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Lactic acid production from corn stover using mixed cultures of *Lactobacillus rhamnosus* and *Lactobacillus brevis*

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ABSTRACT

Mixed cultures of *Lactobacillus rhamnosus* and *Lactobacillus brevis* was studied for improving utilization of both cellulose- and hemicellulose-derived sugars from corn stover for lactic acid production. During simultaneous saccharification and fermentation (SSF) of NaOH-treated corn stover by the mixed cultures, a lactic acid yield of 0.70 g/g was obtained, which was about 18.6% and 29.6% higher than that by single cultures of *L. rhamnosus* and *L. brevis*, respectively. Our results indicated that lactic acid yield from NaOH-pretreated corn stover by mixed cultures of *L. rhamnosus* and *L. brevis* was comparable to that from pure sugar mixtures (0.73 g/g of glucose/xylose mixture at 3:1 w/w).

1. Introduction

Lactic acid is a natural organic acid with a long history of application in the food, leather, cosmetic, and pharmaceutical industries (Wee et al., 2006). It has recently emerged as an important feedstock for the production of biopolymers and chemicals such as polylactide (PLA), acetaldehyde, acrylic acid, and 2,3-pentadione (Varadarajan and Miller, 1999). Chemcal synthesis and fermentation are two major methods for commercial production of lactic acid (Hofvendahl and Hahn-Hagerdal, 2000). The chemical synthesis process involves the production of lactonitrile followed by H_2SO_4 hydrolysis and usually produces a racemic mixture of L- and D-lactic acid (Narayanan et al., 2004). In contrast, a desired isomer of lactic acid can be produced via fermentation using selected-lactic acid-producing strains. Lactic acid production from various substrates (e.g., glucose, sucrose, cheese whey, corn starch, sugar cane, beets, and paper sludge) has been studied in the past decades to meet the increasing demands for lactic acid (Buyukkileci et al.,2004; Dumbrepatil et al., 2008; Marques et al., 2008; Mercier et al., 1992).

Lignocellulosic biomass, such as crop residues and forest waste, is a promising feedstock for lactic acid production due to its abundant availability. Pretreatment technologies including physical, chemical and thermochemical processes are necessary to break down the lignin and complex carbohydrates and consequently increase susceptibility of lignocellulosic biomass to hydrolytic enzymes (Hendriks and Zeeman, 2009). Alkaline pretreatment has shown to effectively improve the enzymatic hydrolysis of herbaceous biomass (Lau et al., 2008). The hydrolysate of lignocellusic biomass is a mixture of hexoses such as glucose, and pentoses such as xylose and arabinose. Efficient utilization of both cellulose- and hemicellulose-derived sugars has the possibility to reduce the cost of production of biobased chemicals by as much as 25% (Hinman et al., 1989). Most homofermentative strains of lactic acid bacteria (LAB), including *Lactobacillus delbrueckii* (Monteagudo et al., 1997), *Lactobacillus helveticus* (Tango et al., 2002), and *Lactobacillus acidophilus* (Portilla et al., 2008), can convert glucose to lactic acid, but not hemicellulose-derived sugars such as xylose or arabinose. In contrast, some heterofermentative LAB are capable of utilizing both hexoses and pentoses. For example, *Lactobacillus pentosus* ATCC 8041 has been used to convert hydrolysates of trimming vine shoots (Bustos et al., 2004) and corn cobs to lactic acid (Zhu et al., 2007). *Lactobacillus bifermentans* DSM 20003 was also reported to effectively convert a mixture of glucose, arabinose and xylose in the hydrolysate of wheat bran to lactic acid (Givry et al., 2008).

Mixed cultures or co-cultures of lactic acid-producing microorganisms may increase the conversion efficiency of substrates. Nancib et al. (2009) compared lactic acid production from date juice by single and mixed cultures of *Lactobacillus casei* and *Lactococcus lactis*. Lactic acid concentration of 60.3 g/L and glucose utilization efficiency of 96% were achieved with the mixed cultures

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of *L. casei* and *L. lactis* after 19-h incubation, whereas lactic acid concentrations of 53 and 42 g/L, and glucose utilization efficiency of 82.2% and 93.8% were obtained with single cultures of *L. casei* and *L. lactis*, respectively. When mixed cultures of *L. casei* and *L. delbrueckii* was used to convert cassava bagasse to lactic acid via simultaneous saccharification and fermentation (SSF), the highest lactic acid concentration of 81 g/L was achieved after 60-h cultivation (John et al., 2006). A co-culture of *Enterococcus casseliflavus* and *L. casei* was also reported to produce 95 g/L of lactic acid with consumption of 50 g/L of xylose and 100 g/L of glucose after 192 h of fermentation (Taniguchi et al., 2004).

