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Continuous Fermentation Studies with Xylose-Utilizing Recombinant *Zymomonas mobilis*

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Abstract

This study examined the continuous cofermentation performance characteristics of a dilute-acid "prehydrolysate-adapted" recombinant *Zymomonas* 39676:pZB4L and builds on the pH-stat batch fermentations with this recombinant that we reported on last year. Substitution of yeast extract by 1% (w/v) corn steep liquor (CSL) (50% solids) and Mg (2 m*M*) did not alter the cofermentation performance. Using declared assumptions, the cost of using CSL and Mg was estimated to be 12.5¢/gal of ethanol with a possibility of 50% cost reduction using fourfold less CSL with 0.1% diammonium phosphate. Because of competition for a common sugar transporter that exhibits a higher affinity for glucose, utilization of glucose was complete whereas xylose was always present in the chemostat effluent. The ethanol yield, based on sugar used, was 94% of theoretical maximum. Altering the sugar ratio of the synthetic dilute acid hardwood prehydrolysate did not appear to significantly change the pattern of xylose utilization. Using a criterion of 80% sugar utilization for determining the maximum dilution rate (D_{max}), changing the composition of the feed from 4% xylose to 3%, and simultaneously increasing the glucose from 0.8 to 1.8% shifted D_{max} from 0.07 to 0.08/h. With equal amounts of both sugars (2.5%), D_{max} was 0.07/h. By comparison to a similar investigation with rec Zm CP4:pZB5 with a 4% equal mixture of xylose and glucose, we observed that at pH 5.0, the D_{max} was 0.064/h and shifted to 0.084/h at pH 5.75. At a level of 0.4% (w/v) acetic acid in the CSL-based medium with 3% xylose and 1.8% glucose at pH 5.75, the D_{max} for the adapted recombinant shifted from 0.08 to 0.048/h, and the corresponding maximum

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volumetric ethanol productivity decreased 45%, from 1.52 to 0.84 g/(L·h). Under these conditions of continuous culture, linear regression of a Pirt plot of the specific rate of sugar utilization vs *D* showed that 4 g/L of acetic acid did not affect the maximum growth yield (0.030 g dry cell mass/g sugar), but did increase the maintenance coefficient twofold, from 0.46 to 1.0 g of sugar/(g of cell·h).

Index Entries: Recombinant *Zymomonas*; continuous cofermentation; xylose; biomass hydrolysate; ethanol yield; acetic acid; maintenance coefficient.

Introduction

One of the important barriers to the efficient conversion of biomass hydrolysates to ethanol (i.e., in high yield) is the inability of the yeast cultures presently employed in sucrose and starch-based fermentations toutilize the five-carbon pentose sugars, namely xylose and arabinose, that comprise the hemicellulose component *(1)*. Hemicellulose represents about 30–40% of the dry wt of biomass, and, consequently, technoeconomic analyses have identified the "pentoseconversion problem" as a target with a high economic impact on biomass-derived fuel ethanol *(2)*. Most of the pentose sugar in hemicellulose is xylose, with arabinose being another constituent.

The economic impact of pentose (xylose) conversion is interdependent on three key fermentation parameters (in order of cost sensitivity): (1) yield, (2) ethanol concentration, and (3) productivity *(2)*.

Several recombinant yeasts and bacteria have been developed that are capable of pentose fermentation (for review see refs. *3* and *4*); however, one problem that seems to be universal with all of these recombinant ethanologens relates to cofermentation. These constructs all differ in the degree to which they are capable of simultaneous utilization of glucose and xylose. For example, in batch fermentations with recombinant *Escherichia coli*, the commonly observed sequential utilization of sugars, glucose followed by xylose, has been described by such terms as "preferential utilization" and "xylose sparing" *(5–7)*. This phenomenon has not been systematically studied, and it is not well understood although it could be caused by different mechanisms, such as competition for entry into the cell via a common carrier (as is the case with *Zymomonas*) *(8,9)* or via substrate-specific membrane transporters, or catabolite repression ("glucose effect"). In practical terms, this pattern of sugar utilization means that the ratio of glucose to xylose can profoundly affect the productivity *(10)*.

Another problem common to fermentations with all these recombinant biocatalysts is their sensitivity to inhibition by acetic acid, which is present at different levels in all biomass hydrolysates (for review see ref. *3*). The mechanism of acetic acid toxicity is well understood *(11)*, and its pH-dependent effect on recombinant *Zymomonas* has been documented *(12,13)*. Recently, Joachimsthal et al. *(14)* described the isolation of an acetic acid–tolerant mutant of wild-type *Zymomonas* ZM4.

One of several biomass-to-ethanol processes being investigated at the National Renewable Energy Laboratory (NREL) is a simultaneous saccharification and cofermentation process that involves a dilute-acid pretreatment *(15,16)* and patented, metabolically engineered *Zymomonas mobilis* capable of pentose utilization *(17,18)*. In 1998, we reported on the cofermentation performance capabilities of an "adapted" recombinant *Zymomonas* that had been isolated from a long-term continuous fermentation using a hardwood dilute-acid prehydrolysate medium *(19)*. The present article represents a continuation of that work, and to avoid repetition, it relies heavily on the background information regarding experimental rationale as well as specific methodologies described previously *(12,19)*.

