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Conversion of Aqueous Ammonia-Treated Corn Stover to Lactic Acid by Simultaneous Saccharification and Cofermentation

YONGMING ZHU,[1] Y. Y. LEE,*,[1] AND RICHARD T. ELANDER[2]

[1]*Department of Chemical Engineering, Auburn University, AL 36849, E-mail: yylee@eng.auburn.edu; and* [2]*National Bioenergy Center, National Renewable Energy Laboratory, Golden, CO 80401*

Abstract

Treatment of corn stover with aqueous ammonia removes most of the structural lignin, whereas retaining the majority of the carbohydrates in the solids. After treatment, both the cellulose and hemicellulose in corn stover become highly susceptible to enzymatic digestion. In this study, corn stover treated by aqueous ammonia was investigated as the substrate for lactic acid production by simultaneous saccharification and cofermentation (SSCF). A commercial cellulase (Spezyme-CP) and *Lactobacillus pentosus* American Type Culture Collection (ATCC) 8041 (Spanish Type Culture Collection [CECT]-4023) were used for hydrolysis and fermentation, respectively. In batch SSCF operation, the carbohydrates in the treated corn stover were converted to lactic acid with high yields, the maximum lactic acid yield reaching 92% of the stoichiometric maximum based on total fermentable carbohydrates (glucose, xylose, and arabinose). A small amount of acetic acid was also produced from pentoses through the phosphoketolase pathway. Among the major process variables for batch SSCF, enzyme loading and the amount of yeast extract were found to be the key factors affecting lactic acid production. Further tests on nutrients indicated that corn steep liquor could be substituted for yeast extract as a nitrogen source to achieve the same lactic acid yield. Fed-batch operation of the SSCF was beneficial in raising the concentration of lactic acid to a maximum value of 75.0 g/L.

Index Entries: Aqueous ammonia pretreatment; biomass; cofermentation; lactic acid; simultaneous saccharification; response surface method.

Introduction

Lactic acid is a commodity chemical widely used in the food industry, cosmetics, pharmaceuticals, and plastics. Currently, its commercial production is primarily based on microbial fermentation of starch-derived glucose or sucrose *(1)*. With the concern on feedstock cost, the use of lignocellulosic materials (LCM) as an inexpensive carbon source for lactic acid production has been

*Author to whom all correspondence and reprint requests should be addressed.

pursued *(2–4)*. The most important component in LCM is cellulose, complete hydrolysis of which leads to glucose. Conventional fermentation technology can be integrated with cellulose hydrolysis for lactic acid production *(5–7)*.

Next to cellulose, hemicellulose represents the other important carbohydrate fraction in LCM. Hemicellulose is a heteropolymer made up of a variety of sugar units including glucose, xylose, galactose, arabinose, and mannose. For a lignocellulosic conversion process to be economically viable, both the cellulose and hemicellulose fractions must be utilized effectively. One conversion scheme applicable for this purpose is simultaneous saccharification and cofermentation (SSCF). In this process, cellulose and hemicellulose are hydrolyzed by "cellulase" enzyme to soluble sugars (hexose and pentose sugars), which are converted into desired products by microorganism that are present in the same vessel as the enzymes.

Production of ethanol by SSCF has been investigated rather extensively *(8–10)*. However, few studies have been made on lactic acid production from lignocellulosic substrates by this method. The key to the success of the SSCF is finding the right microorganism because very few organisms are known to efficiently utilize both hexoses and pentoses. A number of studies have been made to develop new strains for lactic acid cofermentation *(11–14)*. Among the promising strains for our purpose is *Lactobacillus pentosus (15–17)*. As a facultative heterofermentative species, *L. pentosus* ferments hexose (glucose) through the Embden–Meryerhof–Parnas (EMP) pathway under anaerobic conditions giving lactic acid as the sole product (homofermentation), and uses the phosphoketolase (PK) pathway for conversion of pentoses (xylose and arabinose) to equal moles of lactic acid and acetic acid (heterofermentation) *(3)*. Therefore, this species has a theoretical yield of 2 mol of lactic acid/mol of hexose and 1 mol of lactic acid/mol of pentose during anaerobic fermentation.

This investigation was focused on SSCF of the cellulose and hemicellulose in corn stover pretreated by aqueous ammonia for lactic acid production using *L. pentosus* bacteria ATCC 8041. Corn stover is one of the most abundant lignocellulosic feedstocks in the United States. In this work, corn stover was pretreated by the aqueous ammonia pretreatment method developed in our laboratory and designated as soaking in aqueous ammonia (SAA) *(10)*. Our recent study proved that SAA-treated corn stover retains most of glucan and xylan in the biomass, becomes highly susceptible to enzyme attack, and has low lignin content *(10,18)*, making the pretreated corn stover suitable as a SSCF substrate. The microorganism *L. pentosus* ATCC 8041 was originally introduced from the Spanish Collection of Type Cultures (Valencia, Spain; No. CECT-4023). It has been reported to work well for the cofermentation of glucose, xylose, and arabinose in hemicellulose hydrolyzate from trimming wastes of vine shoots *(17)*.

The primary objective of this study was to develop a SSCF process for production of lactic acid from SAA-treated corn stover based on the organism above. Attempts were also made to evaluate and refine the

SSCF bioprocess. For this purpose, statistical experimental design and a response surface methodology were used to analyze the effects of key variables on the SSCF process. Fed-batch operation was then applied to improve the process.

