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Development of New Ethanologenic *Escherichia coli* Strains for Fermentation of Lignocellulosic Biomass

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Abstract

Two new ethanologenic strains (FBR4 and FBR5) of *Escherichia coli* were constructed and used to ferment corn fiber hydrolysate. The strains carry the plasmid pLOI297, which contains the genes from *Zymomonas mobilis* necessary for efficiently converting pyruvate into ethanol. Both strains selectively maintained the plasmid when grown anaerobically. Each culture was serially transferred 10 times in anaerobic culture with sugar-limited medium containing xylose, but no selective antibiotic. An average of 93 and 95% of the FBR4 and FBR5 cells, respectively, maintained pLOI297 in anaerobic culture. The fermentation performances of the repeatedly transferred cultures were compared with those of cultures freshly revived from stock in pH-controlled batch fermentations with 10% (w/v) xylose. Fermentation results were similar for all the cultures. Fermentations were completed within 60 h and ethanol yields were 86–92% of theoretical. Maximal ethanol concentrations were 3.9–4.2% (w/v). The strains were also tested for their ability to ferment corn fiber hydrolysate, which contained 8.5% (w/v) total sugars (2.0% arabinose, 2.8% glucose, and 3.7% xylose). *E. coli* FBR5 produced more ethanol than FBR4 from the corn fiber hydrolysate. *E. coli* FBR5 fermented all but 0.4% (w/v) of the available sugar, whereas strain FBR4 left 1.6% unconsumed. The fermentation with FBR5 was completed within 55 h and yielded 0.46 g of ethanol/g of available sugar, 90% of the maximum obtainable.

Index Entries: Alcohol; biofuel; *Escherichia coli*; fermentation; pentoses.

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†Names are necessary to report factually on available data. However, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable.

Introduction

In 1998, approx 1.4 billion gal of fuel ethanol were produced from corn starch and blended with gasoline as an oxygenate *(1)*. Societal and environmental benefits associated with using ethanol as an automotive fuel include lowering carbon dioxide emissions and decreasing air pollution, national reliance on foreign sources of petroleum, and foreign trade imbalance. Significantly increasing fuel ethanol production beyond current limits, however, will necessitate developing an alternative fermentation feedstock. Only lignocellulosic biomass is plentiful and adequately inexpensive to supplement significantly corn starch as a fermentation feedstock *(2)*.

We are using corn fiber as a model substrate for developing a biomass-to-ethanol conversion process. Corn fiber is a low-value coproduct produced during corn wet milling that holds promise as a feedstock for lignocellulosic fermentations. Corn fiber contains approx 70% (w/w) carbohydrates in the form of cellulose and hemicellulose *(3–5)*. About 3.4×10^6 dry t of corn fiber are produced each year in the United States *(1)*, which if all of it was fermented would produce 4 billion gal of ethanol per year, assuming an 80% conversion efficiency.

A major technical bottleneck to commercializing a biomass (such as corn fiber) to ethanol process is strain development. Corn starch hydrolysate is a relatively pure solution of glucose. By contrast, lignocellulose hydrolysate is a mixture of sugars including glucose and xylose, and frequently arabinose, galactose, and mannose. For example, corn fiber hydrolysate contains a mixture of arabinose, glucose, and xylose. Neither arabinose nor xylose is fermented by wild-type *Saccharomyces cerevisiae* or *Zymomonas mobilis*, which are traditionally used by the commercial alcohol industry. Naturally occurring bacteria that do ferment pentose sugars (i.e., arabinose and xylose) produce a mixture of fermentation products. Metabolic engineering has allowed the development of recombinant microorganisms that will selectively ferment glucose and xylose, and in some cases arabinose, into ethanol (for a recent review *see* ref. *6*). Recombinant microorganisms considered as candidates for industrial application include ethanologenic *Escherichia coli (7,8)*, xylose-fermenting *Z. mobilis (9,10)*, and xylose-fermenting *S. cerevisiae (11–13)*. Another recombinant *Z. mobilis* strain has been reported to ferment both xylose and arabinose, but the rate of arabinose fermentation is slow *(14,15)*.

The most widely studied microorganism from the ones just given is *E. coli* K011. This strain also has the advantage of being able to ferment arabinose and xylose into ethanol. Our own work in batch cultures using this strain has been favorable; biomass hydrolysates fermented with this strain were converted to ethanol with high yields *(3)*. However, when K011 is propagated in repeated batch or continuous cultures, its ethanol yields have been reported to decrease with time *(16,17)*. We addressed this issue by developing the novel ethanologenic *E. coli* strain FBR3 *(18,18a)*.