The objective of this study was to describe the fermentation performance characteristics of the adapted recombinant in various bench-scale continuous fermentations using synthetic media formulations as a function of (1) nutrient composition of the medium, (2) the ratio of sugars (glucose to xylose), and (3) the effect of acetic acid. Joachimsthal et al. *(20)* reported on a successful continuous cofermentation of an equal mixture of 4% xylose and 4% glucose using rec ZmCP4:pZB5 at pH 5.0. Therefore, another objective of this work was to examine the effect of pH on the cofermentation performance of this recombinant under the same conditions of chemostat culture.

Materials and Methods

Microorganism

Z. mobilis recombinants CP4:pZB5 *(18)* and the "hydrolysate-adapted" 39676:pZB4L *(19)* (*Z. mobilis* host strains CP4 and ATCC 39676 carrying the xylose assimilation plasmids pZB5 and pZB4L, respectively) were obtained from NREL. Cryovials of frozen concentrated stock culture were maintained in RM medium (10 g/L of yeast extract and 2 g of KH_2PO_4) supplemented with 10 mg/L of tetracycline and 15% (w/w) glycerol at –70°C.

Preparation of Inoculum

A 1-mL aliquot of a glycerol-preserved culture was removed from cold storage (freezer) and transferred to about 100 mL of RM medium, containing about 20 g/L of xylose and 20 g/L of glucose supplemented with tetracycline (10 mg/L), in 125-mL screw-cap flasks and grown statically overnight at 30°C in an incubator. This preseed was subcultured into inoculation flasks containing RM with 20 g/L of glucose, 20 g/L of xylose, and 10 mg/L of tetracycline and grown statically overnight at 30°C in an incubator. This overnight culture was used at a level of ~10% (v/v) to inoculate the chemostats. The initial optical density (1-cm light path at 600 nm) was in the range of 0.2–0.25, corresponding to 60–75 mg of dry cell mass (DCM) per liter.

Chemostat Startup Procedure

The continuous fermentations were started in the batch mode using media with 25 g/L of glucose and 25 g/L of xylose. Flow was started 24 h after inoculation (preferably when the residual xylose concentration was <5 g/L).

Fermentation Media

In fermentations with CP4:pZB5, the yeast extract–based medium contained the following ingredients per liter of glass distilled water: 40 g of xylose, 40 g of glucose, 5 g of Difco yeast extract (Difco, Detroit, MI), 3.48 g of KH_2PO_4, 0.8 g of NH_4Cl, 0.25 g of $MgSO_4$, 0.01 g of $FeSO_4 \cdot 7H_2O$, 0.21 g of citric acid, and 20 mg of tetracycline. Infermentations with the "adapted" recombinant 39676:pZB4L, an alternative, less expensive, corn steep liquor (CSL) medium was used. This CSL-based medium was prepared with distilled water and 1% (v/v) centrifugally clarified CSL (50% solids) (GPC, Muscatine, IA) as a nutrient substitute for yeast extract. This medium was supplemented either with a "Z salts" cocktail to yield a final concentration of 3.48 g/L of KH_2PO_4, 0.25 g/L of $MgSO_4$, 0.01 g/L of $FeSO_4 \cdot 7H_2O$, and 0.21 g/L of citric acid, or, alternatively, with 2 mL/L of a 10% stock solution of $MgSO_4$ (83 m*M*). All media contained 10 mg/L of tetracycline. All media were sterilized by autoclaving at 121°C for 30–45 min. Stock sugar solutions were autoclaved separately. Tetracycline was added to the sterilized medium after cooling.

Continuous Fermentation Equipment

Continuous fermentations were conducted with NBS BioFlo chemostats (Model C30; New Brunswick Scientific, Edison, NJ) or 2-L NBS bioreactors (Model BioFlo 2000; New Brunswick Scientific). The working volume of these chemostats was about 350 and 1500 mL, respectively. Steady state was assumed only after a minimum of 3 vol had exchanged and when samples taken on successive days gave similar values with substrate and product concentrations. The pH was monitored using a sterilizable combination pH electrode (Ingold). The standard pH control set point was either 5.75 or 6.0, and the pH was kept constant by automatic titration with 4 *N* KOH. The temperature was controlled at 30°C using a circulating water bath and the agitation was moderate (approx 100–150 rpm).

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 high-performance liquid chromatography as described previously *(21)*. The ethanol yield ($Y_{p/s}$) was calcu-

lated as the mass of ethanol produced per mass of sugar consumed. The process yield was determined by dividing the ethanol concentration by the total sugar concentration in the feed medium. For chemostat cultures, the maximum mass growth yield (i.e., corrected for "maintenance" metabolism) (max $Y_{x/s}$, g DCM/g sugar) was determined as the inverse of the slope of the best-fit linear regression for specific sugar utilization rate (q_s, g sugar/[g DCM·h]) as a function of the dilution rate (D, 1/h) *(22)*. The maintenance coefficient (m_s, g sugar/[g DCM·h]) was determined as the y-axis intercept of the best-fit linear regression to the q_s vs D data *(22)*.

