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Fermentation Performance Characteristics of a Prehydrolyzate-Adapted Xylose-Fermenting Recombinant *Zymomonas* in Batch and Continuous Fermentations

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Abstract

Long-term (149 d) continuous fermentation was used to adapt a xylose-fermenting recombinant *Zymomonas mobilis*, strain 39676:pZB4L, to conditioned (overlimed) dilute-acid yellow poplar hemicellulose hydrolyzate ("prehydrolyzate"). An "adapted" variant was isolated from a chemostat operating at a dilution rate of 0.03/h with a 50% (v/v) prehydrolyzate, corn steep liquor, and sugar-supplemented medium, at pH 5.75. The level of xylose and glucose in the medium was kept constant at 4% (w/v) and 0.8% (w/v), respectively. These sugar concentrations reflect the composition of the undiluted hardwood prehydrolyzate. The level of conditioned hardwood prehydrolyzate added to the medium was increased in 5% increments starting at a level of 10%. At the upper level of 50% prehydrolyzate, the acetic-acid concentration was about 0.75% (w/v). The adapted variant exhibited improved xylose-fermentation performance in a pure-sugar, synthetic hardwood prehydrolyzate medium containing 4% xylose (w/v), 0.8% (w/v) glucose, and acetic acid in the range 0.4–1.0% (w/v). The ethanol yield was 0.48–0.50 g/g; equivalent to a sugar-to-ethanol conversion efficiency of 94–96% of theoretical maximum. The maximum growth yield and maintenance energy coefficients were 0.033 g dry cell mass (DCM)/g sugars and 0.41 g sugars/g DCM/h, respectively. The results confirm that long-term continuous adaptation is a useful technique for effecting strain improvement with respect to the fermentation of recalcitrant feedstocks.

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Index Entries: Recombinant *Zymomonas*; continuous cofermentation; xylose; hardwood prehydrolyzate; ethanol yield; adaptation; acetic acid.

Introduction

The efficient fermentation of xylose-rich hemicellulose hydrolyzates ("prehydrolyzates") represents an opportunity to improve significantly the economics of large-scale fuel ethanol production from lignocellulosic feedstocks *(1)*. Although biomass-derived fuel ethanol is not yet economically competitive with gasoline, significant technological advances in both process configuration and biocatalyst performance have resulted in a progressive reduction in estimated production costs *(2,3)*. It has been proposed that significant additional processing cost reductions can be achieved through a combination of process consolidation *(4)*, economies of scale *(3)*, and improved energy utilization *(4,5)*. For example, advances in bioconversion-process consolidation would involve reducing the number of bioreactors through a progression from processes based on sequential hydrolysis and fermentation to ones employing simultaneous hydrolysis and fermentation *(6,7)*. Similarly, advanced designs would employ continuous rather than batch operation. Continuous flow systems configured for cell retention or cell recycling offer the potential of increased productivity and reduced capital expense owing to bioreactor size reduction *(8)*.

One of several biomass-to-ethanol processes currently under investigation by the National Renewable Energy Laboratory (NREL) is a simultaneous saccharification and cofermentation (SSCF) process that involves a dilute-acid pretreatment and a patented *(9)*, metabolically engineered *Zymomonas mobilis* that can coferment glucose and xylose *(10–14)*. Encouraging cofermentation performance data with respect to both ethanol yield and productivity have been obtained for recombinant *Zymomonas* using synthetic prehydrolyzate media and pH-stat batch fermentors *(15–17)*. Dilute acid-catalyzed pretreatment is efficient and cost-effective *(18–20)*, but the resulting "prehydrolyzate" contains acetic acid which is known to be inhibitory to ethanologenic microorganisms *(2,21,22)*. The effect of acetic acid on wild-type *Zymomonas* has been documented *(23–25)*. We have studied the effect of acetic acid on xylose utilization by recombinant *Zymomonas* in pH-stat batch *(15)* and glucose fed-batch fermentations *(16)*. The successful results of preliminary batch trials with recombinant *Z. mobilis* in a laboratory SSCF system using 3.5% (w/v) xylose, 6% (w/v) Sigmacell cellulose and cellulase (25 FPU/g cellulose) were presented at the seventeenth Symposium in 1995 *(12)*. At this year's Symposium, NREL is reporting on prepilot scale SSCF trials with recombinant *Z. mobilis* using both pure sugars and a conditioned yellow poplar dilute-acid prehydrolyzate *(26)*.