E. coli FBR3 was created by transforming a xylose-utilizing isolate of strain FMJ39 *(19)* (FMJ39x) with plasmid pLOI297 *(18)*. Strain FMJ39x cannot grow anaerobically because its lactate dehydrogenase (*ldhA*) and pyruvate formate lyase (*pfl*) activities have been inactivated. Therefore, it is unable to fermentatively reduce pyruvate and regenerate the NAD^+ reduced during glycolysis *(20)*. Plasmid pLOI297 contains the genes from *Z. mobilis* necessary to convert pyruvate into ethanol selectively *(21)*. Transformation of FMJ39x with plasmid pLOI297 restored the ability of the strain to grow anaerobically because expression of the genes from *Z. mobilis* allows NAD^+ to be regenerated when pyruvate is converted to ethanol.

Usually, a plasmid-carrying microorganism is not considered a good candidate for an industrial fermentation process because most plasmids depend on the addition of antibiotics for maintenance. However, when FBR3 is cultured anaerobically without antibiotics, it continues to maintain the plasmid, probably because cells that lose the plasmid lose the ability to grow fermentatively. Cultures of FBR3 serially transferred also maintained good ethanol yields. When a culture of FBR3 serially transferred 17 times was used to ferment 9.5% (w/v) xylose solutions, ethanol yields were 90% of theoretical *(3)*.

In the present study, we used this approach to construct two additional *E. coli* strains with different genetic backgrounds. Specifically, we introduced pLOI297 into two new *E. coli* strains (DC1368 and NZN111) that also carry *pfl* and *ldhA* mutations *(22)*. Unlike the parent strain for FBR3 (FMJ39x), the *pfl* mutation in these newer strains was introduced by genetic recombination and includes an antibiotic resistance marker. Therefore, the presence of both of these critical mutations can be conveniently confirmed using selective plates. For the original strain FBR3, reversion of the *pfl* mutation could not be detected for individual cells because it was introduced using chemical mutagenesis. Furthermore, strain NZN111 does not require additional growth factors (i.e., amino acids) for growth. This trait should be beneficial in developing an industrial medium. We tested both new strains for genetic and phenotypic stability. We also tested them for the ability to convert corn fiber hydrolysate into ethanol efficiently.

Materials and Methods

Bacterial Strains, Plasmid, and Growth Media

Table 1 gives the *E. coli* strains and the plasmid used in this study. *E. coli* DC1368 and NZN111 were from D. P. Clark (Southern Illinois University, Carbondale). Plasmid pLOI297 was from L. O. Ingram (University of Florida, Gainesville). Strains were grown aerobically on Luria–Bertani (LB) broth (10 g of tryptone, 5 g of yeast extract, 5 g/L of sodium chloride, and 15 g/L of Bacto agar if solid medium) supplemented with xylose (20 g/L) and, when indicated, either tetracycline (Tc) (20 μg/mL) or chloramphenicol (Cm) (10 μg/mL) and kanamycin (Kn) (50 μg/mL). Tetrazolium solid medium *(23)* (7 g/L of K_2HPO_4, 3 g/L of KH_2PO_4, 0.1 g/L of $MgSO_4$,

Table 1
Bacterial Strains and Plasmid Used

Strain or plasmid	Relevant characteristics	Source/ref.
E. coli K-12		
DC1368	*thr-1 leu 6 thi-1 lacY tonA22 straA* Δ*pfl*::Cm *ldhA*::Kn	*20*
NZN111	Δ*pfl*::Cm *ldhA*::Kn	*23*
FBR4	DC1368(pLOI297)	This work
FBR5	NZN111(pLOI297)	This work
Plasmid		
pLOI297	Ap^r Tc^r pdc^+ $adhB^{+a}$	*21*

[a]Ap, ampicillin; Tc, tetracycline.

2 g/L of proteose peptone, 25 μg/L of tetrazolium) supplemented with xylose (10 g/L) was used to identify xylose-metabolizing colonies. Antibiotics, tetrazolium, and sugars were added separately as sterilized solutions.

For routine anaerobic growth of *E. coli*, LB broth was prepared without NaCl and supplemented with xylose (4 g/L), acetate (1 g/L) as an additional carbon source, sodium bicarbonate (4 g/L) as a buffer, and cysteine HCl (0.5 g/L) as an oxygen scavenger. When indicated, the redox indicator resazurin (10 μg/mL) was added before autoclaving. Anaerobic medium was flushed with a 20% CO_2 and 80% N_2 gas mixture and sealed with butyl rubber stoppers as previously described *(24)*. Xylose and acetate were each prepared as separate stock solutions and autoclaved under 100% nitrogen atmosphere. The pH of the buffered medium was 7.0.

