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The Effect of Glucose on High-Level Xylose Fermentations by Recombinant *Zymomonas* in Batch and Fed-Batch Fermentations

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Abstract

Xylose-fermenting recombinant *Zymomonas mobilis* has been proposed as a candidate biocatalyst for the production of fuel ethanol from cellulosic biomass and wastes. This study documents the effect of glucose on xylose utilization by recombinant *Z. mobilis* CP4:pZB5 using a nutrient-rich synthetic (pure sugar) hardwood dilute-acid prehydrolyzate medium containing 0.8% (w/v) glucose and 4% (w/v) xylose that was enriched with respect to xylose concentration within the range 6–10% (w/v) xylose. Supplementation with glucose to a final concentration of 2% (w/v) resulted in faster xylose utilization of both 6% and 8% xylose; however, higher levels of glucose supplementation (>2%) did not result in a decrease in the time required for fermentation of either 6% or 8% xylose. An improvement in the rate of 8% xylose utilization was also achieved through continuous glucose feeding in which the total glucose concentration was about 1.3% (w/v). This fed-batch experiment was designed to mimic the continuous supply of glucose provided by the cellulose saccharifying enzymes in a simultaneous saccharifying and cofermentation process. The upper limit ethanol concentration at which xylose utilization by recombinant *Z. mobilis* CP4:pZB5 is completely inhibited is about 5.5% (w/v) at pH 5 and >6% at pH 5.75. At pH 5.75, this level of ethanol was achieved with the following media of pure sugar mixtures (each containing the same sugar loading of 12% (w/v):

1. 6% xylose + 6% glucose;
2. 8% xylose + 4% glucose; and
3. 4% xylose + 8% glucose.

At the level of inoculum used in this study, complete fermentation of the 12% sugar mixtures required 2–3 d (equivalent to a volumetric ethanol productivity of 0.83–1.25 g ethanol/L.h). The sugar-to-ethanol conversion efficiency was 94–96% of theoretical maximum.

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Index Entries: Recombinant *Zymomonas*; xylose; ethanol tolerance; cofermentation; prehydrolyzate; glucose feeding.

Introduction

Recombinant *Zymomonas mobilis* (rec Zm) carrying genes for xylose metabolism from *Escherichia coli (1,2)* is one of several biocatalysts for the production of ethanol from biomass that is currently under investigation by the National Renewable Energy Laboratory (NREL, Golden, CO) *(3–6)*. In the different simultaneous saccharification and fermentation process designs for the production cellulosic fuel ethanol currently being assessed by NREL, a prerequisite of the biocatalyst is the ability to coferment, in high yield, the major hemicellulosic and cellulosic sugars, namely xylose and glucose, respectively *(7,8)*. We have already demonstrated that recombinant *Zymomonas* has excellent cofermentation characteristics in laboratory synthetic media and in hardwood prehydrolyzate in both batch and continuous fermentations *(9,10)*. In previous work with different Zm recombinants, the majority of pH-stat batch fermentations were conducted with media that contained 5:1 ratio of xylose to glucose, with a maximum xylose concentration of 4% (w/v) *(10,11)*. We demonstrated that the presence of 0.8% glucose in the medium significantly reduced the time required for complete xylose utilization *(9)*. These concentrations of xylose (4% w/v) and glucose (0.8% w/v) were selected to mimic the composition of NREL's dilute-acid hardwood hemicellulose hydrolyzate *(12,13)*. In *Zymomonas*, glucose and xylose compete for the same membrane transporter *(14)*. Because the affinity of the transport system is much higher for glucose than xylose *(14)*, it is reasonable to expect that batch productivity would be affected by the ratio of these two sugars in the medium. Apart from an investigation into the efficacy of glucose feeding on reducing the inhibitory effect of acetic acid on xylose utilization *(11)*, no attempt had been made previously to optimize the level of glucose supplementation for the purpose of effecting maximal rate of xylose utilization. The objective of this study was to examine the effect of glucose on xylose utilization (i.e., the time required for complete xylose consumption) using a xylose-enriched synthetic (pure sugar) hardwood prehydrolyzate medium in which the xylose concentration was in the range 6–10% (w/v). Furthermore, it was anticipated that these experiments at elevated sugar levels would also afford an opportunity to assess the effect of ethanol on xylose fermentation by recombinant *Zymomonas*.

Materials and Methods

Organism

The xylose-utilizing recombinant *Z. mobilis* strain CP4 carrying the plasmid pZB5 (designated as Zm CP4:pZB5) *(1,2)* was received from M. Zhang (NREL, Golden, CO). Stock cultures were stored in glycerol at –70°C and pre-cultures were prepared as described previously *(9)*.

