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Evaluation of Ethanol Production from Corncob Using *Scheffersomyces* (*Pichia*) *stipitis* CBS 6054 by Volumetric Scale-up

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Abstract In scale-up, the potential of ethanol production by dilute sulfuric acid pretreatment using corncob was investigated. Pretreatments were performed at 170 °C with various acid concentrations ranging from 0% to 1.656% based on oven dry weight. Following pretreatment, pretreated biomass yield ranged from 59% to 67%. More than 90% of xylan was removed at 0.828% of sulfuric acid. At same pretreatment condition, the highest glucose yield obtained from pretreated biomass by enzymatic hydrolysis was about 76%, based on a glucan content of 37/100 g. In hydrolysate obtained by pretreatment, glucose concentration was low, while xylose concentration was significantly increased above 0.368% of sulfuric acid. At 1.656% of sulfuric acid, xylose and glucose concentration was highest. In subsequent, fermentation with hydrolysate, maximal ethanol yield was attained after 24 h with 0.368% of sulfuric acid. The fermentation efficiency of hydrolysate obtained by enzymatic hydrolysis reached a maximum of 75% at an acid charge of 0.368%.

Keywords Pretreatment · Enzymatic hydrolysis · Corncob · Ethanol

Introduction

The US Energy Security and Independence Act (ESIA) of 2007 promotes production of 16 billion gallons of cellulosic ethanol to displace petroleum-based gasoline for transportation.

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Due to the strong recalcitrance of most biomass feedstocks, such as corn stover, switch grass, and wood, significant barriers remain for efficient cellulosic ethanol bioconversion [5]. Corncob, a collectable byproduct from corn (maize) with an energy density of about 5,000 MJ/m^3, is a viable source of biomass for cellulosic ethanol production due to its low lignin and high carbohydrate contents [1]. These characteristics make corncob an ideal entry feedstock for cellulosic ethanol production.

The feasibility of aqueous sulfuric acid pretreatment for efficient sugar production from corncob in a previous study was demonstrated [10]. Based on total glucose and xylose yield and energy efficiency in sugar production, the optimal pretreatment temperature is between 160 °C and 170 °C with acid charge of 1.8–2.2%(*w*/*w*) on an oven dry (OD) solids basis. The pretreatment experiments were conducted at a capacity of 25 g OD corncob per batch. In this study, pretreatment was scaled-up by a factor of 84 to 2.1 kg per batch. The scale-up pretreatments were conducted at 170 °C [10]. The optimal acid charge range from the previous study was used as a starting point in the scale-up experiments, but new optima had to be determined due to significant differences in reactor design, temperature profile, and capacities.

Xylose fermentation has been a challenge to efficient utilization of corncob due to its high xylan content. Earlier work has used *Escherichia coli* B to ferment sulfuric acid corncob hydrolysate [2] and *Saccharomyces cerevisiae* 1400 (pLNH33) to ferment combined corncob hydrolysates from ammonia pretreatment and enzymatic saccharification of the residual solids [3]. Recombinant *S. cerevisiae* [9] and *Kluyveromyces marxianus* [11] have also been used to ferment xylose from corncob, and fed-batch studies have been conducted for high solids simultaneous saccharification and fermentation of pretreated corncob solid substrates [8, 11]. However, relatively few studies have provided complete mass balance data for ethanol production from corncob.

Lee et al. [7] previously reported the use of oxalic acid impregnation and steam to pretreat corncob and *Scheffersomyces* (*Pichia*) *stipitis* CBS 6054 for fermentation. Xylose was recovered by washing. The study was focused on fermentation, and ethanol yield on unit ton of corncob was not reported. In this study, the performance of *S. stipitis* CBS 6054 in fermenting acid and enzymatic hydrolysates produced from sulfuric acid hydrolysis of corncob at a scale of 2.1 kg was investigated.

Experimental

Materials

Corncob pellets (Pestell, New Hamburg, ON, Canada) with 10% moisture content were used. Sulfuric acid was reagent grade (Sigma-Aldrich, St. Louis, MO, USA). Commercial Novozyme Celluclast 1.5 L and Novozyme 188 (β-glucosidase) were generously provided by Novozymes (Franklinton, NC, USA).