Results and Discussion

This study with a prehydrolysate-adapted variant of rec Zm 39676:pZB4L is a continuation of the work presented last year on pH-stat batch and continuous fermentations with nutrient-rich synthetic prehydrolysate media containing 4% (w/v) xylose and 0.8% (w/v) glucose *(19)*. These sugar concentrations were selected because they resembled the composition of NREL's hardwood (yellow poplar) dilute-acid hydrolysate *(23)*.

CSL as an Effective Nutritional Substitute for Yeast Extract

In pH-stat batch fermentations with various xylose-utilizing Zm recombinants, it has been shown that 1% (v/v) clarified CSL (cCSL) is a cost-effective nutritional substitute for yeast extract *(19,20)*. Furthermore, it has been shown in pH-stat batch fermentations that the performance of recombinant Zm is not significantly compromised when the level of CSL is reduced fourfold provided that the medium is supplemented with an appropriate source of assimilable inorganic nitrogen (e.g., 1.2 g/L of diammonium phosphate (DAP) (unpublished observations). Figure 1 shows a typical time course of a continuous cofermentation of pure sugar synthetic prehydrolysate with the "hydrolysate-adapted" recombinant in which the dilution rate (D) was increased incrementally (range 0.04–0.094/h) over 21 d. Figure 2 shows the concentrations of DCM, ethanol, and residual xylose as a function of the steady-state dilution rate. Over the test range of D, glucose was not detected in the effluent. The medium consisted of 1% (v/v) cCSL in distilled water supplemented with a cocktail of inorganic salts (*see* Materials and Methods). The fermentation performance exhibited by the adapted strain in this medium, as reflected by the concentration values shown in Fig. 2, was almost identical to that observed previously with the standard laboratory yeast extract–based medium. In subsequent experiments, we discovered that the salts mixture used as a supplement could be effectively replaced by a single component: magnesium (approx 2 m*M*). It is not known to what extent other divalent cations (e.g., calcium) can replace magnesium. In an industrial process, the makeup water would probably not be deionized. From an analysis of local tap water, we surmise that the amount of magnesium supplementation required for optimal biocatalyst performance could be considerably less for large-scale operation than the level of 2 m*M* used in the present study.

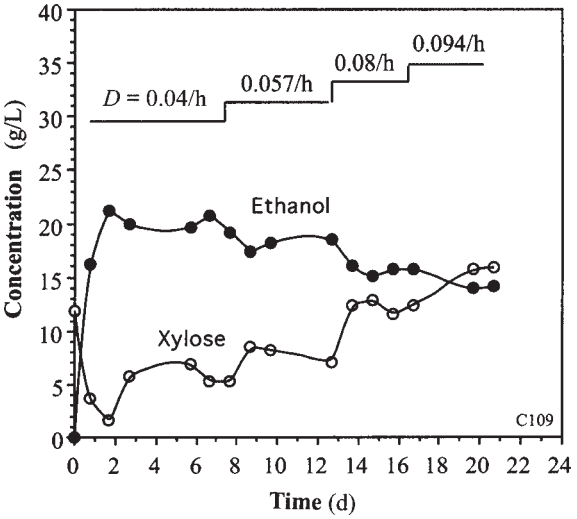

Fig. 1. Time course of continuous cofermentation with adapted rec Zm 39676:pZB4L. The dilution rate (*D*) was increased incrementally as indicated. The synthetic prehydrolysate medium consisted of 1% (v/v) CSL in distilled water supplemented with Z salts (*see* Materials and Methods). The concentration of sugars in the feed was 40 g/L of xylose and 8 g/L of glucose. There was no acetic acid in the medium, and no glucose was detected in the chemostat effluent. The pH was controlled at 5.75 and the temperature at 30°C.

