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Cellulosic Fuel Ethanol

Alternative Fermentation Process Designs with Wild-Type and Recombinant Zymomonas mobilis

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Abstract

Iogen (Canada) is a major manufacturer of industrial cellulase and hemicellulase enzymes for the textile, pulp and paper, and poultry feed industries. Iogen has recently constructed a 40 t/d biomass-to-ethanol demonstration plant adjacent to its enzyme production facility. The integration of enzyme and ethanol plants results in significant reduction in production costs and offers an alternative use for the sugars generated during biomass conversion. Iogen has partnered with the University of Toronto to test the fermentation performance characteristics of metabolically engineered *Zymomonas mobilis* created at the National Renewable Energy Laboratory. This study focused on strain AX101, a xylose- and arabinose-fermenting stable genomic integrant that lacks the selection marker gene for antibiotic resistance. The "Iogen Process" for biomass depolymerization consists of a dilute–sulpfuric acid–catalyzed steam explosion, followed by enzymatic hydrolysis. This work examined two process design options for fermentation, first, continuous cofermentation of C_5 and C_6 sugars by Zm AX101, and second, separate continuous fermentations of prehydrolysate by Zm AX101 and cellulose hydrolysate by either wild-type *Z. mobilis* ZM4 or an industrial yeast commonly used in the production of fuel ethanol from corn. Iogen uses a proprietary process for conditioning the prehydrolysate to reduce the level of inhibitory acetic acid to at least 2.5 g/L. The pH was controlled at 5.5 and 5.0 for *Zymomonas* and yeast fermentations, respectively. Neither 2.5 g/L of acetic acid nor the presence of pentose sugars (C_6:C_5 = 2:1) appreciably affected the high-performance glucose fermentation of wild-type *Z. mobilis* ZM4. By contrast, 2.5 g/L of acetic acid significantly reduced the rate of pentose fermentation by strain AX101. For single-stage continuous fermentation of pure sugar synthetic cellulose hydrolysate (60 g/L of glucose), wild-type *Zymomonas* exhibited a four-fold higher volumetric productivity compared with industrial yeast. Low levels of acetic acid stimulated yeast ethanol productivity. The glucose-to-ethanol conversion efficiency for Zm and yeast was 96 and 84%, respectively.

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Index Entries: Genomic integration; recombinant *Zymomonas* AX101; *Zymomonas mobilis*; arabinose; xylose; ethanol; prehydrolysate; biomass hydrolysate; acetic acid; yeast.

Introduction

Lignocellulosic feedstocks, in the form of either waste materials or designated energy crops, offer an opportunity to greatly expand the capacity of the fuel ethanol industry. Lignocellulose is recalcitrant to enzymatic digestion by cellulase unless it has been "pretreated" to remove the hemicellulose and lignin components. The hemicellulose that comprises 15–25% of the lignocellulosic feedstock is easily hydrolyzed by dilute-acid hydrolysis to its monomeric sugars, the pentose (5-carbon) sugars xylose and arabinose; and, to a smaller extent, the hexose sugars mannose and galactose. The amount of xylose produced is one-third to one-half the amount of glucose produced from the saccharification of lignocellulosic material. Hence, fermentation of the pentose sugars represents an opportunity for major improvement in ethanol yield. Economic analyses have suggested that, to be well positioned in the competitive liquid fuels market, cellulosic ethanol must be produced by the rapid and efficient conversion of all the major sugar components of the hydrolyzed cellulosic feedstock *(1)*. Modern recombinant DNA technology has been successfully used to create many different microbial biocatalysts—both yeast and bacteria—that are capable of fermenting the constituent monosaccharides to ethanol.

Because the fermentation unit operation in the biomass-to-ethanol process is located downstream from the feedstock pretreatment and hydrolysis operations, the fermentation biocatalyst is impacted by both the type of feedstock and the process flow configuration of the process with respect to the distribution of the process streams from the pretreatment (hemicellulose hydrolysis or prehydrolysis) and cellulose digestion operations (saccharifying stage reactor). From a bioengineering perspective, the biocatalyst must conform to the performance characteristics demanded by a particular process with respect to both feedstock and overall process design. The feedstock affects the composition of the prehydrolysate with respect to the type and amount of the different C_6 and C_5 sugars as well as other potentially inhibitory substances such as acetic acid (HAc).

Iogen (Ottawa, Canada) is a major manufacturer of industrial enzymes. Iogen primarily produces cellulase and hemicellulase enzymes for the textiles, pulp and paper, and poultry feed industries. Iogen has recently built a 40 t/d biomass-to-ethanol demonstration plant adjacent to its enzyme production facility *(2)*. The location of the ethanol demonstration plant offers the advantages that the enzyme can be used without the expenses of stabilization and preservation, and that the process sugars can be used for enzyme production.

