Journal of Industrial Microbiology & Biotechnology (1999) 22, 575–581

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Conversion of corn fiber to ethanol by recombinant *E. coli* strain FBR3

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We have developed a novel ethanologenic *Escherichia coli* strain FBR3 that is an efficient biocatalyst for converting mixed sugar streams (eg, arabinose, glucose, and xylose) into ethanol. In this report, the strain was tested for conversion of corn fiber hydrolysates into ethanol. Corn fiber hydrolysates with total sugar concentrations of 7.5% (w/v) were prepared by reacting corn fiber with dilute sulfuric acid at 145°C. Initial fermentations of the hydrolysate by strain FBR3 had lag times of approximately 30 h judged by ethanol production. Further experiments indicated that the acetate present in the hydrolysate could not solely account for the long lag. The lag phase was greatly reduced by growing the pre-seed and seed cultures on corn fiber hydrolysate. Ethanol yields for the optimized fermentations were 90% of theoretical. Maximum ethanol concentrations were 2.80% w/v, and the fermentations were completed in approximately 50 h. The optimal pH for the fermentation was 6.5. Below this pH, sugar consumption was incomplete and above it, excess base addition was required throughout the fermentation. Two alternative neutralization methods (overliming and overliming with sulfite addition) have been reported for improving the fermentability of lignocellulosic hydrolysates. These methods further reduced the lag phase of the fermentation, albeit by a minor amount.

Keywords: alcohol; biofuel; pentoses; corn fiber; ethanologenic; *Escherichia coli*

Introduction

In 1997, approximately one billion gallons of fuel ethanol were produced from cornstarch and blended with gasoline as an oxygenate [21]. Interests exist for increasing ethanol's share of the automotive fuel market because of the opportunity it affords to lower national carbon dioxide emissions, lower air pollutants and decrease reliance on foreign sources of petroleum. Significantly increasing fuel ethanol production beyond current limits will necessitate developing alternative fermentation feedstocks. Only lignocellulosic biomass is plentiful and inexpensive enough to significantly supplement starch as a fermentation feedstock [14]. Corn fiber is one such feedstock that is readily available.

Corn fiber is a low-value co-product produced during corn wet milling that consists of the corn hull of the kernel and residual starch. Corn fiber contains approximately 70% (w/w) carbohydrates [12,13], is uniform in composition and size, and is centrally stockpiled. In addition, because cornstarch converted to ethanol is fermented on site, many corn wet milling plants have fermentation facilities. About 3.4×10^6 dry tons of corn fiber are produced each year in the United States [21]. This quantity translates into a potential source of hundreds of millions of gallons of ethanol per year. Furthermore, corn fiber serves as a model for fermentation of other lignocellulosic biomasses.

A major technical hurdle to converting corn fiber to ethanol is developing an appropriate microorganism for the fermentation of mixed sugars. Over 50% (w/w) of the carbohydrates in corn fiber consist of pentoses (xylose and l-arabinose), which traditional yeasts, used in industrial ethanol fermentations, cannot metabolize. Only a few genetically engineered microorganisms, specifically developed for biofuel production, are capable of converting a mixture of hexoses and pentoses to ethanol efficiently (for a recent review see [18]).

One promising ethanologenic strain capable of fermenting a variety of hexoses and pentoses, which was developed in this laboratory, is *E. coli* strain FBR3. This strain was created by transforming a xylose utilizing clone of strain FMJ39 (FMJ39x) with plasmid pLOI297 [8]. Strain FMJ39x cannot grow anaerobically because it has mutations that prevent it from fermentatively reducing pyruvate [20]. Therefore, under anaerobic growth conditions, the strain is unable to regenerate the NAD^+ consumed when forming pyruvate. Transforming strain FMJ39x with the plasmid pLOI297, which was developed by Alterthum *et al* [1], restores the ability of the strain to grow anaerobically because expression of the plasmid allows for pyruvate to be converted to ethanol and NAD^+ to be regenerated. Furthermore, when grown anaerobically, almost all of the consumed sugar is converted to ethanol.

Normally, a plasmid-carrying microorganism is not considered a good candidate for an industrial fuel fermentation because most plasmids depend upon addition of antibiotics for maintenance. However, we have determined that when FBR3 is grown anaerobically, there is a positive selection for plasmid pLOI297, which is to say, cells that lose the plasmid lose the ability to grow anaerobically and thus die. Cultures of strain FBR3 serially transferred 17 times in

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Received 29 September 1998; accepted 20 February 1999

anaerobic medium not only maintained the plasmid but in pH-controlled fermentations of high sugar concentrations, ethanol yields were 90% of theoretical [8]. The plasmid pLOI297 was also maintained by a similar but earlier version of strain FBR3 (FBR2) when continuously cultured without antibiotic [17].

In a previous study, the fermentation performance of strain FBR3 was tested on glucose, xylose, and arabinose, the major sugars found in corn fiber hydrolysate [8]. Ethanol yields were 90–92% of theoretical. In this study, strain FBR3 was used to convert corn fiber hydrolysates to ethanol.

