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Performance Testing of *Zymomonas mobilis* Metabolically Engineered for Cofermentation of Glucose, Xylose, and Arabinose

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Abstract

IOGEN Corporation of Ottawa, Canada, has recently built a 40t/d biomass-to-ethanol demonstration plant adjacent to its enzyme production facility. It has partnered with the University of Toronto to test the C6/C5 cofermenta-tion performance characteristics of the National Renewable Energy Labora-tory's metabolically engineered *Zymomonas mobilis* using various biomass hydrolysates. IOGEN's feedstocks are primarily agricultural wastes such as corn stover and wheat straw. Integrated recombinant *Z. mobilis* strain AX101 grows on D-xylose and/or L-arabinose as the sole carbon/energy sources and ferments these pentose sugars to ethanol in high yield. Strain AX101 lacks the tetracycline resistance gene that was a common feature of other recombinant Zm constructs. Genomic integration provides reliable cofermentation performance in the absence of antibiotics, another characteristic making strain AX101 attractive for industrial cellulosic ethanol production. In this work, IOGEN's biomass hydrolysate was simulated by a pure sugar medium containing 6% (w/v) glucose, 3% xylose, and 0.35% arabinose. At a level of 3 g/L (dry solids), corn steep liquor with inorganic nitrogen (0.8 g/L of ammonium chloride or 1.2 g/L of diammonium phosphate) was a cost-effective nutritional supplement. In the absence of acetic acid, the maximum volumetric ethanol productivity of a continuous fermentation at pH 5.0 was 3.54 g/L·h. During prolonged continuous fermentation, the efficiency of sugar-to-ethanol conversion (based on total sugar load) was maintained at >85%. At a level of 0.25% (w/v) acetic acid, the productivity decreased to 1.17 g/L·h at pH 5.5. Unlike integrated, xylose-utilizing rec Zm strain C25, strain AX101 produces less lactic acid as byproduct, owing to the fact that the *Escherichia coli* arabinose genes are inserted into a region of the host chromosome tentatively assigned to the gene for D-lactic acid dehydrogenase. In pH-controlled batch fermentations with sugar mixtures, the order

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of sugar exhaustion from the medium was glucose followed by xylose and arabinose. Both the total sugar load and the sugar ratio were shown to be important determinants for efficient cofermentation. Ethanol at a level of 3% (w/v) was implicated as both inhibitory to pentose fermentation and as a potentiator of acetic acid inhibition of pentose fermentation at pH 5.5. The effect of ethanol may have been underestimated in other assessments of acetic acid sensitivity. This work underscores the importance of employing similar assay conditions in making comparative assessments of biocatalyst fermentation performance.

Index Entries: Genomic integration; recombinant *Zymomonas* AX101; arabinose; xylose; ethanol; biomass hydrolysate; acetic acid; corn steep liquor.

Introduction

For more than 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)*. In our earlier work, we focused on exploiting the superior fermentation characteristics of the bacterium *Zymomonas mobilis (7,8)*, and more recently we have been assessing the cofermentation performance of National Renewable Enertgy Laboratories (NREL's) metabolically engineered *Z. mobilis* with a view to their utility for large-scale cellulosic ethanol production *(9–14)*.

Economic analyses have confirmed that, to be well positioned in the competitive liquid fuels market, cellulosic ethanol has to be produced by the rapid and efficient conversion of all the major sugar components of the hydrolyzed cellulosic feedstock *(15)*. Wild-type *Z. mobilis* is limited to fermenting glucose, fructose, and sucrose. Different research groups have developed recombinant *Z. mobilis* for pentose fermentation *(16–18)*. In NREL's first generation of engineered Zm strains, the ability to ferment the hemicellulose C_5 sugars, namely xylose and arabinose, was accomplished through transformation using plasmid vectors containing a combination of *Eschericia coli* genes including xylose isomerase, xylulokinase, L-arabinose isomerase, L-ribulokinase, L-ribulose-5-phosphate 4-epimerase, transaldolase, and transketolase *(18–20)*. Expression of the added genes was under the control of Zm promoters *(18–21)*. Selection of transformants was facilitated by the incorporation of the gene for tetracycline resistance. Our investigations with these plasmid-bearing strains emphasized the importance of genetic stability when pentose sugars were not the dominant component of the fermentation medium *(13)*. Regulatory restrictions on the large-scale use of antibiotics and cultures carrying plasmid genes for antibiotic resistance were the drivers for the development of stable strains that did not carry antibiotic resistance markers. In the more recent second-generation constructs *(22–24)*, genetic stability has been enhanced in the absence of antibiotic selection through the genomic integration of the exogenous *E. coli* pentose metabolism genes.

IOGEN of 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 50t/d biomass-to-ethanol demonstration plant adjacent to its enzyme production facility *(25, 26)*. 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. IOGEN has partnered with the University of Toronto to test the C_6/C_5 cofermentation performance characteristics of NREL's metabolically engineered *Z. mobilis* using its biomass hydrolysates *(27)*. The "IOGEN process" for biomass depolymerization consists of a dilute sulfuric acid–catalyzed steam explosion at 200–250°C, followed by enzymatic hydrolysis using cellulase enzymes. The process stream coming from the digesters contains about 6% (w/v) glucose, 3% xylose, and 0.35% arabinose, with little sugar oligomers. Biomass hydrolysates contain acetic acid (HAc) by virtue of the presence of the acetylated pentosans in hemicellulose *(28)*. Since HAc represents a major limiting factor to high-performance pentose fermentation *(28)*, the IOGEN process incorporates a proprietary hydrolysate conditioning stage that reduces the HAc level to < 0.25% (w/v) prior to fermentation (C. Nicholson, personal communication).

The purpose of the present study was to assess the fermentation performance characteristics of a selected xylose- and arabinose-fermenting integrated strain, designated by NREL as strain AX101, in batch and continuous pH-controlled fermentations using pure sugar synthetic biomass hydrolysate media with a view to making a comparison with the growth and fermentation characteristics of the previously characterized xylose-fermenting recombinants.

Materials and Methods

Organism

Recombinant *Z. mobilis* strain AX101 (derived from Zm ATCC 39676) *(23)* was obtained from Dr. M. Zhang (NREL, Golden, CO). Stock cultures were stored in glycerol at –70°C, and precultures were prepared as described previously *(29)*.