At this meeting last year we demonstrated the cofermentation performance capabilities of recombinant *Zymomonas* 39676:pZB4L in long-term continuous fermentations using both a synthetic pure sugar medium and a conditioned yellow poplar dilute-acid prehydrolyzate medium *(27)*. Because of the presence of toxic byproducts that are produced during dilute acid-

catalyzed hemicellulose hydrolysis, even conditioned prehydrolyzates tend to be recalcitrant to fermentation. In the work that we reported last year *(27)*, the selective pressure of the continuous fermentor yielded an "adapted" variant of 39676:pZB4L, which is the subject of the present investigation. The objective of this work is to describe the physiological characteristics of the prehydrolyzate-adapted recombinant strain in side-by-side comparisons with the nonadapted culture in both batch and continuous fermentations. Hence, this article represents a sequel to, or a continuation of, the work that was presented at the nineteenth Symposium last year, and to avoid repetition, this article relies heavily on the background information regarding experimental rationale, as well as specific methodologies, that have been described previously *(27)*.

Materials and Methods

Microorganism

Zymomonas mobilis 39676:pZB4L (*Z. mobilis* host strain ATCC 39676 transformed with a derivative of the pZB5 plasmid conferring xylose assimilation and fermentation capability, as reported by Zhang et al. *(10)*. Cryovials of frozen concentrated stock culture were maintained in RM medium (10 g/L yeast extract and 2 g/KH_2PO_4) supplemented 10 mg/L tetracycline and 15% (w/w) glycerol at –70°C. Pre-cultures were prepared as described previously *(15)*.

Preparation of Inoculum

Overnight flask pre-cultures were harvested by centrifugation (16,300*g* for 10 min) and the cell pellet resuspended in RM medium without sugar *(15)* to yield a concentrated cell suspension that was used to inoculate the batch fermentors. The initial optical density (OD; 1 cm light path at 600 nm) was in the range 0.2–0.25 corresponding to 60–75 mg dry cell mass (DCM)/L.

Fermentation Media

The nutrient-rich pure-sugar synthetic prehydrolyzate medium used previously *(16)* was modified to contain the following ingredients/L of glass distilled water: 40 g xylose; 8 g glucose; 5 g Difco Yeast Extract (YE) (Difco Laboratories, Detroit, MI); 3.48 g KH_2PO_4; 0.8 g NH_4Cl; 0.5 g $MgSO_4$; 0.01 g $FeSO_4 \cdot 7H_2O$; 0.21 g citric acid; 20 mg tetracycline. Alternatively, a less expensive pure-sugar synthetic prehydrolyzate medium was made with 1% (v/v) centrifugally clarified corn-steep liquor (cCSL) (GPC International, Muscatine, IA) as a nutrient substitute for YE. Production of the dilute-acid hardwood prehydrolyzate in a pilot-scale Sunds hydrolyzer, "conditioning" of the prehydrolyzate by overliming, and the preparation of the prehydrolyzate-containing fermentation medium were as described previously *(27)*. Stock pure-sugar solutions were sterilized separately.

Batch- and Continuous-Fermentation Equipment

Batch fermentations were conducted with about 1500 mL medium in 2 L bioreactors (model F2000 MultiGen, New Brunswick Scientific, Edison, NJ) fitted with agitation (100 rpm), pH, and temperature control (30°C). Continuous fermentations were performed in 750 mL NBS C30 chemostats *(27)* or alternatively with NBS Bioflo 2000 fermentors (1500 mL working volume). The pH was monitored using a sterilizable combination pH electrode (Ingold). The standard pH control set-point was 5.75 and the pH was kept constant by automatic titration with 4 N KOH.

Analytical Procedures, Growth and Fermentation Parameters

Growth was measured turbidometrically at 600 nm (1-cm light path) (Unicam spectrophotometer, model SP1800). In all cases, the blank cuvet contained distilled water. DCM was determined by microfiltration of an aliquot of culture followed by washing and drying of the filter to constant weight under an infrared heat lamp. Fermentation media and cell-free spent media were compositionally analyzed by HPLC as described previously *(15)*. The ethanol yield ($Y_{p/s}$) was calculated as the mass of ethanol produced/mass of sugar consumed. The volumetric ethanol productivity was determined by dividing the final ethanol concentration by the total batch-fermentation time. Bracketed values for volumetric productivity indicate that sugar utilization was incomplete when the experiment was terminated. For chemostat cultures, the maximum mass growth yield (i.e., corrected for maintenance) (max $Y_{x/s}$) was determined as the inverse of the slope in a plot of the specific sugar utilization rate (q_s, g sugar/gDCM/h) as a function of the dilution rate (D, 1/h) where the y-axis intercept represents the value for the maintenance energy coefficient (m_e, g sugar/gDCM/h).