Materials and Methods

Feedstock and Chemicals

Corn stover was supplied by the National Renewable Energy Laboratory (NREL), Golden, CO, and stored at 5°C. The moisture content was 9–14%. The chemical composition of the feedstock was ([w/w], dry basis): 36.8% glucan, 21.7% xylan, 2.6% arabinan, 0.68% galactan, 0.3% mannan, and 17.2% lignin. Ammonia hydroxide (30% [w/w]) and MRS broth, the medium introduced by DeMan et. al. *(19)*, were purchased from Fisher Scientific Co., whereas yeast extract, corn steep liquor (CSL) (containing approx 50% [w/w] solids), and agar were purchased from Sigma Co (St. Louis, MO). The corn steep liquor was centrifuged at 3823 g for 20 min to separate the solids, and the supernatant—clarified corn steep liquor (cCSL)—was used as a nutritional supplement for SSCF. The recovery of the solids for marketing as animal feed has been proposed to improve wet-milling process economics *(20)*.

Aqueous Ammonia Treatment (SAA Pretreatment)

SAA treatment was conducted in a 600-mL stainless steel autoclave. Heating and temperature control were done in a GC oven (Varian Model 3700, Varian, Palo Alto, CA). The treatment conditions were: liquid-to-solids ratio of 10 ([w/w]; air-dried corn stover containing 40.0 g of solids soaked in 400.0 g of 15% [w/w] ammonia solution), 90°C, and 24 h. The treated materials were washed with deionized water until the pH became near neutral. The washed corn stover was then transferred onto a piece of cheesecloth, wrapped, and squeezed by hand to remove most of the free water. By this means the moisture content of the pretreated corn stover was reduced to 69–71%, and the recovery of the corn stover solids was 65% (w/w) on the basis of the untreated solids. The dewatered solids were then subjected to composition analysis and used as the substrate for SSCF experiments. The composition of the treated solids was ([w/w] dry basis): 54.4% glucan, 24.9% xylan, 3.1% arabinan, 1.0% galactan, 0.6% mannan, and 7.7% lignin.

Enzyme

The enzyme used in the SSCF experiments was Spezyme CP cellulase (Genencor Co, Palo Alto, CA). The cellulolytic activity (filter paper units [FPU]/mL) was determined using the NREL Standard Analytic Protocol 007 and found to be 30 FPU/mL *(20)*. This enzyme has proved capability of hydrolyzing both cellulose and hemicellulose in LCM *(22)*.

Inocula Preparation

The *L. pentosus* ATCC 8041 (CECT-4023, Valencia, Spain) strain was grown aerobically on plates made up of 5.5% (w/v) MRS broth and 1% (w/v) agar at 37°C for 36 h in the presence of 10% (v/v) carbon dioxide. A fresh colony was transferred to 10 mL of 5.5% (w/v) MRS medium that was placed in a 20-mL glass tube. The headspace of the test tube was filled with 10% (v/v) of carbon dioxide, capped and placed in an incubator, which was set at 37°C without shaking. After 12 h of growth, 1 mL of the medium was transferred to 100 mL of 5.5% (w/v) MRS medium in a 250-mL Erlenmeyer flask. The headspace of the flask was also filled with 10% (v/v) of carbon dioxide and incubated at the same condition for 12 h, and then the medium was used as inocula for SSCF experiments. The dry cell mass of the inocula was determined to be 2.1–2.3 g/L. All the medium and solutions were sterilized at 121°C for 10 min before inoculation.

SSCF in Batch Mode

SSCF batch experiments were carried out anaerobically in 250-mL Erlenmeyer flasks with a working volume of 100 mL. An Innova 4080 incubator shaker (New Brunswick Scientific Co., NJ) was used to control the temperature (37°C) and provide agitation (150 rpm). The addition of substrate (SAA-pretreated and water-washed corn stover) was based on 3.0 g of glucan input. The pH of the fermentation media was automatically controlled by addition of 6.0 g calcium carbonate. The predetermined, pretreated corn stover, calcium carbonate, yeast extract, and cCSL were added into the flasks and sterilized together at 121°C for 10 min. After the flasks cooled down, inocula and enzyme were added. The flasks were then flushed with sterile nitrogen, capped, and placed in the incubator to start the SSCF. Samples were taken at given times and after sampling, the flasks were flushed with nitrogen again to maintain the anaerobic environment.

SSCF in Fed-Batch Mode

Assays for fed-batch SSCF were run in duplicates. The start of the fed-batch experiments was generally identical to that of the batch experiments with the only exception that a double amount of calcium carbonate (12.0 *g*) was added for pH control. Feeding of solid was applied every 36 h. At each feeding, SAA-treated and water-washed corn stover containing 3.0 g glucan were added into each of the flasks along with 2 mL of diluted Spezyme CP enzyme having a specific activity of 7.5 FPU/mL. By this procedure, the enzyme loading was maintained at 5 FPU/g-glucan throughout the experiments. The flasks were flushed with nitrogen before being placed back into the incubator shaker.

