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Atypical ethanol production by carbon catabolite derepressed lactobacilli

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ABSTRACT

Cost effective use of lignocellulosic biomass for bio-based chemical production requires the discovery of novel strains and processes. *Lactobacillus pentosus* JH5XP5 is a carbon catabolite repression negative mutant which utilizes glucose and pentoses derived from lignocellulosic biomass in the media simultaneously. With a broad range of carbon substrates, *L. pentosus* JH5XP5 produced a significant amount of ethanol without acetate formation. The yields of ethanol were 2.0- to 2.5-fold higher than those of lactate when glucose, galactose or maltose was used either as a single carbon source or simultaneously with glucose. *L. pentosus* JH5XP5 was successfully used in an integrated process of simultaneous saccharification and mixed sugar fermentation of rice straw hydrolysate. During the fermentation, the enzyme activities for the saccharification of cellulose were not diminished. Moreover glucose, xylose, and arabinose sugars derived from rice straw hyrolysate were consumed concurrently as if a single carbon source existed and no sugars or cellulosic fiber remained after the fermentation.

1. Introduction

Currently, the production of many chemicals and energy are dependent on fossil fuels whose reserves are limited. In addition, fluctuations in oil prices necessitate changes in chemical and energy production from fossil fuel-based systems to bio-based systems (Hatti-Kaul et al., 2007). Environmental considerations also demand bio-based chemical production from sustainable feedstocks.

Bio-based production of platform chemical intermediates typically uses two different types of renewable feedstocks: starch-based biomass and lignocellulosic biomass. Although the use of lignocellulosic biomass has significant advantages, particularly by consuming existing agricultural wastes, several barriers still exist to its commercialization (Houghton et al., 2006). The price of the cellulase and the cellobiase enzyme complex that depolymerizes cellulose fiber for fermentation is higher than that for the enzymes to degrade starch biomass. Another barrier is the heterogeneity of lignocellulosic biomass composition. Thermochemically pretreated and hydrolyzed lignocellulose contains mixed sugars (hexoses and pentoses) as well as potential growth inhibitors such as furfural and phenolic compounds from lignin. Given pentoses can represent up to 50% of total sugar in a lignocellulosic hydrolysate, complete utilization of pentose sugars is crucial for economic fermentation of lignocellulosic materials. In addition, concurrent consumption of those mixed sugars is needed when considering product yield, productivity and, particularly, fermentation process design. However, the sequential utilization of sugars by microorganisms, a result of carbon catabolite repression (CCR), prevents the simultaneous utilization of sugars and makes the design of overall process difficult and inefficient. To get around the problems caused by the mixed sugar, many researchers have searched for, or engineered, microorganisms which possess pentose utilization and CCR negative (CCR^-) phenotypes (Dien et al., 2004, 2002, 2000; Ho et al., 1998; Nichols et al., 2003, 2001; Qian et al., 2003; Sibirny et al., 2003; Zhang et al., 1995). Recently, *L. brevis* was shown to lack an apparent CCR and was able to ferment pentose sugars with glucose simultaneously, albeit the ethanol yield was still relatively low (Kim et al., 2009).

Lactic acid and ethanol are promising as potential sustainable platform chemicals and fuels. Currently, ethanol is used as an energy source instead of, or mixed with, gasoline. Ethanol is also used to make ethyl lactate (EL) by esterification with lactate (Benedict et al., 2003). EL is an FDA approved organic solvent that currently is drawing much attention due to the potential applications in the food and pharmaceutical industries (Garlotta, 2001; Hirakawa et al., 1988; Nikles et al., 2001). Another important chemical from lactate is poly-lactate (PLA) (Jacobsen et al., 1999). PLA is a biodegradable plastic synthesized from lactic acid. PLA is a plastic material but is degraded in nature or *in vivo* to lactic acid which can then be further metabolized in most biological systems (Duda and Penczek, 2003; Jacobsen et al., 1999). The application of PLA is widespread from textiles and disposable utensils to drug deliv-

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ery vehicles due to its favorable properties (Datta et al., 1995; Garlotta, 2001).

Lactobacillus pentosus has been studied for the production of lactic acid and surface-active compounds from hemicellulose or lignocellulosic biomass (Portilla-Rivera et al., 2008, 2009; Zhu et al., 2007). As an effective host strain for bio-based chemical production from lignocellulosic biomass, we isolated *L. pentosus* JH5XP5 through a series of selection and enrichment procedures. *L. pentosus* JH5XP5 has a unique CCR^- phenotype showing simultaneous utilization of mixed sugars. As facultative heterofermentative lactobacilli, JH5XP5 ferments a wide range of substrates, and, interestingly, produced a significant amount of ethanol. Here, we examined the fermentation characteristics of *L. pentosus* JH5XP5 which overcome the current barriers for the cost effective utilization of lignocellulosic biomass. Enzymatic hydrolysis of cellulose fiber and simultaneous utilization of mixed sugars were integrated and a new fermentation scheme was evaluated using acid-pretreated rice straw as a carbon source.