Results and Discussion

Generating the "Adapted" Variant of Rec Zm 39676:pZB4L

This study focuses on the fermentation performance of the xylose-utilizing recombinant *Z. mobilis* in a pH-controlled continuous-flow bioreactor (chemostat) using both synthetic and real hardwood prehydrolyzate. Our objective was to use the selective pressure provided by the continuous-growth environment of the chemostat to achieve strain improvement by the "adaptation" resulting from the long-term exposure of the recombinant to incremental increases in the amount of prehydrolyzate in the feed medium. The selective pressure exerted on an organism within the controlled-growth environment of the continuous-flow bioreactor (chemostat) is a powerful research tool for effecting strain improvement through a process of acclimation or adaptation that takes place during the long-term exposure to gradually increasing levels of an inhibitory substance(s).

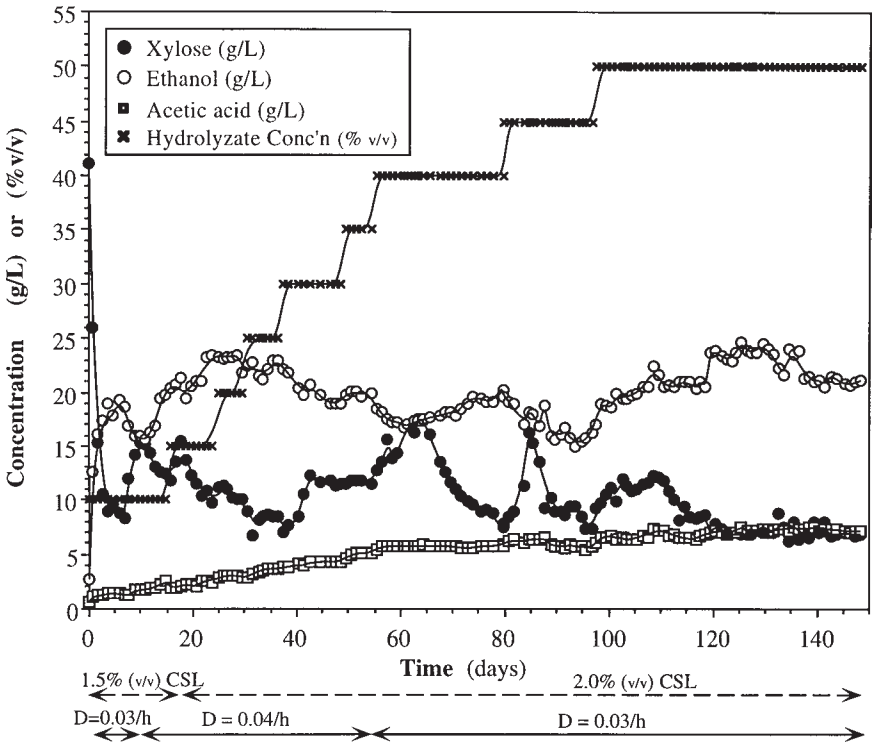

Fig. 1. Adaptation of recombinant *Z. mobilis* 39676:pZB4L to overlimed yellow poplar prehydrolyzate. Experimental details are given in Materials and Methods section. This represents a continuation of the chemostat experiment described in Fig. 5 of ref. *27*.

In the work presented last year on continuous-culture studies with rec Zm 39676:pZB4L *(27)*, we described the initial stages of a long-term chemostat experiment that was intended to generate a prehydrolyzate-adapted variant of rec Zm 39676:pZB4L. At the beginning of the experiment the CSL-based medium contained 10% (v/v) dilute acid-catalyzed yellow poplar prehydrolyzate (with pure-sugar supplementation to achieve glucose and xylose levels of 0.8% [w/v] and 4% [w/v], respectively). These sugar concentrations were selected because they were similar to the concentrations in full strength hydrolyzate liquors obtained by dilute acid pretreatment of hardwood (yellow poplar) sawdust *(28)*. Batch fermentations had shown that 30% hydrolyzate significantly inhibited fermentation performance of the recombinant; however, at the 10% level, performance was not inhibited *(29)*.

