Enzymatic Saccharification and L-lactic Acid Fermentation of Corn Stover Pretreated with Liquid Hot Water by *Rhizopus oryzae*

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Corn stover was pretreated with liquid hot water (LHW) to enhance its enzymatic hydrolysis and L-lactic fermentation. The cellulose conversion rate and L-lactic acid concentration were used to evaluate LHW pretreatment performance. Results showed that the optimum conditions for the LHW pretreatment of corn stover are a reaction temperature of 190 °C for 20 min and a solid-to-liquid ratio of 1:10. The cellulase loading was 30 filter paper units per gram of oven-dried, water-insoluble solid. These conditions resulted in 92.3% conversion of cellulose to glucose. Sequential hydrolysis and fermentation using pretreated water-insoluble solid (WIS) produced an L-lactic acid concentration of 45 g/L. This study indicated that LHW pretreatment of corn stover is a suitable method for achieving high cellulose conversion and L-lactic acid concentration.

Keywords: Enzymatic hydrolysis; L-lactic acid; Corn stover; Liquid hot water pretreatment

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INTRODUCTION

L-lactic acid is a value-added product from renewable biomass. Its production is a present necessity and a potential economically and environmentally feasible process. L-lactic acid is a commodity chemical with a wide range of applications, mainly in the food industry (where it is utilized for approximately 80% of all food production, especially as a microbial preservative or as an acidification, flavoring, and pH buffering agent). L-lactic acid is likewise used in the production of pharmaceuticals, cosmetics, and tanned leather, as well as in the textile and polymer industries (Rivera *et al.* 2007; Xu *et al.* 2006). Cost-effective L-lactic acid with very high stereochemical purity in large-scale production processes is essential for polylactic acid synthesis in the production of biodegradable plastics. The industrial production of L-lactic acid with high stereo-chemical purity can be accomplished using two alternative technologies: chemical synthesis from fossil fuels and biotechnology-based methods. The latter utilizes lignocellulosic biomass as a raw material (Park *et al.* 2004; Oh *et al.* 2005; Marques *et al.* 2008).

Currently, L-lactic acid production by fermentation is the leading technology worldwide. Lignocellulosic biomass represents an abundant natural renewable carbon resource for L-lactic acid production with short- and long-term sustainability. However, this form of biomass is very resistant to enzymatic attack. Thus, pretreatment is required to make the cellulose more accessible to enzymes. Pretreatment improves the rate of conversion, and related research and development will potentially improve the production efficiency and decrease costs. Many pretreatment methods have been proposed and

investigated, *e.g.*, steam explosion (Rocha *et al.* 2012), ammonia fiber explosion (Dien *et al.* 2008; Kim *et al.* 2008), alkaline pretreatment (Zhang *et al.* 2012), wet-oxidation (Garde *et al.* 2002), and liquid hot water (LHW) pretreatment (Mosier *et al.* 2005; Pérez *et al.* 2008; Ingram *et al.* 2009). LHW pretreatment is a promising process for lignocellulosic biomass. LHW is a form of hydrothermal treatment that does not require rapid decompression and does not use any catalysts or chemicals. Pressure is applied to keep water in the liquid state at elevated temperatures (160 °C to 240 °C) to induce alterations in the lignocellulose structure (Alvira *et al.* 2010).

In previous works, many renewable lignocellulose resources can be converted to L-lactic acid. However, most previous investigations have revealed that low L-lactic acid production and productivity is the main limitation associated with pretreatment methods and *Rhizopus* species. Optimization of process parameters of pretreatment and enzymatic hydrolysis was in order to improve L-lactic acid production. Furthermore, studies on the fermentation of L-lactic acid to provide insight into possibilities for enhancement of L-lactic acid production using *Rhizopus oryzae (3.0819). R. oryzae* is a filamentous fungus that is known to exclusively produce L-lactic acid (Açar *et al.* 2007) in a minimal medium utilizing different carbon sources (Saito *et al.* 2012; Yamane and Tanaka 2013). This study investigated the enzymatic hydrolysis of corn stover in LHW as a pretreatment process. Corn stover is a typical lignocellulosic biomass material that is widely found in China and is an ideal feedstock for the production of bio-based chemicals because of its high cellulose content (around 39% of dry weight). This study aimed to evaluate the LHW pretreatment performance in terms of the cellulose conversion of water-insoluble solids (WISs) from corn stover and the efficiency of LHW treatment in enhancing the enzymatic digestibility of corn stover. To obtain the best pretreatment conditions for enzymatic hydrolysis, variations in the pretreatment temperature and time, as well as the solid-to-liquid ratio, were investigated. The cellulase loading and the solid-to-liquid ratio in the subsequent enzymatic hydrolysis were likewise studied. The present study evaluated L-lactic acid production using fermentation media containing the hydrolyzates produced by the enzymatic hydrolysis of LHW-pretreated corn stover WIS.