Materials and methods

Preparation of corn fiber hydrolysates

Corn fiber was obtained from Williams Energy (Pekin, IL, USA; formally Pekin Energy Corporation) and stored at –20°C. The non-cellulosic carbohydrate content of the corn fiber was determined by digesting dried corn fiber in 2 N trifluoroacetic acid for 1 h at 100°C as previously described [7].

The corn fiber was reduced in size using a commercial meat grinder equipped with a cutting blade and 3/32 inch perforated plate on the output side (Biro, Marblehead, OH, USA). Wet corn fiber (25 kg) was mixed with 30 L of dilute 1.1% (v/v) sulfuric acid solution—a 17% (w/w) solids loading of corn fiber—and preheated for approximately 10 min in an autoclave at 121°C. The pre-heating step increased the viscosity of the mixture and kept the solid and liquid phases from separating. Without pre-heating, the corn fiber solution plugged the pump. Next, the slurry was pumped into the reactor using a progressive cavity positive displacement pump (Tarby, Claremore, OK, USA).

The corn fiber was hydrolyzed in a batch reactor made of 316 SS and equipped with an external jet siphon mixer and a steam jacket. The reactor had a working volume of 40 L. Prior to introducing the corn fiber slurry, the reactor was pre-heated to the reaction temperature. The slurry was pumped into the reactor, entering through the jet siphon and thereby pre-heated with direct steam (220°C, saturated). Reactor temperature and pressure were controlled manually by adjusting a variable pressure relief valve. After the corn fiber slurry had been transferred (in 15 min), the jet siphon inlet was changed from the pump to the bottom of the tank. Re-circulation of corn fiber slurry through the jet siphon was used to keep the contents of the tank mixed.

Following hydrolysis, residual solids were removed using a basket centrifuge equipped with a solid bowl. The liquid recovered was neutralized in one of three ways: (1) the pH was adjusted to pH 6.5–7.0 by adding $Ca(OH)_2$; (2) the pH was adjusted to 10 by adding $Ca(OH)_2$, warmed at 90°C for 30 min, and neutralized with H_2SO_4; or (3) treated similar to the last protocol except 1 g L^{-1} of sodium sulfite was added after adjusting the pH to 10. Following neutralization, the resulting precipitates, including gypsum, were removed by centrifugation (20 000 × *g*, 10 min). The liquid recovered was filter sterilized through a 0.22-μm^2 pore-size membrane filter.

Oxygen was removed from the hydrolysate used to grow the seed and pre-seed cultures by treating the hydrolysate with sonic waves (Bransonic Ultrasonic Cleaner, Brauns, Danbury, CT, USA) while under a vacuum for 2 min. The removed gases were next replaced with sterile N_2. Antifoam 289 (Sigma, St Louis, MO, USA; 1:1000 dilution) was added to the hydrolysate before applying the vacuum to minimize foaming and aseptic technique was used throughout.

Bacterial strains, plasmid, and growth media

The development and phenotype of *E. coli* strain FBR3 has been described previously [8]. Strains were routinely grown on LB broth (10 g tryptone, 5 g of yeast extract, 5 g of sodium chloride per liter) supplemented as indicated below with Tet (10 μg ml^{-1}) and either l-arabinose, d-glucose, or d-xylose. Antibiotics and sugars were added separately as filter-sterilized solutions.

For routine anaerobic growth of *E. coli*, LB broth was prepared without NaCl and supplemented with either glucose or xylose (4 g L^{-1}), acetate (1 g L^{-1}) as an additional carbon source, sodium bicarbonate (4 g L^{-1}) as a buffer, and cysteine HCl (0.5 g L^{-1}) as an oxygen scavenger. Anaerobic medium was flushed with carbon dioxide and sealed with butyl rubber stoppers as previously described [8]. The pH of the buffered medium was 7. When hydrolysate was used as the carbon source for pre-seed and seed cultures, LB broth was prepared at 2× concentration, without cysteine or NaCl, and then appropriate amounts of sterile distilled water (flushed with nitrogen) and oxygen-depleted hydrolysate were added.

Batch fermentations of corn fiber hydrolysate and mixed sugars

Minifermentors with automatic pH control were constructed and operated essentially as described previously [5,7]. Each 500-ml Fleaker®culture vessel contained 270 ml of hydrolysate supplemented with 30 ml of a 10× LB solution (10 g L^{-1} tryptone and 5 g L^{-1} yeast extract) and antifoam 289 (0.1 ml L^{-1}). Nitrogen was bubbled through the medium for 30 min subsequent to inoculation to remove oxygen. The fermentation vessels were inoculated with a 5% (v/v) inoculum from an anaerobic culture grown overnight at 37°C. Fermentations were run at 35°C and stirred magnetically with 1×1 inch 'X' shaped stir bars at 350 rpm. The pH was set at 6.5 and maintained by addition of a concentrated base solution (4 N KOH). Ethanol, sugars, organic acids, and OD_{550} were determined periodically with 1.5-ml samples of cultures. Each experiment was run in duplicate.