The chemostat culture experiment shown in Fig. 1 is a continuation of the experiment illustrated in Fig. 5 of our previous study *(27)* in which the chemostat was operated at a dilution rate of 0.04/h for 55 d. Over this period of time, the prehydrolyzate concentration had been increased incrementally from 10–35% (v/v) where the final acetic-acid concentration was

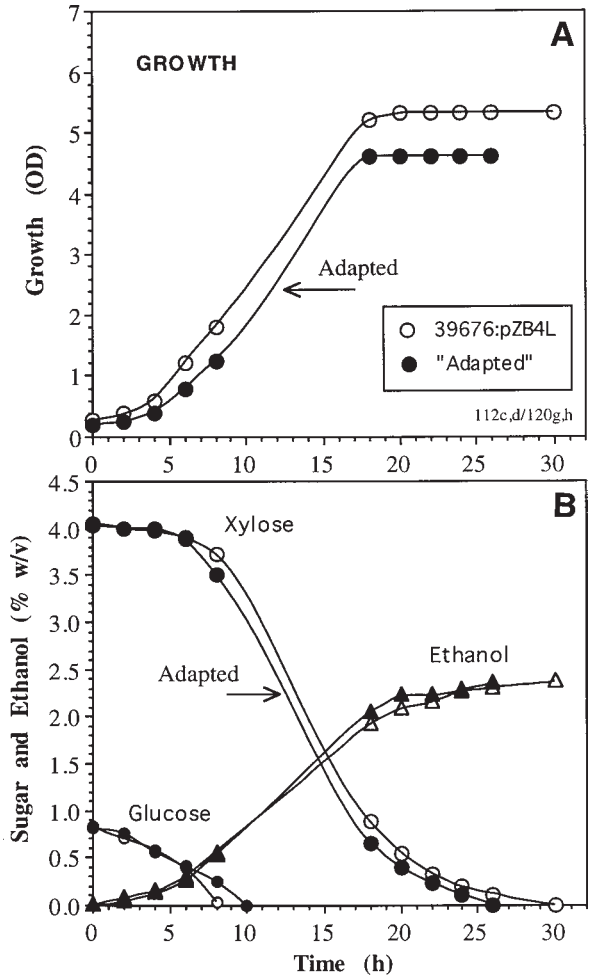

Fig. 2. Comparative performance of rec Zm 39676:pZB4L and adapted variant in pH-stat batch fermentation with a pure-sugar nutrient-rich synthetic prehydrolyzate medium **(A)** growth, and **(B)** sugar utilization and ethanol production. The ZM medium contained 4% (w/v) xylose and 0.8% (w/v) glucose (no acetic acid); the pH was 5.75 and the temperature was 30°C. The values for maximum dry cell mass concentration, ethanol yield, and productivity are given in Table 1.

about 0.5% (w/v). This level of acetic acid is reasonably inhibitory to batch-fermentation performance *(15)*. In this study, the continuous culture was continued at a relatively constant dilution rate of 0.03/h for another 94 d and over this period the prehydrolyzate level of the medium was increased from 35–50% (v/v) in 5% (v/v) increments (Fig. 1). The acetic-acid concentration increased from 0.5% to about 0.75% (w/v) (Fig. 1). After 94 d of continuous operation, the effluent xylose and ethanol concentrations were 0.68 and 2.1% (w/v), respectively (Fig. 1). Over the entire time-course of the experiment, glucose was seldom detected in the chemostat effluent (results not shown). This level of ethanol represents a process ethanol yield (based

Table 1
Summary of Growth and Fermentation Parameters

pH	Acetic acid % (w/v)	Maximum cell mass (g DCM/L)	Maximum ethanol (g/L)	Ethanol yield (g/g)	Ethanol productivity[a] (g EtOH/L/h)
Nonadapted recombinant					
5.75[b]	0	1.38	23.6	0.48	0.79
5.0	0.4	0.73	24.0	0.49	(0.35)
5.5	0.4	0.96	24.9	0.50	0.44
6.0	0.4	1.06	24.2	0.49	0.48
6.0	1.0	0.63	15.8	0.48	(0.22)
Adapted recombinant					
5.75[b]	0	1.34	23.6	0.48	0.90
5.0	0.4	0.88	24.8	0.50	0.51
5.5	0.4	1.04	24.2	0.50	0.55
6.0	0.4	1.18	24.3	0.49	0.61
6.0	1.0	0.72	21.2	0.48	(0.29)

[a]Brackets around values for Ethanol productivity indicate that xylose utilization was incomplete when batch fermentation was terminated.

[b]The medium was "ZM" with 4% (w/v) xylose and 0.8% (w/v) glucose. All other fermentations were with 1% (v/v) clarified CSL-based media (*see* Materials and Methods) with the same sugar concentrations.

on sugars available) of 0.44 g/g (conversion efficiency of 87% theoretical). The culture that was isolated at the termination of this long-term (149 d) experiment was designated as the "adapted" variant of rec Zm 39676: pZB4L. This hardwood prehydrolyzate-adapted strain is the focus of the present comparative physiological assessment.

