Corn Stover Bioconversion by Green Liquor Pretreatment and a Selected Liquid Fermentation Strategy

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Green liquor pretreatments and different liquid-fermentation strategies were tested to establish an effective ethanol production process from corn stover. After pretreatment and enzymatic hydrolysis, 329.7 g of glucose and 126.3 g of xylose were obtained from 1,000 g of dry corn stover. The primary aim of this work was to test the fermentability of the enzymatic hydrolysate of green liquor-pretreated corn stover, for no fermentation has been done on the enzymatic hydrolysate of green liquor-pretreated corn stover before. Three liquid-fermentation strategies, including sequential fermentation, co-fermentation, and co-culture, were carried out and compared to select an appropriate fermentation strategy from green liquor-pretreated corn stover. Among the different liquid-fermentation strategies, sequential fermentation yielded the highest ethanol production (210.7 g ethanol/1,000 g corn stover). Using the sequential fermentation strategy, glucose fermentation by *Saccharomyces cerevisiae* and ethanol distillation was completed prior to xylose fermentation by *Pichia stipitis*, so that the separate utilization of glucose and xylose ensured that each fermentation stage used the suitable microorganism, permitting high ethanol yields. Sequential fermentation was thus considered to be the most promising liquid-fermentation strategy for ethanol production from green liquor-pretreated corn stover.

Key words: Ethanol production; Green liquor pretreatment; Liquid fermentation strategies; Co-fermentation; Co-culture; Sequential fermentation

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INTRODUCTION

The successful development of a bioethanol industry could help to reduce our reliance on fossil resources and mitigate global warming (Bondesson *et al.* 2013). Currently, corn stover is regarded as the most favored lignocellulosic resource for large-scale ethanol production in China because of its abundance and relatively low cost (Zhao and Xia, 2009). It is estimated that about 250 million tons are produced annually in China (Zhao and Xia 2010).

In the bioconversion of lignocellulose to ethanol, pretreatment is an essential procedure. By changing the structural and chemical composition of lignocellulosic materials, pretreatment increases the accessibility of enzymes to cellulose and facilitates hydrolysis of lignocellulose to fermentable sugars (Rossberg *et al.* 2014). Among various pretreatment methods, green liquor is suggested as an ideal first step for bioethanol production from lignocellulosic materials (Jin *et al.* 2010; Wu *et al.* 2010). Green liquor (GL), the mixture of sodium carbonate and sodium sulfide, can be recovered after

combustion of the black liquor in the recovery boiler in a kraft pulp mill. GL pretreatment tends to selectively remove the lignin, leaving as much polysaccharides as possible in pulp, and to expose the cellulose to enzymatic attack producing high sugars yields (Gu *et al.* 2012).

GL pretreatment keeps both cellulose and hemicellulose fraction in the substrate for enzymatic hydrolysis. As a result, the major fermentable sugars from hydrolysis are glucose and xylose. To obtain an economically feasible industrial process for bioethanol production, it is necessary to efficiently convert xylose present in the biomass into ethanol (Chen *et al*. 2010). These years, recombinant strains have been introduced to produce ethanol from cellulose and hemicellulose derived sugars (Meinander *et al*. 1999; Gonçalves *et al.* 2014). However, poor stability of recombinant strains remains a significant challenge (Yoo *et al*. 2012). So far, the effectiveness of these recombinant strains in large-scale fermentations has not been established.

Therefore, two naturally occurring microorganisms, *Saccharomyces cerevisiae* and *Pichia stipitis*, are used to produce ethanol from GL pretreated corn stover. However, *S. cerevisiae* displays higher ethanol and inhibitor tolerance than *P. stipitis* (Guo *et al*. 2008), in spite of the disability of utilizing xylose (Lin *et al*. 2012). According to the different capability of fermenting yeasts, several possible liquid fermentation strategies have been considered, including co-fermentation of glucose and xylose by *P. stipitis* (Nakamura *et al*. 2008), co-cultures of *S. cerevisiae* and *P. stipitis* (Rouhollah *et al*. 2007; Bader *et al*. 2010), and a sequential fermentation process. Co-fermentation of glucose and xylose by *P. stipitis* mean that glucose and xylose were simultaneously utilized by *P. stipitis*, while co-cultures of *S. cerevisiae* and *P. stipitis* mean that glucose and xylose were simultaneously utilized by *S. cerevisiae* and *P. stipitis* to produce ethanol. In the last approach of sequential fermentation, xylose fermentation for ethanol production was carried out after glucose fermentation and ethanol distillation was completed, in order to avoid glucose repression and ethanol inhibition on xylose utilization (Delgenes *et al.* 1988; Laplace *et al.* 1991).

The primary aim of this work was to test the fermentability of the enzymatic hydrolysate of green liquor-pretreated corn stover, for no fermentation was performed on the enzymatic hydrolysate of green liquor-pretreated corn stover before. Three liquid-fermentation strategies, including sequential fermentation, co-fermentation, and co-culture, were carried out and compared to obtain the highest ethanol yield from green liquor-pretreated corn stover.