Culture Growth Conditions and Testing for Maintenance of Antibiotic Markers

Unless stated otherwise, cultures were incubated at 37°C. Cultures were grown anaerobically in either test tubes or serum bottles capped with butyl rubber caps and agitated at 90 rpm. The continued presence of the plasmid and integrated mutations was determined in serially transferred anaerobic cultures. Aliquots from anaerobic liquid cultures were serially diluted and plated onto aerobic LB solid medium supplemented with xylose. Plates were incubated for 12–16 h. Fifty randomly selected colonies were transferred to a plate containing similar medium, but supplemented with the appropriate antibiotic(s) (Cm, Kn, or Tc). The selection plates were examined for growth the next day.

Genetic Procedures

E. coli cells were transformed and plasmids were prepared from cultures (1–10 mL) by routine procedures *(25)*. The purity of plasmid preparations was verified by standard agarose gel electrophoresis of undigested and restriction endonuclease (*Eco*RI)-digested plasmid.

Preparation of Corn Fiber Hydrolysate

Corn fiber was obtained from Williams Energy Service (Pekin, IL) and stored at –20°C. The corn fiber was dried at 65°C for approx 24 h and ground in a hammer mill until it passed through a 28-mesh screen. The corn fiber was mixed with 1% (v/v) H_2SO_4 solution at a ratio of 1 g of biomass to 5.0 mL of acid solution, placed in a shallow Pyrex® dish, covered with aluminum foil, and heated at 121°C for 1 h. When cool, the liquid was separated from the solids using cheesecloth. The recovered liquid portion was then adjusted to a pH of 10.0 by adding $Ca(OH)_2$, reduced by adding sodium sulfite (1 g/L), warmed to and incubated at 90°C for 30 min, and finally neutralized with H_2SO_4 to a pH of 7.0. Following neutralization, the resulting precipitates, including gypsum, were removed by centrifugation (20,000*g*, 10 min). The recovered liquid was filter sterilized through a 0.22-μm membrane filter.

Batch Fermentations of Corn Fiber Hydrolysate, Mixed Sugars, and Xylose

Minibioreactors with automatic pH control were constructed and operated essentially as described previously *(3,25a)*. Each 500-mL Fleaker® (Fisher Scientific, Springfield, NJ) culture vessel contained 270 mL of hydrolysate supplemented with 30 mL of a 10X LB solution (10 g/L of tryptone and 5 g/L of yeast extract) and antifoam 289 (Sigma, St. Louis, MO) (0.1 mL/L). Nitrogen was bubbled through the medium for 30 min to remove oxygen. The fermentation vessels were inoculated with a 5% (v/v) inoculum grown overnight under anaerobic conditions. The inoculum cultures for the corn fiber hydrolysate, mixed sugars, and second group of xylose fermentations were grown on medium supplemented with 15 g/L of xylose. Fermentations were run at 35°C and stirred using magnetic stir bars (2.54 × 2.54 cm "X" shaped) at 350 rpm. The pH was set at 6.5 and maintained by the addition of base (4 *N* KOH). Each experiment was performed in duplicate.

Analytical Procedures

Optical density (OD) was monitored at 550 nm on a Beckman DU-70 spectrophotometer (Beckman Coulter, Inc., Fullerton, CA). Ethanol concentrations were determined by gas-liquid chromatography as described previously *(3)*. Concentrations of sugars, fermentation-generated organic acids, and sugar degradation products were determined by high-pressure liquid chromatography using an Aminex HPC-87H column (Bio-Rad, Richmond, CA) and refractive index detector. Samples were run at 65°C and eluted at 0.6 mL/min with 5 m*M* sulfuric acid. Xylose and galactose (a minor component of corn fiber hydrolysate) coeluted from the column.

Calculation of Fermentation Parameters

Two ethanol yields are reported: metabolic and production yields. Metabolic ethanol yields were calculated from ethanol (grams) produced

per amount (grams) of sugar(s) consumed. Production yields were calculated from ethanol (grams) produced per amount (grams) of sugar(s) added to the fermentation.

Volumetric ethanol production rates (V_{EtOH}) were calculated over the time required for the fermentation to reach 95% of its maximum ethanol concentration. The time required and maximum ethanol concentration were determined by fitting the Mitscherlich model to the ethanol data *(26)*. The model's three parameters were calculated with the Marquardt nonlinear fitting method using the SAS System® (SAS Institute, Cary, NC). Lag phase data were not used for the analysis. Fits with correlations below 0.95 were discarded, and instead, average ethanol productivity was estimated by dividing the maximum ethanol obtained by the sampling time; rates calculated in this manner are marked as such. Both yields and V_{EtOH} were corrected for the dilution of the culture from inoculation and automatic base additions.