Pretreatment of the Corncob

A 23-L rotating stainless steel wood-pulping digester was used to chemically pretreat corncob pellets. Pellets (2.1 kg, OD) were loaded into a screened basket that was fitted into the digester. Sulfuric acid (4.2 L) in deionized water, at the desired concentration, was added, and the digester was sealed. This gave a liquid-to-solid ratio (L/S) of 2 for pretreatment. A low L/S not only reduces thermal energy cost in pretreatment but also improves the sugar concentration in the hydrolysate to reduce downstream ethanol separation and distillation cost. The digester was heated by an external steam jacket and rotated at a speed of 2 rpm, which provided good mixing of the corncob and acid solution during pretreatment. All pretreatments were conducted at 170 °C. The sulfuric acid concentration (*v*/*v*) of the pretreatment solution ranged from 0% to 0.45%, which was equivalent to an acid charge of 0% to 1.656%, OD basis. The maximum acid charge of 1.656% was lower than the 2.2% that is the acid concentration used in a previous study at 170 °C [10]. The reactor had a temperature ramp up time of about 7 min, which effectively increased the pretreatment time or severity and therefore afforded a lower acid charge. After 30 min, the steam and vapor pressure were vented from the top, and the hydrolysate was drained and collected from the bottom of the digester. The pretreatment hydrolysate and substrate were separated, weighed, and allowed to cool to room temperature. Hydrolysates were saved for sugar analysis and fermentation. Portions of the solid cellulosic substrates were weighed then filtered (Ahlstrom grade 617) through a Buchner funnel. After washing, the solid substrates were weighed and saved for subsequent enzymatic hydrolysis. The solids contents of the washed substrates were measured gravimetrically by drying subsets of the washed samples overnight in an oven at 105 °C. Yields of solids and liquids were determined for mass balance and final ethanol yield analysis.

Determination of Sugars, Ethanol, and Byproducts

The chemical composition of the pretreated materials was determined by the TAPPI method (T 222-om-88). Carbohydrate analysis of the pretreated substrates was as previously reported [10]. Concentrations of D-glucose, D-xylose, L-arabinose, and ethanol were determined after separation by HPLC using a BioRad (Hercules, CA, USA) Aminex HPX-87H column (300×7.8 mm) at 55 °C with 5 mM H_2SO_4 as eluant, at a flow rate of 0.3 ml/min, and an injection volume of 20 μL [7]. A refractive index detector (Hitachi High-Technologies Corporation model L-2490, Japan) was used to quantify the products. Samples were appropriately diluted in deionized water, and then filtered through PrepSEP C18 (Fisher Scientific) filters prior to injection. Acetic acid in the pretreatment hydrolysate was measured by the same HPLC for sugar analysis.

Yeast Strain and Inoculum Medium

S. stipitis CBS 6054 was maintained on agar malt medium and stored at 4 °C then transferred to a fresh plate to be used within 24 h of incubation at 30 °C. Cells were grown in 1,000 ml Erlenmeyer flasks containing 400 ml of YPD (10 g/l yeast extract, 10 g/l peptone, and 20 g/l glucose) in an orbital shaker at 30 °C and 200 rpm. Following 24 h growth, cell cultures were harvested, centrifuged (4,068 g, 15 min at 4 °C), and decanted to yield cell pellets, which were washed once with sterile deionized water and adjusted to a calculated concentration of 30 g dry cell weight (DCW) per liter via standard curves relating 600 nm absorbance to DCW per liter concentration. An aliquot was transferred to fresh fermentation medium for an initial cell concentration of 2.0 DCW/l.

