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Effect of Acetic Acid on Xylose Conversion to Ethanol by Genetically Engineered *E. coli*

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ABSTRACT

Efficient utilization of the pentosan fraction of hemicellulose from lignocellulosic feedstocks offers an opportunity to increase the yield and to reduce the cost of producing fuel ethanol. During prehydrolysis (acid hydrolysis or autohydrolysis of hemicellulose), acetic acid is formed as a consequence of the deacetylation of the acetylated moiety of hemicellulose. Recombinant *Escherichia coli* B (ATCC 11303), carrying the plasmid pLO1297 with pyruvate decarboxylase and alcohol dehydrogenase II genes from *Zymomonas mobilis* (CP4), converts xylose to ethanol with a product yield that approaches theoretical maximum. Although other pentose-utilizing microorganisms are inhibited by acetic acid, the recombinant *E. coli* displays a high tolerance for acetic acid. In xylose fermentations with a synthetic medium (Luria broth), where the pH was controlled at 7, neither yield nor productivity was affected by the addition of 10.7 g/L acetic acid. Nutrient-supplemented, hardwood (aspen) hemicellulose hydrolysate (40.7 g/L xylose) was completely fermented to ethanol (16.3 g/L) in 98 h. When the acetic acid concentration was reduced from 5.6 to 0.8 g/L, the fermentation time decreased to 58 h. Overliming, with $Ca(OH)_2$ to pH 10, followed by neutralization to pH 7 with sulfuric acid and removal of insolubles, resulted in a twofold increase in volumetric productivity. The maximum productivity was 0.93 g/L/h. The xylose-to-ethanol conversion efficiency and productivity in $Ca(OH)_2$-treated hardwood prehydrolysate, fortified with only mineral salts, were 94% and 0.26 g/L/h, respectively. The recombinant *E. coli* exhibits a xylose-to-ethanol conversion efficiency that is superior to that of other pentose-utilizing yeasts currently being investigated for the production of fuel ethanol from lignocellulosic materials.

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Index Entries: Fuel ethanol; xylose; recombinant *E. coli;* yield; prehydrolysate; hemicellulose hydrolysate; genetic engineering.

INTRODUCTION

Today's fuel ethanol industry was born out of the "energy crisis" of the 1970s and was developed from the existing technology base of the alcoholic beverage industry (*1,2*). However, the current practice of using sources of carbohydrate that can be utilized alternatively as either food or feed means that the fermentation feestocks account for about two-thirds of the cost of producing ethanol (*3*). The cost of sugar (molasses) or starch-based raw materials (cereal grains) is too expensive to produce fermentation ethanol that is competitive with gasoline (*4*), and the industry today survives largely by virtue of government incentives, subsidies, or tax credits together with the value derived from byproduct credits (*2*). As long as fermentation ethanol has to compete with the refinery gate price of gasoline, its economic feasibility will ride the same roller coaster as the cost of crude oil (*4*). However, growing environmental concerns have created a new opportunity for fuel ethanol (*5*).

The popularity of fermentation ethanol as an "environment-friendly" alternative transportation fuel means that the industry must reduce production costs by expanding beyond its present starch- and yeast-based technology. If there is to be a reduction in the cost of producing fermentation ethanol, then additional lower cost feedstocks are needed to supplement the present supply of surplus corn and cereal grains.

Lignocellulosic materials (biomass) represent a renewable resource that is sufficiently abundant to produce the large volumes of ethanol to meet anticipated transportation fuel requirements (*6,7*). The most economical biomass resource base consists of "wastes," such as agricultural residues (straw, corn stover, cane bagasse), forestry wastes (sawdust, pulp mill residues), and certain separated industrial/municipal solid wastes (MSW—newsprint, paper, and cardboard packing) (*5–7*). In fact, there is often a "negative cost" associated with waste materials. Furthermore, environmental sensitivities have made waste disposal a particularly acute and pressing problem.

However, lignocellulose remains recalcitrant to bioconversion, because the yeast cultures presently employed in starch-based fermentations are unable to utilize the five-carbon pentose sugars that comprise the hemicellulose component (range 10–35% dry wt) of biomass. Technoeconomic analyses have consistently pointed to the "pentose conversion problem" as the target with the highest economic impact on the cost of biomass-derived fuel ethanol (*5–7*). The hemicellulose component of hardwood represents 23% of the dry weight and is particularly rich in xylose (*5*). The theoretical maximum ethanol yield from hardwood is about 474 L/oven-

dried metric t. Without hemicellulose utilization, the expected maximum yield would be only 324 L, so that with hardwood as feedstock, there exists a potential for yield enhancement of 45%. Efficient utilization of xylose could make biomass-derived fuel ethanol cost competitive with corn-based ethanol, which currently costs $0.34/L (*5*).

The economic effect of xylose conversion is interdependent on three key fermentation process parameters (in order of economic sensitivity):

1. Yield;
2. Ethanol concentration; and
3. Productivity (*3*).

For hardwood, current processing technologies employing dilute sulfuric acid are capable of yielding a "prehydrolysis" stream containing a maximum xylose concentration (without concentrating) of about 60 g/L (*8*). Research and development have focused on designing a biosystem capable of producing fuel ethanol from hemicellulose hydrolysate at a pentose conversion efficiency > 70%, thereby surpassing that of current bioconversion technologies that propose to use pentose-fermenting yeasts (*9,10*). Certain thermophilic bacteria are also being studied, but they generally suffer from a relatively low tolerance to ethanol (*11,12*). It is estimated that the use of *Pichia stipitis* yeast for xylose conversion (70% conversion efficiency), in a simultaneous saccharification/fermentation system with a final alcohol concentration of about 30 g/L, would result in a cost reduction of about 18% ($0.36/L) compared to a system in which there was no xylose conversion ($0.44/L) (*8*). Surpassing this xylose conversion efficiency (70%) would constitute success in research directed toward improving ethanol yield from the pentosan fraction of biomass hemicellulose.

