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Improving Fermentation Performance of Recombinant *Zymomonas* in Acetic Acid-Containing Media

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ABSTRACT

In the production of ethanol from lignocellulosic biomass, the hydrolysis of the acetylated pentosans in hemicellulose during pretreatment produces acetic acid in the prehydrolysate. The National Renewable Energy Laboratory (NREL) is currently investigating a simultaneous saccharification and cofermentation (SSCF) process that uses a proprietary metabolically engineered strain of *Zymomonas mobilis* that can coferment glucose and xylose. Acetic acid toxicity represents a major limitation to bioconversion, and cost-effective means of reducing the inhibitory effects of acetic acid represent an opportunity for significant increased productivity and reduced cost of producing fermentation fuel ethanol from biomass. In this study, the fermentation performance of recombinant Z. *mobilis* 39676:pZB4L, using a synthetic hardwood prehydrolysate containing 1% (w/v) yeast extract, 0.2% KH_2PO_4, 4% (w/v) xylose, and 0.8% (w/v) glucose, with varying amounts of acetic acid was examine. To minimize the concentration of the inhibitory undissociated form of acetic acid, the pH was controlled at 6.0. The final cell mass concentration decreased linearly with increasing level of acetic acid over the range 0–0.75% (w/v), with a 50% reduction at about 0.5% (w/v) acetic acid. The conversion efficiency was relatively unaffected, decreasing from 98 to 92%. In the absence of acetic acid, batch fermentations were complete at 24 h. In a batch fermentation with 0.75% (w/v) acetic acid, about two-thirds of the xylose was not metabolized after 48 h. In batch fermentations with 0.75% (w/v) acetic acid, increasing the initial glucose concentration did not have an enhancing effect on the rate of xylose fermentation. However, nearly complete xylose fermentation was achieved in 48 h when the bioreactor was fed glucose. In the fed-batch system, the rate of glucose feeding (0.5 g/h) was designed to simulate the rate of cellulolytic diges-

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tion that had been observed in a modeled SSCF process with recombinant Zymomonas. In the absence of acetic acid, this rate of glucose feeding did not inhibit xylose utilization. It is concluded that the inhibitory effect of acetic acid on xylose utilization in the SSCF biomass-to-ethanol process will be partially ameliorated because of the simultaneous saccharification of the cellulose.

Index Entries: Recombinant *Zymomonas*; acetic acid; xylose; ethanol; SSCF; cofermentation; synthetic biomass prehydrolyzate; glucose fed-batch.

INTRODUCTION

In a survey of industrial biocatalysts, designed to identify promising host strains for genetic transformation directed to rapid and efficient ethanologenic pentose metabolism, the Gram-negative bacterium *Zymomonas mobilis* met the selection criteria, which were based on several fermentation performance characteristics considered to be essential, as well as a number of secondary traits considered to be desirable, for a commercial biomass-to-ethanol process *(1,2)*.

Scientists at the National Renewable Energy Laboratory (NREL) (Golden, CO) have constructed a series of transformation vectors, consisting of a marker gene for tetracycline resistance and four xylose metabolism genes (xylose isomerase, xylulokinase, transaldolase, and transketolase) cloned from *Escherichia coli (3)*, and the proprietary xylose-fermenting *Z. mobilis* recombinants *(4)* are among several biocatalysts for the production of ethanol from biomass that are currently under investigation. The cellulose component of lignocellulosic biomass is recalcitrant to enzymic digestion, unless the impediments caused by the acetylated pentosans (hemicellulose) and lignin are removed by pretreatment *(5)*. Thermochemical pretreatment of lignocellulosic biomass is efficient and cost-effective *(6–8)*, and acetic is a well-known byproduct of dilute-acid prehydrolysis *(9,10)*. The acetic acid concentration of lignocellulosic prehydrolysate can be predicted from the structure and composition of the biomass feedstock *(11)*. NREL is currently working with yellow poplar wood as a feedstock for cellulosic ethanol. The prehydrolysate produced from this hardwood by dilute-acid pretreatment contains about 4% (w/v) xylose, 0.8% (w/v) glucose, and acetic acid in the range of 1.2–1.5% (w/v) *(11–14)*.