Separate Hydrolysis and Fermentation (SHF)

The pretreated material of 5 g dry matter was transferred to a 125-ml Erlenmeyer flask containing 50 ml of 50 mM sodium citrate buffer (pH 6.0). Celluclast 1.5 L (7.5 FPU/g substrate) and Novozyme 188 (11.25 CBU/g substrate) were added for hydrolysis in a shaking incubator at 50 °C, 150 rpm, and incubated for 72 h. Hydrolysate was collected and sterilized by filtration (0.45 μm). The fermentation medium was prepared using hydrolysate after enzymatic hydrolysis. Initially, 5 g/l yeast extract, 5 g/l urea, 0.5 g/l $MgSO_4 \cdot 7H_2O$, and 1 g/l KH_2PO_4 were added to the hydrolysate in 125 mL Erlenmeyer flasks, and finally inoculated at a cell concentration of 2 DCW/l for fermentation.

Fermentation of Pretreatment Hydrolysate

The water-soluble hydrolysate collected after pretreatment was centrifuged to remove the solid part, and pH of the supernatant solution was adjusted to 6.0 with lime. The hydrolysate was sterilized by membrane filtration. Yeast (2 DCW/l) and nutrient (5 g/l yeast extract, 5 g/l urea, 0.5 g/l $MgSO_4 \cdot 7H_2O$, and 1 g/l KH_2PO_4) were added to sterilized hydrolysate for fermentation. Fermentation was performed at 30 °C and 150 rpm and incubated for 120 h.

Results and Discussion

Substrate Solid Yield and Composition

Substrate solid yield ranged from 59% to 67% (Table 1) and decreased with increasing acid. Glucan loss was minimal (less than 10% at an acid charge 1.656%), which was similar to what we observed in small-scale experiments using 50 g of corncob [10]. Xylan removals and glucan losses increased from about 67% to 96% as acid charge on OD corncob increased from 0% to 1.656%. More than 90% of xylan can be removed in pretreatment when acid charge reached 0.83% on OD corncob. Ninety percent of xylan removal was achieved at an acid charge of 1.84% in a small-scale cooking reported by Wang et al. [10], which suggests the temperature ramping time effectively increased the pretreatment severity factor of the digester. According to the report of Chen et al. [4], xylan removal was about 85% when pretreatment was performed at 120 °C for 2 h, which has a similar severity factor with the pretreatment condition used in this study.

Lignin measurements were higher than expected, which was also observed previously. The reason is that lignin fraction contained acid-insoluble lignin and condensation products from hemicelluloses degradation. The accuracy of this measurement is limited by current

Table 1 Corncob components after aqueous sulfuric acid pretreatments for 30 min at 170 °C

Acid charge on corncob (%)	Composition					
	K lignin[a]	Arabinan	Glucan	Xylan	Sum (%)	Solid yield (%)
Untreated	13.9	2.19	37.0	27.8	80.9	100
0	14.1	0.3	37.7	8.5	60.7	67
0.184	13.7	0.4	36.8	9.3	60.4	67
0.368	14.7	0.2	36.8	5.8	57.9	64
0.828	19.2	0.0	35.9	2.1	57.2	62
1.656	19.3	0.0	34.5	1.2	55.1	59

[a] Klason lignin

analytical methods. Researchers at the National Renewable Energy Laboratory have encountered similar problems (personal communication, Dan Schell 2009).