Although *S. cerevisiae* is not capable of utilizing xylose, it can metabolize xylulose, and an alternative approach to the "pentose problem" has been to add glucose isomerase to the fermentation medium to convert the xylose to xylulose (*13*), but the yield and productivity are disappointingly low (*14*). An alternative approach has been to transform an organism exhibiting broad substrate spectrum genetically into an efficient ethanologen through the insertion and expression of foreign genes responsible for ethanol production. Although *Escherichia coli* is capable of metabolizing both hexoses and pentoses, the byproduct of anaerobic fermentations is a mixture of organic acids with only a trace of ethanol (*15*). Using recombinant DNA technology, *E. coli* has been genetically transformed with the "PET" operon (*P*roduction of *ET*hanol) carrying both pyruvate decarboxylase and alcohol dehydrogenase II genes from *X. mobilis* CP4 (*16–19*), and one of several constructs (*E. coli* Luria strain B carrying the plasmid pLO1297) has been judged to possess superior "hardiness" (*19–21*) and ethanologenic characteristics. This construct produces ethanol > 4% (w/v) at near max theoretical efficiency from D-xylose, in a nutrient-rich, synthetic medium (*19–21*). The same genetic approach had been

simultaneously undertaken by Neale in Australia (*22*) using another *E. coli* host (JM101) and a different plasmic system for transformation; however, the xylose-to-ethanol conversion efficiency was lower (71%) compared to Ingram's construct.

In addition to producing monomer sugar residues, thermochemical processing of biomass is known to produce substrates that are inhibitory to both yeast and bacteria (*23–26*). For example, furfural is derived from pentose sugars, whereas hydroxymethylfurfural and levulinic acid come from the hexose sugars. In addition, there are phenolics from lignin degradation (*27*). Acetic acid is present in relatively high concentrations (6–15 g/L) (*28*). Various procedures have been investigated for minimizing the inhibitory effect of these processing byproducts (*23,29,30*). The purpose of this work was to test the tolerance of Ingram's recombinant ethanologenic *E. coli* (ATCC 11303 carrying plasmid pLO1297) to acetic acid and to examine its fermentation performance in hardwood hemicellulose hydrolysate with a view toward assessing its potential to contribute to cost reduction in the production of cellulosic fuel ethanol by surpassing the xylose conversion efficiency of other candidate pentose-utilizing biocatalysts.

MATERIALS AND METHODS

Organisms

Escherichia coli (Luria strain B) ATCC 11303 was obtained from the American Type Culture Collection (Rockville, MD), and *E. coli* ATCC 11303 (carrying the PET plasmid pLO1297) (*19*) was a gift from L. O. Ingram (University of Florida, Gainsville, FL).

Culture Media and Fermentation Systems

Luria broth (LB) (*31*) consisted of tryptone (10 g/L), yeast extract (5 g/L), and NaCl (5 g/L) to which xylose was added at the concentration specified. The defined mineral salts medium (*32*) contained (g/L): NH_4CL, (2.25), $MgSO_4 \cdot 7H_2O$ (0.2), $FeSO_4 \cdot 7H_2O$ (0.5 mg), KH_2PO_4 (2.72), NaCl (5.0), thiamine (0.05 mg) and citric acid (0.21). In the case of media prepared with aspen prehydrolysate (APH), the following additions were made: (i) components of LB (at concentrations stated previously), or (ii) in addition to the components of the mineral salts media, the following substances were added (mg/L): $FeCl_3 \cdot 6H_2O$ (12.1) in place of the $FeSO_4$, $CaCl_3 \cdot 2H_2O$ (7.35), and $MnCl_2 \cdot 4H_2O$ (*32*).

Cultures were stored (−10°C) in LB/glycerol-citrate and were plated on selective media (LB+20 g/L agar+10 mg/L tetracycline and 40 mg/L ampilcillin). Inocula were prepared in buffered media containing antibiotics, but antibiotics were absent from all fermentation media. Batch

fermentations were conducted in MultiGen™ (model F2000) stirred-tank bioreactors having agitation, pH (2*N* KOH), and temperature control (30°C) (New Brunswick Scientific Co., Edison, NJ).

Hardwood Hemicellulose Hydrolysate

Prehydrolyzed aspen (*Populus tremuloides*) hardwood (APH) was obtained from BIO-HOL Developments (Toronto) and was prepared using a single-screw Wenger extruder with SO_2 as catalyst (*33*). The hemicellulose component was completely hydrolyzed with the mass total sugars/dry wood being 23%. Cellulose was minimally hydrolyzed under these conditions (*33*). The extruded wood material was slurried in boiling water (ratio H_2O:extruded wood was 2:1) and filtered. The filtrate was concentrated under vacuum to achieve a xylose concentration of about 4% (w/v). Where indicated, the APH was treated as followed: powdered $Ca(OH_2)$ was added with stirring to pH 10 followed by neutralization (pH 7) with 1*N* H_2SO_4 and centrifugation to remove insolubles.

Analytical Procedures

Growth was measured turbidometrically at 550 nm (1 cm lightpath) and culture dry weight was measured by microfiltration—washing and drying the filter to constant weight under an infrared heat lamp. Compositional analyses of fermentation media, cell-free spent media, and APH were determined using an HPLC equipped with an RI monitor and computer-interfaced controller/integrator (Bio-Rad Labs, Richmond, CA). Separations were performed at 65°C and 85°C on HPX-87H and HPX-87P columns, respectively (Bio-Rad Labs) (injection vol = 0.02 mL). A typical analysis with the HPX-87H column showed that the APH contained DP_2 and DP_3 oligomers, and 6.2 g/L acetic acid, with >90% of the monomer reducing sugar as xylose. The mass ratio of xylose to acetic acid was 6:1. However, the HPX-87H column does not resolve xylose from mannose and galactose. Resolution of the monomer sugars using the HPX-87P column indicated 7.3% hexose (mostly mannose) and 93.7% pentose (mostly xylose).