Analytical procedures

Optical density was monitored at 550 nm on a Beckman DU®-70 spectrophotometer. Ethanol concentrations were determined by gas liquid chromatography as described previously [7]. Concentrations of sugars, fermentation-generated organic acids, and sugar degradation products were determined by high-pressure liquid chromatography (HPLC) using an Aminex HPC-87H column (Bio-Rad, Richmond, CA, USA) and refractive index detector. Samples were run at 65°C and eluted at 0.6 ml m^{-1} with 5 mM sulfuric acid. Hydrolysis samples analyzed for total

solubilized carbohydrates were incubated in 2 N trifluoroacetic acid for 1 h at 100°C to complete the hydrolysis reaction [7]. Xylose and galactose (a minor component) coeluted from the column.

Calculation of fermentation parameters

Ethanol and biomass yields were calculated based on ethanol (wt) or biomass (wt) produced per amount (wt) of sugar consumed. Biomass production was determined from optical density measurements; 1.0 optical density at 550 nm was determined to be equal to a concentration of 0.30 mg ml^{-1} cell dry weight (data not shown). Biomass yields were calculated based on biomass (wt) formed by the total amount of sugar added.

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 [16]. The model's three parameters were calculated with the Marquardt nonlinear fitting method using the SAS System® (SAS Institute, Cary, NC, USA). Lag phase data were not used for the analysis. Both yields and V_{ETOH} were corrected for the dilution of the culture from inoculation and automatic base additions needed to maintain pH 6.5.

A carbon balance was carried out based on sugars (arabinose, glucose, and xylose plus galactose) consumed and products formed (acetate, biomass, ethanol, lactate, and succinate). It was assumed that the amount of carbon dioxide produced was equimolar to ethanol and acetate minus succinate production [6]. Dried *E. coli* biomass was assumed to be 45% (w/w) carbon [3]. Final product and sugar concentrations were corrected for dilution by base addition.

Results and discussion

Hydrolysis of corn fiber

The corn fiber used in this study came from a single lot. The moisture content was 62% (w/w). The non-cellulosic carbohydrate content of dried corn fiber consisted of $11 \pm 0\%$ (w/w) arabinose, $16 \pm 1\%$ (w/w) glucose and $20 \pm 0\%$ (w/w) xylose and galactose with galactose contributing approximately 2% (w/w) of corn fiber [7]. Compositional data are the average of four trials.

The corn fiber was hydrolyzed by mixing it with a dilute sulfuric acid solution and heated at 140–160°C. The optimal hydrolysis temperature and time were determined to be 145°C for 15 min. At 140°C, hydrolysis was incomplete even after 20 min (data not shown). Hydrolysis was completed at 160°C, but the resulting solution was inhibitory to fermentation by strain FBR3 (data not shown). Therefore, 145°C was chosen for subsequent hydrolysis reactions because it was the lowest temperature where hydrolysis of the corn fiber was essentially complete, and it was 15°C below the temperature at which the hydrolysate solution became inhibitory to growth of strain FBR3.

Figure 1 shows the results from a typical hydrolysis run at 145°C. The average total sugar yield from duplicate runs was $7.58 \pm 0.37\%$ w/v, which is 95% of the theoretical yield (excluding cellulose). Most of the non-cellulosic

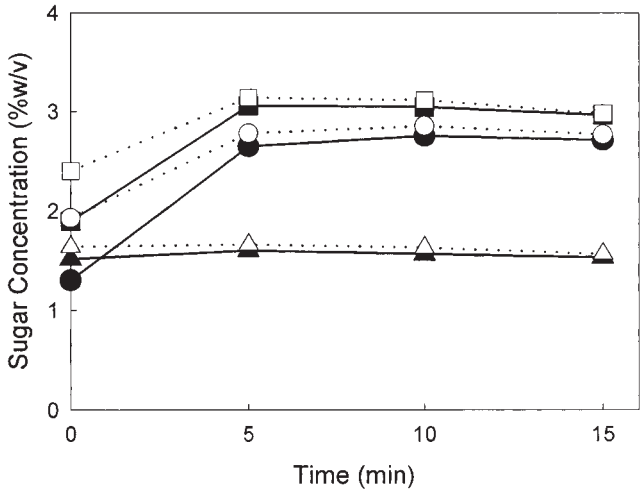

Figure 1 Rate of hydrolysis of corn fiber in dilute sulfuric acid at 145°C. Dotted lines and unfilled symbols are concentrations of total solubilized carbohydrates and solid lines and filled symbols are concentrations of monomeric sugars. Arabinose (▲); glucose (●); and xylose (■).

carbohydrates were solubilized by the time the last of the corn fiber slurry was added to the reactor. Maximum monomeric sugar concentration was obtained by 10 min. In addition to the specified sugars, approximately 3 g L^{-1} acetate was generated from hydrolysis of the acetyl groups [12]. Neither fufural nor hydroxy-methyl-furfural (HMF) products of sugar degradation, were detected by HPLC.

Heating in the presence of dilute sulfuric acid has been the method of choice for hydrolyzing corn fiber [2,4,7,12,23]. In particular, Grohmann and Bothast [12] conducted a systematic kinetic study of corn fiber hydrolysis at 100–160°C with varying sulfuric acid concentrations. They suggested an optimal temperature of 140°C, but also reported longer hydrolysis times. Others reported hydrolysis temperatures of 120–140°C, with longer reaction times for lower temperatures [2,4,7,23]. In agreement with our findings, Grohmann and Bothast [12] also found that corn fiber hydrolyzed at 160°C inhibited fermentation by ethanologenic *E. coli* strain K011. It should be noted that in the previously published methods, corn fiber was manually loaded into a batch reactor in either dry or wet form. In contrast, the grinding and preheating steps used in this study allowed for the corn fiber to be readily pumped.