Preparation of Inoculum

Overnight flask pre-cultures were harvested by centrifugation (16,300*g* for 10 min) and the cell pellet resuspended in rich medium (RM) 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 Medium and Equipment

The fermentation medium (designated as "ZM") was prepared with glass distilled water and contained the following ingredients: 5 g/L Difco Yeast Extract (YE) (Difco Laboratories, Detroit, MI); 3.48 g/L KH_2PO_4; 0.8 g/L NH_4Cl; 0.5 g/L $MgSO_4$; 0.01 g/L $FeSO_4 \cdot 7H_2O$; 0.21 g/L citric acid; 20 mg/L tetracycline. The amount of glucose and xylose added to the medium was variable. The medium and stock sugar solutions were autoclaved separately. Batch and fed-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). 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 the automatic titration with 4 *N* KOH. In fed-batch fermentations, a peristaltic pump was used to deliver 4.85% (w/v) glucose at a constant rate of 2 mL/h through the central agitator shaft of the bioreactor; the flow rate was determined with the aid of an in-line pipet. The glucose feed was initiated after 8 h elapsed fermentation time, when most of the initial 0.8% glucose had been consumed.

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 (HPLC) as described previously *(9)*. 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.

Results and Discussion

Growth and Fermentation Performance of Recombinant Zm in Xylose-Enriched Synthetic Hardwood Prehydrolyzate Medium

In previous work we used a synthetic biomass (hardwood) prehydrolyzate medium (BPH) to assess the growth and cofermentation

performance characteristics of recombinant *Z. mobilis* CP4:pZB5 *(9)* and 39676:pZB4L *(11)*. The nutrient-rich synthetic BPH media were formulated to model the sugar concentration in the NREL hardwood (yellow poplar) dilute-acid prehydrolyzate *(12,13)* and contained 4% (w/v) xylose and 0.8% (w/v) glucose *(9,11)*. In this work, the formulation of the synthetic BPH was modified slightly by reducing the level of yeast extract 50% and by the addition of both ammonium and magnesium salts (*see* Materials and Methods section). Furthermore, in this work the pH was controlled slightly lower at 5.75, compared to 6.0 previously. However, these modifications did not significantly alter the growth and cofermentation performance of recombinant CP4:pZB5 (Fig. 1; open circle symbol) relative to what had been observed previously with 0.8% glucose and 4% xylose *(9)*. The almost superimposable OD trajectories in Fig. 1A show that increasing the xylose loading of the BPH medium to give final xylose concentrations of 6% (w/v) and 8% (w/v) did not appreciably affect either the growth rate or yield. The final cell mass concentrations for the standard 4% xylose BPH, 6% and 8% media were 1.39, 1.46, and 1.48 g DCM/L, respectively (Table 1). Growth was affected when the media contained 10% xylose (Fig. 1A); in this case the final cell concentration was reduced 1.18 g DCM/L (Table 1).

Using the standard 4% xylose BPH medium, what is typically observed is that the rate of xylose utilization slows exponentially when the residual xylose concentration falls below about 0.5% (w/v) (Fig. 1B) and this is most probably owing to the relative low affinity of the membrane transporter for xylose compared to glucose *(14)*. This "tailing off" phenomenon with respect to xylose utilization is a characteristic of recombinant Zm and is independent of any inhibitory effect of ethanol *(1,8,9,11)*.

Utilization of the 0.8% glucose is progressively protracted at increasing levels of xylose supplementation (Fig. 1B). This pattern is consistent with both sugars entering the cell by a common membrane transporter *(14)*. The medium with 6% xylose was completely fermented at 48 h (Fig. 1B). Previously, we had observed with another recombinant strain that when 2% xylose was added to the medium following the almost complete fermentation of the synthetic BPH, the xylose was completely utilized within 48 h *(11)*. Neither the 8% or 10% xylose media were finished when the experiment was terminated at 54 h (Fig. 1B), although in both cases the final ethanol concentration (Fig. 1C, Table 1) was well below what might be considered "inhibitory" *(16)*. The ethanol productivity associated with all these batch fermentations was very similar (Table 1). This may be a consequence of the similarity in cell density for these fermentations (Table 1). The ethanol yield was 0.48 g ethanol/g sugars consumed, which represents 94% of the theoretical maximum sugar-to-ethanol conversion efficiency (Table 1). This series of batch fermentations with 0.8% glucose and increasing amounts of xylose showed the potential for improving the productivity through an increase in the rate of xylose utilization, especially with higher levels of xylose.

