Bioresource Technology 102 (2011) 7451–7456

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Scale-up study of oxalic acid pretreatment of agricultural lignocellulosic biomass for the production of bioethanol

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ARTICLE INFO

Article history:
Received 14 March 2011
Received in revised form 6 May 2011
Accepted 7 May 2011
Available online 14 May 2011

Keywords:
Scale-up
Oxalic acid pretreatment
Hydrolysate
Ethanol production

ABSTRACT

Building on our laboratory-scale optimization, oxalic acid was used to pretreat corncobs on the pilot-scale. The hydrolysate obtained after washing the pretreated biomass contained 32.55 g/l of xylose, 2.74 g/l of glucose and low concentrations of inhibitors. Ethanol production, using *Scheffersomyces stipitis*, from this hydrolysate was 10.3 g/l, which approached the predicted value of 11.9 g/l. Diafiltration using a membrane system effectively reduced acetic acid in the hydrolysate, which increased the fermentation rate. The hemicellulose content of the recovered solids decreased from 27.86% before pretreatment to only 6.76% after pretreatment. Most of the cellulose remained in the pretreated biomass. The highest ethanol production after simultaneous saccharification and fermentation (SSF) of washed biomass with *S. stipitis* was 21.1 g/l.

1. Introduction

With increasing concerns about global warning and depletion of petroleum, development of innovative, sustainable technologies for the production of environmentally friendly fuels is critical for sustained economic development. Lignocellulosic biomass plays a large role in the production of sustainable energy because it is renewable, abundant, and often locally available. Moreover, as CO_2 levels continue to increase along with increasing moisture in the atmosphere, it will become more important to nurture the growth of plants to remove CO_2 from the atmosphere and counter its accumulation. If efficient technologies can be developed to produce renewable fuels from agricultural residues rather than from food grains, sugars and oils, a sustainable biofuel economy could be established alongside agricultural food production (FitzPatrick et al., 2010; Ghosh and Singh, 1993).

The main components of agricultural residues are cellulose, hemicelluloses and lignin. Agricultural residues are easier than wood to use as feedstocks for biofuels due to their lower lignin and higher hemicellulose contents (Barl et al., 1991). Both lignin and cellulose are more recalcitrant to enzymatic or chemical conversion to biofuel precursors. Hemicellulose is much more readily recovered, but presents challenges in use do to simultaneous release of acetic and ferulic acid components along with acid-soluble lignin moieties, all of which can inhibit fermentation and catalytic conversion to more useful products (Oliva et al., 2006).

Corncobs are an important agricultural residue, which are a co-product of corn (maize) production. Traditionally, they have been used as animal bedding or returned to the field (Inglett, 1970). In the USA, about 50 million metric tons of corncobs are available annually based on the 12.1 billion tons of corn production in 2008 (USDA, 2009). Therefore, corncobs could be a significant source of lignocellulosic biomass for biofuel production, if effective and economical collection strategies are developed. Some researchers have studied ethanol production from corncobs because it is a co-product of food production, and thus, does not reduce food supplies (Chen et al., 2010; Lee et al., 2009).

Dicarboxylic acids such as oxalic acid have been suggested as alternatives to sulfuric acid for pretreatment (Kootstra et al., 2009; Lee et al., 2009; Lu and Mosier, 2007) even though they more expensive on a weight basis. This is because dicarboxylic acids exhibit a higher catalytic efficiency for hydrolysis than sulfuric acid when applied under the same severity conditions (Lee and Jeffries, 2011). The severity level is critical because acid catalysts degrade hemicellulosic sugars to furfural and eventually to formic acid if the treatment is carried too far. Ideally, it is better to recover hemicellulosic sugars as oligosaccharides rather than monosaccharides in order to reduce such degradation. One disadvantage of using sulfuric acid in the pretreatment is the generation of large amounts

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doi:10.1016/j.biortech.2011.05.022

of gypsum, which can negatively affect the downstream process. Furthermore, oxalic acid does not inhibit glycolysis and does not produce noxious odors during pretreatment (Mosier et al., 2001). However, these studies have been performed only in the laboratory level, whereas for practical application, the pretreatment of corncobs with oxalic acid for the production of ethanol must be scaled-up.