Comparison of Cofermentation Performance Adapted and Nonadapted Recombinants

Figure 2 compares the growth and cofermentation performance of rec Zm 39676:pZB4L and adapted variant in batch fermentation using a pure-sugar, nutrient-rich synthetic prehydrolyzate medium with the pH controlled at 5.75. The mineral salts and yeast extract-based (ZM) medium contained 4% (w/v) xylose and 0.8% (w/v) glucose, but no acetic acid. Under this condition, the performance exhibited by the two strains was remarkably similar (Fig. 2). Although the adapted strain produced a lower final culture turbidity (Fig. 2A), the final cell-mass concentrations were similar (Table 1). One possible distinguishing feature of the adapted strain revealed in Fig. 2B was the apparent slower rate of glucose utilization. A separate experiment with glucose as the sole sugar confirmed that an idiosyncrasy of the adapted strain is a slower growth with, and metabolism of, glucose, as well as a lower growth yield (Fig. 3). In comparing the time-

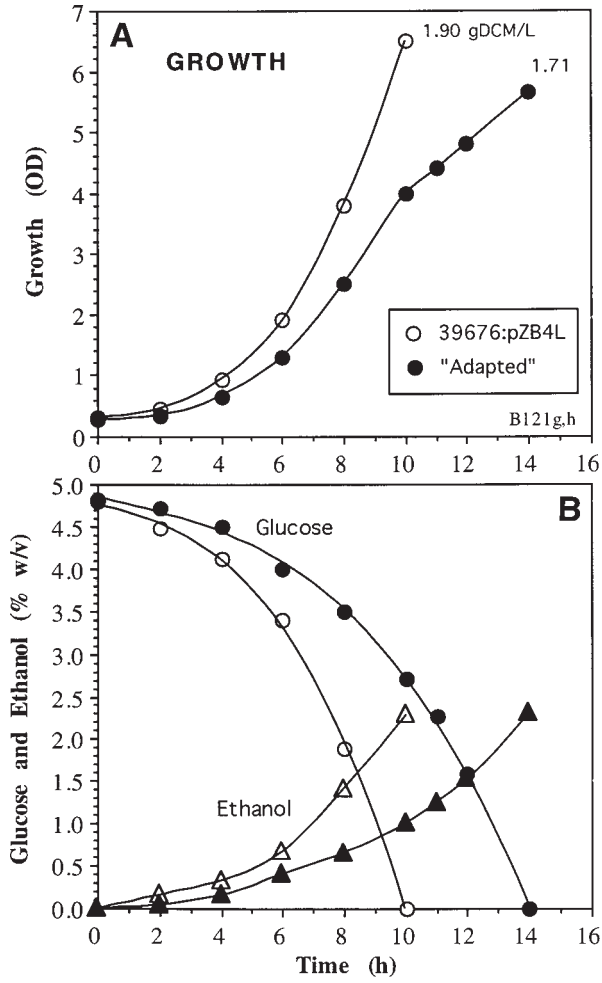

Fig. 3. Comparative performance of rec Zm 39676:pZB4L and adapted variant with glucose as sole sugar source. **(A)** growth, and **(B)** glucose utilization and ethanol production. The ZM medium contained 4.8% (w/v) glucose (no acetic acid); the pH was 5.75 and the temperature was 30°C. The values for maximum dry cell mass concentration are shown in panel (A).

courses of the batch fermentations shown in Figs. 2 and 3, it is important to note the difference in scale of the x-axes. Under these assay conditions, any difference between the two strains was not expected because the medium did not contain any inhibitory substances (principally acetic acid) to which the adapted strain might have become less sensitive.

Comparative Cofermentation Performance in Acetic Acid-Containing Media

Because the adapted strain was isolated from a chemostat that was operating with a feed containing 50% (v/v) prehydrolyzate (0.75% [w/v]

acetic acid), it seemed reasonable to assume that altered sensitivity to acetic-acid inhibition might be one way to characterize the adapted strain. In a previous study, we documented the acetic-acid sensitivity of rec Zm 39676:pZB4L and showed that 0.4% acetic acid caused a 50% inhibition of growth and cofermentation *(16)*. Using a CSL-based synthetic prehydrolyzate medium containing 0.4% (w/v) acetic acid, the adapted and nonadapted recombinants were compared in pH-stat batch fermentations where the pH was controlled at 5.0, 5.5, or 6.0 (Fig. 4). At all three pH values, the adapted strain outperformed the nonadapted recombinant with respect to both growth and xylose fermentation; however, the ethanol yield remained close to theoretical maximum for both strains independent of the pH (Fig. 4, Table 1). The highest ethanol productivity (0.61g/L/h) was achieved by the adapted strain at pH 6 (Fig. 4, Table 1).