RESULTS

Figure 1 shows the typical growth response (as recorded by the change in optical density [OD] of Ingram's (*19*) recombinant *E. coli* B (ATCC 11303 carrying the "PET" plasmid pLO1297) in batch culture conducted in a stirred bioreactor with the pH controlled at 7.0 and the temperature maintained constant at 30°C. The nutrient-rich, complex medium (Luria broth) was supplemented with D-xylose (about 4.4% by wt). Growth in L broth, without added sugar, was logarithmic (results now shown). With

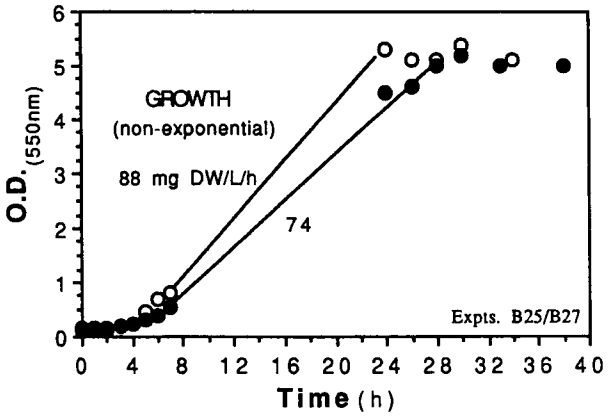

Fig. 1. Effect of acetate on growth of recombinant *E. coli* B: Growth (open circles) was conducted anaerobically in batch mode in a pH-controlled (2*N* KOH) STR at pH 7.0. The medium was Luria broth with added xylose (approx 4.4% by wt). Antibiotics were absent from the medium. The closed circles are for growth with 17.5 g potassium acetate (179 m*M*) added to the LB medium. The "linear" growth rate (mg dry wt cell/L/h) is indicated. Luria broth/xylose: ○ Control pH 7; ● +179 m*M* acetate.

xylose-supplemented L broth, the linear increase in OD (0.26 OD/h) indicates that growth was nonexponential (Fig. 1). From measurements of the relationship between culture dry mass and OD, the linear growth rate was determined to be 88 mg dry wt cells/L/h (Fig. 1).

Figure 1 also shows the effect of acetate (17.54 g potassium acetate) on growth of the recombinant culture. In the presence of this amount of acetate (at pH 7), the growth rate is decreased by 16% to 74 mg dry wt cells/L/h (Fig. 1).

The time-courses for xylose utilization and ethanol production, corresponding to the growth profiles of Fig. 1, are shown in Fig. 2A and B, respectively. The average volumetric productivity (Q_p, g ethanol/L/h) was calculated as the final ethanol concentration divided by the total time of the fermentation (the time from inoculation until complete utilization of the xylose). The maximum volumetric productivity (Q_p^{max}, g ethanol/L/h) was determined from the slope in plots of the ethanol concentration vs the elapsed fermentation time (for the stationary-phase of growth). The specific productivity (q_p, g ethanol/g dry wt cells/h) was estimated by dividing the value for Q_p^{max} by the maximum (stationary-phase) biomass concentration. The product yield ($Y_{p/s}$, g ethanol/g xlylose) was calculated as the maximum ethanol concentration divided by the concentration of xylose initially present in the medium. A product yield of 0.51 g ethanol/g xylose represents a xylose-to-ethanol conversion efficiency of 100%. The values of these key operational parameters were calculated from the data presented in Figs. 1 and 2B, and are summarized in Table 1. Although the final OD appears to be similar when acetate (179 m*M*) is present in the

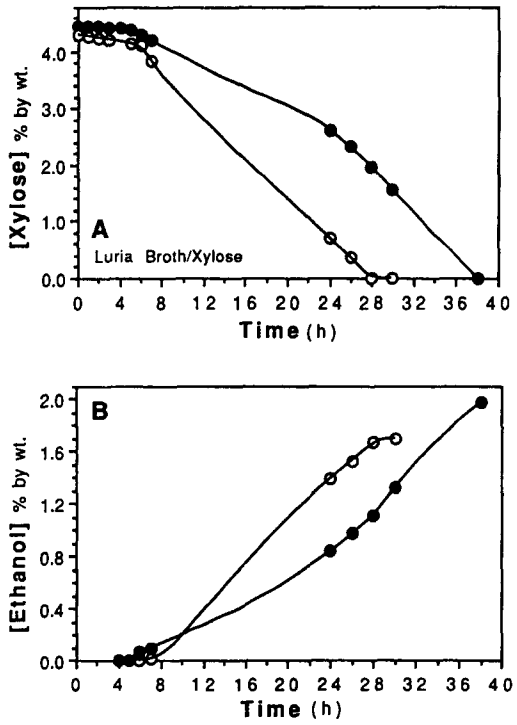

Fig. 2. (A) Effect of acetate on xylose utilization by recombinant *E. coli* B: Conditions were as described for Fig. 1: ○ Control pH 7; ● + 179 m*M* acetate. (B) Effect of acetate on ethanol production by the r E. coli. The rates of ethanol production (both volumetric and specific) are given in Table 1.

Table 1
Effect of Acetate on Xylose Fermentation
by Recombinant *E. coli* B ATCC 11303 (pLO1297)*

			Products		Productivity			Yield	
Conditions medium comp.	Expt.	Xyl, g/L	Biomass, g dry wt/L	EtOH, g/L	Q_p g P/L/h	Q_p^{max} g P/L/h	q_p g P/g cell/h	$Y_{p/s}$, g/g	Conver. effic., %
Luria broth, controlled at pH 7.0									
Control, no acetate	B25	43.0	2.24	17.0	0.60	0.73	0.33	0.40	78
+ Acetate, 179 m*M*	B27	44.6	1.90	19.8	0.52	0.81	0.43	0.44	86

*Potassium acetate (17.5 g) (179 m*M*) was added to the complex Luria broth medium to which D-xylose had been added at the concentration indicated; the concentrations of xylose, ethanol, and acetate were determined by HPLC analysis; the total concentration of acetate did not change appreciably during the course of the batch fermentation; the pH was controlled at 7.0 with KOH; kinetic and yield parameters are defined in the text. P = ethanol.

