0273-2289/01/91–93/0117/$13.75

Fermentation Performance Assessment of a Genomically Integrated Xylose-Utilizing Recombinant of *Zymomonas mobilis* 39676

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Abstract

In pH-controlled batch fermentations with pure sugar synthetic hardwood hemicellulose (1% [w/v] glucose and 4% xylose) and corn stover hydrolysate (8% glucose and 3.5% xylose) lacking acetic acid, the xylose-utilizing, tetracycline (Tc)-sensitive, genomically integrated variant of *Zymomonas mobilis* ATCC 39676 (designated strain C25) exhibited growth and fermentation performance that was inferior to National Renewable Energy Laboratory's first-generation, Tc-resistant, plasmid-bearing *Zymomonas* recombinants. With C25, xylose fermentation following glucose exhaustion was markedly slower, and the ethanol yield (based on sugars consumed) was lower, owing primarily to an increase in lactic acid formation. There was an apparent increased sensitivity to acetic acid inhibition with C25 compared with recombinants 39676:pZB4L, CP4:pZB5, and ZM4:pZB5. However, strain C25 performed well in continuous fermentation with nutrient-rich synthetic corn stover medium over the dilution range 0.03–0.06/h, with a maximum process ethanol yield at D = 0.03/h of 0.46 g/g and a maximum ethanol productivity of 3 g/(L·h). With 0.35% (w/v) acetic acid in the medium, the process yield at D = 0.04/h dropped to 0.32 g/g, and the maximum productivity decreased by 50% to 1.5 g/(L·h). Under the same operating conditions, rec Zm ZM4:pZB5 performed better; however, the medium contained 20 mg/L of Tc to constantly maintain selective pressure. The absence of any need for antibiotics and antibiotic resistance genes makes the chromosomal integrant C25 more compatible with current regulatory specifications for biocatalysts in large-scale commercial operations.

Index Entries: Recombinant *Zymomonas* C25; genomic integrant; xylose; ethanol; biomass hydrolysate; acetate inhibition.

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Introduction

For the past two decades our laboratory has been involved in research and development associated with the production of fuel ethanol—more specifically batch and continuous ethanol fermentations using both wild-type and genetically engineered bacteria and a variety of feedstocks *(1–6)*. Recently, we have been investigating the physiologic characteristics of xylose-utilizing recombinants of *Zymomonas mobilis* that were created at National Renewable Energy Laboratory (NREL) *(7,8)* with a view to their efficacy in the production of cellulosic ethanol *(9–16)*. To be economic, the production of ethanol from cellulosic biomass and wastes must involve the rapid and efficient conversion of both hexose and pentose sugars *(17)*. To exploit the superior fermentation characteristics of *Z. mobilis* for the production of cellulosic ethanol, this bacterium has been genetically engineered to broaden its substrate utilization profile to include pentose sugars *(7,18,19)*. In NREL's first-generation strains, the ability to ferment xylose and arabinose was accomplished using a native Zm plasmid vector with inserted *Escherichia coli* genes coding for both pentose assimilation and pentose phosphate pathways together with an antibiotic resistance gene to facilitate selection *(7,20,21)*. In the more recent second-generation constructs *(22)*, genetic stability has been enhanced in the absence of antibiotic selection through the genomic integration of the *E. coli* xylose-fermenting genes. One of the integrants that was derived from *Z. mobilis* ATCC 39676 lacked the tetracycline (Tc) resistance gene and was designated as strain C25 by NREL *(20)*.

The purpose of the present study was to assess the fermentation performance characteristics of a selected xylose-fermenting integrant, strain C25, in both pH-controlled batch and continuous fermentations using pure sugar synthetic biomass hydrolysate media under conditions similar to those previously employed to study plasmid-bearing *Zymomonas* recombinants *(9–16)*.

Materials and Methods

Organisms

The xylose-utilizing, plasmid-bearing, Tc-resistant, recombinant *Z. mobilis* strains 39676:pZB4L, CP4:pZB5, ZM4:pZB5 *(7,8)*, and the Tc-sensitive genomically integrated strain C25 (derived from Zm ATCC 39676) *(19)* were obtained from M. Zhang (NREL, Golden, CO). Stock cultures were stored in glycerol at –70°C and precultures were prepared as described previously *(9)*.

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 "modified" RM medium (5 g/L of yeast extract and 2 g/L of KH_2PO_4) containing about 20 g/L of xylose and 20 g/L of glucose in 125-mL screw-cap flasks

and grown statically overnight at 30°C in an incubator. The medium was supplemented with 10 mg/L Tc when using Tc-resistant strains. This preseed was subcultured into inoculation flasks containing modified RM with 20 g/L of glucose, 20 g/L of xylose, and 10 mg/L Tc when appropriate 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 batch fermentors. 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)/L.

Fermentation Medium

The fermentation medium (designated as ZM) *(15)* was prepared with glass-distilled water and contained the following ingredients: 5 g/L of Difco yeast extract (Difco, Detroit, MI), 3.48 g/L of KH_2PO_4, 0.25 g/L of $MgSO_4$, 0.01 g/L of $FeSO_4 \cdot 7H_2O$, 0.21 g/L of citric acid, and 20 mg/L of Tc (for Tc-resistant cultures). The amount of glucose, xylose, and acetic acid added to the medium was variable. The medium and stock sugar solutions were autoclaved separately.