EXPERIMENTAL

Corn Stover and Enzymes

Corn stover, which was generously provided by Huhehaote, Neimenggu municipality, China, was cut into pieces 3 to 5 cm in length. Air-dried corn stover was stored in sealed plastic bags. The chemical composition of the corn stover was determined according to NREL standards (NREL 2012), and the corn stover was shown to consist of 38.1% glucan, 22.7% xylan, 2.8% arabinan, and 23.3% lignin.

The M/K pulping digester used in Jiangsu Provincial Key Lab of Pulp and Paper Science and Technology had a capacity of 1,000 g of dry corn stover. The ratio of solid-to-liquid material during the pretreatment step was 1:6 (w/v). The green liquor

prepared for use in laboratory experiments by mixing sodium carbonate and sodium sulfide and a sulfidity of 40%. Corn stover was pretreated with the green liquor at a total titrateable alkaline (TTA) charge of 8% (w/w), which was expressed as the weight of Na_2O on chips (Jin *et al.* 2010).

After impregnation at 80 °C for 30 min, corn stover was pretreated at 140 °C for 1 h. The solid residue was then collected and washed with tap water (dry mass to water ratio of 1:15, g:mL) to remove residual chemicals. Washed corn stover was refined using a laboratory disk refiner (KRK; Japan) with a disk gap of 0.04 mm. Solids recovery was calculated as a percentage of the total solids recovered after pretreatment based on the dry weight of the initial sample. Glucan and xylan recoveries were estimated as the ratio of the amounts of glucan and xylan that remained in the recovered solids relative to those in the untreated original samples.

Enzymatic Hydrolysis

Enzymatic hydrolysis with a substrate loading of 5% (w/v) was performed in a 10-L reactor with a rate of agitation of 150 rpm at 50 °C for 48 h. The pH was adjusted to 4.8 with either sulfuric acid or sodium hydroxide. Enzymatic hydrolysis was carried out using an enzyme cocktail comprising 25 FPIU of cellulase from *Trichoderma reesei* (Sigma C2730), 20 IU of β-glucosidase from *Aspergillus niger* (NZ-188), and 120 IU of xylanase (Fluka-95595), with activities expressed as per gram of glucan. After 48 h of hydrolysis, the hydrolysate was centrifuged at 7,000 rpm for 20 min.

The liquid fraction was subjected to vacuum evaporation in a rotary evaporator (BüCHI R-200, Swiss) at 70 °C and 160 mbar to obtain different concentrations of sugar that could be used for subsequent fermentation (Zhu *et al*. 2013). Samples were taken before and after evaporation to analyze the concentrations of inhibitors and sugars.

Microorganisms and Culture Conditions

The hexose- and pentose-fermenting yeasts used, which were *Saccharomyces cerevisiae* and *Pichia stipitis*, respectively, were provided by the Biochemical Engineering Research Institute of Nanjing Forestry University. *S. cerevisiae* was maintained at 4 °C in a medium that contained 20 g/L glucose, 3 g/L peptone, 5 g/L yeast extract, and 20 g/L agar, and *P. stipitis* was maintained at 4 °C in a medium containing 20 g/L xylose, 3 g/L peptone, 5 g/L yeast extract, and 20 g/L agar.

The inoculation medium of *S. cerevisiae* contained 20 g/L glucose, 5 g/L peptone, and 3 g/L yeast extract at natural pH. The seed culture was prepared by transferring a loop full of cells to 250-mL Erlenmeyer flasks that contained 50 mL of medium and the cells grown at 30 °C on a rotary shaker at 150 rpm for several batches (24 h per batch). Seed culture of *P. stipitis* was performed in 250-mL flasks that contained 50 mL of medium incubated at 30 °C and shaken at 170 rpm. The culture broth contained 30 g/L xylose, 5 g/L peptone, and 3 g/L yeast extract at natural pH. When the optimal level of yeast growth was obtained, as determined by measuring the optical density (OD_{600}), the cells were harvested by centrifugation (3,000 rpm for 10 min), washed, and centrifuged again another two times to remove residual sugar and ethanol. The pelleted cells were inoculated into the fermentation media.

Fermentation

The sequential glucose fermentation was carried out at 30 °C, pH 5.5, and 100 rpm. The culture medium included 0.24 g/L urea, 0.08 g/L $MgSO_4$, 0.08 g/L $ZnCl_2$, and 0.10 g/L $CaCl_2$. The initial inoculum of *S. cerevisiae* was at an OD_{600} of 10. After glucose fermentation, the ethanol generated was distilled, and 0.24 g/L urea, 0.25 g/L MgSO4, 2.5 g/L KH_2PO_4, and 0.25 g/L $CaCl_2$ were added to the xylose fermentation media. The xylose fermentation was carried out at 30 °C, pH 6.0, and 150 rpm with the initial yeast inoculum collected at an OD_{600} of 15.