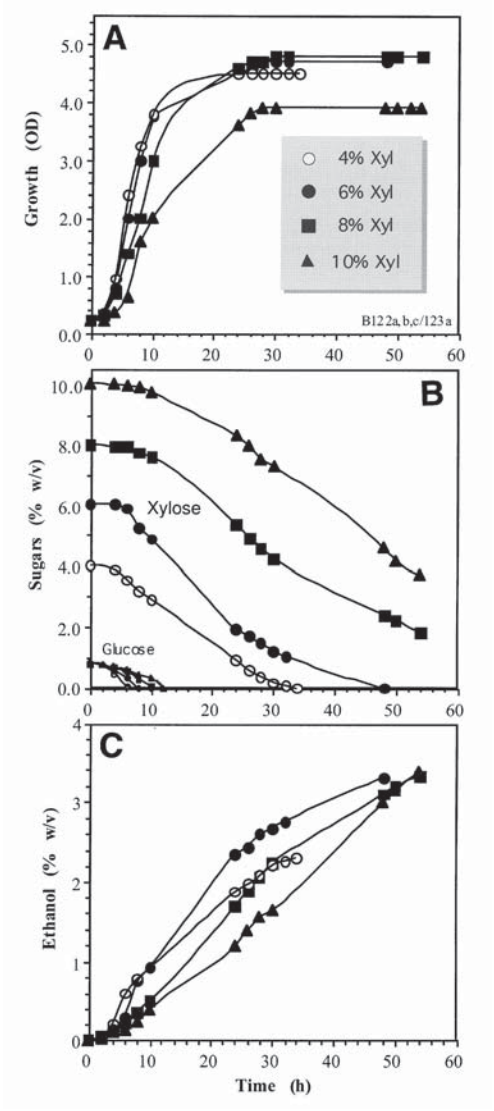

Fig. 1. Fermentation of synthetic hardwood prehydrolyzate fortified with different amounts of xylose: **(A)** Growth, **(B)** glucose and xylose utilization, and **(C)** ethanol production. The ZM medium (see Methods) contained 0.8% (w/v) glucose and was supplemented with 4, 6, 8, or 10% (w/v) xylose. The temperature was kept constant at 30°C. The pH-control set-point was 5.75. The maximum dry cell mass concentrations and values for both ethanol yield and productivity are given in Table 1.

Fermentation of 6% Xylose Supplemented with Different Amounts of Glucose

In this series of pH-stat batch fermentations, we explored the effect of increasing levels of glucose supplementation of a medium containing 6% xylose. In the absence of glucose, the recombinant grows relatively slowly

Table 1
Summary of Growth and Fermentation Parameters

Medium (sugar conc'n) xylose % (w/v)	Glucose % (w/v)	Maximum cell mass (g DCM/L)	Maximum ethanol (g/L)	Ethanol yield (g/g)	Ethanol productivity (g EtOH/L/h)[a]
Expts. for Fig. 1					
4	0.8	1.39	23.0	0.48	0.68
6	0.8	1.46	33.0	0.48	0.69
8	0.8	1.48	33.6	0.48	(0.62)
10	0.8	1.18	33.9	0.47	(0.63)
Expts for Fig. 2					
6	0	0.74	23.0	0.48	(0.32)
6	0.8	1.46	33.0	0.48	0.69
6	2	1.69	39.7	0.49	0.83
6	4	1.85	49.5	0.49	0.69
6	6	2.03	58.6	0.49	0.95
Expts. for Fig. 3					
8	0.8	1.48	36.6	0.48	(0.51)
8	2	1.79	47.7	0.48	0.66
8	4	2.03	57.2	0.48	0.79
4	8	2.37	58.2	0.49	0.97
Fed-batch (0.48% w/v Glc added over 64 h)					
8	0.8	1.66	44.7	0.48	0.62
Expts. for Fig. 4					
8	2	1.79	47.7	0.48	0.66
8[b]	2[b]	1.73	56.2	0.48	(0.57)
Expts. for Fig. 5					
6.6	6.7	2.46	61.9	0.46	1.29
6.5[c]	6.5[c]	2.44	58.0	0.47	1.18

[a]Brackets around values for Ethanol Productivity indicate that xylose utilization was incomplete when batch fermentation was terminated.
[b]1.5% (w/v) ethanol added.
[c]pH 5.0.