Fermentation Equipment

pH-stat batch fermentations were conducted with about 1500 mL of medium in 2-L bioreactors (model F2000 MultiGen; New Brunswick Scientific, Edison, NJ) fitted with agitation (150 rpm), pH, and temperature control (30°C). Continuous fermentations were conducted with either NBS C30 BioFlo chemostats or 2-L NBS Bioflo 2000 bioreactors. 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 of effluent taken on successive days gave similar values for cell mass, and sugar and ethanol 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. In some batch fermentations the pH set point was adjusted after 24 h from 5.75 to 6.5. The temperature was controlled at 30°C using a circulating water bath and the agitation was moderate (approx 100–150 rpm). The continuous fermentations were started in the batch mode using ZM medium with concentrations of glucose and xylose as determined by the condition specified for individual experiments. Flow was started 24 h after inoculation (preferably when the residual xylose concentration was <10 g/L) *(16)*.

Analytical Procedures, Growth, and Fermentation Parameters

Growth was measured turbidometrically at 600 nm (1-cm light path). 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

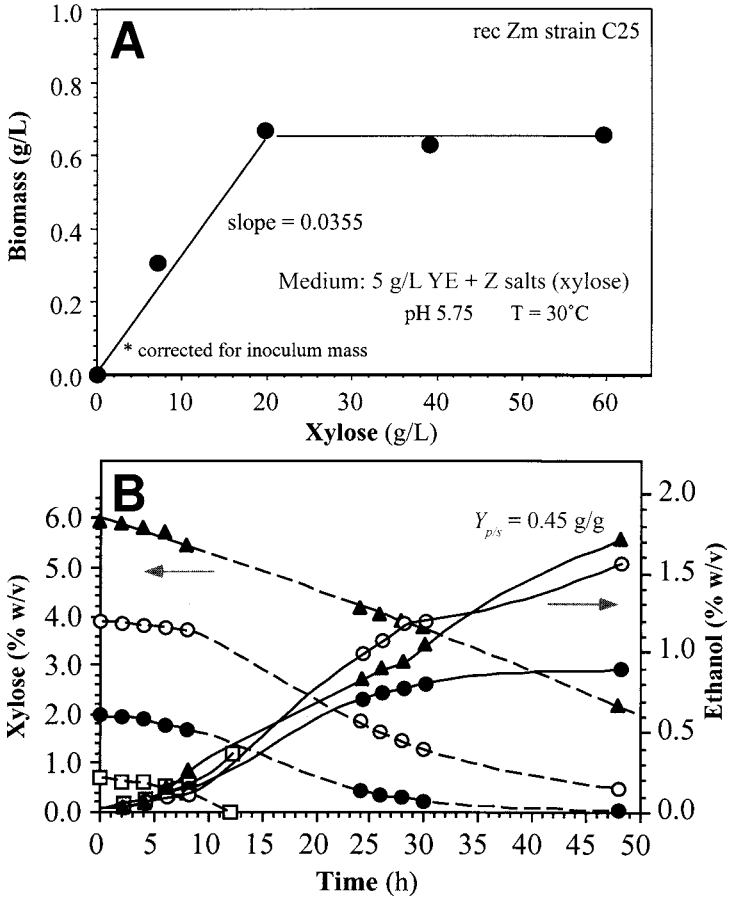

Fig. 1. Growth and fermentation of xylose as the sole sugar by rec Zm strain C25. **(A)** Cell mass as a function of xylose in the medium; **(B)** time course of xylose utilization and ethanol production: (□) 0.8% (w/v) xylose, (●) 2% xylose, (○) 4% xylose, (▲) 6% xylose.

high-performance liquid chromatography as described previously *(9)*. The ethanol yield ($Y_{p/s}$) was calculated as the mass of ethanol produced per mass of sugar consumed; the "process" yield (proc $Y_{p/s}$) was calculated as the mass of ethanol produced per mass of fermentable sugar in the medium (i.e., glucose + xylose).

Results and Discussion

Batch Fermentations

Experiments with Strain C25 and Xylose as Sole Fermentable Sugar

The growth characteristics of strain C25 with xylose as the sole fermentable sugar (Fig. 1) were of interest to us because previously we reported on the growth yield for the plasmid-bearing rec Zm CP4:pZB5 and inferred

that the Y_{ATP} was significantly less for xylose than glucose *(15)*. With recombinant CP4:pZB5, at relatively low xylose concentrations (<20 g/L), the final cell mass was proportional to the amount of sugar in the medium *(15)*. Despite the paucity of data, Fig. 1A shows that this also holds for C25. In previous studies with plasmid-bearing recombinants, the maximum cell mass with xylose alone was about 0.75 g of DCM/L *(15)*, and this is also the case with strain C25 (Fig. 1A). The reason for this plateauing of biomass is not yet fully resolved. However, in the context of ethanol production, perhaps the most notable feature of this experiment with strain C25 is that the ethanol yield was only 0.45 g/g (based on sugar used) and the process yield at 48 h was lower with 6% xylose because the fermentation is incomplete (Fig. 1B). Previous work with NREL's xylose-utilizing Zm recombinants by us *(9,11)* and others *(7,23–29)* had shown that these cultures characteristically exhibit a high ethanol yield based on sugar consumed with perhaps xylitol being a byproduct of concern *(24)* because of its putative inhibition of xylulose kinase *(15)*. The reason for the decrease in ethanol yield with C25 relative to the nonintegrated recombinants is primarily that there is increased production of lactic acid (results not shown). Figure 2 illustrates the effect of pH on xylose utilization by strain C25. When the pH set point on the controller was adjusted from 5.75 to 6.5 at 24 h, the final cell density increased from 0.74 to 0.92 g of DCM/L (Fig. 2A), and the process yield for ethanol at 48 h increased from 0.38 to 0.45 g/g (Fig. 2B).