EXPERIMENTAL

Materials

Corn stover with a moisture content of 10.4% was collected from a field near Jinzhou New District (Dalian, China). The collected materials were manually cut into pieces, milled, and sieved to obtain 40- to 60-mesh fractions. The corn stover was homogenized and stored in a plastic bag for further pretreatment experiments. The corn stover contained benzene–alcohol (2:1; 10.9%), glucan (38.8%), xylan (23.5%), acid-insoluble lignin (15.6%), acid-soluble lignin (2.4%), and ash (3.7%), based on the oven-dried weight.

The commercial cellulase used in the enzymatic hydrolysis was purchased from the Imperial Jade Biotechnology Co., Ltd, Ningxia, China. The cellulase was derived from *Trichoderma longibrachiatum.*

R. oryzae (3.0819) was obtained from the China General Microbiological Culture Collection Center. The strain was grown in Erlenmeyer flasks containing 50 mL of liquid media (glucose, 20 g/L; $(NH_4)_2SO_4$, 2 g/L; KH_2PO_4, 0.3 g/L; $MgSO_4$, 0.3 g/L; $ZnSO_4{\cdot}7H_2O$, 0.05 g/L; $FeSO_4$, 0.018 g/L) at 34 °C and 160 rpm for 18 h. Approximately

10% inoculum was added to 50 mL of the media for the fermentation.

LHW Pretreatment

The LHW pretreatment was conducted in a 15-L digester with four small tanks (mechanical mill of the Shanxi University of Science and Technology, China). Approximately 40 g of corn stover and solid-to-liquid ratio in the range 1:10 to 1:25 (w/v) of deionized water were loaded into the small tanks. The pretreatment temperature was controlled at 170, 180, 190, 200, or 210 °C. The pretreatment time was set at either 20 or 40 min. After pretreatment, the WIS and the prehydrolyzates were separated by filtration using a Büchner funnel. The WIS samples were washed with deionized water to a pH of approximately 7 and used for subsequent enzymatic hydrolysis and L-lactic acid fermentation.

Enzymatic Hydrolysis

The enzymatic hydrolysis of washed WIS was performed at 50 °C for 72 h in 100-mL Erlenmeyer flasks. Each flask contained 20 to 50 mL of 0.05 M sodium citrate buffer (pH 4.8) and had a WIS solid-to-liquid ratio of 1:20 to 1:50 weight per volume (w/v). The enzyme loading ranged from 10 to 30 filter paper units (FPU)/g oven-dried WIS. Samples were collected at 1, 5, 9, 12, 24, 36, 48, and 72 h for glucose concentration determination.

L-lactic Acid Fermentation

The enzymatic saccharification hydrolyzates were concentrated using a rotary evaporator until the glucose concentration reached 80 g/L. The hydrolyzates were then neutralized with $CaCO_3$ to a final pH of 6.5. The hydrolyzate liquor was supplemented with 2 g $(NH_4)_2SO_4$/L, 0.3 g KH_2PO_4/L, 0.3 g $MgSO_4$/L, and 0.05 g $ZnSO_4 \cdot 7H_2O$/L. The mixture was sterilized and directly used as fermentation media. The experiments were conducted in 250-mL Erlenmeyer flasks with a fermentation media volume of 50 mL. Fermentation was performed in shaker incubators at 160 rpm at 36 °C for 72 h. Samples (1 mL) were obtained from the cultures at given fermentation times and centrifuged at 5000 rpm for 10 min. The supernatants were stored for glucose and L-lactic acid analyses.

Chemical Analysis Methods

The xylan, acid-insoluble lignin, ash, and benzene–alcohol (2:1) contents were determined using the Chinese National Standard methods GB/T2677.9-1994, GB/T2677.8-1994, GB/T2677.3-1993, and GB/T2677.6-1994, respectively. The acid-soluble lignin and glucan contents were determined using the methods described in GB/T10337-1989 and NREL, respectively. The glucose and L-lactic acid contents were quantified using an SBA-40D Biological Sensing Analyzer (Biology Institute of the Shandong Academy of Sciences, Jinan, China). The glucan content was calculated using the following formula,

$$\text{Glucan content (\%)} = \frac{[\text{glucose}] \times 0.087 \times 0.9}{m} \times 100\% \quad (1)$$

where [glucose] is the glucose concentration (g/L), m is the mass of oven-dried WIS (g), 0.087 is the volume of acid hydrolysis liquid (L), and 0.9 is the conversion factor for

glucose to glucan.

The conversion of cellulose to glucose during enzymatic hydrolysis was determined by the ratio of the glucose concentration that was released during enzymatic hydrolysis to the total glucose in the substrate. The conversion of cellulose to glucose was calculated using the formula,

$$\text{Conversion of cellulose to glucose (\%)} = \frac{[\text{glucose}] \times \text{V} \times 0.9}{\text{glucan content} \times \text{m}} \times 100\% \qquad (2)$$

where [glucose] is the glucose concentration in the enzymatic hydrolysis liquor (g/L), *V* is the volume of enzymatic hydrolysis liquor (L), and *m* is the mass of oven-dried WIS (g).