In our previous work, we determined the optimal pretreatment condition for the production of ethanol based on laboratory-scale statistical analysis (Lee et al., 2009). In this study, therefore, we scaled-up the optimal pretreatment condition and compared the experimental results with those of the laboratory-scale pretreatment.

2. Methods

2.1. Biomass

Corncobs were provided by Pestell Pet Products Co. (New Hamburg, Canada) as the raw material. Standardized corncob pellets were air dried until the moisture content was reduced to 5% for further processing.

2.2. Reactor setup and operation for large-scale pretreatment

Optimal pretreatment condition using oxalic acid and corncob for bioethanol production was chosen by factorial design in a previous study (Lee et al., 2009). Corncob pellets (80.6 kg dry matter) were impregnated under vacuum with a solution of 30 g/l of oxalic acid in a 500-l reactor for 20 min at room temperature. The solid:liquid ratio during impregnation was 1:6. The excess oxalic acid solution after impregnation was drained out of the system and stored for analysis. The pellets impregnated with oxalic acid were then rapidly heated to 168 °C and held for 26 min. The pretreatment process is shown in Fig. 1. The pretreated pellets were then washed with approximately 300 l of water to extract the sugars. The resulting hemicellulose hydrolysates were stored at 4 °C. The solid residue was used as a substrate for simultaneous saccharification and fermentation (SSF), as described herein.

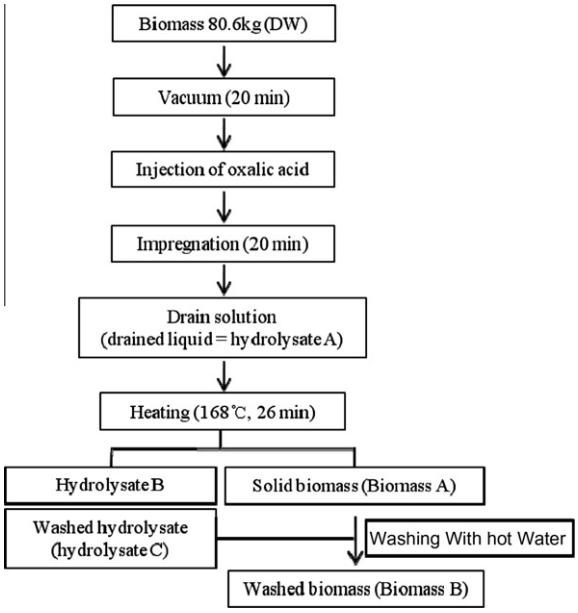

Fig. 1. Scheme of large scale pretreatment by corncob and oxalic acid (hydrolysate A: the drained oxalic acid solution before pretreatment, hydrolysate B: the drained liquid fraction after heating for 26 min, hydrolysate C: the liquid fraction obtained after washing the pretreated biomass).

2.3. Analysis of hydrolysate

The concentrations of D-glucose, D-xylose, L-arabinose and acetic acid in the hydrolysate were determined by high performance liquid chromatography (HPLC; Gilson, Middleton, USA). An Aminex HPX-87H column (300 × 7.8 mm) was used 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. 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. For quantitative analysis, 5-hydroxymethyl furfural (5-HMF) and furfural in the hydrolysates were examined through an HP series II 1090 HPLC using C18 (2) 5 μ 250 × 4.6 mm column (Phenomenex, Torrance, USA) at a flow rate of 0.8 ml/min (isocratic). The mobile phase was composed of 1:8 of acetonitrile: H_2O and 1% acetic acid. The amount of total phenols in hydrolysate was measured by Scalbert's method (1989) and inductively coupled plasma (ICP) analysis of metal ions was carried out using ULTIMA (Horiba Jobin Yvon, France).