Effect of Glucose on Xylose Utilization

By far the majority of our previous work with rec Zm has been done with synthetic hardwood hemicellulose hydrolysate in which the concentration of xylose was 4% (w/v) and glucose was 0.8–1% (w/v). Figure 2 also shows the growth and fermentation performance of C25 in this pure sugar mixture, which was formulated to mimic the NREL hardwood prehydrolysate. The addition of glucose results in an increase in cell density (Fig. 2A), which probably accounts for the increase in xylose utilization (Fig. 2B). Note also that C25 performs well with equal concentrations of xylose and glucose, although above 3.5% (w/v) of each sugar, xylose utilization is not complete at 48 h (results not shown). It is important to understand that these fermentations were conducted in the absence of acetic acid in the medium and, therefore, are not representative of true fermentation performance in unconditioned biomass hydrolysate medium.

Comparative Effect of Acetic Acid on C25 and Other Xylose-Utilizing rec Zm Strains

Acetic acid is a component of biomass hydrolysates and its inhibitory effects on ethanologenic microorganisms are well documented *(30)*. There are several reports on the effect of acetic acid on rec Zm *(11,14,24)*. In the present study, the pure sugar synthetic hardwood prehydrolysate medium containing 4% xylose, 1% glucose, and varying amounts of acetic acid (HAc) over the range 0–1% (w/v) was used to assess the growth and fermentation performance of different Zm recombinants. We were interested to compare

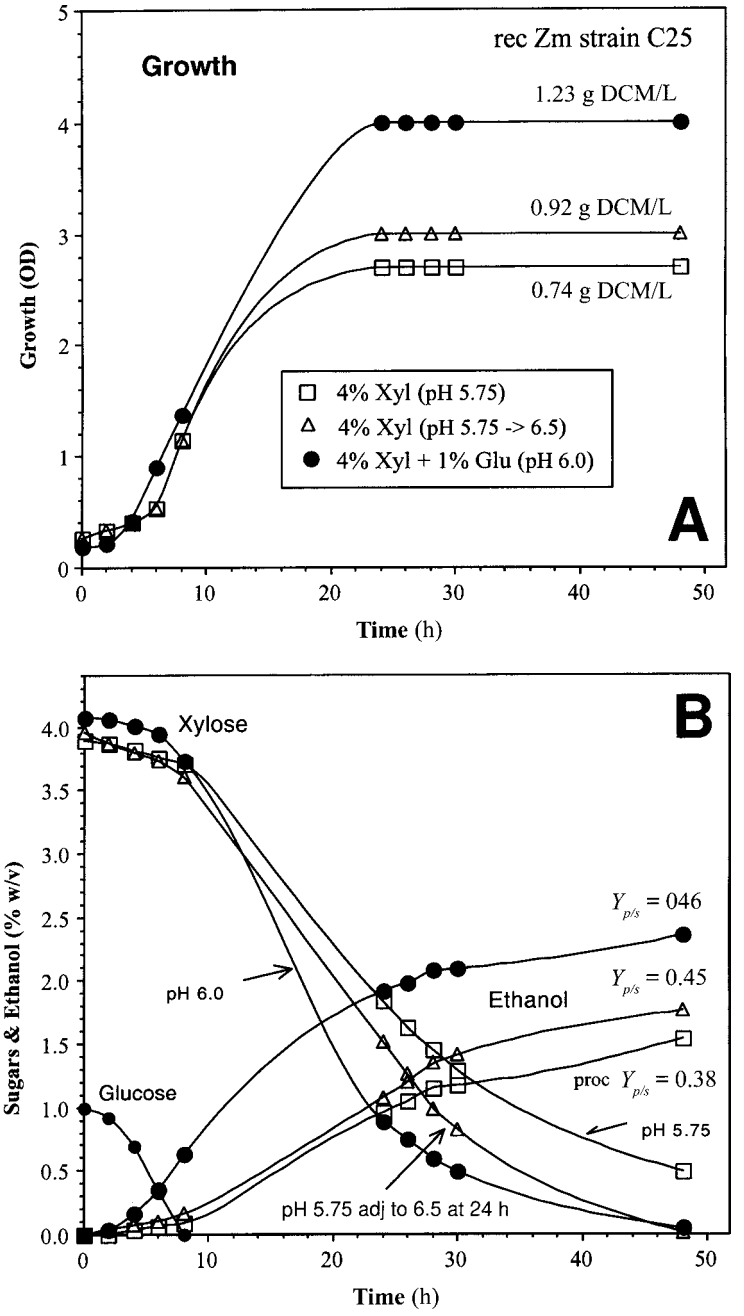

Fig. 2. Effect of pH and glucose on xylose fermentation by rec Zm C25. **(A)** Growth; **(B)** sugar utilization and ethanol production.

the effect of acetic acid on strain C25 and a closely related strain, 39676: pZB4L, and a nonrelated strain, CP4:pZB5. Under these assay conditions, we observed that C25 behaved quite similarly in terms of growth (as final