A carbon balance was carried out based on sugars (arabinose, glucose, xylose, and galactose) consumed and products formed (acetate, biomass, ethanol, lactate, and succinate). Carbon dioxide production was calculated to be equal to moles of ethanol plus acetate minus succinate production *(20)*. Cell mass production was determined from OD measurements; an OD of 1.0 at 550 nm was determined to be equal to a concentration of 0.30 mg/mL of cell dry wt (data not shown). Cell mass production for corn fiber hydrolysate fermentations, in which OD could not be directly measured, was estimated from the mixed sugar control fermentations. Dried *E. coli* biomass was assumed to be 45% (w/w) carbon *(27)*. Final product and sugar concentrations were corrected for dilution by base addition.

Results and Discussion

Construction and Characterization of E. coli *FBR4 and FBR5*

E. coli FBR4 and FBR5 were constructed by transforming strains DC1368 and NZN111, respectively, with pLOI297 (Table 1). Transformants were next cultured in liquid medium under anaerobic conditions (*see* Materials and Methods). The NZN111 transformant initially did not grow well on the xylose-containing medium; however, a xylose-utilizing mutant (FBR5) was readily isolated using tetrazolium plates supplemented with xylose. Tetrazolium is a redox-sensitive dye; colonies that metabolized xylose were identified by color changes in the surrounding medium.

Next strains FBR4 and FBR5 and their parent strains DC1368 and NZN111 were tested for growth on anaerobic LB. Each strain was grown on aerobic LB supplemented with xylose overnight. A 1% (v/v) inoculum was transferred to a culture containing anaerobic LB supplemented with xylose and resazurin (an oxygen indicator). The ODs of the cultures were measured after 24 and 48 h; Table 2 presents the results. Neither DC1368 nor NZN111 showed significant growth in the 48-h period. By contrast, both FBR4 and FBR5 reached their maximum OD within 24 h.

Table 2
Comparison of Growth
of FBR Strains and Parental Strains
in Anaerobic Medium

	OD[a]	
Strain	24 h	48 h
DC1368	0.070	0.070
FBR4	0.270	0.250
NZN111	0.020	0.050
FBR5	0.250	0.240

[a]OD measured at 550 nm.

Neither *E. coli* DC1368 nor NZN111 was expected to grow anaerobically. The *pfl* and *ldhA* mutations carried by these strains prevent them from recycling the NADH,H generated from oxidation of the sugar(s) to pyruvate. In FBR4 and FBR5, introduction of the *Z. mobilis* genes on pLOI297 (encoding pyruvate decarboxylase and alcohol dehydrogenase) allowed selective conversion of pyruvate to ethanol and supplied the needed electron sink for growth under anaerobic conditions. A similar finding was obtained when strain FMJ39 *(18)*, which also carries *pfl* and *ldhA* mutations, was transformed with pLOI297 (strain FBR3).

Evidence that *E. coli* NZN111 will not grow in anaerobic culture has also been observed by other investigators *(22,28,29)*. Donnelly et al. *(29)*, was able to isolate a mutant of NZN111, using directed evolution, that grew anaerobically. The mutant grew very slowly and produced succinate, acetate, and ethanol, but not formate or hydrogen, which indicates it is not a *pfl* revertent. If a similar mutant strain arose in our anaerobic cultures of FBR4 or FBR5, it did not grow to form a significant fraction of the population. As we show in a later section, acetate production was not detected in fermentations with either FBR4 or FBR5.

Genetic Stability of FBR4 and FBR5 in Serial Batch Cultures

Strains FBR4 and FBR5 were tested for continued plasmid maintenance in the absence of antibiotics using serial batch cultures. Cultures of FBR4 and FBR5 were grown anaerobically in media supplemented with xylose and acetate. Acetate is required as a carbon source, but does not serve as an energy source. Cultures were transferred twice per 24-h period using a 2% (v/v) inoculum. Once per day, a sample was removed, diluted, and grown on solid medium. The plates were incubated overnight, and 50 isolated colonies replicate-plated onto solid medium supplemented with Tc to select for the presence of pLOI297 (Table 1). Cultures were transferred a total of 10 times. The ratio of cells carrying and not carrying the plasmid pLOI297 was determined from the number of transferred colonies that grew on the selective plates (Table 3). The results show that an average of

Table 3
Stability of pLOI297 and Chromosomal Mutations
Under Anaerobic Growth Conditions

Strain selection transfers[a]	Colonies that maintained selective marker (%)[b]			
	FBR4		FBR5	
	Tc	Cm/Kn	Tc	Cm/Kn
2	77 ± 11[c]	100 ± 0	94 ± 0	100 ± 0
4	90 ± 0	100 ± 0	98 ± 0	100 ± 0
6	99 ± 0	100 ± 0	93 ± 1	100 ± 0
8	100 ± 0	100 ± 0	95 ± 0	100 ± 0
10	100 ± 0	100 ± 0	94 ± 0	100 ± 0
Average	93 ± 10	100 ± 0	95 ± 2	100 ± 0

[a]Cells were serially transferred in anaerobic LB supplemented with xylose.
[b]Cells were plated onto LB plates supplemented with xylose, incubated for 24 h, and 50 colonies were transferred to selective media plates.
[c]Average of duplicate runs.