Table 1
Factors and Levels in Statistical Experimental Design

		Levels				
Factor	Label	–2	–1	0	1	2
X1	Enzyme loading (FPU/g-glucan)	2.5	5	7.5	10	12.5
X2	Inoculum (% [v/v])	1	2	3	4	5
X3	Yeast extract (% [w/v])	0	0.2	0.4	0.6	0.8
X4	cCSL (% [w/v])	0	0.5	1.0	1.5	2.0

Statistical Experimental Design and Result Analysis

A central composite design *(23)* with eight star points and four replicates in the center point was used to identify the significance of the variables to lactic acid yield. The experimental runs were carried out in random order. The four independent variables were enzyme loading (X1), inocula size (X2), yeast extract concentration (X3), and cCSL concentration (X4); and their respective levels (uncoded and coded) are listed in Table 1. The results were assessed by using the SAS 9.1 ADX program (SAS Institute Inc., Cary, NC). A response surface analysis was applied to examine the feasibility of substituting an inexpensive nitrogen source (cCSL) for the more expensive yeast extract in SSCF.

Analysis

Vials containing slurry samples from SSCF flasks were boiled for 5 min to denature the enzyme and kill the cells. The boiled slurry samples were centrifuged at 60,000 g for 5 min, and the supernatant was taken for analyses of sugars and acids by high-performance liquid chromatography (HPLC) operated with Bio-Rad Aminex HPX-87H column (Bio-Rad Laboratories, Inc., Hercules, CA). The HPLC system includes Liquid Pump (LabAlliance, Accuflow Series III, State College, PA), RI detector (Shodex Model-71, NY), Autosampler (Alcott Chromatography, Model-718, Norcross, GA), and PeakSimple Chromatography Data System. The HPLC was operated at 85°C with a flow rate of 0.55 mL/min of DI water for sugar analysis, and at 65°C and 0.05 M H_2SO_4 as mobile phase for acid analysis. The carbohydrate, acetyl, and lignin contents in the solids were measured by following the NREL Standard Analytical Procedure *(21)*.

Results and Discussion

Formation of Sugars and Acids in SSCF Process

Figure 1 shows the time-course of sugars and acids in a replicated SSCF conducted in batch mode with 7.5 FPU/g-glucan, 3% (v/v) inoculum,

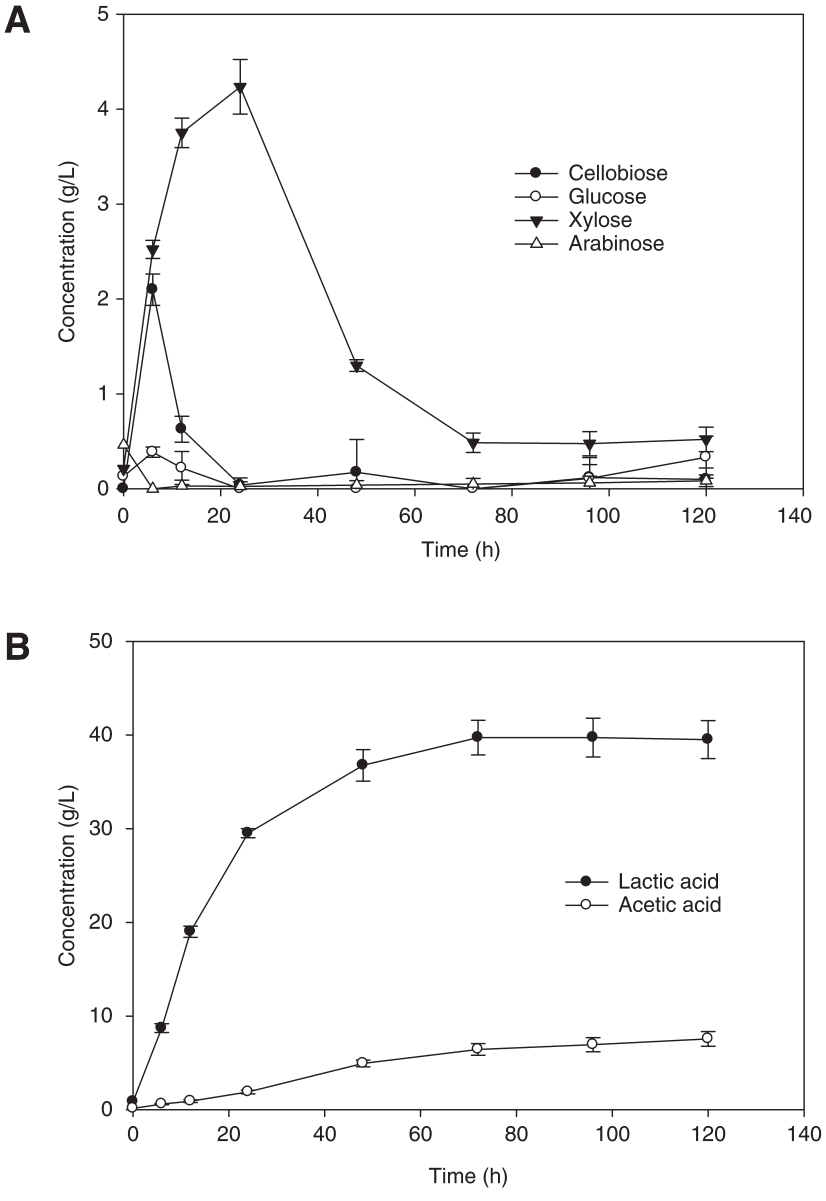

Fig. 1. Profiles of sugars **(A)** and acids **(B)** in SSCF of aqueous ammonia-treated corn stover. Average data from four replicates with standard deviations are presented. SSCF conditions: 3% (w/v) glucan loading, 7.5 FPU/g-glucan, 3% (v/v) inocula, 0.4% (w/v) yeast extract, 1% (w/v) cCSL, 37°C, pH 5.7, and 150 rpm.