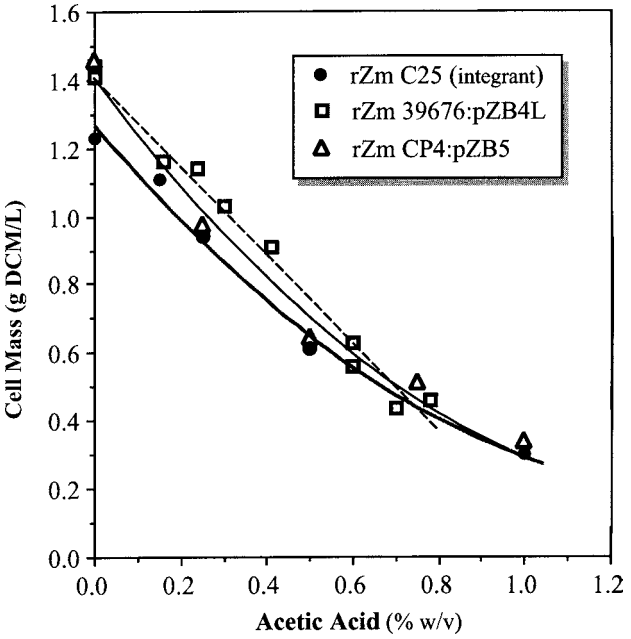

Fig. 3. Comparative relationship between final cell mass concentration and level of acetic acid in the medium for three xylose-utilizing *Zymomonas* recombinants.

cell density) to the plasmid-bearing recombinants 39676:pZB4L and CP4:pZB5 (Fig. 3). In terms of fermentation performance, Fig. 4 shows that strain C25 is more sensitive to inhibition of xylose utilization by acetic acid than the closely related strain 39676:pZB4L. In terms of xylose utilization, recombinant CP4:pZB5 is the most HAc tolerant strain that was tested in this series of experiments with the synthetic hardwood prehydrolysate medium (Fig. 5).

Batch Fermentations with Synthetic Corn Stover Hydrolysate

Various agricultural wastes are being considered for the production of cellulosic ethanol, and among these corn stover is a strong contender. Our work with corn stover fermentations was initiated when Iogen selected this as one of the feedstocks to be processed in its demonstration facility *(31)*. In a separate but closely related study on oat hull hydrolysate, we concluded that rec Zm ZM4:pZB5 was the best of the xylose-utilizing NREL Zm recombinants tested *(32)*. The concentration of glucose and xylose in the Iogen corn stover hydrolysate is 8% glucose and 3.5% xylose and the acetic acid is about 1% (w/v). Proprietary technologies will be employed by Iogen to reduce the acetate content to 0.3% or less (w/v) (Tolan, J., personal communication). The objective of the present study was to compare the effect of acetic acid on the growth and fermentation performance of ZM4:pZB5 and the integrant C25 using pure sugar synthetic corn stover hydrolysate (8% glu + 3.5% xyl) in which the pH was controlled within the range 5.75–6.5. Figure 6 shows the relationship between final cell mass and

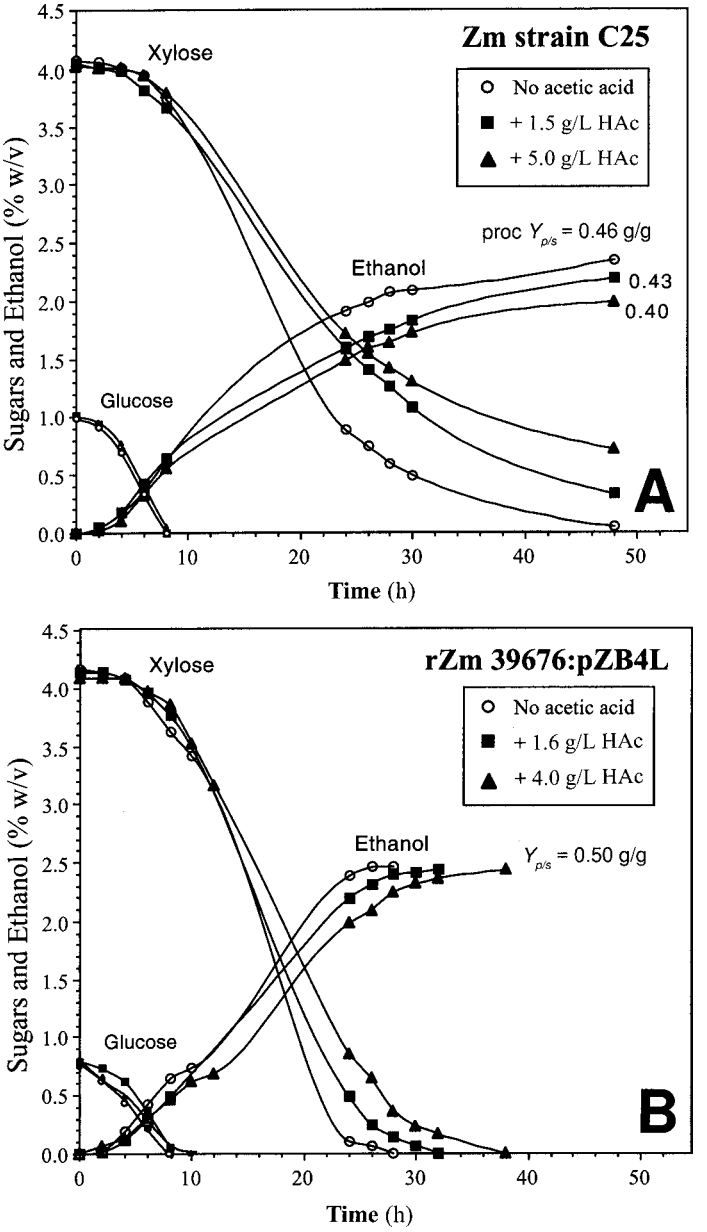

Fig. 4. Sugar utilization and ethanol production using a pure sugar synthetic hardwood prehydrolysate medium. **(A)** Strain C25; **(B)** rec Zm 39676:pZB4L.

the level of acetic acid for ZM4:pZB5 and C25. Over the range of acetate tested, the growth yield for C25 was less than for ZM4:pZB5 (Fig. 6).