RESULTS AND DISCUSSION

Effect of Pretreatment Conditions on Enzymatic Hydrolysis of Corn Stover WIS

Effect of pretreatment temperature and time

Previous reports have shown that lignocellulosic biomass pretreatment at higher temperatures can result in higher enzymatic digestibility than that with untreated biomass (Mood *et al.* 2013; Wang *et al.* 2012; Wan *et al.* 2011). Pretreatment can produce a higher glucose concentration during enzymatic hydrolysis (Donohoe *et al.* 2008). The conditions for the LHW pretreatment included a temperature range of 170 °C to 210 °C for 20 or 40 min. Enzymatic hydrolysis was conducted on corn stover without pretreatment for comparison. The glucan contents of WIS samples after LHW pretreatment at different temperatures and times are summarized in Table 1.

Table 1. Glucan Content, Xylan Content, and Lignin (Acid-insoluble Lignin and Acid-soluble Lignin) Content of WIS after LHW Pretreatment at Different Temperatures for Different Pretreatment Times

Pretreatment temperature (°C)	Glucan content (% WIS)		Xylan content (%WIS)		Lignin content (%WIS)	
	after 20 min	after 40 min	after 20 min	after 40 min	after 20 min	after 40 min
170	42.33	47.42	26.02	17.13	18.84	19.61
180	46.03	50.16	15.29	10.62	18.77	18.76
190	53.67	54.60	9.23	5.43	16.40	16.22
200	53.31	54.73	5.23	5.12	15.98	17.47
210	54.33	54.78	5.10	4.03	17.06	20.39

The glucan content of all WIS ranged from 42% to 55% of WIS, which is higher than that of the untreated corn stover (38.8%). This value depended on the pretreatment temperature and time. The glucan content slightly increased with increasing pretreatment time at the same pretreatment temperature. This value likewise increased with increasing pretreatment temperature. The glucan content rapidly increased when the pretreatment temperature ranged from 190 °C to 210 °C, reaching 11.3% (at 20 min) and 7.2% (at 40 min). However, the glucan content was only slightly increased, by 0.66% (at 20 min) and 0.18% (at 40 min), when the pretreatment temperature ranged from 170 °C to 190 °C. The highest glucose content was obtained at 210 °C for 40 min. This result can be

attributed to the solubilized water-soluble components in the untreated corn stover. For instance, low-molecular-weight lignin and the extractives produced a fraction of glucan-enriched WIS as the incubation time and temperature increased. The hemicellulose components of the corn stover were partly degraded and dissolved, which further increased the WIS glucan content. The enzymatic hydrolysis profile of WIS-pretreated corn stover at different pretreatment temperatures and times is shown in Fig. 1.