Figure 5 compares the growth and fermentation performance at pH 6 using the same medium but with the acetic-acid concentration increased to 1% (w/v). At this relatively high level of acetic acid, the adapted strain achieved a slightly higher cell-mass concentration (Fig. 5A); however, the rate of xylose utilization was significantly faster with the adapted strain relative to the nonadapted strain (Fig. 5B). For both strains, the ethanol yield (based on sugar consumed) was 0.48 g/g (conversion efficiency of 94% theoretical maximum) (Table 1). In the context of rec Zm strain specificity with respect to acetic-acid sensitivity, it is interesting to note that our previous work with rec Zm CP4:pZB5 *(15)* indicates that it possibly rivals the adapted strain in terms of resistance to acetic inhibition.

Characterization of Continuous Cofermentation with Adapted Recombinant

Figure 6 shows the steady-state concentrations of xylose, ethanol, and cell mass as a function of dilution rate for a chemostat culture of the adapted recombinant using a CSL-based pure-sugar synthetic prehydrolyzate medium. The results shown in Fig. 6 are very similar to those observed under the same conditions using the nonadapted recombinant. The similar pattern observed in continuous cofermentation was expected both from the similar performance behavior observed in batch cofermentation and the fact that the medium did not contain any potentially inhibitory substances. In the context of continuous cofermentations with recombinant *Zymomonas*, we noted that in a recent report on performance assessment of rec Zm CP4:pZB5, Rogers et al. *(30)* stated

> "in chemostat culture for 40 g/L glucose plus 40 g/L xylose medium, that full sugar utilization occurred in the dilution rate range 0.05–0.06/h with ethanol concentrations close to 40 g/L" (p. 305).

This observation by Rogers et al. *(30)* is of particular interest because recombinant CP4:pZB5 exhibits more tolerance to acetic acid *(15)* than rec Zm 39676:pZB4L *(16)*.