Lactobacillus rhamnosus has been shown to produce lactic acid as the sole product from cheese whey (Petrov et al., 2006; Ho et al., 1997). *Lactobacillus brevis* ATCC367, a well-known heterofermentative strain, was reported to metabolize monomeric sugars such as glucose, xylose and lactose to lactic acid through the phosphoketolase (PK) pathway with acetic acid as a byproduct (Saier, 1998; Chaillou et al., 1998). We have not found any report on the lactic acid production from lignocellulosic biomass with mixed cultures of *L. rhamnosus* and *L. brevis*. It is our hypothesis that in the mixed culture system of *L. rhamnosus* and *L. brevis,* glucose can be converted to lactic acid by homofermentative LAB (*L. rhamnosus*) via the Embden-Meyerhof Pathway (EMP), while xylose and a limited amount of glucose can be converted to lactic acid and acetic acid by a heterofermentative strain (*L. brevis)* via the PK pathway. As a result, both cellulose- and xylan- derived sugars from lignocellulosic biomass can be utilized with higher efficiency and productivity. To test this hypothesis, single and mixed cultures of *L. rhamnosus* and *L. brevis* were studied for lactic acid production from a mixture of glucose and xylose (with ratio of 3:1 to mimic the composition of cellulosic biomass), and corn stover, respectively. The specific objectives of this study were to: (1) evaluate the performance of single and mixed cultures of *L. rhamnosus* and *L. brevis* for lactic acid production from a mixture of glucose and xylose; and (2) study lactic acid production from NaOH-treated corn stover with single and mixed cultures of *L. rhamnosus* and *L. brevis.*

2. Methods

2.1. Materials

Corn stover was obtained from a local farm in Wooster, OH, USA. It was air-dried, ground to pass through a 5 mm screen and stored at room temperature (22 °C) prior to use. The ground corn stover was pretreated with 1 N NaOH solution at room temperature for 12 h. The ratio of corn stover (dry basis) to NaOH solution was 1:10 (w/v). The pretreated corn stover was then washed with tap water to obtain a pH of approximately 7.0 (Li et al., 2004). The solid fraction was stored in a freezer at −20 °C for SSF tests. The main components of pretreated corn stover were 64.83% cellulose, 23.89% hemicellulose (20.20% xylan) and 11.61% lignin, which was determined based upon the laboratory analytical protocol (LAP) developed by National Renewable Energy Laboratory (NREL) (Sluiter et al., 2008). Spezyme CP with cellulase activity of 50 FPU/mL was kindly provided by Genencor International (Palo Alto, CA, USA). HPLC grade glucose, xylose, cellobiose, and lactic acid obtained from Sigma chemicals (St. Louis, MO, USA) were used for the preparation of HPLC standards. Other chemicals and media components were obtained from Fisher Scientific (Hanover Park, IL, USA).

2.2. Microorganism culture and media preparation

L. brevis ATCC 367 was kindly provided by the National Center for Agricultural Utilization Research, Peoria, IL, USA. *L. rhamnosus* strain was obtained from North Carolina A&T State University, Greensboro, NC, USA. It was streak purified and confirmed by Gram staining. Several independent colonies were analyzed by specific primers RhaI and RhaII of *L. rhamnosus* species (Tynkkynen et al., 1999). Further, the identity of this strain was confirmed by 16S rDNA sequencing to be *L. rhamnosus* (Hugenholtz et al., 1998). Stock cultures of these two strains were maintained at −20 °C in Man Rogosa Sharpe (MRS) media plus glycerol (20%, v/v).