2.4. Chemical analysis of pretreated corncob

The chemical composition of the pretreated corncob was determined by the TAPPI method (T 222-om-88). Samples were milled to pass a 1 mm screen and vacuum dried at 45 °C. Pretreated corncob was hydrolyzed with 3 ml (w/w) H_2SO_4 for 1 h at 30 °C. Hydrolysates were diluted to 4% (w/w) H_2SO_4 with distilled water and then heated at 120 °C for 1 h. After hydrolysis, 2 ml supernatant samples were centrifuged and filtered through a 0.45 μl filter, and the solution was analyzed for monosaccharides by HPLC, as described in section 2.3.

2.5. Removal of acetic acid in hydrolysate by reverse osmosis

The in-house built reverse osmosis unit used an AF20 spiral-wound membrane supplied by PCI Membranes Inc. 1.2 m^2 active surface area. The unit was designed for a maximum operating pressure of 800 PSIG, and a feed flow rate of 5 l/min. Typical permeate flow rates were 8–12 l/h.

2.6. Hydrolysate fermentation

Scheffersomyces (Pichia) stipitis CBS 6054 was used for fermentation. Yeast cells were grown in 1000 ml Erlenmeyer flasks containing 400 ml of yeast peptone dextrose (YPD; 10 g/l yeast extract, 10 g/l peptone, and 20 g/l glucose) in a shaking incubator for 24 h, after which the cell cultures were harvested and washed with sterile deionized water. The hydrolysate was adjusted to pH 6.0 with calcium hydroxide and filtered through a sterilized 0.45 μl filter to remove any remaining calcium hydroxide. Washed cells at a dry cell weight of 2.0 g/l were transferred to hydrolysate with 5 g/l yeast, 5 g/l urea, 0.5 g/l $MgSO_4$ $7H_2O$ and 1 g/l KH_2PO_4. Fermentation was performed at 30 °C in an orbital shaker at 150 rpm. Samples were taken at 24, 48, 72, 96, 120 h and the amounts of monosaccharides remaining and ethanol produced were analyzed by HPLC (as described in Section 2.3).

2.7. Simultaneous saccharification and fermentation (SSF) of pretreated corncob

Five grams of pretreated corncob based on dry weight was transferred to a 125 ml Erlenmeyer flask containing 50 ml of

fermentation medium, which consisted of 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 in 50 mM sodium citrate buffer. For saccharification, cellulase (500 CMC U/g) and β-glucosidase (80 pNPG U/g) of Accellerase 1000 from Genencor (Danisco, Rochester, NY) were added to the fermentation medium. SSF was performed at 30 °C in an orbital shaker at 150 rpm. Samples were taken at 24, 48, 72 h, and the amounts of remaining monosaccharides, acetic acid and ethanol produced were analyzed by HPLC (as described in Section 2.4).

3. Results and discussion

3.1. Chemical composition of hydrolysate and pretreated biomass

The compositional analysis of hydrolysate is shown in Table 1. Three types of hydrolysate were obtained during the pretreatment process. A mass balance is shown in Fig. 2. Hydrolysate A had a high concentration of oxalic acid as it was the drained pretreatment solution. Approximately 15% of the oxalic acid was impregnated in the biomass during the vacuum step. It also contained a small amount of glucose and xylose released from the biomass. Hydrolysate B contained a considerable amount of sugar released from hemicelluloses as it was the drained liquid fraction after heating. The majority of sugar was xylose at a concentration of 49.8 g/l. This result is very similar to previous (Lee et al., 2009). However, hydrolysate B had a higher concentration of acetic acid and furfural than other hydrolysates (A and C). Furfural and 5-HMF were generated from the thermo-conversion of pentose and hexose, respectively. Acetic, lactic, levulinic, malonic and formic acids are the by-products of acid pretreatment released from hemicellulose (Cara et al., 2008; Nichols et al., 2008). Delgenes et al. (1996) suggested that more than 5 g/l acetic acid inhibit fermentation because it is capable of penetrating the microorganism cell wall and hindering the cell activity by acidifying the cytoplasm and disrupting the protein gradient across the cell membrane (Takahashi et al., 1999). Therefore, the removal of acetic acid in hydrolysate B is required before ethanol fermentation. Hydrolysate C was the liquid fraction obtained from washing the pretreated biomass after pretreatment. It consisted mostly of xylose because it was extracted from adsorbed monosaccharide on the surface of pretreated biomass during the washing step. The concentration of fermentable sugars and inhibitors was lower than that of hydrolysate B.