Fermentation of corn fiber hydrolysate

For the fermentation step, the corn fiber hydrolysate was mixed with a 10× solution of LB and inoculated with a 5% (v/v) overnight culture of strain FBR3 that had been grown anaerobically on LB supplemented with 4 g L^{-1} xylose. As a control, strain FBR3 was used to ferment a mixed sugar medium with similar concentrations of arabinose, glucose, and xylose as the hydrolysate. The results from strain FBR3's fermentation of the corn fiber hydrolysate were disappointing (Figure 2). Fermentation of the hydrolysate took approximately twice as long to be completed compared to fermentation of the mixed sugars. The fermentation of hydrolysate also appeared, based upon ethanol production, to have an extended lag phase. However, the eventual ethanol yields were similar (Table 1) and were 88–92% of

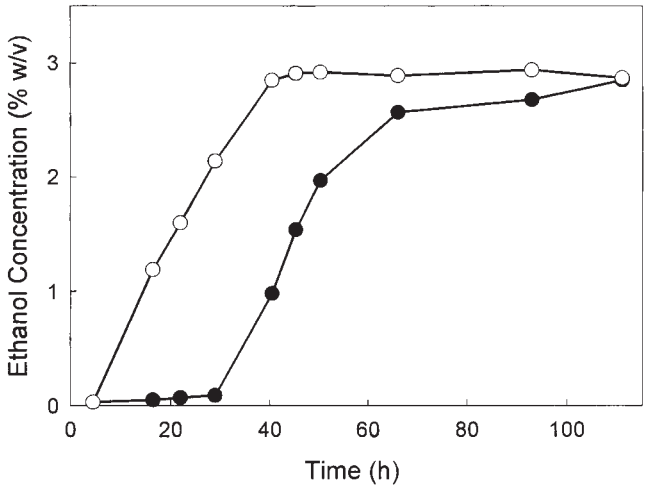

Figure 2 Ethanol measurements from pH-controlled batch fermentations of corn fiber hydrolysate (●) and a mixed sugar control (○). Each plot is the average of duplicate runs.

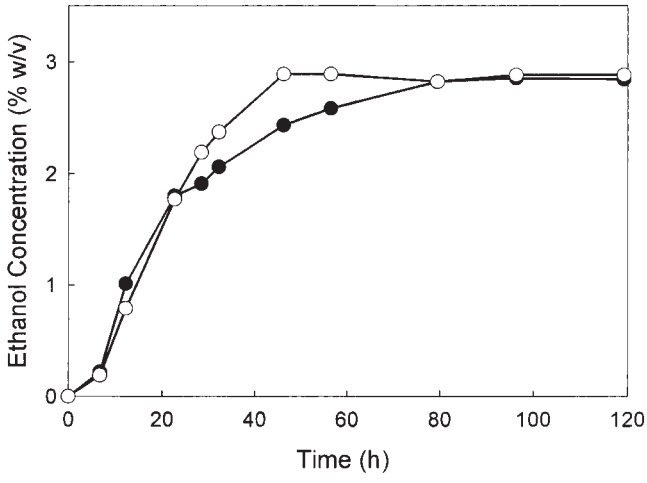

Figure 3 Ethanol measurements from fermentations of mixed sugars (○) and mixed sugars supplemented with 3 g L^{-1} acetate (●). Each plot is the average of duplicate runs.

Table 1 Fermentation of corn fiber hydrolysate by *E. coli* strain FBR3 and effect of acetate addition

Carbon source	Acetate addition	Total sugar (% w/v)	Maximum ethanol (% w/v)	Residual sugar (% w/v)	Base addition (mmol L^{-1})	Succinate (g L^{-1})	$Y_{E/S}$ (g g^{-1})	V_{EtOH} (g L^{-1} h^{-1})	Carbon recovery (%)
Fermentation of corn fiber hydrolysate									
Corn fiber hydrolysate	None	6.66	2.85 ± 0.01	0.49 ± 0.27	190 ± 38	3.5 ± 1.2	0.47 ± 0.01	0.35 ± 0.015	105 ± 2
Mixed sugars	None	6.64	2.87 ± 0.07	0.00 ± 0.00	146 ± 19	3.4 ± 0.2	0.45 ± 0.01	0.71 ± 0.00	101 ± 3
Effect of acetate on fermentation									
Mixed sugars	None	6.21	2.90 ± 0.07	0.00 ± 0.00	111 ± 22	3.1 ± 0.9	0.47 ± 0.01	0.55 ± 0.08	107 ± 1
Mixed sugars	3 g L^{-1}	6.21	2.86 ± 0.01	0.00 ± 0.00	121 ± 0	4.6 ± 0.2	0.46 ± 0.00	0.43 ± 0.00	106 ± 4

theoretical. While the hydrolysate contained no detectable fufural or HMFs, it contained acetate (3 g L^{-1}), which is known to be inhibitory to many microorganisms.