Acetic acid is known to be an effective antimicrobial agent, and is used as such in the food and beverage industries *(15)*. The inhibitory effect of acetic acid on ethanologenic biocatalysts is well-documented (for review, *see* ref. *14*). The mechanism of acetic acid toxicity is also well-understood (for review, *see* ref. *16*), and relates to the ability of the undissociated (protonated) form of the weak acid (pK_a = 4.75) to traverse the cell membrane and to act as a membrane protonophore (i.e., proton transporter), thereby causing an acidification of the cytoplasm. Hence, the inhibitory

effect of acetic acid is pH-dependent *(17–19)*, and derives from its ability to interfere with the homeostatic mechanisms related to the maintenance of a constant intracellular pH *(18,19)*.

The authors have studied the effect of acetic acid on ethanologenic recombinant *E. coli (13,18,20–22)*, and both wild-type *(19,23)* and recombinant *Z. mobilis (24)*. Using recombinant *E. coli*, observed that the energetic uncoupling effect of acetic acid, as reflected in the decreased growth yield, is more pronounced with xylose as energy source, compared to glucose *(18)*. For *Z. mobilis* ATCC 29191 growing in a glucose-based semisynthetic medium, with the pH controlled at 6.0, the final cell mass concentration was reduced about 25% at an acetic acid concentration of 0.5% (w/v) *(19)*.

At this symposium last year, the authors reported on the growth and fermentation characteristics of recombinant *Zymomonas* CP4:pZB5 using synthetic hardwood prehydrolysate media, and in a systematic factorial analysis showed that acetic acid was the most statistically significant limiting factor for xylose utilization and seed production from a corn-steep liquor-based medium in which xylose was the principal sugar *(24)*. With the pH controlled at 6.0, the final cell mass concentration was reduced about 50% at an acetic acid concentration of 0.5% (w/v) *(24)*.

NREL is currently assessing a variety of bioconversion processes for converting lignocellulosic biomass to ethanol at an industrial scale. In the general process design, feedstock comminution is followed by a dilute-acid pretreatment process. Economic sensitivity analysis of several process designs has demonstrated the substantial cost reduction that accompanies modifying the design from one of sequential hydrolysis and fermentation (SHF) to an (simulataneous saccharification and fermentation (SSF) process *(1,25–27)*. Furthermore, there is potential for additional cost reduction (capital and operating costs) by combining the pentose fermentation and cellulose conversion (SSF) unit operations of the process *(2)*, using a biocatalyst with broad substrate specificity. At the seventeenth symposium in 1995, S. Picataggio presented a paper on NREL's proposed simultaneous saccharification co-fermentation (SSCF) process, which employed a proprietary *(28)* recombinant *Z. mobilis* strain CP4:pZB5. Those preliminary observations on the co-conversion efficiency of the recombinant *Zymomonas* were based on a model fermentation medium containing 4% (w/v) xylose, 0.8% (w/v) glucose, 6% (w/v) cellulose and cellulase, but there was no acetic acid in the medium *(28)*.

Using a hardwood prehydrolysate medium, NREL has screened several *Zymomonas* isolates as potential hosts for transformation *(1)*. Ongoing *Zymomonas*-related research and development at NREL is now focused on another similarly engineered recombinant in which *Z. mobilis* ATCC 39676 was transformed with a plasmid designated as "pZB4L". For the past year, our research at the University of Toronto, in collaboration with NREL, has involved studies on the physiological characteristics of this recombinant *Zymomonas* variant, and in a separate paper presented at this

symposium, the authors are reporting on the continuous fermentation performance of this same recombinant culture *(29)*.

The purpose of this study was to examine the effect of simultaneous cellulose saccharification on xylose fermentation, using an acetic acid-containing synthetic biomass prehydrolysate medium. The author used a fed-batch system in which glucose feeding mimicked a constant rate of cellulose hydrolysis. The synthetic prehydrolysate medium and the glucose feed medium were balanced with respect to acetic acid concentration. It was observed that, at relatively low rates of glucose loading, the inhibitory effect of acetic acid was apparently reduced, as reflected in the reduced amount of time required for nearly complete xylose utilization.

