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BIOTECHNOLOGICAL PRODUCTS AND PROCESS ENGINEERING

Conversion of rice straw to bio-based chemicals: an integrated process using *Lactobacillus brevis*

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David A. Mills

Abstract Commercialization of lignocellulosic biomass as a feedstock for bio-based chemical production is problematic due to the high processing costs of pretreatment and saccharifying enzymes combined with low product yields. Such low product yield can be attributed, in large part, to the incomplete utilization of the various carbohydrate sugars found in the lignocellulosic biomass. In this study, we demonstrate that *Lactobacillus brevis* is able to simultaneously metabolize all fermentable carbohydrates in acid pre-processed rice straw hydrolysate, thereby allowing complete utilization of all released sugars. Inhibitors present in rice straw hydrolysate did not affect lactic acid production. Moreover, the activity of exogenously added cellulases was not reduced in the presence of growing cultures of *L. brevis*. These factors enabled the use of *L. brevis* in a process termed simultaneous saccharification and mixed sugar fermentation (SSMSF). In SSMSF with *L. brevis*, sugars present in rice straw hydrolysate were completely utilized while the cellulase maintained its maximum activity due to the lack of feedback inhibition from glucose and/or cellobiose. By comparison to a sequential hydrolysis and fermentation process, SSMSF reduced operation time and the amount of cellulase enzyme necessary to produce the same amount of lactic acid.

Keywords *Lactobacillus brevis* · Carbon catabolite repression · Simultaneous carbohydrate utilization · Lignocellulosic biomass · SSMSF

Introduction

Commercial lactic acid production largely relies on bacterial fermentation of glucose or maltose derived from starch biomass (Datta et al. 1995). However, use of lignocellulosic biomass has some additional advantages (Galbe and Zacchi 2002). Lignocellulosic biomass is an inexpensive, abundant, and underutilized renewable feedstock derived from a number of sources including agricultural and forestry residues, food and beverage processing wastes, municipal wastes, pulp and paper industry wastes, and dedicated energy crops. The available lignocellulosic biomass in US is estimated at 200 million tons and is increasing (Bashir and Lee 1994). In addition to the large supply of biomass, utilization of lignocellulose can augment waste treatment and provide economical benefit to society in terms of reducing solid wastes, carbon dioxide, and greenhouse gases (Hatti-Kaul et al. 2007).

Lignocellulosic biomass is a complex polymer composed of cellulose, hemicelluloses, and lignin. One of the major challenges in using lignocellulosic biomass as a feedstock is the inherent heterogeneity of its sugar composition. Cellulose

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is a glucose polymer with β(1→6) glycoside linkage and hemicellulose is a heteropolymer containing hexose and pentose sugars. To maximize product yield and productivity from lignocellulose, the complete utilization of mixed sugars is essential (Saha 2003). In addition, lignocellulosic hydrolysate contains cell growth inhibitors such as polyphenolics and furfurals (Nilvebrant et al. 2001; Bjorklund et al. 2002). Ideally, the saccharification of lignocellulosic materials and fermentation of mixed sugars could be integrated into a single processing step in order to increase the productivity and to reduce the requirement of enzymes for hydrolysis. An approach taken to achieve this goal involves both strain and process development. The ideal strain would be able to consume all sugars present simultaneously and operate at the optimal temperature conditions needed for cellulase activity. Moreover, the strain would need to be resistant to any and all inhibitors present in lignocellulose (Houghton et al. 2006).

Many microorganisms are able to utilize the pentose sugars derived from lignocellulose; however, in most cases, pentose catabolism is suppressed by glucose also present in the mixture (Stulke and Hillen 1999). This phenomenon is termed carbon catabolite repression (CCR). When a CCR-positive strain is used for bio-based chemical production from mixed sugar substrates, the overall process design is restricted and productivity is reduced because of the sequential utilization of the sugars. Recently, we demonstrated that *L. brevis* exhibits an atypical utilization of mixed sugars, with no apparent CCR, and is able to simultaneously consume all sugars that can be utilized as a single carbon source (Kim et al. 2009).

In this study, *L. brevis* was examined as a fermentative host under the conditions of simultaneous saccharification and fermentation of acid-pretreated rice straw in a fed-batch mode. The fermentation kinetics were evaluated for simultaneous mixed sugar fermentation. The impact of potential inhibitory compounds from acid-pretreated rice straw on lactic acid production by *L. brevis* was also investigated. Finally, enzyme hydrolysis and mixed sugar fermentation by *L. brevis* was integrated and optimized. The integrated process, simultaneous saccharification and mixed sugar fermentation (SSMSF), was performed in batch and fed-batch mode with rice straw hydrolysate.

Materials and methods

Bacterial strains, culture media and conditions

L. brevis IFO 3960 was obtained from the Institute for Fermentation, Osaka (Osaka, Japan). *L. brevis* NRRL 1834 and NRRL 1836 were obtained from the Agricultural Research Service culture collection (Peoria, IL, USA).

L. brevis ATCC 14869 (type strain) were purchased from the American Type Culture Collection (Manassas, VA, USA). MRS medium (15.0 g l^{-1} of bactopeptone, 5.0 g l^{-1} of yeast extract, 2.0 g l^{-1} of ammonium citrate, 5.0 g l^{-1} of sodium acetate, and 2.0 g l^{-1} of dipotassium phosphate) with 20.0 g l^{-1} of glucose was used for the cell growth and maintenance. In this study, glucose was not included in MRS unless stated. Carbon sources were prepared separately and mixed with inoculum. The initial pH of the medium was set at 6.0 and the temperature was maintained at 37 °C. Fermentations were initiated by adding a 5% (v/v) inoculum.

Preparation of rice straw hydrolysate

Acid-pretreated rice straw was prepared by BC International (now Verenium Corporation). Rice straw was cleaned, chopped, and then hydrolyzed by diluted sulfuric acid. During the acid treatment, lignin and hemicelluloses in rice straw were hydrolyzed. The resulting mixture contained cellulose fiber, solid silica particles, phenolic compounds from lignin, and xylose and arabinose from hemicellulose. The total carbohydrate content in the acid-pretreated rice straw was approximately 50% (w/w) based on dry substrate mass (Table 1).