2. Methods

2.1. Bacterial strains, culture conditions and media

L. pentosus JH5 was isolated as a contaminant from a *L. brevis* culture obtained from a national collection. Once the strain was isolated, the species identity was determined by 16S rDNA sequencing. *L. pentosus* NRRL B-227 was obtained from the Agricultural Research Service culture collection (Peoria, IL, USA). Modified MRS medium (15 g l^{-1} bactopeptone, 5.0 g l^{-1} yeast extract, 2.0 g l^{-1} ammonium citrate, 5.0 g l^{-1} sodium acetate, and 2.0 g l^{-1} dipotassium phosphate) with 20 g l^{-1} of glucose was used for the cell maintenance. In this study, glucose was not included in modified MRS unless stated. Carbon sources were prepared separately and mixed with inoculums initially. The initial pH of the medium was set at 6.0 and temperature was maintained at 37 °C. Fermentations were initiated by adding a 5% (v/v) inoculum.

2.2. Isolation of carbon catabolite repression negative (CCR^-) strains

L. pentosus JH5 was grown in 100 ml of the modified MRS with 20 g l^{-1} of xylose for growth substrate and 5 g l^{-1} of 2–deoxyglucose (2-DG) as a CCR inducer. For enrichment, 10% of the initial volume of media was used as inoculums for fresh media containing 2-DG and xylose. After five consecutive cultivations, culture broth was spread on MRS-xylose-2-DG agar plates and several colonies were isolated. Each isolated colony was inoculated and cultivated for 24 h in 100 ml of modified MRS with 10 g l^{-1} of glucose and 10 g l^{-1} of xylose. Cell growth and simultaneous utilization of glucose/xylose were monitored every 4 h. The strain was selected based on the specific cell growth rate and sugar utilization rates. Once the identity of species was determined by 16S rDNA sequencing, the selected strain was designated *L. pentosus* JH5XP5.

2.3. Fed-batch fermentation with mixed sugars

The fermentation was carried out in a BioFlo 3000 Bioreactor (New Brunswick Scientific, Edison, NJ, USA). Temperature was maintained at 37 °C and pH was controlled at 6.0 by addition of 10 N NaOH during the fermentation. Agitation was maintained at 100 rpm without aeration. Modified MRS medium and the carbohydrate(s) were prepared separately to make the final volume of 2.5 l. Initial concentration of mixed sugars were 30 g l^{-1} of glucose, 10 g l^{-1} of xylose and 10 g l^{-1} of arabinose. When sugars were depleted, a concentrated mixed-sugar solution was supplemented reaching initial concentrations of sugars.

2.4. Acid-pretreated rice straw

Acid-pretreated rice straw was prepared by BC International (now Verenium Corporation: Cambridge, MA, USA). Rice straw was cleaned, chopped, and then hemicellulose was hydrolyzed by diluted sulfuric acid. After drying, the acid-pretreated rice straw has a soil like texture properties with dark brown color (Fig. 1a). The resulting mixture contained cellulose fiber, xylose and arabinose from hemicelluloses, and phenolic compounds from lignin. The total carbohydrate content in hydrolyzed rice straw was approximately 50% (w/w) based on dry substrate mass. Remaining 50% dry mass (w/w) of acid-pretreated rice straw was ashes, mostly silica particle (Fig. 1b). Due to the cellulose fiber and lignin, resuspended acid-pretreated rice straw was a mud-like paste with high viscosity (Fig. 1b). This high viscosity limited the initial substrate concentration to 100 g-dry mass l^{-1} (172 g-wet mass l^{-1}) above which agitation could not be maintained to ensure proper mixing.

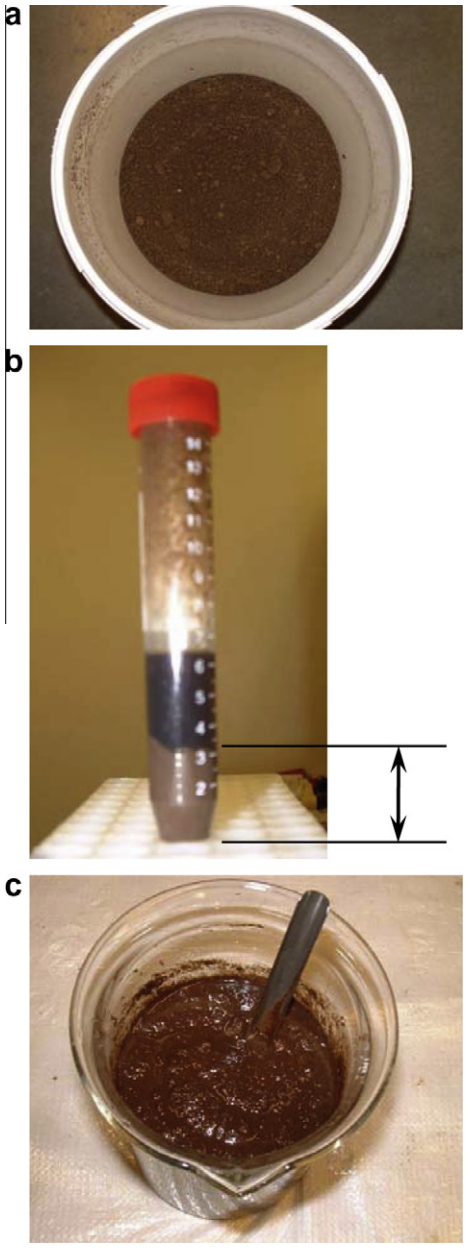

Fig. 1. Acid pretreated rice straw (a) pretreated rice straw prepared by BC International. (b) after fermentation. Volume indicated by arrow is silica beads from rice straw. (c) resuspended in the medium. The concentration is 100 g-dry mass l^{-1}.

