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ORIGINAL PAPER

Production of ethanol from corn stover hemicellulose hydrolyzate using *Pichia stipitis*

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Abstract Hemicellulose liquid hydrolyzate from dilute acid pretreated corn stover was fermented to ethanol using *Pichia stipitis* CBS 6054. The fermentation rate increased with aeration but the pH also increased due to consumption of acetic acid by *Pichia stipitis*. Hemicellulose hydrolyzate containing 34 g/L xylose, 8 g/L glucose, 8 g/L Acetic acid, 0.73 g/L furfural, and 1 g/L hydroxymethyl furfural was fermented to 15 g/L ethanol in 72 h. The yield in all the hemicellulose hydrolyzates was 0.37–0.44 g ethanol/g (glucose + xylose). Nondetoxified hemicellulose hydrolyzate from dilute acid pretreated corn stover was fermented to ethanol with high yields, and this has the potential to improve the economics of the biomass to ethanol process.

Keywords Acetic acid · Pretreatment · Inhibitors · Ethanol · Fermentation · Biomass · Xylose

Introduction

Lignocellulosic biomass is an attractive feedstock for producing liquid fuels and chemicals [1]. Plant biomass, which consists of agricultural residues, waste paper, and forestry residues, are wastes already generated that need to be disposed. Other benefits of using lignocellulosic biomass are that they are the most abundant carbohydrate on earth [2], they are renewable [3] and are not in competition with food sources. The major components in lignocellulosic biomass are cellulose, hemicellulose and lignin. Corn stover, which includes the leaves, stalks, and cobs of the corn plant, may be available in quantities that can support significant ethanol production in the Midwestern regions of the US [4, 5]. Corn stover contains about 37% cellulose, 20% Xylan and 21% lignin by weight [5]. Cellulose is a glucose polymer and xylan hydrolysis produces D-xylose as the major sugar [6].

The structural features of biomass and presence of lignin are known to inhibit biomass hydrolysis [7, 8]. Conversion of biomass to sugars involves pretreatment to enhance digestibility and enzymatic hydrolysis of the fibers to produce fermentable sugars. Pretreatment may involve the use of chemicals, high temperature and pressure to disrupt the structure of biomass and remove or modify the lignin so as to enhance biomass digestibility [9]. Dilute acid pretreatment solubilizes hemicellulose sugars and increases plant cell wall porosity for enzymatic hydrolysis [10]. The solid fraction from dilute acid pretreatment contains cellulose and lignin as the major components. The liquid stream contains D-xylose as the major sugar, and small concentrations of other sugars such as glucose [5, 10]. However, the liquid stream contains inhibitors such as acetic acid from the acetyl group in hemicellulose, hydroxymethylfurfural (HMF) from glucose degradation, furfural from xylose degradation, and phenolic compounds from lignin degradation [11]. These inhibitors are known to affect the growth rate of yeasts [12].

The fermentation of the liquid stream to ethanol is important for a commercially feasible process [13]. Naturally occurring yeasts such as *Pichia stipitis*, *Candida shehatae*, and *Pachysolen tannophilus* are able to ferment both glucose and xylose to ethanol [14, 15]. Among the xylose-fermenting yeasts, *Pichia stipitis* has shown the most promise for industrial application, because it ferments xylose with a high ethanol yield [16, 17]. In this work, the liquid

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stream from dilute acid pretreated corn stover was fermented to ethanol using *Pichia stipitis* CBS 6054. The effect of the rotation speed in a shaker incubator and acetic acid on fermentation by *Pichia stipitis* was studied.

Materials and methods

Microorganism and nutrients

Pichia stipitis CBS 6054 was generously supplied by Dr. Thomas Jeffries of the Forest Products Laboratory, USDA. Stock cultures were maintained on 20% glycerol at 4 °C. *Pichia stipitis* (100 µL of stock culture) was cultivated on YEPX agar plates: 10 g/L yeast extract, 20 g/L peptone, 20 g/L xylose, and 20 g/L agar at 30 °C for 3 days. Colonies from the plates were grown overnight in a filter-sterilized fermentation medium containing 1.7-g/L yeast nitrogen base (without amino acid or ammonium sulfate), 2.27 g/L urea, 6.56 g/L peptone, and 20 g/L xylose. The cells were centrifuged at 3,000 rpm for 5 min and resuspended in water to a final concentration of 50 g dry cells/L (serves as inocula). Nutrient solution (50× the concentration used) was prepared by dissolving 1.7 g of yeast nitrogen base, 2.27 g of urea and 6.56 g of peptone in 20 mL of water.