L. rhamnosus or *L. brevis* was grown in modified MRS media at 37 °C for 18 h. The starting cell density of these strains for fermentation tests was adjusted to approximately 1.9×10^{11} CFU/mL for inoculation. MRS media contained 20 g/L glucose, 5 g/L yeast extract, 2 g/L K_2HPO_4, 0.05 g/L $MnSO_4{\cdot}H_2O$, 0.1 g/L $MgSO_4{\cdot}7H_2O$, 2 g/L NaAc, and 0.5 g/L L-cysteine-HCl·H_2O. When a mixture of glucose and xylose was fermented, the starting glucose and xylose concentrations were 15 and 5 g/L, respectively, which mimicked the ratio of glucan to xylan in lignocellulosic biomass. In the SSF test, pretreated corn stover at solid loading of 30 g/L was used as the carbon source. All the fermentation media were supplemented with 5 g/L yeast extract, 2 g/L K_2HPO_4, 0.05 g/L $MnSO_4{\cdot}H_2O$, and 0.1 g/L $MgSO_4{\cdot}7H_2O$.

2.3. Fermentation

For mixed sugar fermentation by a single strain of *L. rhamnosus* or *L. brevis*, 2 mL of inoculum was added into 45 mL vials containing 38 mL of fermentation medium. For the mixed culture test, 1 mL of *L. rhamnosus* and 1 mL of *L. brevis* were added. $CaCO_3$ (10 g/L) was added at the beginning of the lactic acid fermentation process to maintains the pH at around 5.0 (Hongo et al., 1986). The fermentation tests were conducted at 37 °C with shaking at 100 rpm for 36 h. Samples were taken at 0, 6, 12, 24, and 36 h for analysis using high performance liquid chromatography (HPLC).

Lactic acid production from corn stover by SSF was conducted in 250 mL flasks containing 100 mL media with 3% pretreated corn stover (w/v). SSF tests were started with the addition of cellulase at 25 FPU/g glucan and the single or mixed cultures of *L. rhamnosus* and *L. brevis* (2% inoculum size). The media was incubated at 37 °C with shaking at 100 rpm for 36 h. Samples were taken at 0, 6, 12, 24, and 36 h for HPLC analysis.

2.4. Analytical methods

The concentrations of glucose, xylose, and cellobiose were measured using HPLC (Agilent 1200 series, Santa Clara, CA, USA) equipped with a Biorad Aminex HPX-87P column and a refractive index detector (RID). The mobile phase was HPLC grade water at a flow rate of 0.6 mL/min. The temperatures of the column and detector were maintained at 80 °C and 55 °C, respectively. A Phenomenex Rezex RFQ-Fast Fruit H^+ column (Phenomenex Inc., Torrance, CA, USA) was used for determination of concentration of organic acids. The mobile phase for this column was 0.005 N H_2SO_4 at a flow rate of 0.6 mL/min. The temperatures of the column and detector were maintained at 55 °C and 45 °C, respectively.

Lactic acid productivity was defined as the amount of lactic acid produced per liter per hour. Lactic acid yield from the pure sugar was calculated by dividing the amount of lactic acid produced by the amount of sugar consumed. Lactic acid yield from corn stover was calculated by dividing the amount of lactic acid produced by the dry matter of NaOH-treated corn stover. The pretreatment and fermentation tests were run in triplicate. One-way analysis of variance was performed with SAS (Version 8.1, SAS Institute Inc, Cary, NC, USA).

3. Results and discussion

3.1. Lactic acid production from a mixture of glucose and xylose

Lactic acid production by *L. rhamnosus* and/or *L. brevis* from mixed glucose and xylose in the ratio of 3:1 (w/w) was first studied to mimic lactic acid production from lignocellulosic biomass. The lactic acid production from glucose and xylose mixtures is shown in Fig. 1 and summarized in Table 1. Glucose was completely utilized by *L. rhamnosus* or *L. brevis* after 24 h of fermentation. The lactic acid yield reached 0.79 and 0.58 g/g for *L. rhamnosus* and *L. brevis*, respectively. During 36-h fermentation, 13.44 g/L of lactic acid and 2.19 g/L of acetic acid were produced by *L. brevis* from the sugar mixture (Fig. 1b). Lactic acid productivity during 12 h reached 0.78 and 0.58 g/L.h by *L. rhamnosus and L. brevis*, respectively. *L. brevis* preferred glucose to xylose. However, *L. brevis* began to utilize xylose rapidly after 12 h of fermentation when the remaining glucose was 6.1 g/L. This fermentative pattern differs from other LAB reported in the literature. *L. brevis* ATCC 14869 was reported to consume glucose and xylose simultaneously by Kim et al. (2009), while *L. pentosus* ATCC 8041 only started to utilize xylose after depletion of glucose (Zhu et al., 2007).