Fermentation Media

The compositions of the different semisynthetic fermentation media (designated as ZM, ZM1, ZM3, or CSL) are described in Table 1. Yeast extract was obtained from Difco (Detroit, MI). Corn steep liquor (CSL) medium was prepared by dissolving 30 g of CSL dry solids powder (Kingsley and Keith, Longueil, Quebec, Canada) into 1 L of distilled water. The CSL suspension was clarified by centrifugation and then diluted 10-fold with distilled water. Salts were added as described in Table 1. In some experiments, 1.2 g/L of diammonium phosphate was substituted for

Table 1
Zymomonas Media Formulations

Ingredient	Medium designation and formulation			
	ZM	ZM3[a]	ZM1	CSL
Yeast extract (g)	5.0	3.0	1.0	—
CSL (dry solids) (g/L)[b]	—	—	—	3.0
NH_4Cl (g)	—	0.8	0.8	0.8
KH_2PO_4 (g)	3.48	3.48	3.48	3.48
$MgSO_4 \cdot 7H_2O$ (g)	0.5	0.5	0.5	0.5
$FeSO_4 \cdot 7H_2O$ (g)	0.01	0.01	0.01	0.01
Citric acid (g)	0.21	0.21	0.21	0.21
Distilled water (L)	1.0	1.0	1.0	1.0

[a]Reproduced from ref. 3.

[b]CSL dry solids were suspended in distilled water and the liquid was clarified by centrifugation (see Materials andMethods).

0.8 g/L of ammonium chloride to achieve an equivalent amount of nitrogen for growth. 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 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 g/L of glucose in loosely capped 250-mL Erlenmeyer flasks and grown in a water bath shaker overnight at 30°C. This preseed was subcultured into inoculation flasks containing ZM with 30 g/L of glucose, 10 g/L of xylose, and 10 g/L of arabinose and grown in a waterbath shaker overnight at 30°C. This overnight culture was used at a level of ~10% (v/v) to inoculate the batch fermentors. The initial optical density (OD), (1-cm light path at 600 nm) was in the range 0.25–0.5, corresponding to 70–140 mg of dry cell mass (DCM)/L.

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 (200 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 1500mL, 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, 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 either 5.0 or 5.5 and the pH was kept constant by automatic titration with 4 *N* KOH. The temperature was controlled at 30°C using a circulating water bath, and the agitation was moderate (approx 200 rpm). Continuous fermentations were started in the batch mode, and flow was started about 24 h after inoculation (preferably when the residual xylose concentration was <10 g/L) *(29)*.

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 high-performance liquid chromatography as described previously *(10)*. The initial values of lactic acid measured in CSL media were subtracted from the final measurement to obtain lactic acid produced by the fermentation. 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 and xylose). Carbon recovery was calculated based on the method described previously *(30)*.

Results and Discussion

pH-Controlled Batch Fermentations

Comparative Growth Characteristics of Integrated Zm Strain AX101 with Glucose, Xylose or Arabinose as Sole Fermentable Sugars

Figure 1 shows that genomically integrated Zm strain AX101 is capable of growth on either D-xylose or L-arabinose as sole fermentable sugar, albeit at considerably slower rates compared to glucose. Wild-type (i.e., non-transformed) *Z. mobilis* is unable to grow on, or metabolize, pentose sugars. The ability of strain AX101 to grow on and ferment xylose or arabinose supports claim 1 of US patent 5,843,760, which states, in part, "wherein said organism is capable of growing on arabinose and/or xylose, alone or in combination, as the carbon source and fermenting said arabinose and xylose to ethanol" *(24)*. The growth yield for xylose exhibited by strain AX101 (Table 2) compares well with our previous observations with other Zm recombinants, both the plasmid-bearing strain CP4 (pZB5) *(13)* and the integrated strain C25 *(29)*. Of particular interest to us was the improved ethanol yield exhibited by strain AX101 compared with integrated strain C25: 0.49 and 0.46 g/g, respectively *(29)*. In batch fermentations, xylitol (arabitol), lactic acid, and HAc are produced as byproducts (Table 3) that

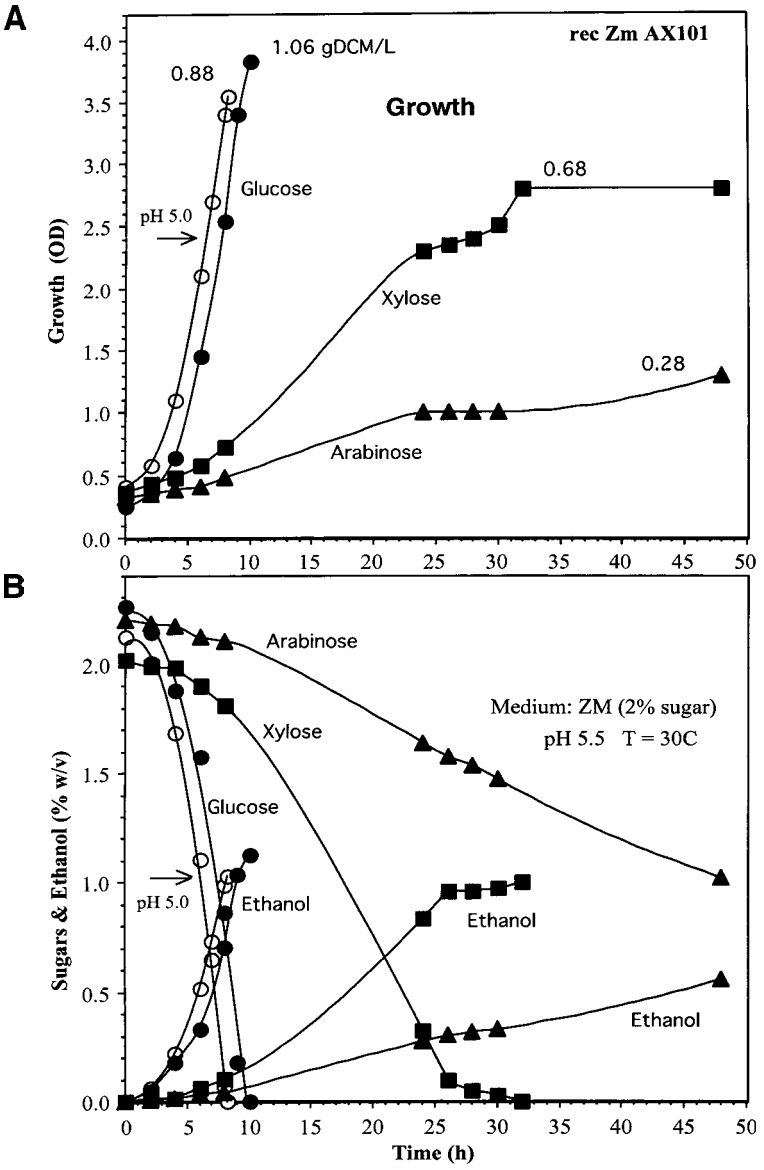

Fig. 1. Growth and fermentation of D-glucose, D-xylose or L-arabinose as sole sugar by integrated Zm strain AX101. **(A)** Growth: time course of culture turbidity; **(B)** time course of sugar utilization and ethanol production. The ZM medium (see Table 1) contained about 2% sugar. The pH was controlled at 5.5 except for glucose fermentations in which it was also controlled at 5.0. The growth and ethanol yields are summarized in Table 2. Concentrations of metabolic end products are given in Table 3.

both lower the ethanol yield and reduce productivity by virtue of the inhibitory effects of xylitol and acetate *(13)*. Compared with integrated strain C25, strain AX101 seems to produce less lactic acid, which can be attributed to the fact that the *E. coli* arabinose genes (araBAD) are inserted into a region

Table 2
Yields for Cell Mass and Ethanol for
Zm AX101 Single Sugar pH-Stat Batch Fermentations [a]

Sugar	Amount (g/L)	Final cell mass (g DCM/L)	Growth yield (g DCM/g sugar)	Ethanol yield (g EtOH/g sugar)
Glucose				
pH 5.0	21.23	0.88	0.041	0.49
pH 5.5	22.60	1.06	0.047	0.50
Xylose	20.17	0.68	0.034	0.50
Arabinose	22.00	0.28	0.024[b]	0.48

[a]Time-courses for growth and sugar consumption are shown in Fig. 1.