0.4% (w/v) yeast extract, and 1% (w/v) cCSL. These data were used as the basis of the statistical experimental design (Table 1). The sugars and acids were traced from time zero when the inocula and enzyme were introduced. Glucose and arabinose remained at low levels throughout (<0.5 g/L), indicating that these sugars were efficiently assimilated after release from

biomass by hydrolysis (Fig. 1A). The cellobiose profile showed a similar pattern except that before 6 h, cellobiose accumulated up to 2.0 g/L because of the insufficient cellobiase activity of the cellulase enzyme and the lack of cellobiose-assimilating capability of this microorganism. The profiles of these three sugars (glucose, arabinose, and cellobiose) in Fig. 1 resemble those of most simultaneous saccharification and fermentation (SSF) or SSCF processes, wherein consumption of sugars are much more rapid than hydrolysis, the latter being the controlling step *(8,23,24)*. However, the assimilation of xylose was rather slow compared with that of glucose or arabinose. As the curve indicates, the xylose concentration increased almost linearly before 12 h and further increased up to 4.2 g/L before it started to decrease. Xylose consumption occurred shortly after depletion of glucose. This is a classic example of diauxic consumption of substrates wherein glucose is preferred to xylose. At this stage, glucose (and probably arabinose as well) was depleted, and xylose assimilation became the predominant reaction. Coinciding with the transition in sugar consumption, lactic acid production decelerated, whereas acetic acid accumulation accelerated (Fig. 1B). This is because xylose was converted into acetic acid in addition to lactic acid, whereas glucose assimilation under anaerobic conditions gave only lactic acid *(3)*.

Table 2 shows lactic acid and acetic acid yields as a percent of theoretical maximum under different SSCF conditions, as defined by the central composite design and represented by coded factors. Because galactose and mannose exist in very small quantities relative to glucose, xylose, and arabinose, only the latter three were used as the basis for yield calculations. The yields were calculated after deducting the contributions of acids at time zero, and assuming the assimilations of hexose (glucose) and pentoses (xylose and arabinose), respectively, follow EMP and PK pathways. The data indicate that the final (120 h) lactic acid yield fell between 0.79–0.92. It is also seen that the lactic acid yield can easily reach above 0.85 under most of the reaction conditions applied in this study. In addition, Table 2 shows that the acetic acid yield varies between 0.7 and 1.2, depending on the reaction conditions. Some of the acetic acid yields exceeded 1.0 probably because of difficulty of tightly controlling the anaerobic condition, which allowed seepage of small amount of oxygen into the flasks. This could divert part of the glucose to take PK pathway, leading to the production of acetic acid in addition to lactic acid *(1)*. Another source of the excess acetic acid production might be the conversion of galactose and mannose, which were present in small amount in the substrate and thus not accounted for in the yield calculation.

Figure 2 depicts the average yields of lactic acid and acetic acid from all 28 runs at different time intervals. The average lactic acid yield leveled off after 72 h, whereas the acetic acid yield continuously increased even after 120 h. The reason for the discrepancy is unclear at this point. We speculate that under high lactate/acetate concentrations, the metabolic pathway for

Table 2
Statistical Experimental Design and Acid Yields

Coded levels				Lactic acid (h)							Acetic acid (h)						
X1	X2	X3	X4	6 h	12	24	48	72	96	120	6	12	24	48	72	96	120
–1	–1	–1	–1	0.11	0.22	0.40	0.66	0.74	0.82	0.87	0.04	0.08	0.11	0.21	0.38	0.58	0.72
–1	–1	–1	1	0.11	0.28	0.53	0.77	0.88	0.89	0.90	0.05	0.10	0.17	0.47	0.72	0.85	0.95
–1	–1	1	–1	0.13	0.38	0.61	0.80	0.83	0.82	0.83	0.06	0.16	0.38	0.77	0.94	1.03	1.13
–1	–1	1	1	0.11	0.38	0.62	0.78	0.85	0.86	0.87	0.07	0.13	0.32	0.70	0.84	0.89	0.95
–1	1	–1	–1	0.14	0.27	0.52	0.73	0.82	0.86	0.90	0.07	0.10	0.18	0.43	0.65	0.79	0.86
–1	1	–1	1	0.15	0.32	0.56	0.71	0.76	0.77	0.80	0.09	0.15	0.26	0.60	0.92	1.10	1.21
–1	1	1	–1	0.16	0.40	0.61	0.77	0.85	0.83	0.83	0.08	0.17	0.39	0.73	0.95	1.02	1.19
–1	1	1	1	0.17	0.42	0.64	0.78	0.87	0.86	0.87	0.08	0.15	0.37	0.70	0.92	0.95	1.05
1	–1	–1	–1	0.12	0.26	0.49	0.70	0.78	0.81	0.85	0	0.03	0.06	0.24	0.47	0.66	0.80
1	–1	–1	1	0.13	0.33	0.60	0.76	0.89	0.91	0.91	–0.01	0.04	0.10	0.37	0.69	0.89	0.97
1	–1	1	–1	0.13	0.45	0.70	0.86	0.89	0.90	0.91	0.03	0.11	0.30	0.71	0.82	0.83	0.89
1	–1	1	1	0.13	0.48	0.74	0.89	0.92	0.88	0.90	0.03	0.11	0.35	0.76	0.93	0.96	1.18
1	1	–1	–1	0.15	0.30	0.52	0.73	0.8	0.86	0.91	0.03	0.05	0.05	0.25	0.48	0.66	0.77