Although *Saccharomyces* yeast currently enjoys a monopoly as the fermentation process biocatalyst in the fuel ethanol industry, it is not the only ethanol-producing microorganism. By virtue of its demonstrated superior

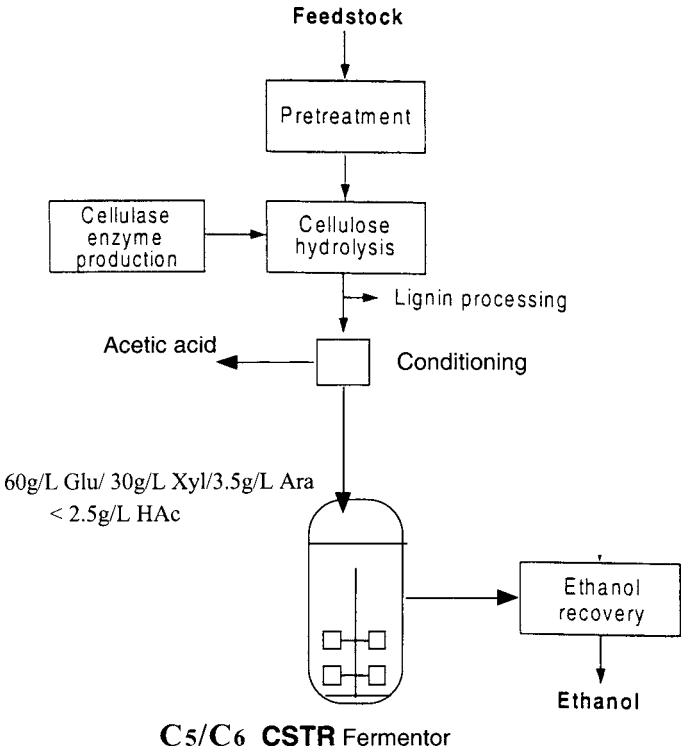

Fig. 1. Iogen biomass-to-ethanol process as proposed in 1999 *(11)*. The original diagram has been modified to include a hydrolysate conditioning operation primarily for the reduction in level of acetic acid. CSTR, continuous-flow stirred-tank reactor.

fermentation performance characteristics, the bacterium *Zymomonas mobilis* (Zm) offers an opportunity for process improvement with respect to both conversion efficiency (yield) and productivity *(3)*. It has the potential to revolutionize the fuel ethanol industry. Although Zm is not used commercially (for fermentation trials at industrial scale, *see* ref *4*; for pilot-scale trials *see* review by Doelle et al. *[5]*), laboratory- and pilot-scale operations indicate that it can generate near-theoretical maximum yields from diverse feedstocks including cellulosics. Iogen has partnered with the University of Toronto to assess the fermentation performance of genotypic variants of the bacterium *Z. mobilis (6,7)*. Wild-type Zm and *Saccharomyces* ferment hexoses but cannot ferment pentoses. Our work with Iogen is a continuation of our ongoing collaboration with the National Renewable Energy Laboratory (NREL) directed at assessing the physiologic and biochemical characteristcs of NREL's patented, pentose-fermenting, recombinant *Zymomonas* cultures *(8–10)*.

The "Iogen Process", as it was originally proposed in 1999 *(11)*, is schematically represented in Fig. 1. Biomass depolymerization consists of "pretreatment" by a dilute–sulfuric acid–catalyzed steam explosion at 200–250°C followed by enzymatic hydrolysis using on-site-generated cel-

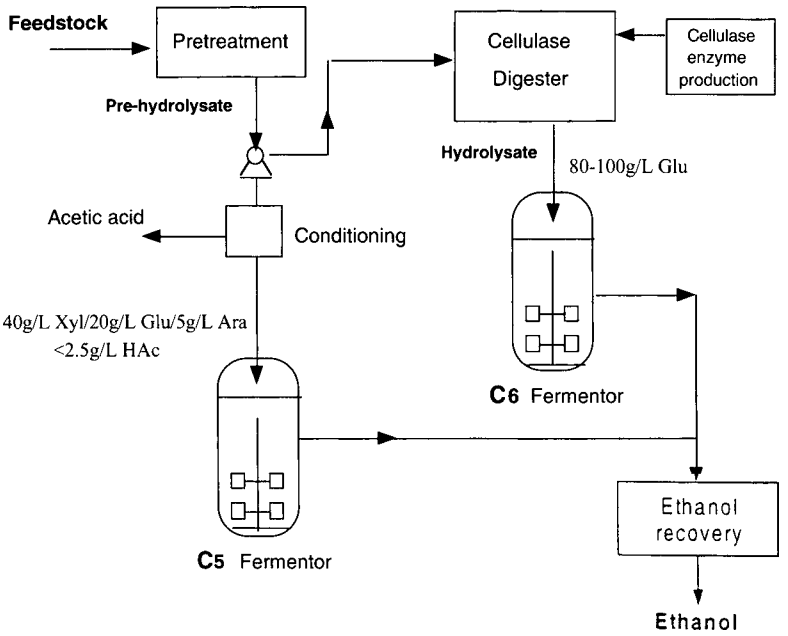

Fig. 2. Revised Iogen process flow diagram. The process involves the separate hydrolysis and continuous fermentation of C_5 and C_6 components of lignocellulosic biomass for the production of fuel ethanol.