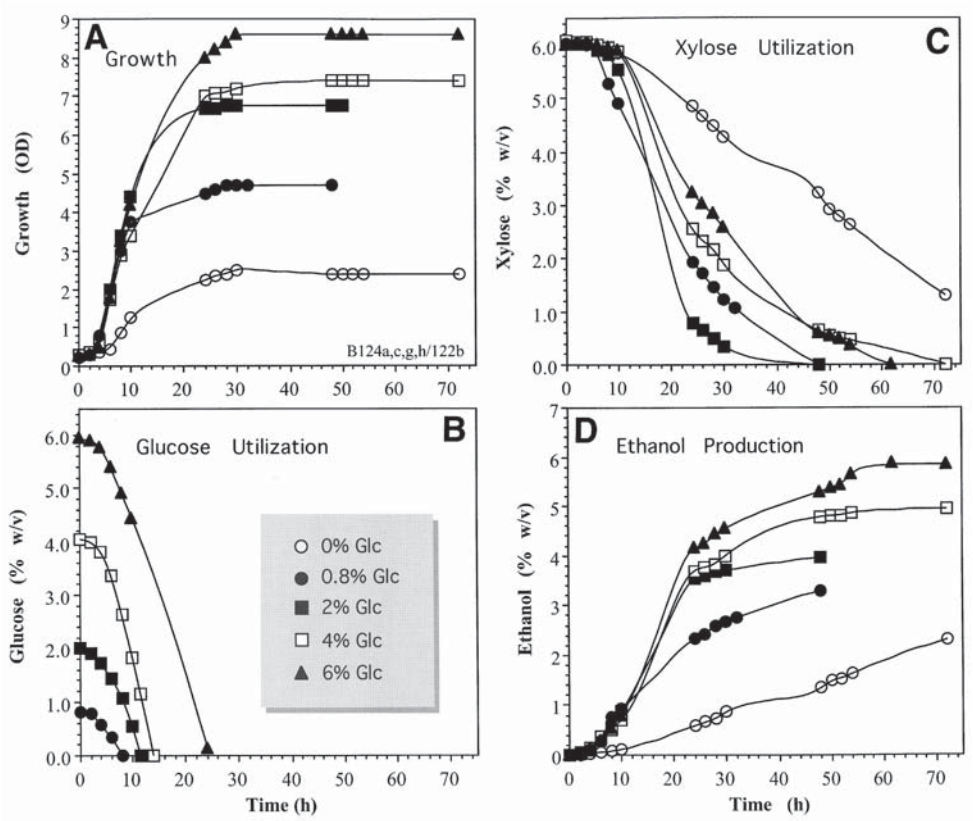

Fig. 2. Fermentation of 6% xylose medium supplemented with different amounts of glucose. **(A)** Growth, **(B)** glucose utilization, **(C)** xylose utilization, and **(D)** ethanol production. The ZM medium contained 6% (w/v) xylose and was supplemented with 0, 0.8, 2, 4, or 6% (w/v) glucose. The temperature was 30°C and the pH was 5.75. The maximum dry cell mass concentrations and values for both ethanol yield and productivity are given in Table 1.

(Fig. 2A) and the final cell mass concentration is 0.74 g DCM/L (Table 1). Slow growth in the absence of glucose is a recognized characteristic of this recombinant *(1)*, but it was interesting to note that the final cell concentration with 6% xylose was very similar to what was observed previously with either 2.5% (unpublished results) or 4% xylose *(9)*. This phenomenon of growth limitation cannot be explained in terms of nutrient limitation, because the medium used was nutrient-rich and clearly capable of supporting higher cell-mass concentrations than the limit of 0.74 achieved with xylose as the sole sugar and energy source. Hence the explanation of this observation remains problematic.

Supplementation of the 6% xylose medium with glucose resulted in faster growth (Fig. 2A) and a final cell-mass concentration that was proportional to the amount of glucose added (Table 1). In the absence of glucose supplementation, about 1.5% xylose remained unconsumed when the experiment was terminated at 72 h, whereas all the xylose was completely

consumed in 48 h when 0.8% glucose was added to the 6% xylose medium (Fig. 2C). The rate of xylose utilization is improved by the addition of 2% glucose to the medium; however, levels of glucose supplementation >2% caused the time required for complete 6% xylose utilization to increase from 48–60 h (Fig. 2C). In the case of supplementation at the level of 4% and 6% glucose, the utilization of xylose may have been initially retarded because of competitive inhibition of xylose uptake by glucose.