1	1	−1	1	0.17	0.38	0.64	0.79	0.85	0.89	0.90	0.06	0.11	0.17	0.41	0.69	0.89	1.01
1	1	1	−1	0.21	0.52	0.73	0.82	0.89	0.89	0.89	0.09	0.15	0.39	0.75	0.89	1.03	1.07
1	1	1	1	0.20	0.53	0.78	0.91	0.93	0.92	0.92	0.08	0.15	0.44	0.82	0.89	0.89	0.98
−2	0	0	0	0.11	0.26	0.42	0.59	0.68	0.74	0.80	0.07	0.11	0.27	0.50	0.66	0.87	0.98
2	0	0	0	0.19	0.46	0.73	0.84	0.89	0.88	0.90	0.05	0.14	0.35	0.65	0.84	0.98	1.13
0	−2	0	0	0.09	0.35	0.62	0.76	0.91	0.84	0.90	0.02	0.10	0.22	0.64	0.92	0.91	1.03
0	2	0	0	0.20	0.45	0.68	0.89	0.87	0.90	0.85	0.09	0.15	0.33	0.80	0.96	1.08	1.25
0	0	−2	0	0.08	0.17	0.33	0.62	0.71	0.72	0.79	0.02	0.04	0.06	0.07	0.16	0.25	0.34
0	0	2	0	0.15	0.48	0.72	0.87	0.88	0.91	0.86	0.08	0.15	0.45	0.80	0.91	1.00	1.08
0	0	0	−2	0.16	0.36	0.59	0.68	0.82	0.81	0.86	0.08	0.13	0.25	0.60	0.88	0.91	0.99
0	0	0	2	0.15	0.43	0.69	0.88	0.89	0.84	0.86	0.07	0.12	0.30	0.74	1.02	1.09	1.24
0	0	0	0	0.18	0.41	0.65	0.78	0.79	0.80	0.79	0.09	0.12	0.30	0.76	1.01	1.10	1.19
0	0	0	0	0.17	0.41	0.64	0.84	0.90	0.90	0.92	0.07	0.15	0.23	0.65	0.80	0.84	0.92
0	0	0	0	0.15	0.40	0.63	0.79	0.88	0.89	0.88	0.06	0.10	0.26	0.66	0.90	0.98	1.09
0	0	0	0	0.16	0.38	0.64	0.83	0.88	0.90	0.89	0.07	0.11	0.26	0.70	0.91	0.99	1.11

Note: The assimilations of glucose and xylose are assumed to follow the EMP and PK pathways, respectively.

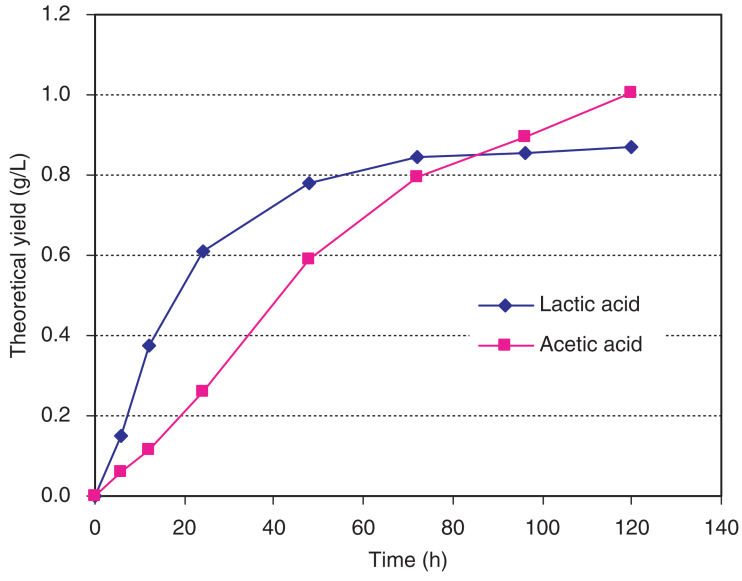

Fig. 2. Average acid yields from 28 runs at different time intervals. The assimilations of glucose and xylose are assumed to follow the EMP and PK pathways, respectively.

xylose utilization shifts significantly in such a way that acetic acid becomes the primary, if not the sole, product of the fermentation. As lactic acid is the product of interest in this study, the data obtained at 72 h is taken for response surface study.

Statistical Analysis and Response Surface

The data associated with lactic acid yield were analyzed, and the estimates of the main effects and the interactions of the factors are listed in Table 3, with the significance of each factor represented by probability levels (*p*-values). Among the four factors under investigation, enzyme loading (X1) and yeast extract concentration (X3) were the most important for lactic acid production, followed by cCSL concentration (X4) and inoculum size (X2). Besides the main effects, the quadratic term of enzyme loading (X1) also was found to be important in lactic acid production. It is notable that the inoculum size was insignificant for final lactic acid yield despite the fact that it affected lactic acid productivity during the early stage (<24 h), implying that inocula addition for the SSCF process can be maintained at a rather low level.