93 and 95% of the cells sampled from the FBR4 and FBR5 cultures, respectively, maintained the plasmid. Furthermore, no decline was detected in the number of cells maintaining the plasmid with culture transfers. In particular, strain FBR4 had an initial percentage of 77% and ended with a percentage of 100%.

As we have detailed, strains FBR4 and FBR5 are dependent on the genes encoded on the pLOI297 plasmid for anaerobic growth. Cells that lose the plasmid and the ability to convert pyruvate into ethanol would be expected to lose the ability to grow anaerobically. Therefore, the plasmid would be expected to be selectively maintained by cultures of FBR4 and FBR5. Those cells sampled that appeared to have lost the plasmid can be attributed to the method used for measuring plasmid maintenance and cytoplasmic inheritance. First, the method used to measure plasmid maintenance overestimates plasmid loss because the sampled cells were initially grown on nonselective plates under aerobic conditions. Therefore, cells that appear to have lost the plasmid might have lost it after being transferred to the solid nonselective medium. Second, cells that lose the plasmid could continue growth for a short time if the cells inherited sufficient Pdc and Adh activity.

Strains FBR4 and FBR5 also carry antibiotic resistance markers for their *pfl* and *ldhA* chromosomal mutations. Cm resistance is associated with the *pfl* mutation and Kn resistance with the *ldhA* mutation (Table 1). These selective markers were used to verify maintenance of the *pfl* and *ldhA* mutations in the serially transferred cultures. The same colonies examined on Tc^r selection plates for the presence of pLOI297 were also plated onto Cm^r and Kn^r selection plates. All colonies transferred to Cm^r and Kn^r selection plates grew (Table 3), indicating stable maintenance of the Δ*pfl*::Cm and *ldhA*::Kn mutations.

Batch Fermentation of Xylose

For the FBR strains to have commercial potential, the cultures must be able to retain their high ethanol yields after repeated transfers. Retaining the pLOI297 plasmid and chromosomal mutations, although necessary for achieving high ethanol yields, does not guarantee that the cultures will still convert highly concentrated sugar(s) streams to ethanol with good yields. For example, repeated batch and continuous cultures of *E. coli* strain K011 were observed to have decreasing ethanol yield while still maintaining the relevant selective marker *(16,17)*. To observe what effect the serial transfers had on the yields of FBR4 and FBR5, they were used to inoculate pH-controlled batch fermentations. The batch fermentation medium containing LB is supplemented with 9.5% (w/v) xylose. The batch fermentations were inoculated with FBR4 and FBR5 cultures that had been transferred 13 times each. As controls, similar fermentations were inoculated with cultures of FBR4 and FBR5 that had been grown on Tc^r plates and transferred only twice.

Table 4 presents the results from the fermentations. Both fermentations used all the xylose and the only detectable side product was succinate (0.047–0.082% [w/v]); acetate, formate, and lactate production were not detected. The ethanol yields varied from 88–92% of theoretical (0.51 g of ethanol/g of sugar) and were slightly higher for the fermentations inoculated from the serially transferred cultures (transferred) compared to the controls (stock). The control cultures also produced more succinate and, as a result, required more base addition to control pH. Therefore, at the least, it can be concluded that serially transferring FBR4 and FBR5 did not decrease their ability to ferment xylose selectively to ethanol.

Batch Fermentations of Mixed Sugars and Corn Fiber Hydrolysate

FBR4 and FBR5 were tested for their ability to ferment corn fiber hydrolysate. The noncellulosic fraction of corn fiber can be easily and efficiently hydrolyzed by treating at elevated temperatures with dilute sulfuric acid *(3,5,30–33)*. The resulting hydrolysate contains a mixture of arabinose, glucose, and xylose (*see* Table 5). It also contains microbial inhibitors formed during the hydrolysis reaction (e.g., acetate) that often prevent the sugars from being readily fermented into ethanol. A common method for partially mitigating these inhibitors is to "overlime" the hydrolysate prior to fermentation *(33–37)*. For this study, corn fiber was hydrolyzed using dilute sulfuric acid (1% [v/v]) for 1 h at 121°C. The hydrolysate (i.e., liquid fraction) was overlimed (*see* Materials and Methods), supplemented with yeast extract and tryptone, and filter sterilized. The fermentations were run in minibioreactors and controlled at a pH of 6.5 and temperature of 35°C.