Co-fermentation by *P. stipitis* was carried out with a cell concentration at an OD_{600} of 15. For the co-culture of *S. cerevisiae* and *P. stipitis*, the initial inoculum consisted of *S. cerevisiae* with an OD_{600} of 5 and *P. stipitis* with an OD_{600} of 10. Both the co-fermentation and the co-culture were performed at 30 °C, pH 6.0, and 150 rpm with the addition of the same nutrients used for xylose fermentation. Samples were collected during fermentation to determine the biomass and quantify sugars, ethanol, and fermentation inhibitors. All experiments were conducted in replicate. Calculations were as follows:

Sugar consumption ratio (%) = consumed glucose and/or xylose (g) / total glucose and/or xylose (g) × 100

Ethanol yield (%) = obtained ethanol (g) / (consumed glucose × 0.51 + consumed xylose × 0.46) (g) × 100

After green liquor pretreatment and subsequent enzymatic hydrolysis, 329.7 g of glucose and 126.3 g of xylose could be produced from 1,000 g of dry corn stover.

Ethanol productivity (g/1,000 dry corn stover) = 329.7 ×glucose utilization ratio ×0.51×ethanol yield +126.3 ×xylose utilization ratio ×0.46 ×ethanol yield

Analytical Methods

The sugars (glucose, xylose, and arabinose), fermentation products (ethanol), and inhibitors (formic acid, acetic acid, levulinic acid, 5-hydroxymethylfurfural, and furfural) were determined by high-performance liquid chromatography with a refractive index (RI) detector (HPLC, Agilent technology 1100 series, Palo Alto, CA). Separations were performed using a Bio-Rad Aminex HPX-87H column (300 mm × 7.8 mm, USA) at 55 °C, using 5 mM sulfuric acid as the mobile phase (0.6 mL/min). Samples were filtered through 0.22-μm syringe filters prior to injection (Tengborg *et al*. 2001).

RESULTS AND DISCUSSION

Fermentable Sugars Production from Corn Stover

The composition of green liquor-pretreated corn stover and enzymatic hydrolysate are summarized in Table 1. As can be seen in the Table, 633.1 g of solid was recovered from 1,000 g of dry corn stover after pretreatment, including 508.2 g of polysaccharides and 106.1 g of lignin, equal to a polysaccharide recovery yield of approximately 80% and lignin removal of 54.5%. The high polysaccharides recovery in the solid indicated that fewer polysaccharides were degraded to monomers or further into fermentation inhibitors, which would serve to increase the overall yield of ethanol (Kim and Lee 2007) and is considered a prerequisite for economical ethanol production. After enzymatic hydrolysis at a substrate loading of 5% (w/v), 26.1 g/L glucose, 9.97 g/L xylose, 1.63 g/L arabinose,

and 0.22 g/L acetic acid were detected in the hydrolysate, equal to a glucan hydrolysis yield of 84.6% and xylan hydrolysis yield of 77.2%. According to these results, 329.7 g of glucose and 126.3 g of xylose could be produced from 1,000 g of dry corn stover subject to green liquor pretreatment and subsequent enzymatic hydrolysis. Few fermentation inhibitors were detected in the enzymatic hydrolysate, as the washing operation following the pretreatment to separate black liquor and the pretreated substrate could remove inhibitors to some extent. The acetic acid (0.22 g/L) probably formed from the further release of acetyl groups on hemicellulose during enzymatic hydrolysis. Additionally, another advantage of green liquor pretreatment is that all fermentable sugars could be recovered in a single step, the enzymatic hydrolysis stage (Jin *et al*. 2010; Wu *et al*. 2010). Green liquor pretreatment has been considered an effective first step for sugar production from corn stover due to the selective removal of lignin, high polysaccharides recovery yield, and high yield of enzymatic hydrolysis after pretreatment (Van Dyk and Pletschke 2012).

Table 1. Composition of Green Liquor-pretreated Corn Stover and Enzymatic Hydrolyzate (Based on One Gram of Dry Corn Stover)

	Solid (g)	Polysaccharides (g)	Glucan (g)	Xylan (g)	Arabinan (g)	Total lignin (g)	Ash (g)
Corn stover before GL pretreatment	1000	636.0	381.4	226.8	27.8	233.4	67.9
Corn stover after GL pretreatment	633.13	508.3	350.7	143.9	13.7	106.1	23.7
Sugars after enzymatic hydrolysis[a]			296.7	111.1	9.8		

[a] The sugars in the enzymatic hydrolyzate were converted to glucan and xylan.