Hydrolyzate and fermentation

The liquid hemicellulose hydrolyzate stream was produced from dilute H_2SO_4 acid pretreated corn stover as reported elsewhere [5]. The acid pretreated corn stover was neutralized with NH_4OH to pH 6 and filter-sterilized. Two liquid streams with different sugar and inhibitor levels were prepared and their compositions are shown in Table 1. Synthetic media composition used in this study is shown in Table 1. Fermentations were performed in sterile 125 ml Erlenmeyer flasks (with 0.2 µm vent cap) in an air-shaker incubator at 30 °C at 100 rev/min and 150 rev/min. Each Erlenmeyer flask contained 50 mL of sugar media, 1 mL of nutrient solution, and 2 mL of inocula to give an initial cell concentration of 2 g/L. Phosphate buffer (1.5 mL of 1 M KH_2PO_4/NaOH, pH 6) was added to some of the fermentation media to give a final buffer concentration of 27.5 mM. All these experiments were performed in triplicate at the same initial cell concentration of 2 g/L.

Analytical methods

Fermentation was monitored by taking 1 mL of sample for analyses. The concentration of glucose, xylose, acetic acid and ethanol were determined using Agilent HPLC System with analytical BIO-RAD Aminex HPX-87H column and a BIO-RAD Cation H refill guard column. The cell concentrations were determined from optical density (OD) measurement of the cells using HP 845 UV-Visible system at 600 nm (1 OD = 0.11 g/L of dry cells). The pH during fermentation was measured using Orion portable pH meter.

Results and discussion

Comparison of *Pichia stipitis* fermentation rate on Liquid stream A at rotation speeds of 100 and 150 rpm

Fermentation results for Liquid Stream A at 100 and 150 rpm are shown in Fig. 1. The time it takes to complete fermentation was reduced from 96 to 48 h by increasing the rotation speed from 100 to 150 rpm. The increase in fermentation rate was due to increased cell growth rate (Fig. 2) and high xylose consumption rate (Fig. 1). Increasing the rotation speed increased the rate of aeration in the flasks leading to a higher cell growth rate and subsequently a faster ethanol production rate. Our result is similar to observations in synthetic media where aeration rate has been shown to increase biomass production rate [18] and the xylose transport rate [19] in *Pichia stipitis*. However,

Table 1 Dilute H_2SO_4 acid pretreated corn stover liquid and synthetic media compositions

Media (g/L)	Corn stover liquid stream A	Corn stover liquid stream B	Synthetic media A	Synthetic media B*
Glucose	6.27 ± 0.12	8.19 ± 0.18	5.85 ± 0.25	6.37 ± 0.52
Xylose	24.96 ± 0.03	33.54 ± 0.04	21.33 ± 0.97	22.46 ± 1.78
Acetic acid	6.09 ± 0.07	7.93 ± 0.08	–	5.34 ± 0.15
Furfural	0.63 ± 0.06	0.73 ± 0.02	–	–
Hydroxymethylfurfural	0.76 ± 0.05	1.00 ± 0.03	–	–

** All errors are ±1 SD

* The media with acetic acid was neutralized with NH_4OH to pH 6

* Fermentations were performed using 50 mL media in 125 mL Erlenmeyer flasks at 30 °C in a shaker incubator at 100 and 150 rpm

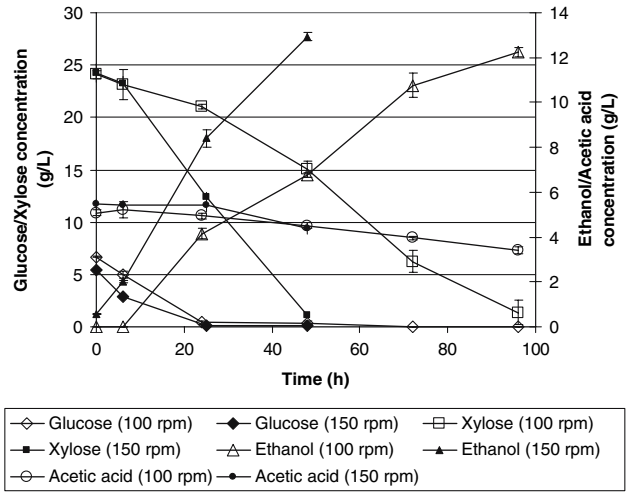

Fig. 1 Comparison of glucose consumption, xylose consumption, ethanol production, and acetic acid in liquid stream A at rotation speeds of 100 and 150 rpm