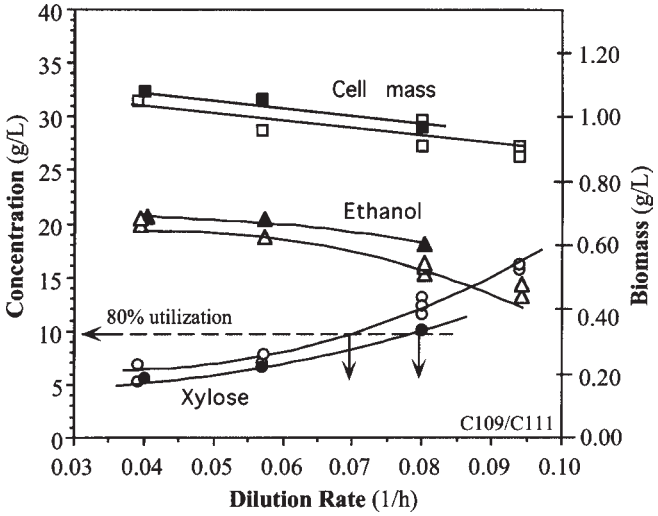

Fig. 2. Steady-state concentrations of xylose, ethanol, and DCM as a function of dilution rate for continuous cofermentation using adapted rec Zm39676:pZB4L. The CSL-based medium was the same as described in Fig. 1. The *y*-axis arrow indicates the residual xylose concentration (9.6 g/L) for 80% total sugar utilization (*see* Table 2). Open symbols represent medium with 40 g/L of xylose + 8 g/L of glucose; solid symbols represent a medium with 30 g/L of xylose + 18 g/L of glucose. There was no acetic acid in the medium, and no glucose was detected in the chemostat effluent. The pH was controlled at 5.75 and the temperature at 30°C.

Table 1
Cost of Alternative Nutrients in Recombinant *Zymomonas* Fermentations[a]

Nutrient	Amount (% [w/v])	Cost (US$)	
		$/1000 L medium	$/gal EtOH
CSL (50% solids)	1.000	1.65	0.1130
CSL	0.250	0.41	0.0283
$MgSO_4$ (2 m*M* Mg)	0.025	0.18	0.0124
Inorganic N $(NH_4)_2HPO_4$ (DAP)	0.100	0.23	0.0158
Option 1 1% CSL + Mg		1.83	0.1250
Option 2 0.25% CSL + Mg + DAP		0.82	0.0570

[a]Costs are based on NREL estimates for whole slurry CSL at $150/t (M. Ruth, personal communication); current selling price of bulk, fertilizer-grade chemicals; a total sugar loading of 10% (w/v); and process ethanol yield of 0.434 g/g (85% conversion efficiency) and 100% ethanol recovery whereby it takes 68.72 L of fermentation medium to produce 1 gal of absolute ethanol.

Economic Impact of Using CSL

CSL has been shown to be an effective alternative to yeast extract for industrial-scale fuel ethanol fermentations using ethanologenic bacteria (for review see ref. *24*). Industrial-grade yeast extract sells for $3/lb, and at the level of 0.5% (w/v) (modified RM) *(25)* the cost associated with this component alone would be $33/1000 L of medium. Clearly this cost is prohibitive for large-scale production. von Sivers et al. *(26)* estimated that the cost of a mineral salts medium suitable for rec *E. coli* fermentations would be 71¢/1000 L, but they did not assess the efficacy of their medium formulation in terms of growth and fermentation performance. In previous studies, we have commented on the economic impact of using 1% (w/v) CSL as a nutritional adjunct in which the cost of 1000 L of fermentation medium was 55¢ *(24)*. This cost was based on an average current selling price of $50/t (whole slurry) for CSL (50% solids) FOB, the wet-miller's rate. We have now revised these estimates based on a more realistic cost of CSL of $150/t (M. Ruth, personal communication) that takes into account transportation costs and the amount required to satisfy large-scale cellulosic ethanol production plants. This increases the cost of 1000 L of fermentation medium from 55¢ to $1.65 (Table 1). For a total sugar loading of about 10% (w/v) and an overall sugar-to-ethanol conversion efficiency of 85%, the cost of CSL as a sole nutrient supplement would be 11.3¢/gal of ethanol. This cost would increase to 12.5¢/gal if 2 m*M* Mg were also added (Table 1) (note the comment in the previous section concerning composition of process makeup water in the industrial plant and the requirement for Mg). Table 1 shows that there is a 50% cost reduction incentive for reducing the level of CSL fourfold, even though this would require the addition of inorganic nitrogen to prevent growth limitation by nitrogen exhaustion. Although we have demonstrated the feasibility of doing this in batch fermentations with rec Zm in pure sugar synthetic prehydrolysate media

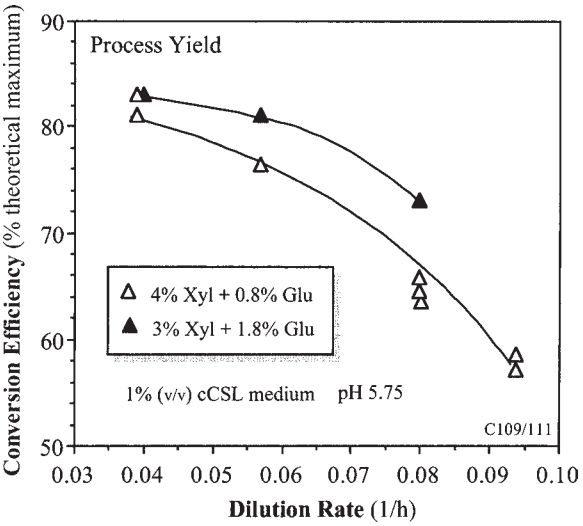

Fig. 3. Sugar-to-ethanol conversion efficiency as a function of steady-state dilution rate. Data are taken from experiments shown in Figs. 1 and 2. Conversion efficiency is calculated as the percentage ratio of process yield to theoretical maximum ethanol yield. Process yield is based on total sugar loading. Theoretical maximum ethanol yield is 0.51 g/g.

containing acetic acid, we have not yet attempted to operate a continuous cofermentation under these conditions of reduced CSL; however, this is clearly an area that deserves further investigation.