lulase enzymes. In this continuous-flow separate hydrolysis and fermentation (SHF) process, the total hydrolysate coming from the digesters contained about 6% (w/v) glucose, 3% xylose, and 0.35% arabinose, with little sugar oligomers *(6)*. The lignin was removed prior to fermentation (Fig. 1). Biomass hydrolysates contain acetic acid by virtue of the presence of the acetylated pentosans in hemicellulose *(12)*. The pH-dependent inhibitory effect of acetic acid on ethanologenic biocatalysts is well documented (for a review *see* ref. *13*). We have studied the effect of acetic acid on both wild-type *(14,15)* and recombinant *Z. mobilis (16)*. Since acetic acid represents a major limiting factor for high-performance pentose fermentation, the Iogen process incorporates a proprietary hydrolysate conditioning stage that reduces the acetic acid level to <0.25% (w/v) prior to fermentation (C. Nicholson, February 2000, personal communication).

Figure 2 illustrates an alternate process that is currently being contemplated by Iogen in its biomass-to-ethanol demonstration plant. Although the overall process remains a continuous-flow SHF process, the pentose-rich prehydrolysate is fermented separately (Fig. 2). Adjustments to mass loadings and other operational parameters result in separate C_5 and C_6 streams of the approximate composition shown in Fig. 2.

Given these two process design options, the purpose of the present study was to examine the fermentation performance characteristics of both recombinant and wild-type *Z. mobilis* cultures in pH-controlled continuous

fermentations using synthetic media formulated to mimic Iogen's various process streams. NREL's patented recombinant *Z. mobilis* strain AX101 is a genomic integrant that ferments both xylose and arabinose, and because it lacks the selection marker gene for Tc resistance *(17,18)*, it has a more acceptable regulatory approval status *(7)*. Another objective of this work was to compare the C_6 fermentation performance of the well-known wild-type *Zymomonas* strain ZM4 *(3,19)* with that of an industrial *Saccharomyces cerevisiae* yeast commonly used in the production of fuel ethanol from corn *(20)*.

Materials and Methods

Organisms

Recombinant *Z. mobilis* strain AX101 (derived from Zm ATCC 39676) *(17)* was obtained from M. Zhang (NREL, Golden, CO). Stock cultures were stored in glycerol at –70°C and precultures were prepared as described previously *(7)*. Wild-type *Z. mobilis* strain ZM4 was obtained from the American Type Culture Collection (ATCC) (Rockville, MD) as ATCC 31821. Allyeast™ is an industrial strain of *S. cerevisiae* and was a gift from Alltech (Nicholasville, KY).

Fermentation Media

The synthetic biomass hydrolysate media contained 3 g/L of Difco yeast extract (Difco Laboratories, Detroit, MI), 0.8 g/L of NH_4Cl, and "Zymo salts" as described previously *(6)*. The amounts of D-glucose, D-xylose, L-arabinose (Sigma, St Louis, MO), and HAc that were added to the different fermentation media were variable. The media and stock sugar solutions were autoclaved separately.

Preparation of Inoculum

A 1-mL aliquot of a glycerol-preserved AX101 culture was removed from cold storage (freezer) and transferred to about 200 mL of RM medium (10 g/L of yeast extract and 2 g/L of KH_2PO_4) containing about 10 g/L of xylose, 10 g/L of arabinose, and 30 of g/L glucose in loosely capped 250-mL Erlenmeyer flasks and grown in a waterbath shaker overnight at 30°C. This preseed was subcultured into inoculation flasks containing the synthetic hydrolysate medium with 30 g/L of glucose, 10 g/L of xylose, and 10 g/L of arabinose and grown in a water bath shaker overnight at 30°C . This overnight culture was used as inoculum at a level of approx 10% (v/v). The initial optical density (1-cm light path at 600 nm) was in the range of 0.25–0.5, corresponding to 70–140 mg of dry cell mass/L. Inocula for both wild-type *Zymomonas* and the Alltech yeast culture were prepared by overnight growth in RM medium containing 60 g/L of glucose.