MATERIALS AND METHODS

Organism

The xylose-utilizing recombinant *Z. mobilis* strain ATCC 39676, carrying the plasmid pZB4L (designated as Zm 39676:pZB4L), was received from M. Zhang (NREL, Golden, CO). Stock cultures were stored in glycerol at −70°C, and precultures were prepared as previously described *(24)*.

Fermentation Medium and Equipment

The fermentation medium was a nutrient-rich, complex medium (designated as RM) consisting of distilled water with 10g/L Difco Yeast Extract (YE) (Difco, Detroit, MI) and 2g/L KH_2PO_4 *(30)*. In all experiments, the medium also contained 4% (w/v) xylose and tetracycline (10 mg/L), but the amount of glucose and acetic acid was variable. Batch and fed-batch fermentations were conducted 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), and was controlled at a set-point of 6.0 by automatic titration with 4 *N* KOH (NBS model pH-40 controller). Fermentations were initiated by directly transferring about 100 mL of a flask preculture into the bioreactor containing 1400 mL of sterilized medium. The initial cell density was monitored spectrophotometrically to give an OD_{600} (1-cm light path) in the range 0.1–0.2, corresponding to 25–50 mg dry wt cells/L. In fed-batch fermentations, the concentration of acetic acid in the feed medium was identical to that in the fermentation medium. A peristaltic pump was used to deliver sterile medium at a constant rate through the central agitator shaft of the bioreactor, and the flow rate (range 8–10 mL/h) was determined with the aid of an in-line pipet.

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 dH_2O. Dry cell mass (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. Neither the OD or the final cell mass were corrected for dilution in fed-batch experiments. Fermentation media and cell-free spent media were compositionally analyzed by HPLC as previously described *(24)*. The ethanol yield ($Y_{p/s}$) was calculated as the mass of ethanol produced per mass of sugar added to the medium. In the plots of fermentation time-courses, the values given for the concentrations of xylose, glucose, and ethanol were those of the sample medium, and they were not corrected for dilution in the case of fed-batch fermentations.

RESULTS AND DISCUSSION

Effect of Acetic Acid in pH-Stat Batch Fermentations

Since much of the previous work with another similarly engineered NREL recombinant, namely *Z. mobilis* CP4:pZB5 *(3,24)*, had been performed using RM medium *(30)*, for purposes of direct comparison, this same RM medium was selected for use in this study. The RM medium was supplemented with 4% (w/v) xylose and 0.8% (w/v) glucose, to mimic the composition of a dilute-acid hardwood hemicellulose hydrolysate which is known to also contain about 1.5% (w/v) acetic acid *(14)*. The pH-dependent effect of acetic acid on ethanologenic recombinant *E. coli (18)*, was previously examined as well as wild-type *Z. mobilis (19)* and recombinant Zm CP4:pZB5 *(24)*. Because the causative agent in acetic acid toxicity is the undissociated form of the acid, the inhibitory effect can be reduced by operating the fermentor at a pH control set-point that is elevated above the pK_a value of 4.75 *(19,24)*. The pH optimum for both growth rate and cell mass yield for wild-type *Z. mobilis* is close to 6.0 *(31,32)*, and therefore this pH value was selected as the control set-point in this study.

Figure 1 (Expt. B1) shows a typical growth and fermentation time-course, using the recombinant culture Zm 39676:pZB4L in the nutrient-rich RM medium. The final cell mass concentration was 1.44 gDCM/L, and the ethanol yield was 0.50 g/g, equivalent to a conversion efficiency of 98% of theoretical maximum (Table 1). Under these conditions, the growth and fermentation performance of NREL recombinants Zm CP4:pZB5 and 39676:pZB4L are identical *(24)*. The addition of 0.4% (w/v) acetic acid to the RM medium (Expt. B2) resulted in a decrease in both growth rate and yield, but the ethanol yield remained unaffected (Fig. 1 and Table 1). With 0.4% acetic acid in the medium (at pH 6), the final cell mass decreased from 1.44 to 0.91 gDCM/L (Table 1); the time required for complete xylose utilization increased from 24 to 34 h (Fig. 1B). These observations on the effect of acetic acid on recombinant Zm 39676:pZB4L are very similar to those previously reported for recombinant Zm CP4:pZB5 using a medium with corn-steep liquor as the sole nutritional supplement *(24)*.