Mixed cultures of *L. rhamnosus* and *L. brevis* were also tested for lactic acid production from mixed glucose and xylose. During both fermentation processes (Fig. 1c and 1d), glucose was completely converted after 24 h of inoculation, which is similar to single strain cultivations. For simultaneous inoculation of *L. rhamnosus* and *L. brevis,* xylose conversion was initiated at 6 h and almost completed at 24 h. Lactic acid concentration reached 14.80 g/L after 36 h of fermentation. A lactic acid yield of 0.73 g/g was obtained in 36 h of fermentation (Fig. 1c). When *L. brevis* was inoculated after 12 h of fermentation, the glucose conversion rate in the first 12 h was lower than that of the simultaneous inoculation. After 36 h of fermentation, 13.44 g/L of lactic acid was produced, which was lower than that obtained with simultaneous inoculation of the two strains (14.80 g/L). There was about 1.98 g/L xylose left (Fig. 1d). The acetic acid produced with mixed cultures of *L. rhamnosus* and *L. brevis* was about 1.2 g/L for both strategies, which was lower than that of the single culture of *L. brevis* (2.2 g/L). In the study by Taniguchi et al. (2004), lactic acid production of 95 g/L was obtained in 192 h with two-stage inoculation of *E. casseliflavus* and *L. casei (E. casseliflavus* was added 49 h after the inoculation of *L. casei)* with the complete consumption of 50 g/L of xylose and 100 g/L of glucose. The lactic acid production of 70 g/L was obtained with simultaneous inoculation of these two strains and the residual concentration of xylose was about 28 g/L after 192 h fermentation (Taniguchi et al., 2004). It can be concluded that selection of LAB strains for the mixed cultures and inoculation strategy was essential for the high efficient conversion of lignocellulosic hydrolyzed sugars to lactic acid.