Fermentation Equipment

pH-controlled continuous fermentations were conducted with either NBS C30 BioFlo chemostats or 2-L NBS Bioflo 2000 bioreactors. The work-

ing volumes of these chemostats were about 350 and 1500 mL, respectively. Flow from the medium reservoir to the batch culture in the chemostat vessel was initiated when the level of fermentable sugar had decreased to about 3–5 g/L. For strain AX101, the initial dilution rate was 0.025–0.03/h; for wild-type Zm, approx 0.1/h; and for the yeast culture, 0.05/h. 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, sugar, and ethanol concentrations. The pH was monitored using a sterilizable combination pH electrode (Broadley James, Irvine, CA). The standard pH control set point was 5.5 for *Zymomonas* and 5.0 for yeast. The pH was kept constant by automatic titration with 4*N* KOH. Temperature was controlled at 30°C using a circulating water bath, and the agitation was moderate.

Analytical Procedures and Growth and Fermentation Parameters

Growth was measured turbidometrically at 600 nm (1-cm light path). In all cases, the blank cuvet contained distilled water. Dry cell mass 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 *(10)*. The "process" ethanol yield was calculated as the mass of ethanol produced per mass of fermentable sugar in the medium (i.e., glucose and xylose). The maximum dilution rate (D_{max}) was achieved when the steady-state level of unfermented sugar in the chemostat effluent was 15% of the total amount of fermentable sugar in the medium. The volumetric productivity was calculated as the product of D_{max} and the steady-state ethanol concentration at D_{max}.

Results and Discussion

The major portion of our previous work with NREL's metabolically engineered pentose fermenting *Z. mobilis* contemplated a consolidated process design with simultaneous hydrolysis and cofermentation of the C_5 and C_6 sugars in a single flow through bioreactor *(9)*. By contrast, the biomass-to-ethanol process originally proposed by Iogen *(11)* involved separate hydrolysis of the cellulose and subsequent continuous fermentation of the combined prehydrolysate and cellulose hydrolysate in the same vessel (Fig. 1). One of the principle differences in these two designs is the concentration of glucose in the fermentation medium. The composition of the hydrolysate coming from the Iogen digester was approx 60 g/L of D-glucose, 30 g/L of D-xylose, and 3.5 g/L of L-arabinose. The continuous fermentation experiments employed a yeast extract-based pure sugar synthetic biomass hydrolysate formulated to mimic the sugar composition of the Iogen hydrolysate. Iogen specified a target of 85% utilization of the xylose (equivalent to 5 g/L in the fermentor effluent) and a pH set point no

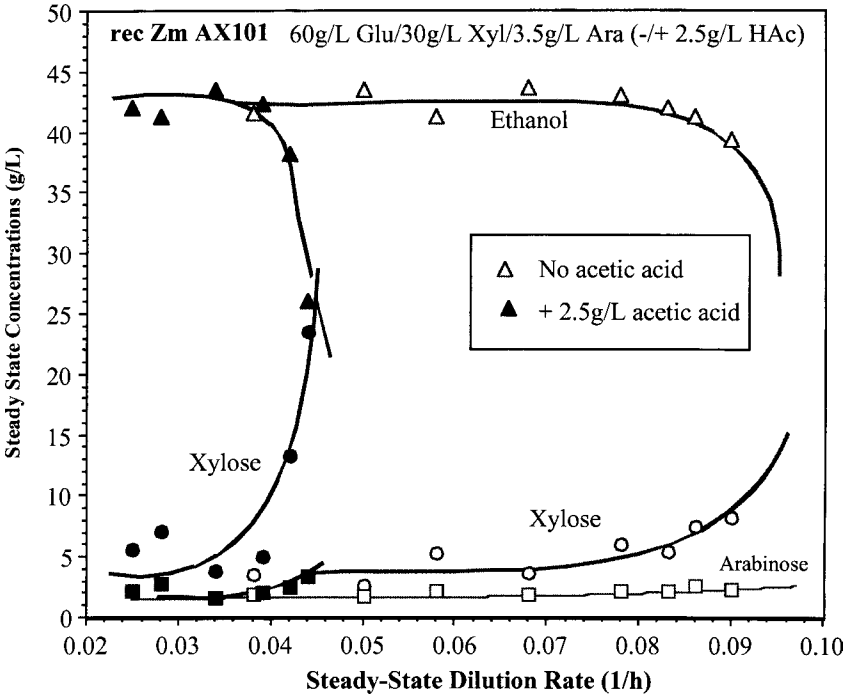

Fig. 3. Steady-state levels of xylose, arabinose and ethanol as function of dilution rate in continuous fermentations of pure sugar synthetic hydrolysates by integrated Zm strain AX101. The yeast extract–based medium (*see* Materials and Methods) contained 60 g/L of glucose, 30 g/L of xylose, and 3.5 g/L of arabinose. The pH was controlled at 5.5. When acetic acid was added, the concentration was 2.5 g/L. No glucose was detected in the effluent over the course of the experiment. Fermentation parameters are summarized in Table 1.