Two different kinds of enzyme-hydrolyzed rice straw were prepared for the inhibition study. Acid-pretreated rice straw was suspended in the 0.05 M sodium acetate (pH 5.0) and the insoluble fraction was separated by centrifugation. The insoluble pellet, mainly cellulose fiber and silica particles, was transferred to filter paper (Whatman paper No.1) and washed twice with distilled water followed by the ethanol. After washing, the insoluble fraction was hydrolyzed in 0.05 M sodium acetate buffer (pH 5.0) with

Table 1 Composition of acid-pretreated rice straw

Components			Contents (w/w)	
Water[a]			41.6±0.9	
Sugar[b]	Cellulose	Glucose[c]	10.6±2.2	(25.7±2.2)[f]
	Hemicellulose[d]	Xylose	4.4±1.3	(10.6±0.9)[f]
		Arabinose	4.9±0.4	(11.8±0.4[f]
Lignin, ashes[e]			21.5±0.9	(51.9±0.9)[f]

[a] Measured by the weight difference between before and after drying at 120 °C for 24 h

[b] No other carbohydrate was observed by HPLC analysis (<0.1 g/L)

[c] Cellulose was completely hydrolyzed by cellulase/cellobiase complex

[d] Hemicellulose was completely hydrolyzed during acid pretreatment

[e] Ashes includes silica particles

[f] Weight percentage based on dry mass

50 filter paper unit per gram substrate (FPU g-substrate^{-1}) of cellulase (Spezyme CP; Genencor International, Palo Alto, CA, USA) and 50 cellobiose unit per gram substrate (CBU g-substrate^{-1}) of cellobiase (Novozyme 188; Novozymes, Franklinton, NC, USA) at 50 °C for 24 h (Vlasenko et al. 1997). This hydrolysate was designated 'washed hydrolysate'. In a second treatment, acid-pretreated rice straw was simply hydrolyzed without filtration in 0.05 M sodium acetate buffer with 50 FPU g-substrate^{-1} of cellulase and 50 CBU g-substrate^{-1} of cellobiase at 50 °C for 24 h. This hydrolysate was designated 'unwashed hydrolysate'. Since free xylose and arabinose were washed away during filtration step, 'washed hydrolysate' contains only glucose (bound as cellulose) as a potential fermentation carbon source. Conversely, 'unwashed hydrolysate' contained all the components from rice straw including glucose, xylose, arabinose, phenolic derivatives from lignin and solid particles.

Enzyme hydrolysis of acid-pretreated rice straw for fermentation studies

The acid-pretreated rice straw was suspended in 0.05 M sodium acetate buffer (pH 5.0) with 50 FPU g-substrate^{-1} and 50 CBU g-substrate^{-1} of cellulase and cellobiase, respectively. After enzyme hydrolysis, MRS medium was added and *L. brevis* was inoculated. Fermentation was carried out with 100 g-dry mass l^{-1} of acid-pretreated rice straw using a BioFlo 3000 Bioreactor (New Brunswick Scientific, Edison, NJ, USA). Temperature was maintained at 37 °C and pH was controlled at 6.0 by 10 N NaOH. Agitation rate was set at 100 rpm without aeration.

Simultaneous saccharification and mixed sugar fermentation

A fed-batch operation of SSMSF was carried out in a BioFlo 3000 Bioreactor (New Brunswick Scientific, Edison, NJ, USA) with 2.5 L of initial volume. In MRS medium, 80 g-dry mass l^{-1} of acid-pretreated rice straw was suspended and fermentation was initiated by adding a 2% (*v/v*) inoculum with 10 FPU g-substrate^{-1} of cellulase and 10 CBU g-substrate^{-1} of cellobiase. Sixty grams (dry mass) per liter of acid-pretreated rice straw was added directly into the fermentation for second round of fermentation. The fermentation pH and temperature were maintained at 6.0 by 10 N NaOH and 37 °C, respectively. To measure the substrate and the end-product concentrations, samples were taken from fermentation broth and solid materials were removed by centrifugation at 13,000 rpm (Eppendorf, Hamburg, Germany). The supernatant was filtrated by 0.2 μm syringe filter (Millipore, Billerica, MA, USA) for further analysis by HPLC.

Enzyme stability tests

To examine the stability of enzyme mixture during *L. brevis* cell growth, 800 FPU and 800 CBU of cellulase and cellobiase were added to 450 ml of modified MRS medium adjusting the final volume to 500 ml. To prevent feedback inhibition of cellulase or cellobiase by glucose, 20 g l^{-1} of xylose was used to support *L. brevis* growth instead of glucose. Culture medium was taken every 2 h and optical density of cells and total enzyme activity were measured as described previously by Vlasenko and coworkers with the following modification (Vlasenko et al. 1997). After removal of cells by centrifugation, 1.0 ml of supernatant was mixed in 3.0 ml of 0.05 M sodium acetate buffer (pH 5.0) with the addition of 0.4 g of filter paper. The final enzyme and substrate concentrations were 1.44 FPU (or CBU) g-substrate^{-1} and 100 g l^{-1} substrate, respectively. Hydrolysis was performed at 50 °C. One hundred microliters of supernatant was taken from this sample every 30 min for a 2 h period. The reaction was stopped by boiling, and the glucose concentration of supernatant was measured by HPLC. The total enzyme activity was defined as an initial glucose production rate (g-glucose l^{-1} h^{-1}). All experiments were performed in triplicate.

Analysis of substrate and fermentation end-products

The concentrations of substrates and fermentation end-products were analyzed by HPLC (Shimadzu, Kyoto, Japan) using a BioRad HPX-87H column (BioRad, Hercules, CA, USA). One milliliter of fermentation broth was centrifuged at 13,000 rpm for 10 min and the supernatant was transferred to a new microcentrifuge tube prior to analysis. For HPLC analysis of supernatant, the BioRad HPX-87H column was heated at 65 °C and a refractive index (RI) detector was used for the identification of substrate(s) and product(s). As a mobile phase, 0.01 N H$_2$SO$_4$ was used, and the flow rate was 0.6 ml min^{-1}. The standard deviation of the HPLC concentration determinations for any substrate or end-product was within ±5 mM. To determine cell density, the cell pellet was resuspended in the same volume of deionized water and the optical density (OD) was measured using a Beckman DU 7400 spectrophotometer (Beckman, Fullerton, CA, USA) at 600 nm.