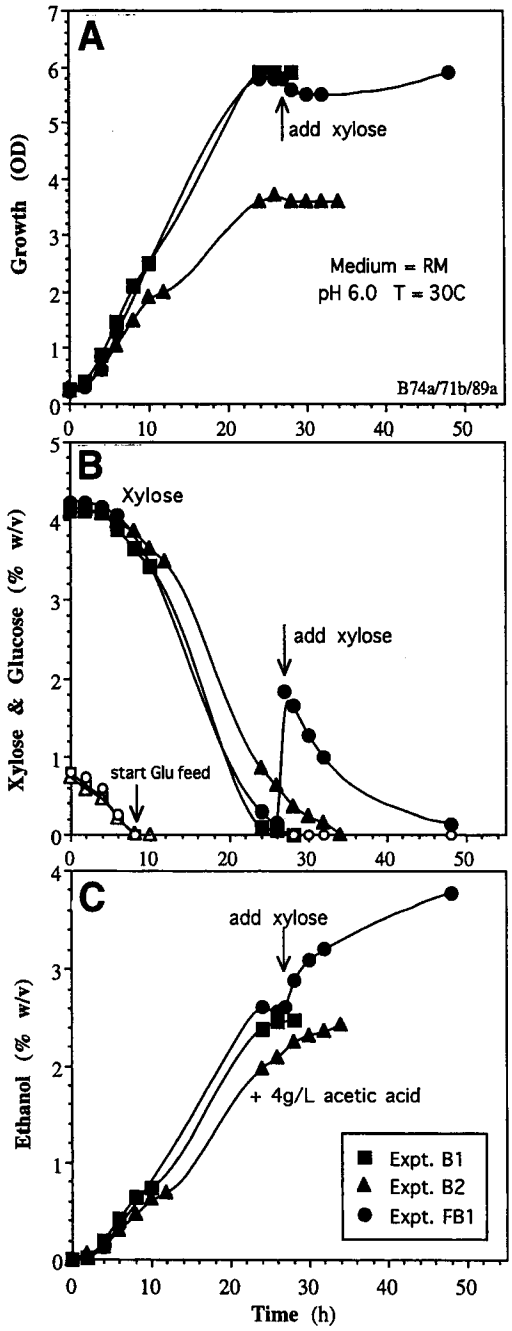

Fig. 1. Time-course of pH-stat batch and fed-batch fermentations with recombinant *Z. mobilis* 39676:pZB4L. **(A)** Growth, **(B)** glucose and xylose utilization, and **(C)** ethanol production. The medium was RM and was supplemented with 4% (w/v) xylose and 0.8% (w/v) glucose. The temperature was kept constant at 30°C. The pH-control set-point was 6.0. The observed final cell mass concentrations, ethanol yield values, and the conditions for the fed-batch Expt. FB1, are given in Table 1.

Table 1
Effect of Acetic Acid on Cofermentation of Xylose and Glucose by Recombinant *Zymomonas* 39676:pZB4L in Batch and Fed-Batch Systems