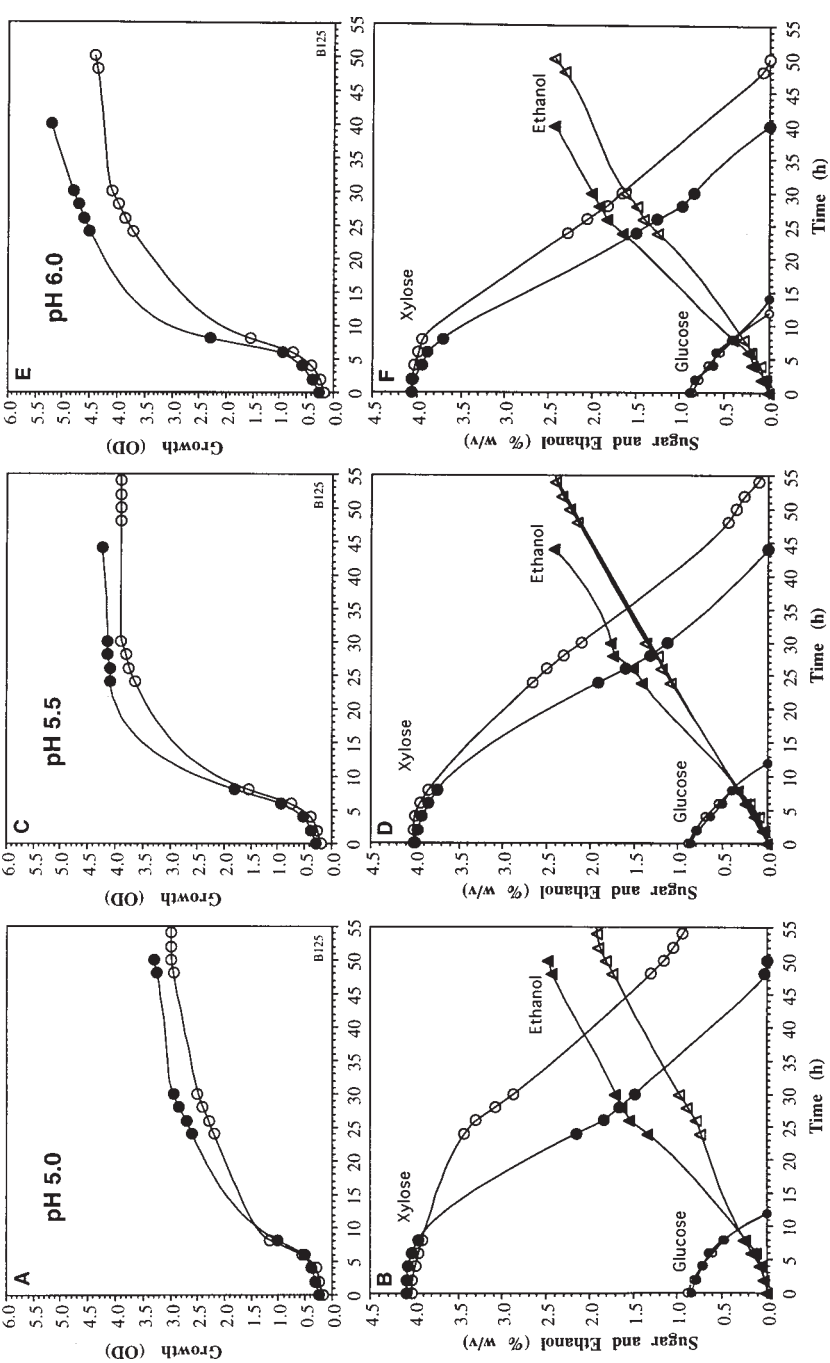

Fig. 4. Effect of 0.4% (w/v) acetic acid on rec Zm 39676:pZB4L and adapted variant in batch fermentations as a function of pH. **(A)** growth, pH 5, **(B)** sugar utilization and ethanol production, pH 5, **(C)** growth, pH 5.5, **(D)** sugar utilization and ethanol production, pH 5.5, **(E)** growth, pH 6, **(F)** sugar utilization and ethanol production, pH 6. Symbols: ○, nonadapted rec Zm; ●, adapted recombinant. The mineral salts medium contained 1% (v/v) clarified CSL with 4% (w/v) xylose and 0.8% (w/v) glucose (Materials and Methods). The values for maximum dry cell mass concentration, ethanol yield, and productivity are given in Table 1.

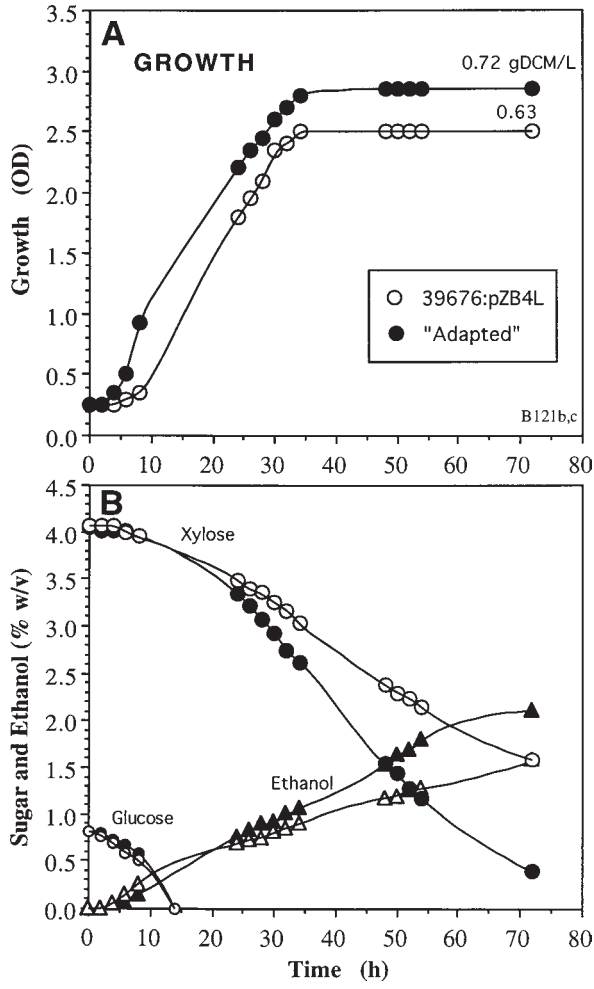

Fig. 5. Effect of 1% (w/v) acetic acid on rec Zm 39676:pZB4L and adapted variant in a CSL-based pure-sugar synthetic prehydrolyzate medium. **(A)** Growth, **(B)** sugar utilization and ethanol production. The medium was the same as described in Fig. 3. The pH was 6 and the temperature was 30°C. The values for maximum dry cell mass concentration, ethanol yield, and productivity are given in Table 1.