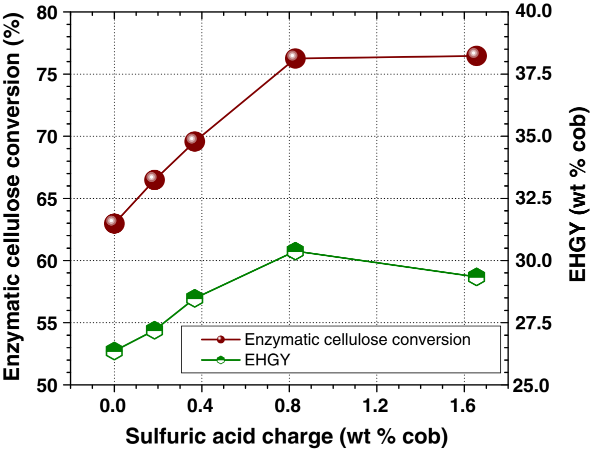
Glucose Yield in Enzymatic Hydrolysate

Enzymatic conversion of the cellulose substrate was calculated based on the measured glucose concentration in the enzymatic hydrolysate and substrate glucan content. Figure 1 shows the effect of acid charge on enzymatic cellulose conversions and enzymatic hydrolysis glucose yield (EHGY) in weight percentage of untreated corncob. Digestibility of the substrate increased as acid charge on corncob in pretreatment increased. Enzymatic cellulose conversion to glucose reached 76% of the total available glucan at an acid charge of 0.8%. The yield of glucose from enzymatic hydrolysis in weight percentage of OD corncob was calculated based on measured glucose concentration in the enzymatic hydrolysate, initial solids consistency of pretreated corncob during enzymatic hydrolysis, and the pretreatment corncob solid yield. The maximum glucose yield from enzymatic hydrolysis was attained with 30 wt.% of corncob at an acid charge of 0.8% (Fig. 1).

Sugar Yield in Pretreatment Hydrolysate

The glucan and xylan removed in pretreatment were dissolved in the pretreatment hydrolysate and can be partially recovered as ethanol through fermentation of the hydrolysate. The glucose and xylose yields from pretreatment hydrolysates were calculated from the measured glucose and xylose concentrations in the hydrolysates and the pretreatment liquid-to-solid corncob ratio of 2. As shown in Fig. 2, glucose yields in the pretreatment hydrolysate increased from 1 to 4 wt.% corncob as pretreatment acid charge on OD corncob increased from 0% to 1.656%. This is because more glucan was removed as pretreatment severity increases, as shown in Table 1. The data of glucan loss in pretreatment (from data in Table 1) and glucose yields from the hydrolysates (Fig. 2) indicates complete recovery of the removed glucan during pretreatment, which is not possible and perhaps due to small measurement errors in carbohydrate that produced a high glucan content or low losses in glucan through pretreatment. This also helps to explain the yield following

Fig. 1 Effects of pretreatment acid charge on enzymatic hydrolysis cellulose conversion efficiency and enzymatic hydrolysis glucose yield (EHGY)



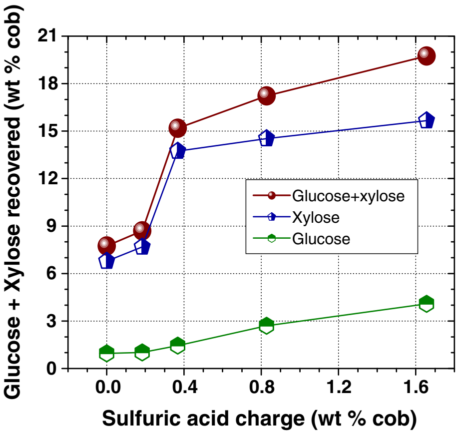


Fig. 2 Effects of pretreatment acid charge on xylose and glucose yield from pretreatment hydrolysate (water-soluble fraction)

enzymatic conversion of cellulose (76%, Fig. 1), which was lower than expected based on small-scale experiments [10].

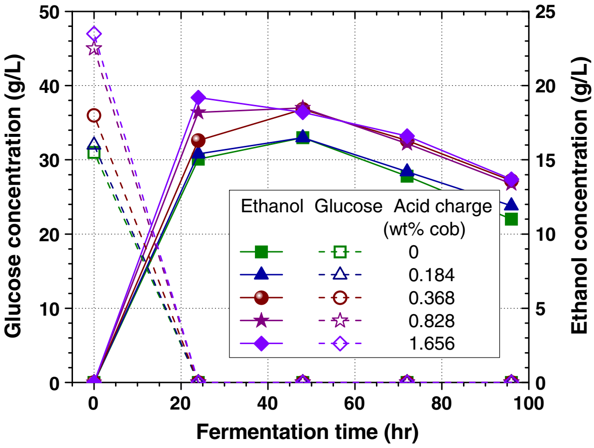
Xylan removal increases almost linearly with an increase in acid charge (Table 1). Not all xylan were converted to xylose. Some were degraded to other byproducts, such as furfural, a fermentation inhibitor. Figure 2 shows the effect of pretreatment acid charge on the xylose yield. The nonlinear relationship of xylose yield with pretreatment acid charge suggests the complex processes among xylan removal and subsequent conversion reactions to xylose, and other by-products. The results in Fig. 2 indicate that xylose recovery was only about 8 wt.% corncob (≈25% of the total xylan) at an acid charge below 0.184%. Xylose recovery increased rapidly to 14 wt.% corncob or 45% of the total xylan present when acid charge was increased to 0.386%. This rapid increase is due to the increased removal of xylan and subsequent conversion to xylose. The increase in xylose recovery slowed when acid charge was further increased, which perhaps is due to the increased production of furfural as acid charge increases [6]. Xylose recovery was lower than we previously obtained in small-scale experiments reported [10], which might have been due to the heating-up period and mixing process. The prolonged pretreatment time due to initial temperature ramping effectively increased the pretreatment severity factor, which may have caused the increased degradation of xylan to by-products, such as furfural other than hydrolyzing to xylose. The optimization of pretreatment time may be required in the future scale-up experiments.