Effect of Sugar Ratio on Continuous Cofermentation Performance

Figure 2 shows the effect of altering the sugar ratio while maintaining the same total sugar loading of 4.8% (w/v). Surprisingly, an increase in the input glucose from 0.8 to 1.8% did not result in an appreciable increase in the cell mass concentration (Fig. 2). The dotted line in Fig. 2 shows the threshold limit for effluent xylose at 80% utilization of the sugars in the feed. When the input level of xylose is reduced from 4 to 3%, the effluent xylose curve is shifted to the right and the maximum *D* value increases from 0.07/h to 0.08/h (Fig. 2). In these experiments the yield of ethanol, based on sugar used, remains relatively constant at about 0.47 g/g (equivalent to 92% of theoretical maximum conversion efficiency), and, therefore, for 80% total sugar utilization, the conversion efficiency is 73.7%.

Figure 3 shows the relationship between the sugar-to-ethanol conversion efficiency and the steady-state dilution rate. The improved process yield at the higher dilution rates is owing to the increased level of glucose that is completely utilized over the entire dilution range tested. Figure 4 shows the best fit for linear regression for specific rate of sugar utilization as a function of the dilution rate for the CSL-based media with different sugar mixtures. Values for the maintenance coefficient and maximum growth yield that were derived from these Pirt plots *(22)* are given in Table 2

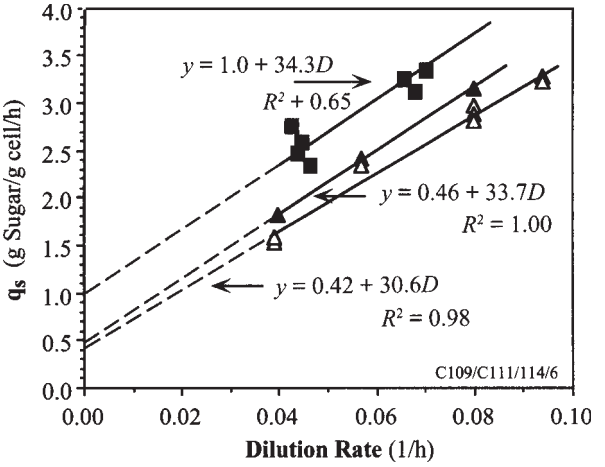

Fig. 4. Specific rates of sugar utilization by adapted rec Zm 39676:pZB4L as a function of dilution rate. The CSL-based medium is described in Fig. 1. △, 40 g/L of xylose + 8 g/L of glucose; ▲, 30 g/L of xylose +18 g/L of glucose; ■, 30 g/L of xylose + 18 g/L of glucose + 4 g/L of acetic acid (*see* Fig. 7). The pH was controlled at 5.75 and the temperature at 30°C. Straight lines were based on linear regression analysis of the data (R = regression correlation). The y-axis intercept represents the maintenance coefficient (m_s), and the inverse of the slope represents the maximum growth yield (*see* Table 2).

and are similar to the values for these parameters reported in our previous study using the same recombinant with ZM medium (4% xylose + 0.8% glucose) *(19)*.

Others have reported on continuous cofermentations with rec Zm using equal amounts of glucose and xylose *(20,27)*. The decision, by these investigators, to use equal amounts of glucose and xylose appears to be based on the fact that the original shake flask experiments reported by Zhang et al. *(18)* in the characterization of NREL's xylose-utilizing recombinants employed a mixture of 2.5% glucose and 2.5% xylose. Figure 5A shows the concentrations of DCM, ethanol, and residual xylose as a function of the steady-state dilution rate for the adapted strain with 2.5% xylose and 2.5% glucose. The results are quite similar to those reported in Fig. 2 underidentical conditions with the unexpected exception that the cell mass concentration with the 2.5% sugar mixture was lower. The lower cell mass translates into higher q_s values (Fig. 6A). The higher maintenance coefficient observed with the continuous cofermentation of 2.5% mixture (Fig. 6A) is consistent with the trend observed with the effect of an increased level of glucose in the medium (Fig. 4).

The continuous cofermentation experiments with a 4% mixture of glucose and xylose shown in Fig. 5B were performed under the same conditions as reported recently by Joachimsthal et al. *(20)* for rec Zm CP4:pZB5 using a nutrient-rich yeast extract–based salts medium but with the pH controlled at either 5.0 or 5.75. The ethanol yield based on sugar used is 0.49 g/g (Fig. 5B). For 80% sugar utilization (16 g/L of residual xylose),