Figure 7 shows the effect of acetic acid on the cofermentation performance of these two recombinants. Even in the absence of acetic acid, strain C25 was unable to complete the fermentation within 48 h (Fig. 7A). The rate

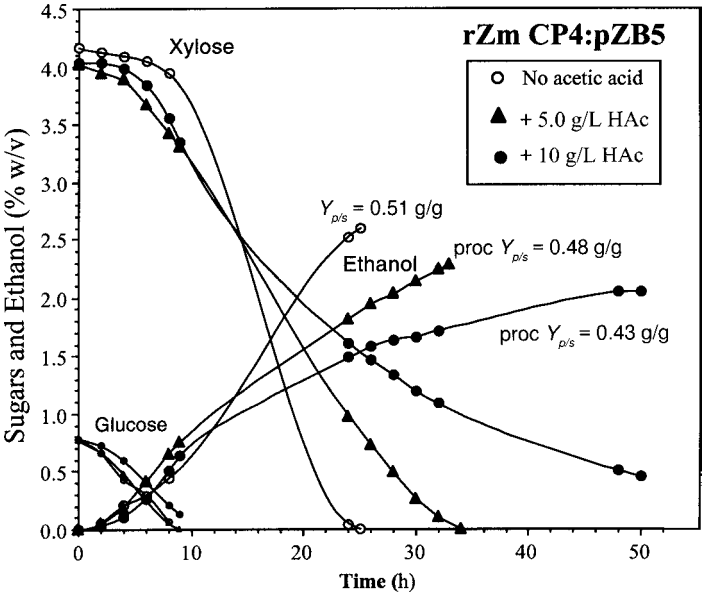

Fig. 5. Sugar utilization and ethanol production by rec Zm CP4:pZB5 using a pure sugar synthetic hardwood prehydrolysate medium. Compare with experiments shown in Fig. 4. Note that higher levels of acetic acid were used in these fermentations with CP4:pZB5.

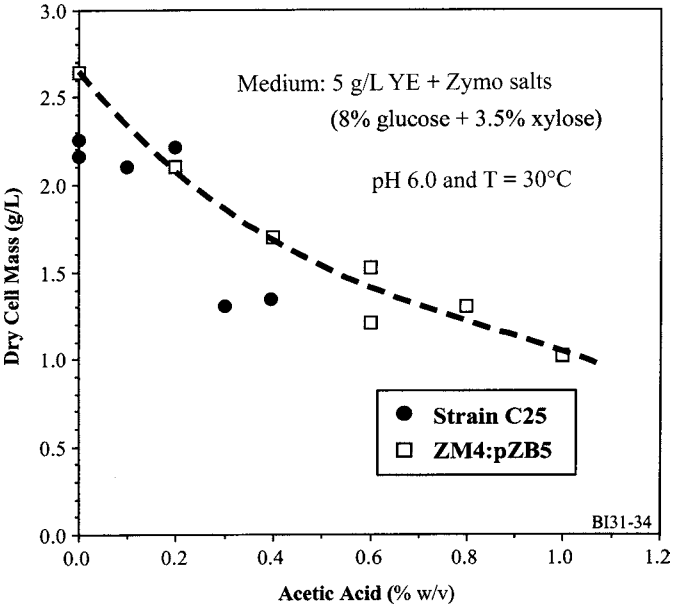

Fig. 6. Comparative relationship between final cell mass concentration and level of acetic acid in a synthetic corn stover hydrolysate medium for *Zymomonas* recombinants ZM4:pZB5 and C25.