The final corn fiber hydrolysate medium contained a total sugar concentration of 8.5% (w/v) (2.0% [w/v] arabinose, 2.8% [w/v] glucose, and 3.7% [w/v] xylose). Table 5 gives the results from the fermentations. Both

Table 4
Phenotypic Stability for Ethanol Production

Strain	Inoculum[a]	Total sugar (% w/v)	Maximum ethanol (% w/v)	Residual sugar (% w/v)	Base addition (mmol/L)	Succinate (% w/v)	Y_M[b] (g/g)	Y_P[c] (g/g)	V_{EtOH}[d] (g/[L·h])	Carbon recovery (%)
FBR4	Transfer	9.50	4.23 ± 0.05	0.00 ± 0.00	181 ± 24	0.056 ± 0.00	0.47 ± 0.01	0.47 ± 0.01	0.57 ± 0.04	105 ± 2
FBR5	Transfer	9.50	4.15 ± 0.04	0.00 ± 0.00	170 ± 0	0.047 ± 0.02	0.46 ± 0.00	0.46 ± 0.00	0.59 ± 0.03	101 ± 0
FBR4	Stock	9.50	3.92 ± 0.07	0.00 ± 0.00	255 ± 6	0.064 ± 0.02	0.44 ± 0.01	0.44 ± 0.01	0.51 ± 0.01	99 ± 2
FBR5	Stock	9.50	3.95 ± 0.05	0.00 ± 0.00	220 ± 30	0.082 ± 0.01	0.44 ± 0.00	0.44 ± 0.00	0.59 ± 0.02	98 ± 0

[a]See text.
[b]Metabolic yield, grams of ethanol produced per gram of sugar(s) consumed.
[c]Process yield, grams of ethanol produced per gram of sugar(s) added to medium.
[d]Average ethanol productivity, grams of ethanol produced per liter of culture per hour.

Table 5
Fermentation of Mixed Sugars and Corn Fiber Hydrolysate[a]

Strain	Carbon source	Total sugar (% w/v)	Maximum ethanol (% w/v)	Residual sugar (% w/v)	Base addition (mmol/L)	Succinate (% w/v)	Y_P (g/g)	Y_M (g/g)	V_{EtOH} (g/[L·h])	Carbon recovery (%)
FBR4	Xylose	7.62	3.37 ± 0.01	0.00 ± 0.00	135 ± 00	0.036 ± 0.00	0.46 ± 0.00	0.46 ± 0.00	0.67 ± 0.01[d]	100 ± 0
FBR5	Xylose	7.62	3.34 ± 0.04	0.00 ± 0.00	158 ± 25	0.038 ± 0.02	0.46 ± 0.00	0.46 ± 0.00	0.66 ± 0.01[d]	99 ± 0
FBR4	Mixed sugars[b]	7.62	3.26 ± 0.09	0.00 ± 0.00	190 ± 38	0.056 ± 0.03	0.45 ± 0.01	0.45 ± 0.01	0.61 ± 0.07	100 ± 1
FBR5	Mixed sugars[b]	7.62	3.40 ± 0.05	0.00 ± 0.00	159 ± 6	0.050 ± 0.04	0.46 ± 0.01	0.46 ± 0.01	0.92 ± 0.04	102 ± 1
FBR4	CFH[c]	8.46	3.30 ± 0.06	1.55 ± 0.10	95 ± 19	0.044 ± 0.05	0.40 ± 0.01	0.50 ± 0.02	0.50 ± 0.06	108 ± 2
FBR5	CFH[c]	8.46	3.74 ± 0.01	0.31 ± 0.01	159 ± 32	0.059 ± 0.06	0.46 ± 0.00	0.48 ± 0.00	0.77 ± 0.05	104 ± 1

[a]Duplicate fermentations were performed for each trial.
[b]Control prepared with reagent grade sugars: 1.52% arabinose, 3.05% glucose, and 3.05% xylose.
[c]Corn fiber hydrolysate: 2.0% arabinose, 2.8% glucose, and 3.7% xylose.
[d]Average productivity was estimated by dividing the maximum ethanol concentration by the sampling time because attempts to fit the Mitscherlich model were unsuccessful.