Calculation of kinetic values

The specific cell growth rate was calculated using linear regression of the natural log of cell growth (OD) versus time during exponential growth phase ($r^2 > 0.95$). The average specific substrate utilization (q_s) and product

formation rates (q_p) were approximated during the exponential phase using following equations:

$$q_s = \frac{1}{x}\frac{dS}{dt} \approx \frac{\Delta S}{X \times \Delta t} = \frac{1}{n}\sum_{i=1}^{n}\frac{(S_i - S_{i+1})}{(t_{i+1} - t_i)\{(X_i + X_{i+1})/2\}}$$

$$q_p = \frac{1}{x}\frac{dP}{dt} \approx \frac{\Delta P}{X \times \Delta t} = \frac{1}{n}\sum_{i=1}^{n}\frac{(P_{i+1} - P_i)}{(t_{i+1} - t_i)\{(X_i + X_{i+1})/2\}}$$

where n, X, S, and P stand for the number of segments, cell mass (optical density of cell), the concentration of substrates, and the concentration of products, respectively. All experiments were performed in duplicate or triplicate.

Results

The effects of substrate composition: co-utilization of xylose, arabinose, and glucose

The characteristics of mixed sugar fermentation derived from lignocellulosic biomass were investigated in MRS medium. *L. brevis* was able to co-metabolize glucose/xylose (Fig. 1a), glucose/arabinose (Fig. 1b), and glucose/xylose/arabinose (Fig. 1c). Lactic acid and acetic acid (and/or ethanol) were produced in an equimolar ratio. No other major by-products were observed by HPLC analysis. In all cases, cell growth continued exponentially for an initial 24 h and then ceased. The specific cell growth rates in exponential phase were ~0.17 h^{-1} regardless of sugar compositions in the media (Table 2).

Although *L. brevis* simultaneously utilized these three sugars without apparent catabolite repression, the preferences between sugars were clearly observed. As shown in Table 2, the molar ratios of substrate consumed were 1.0:1.8 for glucose/xylose and 1.0:2.7 for glucose/arabinose indicating the preference for pentose sugars over glucose. The sugar consumption ratios were maintained in the glucose/xylose/arabinose mixture showing the ratio of 1.0:1.3:2.4 (glucose/xylose/arabinose). Meanwhile, the specific consumption rates of total sugars were similar in any composition of sugar mixtures (Table 2).

Interestingly, the pentose preference was consistently observed regardless of the concentration ratio between glucose and xylose (Table 3). Transport of both glucose and xylose in *L. brevis* is mediated by facilitated diffusion, using the concentration gradient across the cell membrane (Ye and Saier 1995; Chaillou et al. 1998). Regardless of the concentration ratio between glucose and xylose, the specific consumption rates of glucose and xylose did not change (1.0:1.6 glucose:xylose). Moreover, the total sugar consumption rates were similar as 5.0 mM sugar OD^{-1} h^{-1}
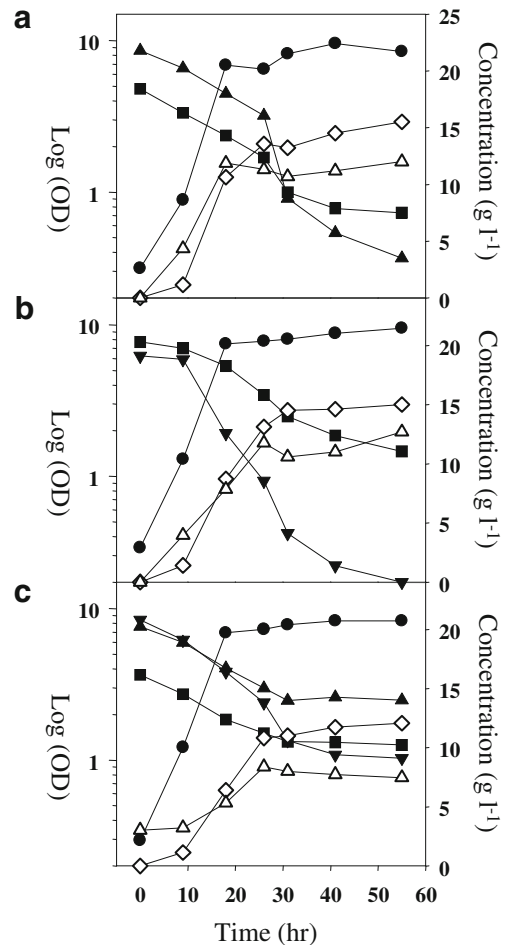

Fig. 1 Simultaneous utilization of **a** glucose/xylose, **b** glucose/arabinose, and **c** glucose/xylose/arabinose mixture by *L. brevis* in MRS medium. *filled circle* Optical density of cell at 600 nm, *filled square* glucose, *filled upright triangle* xylose, *filled inverse triangle* arabinose, *empty diamond* lactic acid, *empty upright triangle* acetic acid

regardless of the concentration ratio between glucose and xylose. Moreover, these total sugar consumption rates of glucose and xylose mixtures were also similar to that of glucose when employed as a single carbon source. As expected, the utilization of pentose shifted the production of two-carbon products from ethanol to acetate. The final concentration of ethanol was four times higher than that of acetate when glucose was used as a single carbohydrate. Conversely, acetate was produced three times more than ethanol when xylose and glucose were co-utilized. The cell growth rates were similar regardless of the composition of sugars and their concentrations suggesting that *L. brevis* maintains a constant metabolic activity.

The impact of temperature on the growth of *L. brevis*

In order to integrate enzyme hydrolysis and fermentation, new operational variables needed to be determined. For

Table 2 Kinetic parameters and substrate consumption ratio of simultaneous fermentation of glucose, xylose and arabinose mixture

Carbon source	Specific cell growth rate[a] (h^{-1})	Specific total carbohydrate consumption rate[b] (nM OD^{-1} h^{-1})	Used substrate ratio[c]		
			Glucose	Xylose	Arabinose
Glucose/xylose	0.17	0.81±0.07	1	1.83	
Glucose/arabinose	0.17	0.82±0.26	1		2.69
Glucose/xylose/ arabinose	0.18	0.82±0.38	1	1.26	2.40

[a] Specific growth rate was measured at the exponential growth phase

[b] The values were obtained by averaging the data during one batch fermentation. Repeated experiments confirmed same trait

[c] Individual sugar substrates were consumed more than 80% of initial content

example, the optimum temperature of the enzyme hydrolysis of cellulosic biomass is 50 °C where most of lactic acid bacteria cannot survive. Four independent strains of *L. brevis* were cultivated at three different temperatures from 30 to 50 °C. None of the strains survived at 50 °C. As shown in Table 4, *L. brevis* exhibited higher activity for carbohydrate metabolism, but a slower cell growth rate, at 40 °C than those at 30 °C. The specific cell growth rates at 30 °C were 1.5~1.8-fold higher than at 40 °C. However, the specific rates of both substrate utilization and lactic acid production by *L. brevis* NRRL 1836 and NRRL 1834 increased as much as up to 2.6-fold at 40 °C.