[b]Based on sugar consumed at 48 h.

of the host chromosome that is attributed to the gene for D-lactic acid dehydrogenase *(23, 24)*.

Effect of Glucose on Xylose Utilization

Several different types of biomass and wastes are being considered as potential feedstocks for cellulosic ethanol. The composition of the hydrolysate to be fermented is a function of both the type of feedstock and the procedure (e.g., thermochemical and/or enzymatic digestion) by which the cellulose and hemicellulose are hydrolyzed. Previously we have examined the fermentation performance of different Zm recombinants using pure sugar media formulated to mimic the composition of hydrolysates of two agricultural wastes—oat hulls *(27)* and corn stover *(29)*—but 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) *(10,12,14)*.

At IOGEN's demonstration plant, the fermentor feed stream from the digesters contains about 6% (w/v) D-glucose, 3% D-xylose, and 0.35% L-arabinose. After conditioning the hydrolysate, the level of HAc does not exceed 0.25% (w/v) and can be significantly less depending on the efficiency of the detoxification process (C. Nicholson, personal communication). The pH-stat batch fermentations shown in Fig. 2 were designed to examine the effect on AX101 growth and fermentation performance caused by varying the amount of glucose in a nutrient-rich medium containing a fixed concentration of xylose (3%) and arabinose (0.35%). In terms of growth (Fig. 2A), the final cell density was directly proportional to the level of glucose in the medium (see inset in Fig. 2B); however, in terms of the rate of xylose utilization, only a small addition of glucose proved to be the most beneficial (Fig. 2C). Note also that AX101 performed well with equal concentrations of xylose and glucose, which augers well for corn fiber fermentations in which the levels of glucose and xylose are similar. It is important to understand that these fermentations were conducted in the absence of

Table 3
Summary of Growth and Fermentation Parameters for ZM AX101 pH-Stat Batch Fermentations [a]

Conditions					Cell			Lactic			proc	Conversion	Carbon
Medium[b]		G/X/A (% [w/v])	HAc[c] (g/L)	pH	mass (g DCM/L)	EtOH (g/L)	Xylitol (g/L)	Acid (g/L)	HAc[d] (g/L)	Q_p [e] (g/ [L·h])	$Y_{p/s}$ [f] (g/g)	efficiency (%)	recovery (%)[g]
Fig. 1	ZM3	2/0/0	0	5.0	0.88	10.34	0.00	0.07	0.09	0.47	0.49	95	101
	FM	2/0/0	0	5.5	1.06	11.24	0.00	0.07	0.04	1.18	0.50	98	103
	ZM	0/2/0	0	5.5	0.68	10.04	0.28	0.18	0.73	0.31	0.50	98	110
	ZM	0/0/2	0	5.5	0.28	5.63	0.00	0.04	0.50	0.04	0.26	50	103
Fig. 2	ZM	0/3/0	0	5.5	0.96	15.28	0.35	0.17	1.20	0.45	0.50	98	111
	ZM	0.5/0.35	0	5.5	1.10	19.45	0.84	0.00	0.40	0.41	0.49	96	106
	ZM3	3/3/0.35	0	5.5	1.48	27.06	1.30	1.05	1.37	0.56	0.44	87	107
	ZM3	6/3/0.35	0	5.5	1.91	41.40	2.01	0.04	0.38	0.86	0.46	90	101
Fig. 3	ZM	4/4/2	0	5.5	1.80	46.33	3.35	0.21	0.84	0.97	0.46	91	106
Fig. 4	ZM3	6/3/0.35	0	5.5	1.91	41.40	2.01	0.04	0.38	0.89	0.46	90	101
	ZM1	6/3/0.35	0	5.5	1.49	42.17	1.85	0.03	0.27	0.88	0.46	90	100
	CSL	6/3/0.35	0	5.5	1.45	41.37	1.89	0.07	0.51	0.86	0.46	91	99
Fig. 5	CSL	6/3/0.35	0	5.5	1.45	41.37	1.89	0.07	0.51	0.86	0.46	91	99
			0.5	5.5	1.35	37.54	1.59	2.92	0.55	0.78	0.41	80	103
			2.0	5.5	1.20	35.18	1.96	0.75	0.76	0.73	0.38	75	100
Fig. 6	ZM	0.5/3/0.35	0	5.5	1.10	16.72	1.04	0.11	0.27	0.70	0.49	96	106
			0+3%E	5.5	1.10	18.08	1.35	0.00	0.35	0.38	0.45	88	108
			2.5	5.5	0.69	17.83	1.00	0.02	0.10	0.37	0.46	91	110
			2.5 +3%E	5.5	0.57	11.65	1.42	0.07	0.50	0.24	0.31	60	106

[a] DCM, dry cell mass; E, ethanol added (% [w/v]); EtOH, ehtanol produced (% [w/v]); HAc. acetic acid; CSL, corn steep liquor.
[b] Designation and composition are as described in Table 1 with G/X/A as the ratio of sugars of d-glucose: d-xylose: l-arabinose (% [w/v]).
[c] HAc added to medium.
[d] HAc produced during fermentation.
[e] Volumetric ethanol productivity based on ethanol at 48 h or when sugars were completely consumed.
[f] Process yield based on total sugars in the medium.
[g] Method for calculating carbon recovery as described previously *(30)*.