higher than 5.5. Using integrated Zm strain AX101, the maximum volumetric productivity in the absence of acetic acid was 3.3 g/(L·h) (Table 1). For 85% xylose utilization, D_{max} was 0.078/h and the ethanol concentration was 42 g/L (Fig. 3), representing a process yield of 0.45 g/g or 88% overall conversion efficiency (Table 1). The pH was 5.5 and the temperature was 30°C. Although the Iogen process incorporates a hydrolysate conditioning stage, not all the acetic acid is removed. Figure 3 shows the sensitivity of strain AX101 to inhibition by acetic acid. With the estimated upper level of 2.5 g/L of HAc in the synthetic biomass hydrolysate feed, the maximum volumetric productivity decreased to 1.7 g/(L·h) (D_{max} was 0.04/h), but the overall conversion efficiency remained at the 88% level (Table 1). Previous experimentation has shown that the fermentation performance can be slightly improved by elevating the pH set point from 5.5 to 6.0 *(7)*. In a study reported at last year's symposium, Mohagheghi et al. *(18)* compared C_6/C_5 cofermentation results obtained with strain AX101 with those reported by Toon et al. *(21)* for recombinant *Saccharomyces* yeast strains and noted that AX101 achieved significantly higher process yields.

Table 1
Summary of Steady-State Fermentation Performance Parameters for Zm AX101, Wild-Type ZM4, and Commercial Yeast in Continuous Fermentations Using Synthetic Biomass Hydrolysates

Biocatalyst	Feed composition (g/L)	HAc (g/L)	EtOH (g/L)	D_{max} (1/h)	Productivity (g/[L·h])	Process yield (g/g)
Process design option no. 1						
Stage 1 (synthetic total hydrolysate)						
Rec ZM AX101	60G/30X/3.5A	0	42.0	0.078	3.3	0.45
Rec ZM AX101	60G/30X/3.5A	2.5	42.5	0.040	1.7	0.45
ZM4	60G/30X/3.5A	2.5	27.0	0.380	10.3	0.29[b]
Process design option no. 2						
C_5 CSTR (synthetic hemicellulose hydrolysate)						
Rec ZM AX101	5G/30X/3.5A	0	16.8	0.060	1.0	0.44
Rec ZM AX101	10G/30X/3.5A	2.5	20.0	0.040	0.8	0.46
C_6 CSTR (synthetic cellulose hydrolysate)						
ZM4	60 Glu	0	29.4	0.395	11.6	0.49
ZM4	100 Glu	0	49.1	0.235	11.5	0.49
S. cerevisiae	60 Glu	0	27.0	0.100	2.6	0.43
S. cerevisiae	60 Glu	2.5	27.4	0.125	3.4	0.43

[a]*Z. mobilis* ZM4 = ATCC 31821; G, glucose; X, xylose; A, arabinose. For Zm fermentations, the pH was controlled at 5.5; for yeast fermentations, the pH was 5.0.
[b]Wild-type Zm culture cannot utilize pentose sugars.