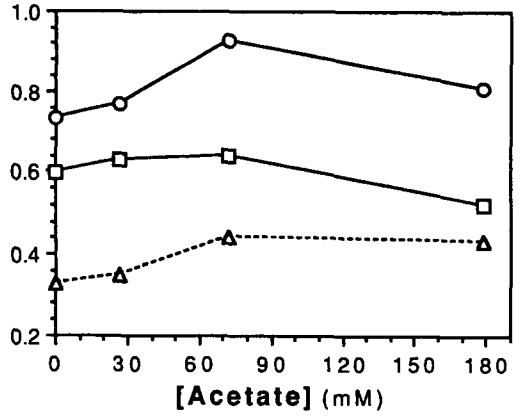

Fig. 3. Effect of acetate on volumetric and specific productivity: Conditions were as described in Fig. 1. The parameters are defined in the text. The pH was controlled at 7.0. Volumetric productivity (g EtOH/L/h): —□— Q_p; —○— Q_p^{max}. Specific productivity (g EtOH/g cell/H): --△-- q_p^{max}.

medium (Fig. 1), the maximum cell density (as dry mass determined by ultrafiltration) is reduced by about 16% (Table 1), suggesting that acetate also has a negative effect on growth yield under these particular growth conditions. Acetate causes a decrease in Q_p (Table 1), which is, in part, a reflection of both the "growth lag" and the slower growth rate caused by acetate (Fig. 1). However, the presence of acetate results in a stimulation of both Q_p^{max} and q_p (Table 1). Figure 3 shows the effect of lesser amounts of acetate on productivity. At pH 7, acetate concentrations of 27 and 72 m*M* stimulated all three productivity parameters; the Q_p, Q_p^{max}, and q_p (Fig. 3). Perhaps of more practical importance, however, is the apparent improvement in product yield that results from the addition of acetate (27–179 m*M*) to the medium under these conditions—an increase in conversion efficiency of 8% (Fig. 4).

HPLC analysis of aspen prehydrolysate (APH) indicated that the mass ratio of xylose to acetic acid was about 6:1. In order to promote good growth in the APH medium, the components of Luria broth were added to a final concentration equal to that of L broth. Such an LB-supplemented APH medium contained about 4% xylose and 100 m*M* acetate. In the case of all APH fermentations (Figs. 5 and 7), inoculation of the media with the recombinant culture was at an initial cell concentration of about 0.5 g dry wt cells/L, which is about a 10-fold higher inoculation density than was used in the control experiments with L Broth (Figs. 2 and 6). The time-course for a typical APH pH-stat (7.0) batch fermentation, with respect to xylose utilization and ethanol production, is shown in Fig. 5A and B, respectively. When the extruded (prehydrolyzed) wood was allowed to air-dry, some of the more volatile substances were removed, and the concentration of acetate in the APH medium prepared from this air-dried material was reduced by about 85%. The effect of reducing the acetate

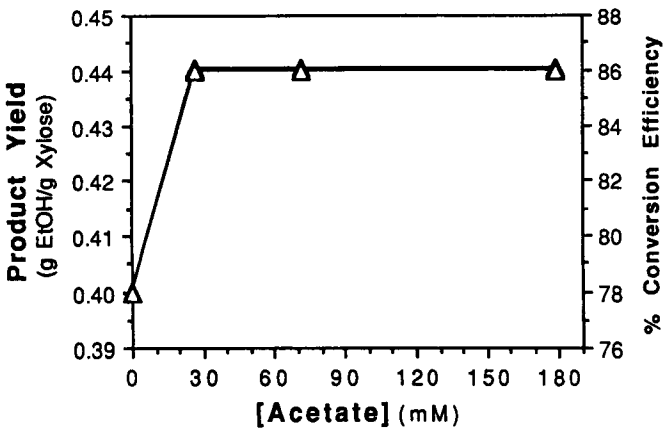

Fig. 4. Effect of acetate on ethanol yield (efficiency of xylose to ethanol conversion) by recombinant *E. coli* B: The fermentation conditions were as described in Fig. 1. The pH was controlled at 7.0. In these batch fermentations, the xylose in the L broth was completely utilized, and the ethanol yield (g EtOH/g xylose) was based on the amount xylose added to the medium. A conversion efficiency of 100% was based on a product yield of 0.51 g EtOH/g xylose utilized.

concentration by this amount on the fermentation performance of the recombinant culture is shown in Fig. 5. In the case of the APH medium with only 13 m*M* acetate, the decrease in fermentation time (from 98 to 57 h) is much more dramatic than could be expected owing to the slight difference in xylose concentration for the two media (Fig. 5). However, even at a 10-fold higher inoculation cell density, the complete fermentation of the low acetate APH medium (57 h) takes twice as long as with the LB medium (28 h—*see* Fig. 2) with about the same amount of xylose. The values of parameters with respect to both productivity and yield, for all APH fermentations, are summarized in Table 2. That there are environmental factors other than acetate contributing to inhibition of productivity in the case of the APH fermentations is suggested by comparing the productivities of the experiments represented in Figs. 2 and 5 (*see also* Tables 1 and 2). For the L broth with 179 m*M* acetate, the time for complete fermentation was only 38 h (Fig. 2), which was 20 h less than for the APH medium with only 13 m*M* acetate. With respect to the yield of ethanol from the aspen prehydrolysate, contrary to the situation with the acetate-supplemented LB medium, it appears that the yield for APH fermentations is decreased significantly at higher acetate concentrations (Table 2). The reduction in yield represents a 12% decrease in xylose-to-ethanol conversion efficiency (Table 2), but interestingly, the ethanol yield observed with the untreated APH medium is equal to the yield produced in the L broth without any added acetate (Tables 1 and 2).