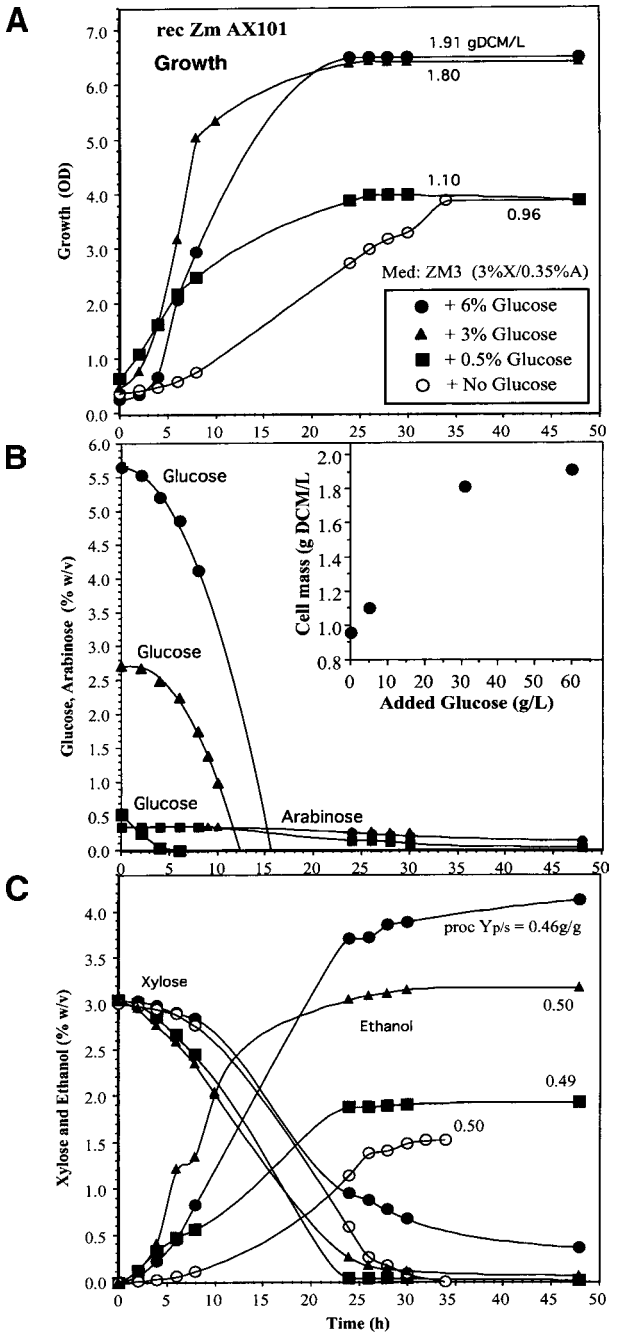

Fig. 2. Effect of C_6:C_5 ratio and amount of glucose on pentose fermentation by Zm AX101. **(A)** growth, **(B)** glucose and arabinose utilization; **(C)** xylose utilization and ethanol production. The medium contained 3% (w/v) xylose, 0.35% arabinose, and different levels of glucose (0, 0.5, 3, and 6%). The inset in 2 (B) shows the final DCM concentration as a function of the amount of glucose added to the medium. The pH was controlled at 5.5 and the temperature was 30°C. Growth and fermentation parameters, including concentrations of byproducts, are summarized in Table 3.

HAc in the medium and therefore are not representative of true fermentation performance in unconditioned biomass hydrolysate media or even conditioned hydrolysates in which the efficiency of HAc removal is <100%.

Performance of AX101 in Nutrient-Rich Synthetic Corn Fiber Hydrolysate

Corn fiber hydrolysate has a relatively high arabinose content *(31)*. The yeast extract–based, pure sugar synthetic corn fiber hydrolysate medium contained 4% (w/v) glucose, 4% xylose, and 2% arabinose, but without any HAc. Strain AX101 exhibited good growth and fermentation performance with the corn fiber hydrolysate medium (Fig. 3). Despite the synthesis of significant amounts of xylitol (arabitol) and HAc, the sugar-to-ethanol conversion efficiency was >90%, and the ethanol productivity was close to 1 g/(L·h) (Table 3). These observations are similar to others using this same strain *(32)* or closely related integrated Zm strains *(33)*.

Substituting CSL for Yeast Extract as a Nutritional Supplement

Yeast extract is useful for laboratory work, but it is far too expensive for large-scale production of bulk chemicals. Our previous work with rec Zm has shown that CSL is a cost-effective source of nutrients *(9,10,27)*. Figure 4 shows batch fermentations in which the level of yeast extract in the synthetic IOGEN hydrolysate was reduced from 3 to 1g/L and in which the yeast extract was replaced with 3 g/L of CSL (see Materials and Methods). In this work, we used dry solids CSL, and 3 g/L is equivalent to 0.5% (v/v) supplementation using liquid whole CSL at 50% solids. Although both a reduction in the level of yeast extract and substitution with CSL result in a lower cell density (Fig. 4A), the sugar utilization profiles for strain AX101 with all three media were almost identical (Fig. 4B). The sugar-to-ethanol conversion efficiency was 90% and the ethanol productivity was 0.86–0.88 g/(L·h) (Table 3). Similar amounts of byproducts were produced with the three different media (Table 3). It has been estimated that the economic impact on large-scale ethanol production of using CSL as a nutritional supplement together with inorganic nitrogen is 4.5¢/US gal of ethanol *(14,27)*. However, it is important to emphasize that this is merely a cost estimate because actual costs will depend on availability, proximity of source, and other competitive markets for CSL.

Effect of HAc on Yield and Productivity

The IOGEN process involves conditioning the hydrolysate to reduce the level of HAc. Since the level of HAc in the hydrolysate will depend in part on the efficiency of the conditioning process, we chose to examine the effect of HAc at the anticipated limits of the range of acetate concentrations after conditioning: 0.05% (w/v) and 0.25%. Although in the absence of HAc, strain AX101 performs well at pH 5.0, in fermentations with HAc in the medium, the pH was controlled at 5.5 in order to reduce the inhibitory effect of the HAc *(34)*. The inhibitory effect of HAc on *Zymomonas* is known to be more severe for xylose utilization compared to glucose *(34, 35)*. Using the CSL-based synthetic IOGEN hydrolysate medium, the addition of