In the experiment with 6% xylose and 6% glucose, complete xylose utilization was achieved after 62 h (Fig. 2C). The final ethanol concentration was 5.9% (w/v) (Fig. 2D), which represents an ethanol yield of 0.49 g/g or a sugar conversion efficiency of 96% of theoretical maximum (Table 1). This observation with respect to maximum ethanol concentration has particular relevance in terms of the recent suggestion made by Rogers et al. *(16)* regarding this recombinant:

> "...at least one of the enzymes, which has been cloned into *Z. mobilis* to facilitate pentose metabolism, has been fully inhibited by 55 g/L ethanol" (p. 305).

In another section of this study, we have further explored the effect of ethanol on xylose fermentation.

This series of batch fermentations demonstrate that the time required by the recombinant (at the inoculation level employed) to ferment 6% xylose can be significantly reduced through glucose supplementation, and furthermore they suggest that the optimal effect is achieved within the range of 0.8–2% (w/v) glucose.

Fermentation of 8% Xylose Supplemented with Different Amounts of Glucose

In this series of pH-stat batch fermentations, we explored the effect of increasing levels of glucose supplementation of a medium containing 8% xylose. We were interested to see if the same enhancing effect of glucose on xylose utilization that had been observed with 6% xylose could be achieved at higher levels of xylose.

Supplementation of the 8% xylose medium with 0.8%, 2%, and 4% glucose resulted in proportionately higher final cell-mass concentrations (Fig. 3A, Table 1). With the addition of 0.8% glucose, 1.2% xylose remained unconsumed after 72 h, whereas with 2% glucose added to the medium, the 8% xylose was completely fermented to ethanol (yield = 0.48 g/g) in 72 h (Fig. 3B). Increasing the amount of glucose from 2% to 3% (not shown) or 4% did not shorten the time required for complete xylose utilization (Fig. 3B). With 4% glucose, the final ethanol concentration was 5.7% (w/v) (Fig. 3C, Table 1) and this level of ethanol may have contributed to a retardation of xylose utilization towards the end of the fermentation (Fig. 3B). It was concluded that xylose utilization can be enhanced by means of glucose supplementation with the level of 2% glucose producing the fastest utilization of the 8% xylose.

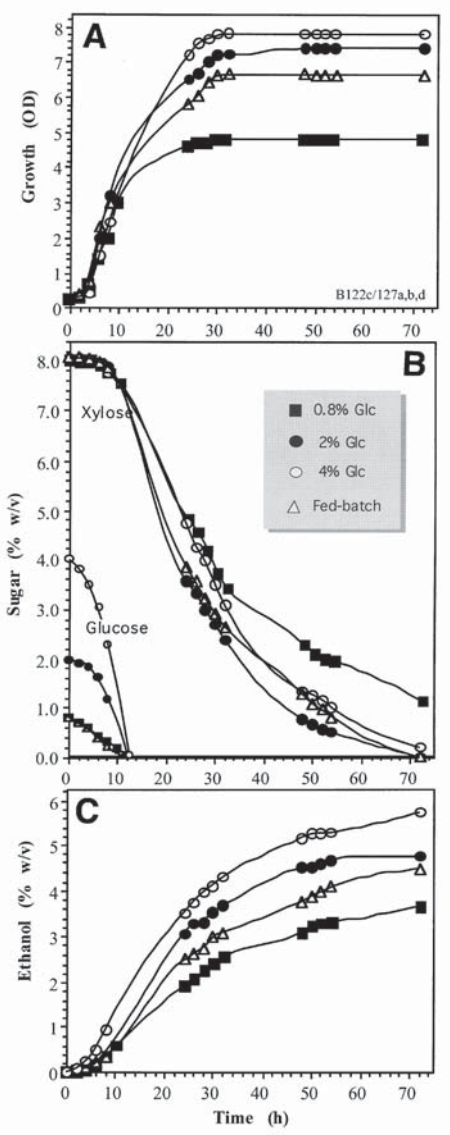

Fig. 3. Fermentation of 8% xylose medium supplemented with different amounts of glucose. **(A)** Growth, **(B)** glucose and xylose utilization, and **(C)** ethanol production. The ZM medium contained 8% (w/v) xylose and was supplemented with 0.8, 2, or 4% (w/v) glucose. In the fed-batch experiment (open triangles), the glucose feed (4.85%) was started after 8 h; the feed rate was 2 mL/h and continued until the fermentation was terminated at 72 h. The temperature was 30°C and the pH was 5.75. The maximum dry cell mass concentrations and values for both ethanol yield and productivity are given in Table 1.