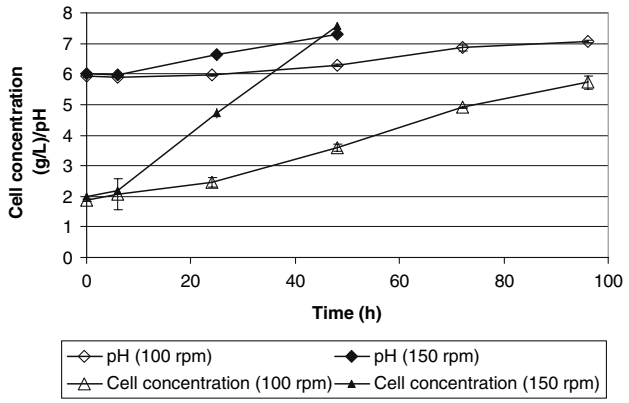

Fig. 2 Comparison of cell growth and pH during fermentation at 100 and 150 rpm

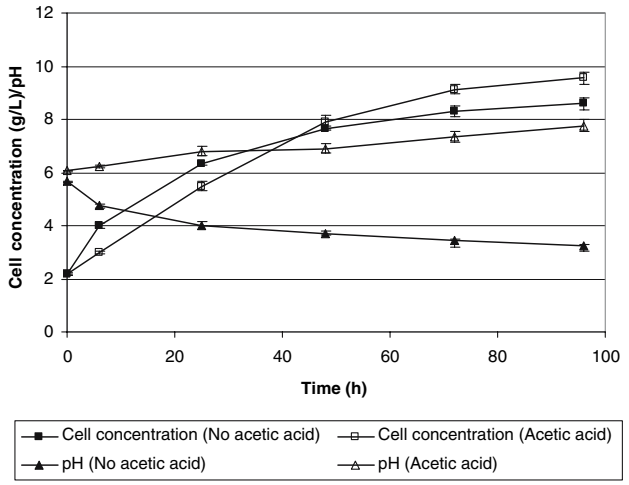

Fig. 3 Comparison of cell growth and pH of synthetic media with acetic acid and without acetic acid

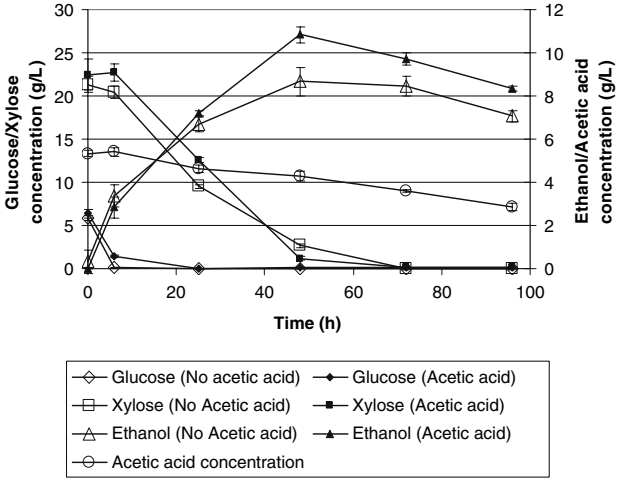

Fig. 4 Comparison of fermentation data of synthetic media with acetic acid and without acetic acid

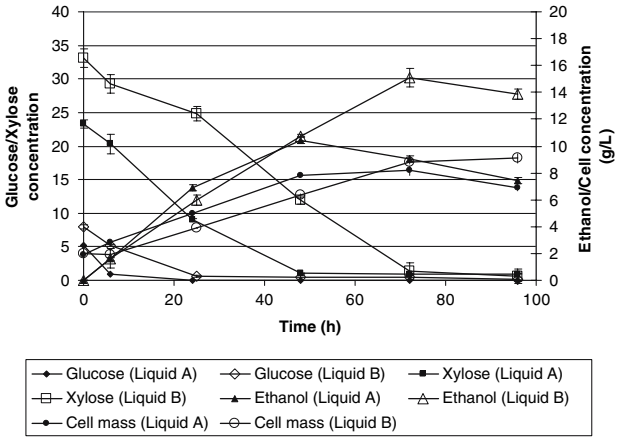

Fig. 5 Fermentation results in Liquid stream A and B at 150 rpm with phosphate buffer

since *Pichia stipitis* is respiro-fermentative [20], excess oxygenation could lead to low yields [21, 22].

The final ethanol concentration and yield at 150 rpm was slightly higher than those at 100 rpm, meaning the level of aeration at 150 rpm was not in excess. Acetic acid was consumed (Fig. 1) and pH increased during fermentation (Fig. 2). A similar observation of an increase in pH during *Pichia stipitis* fermentation was made on sugar cane bagasse hemicellulose hydrolyzate [23] and wood hydrolyzates [24, 25]. The increase in pH may be attributed to the consumption of acetic acid and therefore, the hypothesis was tested.