Due to the strong chelation properties of oxalic acid, metal ions are dissolved from the reactor surface during pretreatment. Furthermore, the corncobs and water can provide calcium and magnesium. The highest concentration metal components were Mg, Fe, Cr, Ca and Ni, followed by Zn, Cu and Al at less than 1 g/l. The concentration of heavy metals such as Ni and Cr above a certain level dissolved in the fermentation media might have affected the physiology of the microorganisms and impede the cell activity. Ni at 10 mg/l was reported to inhibit the ethanol production and hence decrease the specific growth rate of the cell, because nickel ions bind to the cell surface and inhibit the uptake of D-glucose in

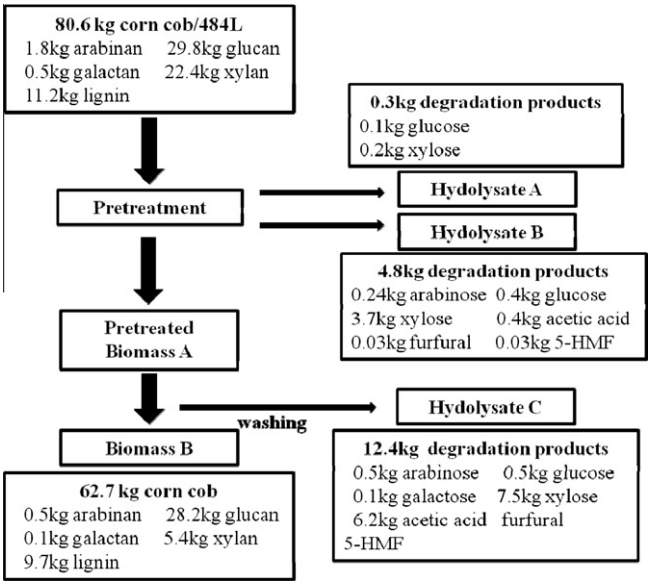

Fig. 2. Preliminary mass balance of a corncob pretreatment.

yeasts (Kleinzeller and Kotyk, 1967). It was found that ions contents of over 500 mg/L of Fe, 100 mg/L of Cr and 4 mg/l of Cu would act as inhibitors for ethanol production by *Pachysolen tannophilus* (Kleinzeller and Kotyk, 1967; Watson et al., 1984). The concentrations of metal ions are shown in Table 2. The concentrations of Cr, Fe and Ni induced from the pretreatment reactor were the highest in hydrolysate B among the three types of hydrolysate. Otherwise, Mg from the biomass or water was highest in hydrolysate A. Especially, the concentration of heavy metals was the lowest in hydrolysate C.

The compositions of the corncob before and after pretreatment by oxalic acid are presented in Table. 3 The raw material contained 13.9% lignin and 67.9% carbohydrate, which agreed with the report of Barl et al. (1991). The hemicellulose content of the pretreated biomass was dramatically decreased compared to the raw material. On the other hand, the rate of cellulose degradation was significantly different from that of hemicellulose, indicating that the cellulose was not easily degraded to fermentable sugar by oxalic acid catalysts under the condition employed (Kenealy et al., 2007; Lee et al., 2009; Scordia et al., 2010). The lignin concentration was decreased from 13.92% to 12.04%, implying that the pretreated material underwent partial delignification during the pretreatment. These results revealed the suitability of the pretreated biomass for SSF due to the high carbohydrate content.