Increased growth temperature impacted the metabolic flux distribution of *L. brevis*. The glucose consumption rates of strains IFO 3960 and NRRL 4527 increased 1.6- and 2.0-fold, respectively. However, the specific lactate formation rates increased only 1.1- and 1.8-fold at 40 °C suggesting that a portion of three-carbon intermediates was redirected away from lactate. Indeed, the lactate yields of strain IFO 3960 and NRRL 4527 decreased from 1.0 and 0.7 at 30 °C to 0.75 and 0.64 at 40 °C, respectively. The increased growth temperature impacted the formation of two-carbon end-products as well (Table 5). The change of acetic acid and ethanol yields indicated that two-carbon flux was redirected from the acetic acid to the ethanol-producing pathway. The acetic acid yields at 40 °C were decreased to 50~80% of those at 30 °C and the ethanol yields at 40 °C were at least two times higher than those at 30 °C. For instance, *L. brevis* NRRL 4527 did not produce ethanol at 30 °C, however the yield at 40 °C was 0.65 (mM/mM).

The change of two-carbon metabolic flux implies that *L. brevis* had an increased requirement for NADH regeneration at the higher temperature. Redirection of metabolic flux from acetate production to ethanol production is expected to result in a reduction of ATP production. Instead, *L. brevis* accelerated the catabolic activity of the cell, and presumably ATP production, at the higher growth temperature since both the specific substrate consumption and product formation rates were increased up to 2.6-fold. However, the cell growth rate of *L. brevis* decreased at the higher temperature suggesting ATP produced from a central metabolism was used for cell maintenance rather than cell growth.

Table 3 The cell growth, specific substrate consumption and specific product formation rates in different concentration of glucose and xylose

Carbon source (mM)		Specific growth rate[a] (h^{-1})	Specific substrate consumption rate[b] (mM substrate OD^{-1} h^{-1})			Specific product formation rate[b] (mM product OD^{-1} h^{-1})		
Glucose	Xylose		Glucose	Xylose	Total carbohydrate[c]	Lactic acid	Acetic acid	Ethanol
300	0	0.18	4.69±1.39	0.00	4.69	4.85±1.69	1.14±0.87	4.60±1.58
200	100	0.19	1.87±0.59	3.08±1.11	4.95	4.15±1.76	3.31±1.69	1.21±0.57
150	150	0.18	1.94±1.05	3.02±1.15	4.97	4.51±2.05	3.51±1.88	1.34±0.66
100	200	0.18	1.90±1.00	3.11±1.18	5.01	4.54±1.96	3.57±1.72	1.20±0.33
0	300	0.16	0.00	4.39±1.68	4.39	3.17±0.75	3.14±0.86	0.04±0.08

Total sugar concentration was maintained at 300 mM

[a] The specific cell growth rate was obtained at the exponential cell growth phase

[b] Specific rates were calculated as described in "Materials and methods" during the exponential phase

[c] The total carbohydrate consumption rate was the sum of specific glucose and xylose consumption rate

Table 4 The effect of temperature on the cell growth, substrate utilization and product formation rates

Strains	Specific cell growth rate[a] (h^{-1})			Specific substrate consumption rate[b] (mM glucose OD^{-1} h^{-1})			Specific product formation rate[c] (mM lactate OD^{-1} h^{-1})		
	30 °C	40 °C	Ratio	30 °C	40 °C	Ratio	30 °C	40 °C	Ratio
IFO 3960	0.34	0.22	0.65	0.28±0.02	0.44±0.14	1.57	0.28±0.16	0.28±0.18	1.11
NRRL 4527	0.36	0.24	0.68	0.24±0.05	0.50±0.01	2.01	0.18±0.05	0.32±0.01	1.78
NRRL 1834	0.45	0.24	0.54	0.29±0.06	0.36±0.12	1.24	0.26±0.08	0.32±0.15	1.23
NRRL 1836	0.44	0.29	0.65	0.32±0.08	0.83±0.32	2.59	0.29±0.08	0.75±0.37	2.58

[a] The specific cell growth rates were measured at the exponential phase. Cell growth was not observed at 50 °C. Duplicated experiments showed the same results within ±0.08

[b] The specific substrate consumption and product formation rates were measured at the exponential phase where the specific cell growth rates were measured

The impact of temperature on enzymatic hydrolysis

The impact of temperature on the enzymatic hydrolysis of cellulose fiber in acid-pretreated rice straw was examined. Acid-pretreated rice straw was resuspended with an initial concentration of 150 g-dry mass l^{-1} at 50 °C and 100 g-dry mass l^{-1} for 40 °C and 30 °C due to the high viscosity of hydrolysates at low temperature. Final glucose concentrations per gram input substrate were similar at the three different temperatures (roughly 0.35~0.38 g-glucose per g-dry mass of substrate). Glucose concentration did not further increase after 24 h of hydrolysis implying the complete digestion of cellulose fiber in the substrate (Fig. 2a). After 12 h of hydrolysis, glucose concentration reached at 90% of maximum regardless of temperature.