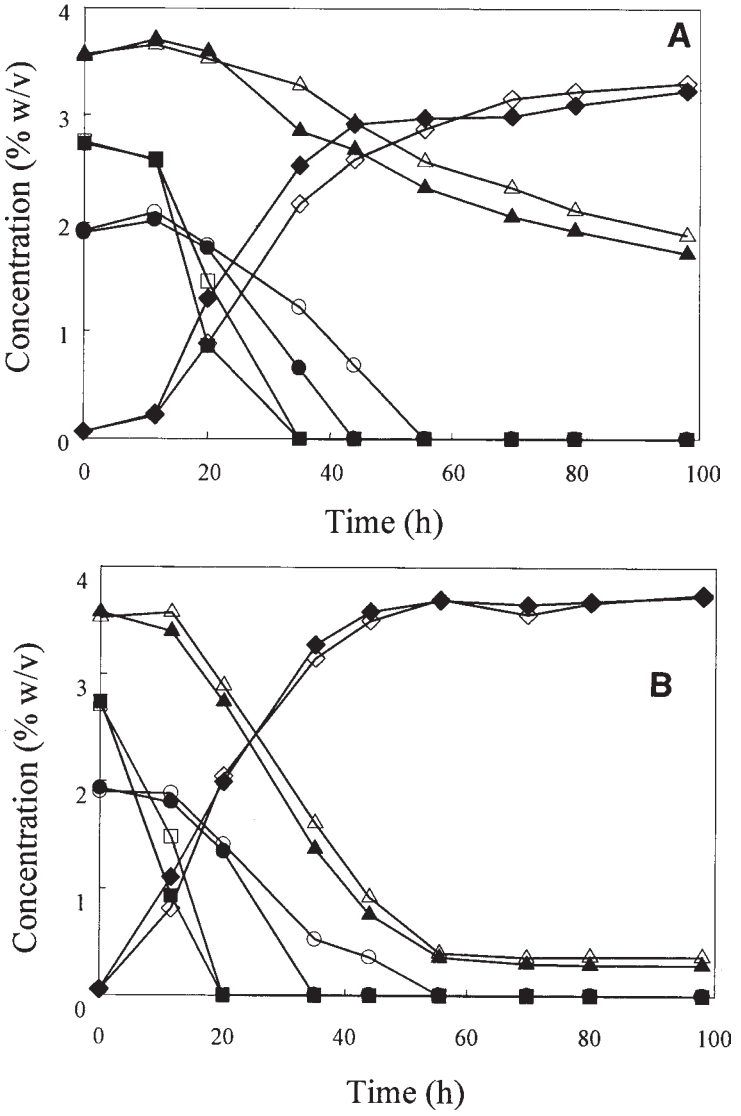

Fig. 1. Ethanol fermentations of corn fiber hydrolysate by **(A)** FBR4 and **(B)** FBR5. Solid and open symbols are data from duplicate runs. ●, Arabinose; ◆, ethanol; ■, glucose; ▲, xylose.

strains failed to completely use all the available sugars in the hydrolysate fermentations. *E. coli* FBR5 consumed all but 0.3% (w/v) of the xylose (3.6% of the available sugars) within 55 h (Fig. 1B). By contrast, FBR4 left behind 1.55% (w/v) of the xylose (18% of the available sugars) after 100 h (Fig. 1A). As a result of the difference in residual xylose, *E. coli* FBR5 had a much higher process yield (92% of theoretical) compared to *E. coli* FBR4 (72% theoretical).

Control fermentations were conducted using either xylose or a mixture of reagent grade sugars (1.52% [w/v] arabinose, 3.05% [w/v] glucose,