Figure 7 shows a Pirt *(31)* plot of q_s and q_p versus D for the chemostat culture with the adapted recombinant. Regression analysis of the specific sugar-utilization rate data gives values of 0.033 g/g and 0.41 g/g/h for max $Y_{x/s}$ and m_e, respectively (Fig. 7). These values compare to values of 0.042 g/g and 1.13 g/g/h observed previously, under the same conditions, using the nonadapted recombinant *(27)*. As well as being influenced by several environmental factors, these physiological and bioenergetic parameters are known to be strain-specific *(27)*. Apart from the recent work of Joachimsthal et al. *(25)* that showed an increase in m_e with increasing amounts of acetate, the literature is silent on the subject of the effect of acetic

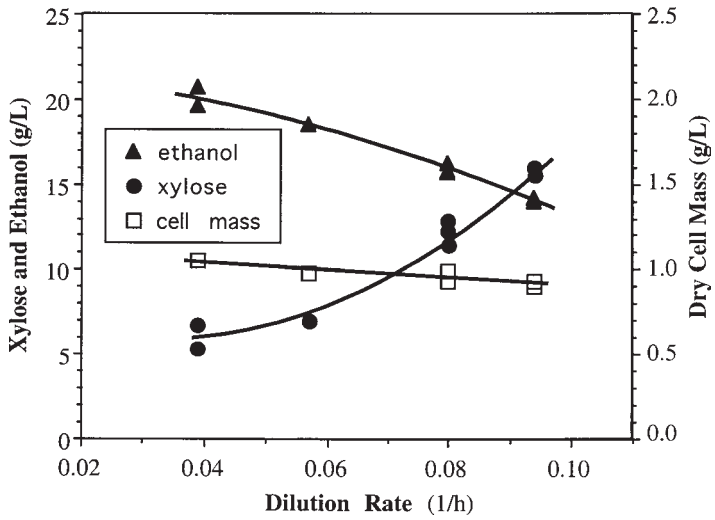

Fig. 6. Steady-state concentrations of xylose, ethanol, and cell mass as a function of dilution rate for pure-sugar continuous cofermentation using "adapted" recombinant *Z. mobilis*: the medium was the same as described in Fig. 2. There was no acetic acid in the medium and no glucose was detected in the chemostat effluent. The pH was 5.75 and the temperature was 30°C.

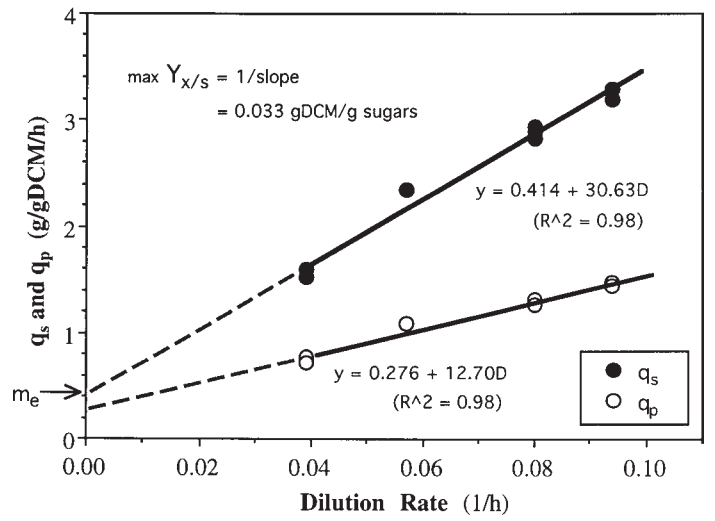

Fig. 7. Specific rates of sugar utilization and ethanol production by recombinant *Z. mobilis* as a function of dilution rate (*see* Fig. 6 for description of experimental conditions).

acid on the maintenance energy coefficient in *Zymomonas*. In this context it is interesting to note that the m_e value for an acetate-tolerant ZM4 mutant was determined to be 1.9 g/g/h at pH 5.4 with about 0.9% (w/v) acetic acid *(25)*. This can be compared to an average value for m_e of 1.6 g/g/h for strain

ZM4 in the absence of acetate *(32)*. The lower maintenance coefficient exhibited by the adapted recombinant is interesting and may provide a clue regarding the mechanism by which this variant is able to tolerate higher levels of acetic acid because this substance is known to act as an energetic uncoupler *(23)*. Clearly, the mechanism by which the adapted recombinant achieves an improved fermentation performance in the presence of acetic acid is an area for further research.

In a separate study being presented at this meeting, the adapted strain has been used in integrated bench-scale SSCF experiments with yellow poplar prehydrolyzate and lignocellulosic solids to total solids loadings of 14% (w/v) *(26)*. The combined results of these two studies certainly auger well for the proposed further testing with this biocatalyst at pilot scale. This study provides support for the contention that long-term continuous culture is an effective technique for effecting strain improvement *(27)*.

Acknowledgments

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