To determine the total activity of cellulase and cellobiase mixtures at different temperatures, glucose production rates during hydrolysis were calculated (Fig. 2b). The initial glucose production rates were 8.0 g l^{-1} h^{-1} at 50 °C and 4.1 g l^{-1} h^{-1} at 30 °C and 40 °C. Although the initial glucose production rate at 50 °C was two times higher than at 30 °C and 40 °C, a significant drop was observed in enzyme activity due to the inhibition by glucose produced. After 4 h of initial hydrolysis, the glucose production rate decreased to 1.0 g-glucose l^{-1} hr^{-1} at all three temperatures.
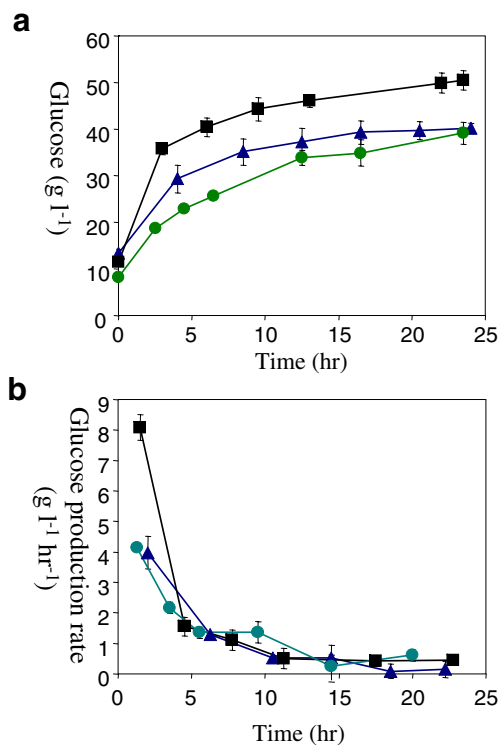
The effect of rice straw hydrolysate: potential growth inhibitors

Plant cell hydrolysate contains inhibitors of microbial growth including soluble phenolic compounds derived

Fig. 2 Enzymatic hydrolysis of acid-pretreated rice straw at 50 °C (*filled square*), 40 °C (*filled upright triangle*) and 30 °C (*filled circle*). **a** The profile of glucose production and **b** glucose production rate (g-glucose production l^{-1} h^{-1})

Table 5 The effect of temperature on the product yields of heterofermentation by *L. brevis* strains

Strain	$Y_{lactate}$		$Y_{acetate}$		$Y_{ethanol}$	
	30 °C	40 °C	30 °C	40 °C	30 °C	40 °C
IFO 3960	1.00	0.70	0.91	0.76	0.12	0.27
NRRL 4527	0.75	0.64	0.68	0.39	0.00	0.65
NRRL 1834	0.90	0.89	0.43	0.20	0.00	0.35
NRRL 1834	0.91	0.90	0.43	0.24	0.27	0.40

Product yields were measured after 24 h at 30 °C and 12 h at 40 °C. Repeated experiments verified the same results within the range of ±0.09. The yields were calculated mole basis

from lignin and acid-induced reaction products of sugars such as furfural or 5-hydroxymethylfurfural (Larsson et al. 2000; Zaldivar et al. 2000; Martinez et al. 2001). Moreover, rice straw possesses large amounts of silica particles that can physically damage microbial cells during the vigorous mixing of a fermentation. Several pretreatment processes have been developed to eliminate such inhibitors and to improve fermentability of hydrolyzed biomass, however, limitations still exist in a large-scale process (Larsson et al. 1999; Nilvebrant et al. 2001; Bjorklund et al. 2002; Persson et al. 2002). These physical and chemical inhibitors can be removed by filtration followed by the acid hydrolysis of rice straw. However, additional filtration step makes the overall process more complicated and expensive. In addition, the pentose sugars from hemicellulose are removed during this process. Given that pentose content up to 50% of available carbohydrates in rice straw hydrolysate, a loss of pentose sugar should not be ignored for the fermentation of lignocellulosic biomass.

To examine the tolerance to the potential inhibitors, *L. brevis* ATCC 14869 was cultivated with "washed" and "unwashed" acid-pretreated rice straw and the lactic acid production was monitored. Surprisingly, the loss of the soluble fraction of acid-pretreated rice straw by washing and filtration resulted in a negative impact on the lactic acid production. Although the production rates of lactic acid were similar between washed and unwashed substrate, the lag time in washed substrate was longer and the final lactic acid concentration was lower than those in unwashed substrate (Fig. 3). Furthermore, the lactic acid production profile in unwashed substrate was similar to that obtained from the rich medium (MRS). These results suggest that any potential inhibitors in acid-pretreated rice straw do not hamper the cell growth and sugar metabolic activity of *L. brevis*.
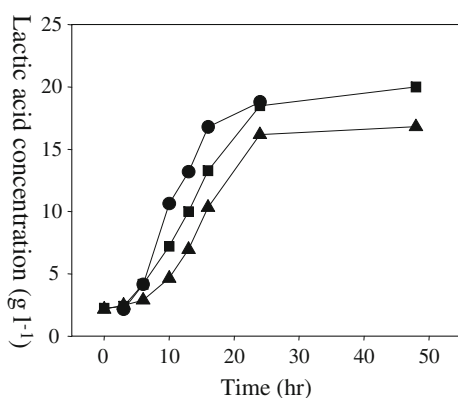
Enzyme stability during the fermentation

Enzyme stability during the fermentation plays a key role in maximizing the operational efficiency and cost effectiveness of an integrated bio-based process. Ideally, glucose production during integrated process has to be high enough to avoid limiting growth of *L. brevis* and yet low enough to avoid feedback inhibition of the cellobiase. In this respect, a constant and optimal glucose production achieved by controlling total enzyme activity is an important factor in determining the overall performance of the process. In order to examine the enzyme stability during fermentation, total enzyme activity was monitored with and without the inoculation of *L. brevis*. Xylose was added as a growth substrate instead of glucose to avoid feedback inhibition of cellobiase by glucose. As shown in Fig. 4, cell growth did not impact enzyme activity. After 24 h of cultivation, total enzyme activity was maintained at 8.0 g-glucose l^{-1} h^{-1} regardless of the *L. brevis* growth phase. Without inoculation, the enzyme activity was maintained constant as well.