Fermentation of mixed sugars with and without acetate addition

To test whether the acetate concentration present in the hydrolysate was sufficient to lower the ethanol productivity of strain FBR3, pH-controlled fermentations were run with mixed sugars and mixed sugars supplemented with 3 g L^{-1} acetate (50 mmol). Both fermentations had similar ethanol productivities for the first 30 h (Figure 3). However, during the second half of the fermentations, the productivity was lower for the fermentation supplemented with acetate compared to those without acetate addition. Other than increasing the duration of the fermentation, acetate addition had no effect on production of ethanol or succinate (Table 1). Therefore, the long lag phase observed for the hydrolysate fermentation cannot be explained by acetate inhibition alone but acetate does appear to slow the fermentation at the concentrations present in the hydrolysate.

Strain FBR3 appears to be less tolerant to acetate than the ethanologenic *E. coli* strain K011 which is tolerant to 7–9 g L^{-1} of acetate at pH 6 [5,12]. This observation is not surprising because of differences in the parent strains. Strain K011 has an *E. coli B* background while strain FBR3 has an *E. coli* K12 background. *E. coli* K12 is extremely sensitive to acetate inhibition, with growth rates slowing significantly in the presence of only 15–20 mmol acetate at pH 6 [9,25]. K12 strains can be adapted to higher acetate concentrations [11].

Growing seed cultures on corn fiber hydrolysate

In an industrial process the use of reagent grade sugars to grow inoculum would be prohibitively expensive. Consequently, the effect of growing pre-seed and seed cultures on hydrolysates was evaluated. Specifically, both the pre-seed and seed cultures were grown on either 10%(v/v), 25% (v/v), or 50% (v/v) hydrolysate supplemented with LB and carbonate buffer (pH 7). The pH-controlled hydrolysate fermentations were run in a similar manner to those described above and each was inoculated with 15 ml (5% v/v) of the appropriate seed culture.

All subsequent hydrolysate fermentations were much improved compared to earlier hydrolysate fermentations except for the fermentation inoculated with the seed culture grown on 10% (v/v) hydrolysate (Figures 2 and 4a). Fermentations inoculated with seed cultures grown on 25–50% (v/v) hydrolysate were 40% faster than those inoculated with cultures grown on 10% hydrolysate or on reagent-grade xylose (Table 2). However, all of the hydrolysate fermentations were still at least 25% slower than mixed sugar

Table 2 Effects of changes in seed culture medium on fermentation of corn fiber hydrolysate (CFH)

Seed growth medium	Total sugar (% w/v)	Maximum ethanol (% w/v)	Residual sugar (% w/v)	Base addition (mmol L^{-1})	Succinate (g L^{-1})	$Y_{E/S}$ (g g^{-1})	V_{EtOH} (g L^{-1} h^{-1})	Carbon recovery (%)
10% CFH	6.66	2.90 ± 0.02	0.58 ± 0.39	95 ± 19	2.7 ± 0.6	0.47 ± 0.01	0.04	108 ± 6
25% CFH	6.66	2.80 ± 0.02	0.21 ± 0.00	127 ± 0	3.6 ± 0.1	0.45 ± 0.00	0.50	97 ± 0
50% CFH	6.66	2.85 ± 0.12	0.21 ± 0.01	248 ± 32	3.9 ± 0.8	0.47 ± 0.02	0.57 ± 0.10	100 ± 3

fermentations, primarily because of longer lag phases. Parameters not related to ethanol productivity were similar for hydrolysate and mixed sugar fermentations. Ethanol yields were 87–92% of theoretical and residual sugars, which consisted exclusively of xylose, were 0.21–0.58% (w/v) (Table 2).

A typical plot of sugar utilization is shown in Figure 4b. Initially, arabinose and glucose were consumed rapidly. Xylose was not consumed until later in the fermentation and was consumed at a slower rate than arabinose or glucose. A similar pattern of sugar consumption was observed for ethanologenic *E. coli* strain K011 on corn fiber hydrolysate [7].