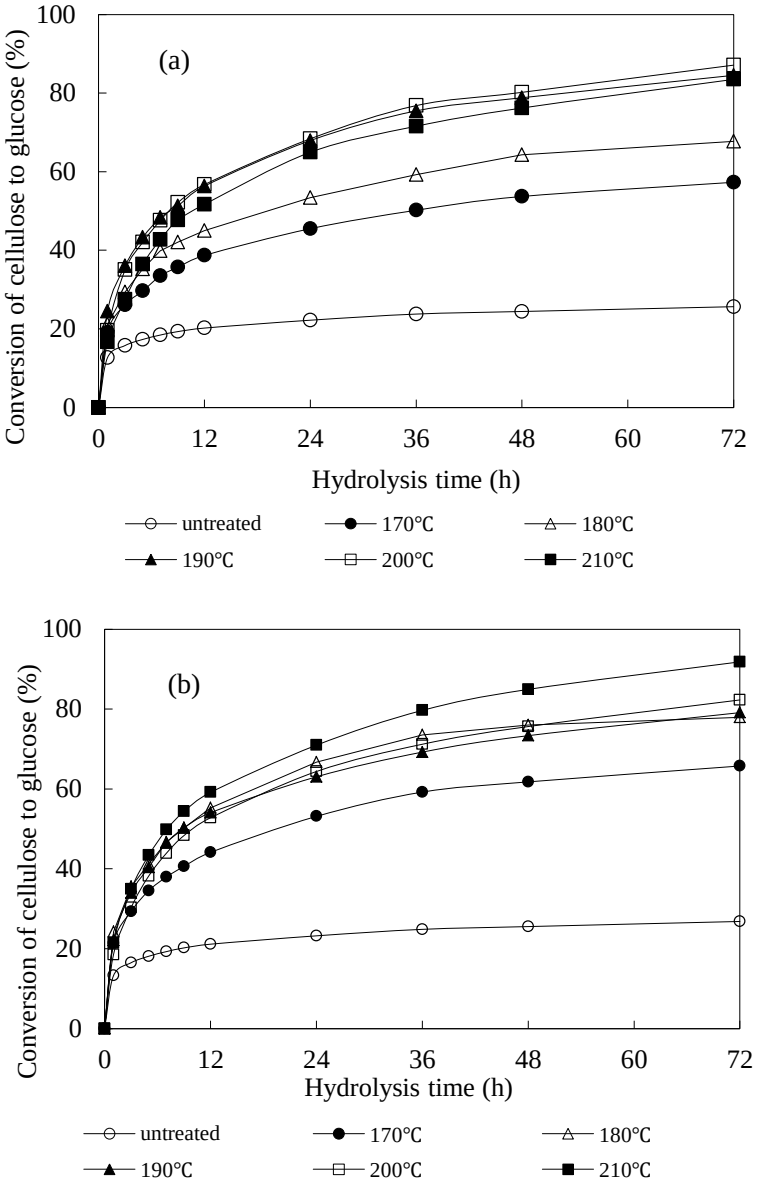


Fig. 1. Conversion of cellulose in the enzymatic hydrolysis of WIS from LHW-pretreated corn stover at different pretreatment temperatures for different pretreatment times: (a) 20 and (b) 40 min. Enzymatic hydrolysis conditions: 25 FPU/g oven-dried WIS, pH 4.8, solid-to-liquid ratio 1:50 (w/v), and 50 °C for 72 h

The enzymatic digestibility of each WIS was different. The conversion of cellulose to glucose rapidly increased at the beginning of the enzymatic hydrolysis process and then slowly declined, which was similar to findings in previous reports (Zaldivar *et al.* 1999). The maximum conversion of cellulose to glucose was only 25.7% after 72 h of enzymatic hydrolysis. However, the pretreatment effects on WIS from the

pretreated samples showed that the efficiency of cellulose conversion during enzymatic hydrolysis ranged from 57% to 92%. The LHW pretreatment may have increased the WIS enzymatic digestibility by increasing the surface area of raw material samples. Consequently, sufficient contact occurred between cellulase and the substrate, which increased in the amount of glucose released. The cellulose conversion decreased from 84.5% to 79.1% at 72 h when the pretreatment time was prolonged from 20 min to 40 min at 190 °C. This decrease in cellulose conversion indicates that a prolonged pretreatment time does not improve WIS enzymatic hydrolysis. Temperatures higher than 190 °C had no significant effects on WIS enzymatic hydrolysis (Fig. 1). High temperatures and prolonged LHW pretreatment time could cause more polysaccharide degradation and loss, which decreases the cellulose yield. Therefore, LHW pretreatment at 190 °C for 20 min is optimal for corn stover. A cellulose conversion of 91.8% after 72 h of enzymatic hydrolysis was obtained from corn stover with LHW pretreatment at these conditions.

Effect of solid-to-liquid ratio in LHW pretreatment

The solid-to-liquid ratio is equal to the quantity of oven-dried WIS divided by the entire liquid volume in the LHW pretreatment. When the corn stover quantity was kept constant, the higher solid-to-liquid ratio resulted in a higher substrate concentration (Table 2). Increasing the solid-to-liquid ratio can decrease processing costs by lowering the reactor size and the amount of heat required during the pretreatment. When the solid-to-liquid ratio was increased from 1:10 to 1:25 (w/v) during LHW pretreatment at 190 °C, the rate of glucose release was slightly increased during enzymatic hydrolysis. The conversion of cellulose to glucose at 72 h by enzymatic hydrolysis increased from 92.3% to 98.1%, with only a difference of 5.8% after 72 h (Fig. 2).

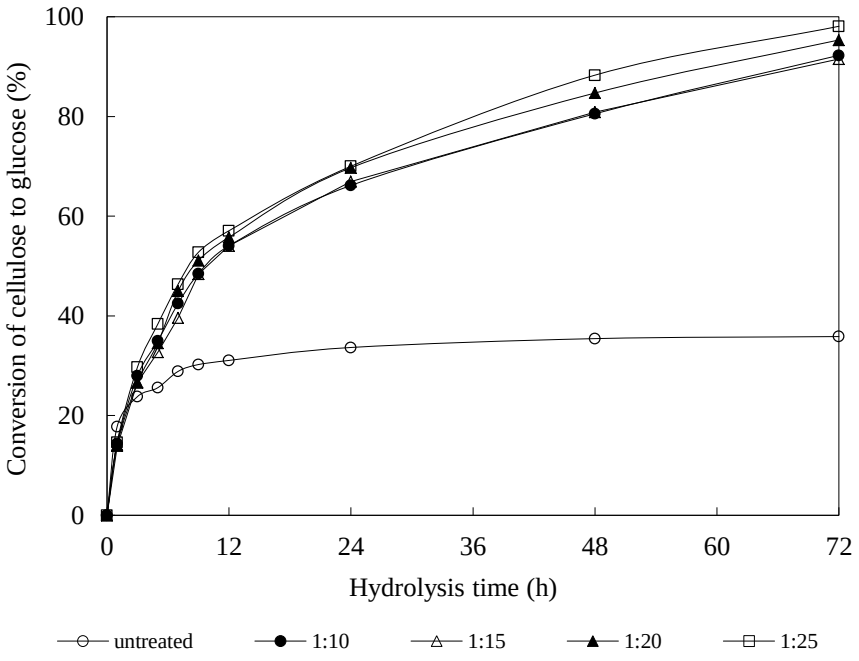


Fig. 2. Enzymatic hydrolysis of corn stover WIS after LHW pretreatment for 20 min at 190 °C with various solid-to-liquid ratios. Enzymatic hydrolysis conditions: 30 FPU/g oven-dried WIS, pH 4.8, solid-to-liquid ratio 1:50 (w/v), and 50 °C for 72 h