Simultaneous Fermentation of Glucose and Xylose for Ethanol Production

The enzymatic hydrolyzate was condensed to increase sugar concentration and subsequent ethanol concentration in the fermentation liquor, which was conducted to reduce the cost of subsequent ethanol distillation (Zhu *et al*. 2013). The concentrated liquor contained 74.6 g/L glucose and 28.9 g/L xylose, wherein little inhibitor was detected after the hydrolyzate was condensed to one-third of its original volume. The co-fermentation of glucose and xylose by *P. stipitis* is shown in Fig. 1. Unlike *S. cerevisiae*, *P. stipitis* was able to use all of the glucose and most of the xylose in the hydrolyzate, but at different rates of consumption. As illustrated, 74.6 g/L glucose was completely consumed at 24 h, equal to a utilization rate of 3.1 g/(L·h). After the exhaustion of glucose, xylose began to be used. However, only 19.4 g/L of xylose was consumed from 12 h to 72 h, equal to a utilization rate of 0.32 g/(L·h). Glucose repression on xylose utilization was obvious, for that in the presence of glucose, the expression levels of both xylose transporters and key enzymes for xylose metabolism were reduced (Görke and Stülke 2008; Ren *et al*. 2009). The highest ethanol concentration of 34.8 g/L was obtained at 48 h. After 48 h, xylose was further consumed, whereas the ethanol concentration showed a slight decrease. This may have occurred because both ethanol and xylose can be utilized by *P. stipitis* as carbon sources, facilitating a high cell biomass production (Schirmer-Michel *et al*. 2009; da Cunha-Pereira *et al*. 2011). After 72 h of

fermentation, *P. stipitis* had consumed 100% of glucose and 69.5% of xylose, obtaining 32.9 g/L ethanol, equal to an ethanol yield of 69.6%, and the ethanol productivity of co-fermentation of glucose and xylose by *P. stipitis* was estimated to be 145.1 g/ 1,000 g of dry corn stover.

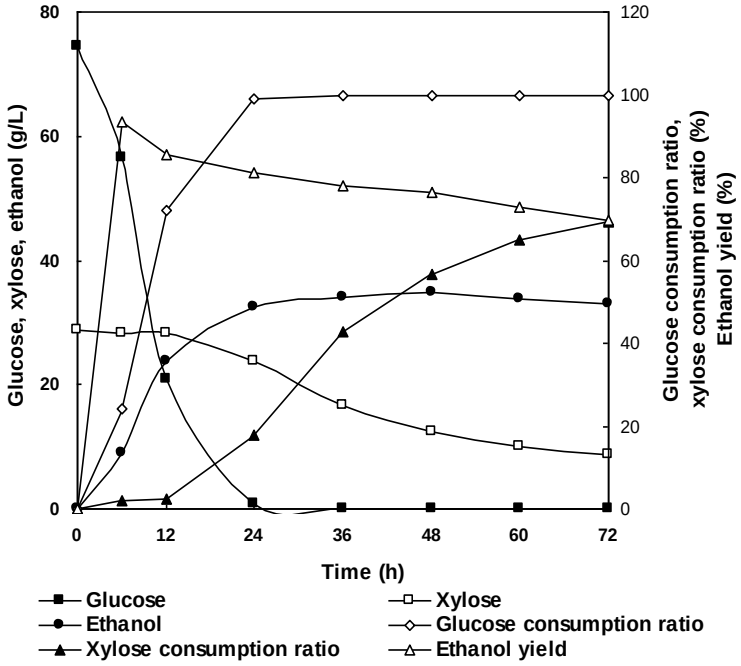

Fig. 1. Co-fermentation of glucose and xylose by *P. stipitis* with OD_{600} 15, at 30 °C, pH 6.0, and 150 rpm

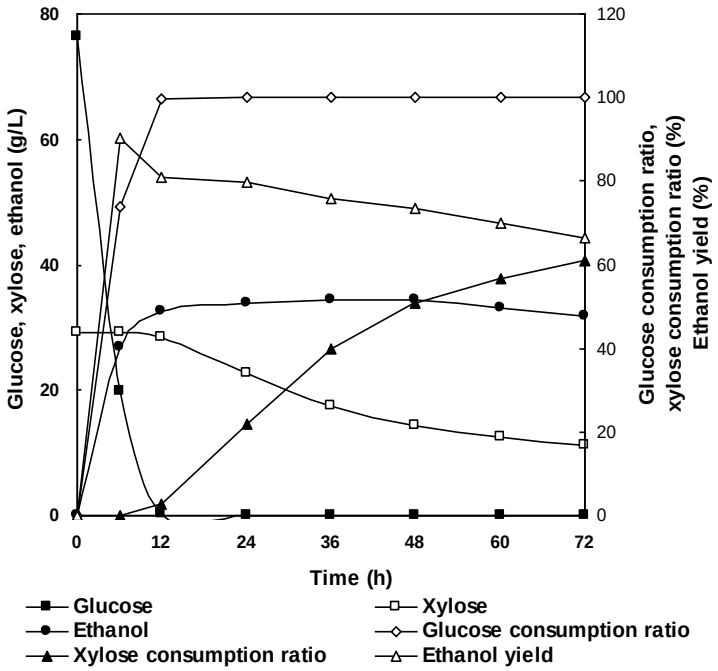

Fig. 2. Co-culture of *S. cerevisiae* and *P. stipitis* with a cell concentration of OD_{600} 15 (*S. cerevisiae* to *P. stipitis* ratio of 1:2), at 30 °C, pH 6.0, and 150 rpm