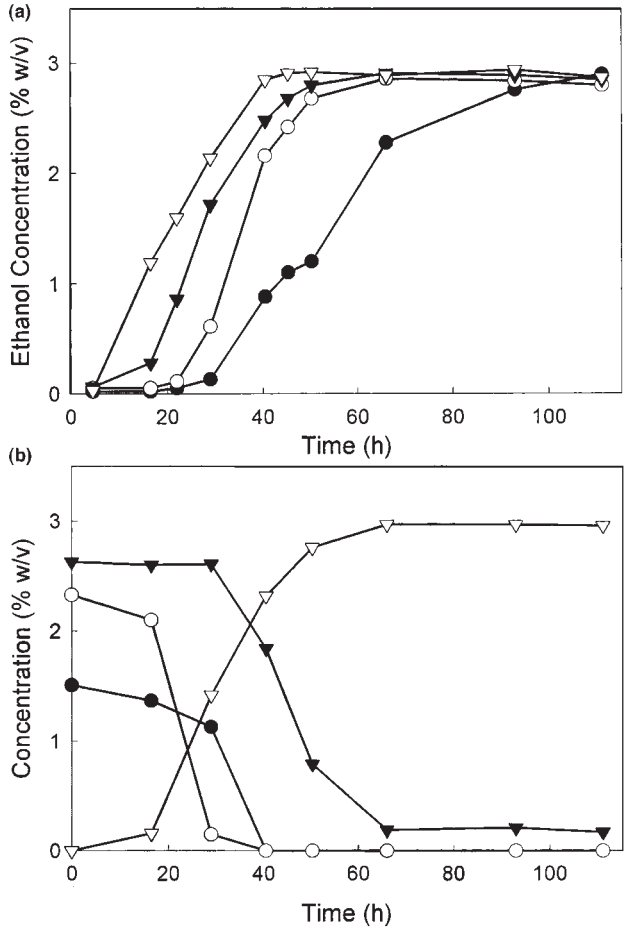

Figure 4 (a) Ethanol measurements from fermentations of corn fiber hydrolysate using pre-seed and seed cultures grown on varying concentrations of hydrolysate. Results from a mixed sugar fermentation control are also plotted. Pre-seed and seed cultures were grown on the following hydrolysate concentrations: 50% (v/v) (▼); 25% (v/v) (○); 10% (v/v) (●); mixed sugars (▽). Each plot is the average of duplicate runs. (b) Ethanol and sugar measurements from a hydrolysate fermentation inoculated from pre-seed and seed cultures grown on 50% (v/v) hydrolysate. Arabinose (●); ethanol (▽); glucose (○); xylose (▼).

Succinate was the primary other fermentation product produced in addition to ethanol. Succinate accounted for 2–6% (w/v) of the sugars consumed (Table 2). Minor amounts of lactate production (less than 1 g L^{-1}) were detected at the end of the fermentations (data not shown). Measured products accounted for 97–103% of the sugars consumed (Table 2).

The vastly improved fermentations of seed cultures grown on hydrolysate, compared to those grown on xylose, can be attributed to two factors: (1) hydrolysate-grown cultures had greater cell densities than those grown on xylose; and (2) those grown on hydrolysate had an opportunity to adapt to the substrate prior to fermentation. In separate experiments, all the hydrolysate seed cultures had a higher cell concentration ($4.5–8.8 \times 10^8$ CFU ml^{-1}) compared to the cultures grown on xylose (3.4×10^8 CFU ml^{-1}). Besides increased cell concentration, growing the inoculum on hydrolysate also allowed the cells to adapt to inhibitors present in the hydrolysate. Adaptation would also be expected to shorten the lag phase. Serially transferring cultures in hydrolysate, albeit usually over a longer time period, is a common method for increasing fermentation efficiency [15].

Effect of pH set-point on hydrolysate fermentation

The optimal pH for fermentations was tested in the range 6.0–7.5 and determined to be 6.5. Fermentations at pH 6.0 produced 1.67% (w/v) ethanol and only consumed a little more than half of the initial sugars (Table 3, Figure 5). By comparison, fermentations run at pH 6.5–7.5 had much less residual sugar and approximately twice the ethanol productivity (Table 3). However, running fermentations above pH 6.5 had the undesirable effect of greatly increasing base consumption (Table 3). The increase in base consumption at the higher pHs cannot be attributed to changes in organic acid production because each culture produced similar amounts of succinate. Instead, the increase in base consumption was probably a consequence of $CaCO_3$ $+2H^+$ being produced from the CO_2 and Ca^{+2}, the latter originating from the $Ca(OH)_2$ used to neutralize the hydrolysate. A white precipitate was observed in the fermentations run at pH 7.0 and 7.5.

Inhibitor mitigation of hydrolysate

A common method for making lignocellulosic hydrolysates more amenable to fermentation, and subsequently reducing

Table 3 Effect of pH on fermentation of corn fiber hydrolysate

pH Setpoint	Total sugar (% w/v)	Maximum ethanol (% w/v)	Residual sugar	Base addition (mmol L^{-1})	Succinate (g L^{-1})	$Y_{E/S}$ (g g^{-1})	V_{EtOH} (g L^{-1} h^{-1})	Carbon recovery (%)
6.0	6.51	1.67 ± 0.02	2.99 ± 0.04	16 ± 3	0.0 ± 0.0	0.46 ± 0.00	0.21 ± 0.00	101 ± 0
6.5	6.51	2.75 ± 0.15	0.32 ± 0.09	124 ± 3	5.7 ± 0.5	0.45 ± 0.02	0.48 ± 0.11	102 ± 3
7.0	6.51	2.25 ± 0.21	0.93 ± 0.59	432 ± 127	5.0 ± 0.9	0.45 ± 0.03	0.52 ± 0.00	103 ± 6
7.5	6.51	2.00 ± 0.07	0.94 ± 0.06	663 ± 16	4.7 ± 0.4	0.42 ± 0.02	0.51 ± 0.13	97 ± 3