2.5. Sequential hydrolysis and fermentation of the acid-pretreated rice straw

For hydrolysis of cellulose fiber from acid pretreated rice straw, 100 g-dry mass l^{-1} of acid-pretreated rice straw was suspended in 0.05 M sodium acetate buffer (pH 5.0) with 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 (Vlasenko et al., 1997). Agitation was maintained at 250 rpm during the hydrolysis of acid-pretreated rice straw. After 6 h of hydrolysis, the rice straw hydrolysate was autoclaved; then, pre-sterilized modified MRS was added with the inoculation. The fermentation was carried out in a BioFlo 3000 Bioreactor (New Brunswick Scientific, Edison, NJ, USA). Temperature was maintained at 37 °C and pH was controlled at 6.0 by addition of 10 N NaOH. Agitation was maintained at 100 rpm without aeration during fermentation.

2.6. Simultaneous saccharification and mixed sugar fermentation of acid-pretreated rice straw

Fed-batch operation of Simultaneous saccharification and mixed sugar fermentation (SSMSF) was carried out in a BioFlo 3000 Bioreactor (New Brunswick Scientific, NJ, USA) with 2.5 l of initial volume as previously described (Kim et al., 2010). With inoculation, 10 FPU g-substrate^{-1} of cellulase and 10 CBU g-substrate^{-1} of cellobiase were added into the modified MRS which contained 100 g-dry mass l^{-1} of acid-pretreated rice straw. The pH was adjusted and controlled at 6.0 by 10 N NaOH and the temperature was maintained at 37 °C throughout the fermentation. The acid-pretreated rice straw was added directly, without sterilization, into the fermentation broth. For analysis of substrate and product concentrations, 5 ml of fermentation broth was taken and centrifuged at 10,000*g* to remove the solid materials. The supernatant was filtrated through the 0.2 μm syringe filter (Millipore, Billerica, MA, USA) and analyzed by HPLC.

2.7. Analysis of sugars and metabolites

The concentration of substrates and endproducts were analyzed by HPLC (Shimadzu, Kyoto, Japan) using a BioRad HPX-87H column (BioRad, Hercules, CA, USA). One ml of fermentation broth was centrifuged at 10,000*g* for 10 min and the supernatant was transferred to a new microcentrifuge tube prior to analysis. For HPLC analysis of the supernatant, the BioRad HPX-87H column was heated at 65 °C and a reflective index (RI) detector was used for the identification of substrate(s) and product(s). As a mobile phase, 0.01 N H_2SO_4 was used with the flow rate of 0.6 ml min^{-1}. The standard deviation of the HPLC concentration determinations for any substrate or endproduct was within ±5 mM. To determine cell density the cell pellet was resuspended in the deionized water and the optical density (OD) was measured using a Beckman DU 7400 spectrophotometer (Beckman, Fullerton, CA, USA) at 600 nm.

3. Results and discussion

3.1. Simultaneous utilization of glucose and xylose by carbon catabolite repression negative (CCR^-) strain

As a facultative heterofermentative lactobacilli, *L. pentosus* is able to metabolize pentose sugars, albeit their utilization is regulated by CCR (Chaillou et al., 2001; Lokman et al., 1997; Mahr et al., 2000; Posthuma et al., 2002). It was reported that *L. pentosus* utilizes xylose sequentially following the complete depletion of glucose, or small amount of xylose, simultaneously with the rapid consumption of glucose (Bustos et al., 2005; Moldes et al., 2006). When *L. pentosus* JH5 (host strain) and *L. pentosus* NRRL B-227 (type strain) were cultivated in 200 ml of modified MRS with 10 g l^{-1} of glucose and 10 g l^{-1} of xylose, the sugar utilization profiles were consistent to the CCR^+ phenotype (Fig. 2a and b).

Xylose was consumed after complete depletion of glucose showing sequential utilization of mixed sugars. Moreover, the xylose utilization rate was slow compared to when xylose was used as a single carbon source. *L. pentosus* JH5 was able to co-utilize xylose with glucose, however, the amount consumed simultaneously was small (Fig. 2b). While 10 g l^{-1} of glucose was consumed, only 2.1 g l^{-1} of xylose was co-utilized by *L. pentosus* JH5. Furthermore, xylose was not completely consumed over 40 h of fermentation.

Conversely, *L. pentoses* JH5XP5, which is isolated through the repeated enrichment and selection in a culture medium containing 2-DG/xylose, is capable of consuming glucose and xylose concurrently (Fig. 2c). Both 10 g l^{-1} of glucose and 10 g l^{-1} of xylose were completely consumed producing 12.0 g l^{-1} of lactic acid, 5.0 g l^{-1} of acetic acid and 2.3 g l^{-1} of ethanol in 24 h. The specific cell growth rate of *L. pentosus* JH5XP5 was similar to that of the host strain *L. pentosus* JH5 as 0.26 h^{-1}.

3.2. Stability of CCR^- phenotype

Given JH5XP5 is a mutant strain, we examined the phenotypic stability of simultaneous sugar utilization without the selective agent (2-DG) being present. Five consecutive fermentations were performed in the modified MRS media at 37 °C with 10 g l^{-1} of glucose and 10 g l^{-1} of xylose as carbon sources. After an individual fermentation, 5% of the culture broth was used as an inoculum for the next round of fermentation. During the five consecutive fermentations, the simultaneous sugar utilization profiles, kinetic values, and product yields were reproduced, indicating that the CCR^- phenotype of *L. pentosus* JH5XP5 was stable (Fig. 3).