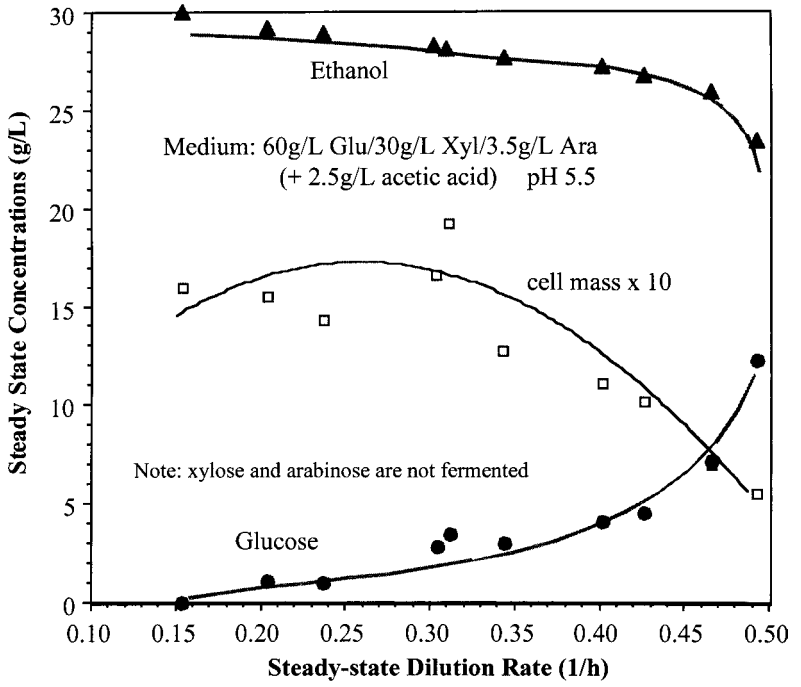

Fig. 4. Steady-state levels of glucose, cell mass, and ethanol as function of dilution rate in continuous fermentation of synthetic biomass hydrolysate by wild-type Zm strain ZM4 (ATCC 31821). The yeast extract–based medium contained 60 g/L of glucose, 30 g/L of xylose, 3.5 g/L of arabinose, and 2.5 g/L of acetic acid. The pH was controlled at 5.5. For 95% utilization of the glucose (effluent contains 3 g/L), the D_{max} = 0.4/h. Fermentation parameters are summarized in Table 1.

Remaining within the constraints of the process design outlined in Fig. 1, an alternative fermentation option that we explored involved using wild-type *Zymomonas* strain ZM4 (ATCC 31821). The continuous fermentation of total hydrolysate (with 2.5 g/L of HAc) by strain ZM4 is shown in Fig. 4. Although the wild-type Zm is unable to ferment either xylose or arabinose, there are advantages to considering its deployment. First, acetic acid is not as inhibitory for glucose fermentation by *Z. mobilis (14,15)*; second, although the ethanol concentration is lower (27 g/L), there is a marked improvement in productivity (10.3 g/[L·h]) because the D_{max} is 0.38/h (Table 1). This design would have merit if the C_5 component could be used for another purpose (e.g., in the enzyme plant) within the context of the proposed biorefinery or "sugar platform" for multiple products. The downside to this option is the potential for fouling of the distillation columns by the unfermented C_5 sugars during ethanol recovery.

Over the course of our work with Iogen, the overall process design evolved from a single fermentation process to a double fermentation process with separate continuous fermentations of the pentose-rich hemicellulose hydrolysate ("prehydrolysate") and the cellulose hydrolysate (*see* Fig. 2).

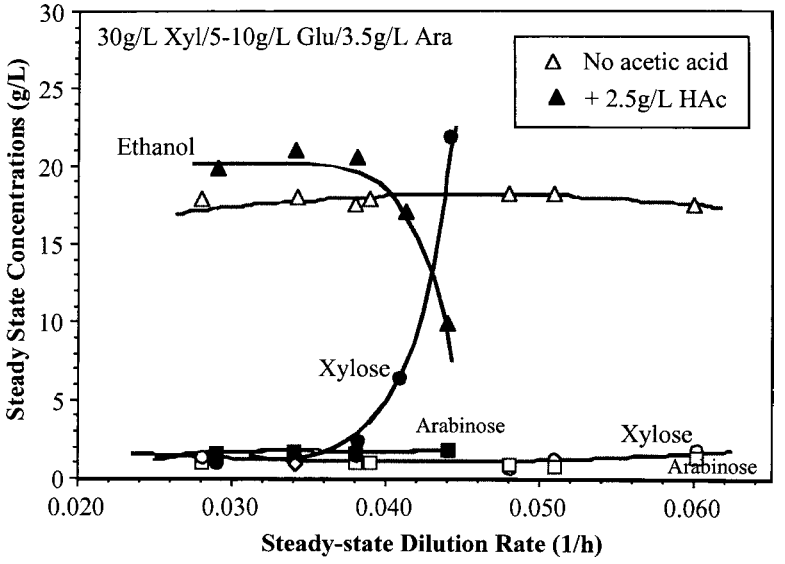

Fig. 5. Steady-state levels of sugars and ethanol as function of dilution rate continuous fermentations of pure sugar synthetic prehydrolysates by integrated Zm strain AX101. The yeast extract–based medium (*see*Materials and Methods) contained 30 g/L of xylose, 5 g/L of glucose, and 3.5 g/L of arabinose. The pH was controlled at 5.5. When acetic acid was added, the concentration was 2.5 g/L and the glucose concentration was 10 g/L. Fermentation parameters are summarized in Table 1.

The conditioned biomass prehydrolysate consisted primarily of xylose at a concentration in the range of 3–4% (w/v) and with glucose and arabinose being minor components. This would be fed to the C_5 continuous-flow fermentor (Fig. 2). Cellulose would be hydrolyzed using cellulase and, depending on the solids loading of the digester, the level of glucose in the feed to the C_6 continuous-flow fermentor could be in the range of 60–100 g/L (Fig. 2). The advantage of this type of design is that it allows for optimization by employing different biocatalysts that are best suited for either C_5 or C_6 fermentation.