	Medium composition			Fermentation parameters							
Expt.	Xylose % (w/v)	Glucose % (w/v)	Ac acid % (w/v)	Glucose in feed % (w/v)	Ac in feed % (w/v)	Feed rate (ml/h)	Feed interval (h)	Total Glu (g)	Final cell mass (gDCM/L)	Ethanol yield (g/g)	Conversion efficiency % (theoret. max)
Batch fermentations											
B1	4.0	0.8	0	–	–	–	–	12	1.44	0.50	98
B2	4.0	0.8	0.40	–	–	–	–	12	0.91	0.50	98
B3	4.0	0.8	0.75	–	–	–	–	12	0.43	0.47	92
B4	4.0	2.2	0.75	–	–	–	–	33	0.49	0.49	96
Fed-batch fermentations											
FB1	4.0[a]	0.8	0	5.2	0	9.6	8–48	32	1.40	0.50	98
FB2	4.0	0.8	0.75	0	0.75	8.9	0–48	12	0.31	0.49	96
FB3	4.0	0.8	0.75	5.0	0.75	8.5	0–48	33	0.53	0.48	94
FB4	4.0	0.8	0.75	13.6	0.75	8.7	0–48	69	0.70	0.48	94

Note: The base medium (RM) contained 10g/L Difco yeast extract and 2 g/L KH_2PO_4. The temperature of the pH-stat stirred-tank fermentors was maintained constant at 30°C, and the pH was controlled at 6.0. Maximum theoretical ethanol yield = 0.51 g/g. DCM was determined by ultrafiltration (*see* Methods), and was not corrected for dilution in fed-batch fermentations.

Abbreviations: Ac, acetic acid; B, batch fermentation; FB, fed-batch fermentation

[a] In Expt. FB1, xylose was added at 27 h, and the concentration was 1.8% (w/v) (*see* Fig. 1).

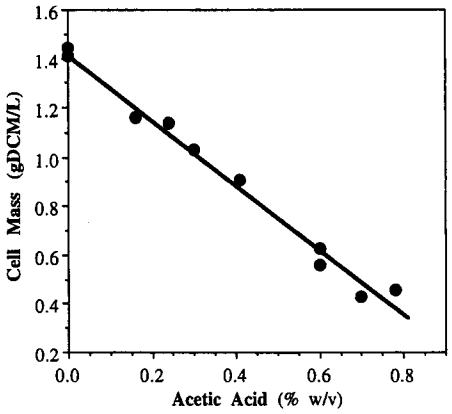

Fig. 2. Final cell mass concentration for recombinant Zm 39676:pZB4L as a function of the acetic acid concentration. These data represent a series of batch fermentations similar to Expt. B2 shown in Fig. 1. DCM was determined by ultrafiltration, as described in Materials and Methods. The RM medium contained 4% (w/v) xylose and 0.8% (w/v) glucose, and varying amounts of acetic acid. The temperature was 30°C and the pH was controlled at 6.0.

The acetic acid concentration of the dilute-acid poplar wood prehydrolysate produced by NREL is approx 1.5% (w/v) *(14)*. However, based on observations with recombinant Zm CP4:pZB5 in acetic acid-containing synthetic biomass prehydrolysate media, the recommended upper limit for acetic acid for the purpose of seed production (i.e., preparation of inoculum) was 0.75% (w/v), equivalent to that of a 50% prehydrolysate medium *(24)*. Figure 2 shows a plot of the final cell mass concentration as a function of the acetic acid concentration (range 0–0.8%) in RM medium containing 4% (w/v) xylose and 0.8% (w/v) glucose. The results shown in Figure 2 for recombinant Zm 39676:pZB4L are very similar to those previously observed with recombinant Zm CP4:pZB5 under the same operating conditions (unpublished results). In Fig. 2, the line created by linear regression analysis is represented by the following relationship: DCM concentration (g/L) = 1.42 − 1.33 (% w/v acetic acid) (with a regression coefficient of 0.985).