The time course of the co-culture of *S. cerevisiae* and *P. stipitis* in the condensed liquor is shown in Fig. 2. The condensed liquor contained 76.3 g/L of glucose and 29.1 g/L of xylose at 0 h. During the first phase of fermentation (initial 12 h), glucose was consumed rapidly by *S. cerevisiae* and *P. stipitis* and the rate of glucose consumption (6.4 g/(L·h)) was much higher than that in the presence of *P. stipitis* alone. However, 17.0 g/L xylose was consumed from 12 h to 72 h, equal to a consumption rate of 0.28 g/(L·h). After 72 h of co-culture, the glucose consumption ratio, xylose consumption ratio and ethanol yield were 100%, 61.2%, and 66.5%, respectively, while the ethanol productivity of co-culture of *S. cerevisiae* and *P. stipitis* was estimated to be 135.4 g/1,000 g of dry corn stover. Compared with co-fermentation by *P. stipitis* alone, the ethanol yield decreased, probably because the oxygen uptake required for xylose fermentation facilitated a high cell biomass production of *S. cerevisiae* by channeling glucose into the cell biomass rather than ethanol (Zhu *et al*. 2013).

The preferential use of glucose over xylose was observed in both co-fermentation and co-culture, which means a relatively high yield of ethanol could be produced before xylose utilization, which might have a negative influence on the xylose utilization. Thus, the ethanol tolerance of the xylose-fermenting strain was the key factor for co-fermentation and co-culture, which also limited the initial sugar concentration of these two strategies. Table 2 illustrates the effect of additional ethanol on xylose fermentation. When the xylose medium (30 g/L xylose) was fermented by *P. stipitis* with no additional ethanol, 99.2% of xylose was converted to 12.1 g/L ethanol at 24 h, equal to an ethanol yield of 88.3%. The xylose consumption ratio decreased slightly with the addition of 30 g/L ethanol, but dropped to 11.9% when the amount of added ethanol further increased to 60 g/L. However, ethanol addition had a greater influence on the ethanol yield, as the ethanol yield decreased rapidly with increasing amounts of ethanol. With an ethanol addition of 60 g/L, no additional ethanol was produced. According to a previous report, ethanol can damage cell membranes, resulting in altered membrane organization and permeability. Additionally, transmembrane proton flow is destroyed, leading to cellular death upon extreme cytoplasmic acidification (Meyrial *et al*. 1995). The results indicated that *P. stipitis* may have an ethanol tolerance of approximately 30 g/L, consistent with the results reported by Laplace (1991), and was completely depressed at an ethanol concentration of 60 g/L. This may restrict the process to the use of only low initial sugar concentrations, which is not compatible with distillation requirements (Galbe *et al*. 2007).

Table 2. Effect of Ethanol Addition on Xylose Fermentation by *P. stipitis*[a]

Ethanol addition (g/L)	Fermentation Time (h)	Xylose consumption rate (g/(L·h))	Ethanol produced (g/L)	Xylose consumption ratio (%)	Ethanol yield (%)
0	24	1.3	12.1	99.2	88.3
15	24	1.3	11.0	99.6	80.2
30	36	0.8	9.4	97.7	69.6
45	48	0.3	2.8	39.3	52.2
60	60	0.06	0	11.9	0

[a]Initial xylose concentration of 30 g/L, OD_{600} 15, at 30 °C, pH 6.0 and 150 rpm

Sequential Fermentation of Glucose and Xylose for Ethanol Production

S. cerevisiae is more tolerant of ethanol, sugars, and inhibitors than is *P. stipitis* (Meyrial *et al*. 1995; Guo *et al*. 2008). As a result, a sequential fermentation strategy is proposed in which glucose fermentation by *S. cerevisiae* and ethanol removal must be completed prior to xylose fermentation by *P. stipitis* to avoid glucose inhibition of xylose uptake and ethanol inhibition of xylose fermentation.

In this strategy, the initial sugar concentration was not limited by the ethanol tolerance of *P. stipitis*, so that the enzymatic hydrolyzate was condensed to 1/4, 1/5, 1/6, 1/7, and 1/8 of the original volume (condensed sugar solutions 1 through 5, illustrated in Table 3), to evaluate the sugar tolerance of this strategy. Because *S. cerevisiae* was previously reported to have an ethanol tolerance in the range of 70 to 110 g/L, a sugar solution containing the highest sugar content (195.3 g/L glucose and 75.8 g/L xylose) was designed.