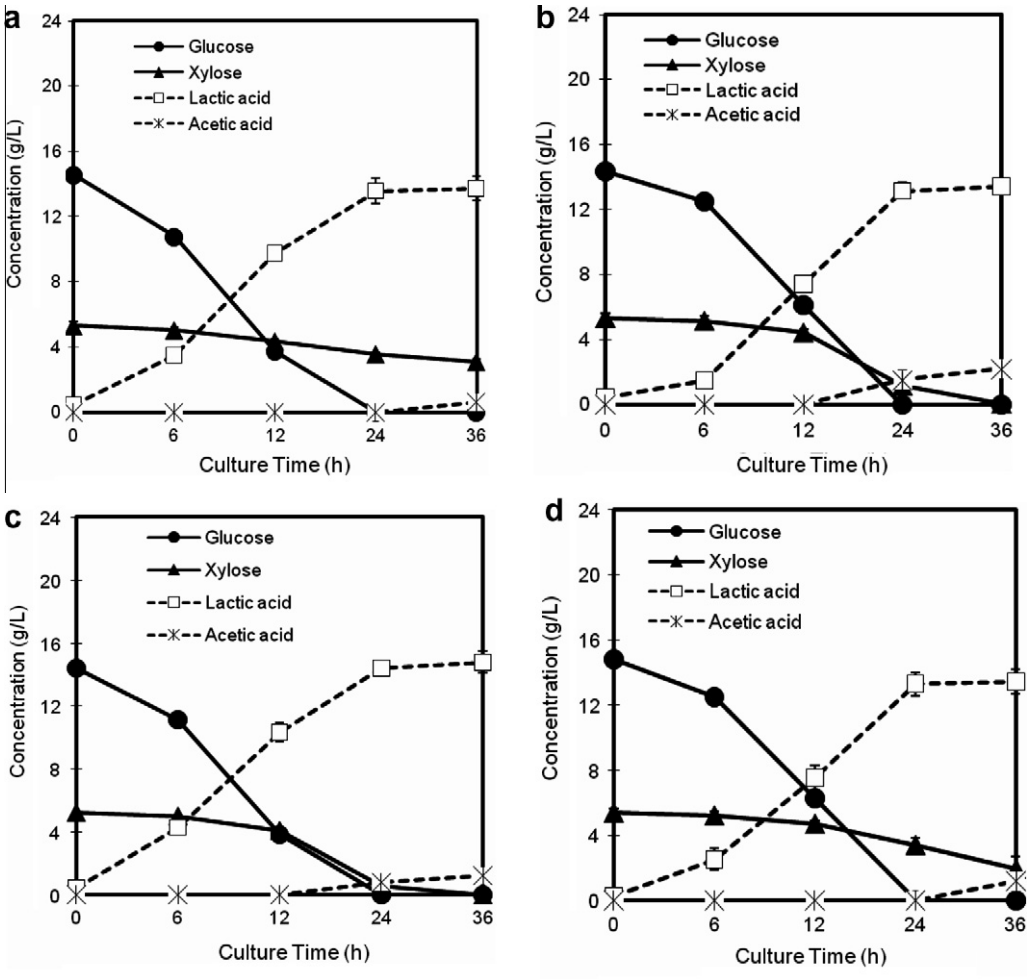

Fig. 1. Lactic acid production from the mixture of glucose and xylose by *L. rhamnosus* (a), *L. brevis* (b), simultaneous inoculation of *L. rhamnosus* and *L. brevis* (c), or inoculation *L. brevis* 12 h after *L. rhamnosus* (d).

Table 1
Summary of lactic acid production from the mixture of glucose and xylose by single strain or mixed cultures of *L. rhamnosus* and *L. brevis*.

Culture	Lactic acid concentration (g/L)	Acetic acid concentration (g/L)	Lactic acid yield* (g/g)	Lactic acid productivity (g/L.h)
L. rhamnosus	13.73 ± 0.75	0.60 ± 0.03	0.79 ± 0.04	0.37 ± 0.02
L. brevis	13.44 ± 0.49	2.19 ± 0.01	0.67 ± 0.04	0.36 ± 0.01
L. rhamnosus + *L. brevis*	14.80 ± 0.69	1.22 ± 0.02	0.73 ± 0.02	0.40 ± 0.05
L. rhamnosus + *L. brevis* (12 h)	13.44 ± 0.77	1.21 ± 0.24	0.72 ± 0.05	0.37 ± 0.02

* Lactic acid yield was based on the total consumed glucose and xylose.

3.2. Lactic acid production from corn stover

One of the barriers for lactic acid production from lignocellulosic biomass is the low efficiency in conversion of hemicellulose-derived sugars to lactic acid. After NaOH treatment, the lignin content in the solid fraction of corn stover was reduced from 21.16% to 11.61%. As a result, the cellulose content increased from 41.15% to 64.83%. The theoretical sugar yields from NaOH-pretreated corn stover were 21.60 g/L of glucose and 6.89 g/L of xylose. Single strain or mixed cultures of *L. rhamnosus* and *L. brevis* were inoculated with two strategies (simultaneous inoculation and two-step inoculation) for lactic acid production from corn stover similar to those used for mixed sugar fermentation.

Lactic acid production from NaOH-pretreated corn stover during SSF by single or mixed cultures is shown in Fig 2. There was no sugar detected at the beginning of the SSF tests. Glucose and cellobiose accumulated to the maximum level at 6 h in all experiments, indicating that at the initial SSF stage the conversion rates of sugars were lower than the releasing rates of sugars from NaOH-treated corn stover. Concentrations of glucose and cellobiose started to decrease after 6 h of cultivation. The products of enzymatic hydrolysis, such as glucose and cellobiose, are the main inhibitors to cellulase (Adsul et al., 2007). Inhibition by glucose was reduced as a result of conversion to lactic acid, but inhibition by cellobiose remained. To reduce the cellobiose concentration, cellobiase might have to be added to the fermentation (Moldes et al., 2001), but inclusion of cellobiase will increase production costs.