In general, lactic acid bacteria are nutritionally fastidious, and costly nitrogen sources such as yeast extract and peptone have been commonly provided for lactic acid bacteria to grow fast and function effectively *(26–28)*. In order to improve the process profitability, a number of efforts have focused on substituting inexpensive nitrogen sources. Corn steep liquor has been proposed as an alternative cost-effective nitrogen source for

Table 3
Coefficient Estimates of Polynomial Models (Using Coded Levels) for Lactic Acid Yield and Significance Examination

Term	12 h			24 h			48 h			72 h		
	Estimate	Standard error	*t* value	Estimate	Standard error	*t* value	Estimate	Standard error	*t* value	Estimate	Standard error	*t* value
X1	0.040408	0.002464	16.3964[a]	0.055415	0.006664	8.315467[a]	0.040398	0.009114	4.432355[a]	0.031773	0.008461	3.755105[b]
X2	0.022379	0.002464	9.080788[a]	0.017545	0.006664	2.63269[c]	0.011352	0.009114	1.245449	–0.003163	0.008461	–0.37387
X3	0.07632	0.002464	30.96807[a]	0.081493	0.006664	12.22858[a]	0.052529	0.009114	5.763356[a]	0.035861	0.008461	4.238344[a]
X4	0.020053	0.002464	8.136795[a]	0.030001	0.006664	4.501869[a]	0.029809	0.009114	3.270491[b]	0.020496	0.008461	2.422365[d]
X1 × X1	–0.00944	0.002464	–3.82926[b]	–0.01531	0.006664	–2.29738[c]	–0.020952	0.009114	–2.29878[c]	–0.016618	0.008461	–1.96404[d]
X1 × X2	0.003612	0.003018	1.196645	–0.002035	0.008162	–0.24932	0.004215	0.011163	0.377627	1.62E-05	0.010363	0.001562
X1 × X3	0.013821	0.003018	4.578959[a]	0.015459	0.008162	1.894112	0.014918	0.011163	1.336404	0.006814	0.010363	0.657579
X1 × X4	0.00314	0.003018	1.040322	0.007216	0.008162	0.884107	0.009693	0.011163	0.868298	0.007069	0.010363	0.68215
X2 × X2	0.000212	0.002464	0.086155	0.003968	0.006664	0.595405	0.006501	0.009114	0.71331	0.009067	0.008461	1.071636
X2 × X3	–2.20E-05	0.003018	–0.00727	–0.008185	0.008162	–1.00283	–0.007012	0.011163	–0.62817	0.008594	0.010363	0.8293
X2 × X4	0.000964	0.003018	0.319473	–0.002577	0.008162	–0.3157	–0.001427	0.011163	–0.12781	–0.015373	0.010363	–1.48345

(Continued)

Table 3 (Continued)

Term	12 h			24 h			48 h			72 h		
	Estimate	Standard error	*t* value	Estimate	Standard error	*t* value	Estimate	Standard error	*t* value	Estimate	Standard error	*t* value
X3 × X3	–0.01763	0.002464	–7.15521[a]	–0.026139	0.006664	–3.92241[b]	–0.012758	0.009114	–1.39975	–0.014194	0.008461	–1.67758
X3 × X4	–0.0109	0.003018	–3.61063[b]	–0.016209	0.008162	–1.98593	–0.007115	0.011163	–0.6374	–0.007789	0.010363	–0.75163
X4 × X4	–0.00017	0.002464	–0.07025	0.002656	0.006664	0.398492	–0.005246	0.009114	–0.57559	0.00112	0.008461	0.132371
R^2	–	0.9913	–	–	0.9554	–	–	0.8555	–	–	0.7992	–

[a] $p < 0.001$.
[b] $p < 0.01$.
[c] $p < 0.05$.
[d] $p < 0.1$.

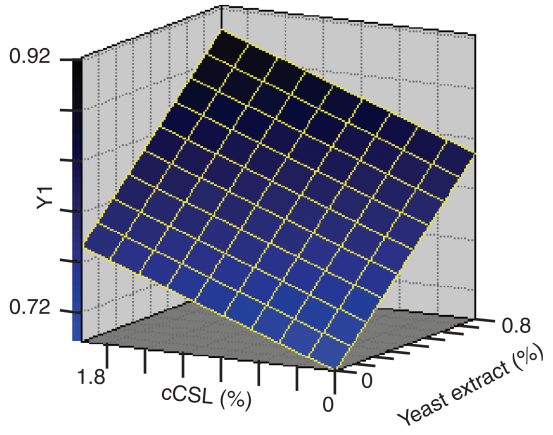

Fig. 3. Response surface representing lactic acid yield (Y1) as a function of yeast extract and cCSL additions. Enzyme loading: 5 FPU/g-glucan; inoculum size: 1% (w/w).

a range of microbes *(20,24,29,30)*. For example, Lawford and Rousseau *(20)* reported that in the SSCF biomass-to-ethanol process using recombinant *Zymomonas* yeast extract could be entirely replaced with corn steep liquor without affecting the growth and fermentation performance of the microorganism. Patel et al. *(13)* reported that in the lactic acid fermentation of hemicellulose hydrolyzate obtained from acid hydrolysis of sugar cane bagasse, a theoretical yield of 89% was attainable by using 0.5% corn steep liquor as the only organic nitrogen source for a thermotolerant acidophilic *Bacillus* sp.

In this study, cCSL was tested as a nutritional supplement for *L. pentosus* ATCC 8041. Figure 3 shows the response surface representing the 72-h lactic acid yield as a function of yeast extract and cCSL concentrations. The lactic acid yield increased linearly with both yeast extract and cCSL concentration, consistent with the almost linear effect of yeast extract concentration on lactic acid production by *L. casei* reported by Hujanen and Linko *(27)* using the same statistical analysis method. Further examination of the response surface shows that yeast extract can be replaced by cCSL, with an estimated ratio of 1 : 5 (g yeast extract/g cCSL), to achieve equivalent lactic acid yield.

