellulose accessibility to cellulase
 EG = ethylene glycol
 GE = glucose equivalent
 NCAC = noncellulose accessibility to cellulase
 MC = moisture content (%)
 QS = quantitative saccharification
 RSM = response surface methodology
 TGC = a nonhydrolytic fusion protein, containing a green fluorescence protein and a cellulose-binding module
 TSAC = total substrate accessibility to cellulase

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Received for review February 19, 2009
 Revised manuscript received April 30, 2009
 Accepted May 8, 2009