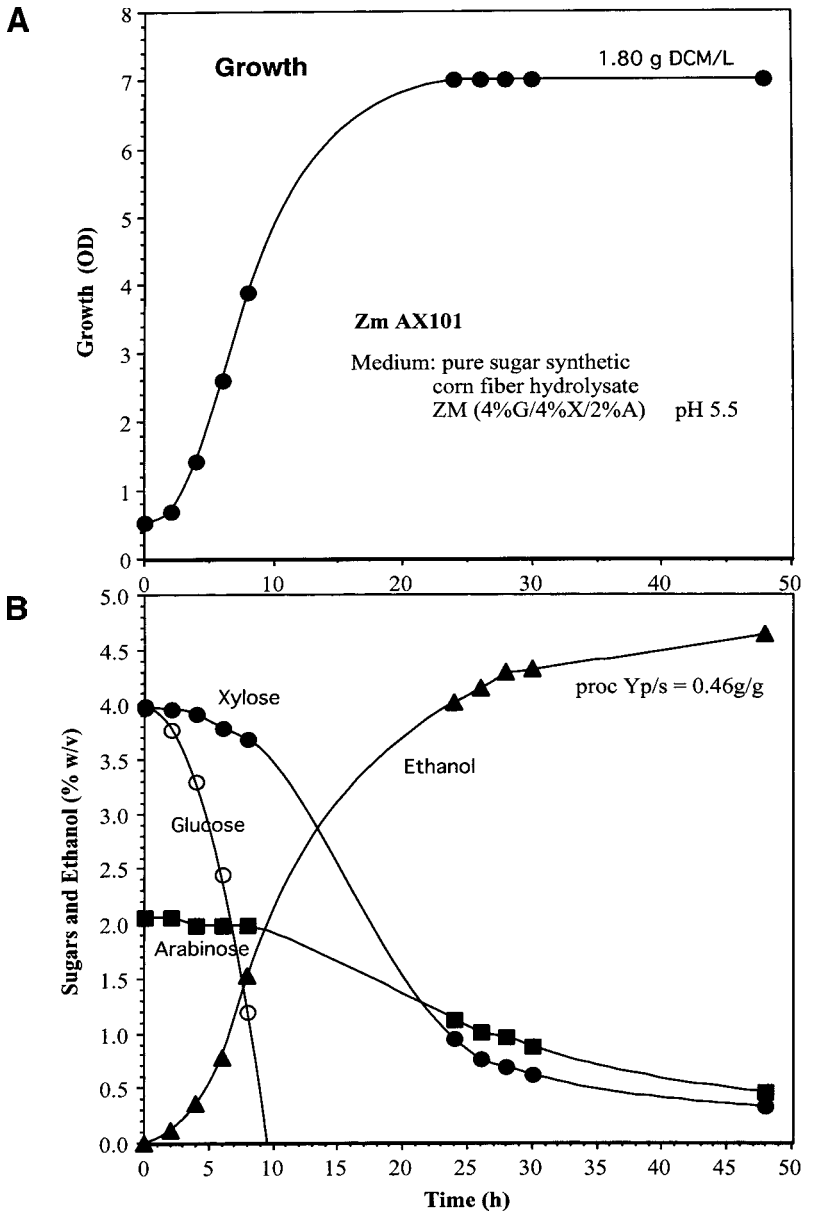

Fig. 3. Growth and fermentation performance of integrated Zm AX101 in arabinose-rich synthetic corn fiber hydrolysate. **(A)** growth; **(B)** sugar utilization and ethanol production. There was no HAc in the ZM medium (4% glucose, 4% xylose, 2% arabinose). The pH was controlled at 5.5. Growth and fermentation parameters, including concentrations of byproducts, are summarized in Table 3.

0.05% (w/v) HAc had almost as detrimental an effect on xylose utilization as the addition of 0.2% HAc (Fig. 5). The overall sugar-to-ethanol conversion efficiency decreased from 91% in the control without HAc to 80 and 75% with 0.05 and 0.2% HAc, respectively (Table 3). The ethanol productivity

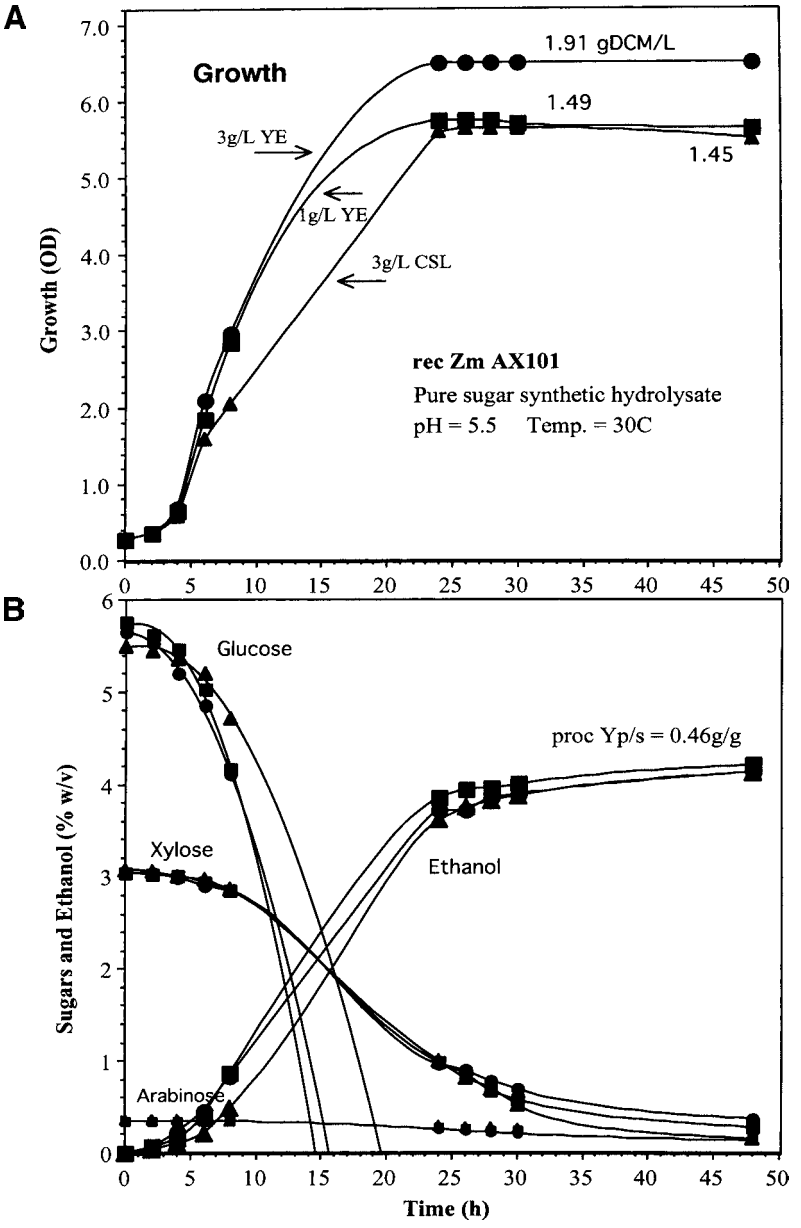

Fig. 4. Effect of nutrient supplementation on growth and fermentation performance of Zm AX101 in pure sugar synthetic hydrolysate. **(A)** growth; **(B)** sugar utilization and ethanol production. Growth and fermentation parameters, including concentrations of byproducts, are summarized in Table 3. YE, yeast extract.