Pure sugar synthetic media were used in experiments designed to assess the performance of rec Zm AX101 in the prehydrolysate fermentation and either wild-type *Zymomonas* (ZM4) or Alltech's *S. cerevisiae* yeast in the fermentation of the cellulose hydrolysate. For the C_5 fermentation, the feed contained 30 g/L of xylose, 5 or 10 g/L of glucose, and 3.5 g/L of arabinose (with or without 2.5 g/L of HAc) at pH 5.5 and 30°C (Fig. 5). The maximum volumetric productivity for the C_5 fermentor in the absence of acetic acid was 1 g/(L·h) (Table 1). For 85% xylose utilization, D_{max} was 0.06/h and the overall conversion efficiency (based on sugar input) was 90% (Table 1). With 2.5 g/L of HAc in the synthetic biomass hydrolysate feed (1% glu), the maximum volumetric productivity decreased to 0.8 g/(L·h) (D_{max} was 0.04/h); however, the overall conversion efficiency

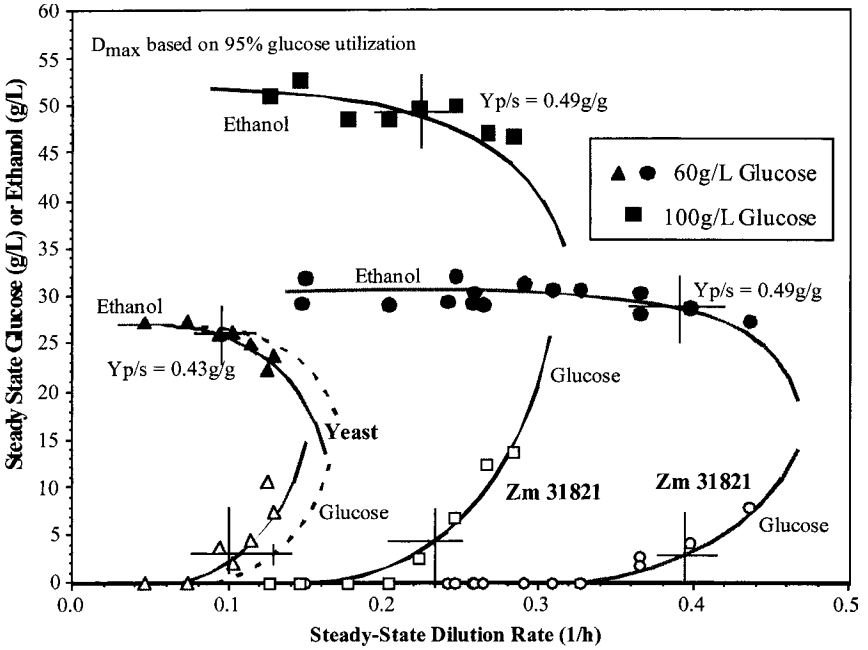

Fig. 6. Steady-state levels of glucose and ethanol as function of dilution rate in continuous fermentations of pure sugar synthetic cellulose hydrolysates. The biocatalyst was either wild-type *Z. mobilis* 31821 or an industrial strain of *S. cerevisiae* yeast. The yeast extract-based medium (*see* Materials and Methods) contained either 60 g/L or 100 g/L of glucose. The pH was controlled at 5.5 for Zm cultures and at 5.0 for yeast fermentations. When acetic acid was added (dashed lines for yeast), the concentration was 2.5 g/L. Lines indicate dilution rate at 85% sugar utilization (*see* Materials and Methods). Fermentation parameters are summarized in Table 1.

remained at the 90% level (Fig. 5 and Table 1). For glucose fermentation in the C_6 fermentor, at a sugar loading of 60 g/L, the wild-type Zm by far outperformed the industrial yeast in terms of both conversion efficiency (96% for Zm vs 84% for Alltech yeast) and productivity (11.2 g/(L·h) for Zm vs 2.6 for the yeast) (Fig. 6 and Table 1). For strain ZM4 and Allyeast the respective D_{max} values were 0.385/h and 0.1/h (Table 1). A particularly interesting observation was the seemingly stimulatory effect of 2.5 g/L of acetic acid (pH 5.0) on the productivity of the yeast fermentation (Fig. 6 and Table 1). The stimulatory effect of low levels of HAc on yeast ethanol productivity has been reported by others *(22,23)*. At a sugar loading of 10% glucose, D_{max} for *Zymomonas* decreased to 0.235/h (Fig. 6), but the productivity remained at 11.5 g/(L·h) (Table 1). In the present work, replacing the more traditional yeast biocatalyst in the C_6 fermentor with wild-type *Zymomonas* resulted in a significant increase in yield and more than a fourfold improvement in productivity (Table 1). It was concluded that, for this type of SHF process (i.e., with separate C_5 and C_6 fermentation), the biocatalyst of choice would be NREL's metabolically engineered stable

integrant Zm AX101 for prehydrolysate fermentation and nontransformed *Z. mobilis* for fermentation of the cellulose hydrolysate. This work supports our previous claims regarding the superior fermentation characteristics of *Zymomonas* in glucose-based fermentations *(24,25)*.