The hydrolysis of hemicellulose to yield fermentable sugars is also known to produce other degradation products that can be inhibitory to microbial growth and fermentation (*23–28*). Various treatments have

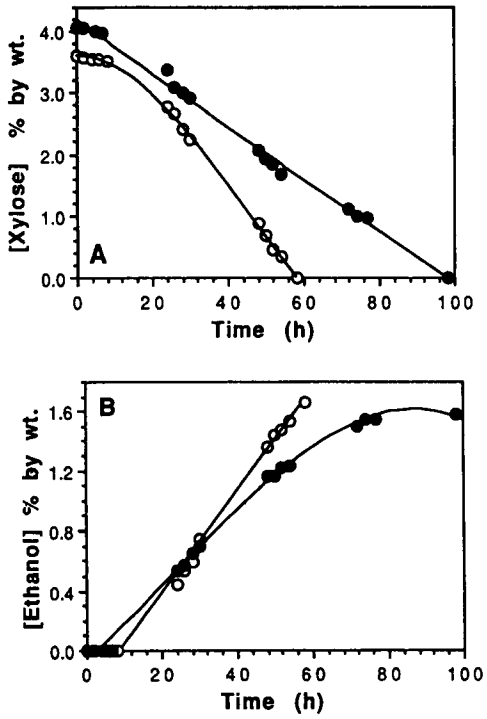

Fig. 5. Hardwood hemicellulose hydrolysate fermentation by recombinant *E. coli* B—effect of removing volatiles by air-drying the extruded wood: (A) Xylose utilization from aspen prehydrolysate (APH). The production of the APH is described in Materials and Methods. The APH was supplemented with the nutrients/salts comprising Luria broth. The concentration of acetate in the APH was determined by HPLC. The closed circles are for supplemented, but "untreated" APH. The open circles represent a sample of extruded aspen wood that was allowed to air-dry in order to reduce the amount of volatile substances. Batch fermentations were conducted anaerobically in pH-controlled (7.0) stirred reactors. ● APH—control untreated, 93 m*M* acetate. ○ APH—air-dried, 13 m*M* acetate. (B) Ethanol production from aspen prehydrolysate. ● APH—control untreated, 93 m*M* acetate. ○ APH—air-dried, 13 m*M* acetate.

been developed in order to decrease the toxicity of the prehydrolysate for fermentation by a variety of different biocatalysts (*23,29,30*). The procedure used in this study was modified from one originally developed by Johnson and Harris (*29*). It involves overliming the APH with powdered calcium hydroxide to pH 10, followed by neutralization to pH 7 with 1*N* sulfuric acid and centrifugation to remove the $CaSO_4$ and other insolubles formed during the neutralization process.

As a control for the possible effects of this particular $Ca(OH)_2$ treatment procedure on the growth and fermentation performance of the recombinant *E. coli* B culture, L broth was treated in a similar fashion to

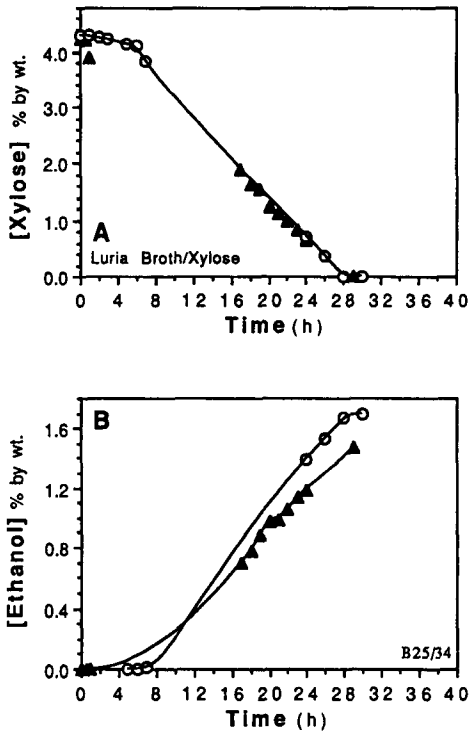

Fig. 6. Effect of $Ca(OH)_2$ treatment of L broth on ethanol production from xylose by recombinant *E. coli* B; (A) Xylose utilization; (B) ethanol production. The conditions were as described in Fig. 1. The open circles are for the "control"—L broth with xylose controlled at pH 7.0. The process whereby $Ca(OH)_2$ is used to "pretreat" the fermentation medium (filled triangles), is described in Materials and Methods. ○ Control pH 7; ▲ + $Ca(OH)_2$.

the APH medium. Figure 6 shows that this procedure had very little effect on the fermentation performance of the recombinant, with the exception that the ethanol yield was slightly reduced. This is in contrast to the very marked improvement in fermentation performance in the $Ca(OH)_2$-treated APH, both with respect to productivity and yield (Fig. 7). In APH media with comparable levels of acetate, the fermentation time was decreased from 98 to 28 h, and the xylose-to-ethanol conversion efficiency was improved by 14%, from 78% with untreated APH to 92% after $Ca(OH)_2$ treatment (Table 2). Partial air drying of a sample of the extruded wood produced an APH medium of intermediate acetate concentration (43 m*M*). The effect of the $Ca(OH)_2$ treatment of this APH (lower acetate) medium on fermentation is shown in Fig. 8. In this case, the fermentation time of 37 h was less than with the untreated medium (58 h) prepared from the fully dried extruded wood (Fig. 8), but was longer than for the $Ca(OH)_2$-treated APH medium (28 h) containing the higher concentration