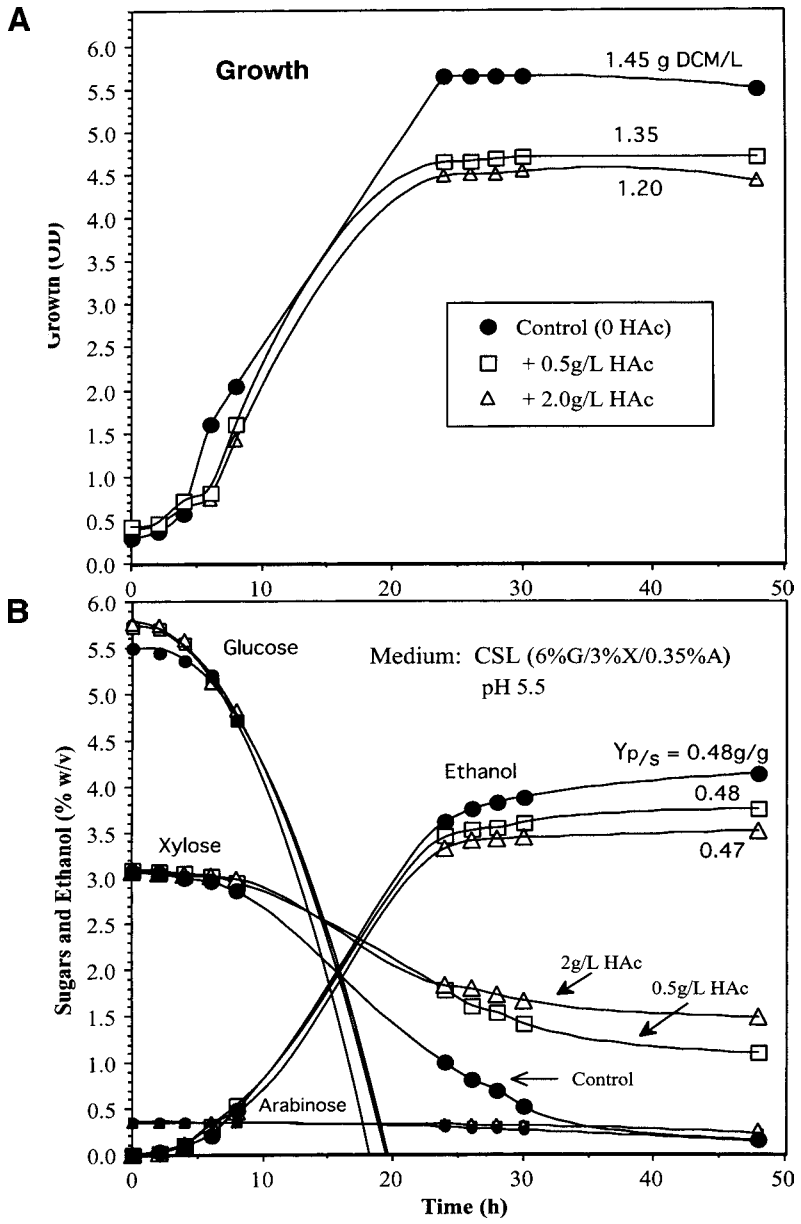

Fig. 5. Effect of HAc on growth and fermentation performance of Zm AX101 in pure sugar synthetic hydrolysate. **(A)** growth; **(B)** sugar utilization and ethanol production. The CSL-based medium (6% glucose, 3% xylose, and 0.35% arabinose) was supplemented with different amounts of HAc (0, 0.5, and 2.5 g/L). The pH was controlled at 5.5. Growth and fermentation parameters, including concentrations of byproducts, are summarized in Table 3.

decreased from 0.86 without HAc to 0.73 g/(L·h) with 0.2% HAc (Table 3). The inhibition by HAc was exacerbated by the fact that strain AX101 produces about 0.05% HAc as a byproduct (Table 3).

Inhibitory Effect of Ethanol and HAc on AX101 Pentose Fermentation

The inhibition caused by low levels of HAc (0.5 g/L) (Fig. 5B) was not anticipated from other experiments that we had conducted with media containing lesser amounts of glucose (unpublished results), and this prompted us to examine the possible effect of ethanol in potentiating the effect of HAc. To test the effects of ethanol and HAc on xylose utilization (secondarily on arabinose utilization), we used nutrient-rich synthetic hydrolysate medium with a reduced level of glucose (0.5% glucose) and standard levels of both xylose (3%) and arabinose (0.35%) similar to the medium used in the experiment shown in Fig. 2. Since the expected yield of ethanol from 6% glucose is about 3% after 16 h of elapsed batch fermentation, this same amount of ethanol was added to the medium to assess the effect of ethanol *per se* on xylose (and arabinose) utilization (Fig. 6). In the context of the effect of ethanol on xylose fermentation by recombinant ZM, studies with NREL's plasmid-bearing strains CP4 (pZB5) and ZM4 (pZB5) showed that at an ethanol concentration of about 5.5–6.0% (w/v), xylose utilization was completely inhibited *(11,36)*. In this work, the amount of HAc used in these batch fermentations was 2.5 g/L (Fig. 6). The HAc had a more pronounced inhibitory effect on growth than did the ethanol (Fig. 6A), although the combination of HAc and ethanol inhibited growth much more than the HAc alone (Fig. 6A). At the concentrations and pH used in this series of experiments, HAc and ethanol each produced about the same effect on the rate of xylose and arabinose utilization (Fig. 6B), but significantly, the inhibition by even a lesser amount of HAc (2 g/L) caused a more pronounced inhibition of xylose utilization when the medium contained 6% glucose rather than the 0.5% that was used in this assay (Figs. 5 and 6). However, the inhibition of xylose utilization caused by the combination of ethanol and HAc (Fig. 6B) resembles the xylose utilization trajectories of Fig.5B. From this observation, we conclude that ethanol potentiates the inhibitory effect of HAc on pentose utilization. Hence, when assessing the sensitivity of a particular strain to HAc, it is important that the assay conditions be similar, with respect to both pH and ethanol concentration—the latter being primarily a function of the glucose concentration since this sugar is metabolized much faster than the C_5 sugars.

Continuous Fermentations with rec Zm Strain AX101

Since IOGEN is contemplating a continuous fermentation process *(26)*, it was important to assess the performance of the integrated recombinant in the continuous fermentation mode. Like integrated strain C25, strain AX101 lacks the tetracycline resistance gene, making it compatible with current regulatory requirements. Figure 7 shows the continuous fermentation of a pure sugar synthetic IOGEN hydrolysate using strain AX101. The