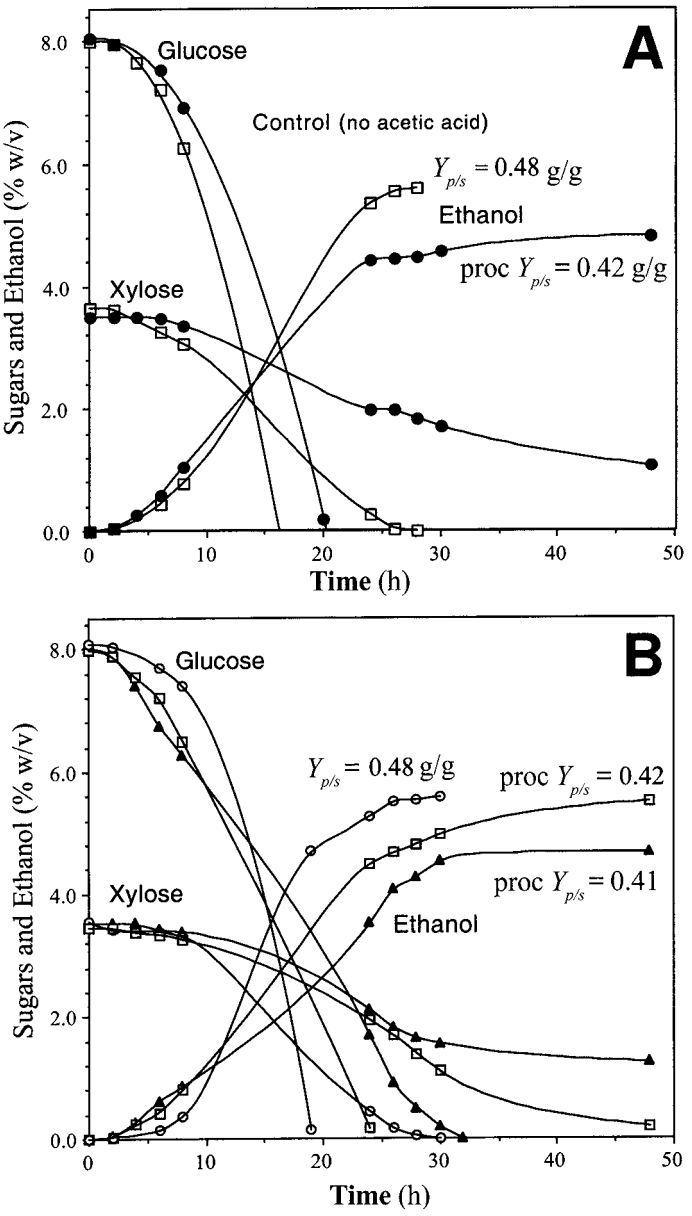

Fig. 7. Time course of sugar utilization and ethanol production using a synthetic corn stover hydrolysate medium and Zm recombinants ZM4:pZB5 and C25. **(A)** Without added acetic acid: (●) strain C25 (no HAc), (□) ZM4:pZB5 (no HAc); **(B)** with added acetic acid (▲) strain C25 (0.4% HAc), (○) ZM4:pZB5 (0.4% HAc), (□) ZM4:pZB5 (0.8% HAc).

of xylose utilization with strain C25 was markedly slower following glucose exhaustion (Fig. 7A). However, ZM4:pZB5 completed the batch fermentation in 24 h with an excellent product yield of 0.48 g/g (Fig. 7A).

At a level of 4 g/L of HAc, it is the rate of glucose utilization that is affected with both strains, whereas the rate of xylose utilization for both strains is relatively unaffected (Fig. 7). With ZM4:pZB5, the fermentation is essentially complete in 48 h even with 8 g/L of acetic acid in the medium (Fig. 7B). For C25 to achieve complete fermentation within 48 h, we recommend that the corn stover hydrolysate be diluted to 85% strength (i.e., 68 g/L of glucose and 30 g/L of xylose). These results point to the superiority of recombinant ZM4:pZB5 for batch fermentations with media that contain acetic acid.

Continuous Fermentations with rec Zm ZM4:pZB5 and C25: Pure Sugar Synthetic Corn Stover Hydrolysate (8% glu + 3.5% xyl)

Since Iogen is contemplating a continuous fermentation process using corn stover hydrolysate *(33)*, it was important to assess the performance of the chromosomal integrated recombinant in the continuous fermentation mode. Because strain C25 lacks the Tc resistance gene, it is more compatible with current regulatory requirements. Figure 8 shows the continuous fermentation of a pure sugar synthetic corn stover hydrolysate using strain C25. The medium was a standard 5 g/L of yeast extract with Zymo salts (ZM medium) *(15)*, the pH was controlled at 6.0, and the temperature was 30°C. Over the 21 d the chemostat was operated, the dilution rate was increased in increments of 0.01 from 0.03 to 0.06/h (Fig. 8). The maximum ethanol productivity was 3 g/(L·h). Figure 8B shows the steady-state levels of both ethanol and xylose; glucose was not detected in the effluent over this time period. At $D = 0.06$/h, approx 25% of the xylose was unfermented, the process yield was 0.434 g/g (85% efficiency), and the experiment was terminated. The effect of starting at a higher D value is not known, but since 0.06/h is probably close to washout with respect to xylose utilization, it would seem prudent to start at a lower D value and progress toward the upper flow rate of 0.06/h.

Figure 9 demonstrates the effect of adding 3.5 g/L of acetic acid to the nutrient-rich synthetic corn stover medium. After operating for about a week at $D = 0.03$/h, the residual xylose was about 3 g/L; however, when the dilution rate was increased to 0.04/h, the level of unfermented xylose increased to 25 g/L (Fig. 9A). The process yield at $D = 0.04$/h was 0.32 g/g and the productivity was 1.5 g/(L·h). Curiously, the level of xylose did not decrease after the dilution rate was lowered to 0.016/h (Fig. 9A); this suggested that the culture was somehow diminished in its capacity to utilize xylose. Steady-state values for effluent ethanol and xylose are shown in Fig. 9B. Glucose was not detected in the effluent. For comparison, Fig. 9B also shows data from a chemostat experiment with rec Zm ZM4:pZB5 under identical operating conditions. Note that with this Tc-resistant recombinant, Tc (20 mg/L) was added to the medium for maintenance of selective pressure. Although previously we have operated chemostats with other plasmid-bearing xylose-utilizing recombinants without Tc, the medium contained a higher proportion of xylose relative to glucose, and under this

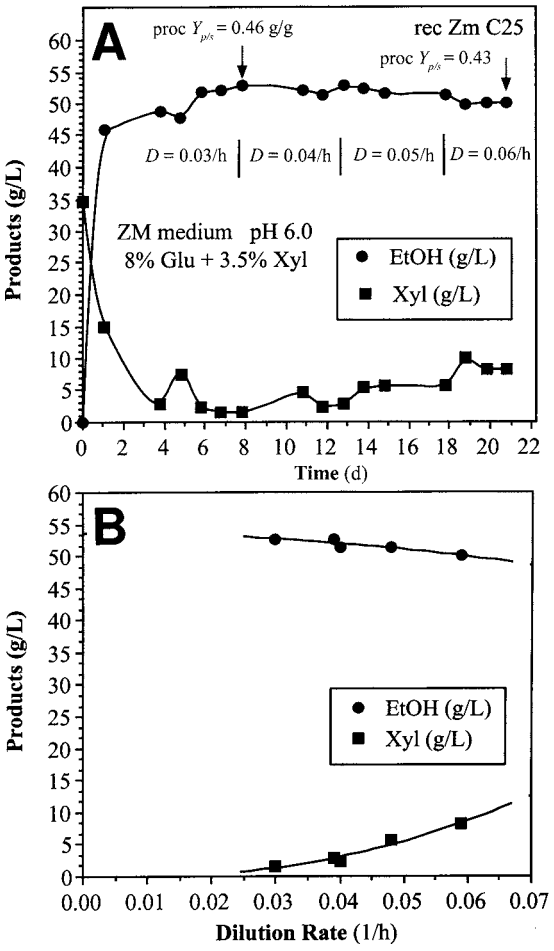

Fig. 8. Continuous fermentation of synthetic corn stover hydrolysate using rec Zm strain C25. **(A)** Time course of ethanol and residual xylose in effluent stream; **(B)** steady-state concentrations of ethanol and xylose as a function of dilution rate.