Effect of Acetic Acid in pH-Stat Fed-Batch Fermentations

One of several process designs currently under assessment at NREL for the conversion of biomass to ethanol on an industrial scale is simultaneous saccharification and cofermentation (SSCF) *(2)*. In such a process, the saccharifying enzymes release glucose from cellulose and cellobiose. In this study, the effect of simultaneous saccharification of cellulose on the efficiency of xylose fermentation by recombinant Zm 39676:pZB4L was examined using a fed-batch system in which glucose is fed to the pH-stat bioreactor at rates commensurate with anticipated rates of cellulose

hydrolysis by the exogenous enzymes in the SSCF, as proposed by NREL *(28)*. In the fed-batch fermentation experiment, designated as FB1, 5.2% (w/v) glucose was fed at a rate of 9.6 mL/h, commencing after 8 h of batch fermentation. To prevent complete exhaustion of xylose from the medium, additional xylose (1.8%) was added after 27 h (Fig. 1). In this experiment, there was no acetic acid in the medium. This level of glucose feeding did not appear to affect the rate of xylose conversion (Fig. 1B). After 48 h, the final ethanol concentration was 3.7% (w/v), representing an ethanol yield from both xylose and glucose of 0.50 g/g (98% conversion efficiency) (Fig. 1C). The tailing off that is observed in the xylose concentration trajectory in the latter stages of the fermentation (Fig. 1B) is probably caused by the competition by glucose and xylose for uptake by the common transporter *(33)*. Based on a cell mass concentration of about 1.4 gDCM/L, the specific productivity associated with cofermentation by recombinant 39676:pZB4L over the fed-batch fermentation interval of 28–34 h is estimated at approx 0.7 g ethanol/gDCM/h (Fig. 1C).

In this study, the highest level of acetic acid tested was 0.75% (w/v), which represents about a 50% dilution of the level anticipated in hardwood prehydrolysate. The effect of this level of acetic acid in batch fermentation, with the pH controlled at 6.0, is represented by Exp. B3 (Fig. 3). The final cell mass concentration is only about one-third of that observed in the absence of acetic acid (Fig. 3), and the ethanol yield (based on sugar consumed) is also reduced from 0.50 to 0.47 g/g (Table 1). With 0.75% acetic acid in the medium, only about one-third of the xylose is consumed after 48 h (Fig. 3B). However, when a solution of 5% glucose and 0.75% acetic acid is fed to the bioreactor (Expt FB3), at a similar rate to that used in experiment FB1, the xylose was almost completely fermented after 48 h (Fig. 3B). Since the xylose concentration trajectories in Fig. 3B were not corrected for the volumetric dilution effect produced by glucose feeding, a control experiment (Expt. FB2) was performed in which the bioreactor was fed with a solution of 0.75% acetic acid (Fig. 3B). Fed-batch experiment FB2 shows that the dilution of xylose, caused by feeding, produced an artificial or apparent improvement in the rate of xylose utilization; however, this was not significant compared to the enhancing effect produced by the presence of glucose in the feed (Fig. 3B).

Supplementing the medium with glucose can be expected to result in an increase in the cell mass concentration, and the rate of xylose utilization can be expected to be proportional to the cell concentration. Increasing the concentration of glucose in the feed about 2.5-fold (Expt. FB4) did not decrease the time required for nearly complete xylose fermentation (Fig. 3), despite the increase in the observed final cell density from 0.53 to 0.70 g DCM/L (Fig. 2A and Table 1). In *Zymomonas*, the uptake of glucose and xylose is by a common transporter that exhibits much higher affinity for glucose than xylose *(33)*. The failure of extra glucose to further improve the rate of xylose utilization is probably caused by the preference of