In another related experiment, which was part of a separate study (data not plotted), we observed that a mixture of 4% xylose and 8% glucose was completely fermented in 60 h with a final ethanol concentration of

5.82% (w/v) and volumetric productivity of 0.97 g/L/h (Table 1). With this mixture the final cell density was 2.37 g DCM/L; the 8% glucose was completely fermented in 34 h and the 4% xylose was completely utilized in 60 h (Table 1). Like the experiment with the 8% xylose and 4% glucose (Fig. 3C), the relatively high level of ethanol generated from the 8% glucose and 4% xylose medium may have protracted the time required to complete the fermentation by inhibiting xylose utilization in the final stages of the batch fermentation (50–60 h) (results not shown).

In the simultaneous saccharification cofermentation (SSCF) process design proposed by NREL *(5,7)* the saccharification of cellulose would provide a continuous supply of glucose to the ethanologenic biocatalyst. In order to model this situation whereby the continuous supply of glucose might not be expected to cause the same level of competitive inhibition of xylose uptake produced by the higher levels of glucose supplementation previously tested, we performed a fed-batch experiment in which glucose feeding was initiated after the 0.8% glucose had been consumed in batch mode. The feed reservoir contained 4.85% (w/v) glucose and the feed rate was constant at 2 mL/h over the period from 8–72 h (Fig. 3; open triangle symbol). In the fed-batch experiment, the level of glucose supplementation was equivalent to 1.28% (w/v) (0.8% + 0.48%). Glucose feeding promoted growth beyond the level observed with the standard BPH medium (Fig. 3A); the final cell-mass concentration was 1.66 g DCM/L (Table 1). However, perhaps most significant was observation that the xylose utilization trajectories for the 2% glucose supplemented medium and the fed-batch fermentation were very similar (Fig. 3B). In a previous study with recombinant Zm, we demonstrated the benefit of providing a continuous supply of glucose for xylose fermentation in reducing the effect of inhibition by acetic acid *(11)*. The results of the present work with the fed-batch fermentation auger well for the performance of recombinant Zm in the SSCF process for the production of cellulosic ethanol where the xylose concentration is expected to be in the range 4–6%.

Effect of Ethanol on Xylose Utilization

Wild-type *Zymomonas* is known to be as ethanol-tolerant as yeast *(17–20)*; however, xylose utilizing recombinant *Zymomonas* appears to be more sensitive to end-production inhibition in terms of xylose fermentation *(16)*. This study at elevated levels of xylose provided an opportunity to assess the effect of ethanol on xylose utilization by recombinant Zm CP4:pZB5. Both the fermentation with 6% xylose and 6% glucose and the fermentation with 8% xylose and 4% glucose produced final ethanol concentrations that exceeded the xylose fermentation limit of 5.5% (w/v) (Table 1) proposed recently by Rogers et al. for this recombinant strain *(16)*.

Because it had been demonstrated that the recombinant produced 4.8% (w/v) ethanol from a medium containing 8% xylose and 2% glucose, the addition of 1.5% (w/v) ethanol to a medium containing 8% xylose and