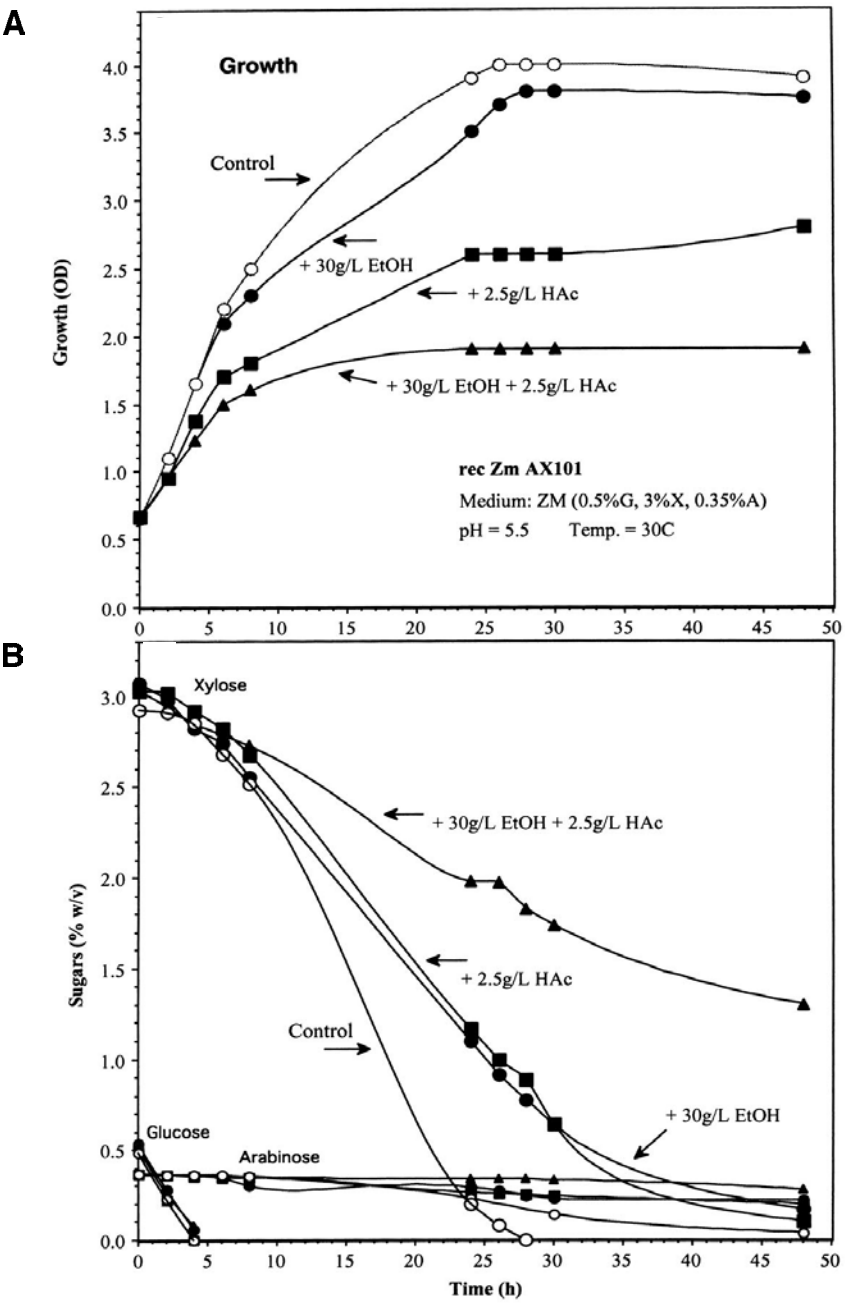

Fig. 6. Separate and combined effects of ethanol and HAc on xylose and arabinose fermentation by Zm AX101. **(A)** growth; **(B)** sugar utilization and ethanol production. The CSL-based medium (0.5% glucose, 3% xylose, and 0.35% arabinose) was supplemented with 3% (w/v) ethanol and/or 0.25% (w/v) HAc. The pH was controlled at 5.5. Growth and fermentation parameters, including concentrations of byproducts, are summarized in Table 3.

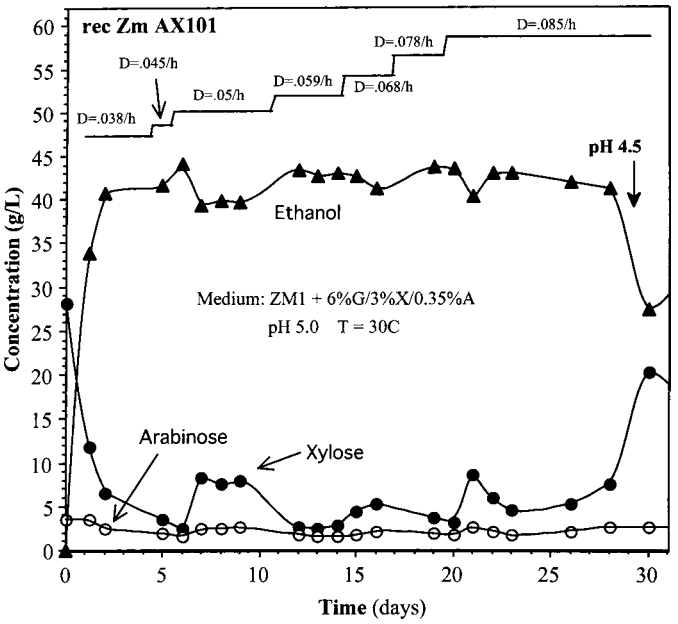

Fig. 7. Time course of continuous fermentation of pure sugar synthetic hydrolysate by integrated Zm strain AX101. The ZM1 medium (Table 1) contained 6% glucose, 3% xylose, and 0.35% arabinose. The pH was controlled at 5.0, except where indicated. The dilution rate (D) was increased incrementally over the range of 0.038/h to 0.085/h. Glucose was not detected in the fermentor effluent stream. The steady-state fermentation parameters are summarized in Table 4.

ZM1 medium (Table 1) contained 6% glucose, 3% xylose, and 0.35% arabinose. The pH was controlled at 5.0 and the temperature at 30°C. Over the 30 d that the chemostat was operated, the dilution rate was increased in small increments from 0.038 to 0.085/h (Fig. 7). After 28 d, we experienced a problem with the pH controller, and, as a consequence, the pH fell and stabilized at 4.5 (Fig. 8A). We decided to include this datum even though better performance could most likely have been achieved had the dilution rate been simultaneously reduced at pH 4.5. At pH 5.0, the sugar-to-ethanol conversion efficiency varied over the dilution range from 85 to 90% (Table 4). Figure 8A shows the steady-state levels of cell mass, ethanol, xylose, and arabinose; glucose was not detected in the effluent over this time period except when the pH was 4.5 (results not shown). The increased cell mass at the higher D values probably reflects the wall growth that proved difficult to control after 3 to 4 wk of continuous operation. Both the volumetric ethanol productivity and specific productivity increased linearly with increased steady-state dilution rate, with the more erratic behavior in terms of q_p at the higher D values likely owing to wall growth (Fig. 8B). The maximum ethanol productivity at pH 5.0 was 3.54 g/(L·h) (Fig. 8B). The levels of by-products produced by this continuous culture are summarized in Table 4.