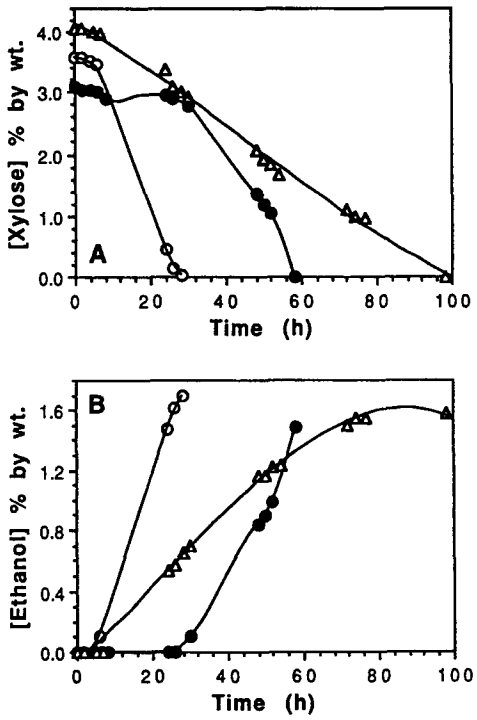

Fig. 7. Effect of $Ca(OH)_2$ treatment of aspen prehydrolysate on the fermentation performance of recombinant *E. coli* B: (A) Xylose utilization. △ APH—control, no $Ca(OH)_2$, 93 m*M* acetate. ○ + $Ca(OH)_2$, 103 m*M* acetate. ● + $Ca(OH)_2$ with mineral salts, 96 m*M* acetate. (B) ethanol production. △ APH—control, no $Ca(OH)_2$, 93 m*M* acetate. ○ + $Ca(OH)_2$, 103 m*M* acetate. ● + $Ca(OH)_2$ with mineral salts, 96 m*M* acetate. The pH was controlled at 7.0. The concentration of acetate in the medium was determined by HPLC and did not change appreciably during the fermentation. The APH was supplemented with the nutrients comprising Luria broth, except for the experiment indicated by the closed circles where the APH was supplemented by the components of the "defined minerals salts medium" (*see* Materials and Methods).

of acetate (Table 2). The increase in fermentation time was owing, at least in part, to the higher concentration of xylose in the 43 m*M* acetate APH medium (Table 2). However, interestingly enough, the yield of ethanol was about the same for these three different media (Table 2).

Finally, an experiment was conducted to examine the contribution of the nutrient supplementation on both productivity and ethanol yield. Instead of adding the organic nutrient components of Luria broth to the APH, only certain essential inorganic salts were used to supplement the $Ca(OH)_2$-treated prehydrolysate (Fig. 7). Whereas the productivity was reduced by bout 50%, the product yield was not affected (Table 2). The prolonged fermentation time (58 h) in the case of the mineral salts-supplemented APH medium was caused by the slower growth in the ab-

Table 2
Ethanol Production from Hemicellulose Hydrolysates
by Recombinant *Escherichia coli* B ATCC 11303 (pLO1297)[a]

Conditions medium comp.	Expt.	Xyl, g/L	Products: Biomass, g dry wt/L[c]	Products: EtOH, g/L	Productivity: Q_p g P/L/h	Productivity: Q_p^{max} g P/L/h	Productivity: Ferm. time, h	Yield: $Y_{p/s}$, g/g	Yield: Conver. effic., %
Aspen prehydrolysate + complex nutrients (LB) 13 m*M* acetate, no $Ca(OH)_2$	APH3	36.1	0.5	16.6	0.29	0.34	58	0.46	90
93 m*M* acetate, no $Ca(OH)_2$	APH6	40.7	0.5	16.3	0.17	0.31	98	0.40	78
Aspen prehydrolysate + complex nutrients (LB) 103 m*M* acetate, + $Ca(OH)_2$[b]	APH4	35.9	0.5	16.9	0.60	0.76	28	0.47	92
43 m*M* acetate, + $Ca(OH)_2$[b]	APH8	38.4	0.5	17.2	0.46	0.93	37	0.45	88
Aspen prehydrolysate + mineral salts 96 m*M* acetate, + $Ca(OH)_2$[b]	APH5	31.0	0.5	14.9	0.26	0.65	58	0.48	94

[a]Conditions: The pH was controlled at 7.0 with KOH; the acetate concentration was determined by HPLC analysis and did not change appreciably during the fermentation; the monomer sugars in the "prehydrolysate" were predominantly xylose; the process yield ($Y_{p/s}$) was based on the amount of fermentable sugar in the medium; *P* = ethanol.
[b]Powdered $Ca(OH)_2$ was added to the aspen "prehydrolysate" to pH 10 and neutralized to pH 7 with 1*N* H_2SO_4 followed by centrifugation to remove insolubles.
[c]Approximately initial cell density (inoculum; g dry wt cells/L).

sence of the organic nutrients (Fig. 7). The stationary phase of fermentation activity, which is reflected in the value for Q_p^{max}, was similar in both the LB-supplemented (0.76 g EtOH/L/h) and mineral salts-supplemented (0.65 g EtOH/L/h), $Ca(OH)_2$-treated APH media (Table 2).

DISCUSSION

The efficient utilization of hemicellulose represents an important aspect of biomass processing because it accounts for 10–40% of natural cellulosic biomass. The fact that HC is considerably easier to hydrolyze than cellulose makes it possible to hydrolyze the HC component of biomass selectively. Although dilute acid is one of the attractive methods (*34*), there are alternative processing options that offer economic advantages. Steam-explosion pretreatment (autohydrolysis) effectively solubilizes the HC fraction (*35,36*), but the various thermochemical processes cur-

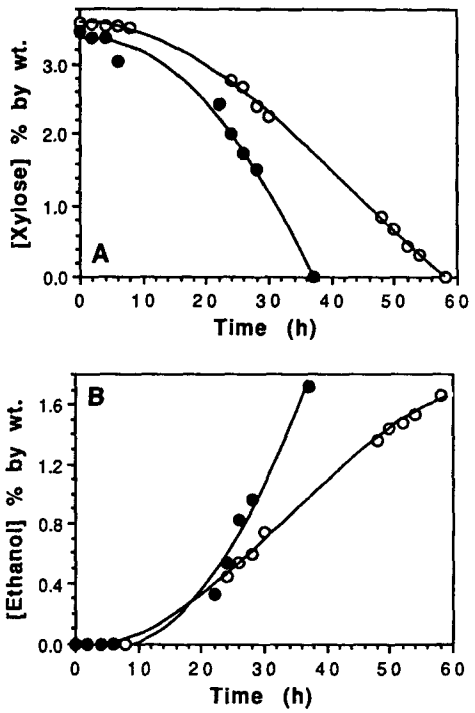

Fig. 8. Effect of $Ca(OH)_2$ treatment of aspen prehydrolysate on the fermentation performance of recombinant *E. coli* B: (A) Xylose utilization. ○ No $Ca(OH)_2$, air-dried; 13 m*M* acetate. ● + $Ca(OH)_2$, 43 m*M* acetate. (B) Ethanol production. ○ No $Ca(OH)_2$, air-dried; 13 m*M* acetate. ● + $Ca(OH)^2$, 43 m*M* acetate. The pH was controlled at 7.0. The different APH media were supplemented with the nutrients comprising Luria broth and were prepared either from fully air-dried extruded wood (open circles) or from a sample that was only partially air-dried (closed circles). The concentration of acetate in each medium was determined by HPLC.