Effect of acetic acid on fermentation

To ascertain whether acetic acid is the cause of increase in pH observed, synthetic media was prepared with and without acetic acid. The results show that acetic acid consumption by *Pichia stipitis* is the cause of the increase in pH

Table 2 Summary of fermentation results

Substrates	Liquid A (100 rpm)	Liquid A (150 rpm)	Liquid A[a] (150 rpm)	Liquid B[a] (150 rpm)	Synthetic media (No acetic)	Synthetic media (Acetic)
Maximum ethanol concentration (g/L)	12.23 ± 0.20	12.94 ± 0.18	10.4 ± 0.27	15.06 ± 0.69	8.67 ± 0.74	10.83 ± 0.35
Fermentation time (h)	96	48	48	72	48	48
Ethanol yield on substrates (g/g)	0.40 ± 0.01	0.44 ± 0.01	0.37 ± 0.01	0.37 ± 0.03	0.32 ± 0.04	0.38 ± 0.02
Xylose consumption rate (g/L h)	0.24 ± 0.03	0.48 ± 0.02	0.46 ± 0.01	0.44 ± 0.01	0.39 ± 0.02	0.44 ± 0.04
Cell growth rate (g/L h)	0.04	0.12	0.12	0.09	0.11	0.12
Final pH	7.07 ± 0.04	7.15 ± 0.05	6.87 ± 0.02	6.67 ± 0.02	3.68 ± 0.11	6.91 ± 0.16

All errors are ±1 SD. The media with acetic acid was neutralized with NH_4OH to pH 6. Fermentations were performed using 50 mL media in 125 mL Erlenmeyer flasks at 30 °C in a shaker incubator at 100 and 150 rpm

[a] These fermentations had phosphate buffer in them

(Fig. 3). In the media without acetic acid, pH decreased during fermentation because, the CO_2 produced from fermentation forms carbonic acid (H_2CO_3) with water [26], which dissociates to acidify the medium and decrease the pH. A drop in pH is also expected from the consumption of NH_4OH. The pH increased in the media containing acetic acid because the acetic acid is consumed by *Pichia stipitis*. However, we do not know the products generated by *Pichia stipitis* when acetic acid is consumed. This result shows that pH variation during *Pichia stipitis* fermentation is dependent on two counteracting factors, the production of CO_2 and consumption of NH_4OH, which decrease the pH and acetic acid consumption which increase the pH.

The final ethanol concentration and yield on the synthetic media containing acetic acid was higher than the media without acetic acid (Fig. 4). The presence of nitrogen from the NH_4OH used to neutralize the acetic acid containing medium is responsible for the higher ethanol concentration and yield. This is very similar to previous studies showing the importance the nitrogen in obtaining high ethanol concentrations [27, 28]. In the synthetic medium containing glucose and xylose at the rotation speed of 100 rpm, the ethanol yield was in the range 0.40–0.44 g/g [17], whereas the ethanol yield in synthetic medium A (without acetic acid) at 150 rpm was 0.32 g/g. Although the increased aeration at 150 rpm resulted in a lower ethanol yield in synthetic media, this was not the case in acid pretreated hemicellulose hydrolysate.

Fermentation results for Liquid streams A and B

Fermentation results for Liquid streams A and B are shown in Fig. 5. Liquid stream B contains higher inhibitor levels compared to Liquid stream A (Table 1). Stream B had a lag phase of 6 h of no cell growth. The ethanol production rate was higher on Liquid stream A compared to Liquid stream B. The high concentration of inhibitors in Liquid stream B such as acetic acid, HMF and furfural is responsible for the lag, the slow cell growth rate (Table 2) and ethanol production rate in Liquid stream B compared to Liquid stream A. The addition of phosphate buffer (27.5 mM) was not high enough to stabilize the pH at 6.

Conclusion

An increase in the rotation speed from 100 rpm to 150 rpm in a shake flask incubator reduced the time it takes to complete fermentation by half. Fermentation of the hemicellulose corn stover hydrolyzate resulted in an increase in pH. The increase in pH during fermentation is due to consumption of acetic acid by *Pichia stipitis*, however, the products generated from the acetic acid consumption is unknown. The use of liquid stream with a higher inhibitor level (Liquid stream A & B) increased lag phase of cell growth and reduced ethanol production rate. However, the use of phosphate buffer at 27.5 mM, did not appear to stabilize the pH. The liquid stream from acid pretreated corn stover has been fermented to ethanol with the yield of 0.37–0.44 g ethanol/g (glucose + xylose). The ethanol yield on corn stover hemicellulose hydrolyzate was not reduced by increased aeration at 150 rpm as observed in synthetic media.

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