Table 3. Concentrated Sugar Solutions Used in this Study

Concentrated sugar solution	Glucose concentration (g/L)	Xylose concentration (g/L)	Acetic acid concentration (g/L)
1	94.8	36.6	0.9
2	119.9	45.9	1.1
3	144.2	57.7	1.3
4	171.7	65.9	1.3
5	195.3	75.8	1.4

Table 4 lists the results of glucose fermentation in sequential fermentation with different initial sugar concentrations. With the depletion of available glucose, 46.2, 57.4, 67.7, 78.4, and 87.8 g/L ethanol was obtained, 95.6, 93.8, 93.2, 91.3, and 89.7% of the theoretical ethanol yield, respectively. A high yield of ethanol was obtained, as *S. cerevisiae* is the most commonly used microorganism in industrial processes to ensure high-product yields and has a longstanding use (van Zyl *et al*. 2007), despite its inability to ferment xylose to ethanol (Hahn-Hägerdal *et al*. 2007). The biomass (OD_{600}) of the cultures remained almost the same throughout the fermentation process, implying that little glucose was used by yeasts for cell growth, which also contributed to the high ethanol yield with glucose fermentation. With the increase in the total sugars concentration, the fermentation time increased from 8 h to 40 h, while the average glucose consumption rate decreased from 11.8 g/(L·h) (solution 1) to 4.8 g/(L·h) (solution 5).

This reduction in average glucose consumption rate might be ascribed to higher osmotic pressure or the small influence of acetic acid, whereas the glucose consumption ratio and ethanol yield were only slightly influenced. However, the existence of acetic acid might affect the subsequent xylose fermentation, which is more susceptible to inhibitors (Guo *et al*. 2008).

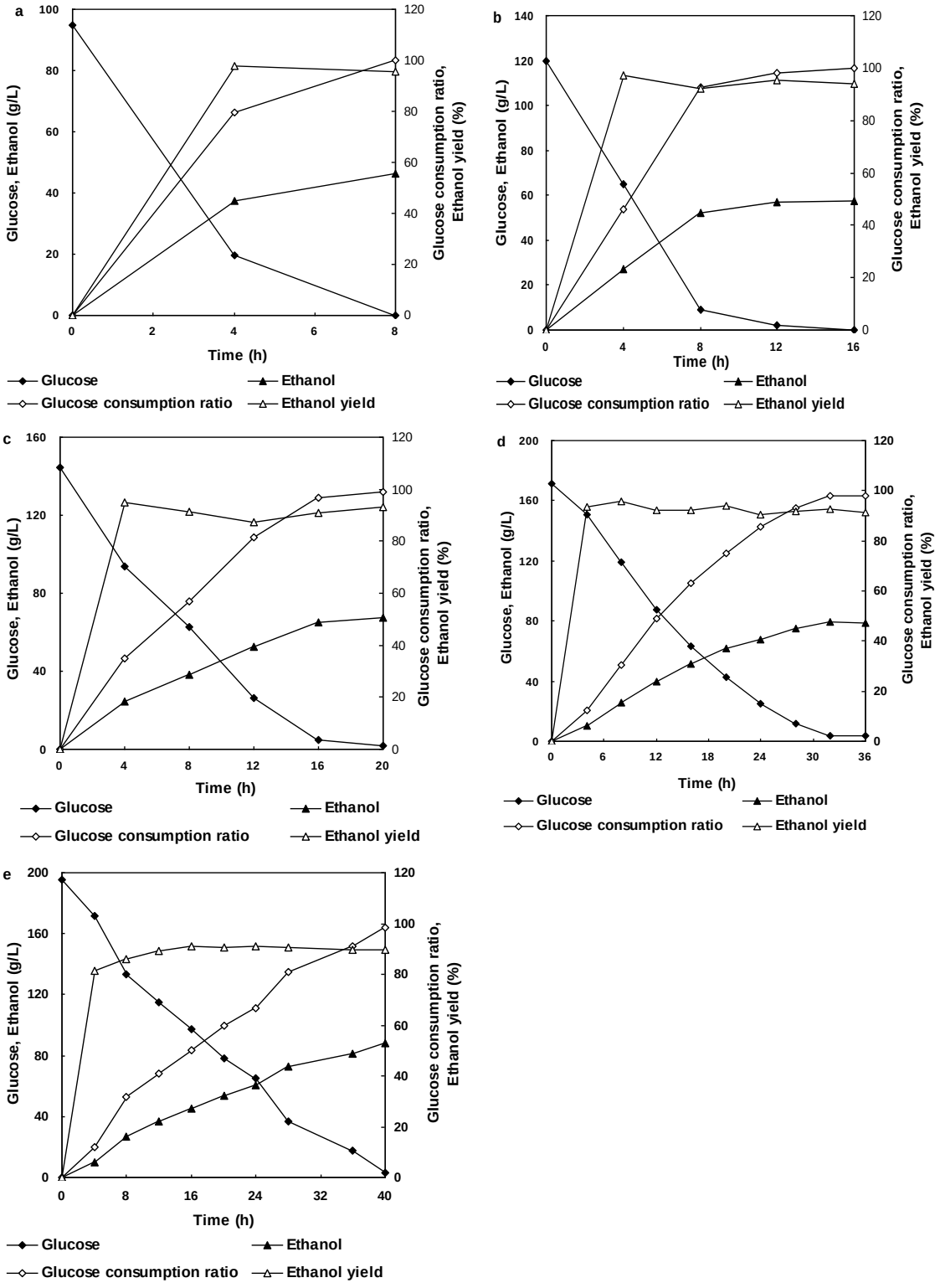

Fig. 3. Time course of glucose fermentation by *S. cerevisiae* with different initial sugar concentration at cell concentration of OD_{600} 10, 30 °C, pH 5.5, and 100 rpm. (a, b, c, d, and e were the fermentation course of sugar solution 1 to 5, respectively)