3.3. Atypical fermentation characteristic of *L. pentosus JH5XP5*

L. penstosus JH5XP5 was able to utilize a wide range of individual sugars, including pentoses (Table 1). When xylose, arabinose, and ribose were used as a single carbon source, lactate and acetate were produced in an equimolar ratio without ethanol formation. Galactose, fructose and maltose were readily metabolized but lactose, sucrose and cellobiase could not be used. As expected, *L. pentosus* JH5XP5 exhibited a CCR^- phenotype in sugar utilization. Any sugar that could be used by *L. pentosus* JH5XP5 as a sole carbon source could be metabolized simultaneously with glucose (Table 2). The consumption rate of second carbohydrate in the medium was similar or faster than that of glucose (consumed simultaneously).

In addition to the expected consumption of broad range of sugar substrates, *L. pentosus* JH5XP5 presented atypical endproduct profiles. During the growth on glucose, neither of parent strain (*L. pentosus* JH5) nor the type strain (*L. pentosus* NRRL B-227) produced ethanol (Fig. 2a and b). Amazingly, *L. pentosus* JH5XP5 produced ethanol when grown on glucose, galactose and maltose. Furthermore, the molar yields (mole per mole) of ethanol from glucose, galactose and maltose were 2.0–2.5 times higher than those of lactate without production of acetic acid (Table 1). These high levels of ethanol production from galactose and maltose were also observed during their simultaneous utilization with glucose as well (Table 2).

The endproduct profile is closely related to the regulation of NAD^+/NADH which is essential in lactic acid bacteria (LAB). *L. pentosus* metabolizes a hexose through a homofermentative path-

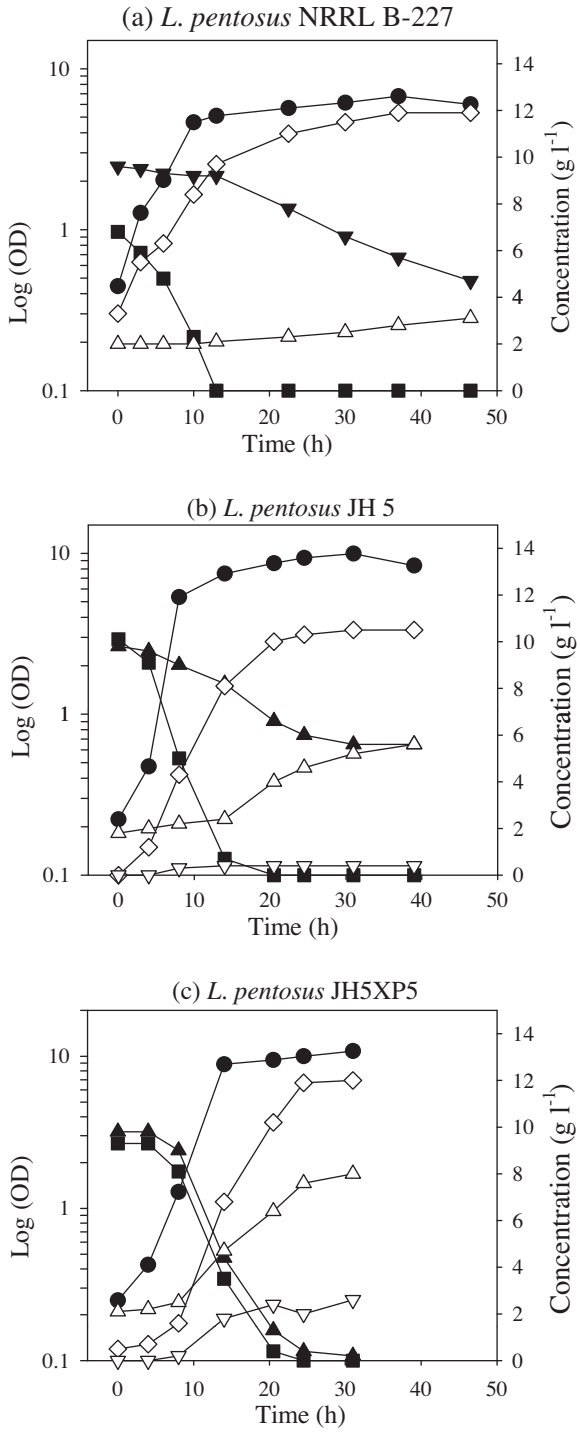

Fig. 2. Fermentation profiles in the modified MRS with a glucose and xylose mixture by (a) *L. pentosus* NRRL B-227, (b) *L. pentosus* JH5, and (c) *L. pentosus* JH5XP5. Symbols: ●, optical density at 600_{nm}; ■, glucose; ▲, xylose; ◇, lactate; △, acetate; ▽, ethanol.

way producing two molecules of lactic acid. Meanwhile, NAD^+ is regenerated by the reduction of pyruvate to lactic acid (Bustos et al., 2005). Considering this, it is uncommon that the ethanol production is twofold higher (on a mole basis) than lactic acid production during growth of *L. pentosus* JH5XP5. When pentoses were utilized, *L. pentosus* JH5XP5 did not produced ethanol. For the pentose metabolism, *L. pentosus* uses a heterofermentative pathway where five-carbon sugars are split to pyruvate (C3) and acetyl-CoA (C2). In this case, the redox balance is fulfilled by the reduction of pyruvate to lactic acid and acetyl Co-A is converted the acetic acid producing extra ATP.