Sequential hydrolysis and fermentation of acid-pretreated rice straw

Sequential hydrolysis and fermentation (SHF) was then performed using acid-pretreated rice straw. To hydrolyze 100 g-dry mass l^{-1} of the acid-pretreated rice straw, 50 FPU g-substrate^{-1} of cellulase and 50 CBU g-substrate^{-1} of cellobiase were added. Hydrolysis was carried out in the fermentation vessel for 12 h to allow the complete degradation of cellulose fiber (Fig. 5, section A). After heat sterilization (Fig. 5, section B), MRS medium lacking glucose was directly added to the fermentation vessel and *L. brevis* was inoculated (Fig. 5, section C). The fermentation was complete 24 h after inoculation. During this time period, 25.3 g l^{-1} of glucose, 18.4 g l^{-1} of xylose and 13.5 g l^{-1} of arabinose were utilized simultaneously and 30.5 g l^{-1} of lactic acid, 16.7 g l^{-1} of acetic acid, and 5.2 g l^{-1} of ethanol

Fig. 3 The effect of soluble growth inhibitors in acid-pretreated rice straw on the lactic acid production by *L. brevis*. MRS Medium with unwashed acid-pretreated rice straw (*filled square*) and washed acid-pretreated rice straw (*filled upright triangle*) were prepared as describe in Materials and Methods. Control fermentation (*filled circle*) was performed in MRS with glucose as sole carbon source
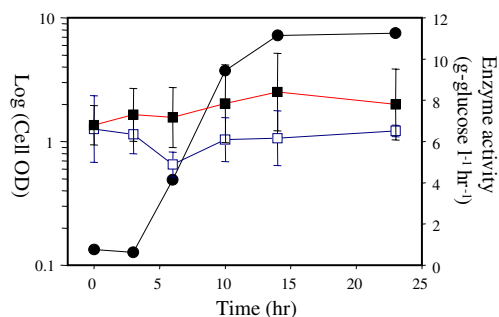
Fig. 4 The stability of cellulase and cellobiase mixture during the fermentation of *L. brevis* in the MRS medium. *filled circle* Cell growth, *filled square* enzyme activity with the cell growth, *empty square* enzyme activity without the cell growth

were produced. The yields of lactic acid, acetic acid, and ethanol were 0.92, 0.63, and 0.32 (mM/mM), respectively. Fermentation profiles and productivity using hydrolyzed rice straw as a substrate were similar to those observed with MRS medium containing an equivalent amount of glucose, xylose, and arabinose (data not shown).

Simultaneous saccharification and mixed sugar fermentation in batch mode

Enzyme hydrolysis of cellulosic fiber and fermentation of lignocellulosic sugars were then integrated into a single process termed "simultaneous saccharification and mixed sugar fermentation". SSMSF was performed in the batch mode at the 200 ml scale with 100 g-dry mass l^{-1} of acid-pretreated rice straw. To take advantage of the integrated process, 10 FPU g-substrate^{-1} of cellulase and 10 CBU g-substrate^{-1} of cellobiase were added in MRS medium instead of 50 FPU (or CBU) g-substrate^{-1} was used in the SHF of acid-pretreated rice straw. Temperature was maintained at 37 °C and pH was set at 6.0 initially.

Although the 40 °C was determined to be optimum temperature for the integrated process (as discussed above), the SSMSF was performed at 37 °C due to the unique characteristics of the initial rice straw hydrolysate medium. When the acid-pretreated rice straw was resuspended in MRS, the initial medium was a viscous semi-solid paste due to the high concentration of cellulose fiber and silica. At low agitation rate, these characteristics impacted temperature control and permitted temperature fluctuations above 40 °C which negatively impacted *L. brevis* cell growth (data not shown). As a consequence, 37 °C was determined to be a better set point when working with this unusual substrate thereby preventing temperature fluctuations rising above 40 °C.

As a control experiment, enzyme hydrolysis was performed in same condition without an inoculation of *L. brevis*. Figure 6a and b shows the fermentation profile of SSMSF and the enzyme hydrolysis, respectively. In SSMSF, glucose concentration increased during the initial 5 h from 1.9 to 5.2 g l^{-1}, then reduced to 2.0 g l^{-1} until 34 h of fermentation (Fig. 6a). During this time period, xylose and arabinose concentration decreased concurrently. Lactic acid, acetic acid, and ethanol concentration increased only for the initial 34 h then remained constant suggesting fermentation arrest. Since
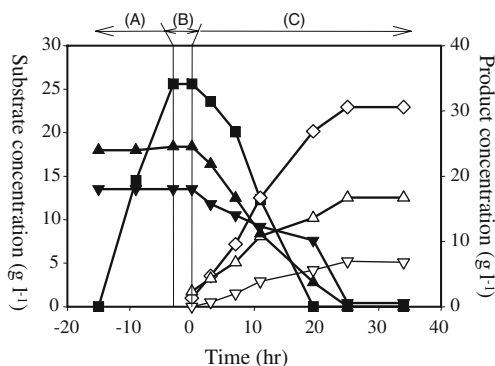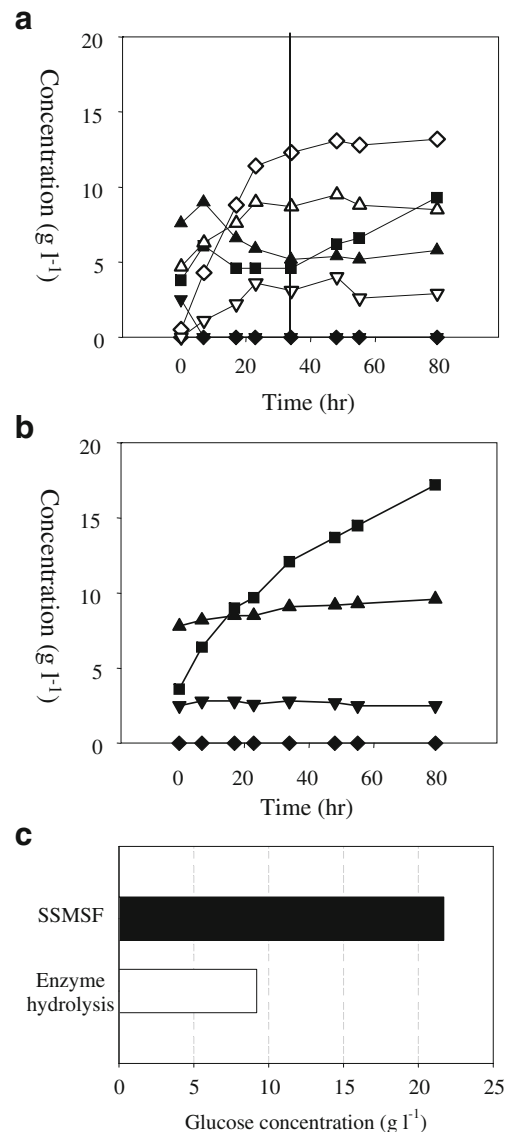