When the single strain of *L. rhamnosus* was inoculated in the SSF process (Fig. 2a), xylose concentration increased during the fermentation process and reached 4.87 g/L at 36 h, which is about 72% of the theoretical hydrolysis yield of xylose. When *L. brevis* was used for the SSF process (Fig. 2b), xylose concentration reached a maximum level of 0.66 g/l at 12 h of cultivation and

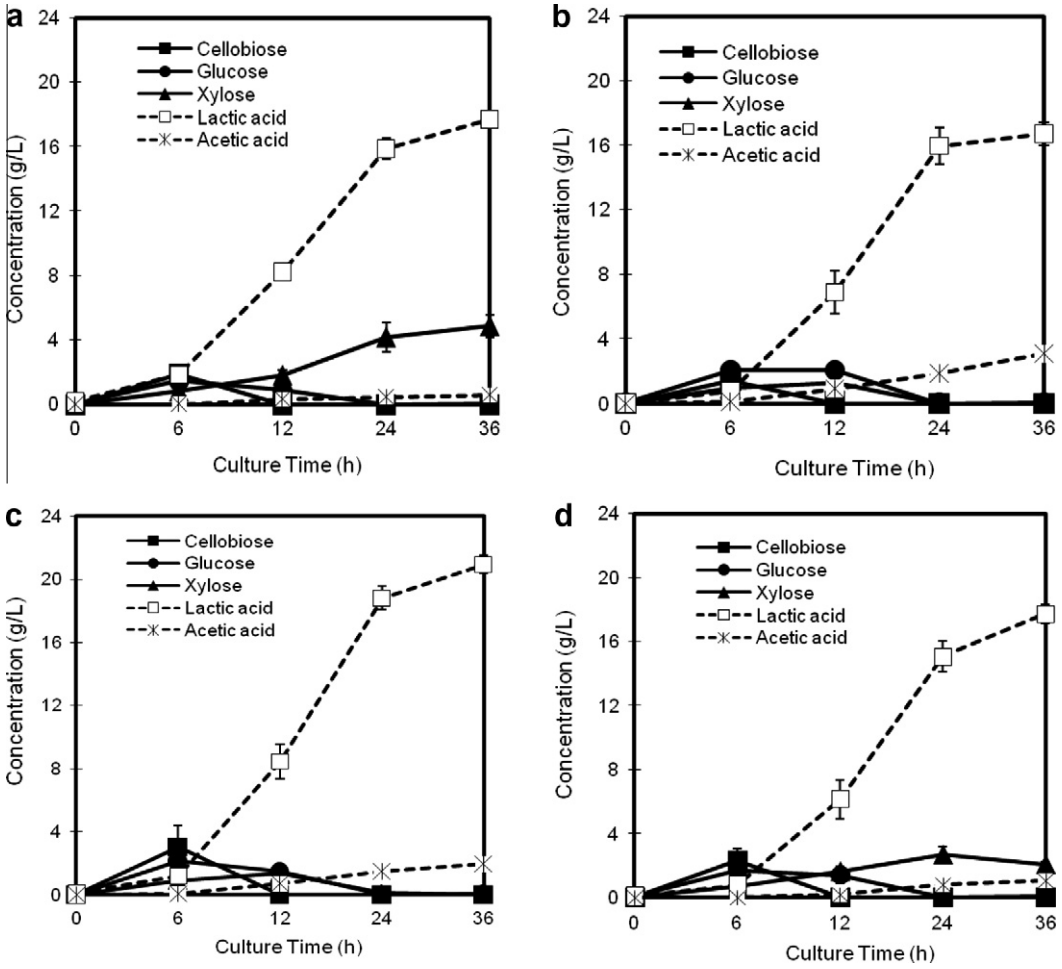

Fig. 2. Lactic acid production from NaOH-treated corn stover by *L. rhamnosus* (a), *L. brevis* (b), simultaneous cultivation of *L. rhamnosus* and *L. brevis* (c), or cultivation *L. brevis* 12 h after *L. rhamnosus* (d).

Table 2
Summary of lactic acid production of single strain *L. rhamnosus* or *L. brevis* or mixed cultures from NaOH-treated corn stover.