This limited increase in the conversion of cellulose to glucose shows that the solid-to-liquid ratio had little effect on the enzymatic digestibility of WIS from corn stover in range of 1:10 to 1:25. The effect of severity remained the same for all solid-to-liquid ratios then with increase of solid-to-liquid ratios we should see an improvement in glucose recovery. Therefore, a solid-to-liquid ratio of 1:10 was used for the LHW pretreatment of corn stover.

Table 2. Glucan Content, Xylan Content, and Lignin (Acid-insoluble Lignin and Acid-soluble Lignin) Content of WIS after LHW Pretreatment with Different Solid-To-Liquid Ratios

Solid-to-liquid ratio	Glucan content (%WIS)	Xylan content (%WIS)	Lignin content (%WIS)
1:10	57.94	2.25	14.18
1:15	58.46	2.30	15.28
1:20	60.03	2.15	15.77
1:25	60.55	2.33	15.62

Optimization of Enzymatic Hydrolysis of Corn Stover WIS

Effect of cellulase loading

In addition to the enzymatic temperature and pH, cellulase loading is another important factor during the enzymatic hydrolysis of cellulosic substrates. Increasing the cellulase loading generally results in increased glucose release and cellulose conversion. The enzymatic hydrolysis curves of WIS from corn stover after LHW pretreatment with different cellulase loadings are shown in Fig. 3. Cellulase loading had a significant effect on the cellulose conversion of WIS by enzymatic hydrolysis. When the cellulase loading was increased from 10 FPU/g oven-dried WIS to 30 FPU/g oven-dried WIS on pretreated corn stover at 190 °C or 210 °C for 20 min, the enzymatic digestibility of corn stover WIS was greatly improved. The cellulose conversion in WIS from corn stover pretreated at 210 °C was higher than that in WIS from corn stover pretreated at 190 °C under the same enzymatic hydrolysis conditions (Fig. 4). The higher cellulose conversion in WIS from corn stover pretreated at 210 °C indicates that higher pretreatment temperatures can improve the enzymatic digestibility of corn stover and increase the amount of glucose released during enzymatic hydrolysis.

Effect of solid-to-liquid ratio

A high glucose concentration is necessary to obtain a high L-lactic acid concentration by fermentation, which subsequently decreases the production cost. Decreasing the solid-to-liquid ratios during the enzymatic hydrolysis of a cellulosic substrate may increase the glucose content. Nevertheless, an extremely low solid-to-liquid ratio indicates an extremely high initial solid concentration. High solid concentrations may cause difficulties in uniformly mixing the enzyme-containing liquid and WIS because the amount of liquid in the system is reduced. The WIS from LHW pretreatment at 190 and 210 °C for 20 min were subjected to enzymatic hydrolysis at various solid-to-liquid ratios, ranging from 1:20 to 1:50 (w/v).