Table 4. Glucose Fermentation by *S. cerevisiae* in Sequential Fermentation Process[a]

Sugar solution	Fermentation Time (h)	Initial glucose (g/L)	Glucose consumption rate (g/(L·h))	Ethanol produced (g/L)	Glucose consumption ratio (%)	Ethanol yield (%)	Ethanol productivity (g/1,000 dry corn stover)
1	8	94.8	11.8	46.2	100	95.6	160.7
2	16	119.9	7.5	57.4	100	93.8	157.7
3	20	144.2	7.2	67.7	98.74	93.2	156.6
4	36	171.7	4.8	78.4	98.07	91.3	153.5
5	40	195.3	4.9	87.8	98.28	89.7	150.8

[a]Initial OD_{600} 10, at 30 °C, pH 5.5, and 100 rpm

Table 5 shows the results of xylose fermentation in the sequential fermentation process after ethanol distillation. As can be seen, 35.4, 44.8, 57.1, 64.8, and 74.2 g/L xylose were converted to 13.4, 16.2, 18.4, 20.6, and 22.8 g/L ethanol, respectively, equal to ethanol yields of 86.1, 81.6, 76.8, 75.7, and 72.2%, respectively. All parameters of xylose fermentation were lower than those for glucose fermentation, probably due to the limited capacity of *P. stipitis*. Overall, the average xylose consumption rate was about 1.0 g/(L·h), which was much higher than that of co-fermentation (0.32 g/(L·h)) and co-culture (0.28 g/(L·h)), probably because of the removal of glucose inhibition on xylose uptake and ethanol inhibition on *P. stipitis*.

After xylose fermentation, the ethanol yield of total sequential fermentation of sugar solutions 1 through 5 was 210.7, 205.1, 201.2, 197.5, and 192.7 g/ 1,000 g of dry corn stover, respectively. All of these results were much higher than that of co-fermentation (157.4 g) and co-culture (150.3 g). From the view of ethanol production, sequential fermentation of glucose and xylose was considered the most suitable strategy. During sequential fermentation, the separate utilization of glucose and xylose ensured each fermentation stage used suitable microorganisms, thereby permitting high ethanol yields during both fermentation steps.

Table 5. Xylose Fermentation by *P. stipitis* in Sequential Fermentation Process [a]

Sugar solution	Fermen-tation Time (h)	Initial xylose (g/L)	Xylose consumption rate (g/(L·h))	Ethanol produced (g/L)	Xylose consumption ratio (%)	Ethanol yield (%)	Ethanol productivity (g/1,000 dry corn stover)
1	30	35.4	1.2	13.4	96.0	86.1	50.0
2	36	44.8	1.2	16.2	96.6	81.6	47.4
3	48	57.1	1.2	18.4	91.1	76.8	44.6
4	60	64.8	1.1	20.6	91.4	75.7	44.0
5	72	74.2	1.0	22.8	92.3	72.2	41.9