condition xylose would appear to provide the necessary selective pressure for sustained biocatalyst stability *(14,16)*. Some preliminary chemostat experiments with ZM4:pZB5 in the absence of Tc suggest that perhaps the high glucose-to-xylose ratio in this medium mitigates against the selective effect of xylose. The extent to which Tc is an absolute requirement of stability of ZM4:pZB5 with respect to xylose utilization has not yet been extensively investigated in our laboratory. The absence of any need for antibiotics and antibiotic resistance genes makes the genomic integrant C25 more compatible with current regulatory specifications for biocatalysts in large-scale commercial operations.

These results suggest that C25 could be used in a continuous fermentation process provided that the acetic acid concentration is not >3.5 g/L, the dilution rate is not >0.03/h, and the pH is controlled at about 6.0.

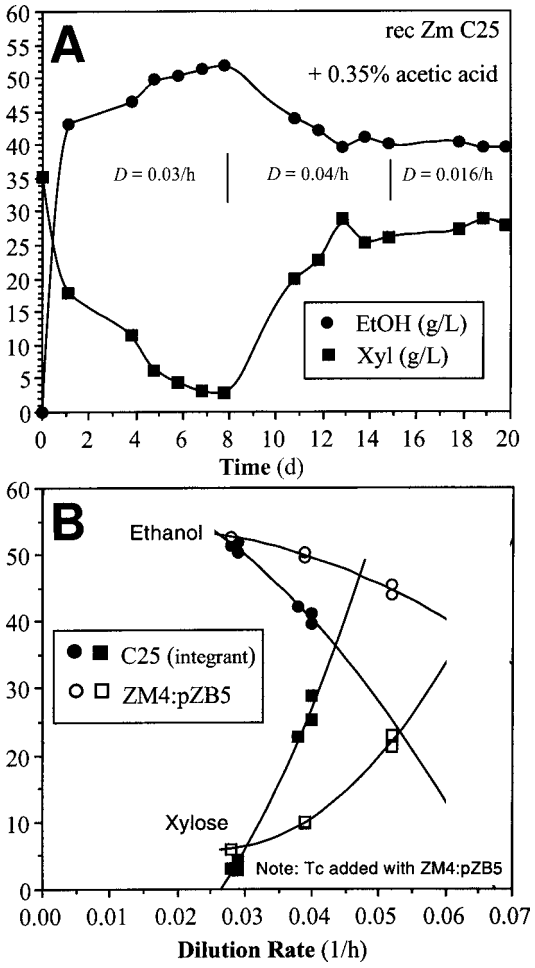

Fig. 9. Continuous fermentation of synthetic corn stover hydrolysate containing 0.35% acetic acid using rec Zm strain C25. **(A)** Time course of ethanol and residual xylose in effluent stream; **(B)** steady-state concentrations of ethanol and xylose as a function of dilution rate. Note that data from a similar experiment with rec Zm ZM4:pZB5 are added to (B) for comparison.

Unfortunately, these conditions are not compatible with current process design parameters being contemplated by Iogen in its new demonstration plant *(33)*.

Conclusion

In making a general conclusion for these collective observations with strain C25, it is important to emphasize that this study focused attention on only one of the many genomic type integrants that have been created *(34)*. The newest generation of integrants contains genes for arabinose as well as xylose fermentation *(35)*, although the fermentation performance of these

recombinants has not yet been thoroughly tested. A promising alternative approach to random insertion that has been explored by NREL for integrating the pentose assimilation and metabolism genes into the Zm chromosome involves site-specific gene targeting for insertion *(22)*. Finally, in future genetic developments, it would seem prudent, in light of previous experience, to exploit the superior qualities of Zm ZM4 as a host for chromosomal integration experimentation.

Acknowledgments

We are grateful to Dr. Min Zhang (NREL) for providing the recombinant *Z. mobilis* strains. This work was funded jointly by Iogen (Ottawa, Canada) and the Biochemical Conversion Element of the Office of Fuels Development of the U.S. Department of Energy (NREL subcontract ZDH-9-29009-02).