Table 4 Influence of hydrolysate neutralization method on fermentations

Carbon source	Over-liming	Total sugar (% w/v)	Maximum ethanol (% w/v)	Residual sugar (% w/v)	Base addition (mmol L^{-1})	Succinate (g L^{-1})	$Y_{E/S}$ (g g^{-1})	V_{EtOH} (g L^{-1} h^{-1})	Carbon recovery (%)
CFH	No	6.56	2.86 ± 0.21	0.21 ± 0.02	165 ± 13	5.3 ± 0.1	0.46 ± 0.01	0.44 ± 0.02	103 ± 2
CFH	Yes	6.56	3.00 ± 0.29	0.29 ± 0.04	92 ± 22	5.4 ± 0.1	0.48 ± 0.00	0.46 ± 0.05	106 ± 0
CFH	Yes + sulfite	6.56	2.88 ± 0.04	0.35 ± 0.10	133 ± 6	5.9 ± 0.6	0.47 ± 0.00	0.66 ± 0.18	105 ± 1

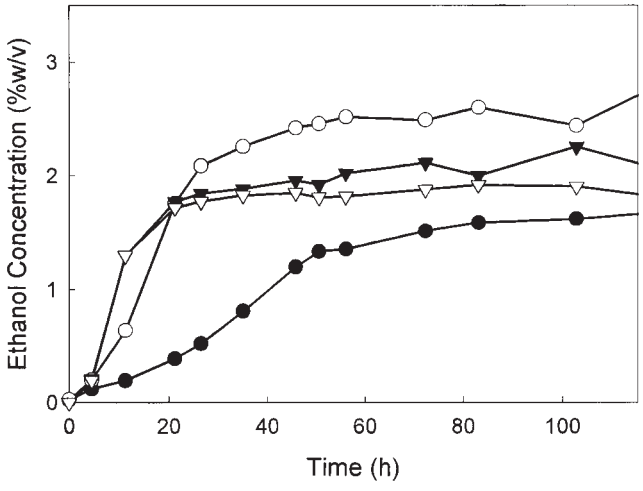

Figure 5 Ethanol measurements from fermentations of corn fiber hydrolysate with pH set-points of 6.0–7.5. pH 6.0 (●), pH 6.5 (○); pH 7.0 (▼); pH 7.5 (▽). Each plot is the average of duplicate runs.

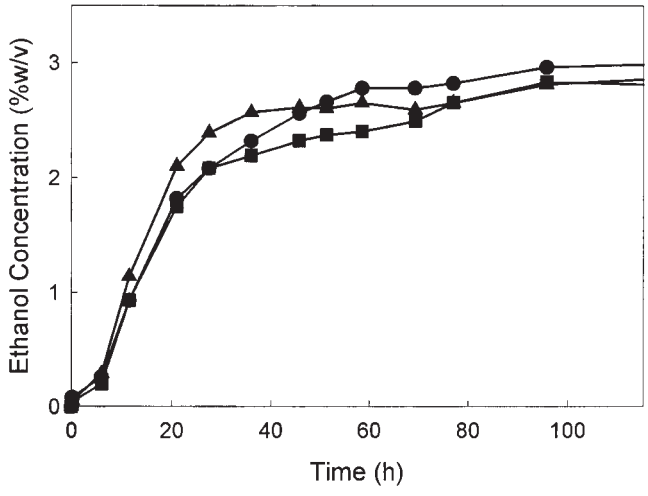

Figure 6 Ethanol measurements from fermentations of hydrolysate that were prepared by neutralizing (■), over-liming (●), or over-liming with sulfite addition (▲). Each plot is the average of duplicate runs.

lag phases, is to 'over-lime.' Over-liming consists of adjusting the pH of the hydrolysate to pH 10 with $Ca(OH)_2$ and incubating it at an elevated temperature (eg, 90°C) before neutralizing the solution with H_2SO_4 [19,22,24]. A variation on this protocol is to add a small amount of sulfite [15,22] to the over-limed solution before warming it. Recently, Olsson *et al* [22] compared four variations of over-liming for preparing willow hydrolysate for fermentation by *E. coli* strain K011 and determined that over-liming with sulfite addition was best. It should be noted that this method does not significantly remove acetate from the hydrolysate [10]. However, over-liming has the significant advantage of being conveniently applied to hydrolysates that contain solids.

Both over-liming and over-liming with sulfite addition were compared to the standard neutralization protocol. The same pre-seed and seed culture, grown on 50% (v/v) neutralized corn fiber hydrolysate, was used to inoculate each culture. Over-liming and sulfite addition had a small beneficial effect upon the fermentations. The lag phase for this hydrolysate was reduced (Figure 6) and average ethanol productivity was increased approximately 10% (Table 4). Ethanol yield did not vary among the fermentations and was 90–94% of theoretical. Residual sugar was also similar [3–5% (w/w) of initial sugar] and consisted entirely of xylose (Table 4). Other products were limited to succinate and a trace amount of lactate (less than 1 g L^{-1}).

Conclusions

E. coli strain FBR3 efficiently converted corn fiber hydrolysate to ethanol with yields that were 92% of theoretical. Supplementing the seed medium with hydrolysate (25–50% v/v) dramatically reduced the lag phase of the fermentation. Fermentations using seed cultures grown on hydrolysate were completed in half the time compared to

fermentations inoculated with seed cultures grown on reagent-grade xylose. Also important was controlling the pH of the fermentation at 6.5. Lowering the pH reduced the ethanol yield and raising it increased base consumption. Neutralizing the hydrolysate using excess $Ca(OH)_2$ (overliming) and adding sulfite improved the ethanol productivity but not the yield.