Fed-Batch Experiment

Lactic acid concentration is a key factor affecting the costs of the downstream recovery process. In this study, the concentration of lactic acid was improved by using fed-batch technique. In accordance with the statistical analysis and taking the costs into consideration, the enzyme loading was set at 5 FPU/g-glucan and inoculum size 1% (v/v). The yeast extract was maintained at a low level of 0.2% (w/v), whereas cCSL was at a comparatively high level (2% [w/v]) to compensate for the low yeast extract concentration. Four batches of SAA-treated and washed corn stover were fed to the vessels at 0, 36, 72, and 108 h, resulting in a cumulative glucan addition of 12.0 g. The time intervals for substrate additions were chosen

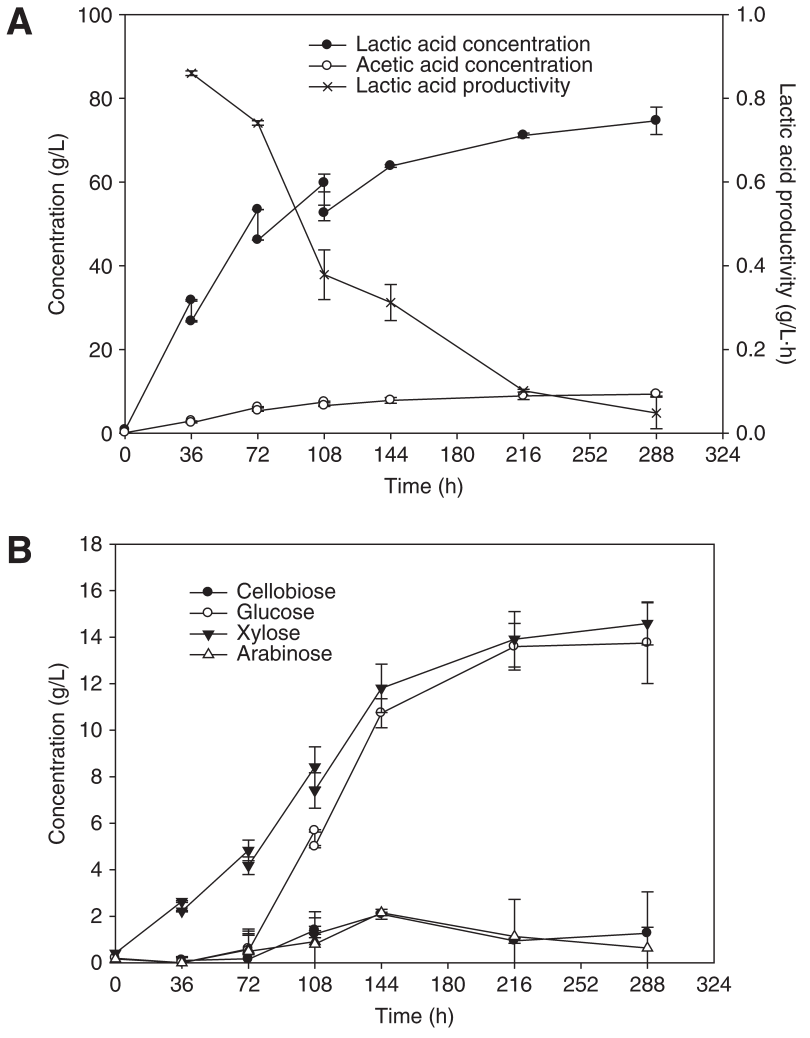

Fig. 4. Changes of sugar concentrations **(A),** acid concentrations and lactic acid productivity **(B)** in fed-batch SSCF experiments. Average data from duplicates with standard deviations are presented.

in such a manner that the previous batch of solids were well liquefied as evidenced by observation. Cellulase enzyme was added in a quantity to maintain its level in the SSCF media at 5 FPU/g-glucan.

Shown in Fig. 4B are the concentrations of lactic acid and acetic acid, as well as the lactic acid productivity in the fed-batch experiment. The abrupt drops at certain points (36, 72, and 108 h) were owing to the feeding operations, which suddenly increased the working volume of the SSCF medium. The maximum lactic acid concentration achieved in these experiments was 74.8 g/L. Lactic acid concentrations increased rapidly before 72 h with an average productivity of more than 0.7 g/L·h, indicating that the microorganisms exerted high activity at the early stage. This value is comparable

with that reported by Bustos et al. *(14)* in the fermentation of hemicellulose hydrolyzate from vine-trimming waste, which was 0.800 g/L·h after 24 h. On the other hand, the productivity of lactic acid in this study rapidly dropped with time. For example, at 108 h, the productivity decreased to 0.38 g/L·h, and further decreased to 0.05 g/L·h at 288 h. The final yield of lactic acid was determined to be 0.65 g/L. It is unlikely that the decrease in productivity resulted from the lack of available carbon sources in the fermentation medium, as evidenced by the accumulations of sugars in the reaction (Fig. 4A). Also, agar plates verified that the cells remained highly viable at the end of the operation (approx 10^6 colony forming units/mL). Therefore, it is concluded that the drastic decrease in lactic acid productivity was because of the strong inhibition of the elevated concentration of lactate/acetate anions to the lactic acid bacteria, consistent with the inhibitory effect of lactic acid (lactate ions) on *Lactobacillus* strains documented by others. Iyer and Lee *(5)* reported that the *L. delbrueckii* NRRL-B445 strain was strongly inhibited when lactic acid reached 65.0 g/L. Bustos et al. *(31)* observed marked inhibition to *L. pentosus* ATCC 8041 by lactic acid as the lactic acid concentration reached up to 46.0 g/L.