[a]Initial OD_{600} 15, at 30 °C, pH 6.0, and 150 rpm

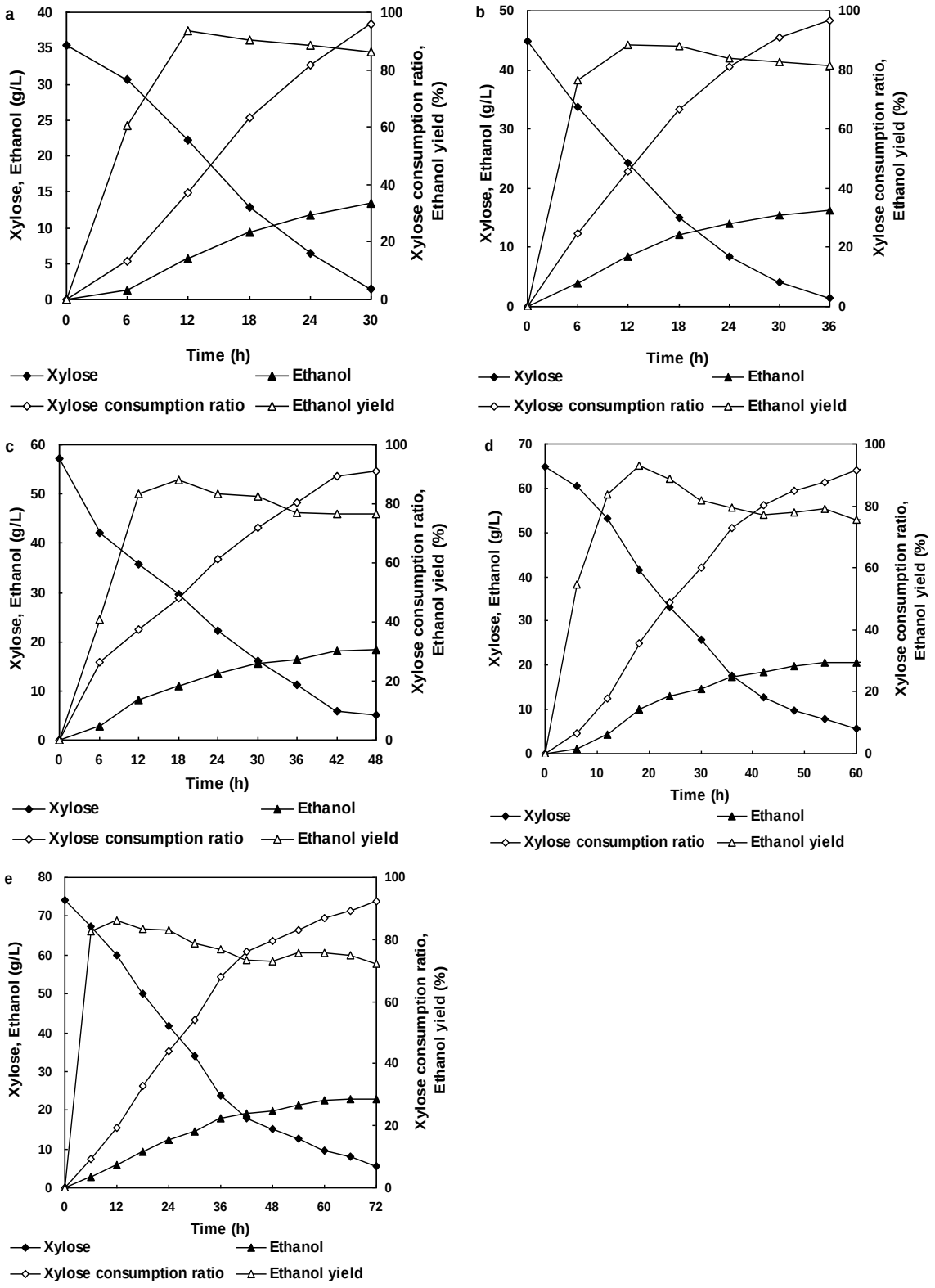

Fig. 4. Time course of xylose fermentation by *P. stipitis* with different initial sugar concentration at cell concentration of OD_{600} 15, 30 °C, pH 6.0, and 150 rpm. (a, b, c, d, and e were the fermentation course of sugar solution 1 to 5, respectively)

The toxicity of acetic acid might hinder xylose fermentation to some extent; the effect of acetic acid on xylose fermentation is shown in Table 6. As the acetic acid addition increased to 2.0 g/L, the ethanol yield decreased from 88.3% to 79.6% and the xylose consumption ratio changed slightly. The average xylose consumption rate was influenced only at the acetic acid concentration of 2.0 g/L. This may occur because a higher pH value of 6.0 was applied in the xylose fermentation. At lower pH values, acetic acid in its undissociated form is able to penetrate the bacteria cell walls and acidify the cytoplasm, disrupting the protein gradient across the cell membrane and interfering with cellular processes (Keating *et al*. 2006). However, the toxicity of acetate is much lower than that of acetic acid (Walton *et al*. 2010).

Table 6. Effect of Acetic Acid Addition on Xylose Fermentation by *P. stipitis*[a]

Acetic acid addition (g/L)	Fermentation Time (h)	Xylose consumption rate (g/(L·h))	Ethanol produced (g/L)	Xylose consumption ratio (%)	Ethanol yield (%)
0	24	1.2	12.1	99.2	88.3
0.5	24	1.2	11.9	99.1	87.3
1.0	24	1.2	11.7	98.8	85.5
1.5	30	1.0	10.9	98.8	79.6

[a]Initial xylose concentration of 30 g/L, OD_{600} 15, at 30 °C, pH 6.0, and 150 rpm

CONCLUSIONS

1. Green liquor pretreatment, followed by enzymatic hydrolysis, enabled highly efficient conversion of polysaccharides to monosaccharides. After enzymatic hydrolysis, 329.7 g of glucose and 126.3 g of xylose were obtained from 1,000 g of dry corn stover.
2. Sequential fermentation was the most suitable liquid-fermentation strategy for green liquor-pretreated corn stover, as the highest ethanol production of 210.7 g was obtained from 1,000 g of dry corn stover.
3. During sequential fermentation, the separate utilization of glucose and xylose ensured that each fermentation process occurred using suitable microorganisms, thereby permitting high ethanol yields during both fermentation steps.
4. Ethanol has a negative influence on xylose fermentation, and the ethanol tolerance of *P. stipitis* was about 30 g/L. This was the reason for the relatively lower ethanol production from co-fermentation (145.1 g) and co-culture (135.4 g).

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