Acknowledgements

The authors thank Patricia J O'Bryan, Matthew Wittke, and Amy M Martin for excellent technical help and Nancy N Nichols for critical suggestions. Justin C Recknor's help with the non-linear fitting of the data is also sincerely appreciated.

References

1 Alterthum F and LO Ingram. 1989. Efficient ethanol production from glucose, lactose, and xylose by recombinant *Escherichia coli*. Appl Environ Microbiol 55: 1943–1948.
2 Asghari A, RJ Bothast, JB Doran and LO Ingram. 1996. Ethanol production from hemicellulose hydrolysates of agricultural residues using genetically engineered *Escherichia coli* strain KO11. J Ind Microbiol Biotechnol 16: 42–47.
3 Bauer S and E Ziv. 1976. Dense growth of aerobic bacteria in a bench-scale fermentor. Biotechnol Bioeng 28: 81–94.
4 Beall DS, LO Ingram, A Ben Bassat, JB Doran, DE Fowler, RG Hall and BE Wood. 1992. Conversion of hydrolysates of corn cobs and hulls into ethanol by recombinant *Escherichia coli B* containing integrated genes for ethanol production. Biotechnol Lett 14: 857–862.
5 Beall DS, K Ohta and LO Ingram. 1991. Parametric studies of ethanol production from xylose and other sugars by recombinant *Escherichia coli*. Biotechnol Bioeng 38: 296–303.
6 Clark DP. 1989. The fermentation pathways of *Escherichia coli*. FEMS Microbiol Rev 63: 223–234.
7 Dien BS, RB Hespell, LO Ingram and RJ Bothast. 1997. Conversion of corn milling fibrous co-products into ethanol by recombinant *Escherichia coli* strains K011 and SL40. World J Microbiol Biotech 13: 619–625.
8 Dien BS, RB Hespell, H Wyckoff and RJ Bothast. 1998. Fermentation of hexose and pentose sugars using a novel ethanologenic *Escherichia coli* strain. Enzyme Microbial Technol 23: 366–371.
9 Diez-Gonzalez F and JB Russel. 1997. The ability of *Escherichia coli* O157:H7 to decrease its intracellular pH and resist the toxicity of acetic acid. Microbiology 143: 1175–1180.
10 Frazer FR and TA McCaskey. 1989. Wood hydrolyzate treatments for improved fermentation of wood sugars to 2,3-butanediol. Biomass 18: 31–42.
11 Goodson M and MJ Wolin. 1989. Resistance of acid-habituated *Escherichia coli* to organic acids and its medical and applied significance. Lett Appl Microbiol 8: 211–214.
12 Grohmann K and RJ Bothast. 1997. Saccharification of corn fiber by combined treatment with dilute sulfuric acid and enzymes. Process Biochem 32: 405–415.
13 Gulati M, K Kohlmann, MR Ladisch, R Hespell and RJ Bothast. 1996. Assessment of ethanol production options for corn products: engineering framework for estimating theoretical yields and practical maxima. Bioresource Technol 58: 253–264.
14 Hacking AJ. 1986. Economic Aspects of Biotechnology. pp 74–92, Cambridge University Press, Cambridge.
15 Hajny GJ. 1981. Biological Utilization of Wood for Production of Chemicals and Foodstuffs. FPL No. 385, pp 1–64. United States Forest Products Laboratory, USDA, Washington, DC.
16 Hall E. 1954. The mitscherlich equation and its constants. Agron J 46: 323–326.
17 Hespell RB, H Wyckoff, BS Dien and RJ Bothast. 1996. Stabilization of *pet* operon plasmids and ethanol production in *Escherichia coli* strains lacking lactate dehydrogenase and pyruvate formate-lyase activities. Appl Microbiol 62: 4594–4597.
18 Ingram LO, PF Gomez, X Lai, M Moniruzzaman, BE Wood, LP Yomano and SW York. 1998. Metabolic engineering of bacteria for ethanol production. Biotechnol Bioeng 58: 204–214.
19 Leonard RH and GJ Hajny. 1945. Fermentation of wood sugars to ethyl alcohol. Ind Eng Chem 37: 390–395.
20 Mat-Jan F, KY Alam and DP Clark. 1989. Mutants of *Escherichia coli* deficient in the fermentative lactate dehydrogenase. J Bacteriol 171: 342–348.
21 National Corn Growers Association. 1998. The World of Corn. pp 1–24. St Louis, MO.
22 Olsson L and B Hahn-Hagerdal. 1995. Kinetics of ethanol production by recombinant *Escherichia coli* K011. Biotechnol Bioeng 45: 356–365.
23 Osborn D and LF Chen. 1984. Corn hull hydrolysis using glucoamylase and sulfuric acid. Starch 11: 393–395.
24 Sjolander NO, AF Langlykke and WF Peterson. 1938. Butyl alcohol fermentation of wood sugar. Ind Eng Chem 30: 1251–1255.
25 Wolin MJ. 1969. Volatile fatty acids and the inhibition of *Escherichia coli* growth by rumen fluid. Appl Microbiol 17: 83–87.