3.2. Improving fermentability by acetic acid removal

As the generation of inhibitors is unavoidable during acid pretreatment, the use of hydrolysates as a fermentation resource necessitates additional processing such as, lime treatment,

Table 1
Sugar and by-products during oxalic acid pretreatment of corncob.

	Glucose (g/l)	Xylose (g/l)	Arabinose (g/l)	Acetic acid (g/l)	5-HMF (g/l)	Furfural (g/l)
Hydrolysate A[a]	0.32	0.74	0	0	0	0.01
Hydrolysate B[b]	5.39	49.78	3.28	5.21	0.34	0.45
Hydrolysate C[c]	2.74	32.55	1.71	3.83	0.19	0.40

[a] The drained oxalic acid solution before pretreatment.
[b] The drained liquid fraction after heating.
[c] The liquid fraction obtained after washing the pretreated biomass.

Table 2
Metal ion concentration in aqueous hydrolysate during pretreatment.

	Al (mg/l)	Ca (mg/l)	Cr (mg/l)	Cu (mg/l)	Fe (mg/l)	Mg (mg/l)	Ni (mg/l)	Zn (mg/l)
Hydrolysate A [a]	0.16	2.83	0.20	0.62	12.52	67.19	0.40	0.45
Hydrolysate B [b]	0.41	5.10	22.34	0.31	44.21	54.61	5.65	0.84
Hydrolysate C [c]	0.26	4.72	4.37	0.21	13.24	20.48	1.83	0.78

[a] The drained oxalic acid solution before pretreatment.
[b] The drained liquid fraction after heating.
[c] The liquid fraction obtained after washing the pretreated biomass.

Table 3
Composition of corncob components before and after pretreatment (based on dry weight).

	Glucan (%)	Xylan (%)	Galactan (%)	Arabinan (%)	Lignin (%)
Raw material	37.07	27.86	0.61	2.19	13.92
Pretreated biomass	35.09	6.76	0.12	0.55	12.04

membrane exchange, organic solvent extraction, and addition of adsorbents (Olsson and Hahn-Hagerdal, 1996). The ethanol production and consumption of fermentable sugars of *S. stipitis* using hydrolysate C are shown in Fig. 3. The maximum ethanol concentration attained after fermentation with an initial fermentable sugar level of 35.29 g/l was 10.3 g/l after 120 h. This low initial ethanol production rate was caused by the effect of the inhibitors that remained in the hydrolysate in reducing the growth and respiration of yeast (Larsson et al., 1999; Palmqvist and Hahn-Hagerdal, 2000). The xylose concentration was rapidly decreased during the ethanol production until 120 h. The highest ethanol production yield was 0.31, corresponding to an ethanol volumetric productivity of 0.09 g/l h.

In order to improve ethanol production, hydrolysate C was treated by reverse osmosis with membrane before fermentation. Hydrolysates with the same fermentable sugars condition but different concentrations of inhibitors were prepared by diafiltration. The hydrolysate was diluted by adding an equivalent volume of deionized water, and then it was returned to approximately its original volume by membrane separation. The permeate was discarded, and the retentate was labeled as hydrolysate 1. This process of dilution followed by membrane separation was repeated twice more to get hydrolysates 2 and 3. As shown in Table 4, acetic acid and furfural were removed by reverse osmosis, while the concentrations of fermentable sugar and phenolic compounds were not affected by the reverse osmosis treatment. The 5-HMF concentration was slightly decreased depending on the reverse osmosis treatment.