Table 2
Summary of Parameters for Continuous Fermentations with Recombinant *Zymomonas*[a]

rec Zm strain	pH	Xylose (g/L)	Glucose (g/L)	N source	Residual xylose (g/L)	D_{max} (1/h)	q_s (g S/[g cell·h])	q_p (g E/[g cell·h])	max $Y_{X/S}$ (g DCM/g S)	m_s (g S/[g cell·h])	Reference
Adapted	5.75	40	8	CSL	9.6	0.070	2.56	1.23	0.033	0.42	This work
39676:pZB4L	5.75	30	18	CSL	9.6	0.080	3.16	1.52	0.030	0.46	This work
	5.75	25	25	CSL	10.0	0.070	3.22	1.50	0.031	0.93	This work
Adapted 39676:pZB4L + 4 g/L of acetic acid	5.75	30	18	CSL	9.6	0.048	2.65	1.25	0.029	1.00	This work
CP4:pZB5	5.00	40	40	YE	16.0	0.064	3.56	1.69	0.022	0.61	This work
	5.00	40	40	YE	16.0	0.090	3.64	1.72	0.079	2.50	*21*
	5.75	40	40	YE	16.0	0.084	4.02	1.90	0.026	0.61	This work
	5.75[b]	40	40	YE	16.0	0.095	4.90	2.30	NA	NA	This work

[a]*D*, dilution rate; CSL, corn steep liquor; YE, yeast extract; S, sugar (xylose + glucose); E, ethanol; q_s, specific rate of sugar utilization; q_p, specific rate of ethanol production; max $Y_{X/S}$, maximum growth yield derived from inverse slope of Pirt plot; m_s, maintenance coefficient derived as *y*-axis intercept of Pirt plot *(22)*. N/A, not available; residual xylose (i.e., the amount of sugar in the effluent), D_{max}, q_s, and q_p are values for >80% utilization of sugars.

[b]Wall growth (i.e., cell mass retention in the continuous-flow fermentor) mimics the immobilized cell system and makes values for residual xylose, *D*, q_s, and q_p nonrepresentative of a truly homogeneous system.

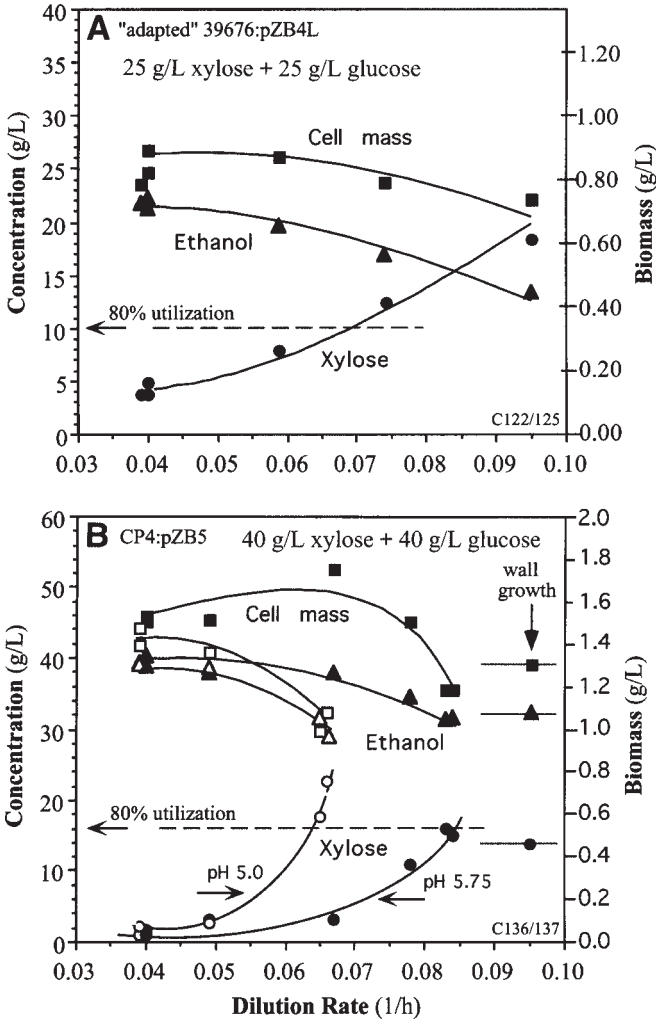

Fig. 5. Steady-state concentrations of xylose, ethanol, and DCM as a function of dilution rate. **(A)** Continuous cofermentation with the adapted recombinant in CSL medium with 25 g/L of xylose + 25 g/L of glucose (the pH was 5.75); **(B)** continuous cofermentation with rec Zm CP4:PZB5 in yeast extract medium (*see* Materials and Methods) and 40 g/L of xylose + 40 g/L of glucose. Open symbols = pH 5.0 and closed symbols = pH 5.75. The arrow at D = 0.095/h indicates that "wall growth" was observed after about 30 d of operation.

the maximum D values are 0.064/h and 0.084/h for pH 5.0 and pH 5.75, respectively (Fig. 5B, Table 2). After about 1 mo, the pH 5.75 chemostat exhibited considerable "wall growth" and therefore the values for "free" cell mass, ethanol, and residual xylose at D = 0.095/h in Fig. 5B are not representative of a truly homogeneous (free cell) system (*see* Table 2). The chemostat operating at pH 5.75 exhibits a similar pattern with respect to DCM, ethanol, and residual xylose as that of the pH 5.0 chemostat of Joachimsthal et al. *(20)*, whereas our pH 5.0 chemostat performed less