Culture strategy	Lactic acid concentration (g/L)	Acetic acid concentration (g/L)	Lactic acid yield on substrate (g/g)*	Lactic acid productivity (g/L.h)
L. rhamnosus	17.70 ± 0.46	0.74 ± 0.04	0.59 ± 0.02	0.49 ± 0.04
L. brevis	16.71 ± 0.73	3.10 ± 0.18	0.54 ± 0.02	0.45 ± 0.02
L. rhamnosus + *L. brevis*	20.95 ± 0.54	1.97 ± 0.12	0.70 ± 0.03	0.58 ± 0.03
L. rhamnosus + *L. brevis* (12 h)	17.75 ± 0.60	1.05 ± 0.10	0.59 ± 0.02	0.49 ± 0.04

* Lactic acid yield was based on the total weight of NaOH-treated corn stover added.

there was no accumulation of xylose after 24 h. The results indicate that *L. brevis* starte to convert xylose rapidly after 12 h.

When *L. rhamnosus* and *L. brevis* were inoculated simultaneously at the beginning of the SSF test, glucose and cellobiose reached a maximum level of 1.69 and 1.49 g/L at 6 h of fermentation, respectively (Fig. 2c) and then were rapidly utilized by the mixed cultures. The xylose reached a maximum concentration of 1.36 g/L at 12 h of cultivation and it was completely converted after 24 h of cultivation (Fig. 2c). When *L. brevis* was inoculated at 12 h (Fig. 2d), xylose was increasing from the beginning of the test and reached a maximum level of 3.34 g/L at 24 h of the test. At 36 h, there was still 2.13 g/L of xylose left. The acetic acid production profile was also affected by the timing of *L. brevis* inoculation. When *L. rhamnosus* and *L. brevis* were inoculated simultaneously at the beginning of the SSF test, the acetic acid was detected at 6 h and increased to 1.97 g/L at 36 h (Fig. 2c). When *L. brevis* was inoculated at 12 h of the test, acetic acid was detected at 12 h and reached 1.05 g/L at 36 h (Fig. 2d).

The performance of *L. rhamnosus*, *L. brevis* or the mixed cultures of these two strains for lactic acid production from NaOH-treated corn stover was similar to the fermentation of the sugar mixture (Table 2). High selective conversion of glucose to lactic acid was obtained with *L. rhamnosus*. The lactic acid and acetic acid concentrations at the end of 36 h fermentation with *L. rhamnosus* were 17.70 and 0.74 g/L, respectively. When *L. brevis* was used, both the glucose and xylose were completely converted in 36 h. At the end of the fermentation, the lactic acid and acetic acid concentrations were 16.71 and 3.10 g/L respectively. The lactic acid productivity was 0.49 and 0.45 g/L·h for *L. rhamnosus* and *L. brevis*, respectively. The lactic acid productivity was lower than that reported by Zhu et al. (2007). An average lactic acid productivity of more than 0.7 g/L·h was obtained with *Lactobacillus pentoses* ATCC-8041 in the first 72-h fermentation when NaOH-treated corn stover was converted (Zhu et al., 2007). The lower lactic acid productivity in our study was mainly attributed to the relatively low substrate concentration (3%).

Simultaneous inoculation with a mixed cultures of *L. rhamnosus* and *L. brevis* substantially improved lactic acid production. A lactic acid yield of 0.70 g/g and a productivity of 0.58 g/L·h were obtained during 36 h of fermentation. This result was comparable with that reported by Nancib et al. (2009) in the fermentation of data juice extract with mixed cultures of *L. casei* and *L. lactis*. The highest lactic acid concentration of 60.3 g/L was obtained in the mixed culture system compared to the single culture fermentations of *L. casei* or *L. lactis* with maximum lactic acid concentrations of 53 and 46 g/L, respectively. Two-stage inoculation did not benefit the lactic acid production and xylose conversion. A lactic acid yield of 0.59 g/g and a productivity of 0.49 g/L·h were obtained during 36 h of fermentation.

4. Conclusion

Efficient utilization of both hexoses and pentoses in cellulose and hemicellulose is of significance for the economical conversion of lignocellulosic biomass to biochemicals. Lactic acid yield of 0.70 g/g and productivity of 0.58 g/L·h were obtained from NaOH-treated corn stover with a mixed culture of *L. rhamnosus* and *L. brevis*. The lactic acid productivity can be further increased with an increase in substrate loading. The acetic acid concentration (1.97 g/L) of the mixed culture system was lower than that obtained with *L. brevis* only (3.10 g/L).

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