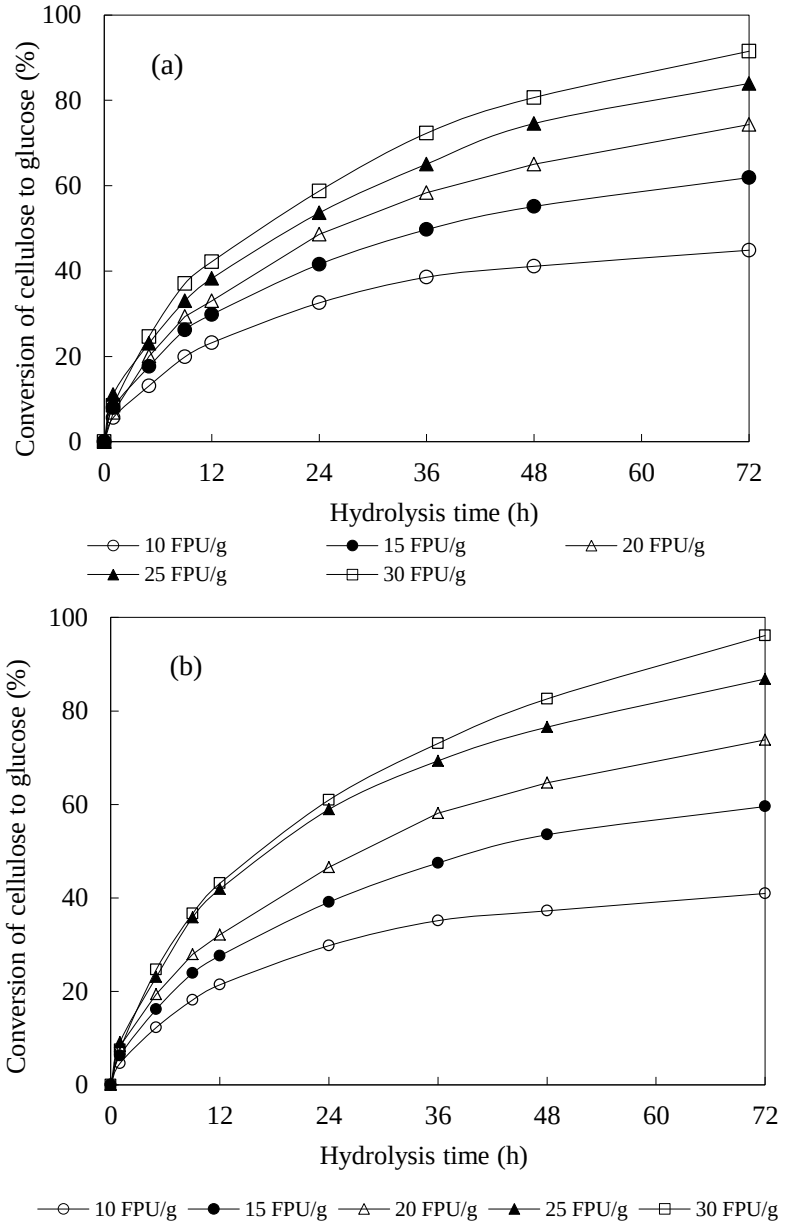
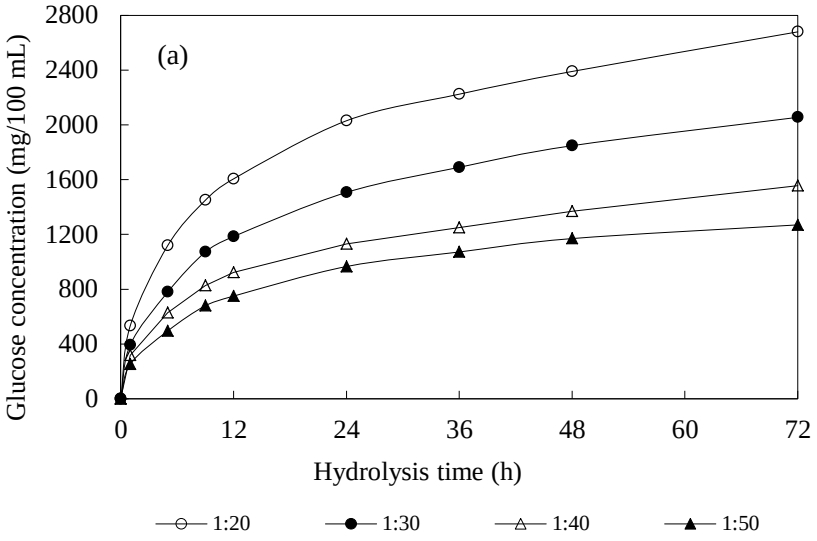
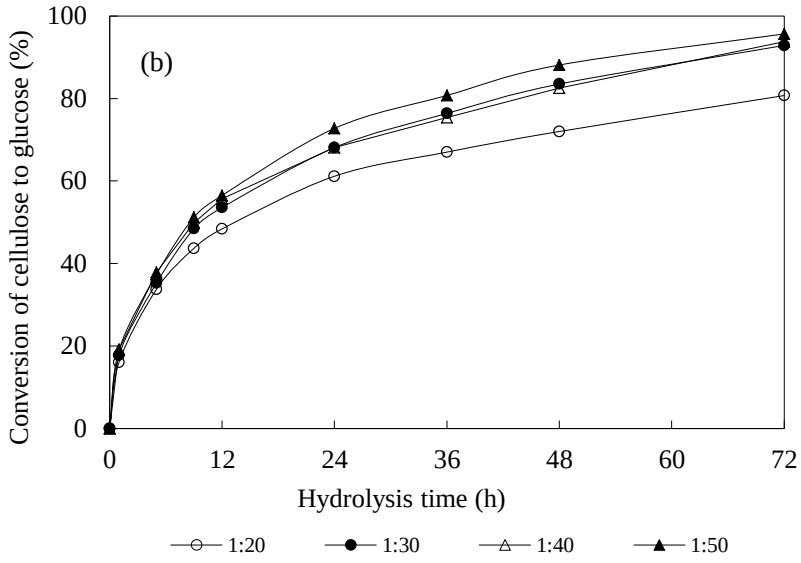
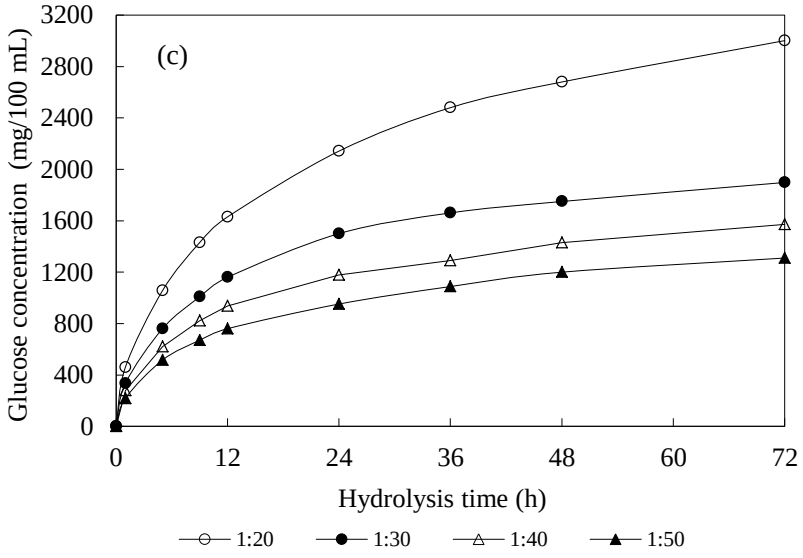