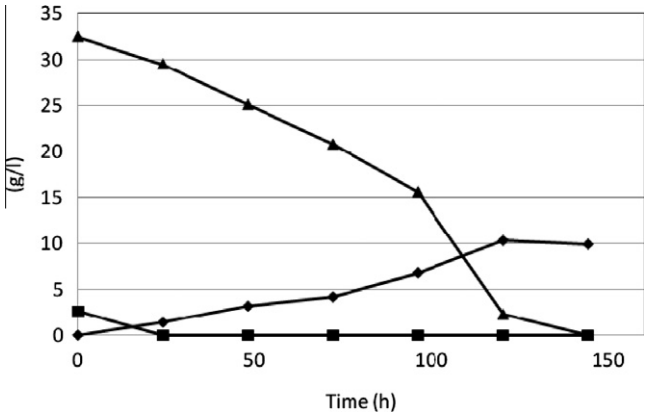

Fig. 3. Time course for ethanol and fermentable sugars concentration during fermentation by *S. stipitis* and hydrolysate C (♦: ethanol, ■: glucose, ▲: xylose).

Four hydrolysates containing almost the same initial fermentable sugar concentration were used for ethanol fermentation, and the results of the ethanol production are shown in Fig. 4. Ethanol production by *S. stipitis* was clearly inhibited by the concentration of the inhibitors present in the hydrolysate. After 48 h of fermentation, the maximum ethanol production was 8.8 g/l when the hydrolysate containing 30.5 g/l of total fermentable sugar and 1.2 g/l of acetic acid was used for fermentation. However, 3.2 g/l of ethanol was obtained after the same time with the untreated hydrolysate (original). Hydrolysate with a low concentration of acetic acid presented a high ethanol volumetric productivity of 0.18 g/l h compared with the other hydrolysates.

3.3. Simultaneous saccharification and fermentation (SSF) of pretreated biomass

The ethanol production by washed biomass (Biomass B) after pretreatment was shown in Fig. 5. Biomass A (unwashed biomass) could not be used to produce ethanol by SSF because high concentration of inhibitors remained on surface of pretreated biomass. The time course of glucose and xylose in Fig. 5 presents the amount of sugars following enzymatic saccharification at 30 °C and 150 rpm which was the same with that of SSF. The ethanol yield was calculated based on the total sugar produced by enzymatic hydrolysis. The glucose production increased with increasing hydrolysis time, but the xylose concentration did not change significantly over time due to the low xylan concentration of 6.76% in the pretreated biomass and the ability of the enzyme used in SSF to selectively react on cellulose. The SSF process was completed after 72 h, at which time the concentrations of glucose and xylose were close to zero (data not shown). The highest ethanol production was 21.1 g/l after 72 h, corresponding to an ethanol yield of 0.72. At that time, the 24.6 g/l of glucose and 4.8 g/l xylose were produced by enzymatic hydrolysis using the same condition with SSF without yeast. The total sugar of 29.4 g/l equated to an ethanol production of 15 g/l. However, during SSF with *S. stipitis*, the experimental ethanol yield was higher than the theoretical value. Lee et al. (2009) has previously explained that β-glucosidase was secreted from *S. stipitis* during SSF, which effectively accelerated the ethanol production.

The expected ethanol production was 21.7 g/l under the same laboratory-scale condition according to the following equation (Lee et al., 2009). The large-scale ethanol production of 21.1 g/l was a similar result to that of the laboratory-scale data.

$$Y = 16.81125 + 2.22702X_2 + 1.63695X_3 - 0.93950X_2X_3$$

where *Y*, *X*2 and *X*3 are the ethanol production, oxalic acid concentration during the vacuum impregnation of corncob, and reaction time, respectively.

Table 4
Fermentable sugar and inhibitors concentration contained in hydrolysate C[a] by reverse osmosis treatment.