rently employed for prehydrolysis all produce different and varying amounts of toxic substances (*23–28*). The major inhibitors produced by autohydrolysis of poplar (aspen) have been shown to be water-soluble lignin degradation products (aromatic monomers, including carboxylic acids, such as hydrobenzoic acid, vanillic acid, and syringic acid) (*26,27*). The toxicity is dependent not only on the concentration of the putative inhibitor, but also on the tolerance of the biocatalyst, be it yeast or bacteria.

Effect of Acetate on Xylose Fermentation

During acid hydrolysis or autohydrolysis of hemicellulose, acetic acid is formed as a consequence of the deacetylation of the acetylated moiety in hemicellulose. Acetic acid is used as an antimicrobial agent in the food industry (*37*), and the acetic acid that is present in hemicellulose hydrolysate is known to be inhibitory to both yeasts (*38,39*) and bacteria (*26*).

Acetic acid inhibition of yeast is minimized by controlling the pH at 6.5 (*40*). Acetic acid is one of the byproducts of metabolism by *E. coli*, and it has been shown to be the cause of growth inhibition (*41*). Under anaerobic conditions at pH 7, growth of *E. coli* K12(S) was 50% inhibited by the addition of 10 m*M* sodium acetate and completely inhibited at 35 m*M* (equivalent to 0.2 m*M* of the protonated acid) (*41*). A recent study suggested that *E. coli* B was more tolerant of acetic acid than other strains (*42*). To examine the sensitivity of the recombinant *E. coli* B to acetic acid, we added increasing amounts of acetate to LB medium, to a maximum of 179 m*M* (equivalent to 10.7 g acetic acid). Using the Henderson-Hasselbalch relationship ($pK_a = 4.75$), it can be calculated that 179 m*M* acetate, at pH 7, is equivalent to only 1 m*M* of the protonated acid (CH_3COOH). By virtue of its ability to traverse the cell membrane freely, the protonated species acts as a membrane protonophore and causes its inhibitory effect by bringing about the acidification of the cytoplasm, thereby collapsing the transmembrane pH gradient and destroying the homeostasis with respect to the intracellular pH (*41,43*). In *E. coli*, the internal cytoplasmic pH is slightly alkaline and is kept constant at about 7.4–7.8 (*44*) by the extrusion of protons through the action of membrane proton pumps (*45–47*). The specific growth rate is about 50% inhibited when the pH_i is 6.85 (*48*). Ethanol is thought to potentiate the inhibitory effect of acetic acid (*49*), which is particularly pertinent to the performance of the recombinant *E. coli* in an acetic-acid-containing medium, since ethanol is the major metabolic product.

Typically, the mass ratio of acetic acid to xylose in the hardwood hemicellulose hydrolysate was about 1:6, but a ratio of 1:4 was reported by Beck (*23*) when processing hardwood chips with dilute sulfuric acid. The maximum amount of acetate used here represents about 1.5 times the amount that would be present in an APH fermentation medium with a xylose concentration of 4%. Nevertheless, it was found to enhance both the maximum volumetric productivity and yield (Table 1). In connection with these observations, it is perhaps interesting to note that Luli and Strohl (*42*) observed a 50% inhibition of growth of *E. coli* with 60 m*M* acetate at pH 7, but noted the apparent superior tolerance of *E. coli* B relative to the other strains they investigated.

Production of Ethanol from Hardwood Hemicellulose Hydrolysate

As part of our initial survey of the potential of this culture for utility in fermentations with lignocellulosic materials, we had previously examined its performance in an aspen prehydrolysate medium, but these preliminary fermentation trials were performed with shake flasks with phosphate added as buffer (*32*). Under those conditions, the recombinant did not perform well (process yield < 0.1 g/g) (*32*). Similarly, one of Ingram's

earlier genetic constructs, *E. coli* S17-1 (carrying plasmid pLO1308-10) (*17*), had been previously investigated using hardwood prehydrolysate prepared with dilute sulfuric acid (*50*). However, the pH of the fermentation was not controlled, and the treatment of the prehydrolysate included the addition of sodium sulphite at 1 g/L (*50*). At an initial pH of 7, there was only 44% utilization of the 2.5% pentose and the 5.9 g/L of ethanol represented a "process" yield of only 0.24 g/g (equivalent to 46% conversion efficiency) (*51*).

Nevertheless, the results of this study clearly show that the poor performance in the buffered APH medium cannot be attributed solely to inhibition by acetate. Encouraged by the success of these control experiments with acetate, we undertook the present reassessment of the fermentation capabilities of the recombinant in hardwood prehydrolysate, but this time, the fermentations were conducted in stirred tanks with pH control and at a higher inoculation cell density. Under these conditions, a sevenfold reduction in the amount of acetate from 93 to 13 m*M*, resulted in an improvement from 78 to 90%, but the productivity was low considering the higher inoculum (0.5 g dry wt cells/L) used.