Fig. 5 Sequential hydrolysis and fermentation of acid-pretreated rice straw by *L. brevis*. The interval indicates *A* the hydrolysis of rice straw by cellulose and cellobiase mixture *B* the heat sterilization and cooling, and *C* the fermentation of rice straw hydrolysate. *filled square* glucose, *filled upright triangle* xylose, *filled inverse triangle* arabinose, *empty diamond* lactate, *empty triangle* acetate, *empty inverse triangle* ethanol

Fig. 6 Simultaneous saccharification and mixed sugar fermentation of acid-pretreated rice straw in flask culture without pH control. **a** Simultaneous enzyme hydrolysis and mixed sugar fermentation, **b** enzyme hydrolysis without inoculation, **c** the comparison of glucose production during 80 h of SSMSF and simple enzyme hydrolysis. *filled square* Glucose, *filled upright triangle* xylose, *filled inverse triangle* arabinose, *empty diamond* lactate, *empty upright triangle* acetate, *empty inverse triangle* ethanol

the pH was not controlled during the process, the fermentation ceased most likely due to the low pH and product inhibition by lactic acid. However, enzyme hydrolysis of cellulose fiber continued after fermentation ceased leading to an increase in glucose concentration after 34 h. During the 34 h of fermentation, 3.8 g l^{-1} of xylose and 2.5 g l^{-1} of arabinose were consumed with 12.6 g l^{-1} of lactic acid, 4.8 g l^{-1} of acetic acid and 4.0 g l^{-1} of ethanol produced.

The control experiment that was performed without inoculation exhibited inefficient hydrolysis of cellulose fiber due to the sub-optimum temperature, improper mixing, and, most likely, feedback inhibition (Fig. 6b). During 80 h of hydrolysis, glucose concentration increased only 14.7 g l^{-1}.

A stoichiometric calculation derived from the lactic acid produced during 34 h of SSMSF indicated that, at a minimum, 21.7 g l^{-1} of glucose was consumed even though the observed change of glucose concentration was only 0.1 g l^{-1}. During the same time frame, only 9.2 g l^{-1} of glucose was produced from enzyme hydrolysis alone indicating that the SSMSF produced 2.4-fold more glucose than the enzyme hydrolysis alone (Fig. 6c).

Fed-batch operation of SSMSF with acid-pretreated rice straw

In order to increase the productivity and reduce the enzyme requirement, SSMSF was carried out in a fed-batch mode using acid-pretreated rice straw. The amounts of cellulase and cellobiase used were reduced to 10 FPU g-substrate^{-1} and 10 CBU g-substrate^{-1} each. Initially, 80 g-dry mass l^{-1} of acid-pretreated rice straw was suspended in the MRS medium. The initial concentrations of glucose, xylose, and arabinose were 2.6, 7.8, and 9.4 g l^{-1}, respectively. During 13 h of lag phase, glucose concentration increased to 4.9 g l^{-1}. Then, the mixture of glucose, xylose, and arabinose was consumed simultaneously by *L. brevis*. No carbohydrates were detected in the medium after 33 h of fermentation. Production of lactic acid ceased at 38 h suggesting that the carbohydrates in initial substrate were completely consumed. At this point, an additional 60 g-dry mass l^{-1} of acid-pretreated rice straw was added as solid powder without sterilization (Fig. 7, point A). Although the glucose was not detected by HPLC 4 h after this addition, the end-product yield suggests that glucose was released from cellulose and metabolized by *L. brevis* simultaneously with xylose and arabinose. The lactic acid concentration increased until 62 h of operation when the fermentation appeared to be finished (Fig. 7, point B). In order to determine whether any cellulose remained, an excessive amount of cellulase and cellobiase were added to the fermentation broth and the temperature was shifted to 50 °C where *L. brevis* cannot survive (Fig. 7, point C). Glucose and lactic acid concentrations did not change for 36 h after this temperature shift implying that the cellulose in
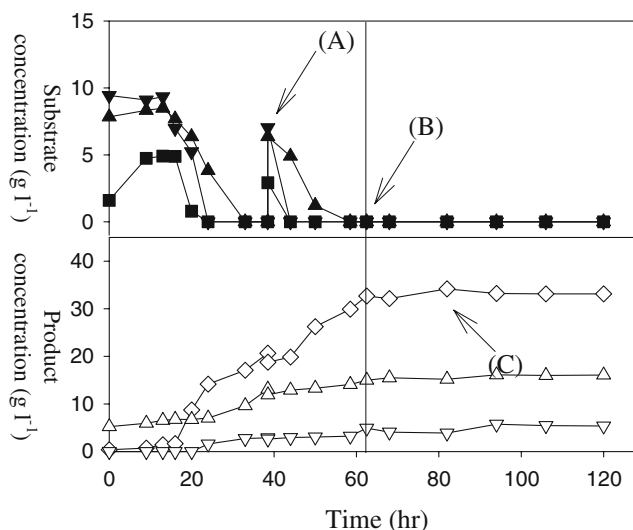

Fig. 7 Simultaneous saccharification and mixed sugar fermentation of acid-pretreated rice straw by *L. brevis* in the fed-batch mode. The points noted indicate *A* the addition of 80 g-dry mass l^{-1} of acid-pretreated rice straw, *B* the completion of fermentation and hydrolysis, *C* the addition of excessive amounts of cellulase and cellobiase. *filled square* Glucose, *filled upright triangle* xylose, *filled inverse triangle* arabinose, *empty diamond* lactate, *empty upright triangle* acetate, *empty inverse triangle* ethanol

the acid-pretreated rice straw was completely hydrolyzed by the enzymes and consumed by *L. brevis*. The final concentrations of lactic acid, acetic acid, and ethanol at 62 h were 34.2, 17.2, and 5.8 g l^{-1} with the yields of 0.96, 0.66, and 0.3 (mM/mM), respectively

Discussion

Estimated renewable biomass in the US is approximately 200 million dry tons/year. As such, lignocellulosic biomass is a most promising substitute for petroleum in chemical and fuel production. However, several technical hurdles limit the use of lignocellulosic biomass as a commercial substrate. The inherent complexity and heterogeneity of lignocellulose complicate the fermentation strategies centered on complete utilization of all sugars present. As a consequence, product yields are lower than that obtained from starch biomass substrates. In order to overcome these critical barriers in commercialization, several conditions need to be satisfied. Mixed sugars from lignocellulosic biomass have to be utilized efficiently and completely in order to maximize the overall yield. Hydrolysis of substrate and fermentation processes should be integrated to reduce the operation time and increase the efficiency of hydrolysis. In order to increase the final product concentration, the fermentation process needs to be operated in fed-batch mode. Considering the efficacy of process design and its operation to meet those conditions, each sugar in the mixture needs to

be utilized simultaneously by the fermentation host microorganism. In addition, the microbial strain should be resistant to the potential inhibitors in lignocellulosic hydrolysate to avoid the additional pretreatment steps. Finally, where possible, the use of cellulase and cellobiase should be minimized since the enzymes are one of the dominant cost factors for the fermentation of lignocellulosic biomass.