Besides the inferior glucose assimilation, xylose utilization was also inefficient, as can be seen from the comparatively low acetic acid concentration throughout the experiment (Fig. 4B) and the low final acetic acid yield (0.51 at 288 h). Therefore, if the complete utilization of carbohydrates is desirable, the inhibitory effect of the acid products must be taken into consideration before substrate additions. As an illustration, another series of fed-batch experiments was conducted with reduced substrate addition. In this experiment, SAA-treated and water-washed corn stover containing 6.0 g of glucan (in contrast to 12.0 g of glucan in the earlier fed-batch SSCF) was evenly added in two batches, one at time zero and the other at 36 h. The results are summarized in Fig. 5. Unlike in the earlier fed-batch SSCF, the xylose concentration decreased at the late stage of this experiment, although at a fairly low rate as compared with that of batch SSCF (Fig. 1A). At the end of the run (144 h), the lactic acid concentration reached 61.8 g/L, and the acetic acid 8.8 g/L. In comparison with the earlier fed-batch experiments, both lactic and acetic yields obtained in this experiment were remarkably higher (0.81 and 0.80 vs 0.65 and 0.61), indicating that inhibition was much less pronounced.

Conclusions

This study showed that SAA-pretreated corn stover is a substrate suitable for lactic acid production. The cellulose and hemicellulose fractions in the pretreated corn stover were effectively converted to lactic acid by SSCF. The maximum lactic acid yield was more than 90% of the theoretical maximum on the basis of all available fermentable sugars. The significances of enzyme, inocula, yeast extract, and cCSL for lactic acid production were

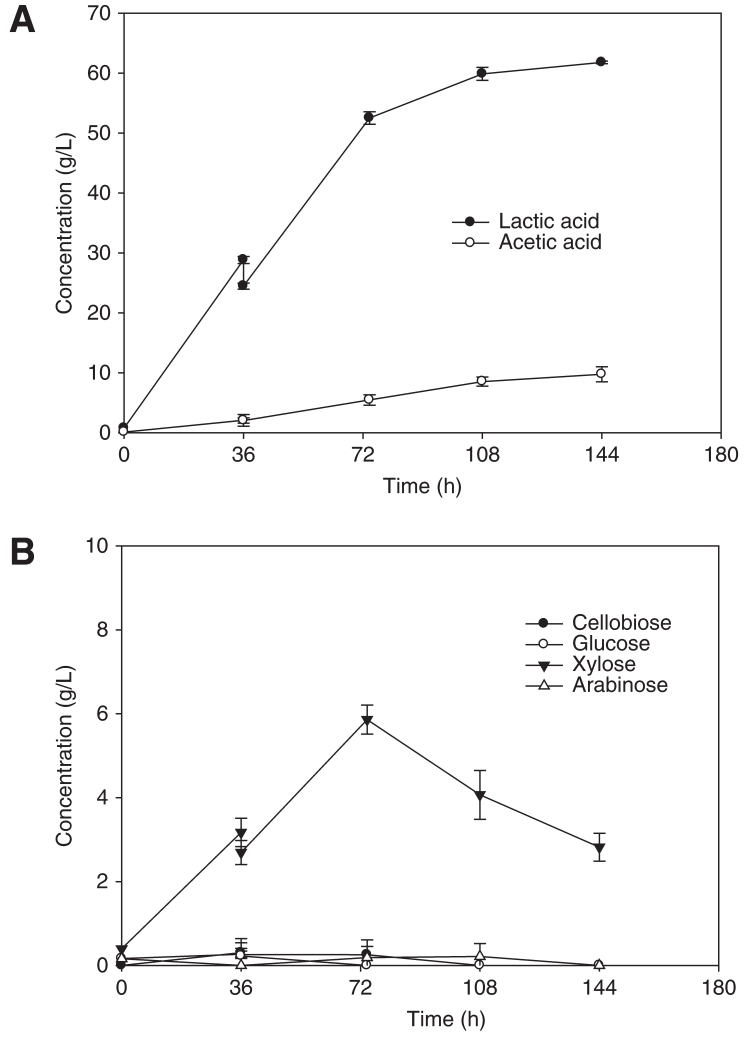

Fig. 5. Changes of sugar concentrations **(A)**, and acid concentrations **(B)** in reduced-feeding fed-batch SSCF experiments. Average data from duplicates with standard deviations are presented.

tested by way of statistical experiment design. It was found that the concentrations of enzyme and yeast extract are the two most significant factors affecting lactic acid yield. On the other hand, inocula size had an insignificant effect within the range of 1–5% (w/w). The response surface study indicated that yeast extract can be replaced by cCSL, without adversely affecting lactic acid yield. Acetic acid was also produced as a result of pentose assimilation (xylose and arabinose) through PK pathway. Fed-batch operation increased the lactic acid product concentration to 74.8 g/L, although at a relatively low yield (65%). Further improvement of the product concentration was difficult owing to end-product inhibition of the organism.

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