Separate Fermentation of Enzymatic and Pretreatment Hydrolysate

Separate fermentation of the enzymatic hydrolysate was conducted using *S. stipitis* CBS 6054. Glucose consumption was rapid in the first 24 h for all the five sets of experiments conducted, as shown in Fig. 3. Maximal ethanol concentrations were attained in 24 to 48 h of fermentation. The data suggest that the higher the acid charge in pretreatment, the higher the maximal ethanol concentration in the fermentation broth. The maximal ethanol production rate in the first 24 h was 0.8 g/L/h, which was achieved at an acid charge of 1.656%.

The pretreatment hydrolysate was also fermented using the same *S. stipitis* CBS 6054 strain. Detoxification of hydrolysate is required for ethanol fermentation because pretreatment was performed with low liquid-to-solid ratio. Therefore, hydrolysate was diluted with water for ethanol fermentation to avoid sugar loss. Maximal ethanol yield was

Fig. 3 Time-dependent glucose and ethanol concentrations during fermentation of glucose from separate enzymatic hydrolysis



obtained from the hydrolysate produced with an acid charge of 0.368% (Fig. 4). The data were presented in weight percentage of corncob to be consistent with the sugar data shown in Fig. 2 and for easy mass balance analysis. Using a pretreatment liquid-to-corncob ratio (L/S) of 2 and a dilution ratio of 2:1 before fermentation, the measured ethanol concentrations in the fermentation broth can be found. The maximal ethanol production rate in the first 24 h was only 0.17 g/L/h.

The maximal fermentation efficiencies were determined based on the obtained maximal ethanol concentrations of initial fermentable sugar concentrations. The fermentation efficiencies in fermenting the enzymatic hydrolysates were from 75% to 82% and were not significantly affected by acid charge in pretreatment as shown in Fig. 5. However, the fermentation efficiency in fermenting pretreatment hydrolysate is significantly affected by the acid charge (Fig. 5). Fermentation efficiency was first increased as acid charge in pretreatment increases and reached a maximum of about 75% at an acid charge of 0.368%. The initial increase in acid charge increases sugar (glucose and xylose) concentrations in the pretreatment hydrolysate (Fig. 2), which facilitates

Fig. 4 Time-dependent ethanol yield from fermentation of pretreatment hydrolysates (water-soluble fraction) obtained at different pretreatment acid charges (*solid line* ethanol production, *dotted line* xylose consumption)