References

1. Lawford, G. R., Lavers, B. H., Good, D., Charley, R. C., Fein, J. E. and Lawford, H. G. (1982), in *Proceedings of the International Symposium on Ethanol from Biomass*, Duckworth, H., ed., Royal Society of Canada, Ottawa, Canada, pp. 482–507.
2. Lawford, H. G. (1987), US Patent 4,647,534.
3. Lawford, H. G. (1988), in *VIII International Symposium on Alcohol Fuels*, New Energy and Industrial Technology Development Organization, Tokyo, Japan, pp. 21–27.
4. Lawford, H. G. (1988), in *Canadian Power Alcohol Conference*, Candlish, B., ed., Biomass Energy Institute, Winnipeg, Manitoba, Canada, pp. 245–251.
5. Lacis, L. S. and Lawford, H. G. (1989), in *Bioenergy—Proceedings of the 7th Canadian Bioenergy R&D Seminar*, Hogen, E., ed., NRC Canada, Ottawa, pp. 411–416.
6. Lawford, H. G. and Rousseau, J. D. (1991), in *Energy from Biomass and Wastes XV*, Klass, D. L., ed., Institute Gas Technology, Chicago, pp. 583–622.
7. Zhang, M., Eddy, C., Deanda, K., Finkelstein, M., and Picataggio, S. K. (1995), *Science* **267,** 240–243.
8. Picataggio, S., Zhang, M., Eddy, C. K., Deanda, K., and Finkelstein, M. (1996), US Patent 5,514,583.
9. Lawford, H. G., Rousseau, J. D., and McMillan, J. D. (1997), *Appl. Biochem. Biotechnol.* **63–65,** 269–286.
10. Lawford, H. G. and Rousseau, J. D. (1997), *Appl. Biochem. Biotechnol.* **63–65,** 287–304.
11. Lawford, H. G. and Rousseau, J. D. (1998), *Appl. Biochem. Biotechnol.* **70–72,** 161–172.
12. Lawford, H. G., Rousseau, J. D., Mohagheghi, A., and McMillan, J. D. (1998), *Appl. Biochem. Biotechnol.* **70–72,** 353–368.
13. Lawford, H. G. and Rousseau, J. D. (1999), *Appl. Biochem. Biotechnol.* **77–79,** 235–249.
14. Lawford, H. G., Rousseau, J. D., Mohagheghi, A., and McMillan, J. D. (1999), *Appl. Biochem. Biotechnol.* **77–79,** 191–204.
15. Lawford, H. G. and Rousseau, J. D. (2000), *Appl. Biochem. Biotechnol.* **84–86,** 277–294.
16. Lawford, H. G., Rousseau, J. D., Mohagheghi, A., and McMillan, J. D. (2000), *Appl. Biochem. Biotechnol.* **84–86,** 295–310.
17. Hinman, N. D., Wright, J. D., Hoagland, W., and Wyman, C. E. (1989), *Appl. Biochem. Biotechnol.* **20/21,** 391–401.
18. Sprenger, G. A. (1993), *J. Bacteriol.* **27,** 225–237.
19. Feldman, S. D., Sahm, H., and Sprenger, G. A. (1992), *Appl. Microbiol.* **38,** 354–361.
20. Picataggio, S. K., Zhang, M., Eddy, C. K., Deanda, K., and Finkelstein, M. (1998), US Patent 5,726,053.
21. Deanda, K. A., Eddy, C., Zhang, M., and Picataggio, S. (1996), *Appl. Environ. Microbiol.* **62,** 4465–4470.

22. Zhang, M., Chou, Y. C., Lai, X. K., Milstrey, S., Danielson, N., Evans, K., Mohagheghi, A., and Finkelstein, M. (1999), 21st Symposium on Biotechnology for Fuels and Chemicals, Fort Collins, CO (abstract no. 2-16).
23. Rogers, P. L., Joachimsthal, E. L., and Haggett, K. D. (1997), *J. Australasian Biotechnol.* **7,** 304–309.
24. Joachimsthal, E., Haggett, K. D., and Rogers, P. L. (1999), *Appl. Biochem. Biotechnol.* **77–79,** 147–157.
25. Krishnan, M. S., Blanco, M., Shattuck, C. K., Nghiem, N. P., and Davison, B. H. (2000), *Appl. Biochem. Biotechnol.* **84–86,** 525–542.
26. Joachimsthal, E. L. and Rogers, P. L. (2000), *Appl. Biochem. Biotechnol.* **84–86,** 343–356.
27. Dennison, E. and Abbas, C. (2000), 22nd Symposium on Biotechnology for Fuels and Chemicals, Gatlinburg, TN (abstract no. 2-04), Humana, Totowa, NJ.
28. Ngheim, N. P., Krishnan, M. S., Davison, B. H., Jackson, A. N., and Cofer, T. M. (2000), 22nd Symposium on Biotechnology for Fuels and Chemicals, Gatlinburg, TN (abstract no. 3-25), Humana, Totowa, NJ.
29. Dowe, N., Newman, M. M., Mohagheghi, A., and McMillan, J. D. (2000), 22nd Symposium on Biotechnology for Fuels and Chemicals, Gatlinburg, TN (abstract no. 6-20), Humana, Totowa, NJ.
30. McMillan, J. D. (1994), in *Enzymatic Conversion of Biomass for Fuels Production*, Himmel, M. E., Baker, J. O., and Overend, R. A., eds., ACS Symposium Series 566, American Chemical Society, Washington, DC, pp. 411–437.
31. Foody, B. F. (2000), 21st Symposium on Biotechnology for Fuels and Chemicals, Fort Collins, CO (abstract no. 6-01), Humana, Totowa, NJ.
32. Lawford, H. G., Rousseau, J. D., and Tolan, J. S. (2000), 22nd Symposium on Biotechnology for Fuels and Chemicals, Gatlinburg, TN, Humana, Totowa, NJ.
33. Foody, B. F. and Tolan, J. S. (2000), 22nd Symposium on Biotechnology for Fuels and Chemicals, Gatlinburg, TN (abstract no. 6-07), Humana, Totowa, NJ.
34. Zhang, M., Chou, Y. C., Mohagheghi, A., Evans, K., Milstrey, S., Lai, X. K., and Finkelstein, M. (2000), 22nd Symposium on Biotechnology for Fuels and Chemicals, Gatlinburg, TN (abstract no. 2-03), Humana, Totowa, NJ.
35. Zhang, M., Chou, Y.-C., Picataggio, S. K., and Finkelstein, M. (1998), US Patent 5,843,760.