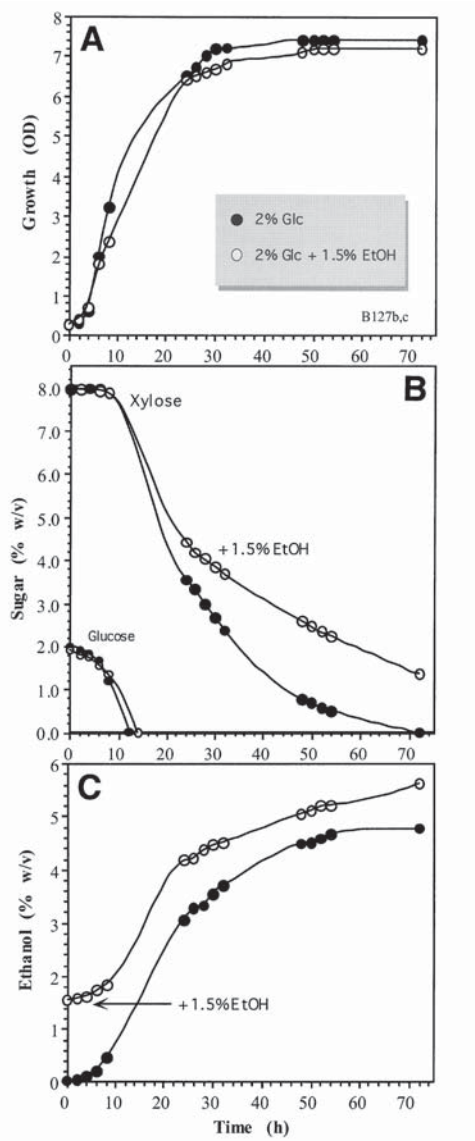

Fig. 4. Effect of 1.5% (w/v) exogenous ethanol on the fermentation of 8% xylose and 2% glucose by recombinant Zm CP4:pZB5. **(A)** Growth, **(B)** glucose and xylose utilization, and **(C)** ethanol concentration. The ZM medium contained 8% (w/v) xylose and was supplemented with 0.8 or 2% (w/v) glucose. In the experiment with added ethanol (open circles), the medium contained 8% xylose, 2% glucose and 1.5% (w/v) ethanol. The temperature was 30°C and the pH was 5.75. The maximum dry cell mass concentrations and values for both ethanol yield and productivity are given in Table 1.

2% glucose should, in theory, yield a final ethanol concentration of about 6.3% (w/v) (4.8% + 1.5%). Figure 4A shows that addition of 1.5% (w/v) ethanol had only a slight inhibitory effect on growth; the final cell-mass concentration fell from 1.79 to 1.73 g DCM/L (Table 1). Likewise, this level

of exogenous ethanol did not appreciably affect glucose utilization (Fig. 4B); however, it did inhibit xylose utilization (Fig. 4B). In the absence of added ethanol, the 8% xylose was completely fermented in 72 h, but in the presence of 1.5% (w/v) exogenous ethanol, about 1.4% xylose remained unconsumed at 72 h (Fig. 4B) when the ethanol concentration was 5.6% (w/v) (Fig. 4C, Table 1). The inhibitory effect of ethanol was already apparent at 24 h when the ethanol concentration was approximately 4% (w/v) (Fig. 4B,C). In the absence of added ethanol, the control fermentation with 8% xylose and 2% glucose does not achieve a level of 4% ethanol until about 40 h elapsed fermentation time (Fig. 4C).

Cofermentation by Recombinant Zm of 6.5% Xylose and 6.5% Glucose

The results of the experiment with 6% xylose and 6% glucose that are shown in Fig. 2 are very similar to the observations reported last year by Rogers et al. *(16)* using this same recombinant and medium; however, whereas our experiments were performed at pH 5.75, it was noted that their experiment was performed with the pH controlled at 5.0 *(16)*. At a sugar-to-ethanol conversion efficiency of 94% (equivalent to an ethanol yield of 0.48 g/g), the expected yield of ethanol from a mixture of 6.5% xylose and 6.5% glucose is 6.24% (w/v) ethanol. This level of ethanol is higher than had been observed in our previous experiments. It was not known to what extent the pH might affect either the rate of xylose utilization or the final ethanol concentration. Figure 5A shows that the final cell-mass concentrations at pH 5.0 and 5.75 are similar. Utilization of glucose is slightly faster at the higher pH (Fig. 5B). The utilization of xylose appears to be affected by the difference in pH only towards the end of the fermentation as the ethanol concentration rises above 5% (w/v) (Fig. 5B). We chose to control the pH at the higher set-point in our experiments with a view to reducing the inhibitory effect of acetic acid that is present in hardwood dilute-acid prehydrolyzate *(9,21)*. However, at more tolerable levels of acetate brought about by some treatment that removes acetic acid from the prehydrolyzate or, alternatively, through the use of an acetate-tolerant mutant *(22)*, operation at the lower pH of 5.0 would be beneficial to the proposed SSCF process to better accommodate the pH optimum of the cellulose saccharifying enzymes *(7)*.

In the context of the effect of ethanol on xylose utilization by the recombinant, it is most interesting to note that the fermentation appeared to stall with a residual of about 1% xylose at pH 5.0 when the ethanol concentration reached 5.5% (w/v) after 32 h, whereas at pH 5.75, all the xylose was consumed and the ethanol concentration was 6.2% (w/v) after 48 h (Fig. 5B). These experiments suggest that, for this rec Zm strain, the upper limit concentration of ethanol, at which xylose utilization is completely inhibited, is pH-dependent. The experiments leading to the previously proposed limit ethanol concentration of 5.5% were performed at pH 5.0 *(16)*.