Various pretreatments have proven successful in enhancing the performance of pentose-utilizing yeasts in prehydrolysates (*23,29,30*). A treatment involving overliming with $Ca(OH)_2$ to pH 10, followed by neutralization with 1*N* H_2SO_4, improves the performance of the recombinant through a 50% reduction in the fermentation time in APH that was supplemented with the nutrients found in LB (tryptone and yeast extract) (Fig. 7), presumably by removing or inactivating some unidentified inhibitor(s); however, the mechanism is not understood. This overliming method has been used by others for yeast fermentations, but it has included the addition of sulfite (*52*). How sulfite treatment (added 1 g/L) improves the fermentation performance of pentose-utilizing yeasts has not been established (*30*). The success of this procedure has been variously ascribed to:

1. The removal of furfural;
2. The conversion of furfural to furfuryl alcohol;
3. Reactions between furfural and other hydrolysate components, such as phenolics; or
4. The removal of heavy metal cations (e.g., nickel from stainless-steel reactor vessels) along with the insoluble calcium sulfate (*30,52*).

As might be expected, APH that had been supplemented with only mineral salts provided a less conducive environment for rapid fermentation, but nevertheless, the yield was equally high (Table 2).

Similar treatments of prehydrolysates with various modifications have been examined recently by different investigators in fermentation trials with hemicellulose hydrolysates using various pentose-utilizing yeasts (*32,52–57;* results summarized in Table 3). Yeasts are sensitive to

Table 3
Production of Ethanol
from Hemicellulose Hydrolysates by Pentose-Utilizing Yeasts

Biomass feedstock, treatment	Culture	Sugars, g/L	Xylose, %	Process yield,[a] g P/gS	Reference
Hardwood (red oak)					
T.V.A.[b]—dilute H_2SO_4 (KOH+HCL/sulfite)	*P. tannophilus* NRRL Y2460	62	70	0.25	Strickland and Beck (1985)
T.V.A.[b]—dilute H_2SO_4 ($Ca[OH]_2$+H_2SO_4 +sulfite)	*P. tannophilus* NRRL Y2460	62	70	0.29	Perego et al. (1990)
	C. shehatae ATCC 22484	62	70	0.21	Perego et al. (1990)
	P. stipitis CBS 5773	62	70	0.02	Perego et al. (1990)
Hardwood (aspen)					
SLR reactor[g] (HCl) (steam stripped)[c]	*P. stipitis* R (ex CBS 5776)	23	62	0.39	Parekh et al. (1987)[d]
Bio-Hol process (Wenger extruder/SO_2) (steam stripped)[c]	*P. stipitis* R	60	80[e]	0.44	Gans et al. (1989)
Hardwood Spent sulphite liquor (steam stripped)[c]	*P. stipitis* R	50	65	0.36	Parekh et al. (1987[d]
Wheat straw	*P. tannophilus* NRRL Y2460	43	-	0.23	Perego et al. (1990)
Sugar cane bagasse acid	*Candida sp.* XF217 mutant[f]	100	65	0.30	Lodics and Gong (1984)
Dilute H_2SO_4— steam explosion (NaOH+charcoal)	*P. stipitis* CBS 5773	75	-	0.34	Roberto et al. (1991)
H_2SO_4 (1.8%) ($Ca[OH]_2$ to pH 6.5)	*P. stipitis* CBS 7126	49	85	0.31	van Zyl and Du Preez (1988)

[a] Process yield (g P/g S) was based on the amount of fermentable sugar in the fermentation medium. P = ethanol, S = sugar.
[b] T.V.A.—Two-stage process (Tennessee Valley Authority); "first stage" is hemicellulose hydrolysis.
[c] Steam stripping (at 1 atm) reduces the acetic acid concentration to < 0.6 g/L.
[d] Used high inoculum (12 g dry wt/L) of wood hydrolysate-adapted ("R" strain) of *Phicia stipitis* (CBS 5776).
[e] Average value (range quoted for xylose-mannose = 77–82% total sugars).
[f] Used wood hydrolysate-adapted strain of *Candida sp.* XF217.
[g] St. Lawrence Reactors Ltd. (Mississauga, Canada); acid/high-temp. continuous plug-flow reactor.

inhibition by acetic acid, and steam stripping has been used to reduce the concentration to < 0.6 g/L (Table 3). In the fuel ethanol industry, the raw materials represent by far the largest component of the total production costs, and for this reason, it is not surprising that the yield (efficiency of sugar to ethanol conversion) has been shown to be the most economically sensitive process parameter (*8*). With respect to ethanol yield from hemicellulose hydrolysates from a variety of sources, including hardwoods, crop residues (wheat straw and sugar cane bagasse), and pulp mill effluent (spent sulfite liquor), the best results to date ("process" yield = 0.44 g EtOH/g sugar) appear to have been generated by Bio-hol Developments (Toronto) by Gans and coworkers (*33*) using a wood hydrolysate-adapted strain of *Pichia stipitis* CBS 5776 (so-called "R" strain) (*55*) (Table 3). Another research and development company, IOGEN Corporation (Ottawa), has examined the performance of the same *P. stipitis* R (CBS 5776) in continuous xylose fermentations, where, at a feed concentration of 50 g/L, the process yield was 0.4 g/g (B. Foody—personal communication); however, because access to this variant is restricted, the literature is silent with respect to independent corroboration of these reports. These results with *P. stipitis* contrast dramatically with those of Perego et al. (*52*) using a hardwood hydrolysate prepared by the TVA dilute acid process (Table 3), where it was concluded that "none of the conditions tested has been able to enhance the insufficient performance of *P. stipitis* (CBS 5773), thus proving this strain to be absolutely unsuitable for hardwood hemicellulose hydrolysate fermentation." Perhaps the observations of Perego et al. (*52*) should be regarded more as a reflection of the uniqueness of the product stream from any given prehydrolysis process than as a testimony to the superiority of *P. tannophilus* in comparison to *C. shehatae* and *P. stipitis*.

Comparing the results of this investigation to those of others (Table 3), it is clear that the recombinant *E. coli* exhibits a xylose-to-ethanol conversion efficiency that is superior to that of the other pentose-utilizing yeasts currently being investigated for the production of fuel ethanol from lignocellulosic materials. In this respect, it merits further research.

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