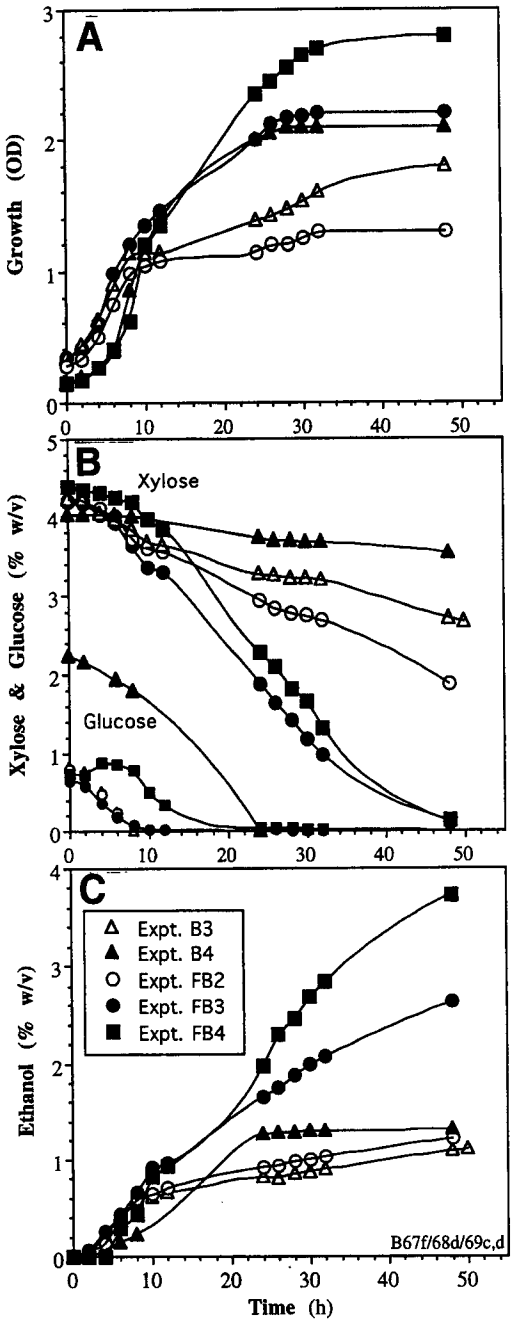

Fig. 3. Time-course of pH-stat batch and fed-batch fermentations with recombinant *Z. mobilis* 39676:pZB4L. **(A)** Growth, **(B)** glucose and xylose utilization, and **(C)** ethanol production. The experimental conditions, as well as the values for final cell mass concentrations and ethanol yields, are presented in Table 1.

the common transporter for glucose (*33*). Hence, the accumulation of glucose over the initial 10 h interval could be expected to have a retarding effect on xylose uptake during that same interval. To examine this phenomenon further, a separate batch fermentation was performed (Expt. B4), in which the initial concentration of glucose in the medium was increased from 0.8 to 2.2% (w/v). Although there was an increase in growth (Fig. 3A), with the final cell mass increasing from 0.43 to 0.49 gDCM/L (Table 1), xylose utilization was significantly decreased with only about 10% of the xylose consumed after 48 h (Fig. 3B). This observation emphasizes the importance of the proper balance between the concentration of the two sugars during cofermentation.

It was concluded that, under certain well-defined conditions, the detrimental effect of acetic acid on xylose utilization by recombinant *Zymomonas* can be reduced, if glucose is supplied for cofermentation at a rate that does not result in glucose accumulation, since this appears to interfere with xylose uptake. Consequently, it is expected that the inhibitory effect of acetic acid in the SSCF process will be partially ameliorated because of the continuous supply of glucose provided by the action of the exogenous cellulolytic enzymes.

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REFERENCES

1. Zhang, M., Franden, M. A., Newman, M., McMillan, J., Finkelstein, M., and Picataggio, S. (1995), *Appl. Biochem. Biotechnol.* **51/52,** 527–536.
2. Picataggio, S. K., Zhang, M., and Finkelstein, M. (1994), in *Enzymatic Conversion of Biomass for Fuels Production*, Himmel, M. E., Baker, J. O., and Overend, R. A., eds., American Chemical Society, Washington, DC, *ACS Symposium Series* 566, pp. 342–362.
3. Zhang, M., Eddy, C., Deanda, K., Finkelstein, M., and Picataggio, S. K. (1995), *Science,* **267,** 240–243.
4. Picataggio, S. K., Zhang, M., Eddy, C. K., Deanda, K. A., and Finkelstein, M. (1996), US Patent 5,514,583.
5. Wright, J. D. (1988), *Chem. Eng. Prog.* **84,** 62–74.
6. Grethlein, H. E. (1985), *BioTechnology* **3,** 155–160.
7. Grethlein, H. E., Allen, D. C., and Converse, A. O. (1984), *Biotech. Bioeng.* **26,** 1498–1505.
8. Torget, R., Werdene, P., Himmel, M., and Grohmann, K. (1990), *Appl. Biochem. Biotechnol.* **24/25,** 115–126.
9. Grohmann, K., Himmel, M., Rivard, C., Tucker, M., Baker, T. Torget, R., and Graboski, M. (1984), *Biotechnol. Bioeng. Symp.* **14,** 139–157.
10. Kong, F., Engler, C. R., and Soltes, E. (1992), *Appl Biochem. Biotechnol.* **34/35,** 23–35.