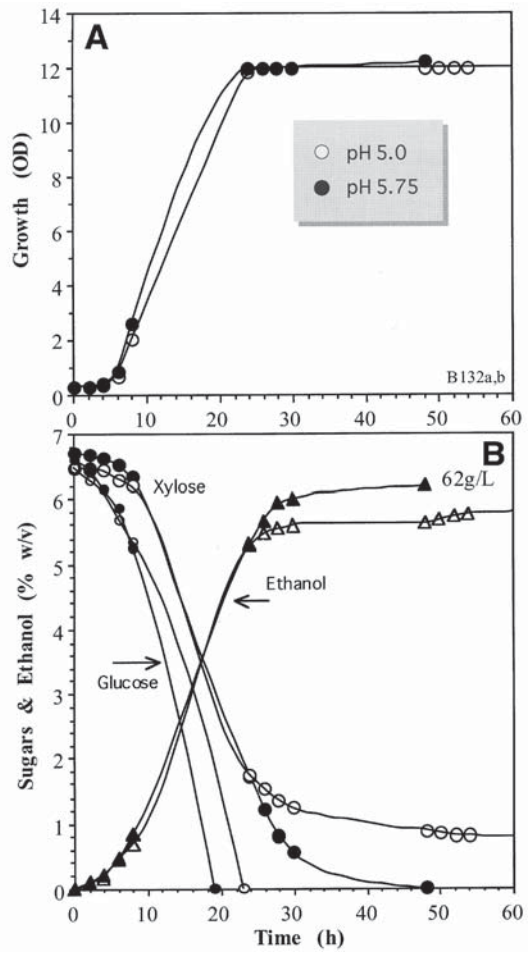

Fig. 5. Cofermentation of 6.5% xylose and 6.5% glucose at pH 5.0 and 5.75. **(A)** Growth, **(B)** glucose and xylose utilization, and **(C)** ethanol production. The ZM medium contained equal concentrations (6.5% w/v) of xylose and glucose. The temperature was 30°C and the pH was controlled at either 5.0 (open circles) or 5.75 (filled circles). The maximum dry cell mass concentrations and values for both ethanol yield and productivity are given in Table 1.

At this meeting, Joachimsthal et al. *(23)* reported that another NREL-generated recombinant Zm strain, namely rec Zm 31821:pZB5 (also known as "ZM4:pZB5"), can completely ferment a mixture of 6.5% xylose and 6.5% glucose in 48 h (at 30°C and pH 5.0) producing "in excess of 60 g/L"; "the yield based on sugars available was in excess of 90% of theoretical" *(23)*. *Z. mobilis* ZM4 (ATCC 31821) is the subject of several patents *(24,25)* and is claimed to be superior to other wild-type strains with respect to several key process techno-ethanologenic traits *(17)*. The volumetric ethanol productivity of 1.25 g/L/h exhibited by ZM4:pZB5 is similar to that observed in our present study under similar experimental conditions and sugar load-

ing (Table 1). Although it may well be that, because of its pH optimum and ethanol tolerance, *Z. mobilis* ZM4 proves to be a more robust host for pentose metabolic engineering, the present study points to the importance of making performance comparisons under identical conditions, because at pH 5.75 rec CP4:pZB5 appears to perform as well as rec ZM4:pZB5 at pH 5.0 *(23)*.

Finally, it is recognized that recombinant *Zymomonas* is not the only candidate biocatalyst for the production of biomass-derived fuel ethanol (for review, *see* ref. *26*). Other promising metabolically engineered ethanologenic biocatalysts include *E. coli* strains KO11 *(27)* and SL40 *(28)*, *Klebsiella oxytoca* M5A1 *(29)*, and the xylose-fermenting yeast recombinant *Saccharomyces cerevisiae* 1400(pLNH33) *(30)*. In a review of recent data concerning the xylose fermentation and cofermentation performance characteristics of these different recombinants, Rogers et al. *(16)* point out that:

> "all have comparable ethanol tolerances at this stage of development (viz., 55–60 g/L) as well as similar yields and productivities" (p. 306).

A proper comparison of productivities is made difficult because of the influence of inoculum size; however, techno-economic sensitivity analyses have shown ethanol yield to be the prime factor in the cost of cellulosic ethanol production *(31,32)*. From this perspective, it is perhaps noteworthy that the most recent published information concerning cofermentation of xylose and glucose by recombinant *S. cerevisae* 1400(pLNH33) indicates that the conversion efficiency is only 80% of theoretical *(33)*. Therefore, at least with respect to yield, it appears that recombinant *Zymomonas* is superior. Nevertheless, we agree with Rogers et al. *(16)* in their statement that:

> "other factors (that are) likely to influence the final selection of the optimal strain include strain stability, resistance to inhibitors, susceptibility to contamination and safety/regulatory issues related to large-scale fermentations with recombinant microorganisms" (p. 306).

Acknowledgments

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