Acknowledgment

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References

1. Hinman, N. D., Wright, J. D., Hoagland, W., and Wyman, C. E. (1989), *Appl. Biochem. Biotechnol.* **20/21,** 391–401.
2. Foody, B. F. and Tolan, J.S. (2001), in *23rd Symposium on Biotechnology for Fuels and Chemicals*, Finkelstein, M. and Davison, B., eds., Breckenridge, Breckenridge, CO, Abstract no. 6–05.
3. Rogers, P. L., Lee, K. J., Skotnicki, M. L. and Tribe, D. E. (1982), *Adv. Biochem. Eng.* **23,** 37–84.
4. Bringer, S., Sahm, H., and Swyzen, W. (1984), *Biotechnol. Bioeng. Symp.* **14,** 311–319.
5. Doelle, H. W., Kirk, L., Crittenden, R., Toh, H., and Doelle, M. (1993), *Crit. Rev. Biotechnol.* **13,** 57–98.
6. Lawford, H. G., Rousseau, J. D., and Tolan, J.S. (2001), *Appl. Biochem. Biotechnol.* **91–93,** 133–146.
7. Lawford, H. G. and Rousseau, J. D. (2002), *Appl. Biochem.Biotechnol.* **98–100,** 429–448.
8. Lawford, H. G. and Rousseau, J. D. (2000), *Appl. Biochem. Biotechnol.* **84–86,** 277–294.
9. Lawford, H. G., Rousseau, J. D., Mohagheghi, A., and McMillan, J. D. (2000), *Appl. Biochem. Biotechnol.* **84–86,** 295–310.
10. Lawford, H. G. and Rousseau, J. D. (2001), *Appl. Biochem. Biotechnol.* **91–93,** 117–131.
11. Tolan, J. S. (1999), in *The Alcohol Textbook,* Jacques, K. A., Lyons, T. P., Kelsall, D. R., eds., Nottingham University Press, Nottingham, UK, pp. 117–128.
12. Timell, T. E. (1964), *Adv. Carbohydr. Chem.* **9,** 247–302.
13. McMillan, J.D.(1994), in *Enzymatic Conversion of Biomass for Fuels Production,* ACS Symposium Series 566, Himmel, M. E., Baker, J. O., and Overend, R. A., eds., American Chemical Society, Washington, DC, pp. 411–437.
14. Lawford, H. G. and Rousseau, J. D. (1994), *Appl. Biochem. Biotechnol.* **45/46,** 437–448 .
15. Lawford, H. G. and Rousseau, J. D. (1993), *Appl. Biochem. Biotechnol.* **39/40,** 687–699.
16. Lawford, H. G., Rousseau, J. D., and McMillan, J. D. (1997), *Appl. Biochem. Biotechnol.* **63–65,** 269–286.
17. Zhang, M., Chou, Y-C., Picataggio, S. K., and Finkelstein, M. (1998), US patent no. 5,843,760.
18. Mohagheghi, A., Evans, K., Chou, Y.-C. and Zhang,M. (2002), *Appl. Biochem. Biotechnol.* **98–100,** 885–898.
19. Roger, P.L. and Tribe, D. E. (1984), US patent no. 4,443,544.
20. Ingledew, W.M. (1999), in *The Alcohol Textbook,* ACS Symposium Series 566, Jacques, K. A., Lyons, T. P., Kelsall, D. R., eds., Nottingham University Press, Nottingham, UK, pp. 49–87.
21. Toon, S., Philippidis, G. P., Ho, N. W. Y., Chen, Z. D., Brainard, A., Lumpkin, R. E., and Riley, C. J. (1997), *Appl. Biochem. Biotechnol.* **63–65,** 243–255.

22. Maiorella, B., Blanch, H. W., and Wolke, C. R. (1983), *Biotechnol. Bioeng.* **25,** 103–111.
23. Vega, J. L., Claussen, E. C., and Gaddy, J. L (1987), *Biotechnol. Bioeng.* **29,** 429–435.
24. Lawford, H. G. (1988), in *VIII International Symposium on Alcohol Fuels,* New Energy and Industrial Technology Development Organization, Tokyo, Japan, pp. 21–27.
25. Beavan, M., Zawadzki, B., Droniuk, R., Fein, J., and Lawford, H. G. (1989), *Appl. Biochem. Biotechnol.* **20/21,** 319–326.