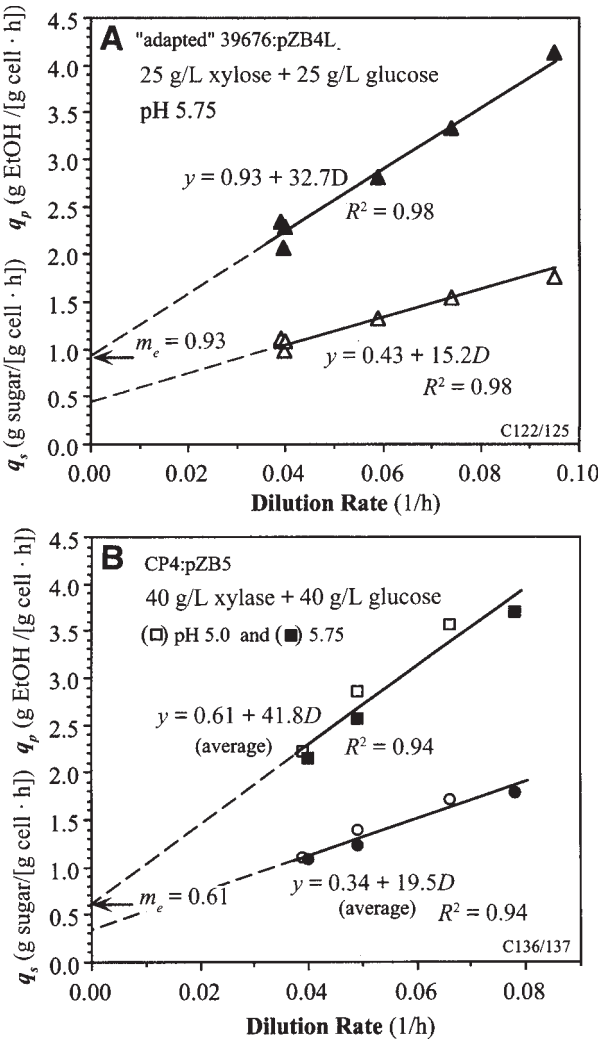

Fig. 6. Specific rates of sugar utilization and ethanol production as a function of steady-state dilution rate. Data are taken from experiments shown in Fig. 5. **(A)** Adapted rec Zm 39676:pZB4L; **(B)** rec Zm CP4:pZB5. Values for maximum growth yields are given in Table 2. In (B), the lines represent average of data for pH 5.0 and 5.75.

favorably in terms of xylose utilization and maximum productivity (Fig. 5B). The reason for this discrepancy in observations at pH 5.0 remains unresolved. Figure 6B shows the best-fit linear regression for both specific rates of sugar utilization and ethanol production as a function of *D*. The values for maintenance coefficient and maximum growth yield are given in Table 2, and comparison with other experiments performed in this study are made difficult because of differences in both the recombinant and the composition of the medium. However, we noted that the values for these parameters are markedly different from those reported by Joachimsthal et al. *(20)* (Table 2).

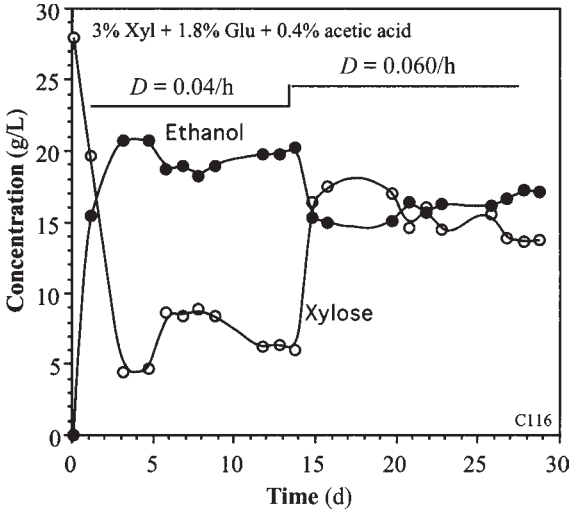

Fig. 7. Time course of continuous cofermentation with adapted rec Zm 39676:pZB4L. The dilution rate (*D*) was increased incrementally as indicated. The CSL-based synthetic prehydrolysate medium contained 30 g/L of xylose, 18 g/L of glucose, and 4 g/L of acetic acid. No glucose was detected in the chemostat effluent. The pH was controlled at 5.75 and the temperature at 30°C.

Effect of 0.4% Acetic Acid

Previously, in pH-stat batch fermentations with different recombinant Zm strains and media containing 4 g/L of acetic acid, we demonstrated that both CP4:pZB5 and the adapted strain outperformed the nonadapted parent strain 38676:pZB4L in terms of productivity *(12,19)*. With the adapted strain and the standard pure sugar synthetic prehydrolysate medium (40 g/L of xylose + 8 g/L of glucose), a shift in the pH control set point from 6.0 to 5.0 resulted in a 12% reduction in the final cell mass concentration and a 32% reduction in ethanol productivity; the ethanol yield was unaffected, and the final ethanol concentration remained constant at 24 g/L *(19)*.