Various researchers have sought to identify or engineer strains capable of efficient utilization of the pentose derived from hemicellulose (Du Preez and Van Der Walt 1983; Zhang et al. 1995; Ho et al. 1998; Jeffries 2006; Rodrigues et al. 2008). In most cases, however, glucose suppressed the consumption of pentoses showing a typical sequential utilization of mixed sugars (Laplace et al. 1993; Lawford et al. 1998, 2000; Mohagheghi et al. 1998). Dien et al. developed an *E. coli* strain which completely lacks carbon catabolite repression (CCR) by deletion mutation of glucose PTS genes (*ptsG*; Dien et al. 1999, 2001, 2002). Recently, we demonstrated that *L. brevis* was able to simultaneously metabolize a large number of sugar substrates including xylose, arabinose, and galactose without the typical hierarchical carbohydrate consumption pattern (Kim et al. 2009).

While the molecular underpinnings for this relaxed control of sugar consumption remain to be determined, *L. brevis* met the demands as an optimal strain for the lignocellulosic utilization as any fermentable sugars in the media could be metabolized simultaneously without suppression by glucose. Moreover, *L. brevis* preferred the pentose sugar over glucose (Table 2 and Table 3). Meanwhile, the specific *L. brevis* growth and total carbohydrate consumption rates remain constant regardless of sugar composition, or their initial concentration ratio, suggesting *L. brevis* maintains a maximum metabolic activity when growing on mixed sugars similar to that of a single sugar like glucose.

We noted that washing the acid-pretreated rice straw hydrolysate to eliminate the soluble inhibitors prolonged the lag time of cell growth, likely due to removal of pentose sugars, and reduced the lactic acid production. By comparison, unwashed hydrolysate supported vigorous growth indicating *L. brevis* is resistant to such inhibitors. The natural resistance to the potential inhibitors in rice straw hydrolysate provides a strong advantage of *L. brevis* as a host microorganism of lignocellulosic fermentation. Others have noted that *L. brevis* was resistant to hops—the plant antibacterial compound (Sakamoto et al. 2001, 2002)—and attributed the resistance to specific ABC transporters (Suzuki et al. 2002). Given that *L. brevis* is often found in lignocellulose-rich environments, such as plant materials and fermented foods or beverages, it is likely this species has evolved/acquired such resistance to dominate these niches.

For the efficient fermentation of lignocellulose, the integration of both enzymatic hydrolysis and fermentation steps offers a possible increase of the productivity combined with a

reduction in enzyme requirement. Others have examined concurrent operation of enzymatic hydrolysis of cellulose fiber or starch biomass, and the fermentation of resultant glucose (Nikolic et al. 2009; Ou et al. 2009; Trovati et al. 2009). However, these simultaneous saccharification and fermentation (SSF) approaches necessarily use feedstock that could be broken down into glucose alone. Lignocellulosic fermentations are more problematic because of the additional pentose sugars which, in the case of *L. brevis*, can be simultaneously fermented. The synergistic effects of integration can only be achieved when two processes, hydrolysis and fermentation, are compatible. For example, the catalytic activity of hydrolysis should not jeopardize, nor be jeopardized by, the bacterial fermentation. As shown in Fig. 4, the stability of cellulase and cellobiase were maintained throughout the fermentation of *L. brevis* and the fermentation was not influenced by the presence of enzymes in the medium.

Not surprisingly, the optimum conditions for the fermentation of *L. brevis* are different from those for enzyme hydrolysis of lignocellulosic starting material. While MRS medium at pH 6.0 was compatible with the hydrolysis of acid-pretreated rice straw (data not shown), temperature strongly impacted both the hydrolysis and the fermentation processes. The initial hydrolysis rates at 40 °C and 30 °C were reduced to a half of that observed at 50 °C, a temperature that does not support growth of *L. brevis*. Nonetheless, the feedback inhibition by glucose and cellobiose produced a larger effect on the hydrolysis process than the temperature shift away from 50 °C. Even at 50 °C, total enzyme activity decreased exponentially approaching only 20% of initial activity after first 4 h of hydrolysis (Fig. 2b). Thus, removal of feedback inhibition by simultaneous saccharification and fermentation at a lower temperature appears to compensate for the reduction of enzyme hydrolysis rate. Indeed, SSMSF produced 2.4-fold more glucose than the simple enzyme hydrolysis at 37 °C (Fig. 6c). In a similar vein, the use of a non-optimal (high) fermentation temperature for *L. brevis* resulted in a decreased cell growth rate yet produced a better outcome in terms of the bio-based chemical production. Both specific product formation and substrate consumption rates increased at 40 °C and the yield of ethanol, which is currently used as fuel chemical, increased as well.

A critical factor for the commercial use of lignocellulosic biomass is the high cost of enzymatic hydrolysis. Reduction of this enzyme requirement can be achieved in two different ways. Removal of feedback inhibition enables maximum activity throughout the process and thus minimizes the need for additional enzyme input. In addition, enzyme stability throughout the fermentation decreases the total amount of enzyme required during the fed-batch operation of SSMSF. As shown in Fig. 6, the lactic acid production during the batch SSMSF ceased after 36 h while the glucose concentration increased continuously for additional 44 h in the similar

pattern to that obtained from direct hydrolysis without cell growth. This enzyme stability within the fermentation process may enable subsequent fed-batch operation of SSMSF without additional enzyme supplement (Fig. 7).

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