The co-consumption of glucose impacted the endproduct profiles of the fructose fermentation. Production of mannitol from fructose has been widely reported in heterofermentative lactobacilli (Aarnikunnas et al., 2002; Grobben et al., 2001; Korakli and Vogel, 2003; Saha, 2004). Heterofermentative LAB typically convert three moles of fructose to two moles of mannitol, one mole of lactic acid and one mole of acetic acid (Salminen and Wright, 1993). The conversion of NADH to NAD^+ by mannitol dehydrogenase enables shunting the two carbon endproduct from ethanol to acetic acid with concurrent production of an extra ATP. Homofermentative LAB, however, converts fructose to fructose-6-phosphate and metabolizes it in the glycolytic pathway without mannitol production (Wisselink et al., 2002). Thus, the redox balance is primarily regulated by the conversion of pyruvate to lactic acid. As a facultative heterofermentative LAB, *L. pentosus* JH5XP5 uses homofermentative pathway for hexose metabolism. However, *L. pentosus* JH5XP5 is able to convert some fructose to mannitol when fructose was used as a single carbon source. Interestingly, such mannitol production disappeared during co-utilization of fructose and glucose. Instead, ethanol, which is not expected to be produced from fructose fermentation, was produced 1.3-fold more than lactic acid on a mole basis. This suggests the possible modulation of a mannitol dehydrogenase by glucose even though *L. pentosus* JH5XP5 exhibited CCR^- phenotype.

3.4. Fed-batch fermentation of glucose, xylose, and arabinose mixture in rich media

To increase the final concentration of lactic acid, the fed-batch fermentation was carried out under controlled conditions (Fig. 4). For the fed-batch fermentation, 2.5 l of modified MRS was prepared. The initial concentration of mixed sugar was 30.0 g l^{-1} of glucose, 10.0 g l^{-1} of xylose and 10.0 g l^{-1} of arabinose, which is equivalent to the available sugars in 100 g-dry mass l^{-1} of rice straw hydrolysate. During the fermentation, all three carbon sources were consumed concurrently and efficiently by *L. pentosus* JH5XP5. The initial carbohydrates were consumed within 16 h and an additional 47.0 g l^{-1} of total sugar (28 g l^{-1} of glucose, 9.5 g l^{-1} of xylose and 9.5 g l^{-1} of arabinose) were metabolized completely during the next 24 h after additional substrate supplementation (point A; Fig. 4). However, after the second round of substrate addition (point B; Fig. 4), 15.0 g l^{-1} of glucose remained unfermented for 28 h of operation. Meanwhile 10 g l^{-1} of xylose and 10 g l^{-1} of arabinose were completely consumed. The final concentrations of products were 67 g l^{-1} of lactate, 27 g l^{-1} of acetate (production of 24.5 g l^{-1}) and 13 g l^{-1} of ethanol.

3.5. Sequential hydrolysis and fermentation of acid-pretreated rice straw

The ability of JH5XP5 to simultaneously consume xylose, arabinose and glucose suggests this strain would be useful for fermentation of a sustainable feedstock. To evaluate the feasibility, rice straw was used as a fermentation substrate. Hemicellulose from rice straw was hydrolyzed to xylose and arabinose by the diluted sulfuric acid. This acid-pretreated rice straw contains carbohydrates, such as glucose, xylose, arabinose and cellulose fibers, as well as possible cell growth inhibitors such as phenolic compounds from lignin and solid particles. The hydrolysis of cellulose fiber in the acid-pretreated rice straw and the fermentation of resulting mixed sugars were performed sequentially in a single fermentation vessel (Fig. 5). Acid-pretreated rice straw (100 g-dry mass l^{-1}) was resuspended in the sodium acetate buffer (0.05 M) and hydrolyzed by a mixture of cellulase and cellobiase for 24 h (Fig. 5, section A).

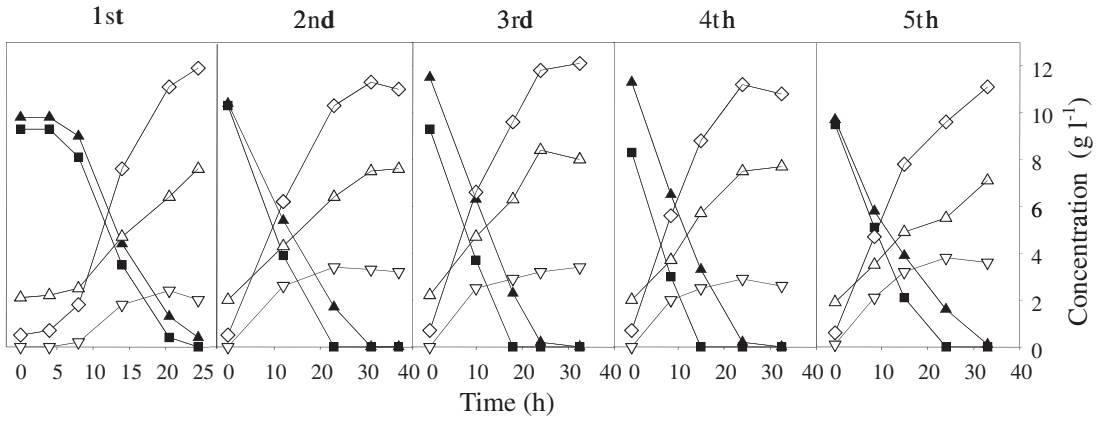

Fig. 3. The stability of CCR$^-$ phenotype of *L. pentosus* JH5XP5 without the selective agent. Fermentations were repeated five times consecutively with a glucose and xylose mixture. Symbols: ■, glucose; ▲, xylose; ◇, lactate; △, acetate; ▽, ethanol.