Fig. 3. Enzymatic hydrolysis of WIS after LHW pretreatment for 20 min at (a) 190 and (b) 210 °C with various cellulase loadings. Enzymatic hydrolysis conditions: pH 4.8, solid-to-liquid ratio 1:50 (w/v), and 50 °C for 72 h

The glucose concentration in the hydrolyzates from enzymatic hydrolysis increased with pretreatment temperature and decreased with the solid-to-liquid ratio (*i.e.*, 1:50 < 1:40 < 1:30 < 1:20; Fig. 4.). The highest glucose concentration (2679 mg/100 mL at 190 °C and 3002 mg/100 mL at 210 °C) was obtained using the highest solid-to-liquid ratio (1:20) at 72 h. The lowest glucose concentration (1270 mg/100 mL at 190 °C and 1310 mg/100mL at 210 °C) was obtained using the lowest solid-to-liquid ratio (1:50) at 72 h. The glucose concentration increased by 52.6% (at 190 °C) and 56.4% (at 210 °C). The conversion of cellulose to glucose was decreased slightly with increasing solid-to-liquid ratios (Fig. 4(b) and 4(d), respectively). This result indicates that an increased solid-to-liquid ratio in range of 1:20 to 1:50 (w/v) is beneficial to subsequent L-lactic acid fermentation.


(a)
Glucose concentration (mg/100 mL)
2800
2400
2000
1600
1200
800
400
0
0
12
24
36
48
60
72
Hydrolysis time (h)
1:20
1:30
1:40
1:50


(b)
Conversion of cellulose to glucose (%)
100
80
60
40
20
0
0
12
24
36
48
60
72
Hydrolysis time (h)
1:20
1:30
1:40
1:50


(c)
Glucose concentration (mg/100 mL)
3200
2800
2400
2000
1600
1200
800
400
0
0
12
24
36
48
60
72
Hydrolysis time (h)
1:20
1:30
1:40
1:50

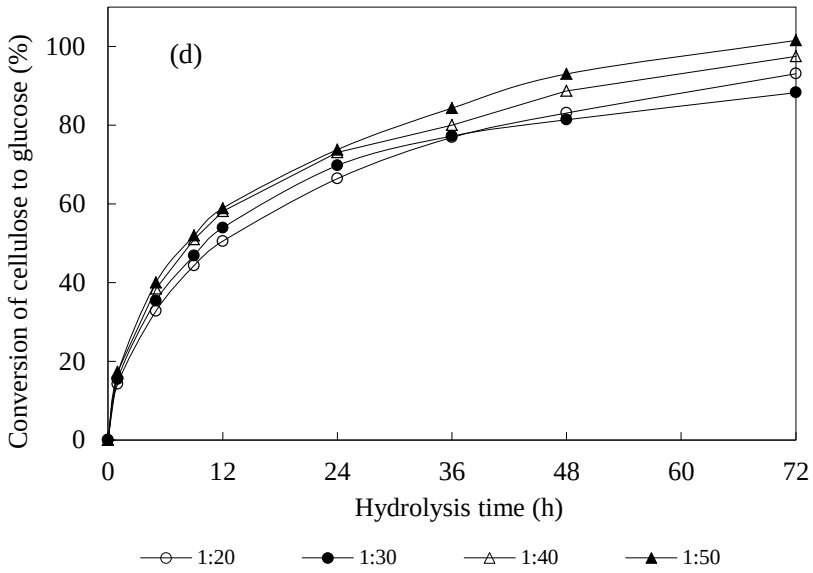


Fig. 4. Enzymatic hydrolysis of WIS after LHW pretreatment at (a, b) 190 and (c, d) 210 °C for 20 min with various solid-to-liquid ratios: (a, c) glucose concentration and (b, d) conversion of cellulose. Enzymatic hydrolysis conditions: 30 FPU/g oven-dried WIS, pH 4.8, and 50 °C for 72 h