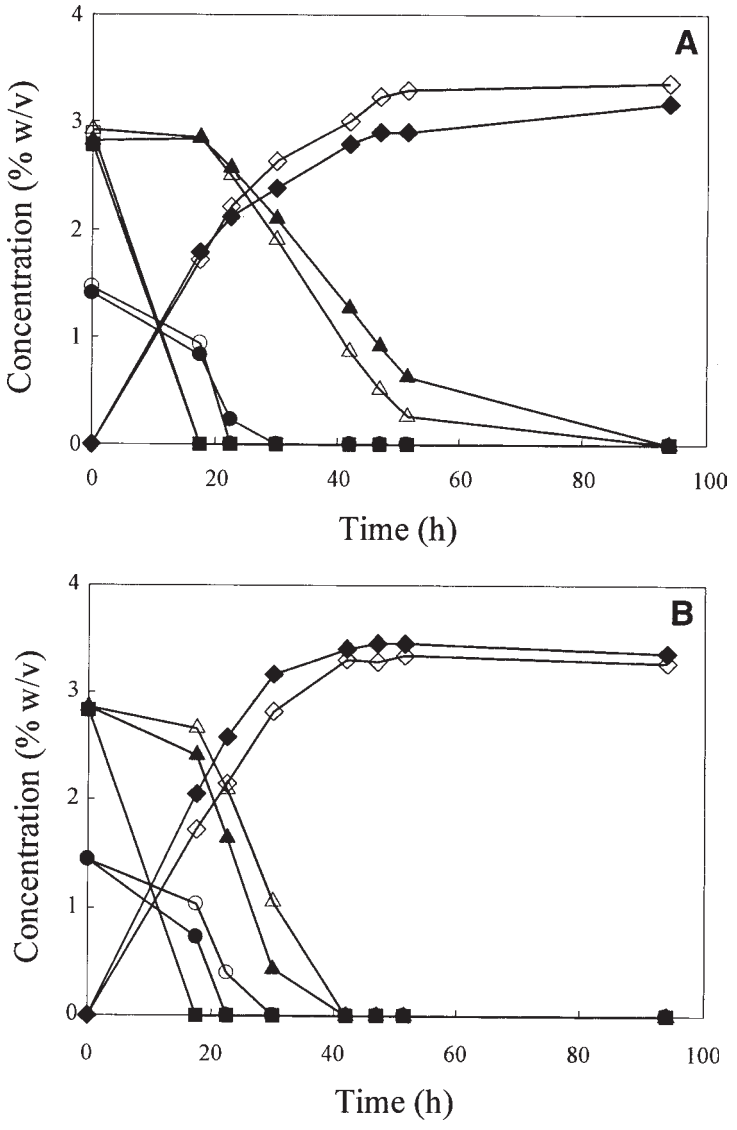

Fig. 2. Ethanol fermentation of sugar mixture (reagent grade) by **(A)** FBR4 and **(B)** FBR5. Solid and open symbols are data from duplicate runs. ●, Arabinose; ◆, ethanol; ■, glucose; ▲, xylose.

and 3.05% [w/v] xylose) as a carbon source. The strains did ferment the control sugar mixture to completion. Ethanol yields for the control fermentations were 88–92% of theoretical, and the only detectable side product produced was succinate (Table 5).

The difference between the strains on corn fiber hydrolysate was also apparent for the control fermentations of mixed sugars. Although final ethanol concentrations were similar for both strains, *E. coli* FBR4 fermented the xylose more slowly than *E. coli* FBR5 (Fig. 2A,B). Whereas strain FBR5

completely fermented the sugars in approx 40 h, strain FBR4 took at least 20 h longer. However, strain FBR4 consumed xylose slower than FBR5 only when other sugars (i.e., arabinose and glucose) were present. This trend is also reflected in the average ethanol production rates (Table 5). Both strains had similar ethanol production rates when xylose was the sole sugar source, but FBR5 had higher production rates for the mixed sugars and hydrolysate fermentations (Table 5).

Comparison of Strains FBR3, FBR4, and FBR5

E. coli FBR5 was superior to strain FBR4 in terms of ethanol productivity and production yield. *E. coli* FBR4 required nearly 20 h longer to complete the fermentation of the reagent grade mixed sugars and left significantly higher concentrations of residual xylose when fermenting the corn fiber hydrolysate than FBR5. As a result of the residual xylose, the production yield of FBR4 on the hydrolysate was only 78% of theoretical, compared with 90% for FBR5.

E. coli FBR5 is also a better strain to use for ethanol fermentation compared to *E. coli* FBR3 *(18,30)* for several reasons. First, *E. coli* FBR5 proved to be more robust for fermenting corn fiber hydrolysate. Strain FBR5 successfully fermented the corn fiber hydrolysate without prior strain adaptation, which was necessary for strain FBR3 *(30)*. Second, *E. coli* FBR5 had a higher ethanol productivity than *E. coli* FBR3 when fermenting corn fiber hydrolysate (0.77 and 0.57 g/[L·h], respectively). Third, as stated previously, the critical *pfl* mutation was introduced into *E. coli* FBR5 by molecular means and therefore can be easily monitored by testing for Cm resistance. Chemical mutagenesis was used to introduce this mutation into the parent of FBR3. Finally, FBR5 is a prototroph, whereas *E. coli* FBR3 is auxotrophic for threonine, leucine, and thiamine *(19)*. Therefore, *E. coli* FBR5 should require a simpler production medium than *E. coli* FBR3.

The batch fermentation results for FBR5 also compare favorably with those reported for the widely used ethanologenic *E. coli* K011. Ethanol yields (0.44–0.47 g/g) reported herein for fermentations of xylose and mixed sugars are similar to those determined for strain K011 *(3)*. *E. coli* K011 has been reported to ferment corn fiber hydrolysate, prepared with overliming, with an average productivity of 0.8 g/(L·h) and a process yield of 0.43 g/g *(5)*, quite similar to the rate (0.77 g/[L·h]) and yield (0.46 g/g) reported herein for strain FBR5.

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