Table 1
Carbohydrate utilization and product formation by *L. pentosus* JH5XP5. Fermentation was performed in the modified MRS with the supplement of 30 g l^{-1} of each carbohydrate.

Substrate			Product					
Name		Consumption mM (%)	Production (mM)			Yield (mmole/mmole)		
			Lactate	Acetate	Ethanol	Lactate	Acetate	Ethanol
Pentose[a]	Xylose	92 (55%)	89	86	2	0.97	0.93	0.02
	Arabinose	78 (39%)	76	76	3	0.97	0.97	0.04
	Ribose	75 (45%)	71	68	0	0.95	0.91	0.00
Hexose	Galactose[b]	45 (27%)	23	1	61	0.51	0.02	1.36
	Glucose	95 (68%)	57	3	134	0.60	0.03	1.41
	Fructose[c]	166 (100%)	33	44	0	0.20	0.27	0.00
	Mannose				n/a			
Hexose dimer	Maltose	67 (72%)	85	2	174	1.27	0.03	2.60
	Sucrose				n/a			
	Lactose				n/a			
	Cellobiolse				n/a			

n/a: not applicable. Carbohydrate was not consumed.
The fermentations were monitored every 4 h for 24 h. The SD of HPLC analysis was <±5 mM. Duplicated experiments exhibited the RSD of yields <±10%.
[a] Initial concentration of pentose was 25 g l^{-1}.
[b] Initial concentration of galactose was 32 g l^{-1}.
[c] Mannitol production was observed.

Table 2
Mixed sugar fermentation of glucose and secondary carbohydrates in modified MRS by *L. pentosus* JH5XP5.

Substrate					Product[d]					
1st carbon source[a]		2nd carbon source[b]			Production (mM)			Yield (mmole/mmole)		
Name	Consumed mM (%)	Name		Consumed mM (%)	Lactate	Acetate	Ethanol	Lactate	Acetate	Ethanol
Glucose	84(69%)	Pentose	Xylose	64(64%)	97	53	80	0.65	0.36	0.54
	67(55%)		Arabinose	70(70%)	88	56	55	0.64	0.41	0.40
	45(37%)		Ribose	85(85%)	90	70	37	0.69	0.54	0.28
	97(79%)	Hexose	Galactose	37(44%)	74	5	145	0.56	0.04	1.09
	24(20%)		Mannose	5(6%)	12	4	31	0.41	0.14	1.05
	111(91%)		Fructose[c]	28(33%)	74	33	96	0.53	0.24	0.69
	56(46%)	Hexose dimer	Lactose	0(0%)	30	0	81	0.53	0.00	1.44
	84(69%)		Maltose	35(79%)	99	4	219	0.83	0.03	1.84
	67(55%)		Cellobiose	1(2%)	43	2	93	0.63	0.03	1.37

Fermentations were monitored (sampled) every 4 h for 24 h. The values were obtained after 24 h of fermentation. The SD of HPLC analysis was <±5 mM. Duplicated experiments exhibited the RSD of yields <±10%.
[a] Initial concentration of glucose was 20 g l^{-1}.
[b] Initial concentration of each carbohydrate was 15 g l^{-1}. Second carbohydrates were consumed simultaneously with glucose.
[c] Mannitol was not produced in simultaneous fermentation of glucose and fructose.
[d] No other by-product was detected in HPLC analysis.

The final carbohydrate concentrations after enzyme hydrolysis were 23 g l^{-1} of glucose, 8.9 g l^{-1} of xylose and 4.5 g l^{-1} of arabinose. No other sugar was found in an amount above 1.0 g l^{-1}. After heat sterilization (Fig. 5, section B), the components of MRS medium were added directly to the hydrolysate.

The fermentation of *L. pentosus* JH5XP5 was not hampered by potential chemical or physical inhibitors in rice straw hydrolysate. The fermentation profiles and kinetic values of sugar consumption and product formation were similar to those obtained from the fermentation in the modified MRS with the same composition of sug-