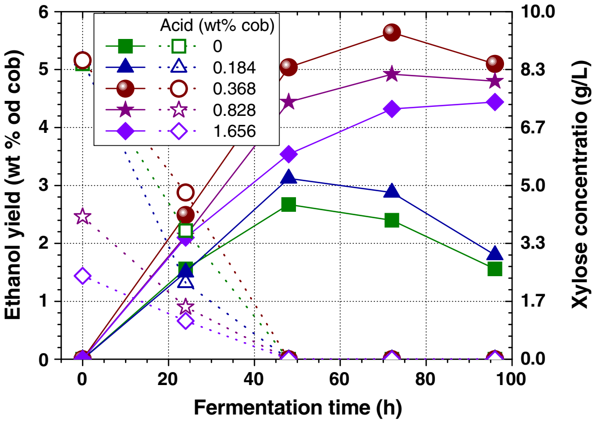
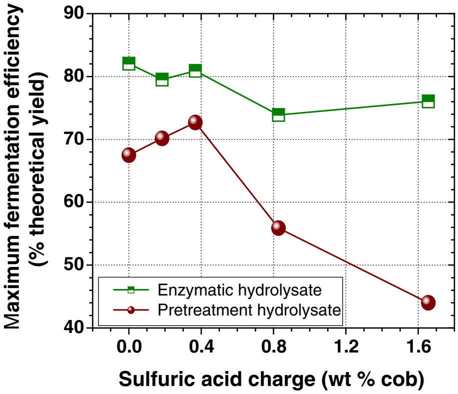


Fig. 5 Effects of pretreatment acid charge on maximal fermentation efficiency of the pretreatment and enzymatic hydrolysates

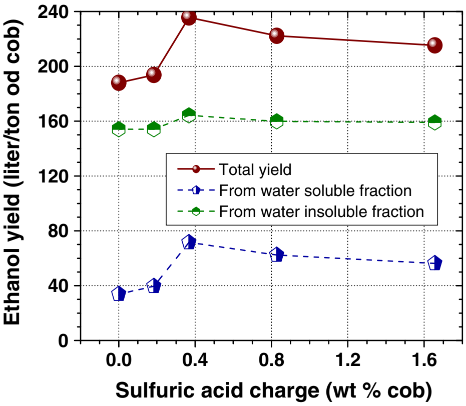


fermentation. Increasing acid charge beyond 0.368% only slightly increased sugar concentration (Fig. 2), but significantly increased acetic acid and furfural concentrations [10]. Acetic acid concentration in the hydrolysate increased from 3 to 6 g/L when acid charge was increased from 0% to 1.656%. As a result, fermentation efficiency decreased rapidly as acid charge further increased.

Ethanol Yield

Based on the ethanol data presented above, the total ethanol yields from both the water-insoluble fraction (enzymatic hydrolysate) and pretreatment hydrolysate were determined. The maximal ethanol yield was achieved at an acid charge of 0.368% from both fractions as shown in Fig. 6. Increased acid charge in pretreatment increased high ethanol concentration in the fermentation broth of the enzymatic hydrolysate but reduced the solid (water-insoluble) yield, which resulted in decreased ethanol yield. The maximal ethanol yield from the water-insoluble fraction was only about 164 L/ton corncob, or about 61% of the theoretical yield based on corncob glucan content of 37% (Table 1). The maximal ethanol yield from the water-soluble fraction was 71 L/ton corncob, or only about 35% of the theoretical yield based on corncob xylan content of 27.8% (Table 1). The total ethanol yield was 235 L/ton corncob, or about 58% of theoretical. Further study is needed to improve ethanol yield especially from the water-insoluble fraction.

Fig. 6 Effects of pretreatment acid charge on total ethanol yield and yields from the water-soluble and water-insoluble fractions



Conclusions

Dilute sulfuric acid pretreatment was applied to corncob for ethanol production using *S. stipitis* CBS 6054. Pretreatment was conducted at relatively mild conditions of 170 °C with acid charge on corncob that ranged from 0% to 1.656%. Maximal xylose recovery was about 55% at an acid charge of 1.656%, based on corncob xylan content of 27.8%, while maximal enzymatic glucose recovery (≈75%) based on corncob glucan content of 37% was achieved at an acid charge of 0.8%. The pretreatment hydrolysate (water-soluble fraction) was fermentable with a maximal efficiency of 73% following a 2:1 dilution with DI water. The maximal ethanol was achieved at an acid charge of 0.368% for the water-soluble and water-insoluble fractions. The maximal ethanol yield through separate enzymatic saccharification and fermentation of the solid cellulosic substrate (water-insoluble) fraction was 164 L/ton corncob. The total maximal ethanol yield was 235 L/ton. Further studies are needed to improve ethanol yields.

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