L-lactic Acid Fermentation of Hydrolyzates from Enzymatic Saccharification of WIS after LHW Pretreatment

The capacity of *R. oryzae (3.0819)* to produce L-lactic acid from LHW-pretreated WIS hydrolyzates was evaluated. This part of the study aimed to produce L-lactic acid using the concentrated hydrolyzates derived from enzymatic saccharification of WIS as an alternative and inexpensive carbon source. To investigate the feasibility of using the corn stover hydrolyzates, preliminary cultures of *R. oryzae (3.0819)* were incubated in shake flasks (see section 2.1). The glucose consumption and L-lactic acid production in these cultures were measured. The profiles of L-lactic acid and glucose utilization for this fermentation experiment are presented in Fig. 5.

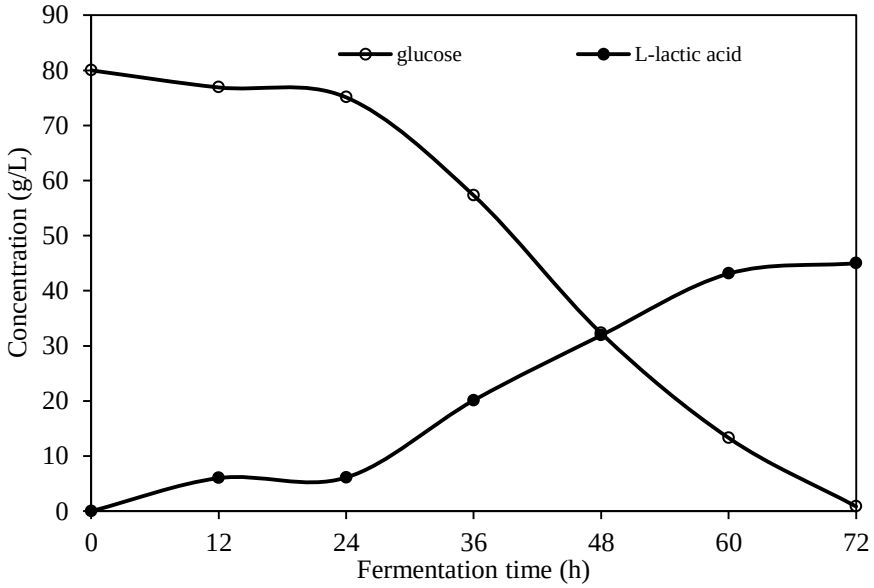


Fig. 5. Profiles of L-lactic acid production and glucose utilization

The maximum amount of L-lactic acid was produced within 60 h of fermentation, wherein the L-lactic acid content reached 45 g/L. Most of the glucose was consumed, and only 0.85 g/L remained after fermentation. Therefore, the conversion of corn stover pretreated with LHW to L-lactic acid by *R. oryzae* (3.0819) was highly efficient.

The mass balance is very important to evaluate the whole process of L-lactic acid fermentation at the optimal conditions using *R. oryzae (3.0819)*. The WIS yield was 59.64% at the optimum conditions of 190 °C for 20 min. The 0.6 kg WIS was obtained from 1 kg corn stover. 0.35 kg glucan, 0.01 kg xylan and 0.09 kg lignin were preserved in the WISs. 1 kg water was used to wash the WISs. 0.1 kg total enzymes were added to hydrolyze the WISs. 0.32 kg glucose was obtained during enzymatic hydrolysis. 0.20 kg L-lactic acid was produced during fermentation. Several methods exist for producing L-lactic acid by the fermentation of biomass feedstock (Zaldivar *et al.* 1999; Zhang *et al.* 2007). Factors such as the pH, temperature, and amount of substrate, as well as the product concentrations of glucose and lactic acid, can affect the fermentation efficiency. Thus, further systematic optimization of the L-lactic acid fermentation method and process should be performed. More efficient methods to obtain a higher yield of L-lactic acid concentration are expected in the near future, having already been started in this preliminary study.

CONCLUSIONS

1. The LHW pretreatment of corn stover significantly enhanced the conversion rate of enzymatic hydrolysis. The results show that when corn stover is pretreated by LHW with a 1:10 solid-to-liquid ratio at 190 °C for 20 min, its enzymatic hydrolysis produces a 92.3% yield for 72 h; its cellulase loading is 30 FPU/g oven-dried WIS at pH 4.8 and 50 °C.
2. The hydrolyzates and *R. oryzae (3.0819)* were successfully used for the L-lactic acid production from LHW-pretreated corn stover. The highest concentration of L-lactic acid produced was 45 g/L. This study proved that corn stover pretreated with LHW could successfully be converted to L-lactic acid.

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