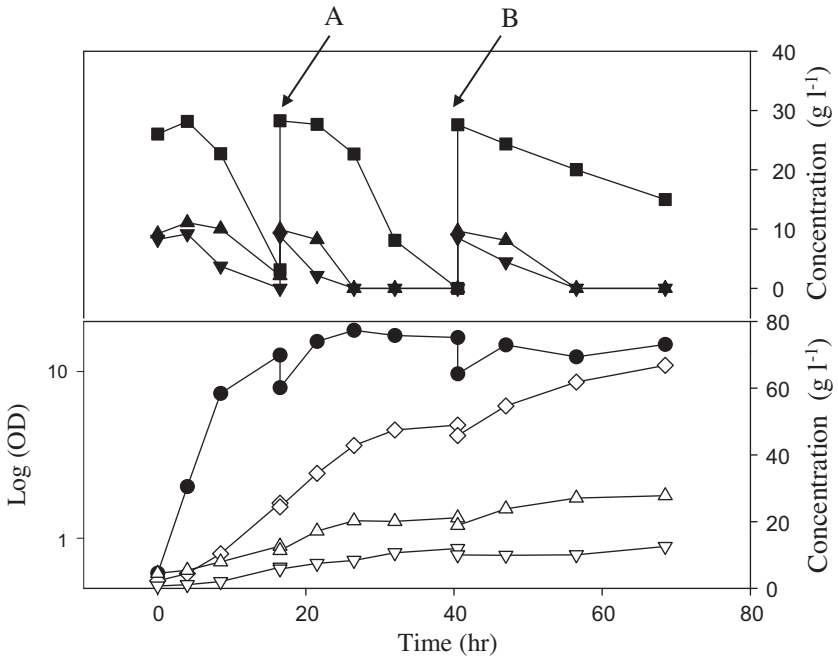

Fig. 4. Simultaneous utilization of glucose, xylose, and arabinose in the fed-batch fermentation by *L. pentosus* JH5XP5. Fermentation was carried out in modified MRS. The arrows indicate the additions of mixed sugar substrate. Symbols: ●, optical density at 600 nm; ■, glucose; ▲, xylose; ▼, arabinose; ◇, lactate; △, acetate; ▽, ethanol.

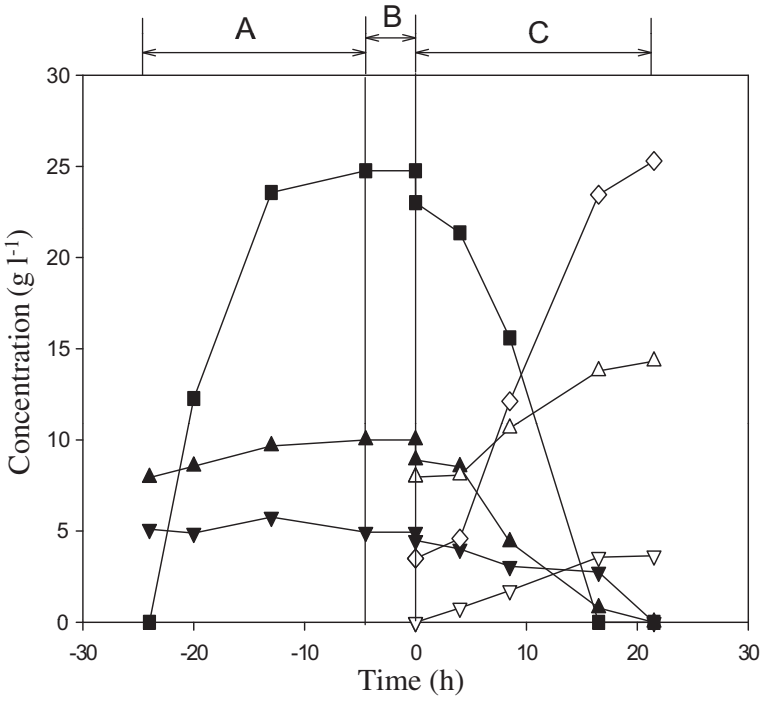

Fig. 5. Sequential hydrolysis and fermentation of the acid-pretreated rice straw by *L. pentosus* JH5XP5. A: Enzyme hydrolysis of cellulose fiber in the acid-pretreated rice straw by a cellulase and cellobiase mixture, B: Heat sterilization and cooling, C: Fermentation of the rice straw hydrolysate. Symbols: ■, glucose; ▲, xylose; ▼, arabinose; ◇, lactate; △, acetate; ▽, ethanol.

ars. It has been previously reported that *L. pentosus* is resistant to the hemicellulose hydrolysates (Garde et al., 2002; Moldes et al., 2006; Zhu et al., 2007). Fermentation was complete in 21 h. The final concentrations of lactic acid, acetic acid, and ethanol were 25.3 g l^{-1}, 14.3 g l^{-1} (production of 11.8 g l^{-1}), and 3.7 g l^{-1}, respectively. The product yields of lactic acid, acetic acid and ethanol from total carbohydrates were 1.12 (mol/mol), 0.36 (mol/mol) and 0.39 (mol/mol), respectively.

3.6. Simultaneous saccharification and mixed sugar fermentation in rice straw

Previously we designed a process termed simultaneous saccharification and mixed sugar fermentation (SSMSF) to increase the productivity by integrating the enzyme hydrolysis of cellulose fiber and the fermentation of mixed sugars derived from lignocellulosic biomass (Kim et al., 2010). Taking advantage of the simultaneous