Hyrolysate type	Glucose (g/l)	Xylose (g/l)	Acetic acid (g/l)	5-HMF (g/l)	Furfural (g/l)	Total phenolic compound (g/l)
Original	3.9	29.4	3.8	0.19	0.40	2.38
No. 1[b]	3.6	27.3	2.4	0.17	0.20	2.74
No. 2[c]	3.6	26.7	1.7	0.15	0.02	2.66
No. 3[d]	3.6	26.9	1.2	0.12	0.00	2.56

[a] The liquid fraction obtained after washing the pretreated biomass.
[b] Retained hydrolysate after pass the membrane.
[c] Dilution process followed by membrane separation was repeated twice.
[d] Dilution process followed by membrane separation was repeated three times.

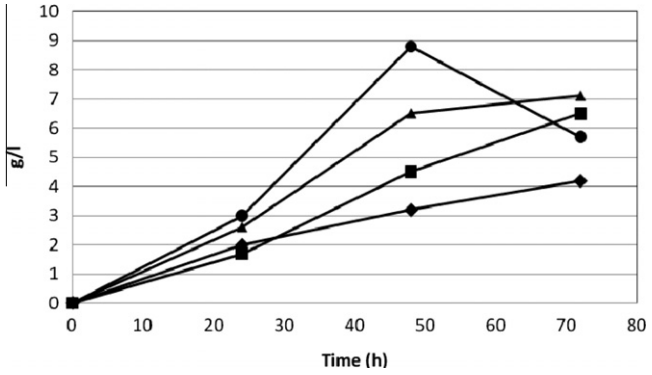

Fig. 4. Ethanol production depends on acetic acid concentration (♦: without reverse osmosis treatment as original hydrolysate C (original), ■: total fermentable sugar 30.9 g/l and acetic acid 2.4 g/l (No. 1), ▲: total fermentable sugar 30.3 g/l and acetic acid 1.7 g/l (No. 2), ●: total fermentable sugar 30.5 g/l and acetic acid 1.2 g/l (No. 3)).

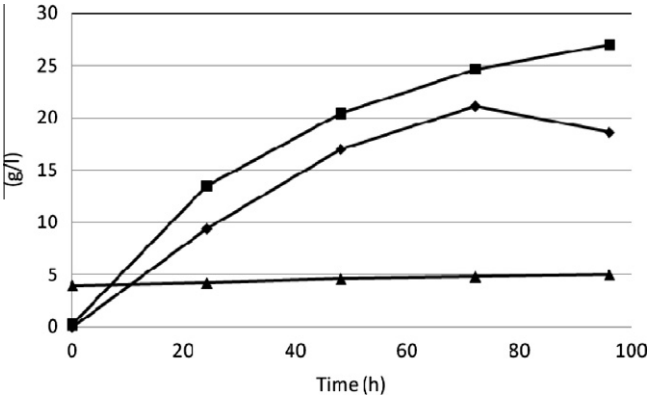

Fig. 5. Measured glucose, xylose and ethanol concentration during enzymatic hydrolysis and fermentation in the same condition (♦: ethanol, ■: glucose, ▲: xylose).

4. Conclusion

In this study, pilot-scale pretreatment of oxalic acid was performed. Pretreatment was accomplished with 80.6 kg of corncobs at the same conditions with laboratory scale experiment. Among the three kinds of hydrolysates obtained from pretreatment, Hydrolysate C (obtained by washing the pretreated biomass) contained 35.29 g/l of fermentable sugar and produced 10.3 g/l of ethanol by fermentation, which was very similar to the expected data of 11.9 g/l. The highest ethanol production by washed biomass was 21.1 g/l. Finally, these results suggest that oxalic acid pretreatment should be considered further for pilot scale studies, and finally industrial scale processes.

Acknowledgements

This work was supported by Priority Research Centers Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (Project No. 2011-0018393) to J.-W. Lee. We thank Frederick J. Matt of the Analytical Chemistry and Microscopy Laboratory of the USDA Forest Products Laboratory (FPL) for carrying out the carbohydrate determination.

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