11. Timell, T. E. (1964), *Adv. Carbohydr. Chem.* **19,** 247–302.
12. Lawford, H. G. and Rousseau, J. D. (1993), in *Energy from Biomass and Wastes XVI.* (1992) Klass, D. L., ed., Institute of Gas Technology, Chicago, pp. 559–597.
13. Lawford, H. G. and Rousseau, J. D. (1993), *Appl. Biochem. Biotechnol.* **39/40,** 667–685.
14. McMillan, J. D. (1994), in *Enzymatic Conversion of Biomass for Fuels Production,* Himmel, M. E., Baker, J. O., and Overend, R. A., eds., American Chemical Society, Washington, DC, *ACS Symposium Series* 566, pp. 411–437.
15. Freese, E., Sheu, C. W., and Galliers, E. (1973), *Nature* **241,** 321–326.
16. Booth, I. R. (1985), *Microbiol. Rev.* **49,** 63–91.
17. Prior, B. A., Killan, S. G., and du Preez, J. C. (1989), *Process Biochem.* **24,** 21–32.
18. Lawford, H. G. and Rousseau, J. D. (1993), *Appl. Biochem. Biotechnol.* **39/40,** 301–322.
19. Lawford, H. G. and Rousseau, J. D. (1994), *Appl. Biochem. Biotechnol.* **45/46,** 437–448.
20. Lawford, H. G. and Rousseau, J. D. (1991), *Biotechnol. Letts.* **13,** 191–196.
21. Lawford, H. G. and Rousseau, J. D. (1993), in: *Energy from Biomass and Wastes XVI,* Klass, D. L., ed., Institute of Gas Technology, Chicago, IL, pp. 559–597.
22. Lawford, H. G. and Rousseau, J. D. (1992), *Appl. Biochem. Biotechnol.* **34/35,** 185–204.
23. Lawford, H. G. and Rousseau, J. D. (1993), *Appl. Biochem. Biotechnol.* **39/40,** 687–699.
24. Lawford, H. G., Rousseau, J. D., and McMillan, J. D. (1997), *Appl. Biochem. Biotechnol.* **63–65,** 269–286.
25. Hinman, N. D., Wright, J. D., Hoagland, W., and Wyman, C. E. (1989), *Appl. Biochem. Biotechnol.* **20/21,** 391–401.
26. Hinman, N. D., Schell, D. J., Riley, C. J., Bergeron, P. W., and Walter, P. J. (1992), *Appl. Biochem. Biotechnol.* **34/35,** 639–649.
27. Lynd, L. R. (1990), *Appl. Biochem. Biotechnol.* **24/25,** 695–719.
28. Picataggio, S. K., Eddy, C., Deanda, K., Franden, M. A., Finkelstein, M., and Zhang, M. (1996), *Seventeenth Symposium on Biotechnology for Fuels & Chemicals* (Paper #9).
29. Lawford, H. G., Rousseau, J. D., Mohagheghi, A., and McMillan, J. D. (1998), *Appl. Biochem. Biotechnol.,* (19th Symp) **70–72.**
30. Goodman, A. E., Rogers, P. L., and Skotnicki, M. L. (1982), *Appl. Environ. Microbiol.* **44,** 496–498.
31. Lawford, H. G. and Ruggiero, A. (1990), *Biotechnol. Appl. Biochem.* **12,** 206–211.
32. Lawford, H. G., Holloway, P., and Ruggiero, A. (1988), *Biotechnol. Letts.* **10,** 809–814.
33. DiMarco, A. and Romano, A. H. (1985), *Appl. Environ. Microbiol.* **49,** 151–157.