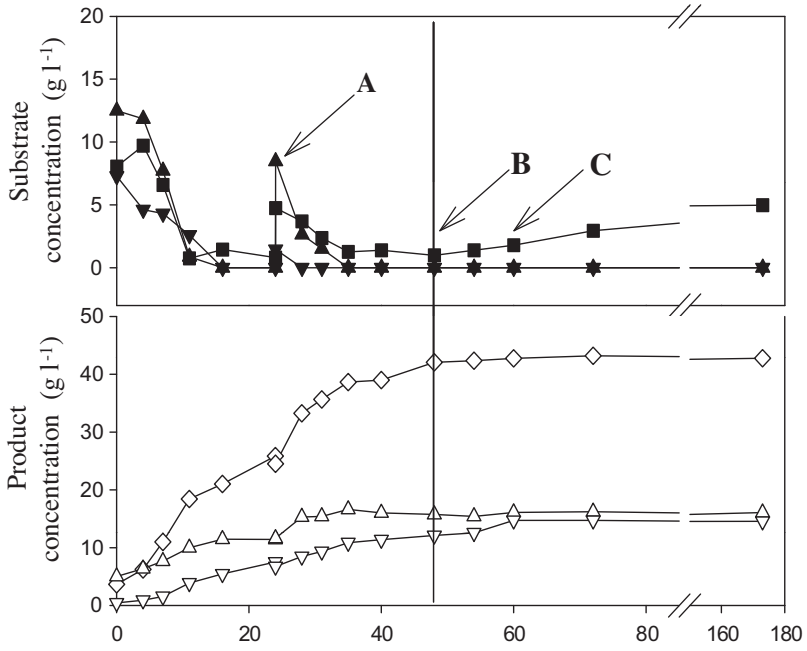

Fig. 6. Fed-batch operation of simultaneous saccharification and mixed sugar fermentation (SSMSF) using acid-pretreated rice straw. A: 80 g-dry mass l^{-1} of acid-pretreated rice straw was added directly to the fermentation medium, B: fermentation was finished, C: excessive amount of cellulase and cellobiase was added and the temperature was shift to 55 °C. Symbols: ■, glucose; ▲, xylose; ▼, arabinose; ◇, lactate; △, acetate; ▽, ethanol.

mixed sugar utilization of *L. pentoses* JH5XP5, SSMSF was operated in a fed-batch mode using acid-pretreated rice straw as a carbon source (Fig. 6). Modified MRS was prepared with 100 g-dry mass l^{-1} of acid-pretreated rice straw. A mixture of enzymes, 10 FPU g-substrate^{-1} of cellulase and 10 CBU g-substrate^{-1} of cellobiase, was added upon inoculation. After 24 h of fermentation, 200 g-dry mass of acid-pretreated rice straw was added to the fermentation broth as a solid powder without an additional sterilization step (Fig. 6, point A). Due to the amount of substrate added (487 g-wet mass) the fermentation volume increased to three liters. After 24 h of lactic acid production, no additional increase in glucose or lactic acid concentration was observed indicating the arrest of both the hydrolysis of cellulose fiber and its fermentation (Fig. 6, point B). At 12 h from this fermentation end point, an excessive amount of enzyme mixture (50 FPU g-substrate^{-1} of cellulase and 50 CBU g-substrate^{-1} of cellobiase) was added and the temperature was raised to 55 °C at which *L. pentosus* was not able to grow (Fig. 6, point C). This final hydrolysis of the remaining cellulose fiber continued for 113 h resulting in an increase of only 3.2 g l^{-1} of glucose. Thus, we concluded that the fermentation was finished at 48 h (Fig. 6, point B) upon near-complete consumption of the cellulose in the rice straw.

The simultaneous consumption of two different pentose sugars and glucose was clearly observed during the fermentation. After complete co-consumption of measurable carbohydrates in the media, however, the lactic acid concentration increased continuously demonstrating the saccharification of cellulose fiber and its simultaneous fermentation by *L. pentosus* JH5XP5. The final product concentrations were 43.0 g l^{-1} of lactic acid, 15.7 g l^{-1} of acetic acid (13.2 g l^{-1} of production) and 12.1 g l^{-1} of ethanol.

The enzyme requirement, which is critical for cost effectiveness, was reduced in two different ways. First, the enzyme requirement per unit substrate was reduced by increasing the total enzyme activity, a result achieved by removing feedback inhibition by glucose thus maintaining the maximum enzyme activity throughout the fermentation. Second, the enzyme requirement per unit product was reduced by a combination of the fed-batch operation and enzyme stability during the operation. Because the enzymes were maintained intact without degradation during the entire fed-batch fermentation, the final lactic acid concentration could be increased without further addition of the enzyme mixture.

Another advantage of process integration is the reduction in overall operation time. Sequential hydrolysis and fermentation required 24 h of hydrolysis time and an additional 24 h of fermentation to produce 27 g l^{-1} of lactic acid (Fig. 5, section C). However, the same amount of lactic acid was produced through SSMSF in only 24 h (Fig. 6, point A). An additional reduction in process operation time can be achieved by the omission of the pretreatment and sterilization of the substrate (Fig. 5, section B). Since *L. pentosus* JH5XP5 is resistant to the potential inhibitors in lignocellulosic biomass, rice straw hydrolysate can be used directly without fractionation or cleanup steps. Furthermore, sterilization of the substrate is not necessary because rice straw was hydrolyzed with diluted sulfuric acid and was maintained at a low pH (initial pH < 2.0). Finally, anaerobic fermentation reduces the burden of aeration, which is problematic in the acid-pretreated rice straw fermentations due to high viscosity and foaming of the media.

4. Conclusion

L. pentosus JH5XP5 has atypical but favorable fermentation characteristics for the bio-based chemicals and energy production from lignocelluloses. As a stable carbon catabolite negative (CCR$^-$) mutant, *L. pentosus* JH5XP5 was able to co-utilize glucose and any fermentable carbon sources which enables to design a mixed sugar fermentation process as if only a single carbohydrate existed in the medium. In addition, *L. pentosus* JH5XP5 produced a significant amount of ethanol from hexose sugar as a single carbon source or simultaneously with pentose sugar. Fed-batch mode of SSMSF was successfully operated using rice straw hydrolysate showing no inhibition by potential growth inhibitors. The integrated process

was able to reduce the requirement of cellulase and cellobiase, and overall operation time.

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