The present study assessed the performance of the adapted recombinant in a continuous cofermentation using the CSL-based medium (30 g/L of xylose + 8 g/L of glucose) containing 4 g/L of acetic acid. Figure 7 represents a 1-mo time course of a pH 5.75 continuous cofermentation. At $D = 0.04$/h, there was good sugar utilization and the ethanol yield was 0.48 g/g. However, when the dilution rate was increased from 0.04/h to 0.06/h at d 14, the residual xylose surpassed the 80% total sugar utilization threshold (Fig. 7). From another chemostat experiment (not shown), the maximum dilution rate for 80% sugar utilization was estimated to be 0.048/h, whereas in the absence of acetic acid, it was 0.08/h (Table 2). At these respective *D* values, this level of acetic acid at pH 5.75 caused a 30% decrease in the cell mass concentration, from 0.95 to 0.67 g of DCM/L, and a 45% reduction in ethanol productivity, from 1.52 to 0.84 g of EtOH/(L·h)

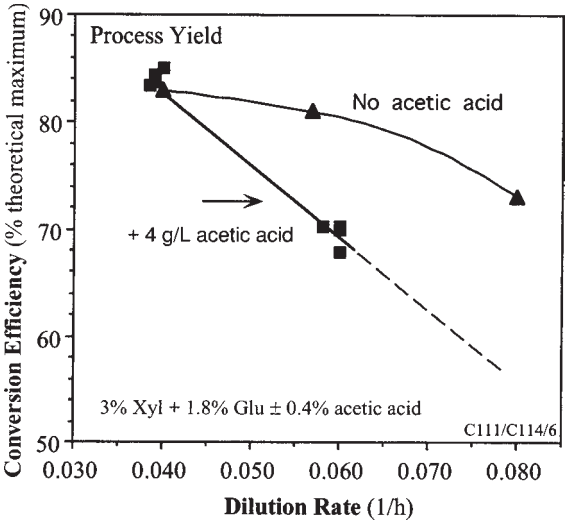

Fig. 8. Sugar-to-ethanol conversion efficiency as a function of steady-state dilution rate. Data are taken from experiments similar to that shown in Fig. 7. Conversion efficiency was calculated as described in Fig. 3.

(not shown). Even at the more permissive pH of 5.75 *(19)*, this amount of acetic acid had a dramatic effect on the process yield (Fig. 8).

The influence of acetic acid on the specific rate of sugar utilization is shown in Fig. 4. As expected from previous observations on the effect of acetic acid on rec Zm in pH-stat batch fermentations *(12,13,19)*, the maintenance coefficient is higher in the presence of acetic acid, perhaps because energy is dissipated in expelling protons to counteract the lowering of the intracellular pH by the permeant undissociated acetic acid (Fig. 4 and Table 2).

The unconditioned dilute-acid hardwood prehydrolysate produced by NREL contains about 3.5-fold higher levels of acetic acid than tested in the present study *(23)*, and, consequently, these observations underscore the strong inhibitory effect of this component of biomass hydrolysates on cofermentation performance of recombinant *Zymomonas*. This study did not involve biomass hydrolysates, and, therefore, the possibility remains that the physiological characteristic that distinguishes the adapted strain may not have been exposed. Although this strain appears to be more acetic acid tolerant than its parent (39676:pZB4L) *(19)*, in this respect it may not be superior to strain CP4:pZB5 or other rec Zm strains, e.g., ZM4:pZB5 *(10,20)*. However, it is also conceivable that the adaptation that this strain experienced during its long-term continuous culture in prehydrolysate medium relates to a decrease in sensitivity to some other inhibitory component of the biomass hydrolysate.

Significance of the "Maintenance Coefficient"

In this and previous studies *(19,21)*, we have plotted q_s as a function of the specific growth rate in chemostat cultures as a means of estimating a

value for Pirt's "maintenance coefficient" (*m*) *(22)* by requiring the data to conform to linearity. Others have done likewise *(20)*, and it has been fashionable to report a value for *m* without critically assessing its possible physiological significance. Pirt *(22)* asserted that the determination of *m* was only valid under a condition of energy-limited growth, and when there are significant amounts of sugar in the chemostat effluent, this condition is probably not met. We have also explored this issue of the meaning of the maintenance coefficient *(28)*. Because of observed deviations from linearity under certain conditions, we concluded that *m* (as defined by Pirt) *(22)* is probably not a constant that is independent of the growth rate and that its value is only of limited significance in terms of describing either nongrowth-related metabolism or the varying degrees of "energetic uncoupling" under different environmental conditions *(28)*. This is a subject that will be explored further in a cybernetic model for cofermentation of glucose and xylose by recombinant Zm being proposed by Kompala et al. *(29)*.

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