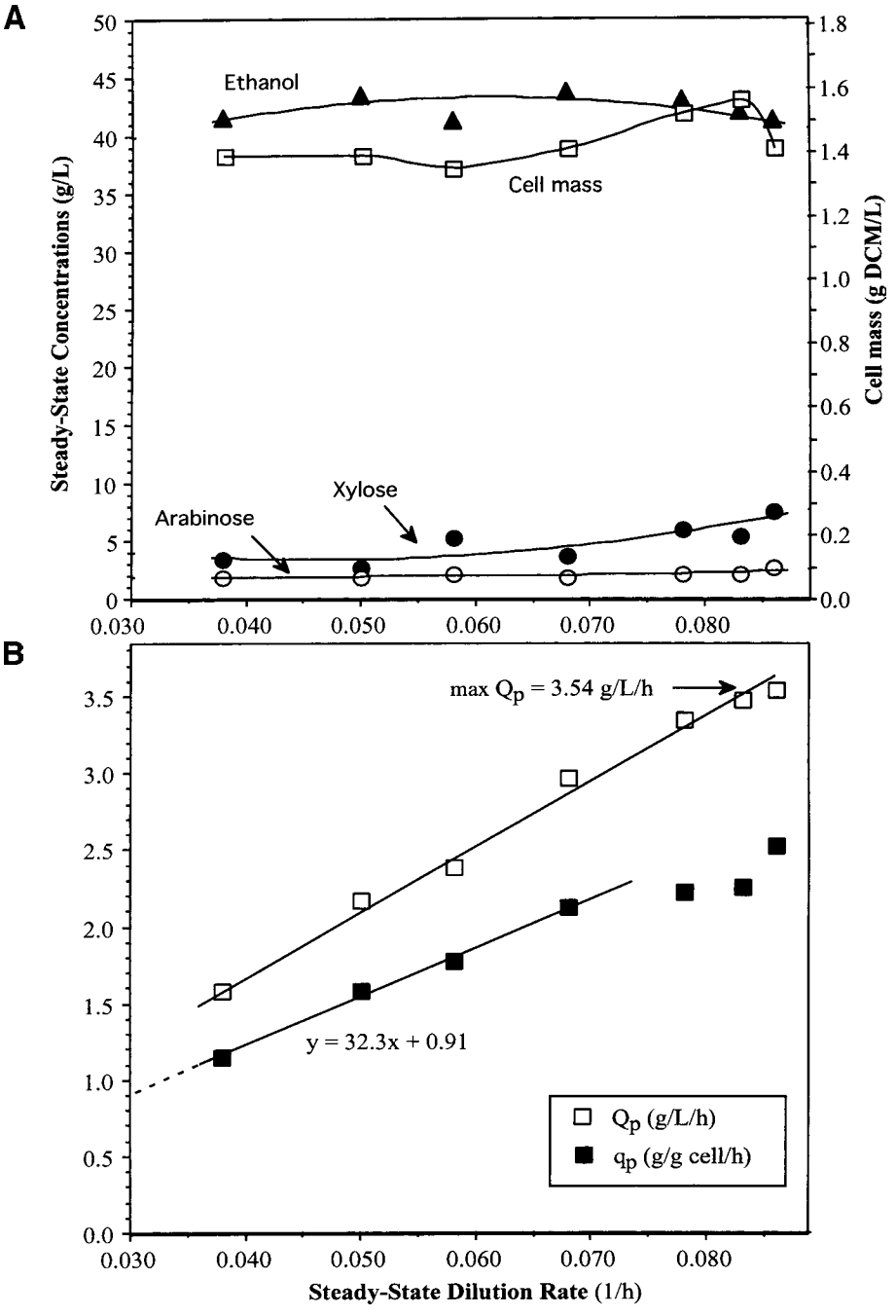

Fig. 8. Steady-state conditions in continuous fermentation of pure sugar synthetic hydrolysate by integrated Zm strain AX101. **(A)** Steady-state concentrations of cell mass, ethanol, xylose and arabinose (glucose was not detected) as a function of dilution rate; **(B)** volumetric and specific ethanol productivities as a function of dilution rate. Data were taken from the experiment shown in Fig. 7. The steady-state fermentation parameters are summarized in Table 4.

Table 4
Steady-State Growth and Fermentation Parameters for Zm AX101 in Continuous Cofermentations Using Synthetic Hydrolysate[a]

	Effluent sugars		Fermentation products						Productivity				Yield	
PH	[Xyl] (g/L)	[Ara] (g/L)	Cell Mass (g DCM/L)	EtOH (g/L)	Xylitol (g/L)	Lactic Acid (g/L)	Glycerol (g/L)	HAc (g/L)	D[b] (h-1)	Q_p (g/[L·h])	q_p (g/[g cell·h])	proc $Y_{p/s}$[c]	Conversion efficiency (%)	Carbon[d] recovery (%)
Batch														
5.0	11.8[e]	3.5[e]	1.67	33.85	0.96	0.01	0.17	0.21	0.000	—	—	0.36	70.1	102
Continuous														
5.0	3.5	1.9	1.38	41.61	0.99	0.30	0.11	0.65	0.038	1.58	1.15	0.452	88.6	99
5.0	2.7	1.8	1.38	43.47	0.87	0.32	0.00	0.74	0.050	2.17	1.58	0.464	90.0	100
5.0	5.3	2.2	1.34	41.23	1.16	0.88	0.00	0.66	0.058	2.39	1.78	0.444	87.0	100
5.0	3.7	1.9	1.40	43.7	0.92	0.70	0.00	0.63	0.068	2.97	2.12	0.463	90.7	101
5.0	6.0	2.2	1.51	43.01	1.34	0.74	0.00	0.52	0.078	3.35	2.22	0.457	89.6	103
5.0	5.4	2.2	1.55	41.96	1.04	0.87	0.21	0.66	0.086	3.54	2.53	0.438	85.9	102
4.5	20.4	2.7	1.0	27.5	0.9	0.19	0.24	0.36	0.086	2.47	2.45	0.291	57.0	100
5.5	2.6	1.7	1.25	41.84	0.48	8.01	0.00	2.78[f]	0.28	1.17	0.94	0.454	89.0	104

[a] Rec Zm strain AX101 in medium ZM1 (6% Glu; 3% Xyl: 0.35% Arab).
[b] D = steady-state dilution rate (at least three volume changes).
[c] *Process* ethanol yield based on total fermentable sugar in fermentor feed.
[d] Method for calculating carbon recover as described previously. *(30)*
[e] Residual levels of xylose and arabinose when flow started after 29 h of elapsed batch fermentation time.
[f] Separate experiment with 2.5 g/L of HAc added to the feed medium.

In a separate continuous fermentation, 0.25% (w/v) HAc was added to the feed and the pH was raised from 5.0 to 5.5 to reduce the inhibitory effect of the HAc. With 0.25% HAc added to the synthetic hydrolysate medium, the volumetric productivity at a dilution rate of 0.028/h was 1.17 g/(L·h), which was about one-third the value of the maximum productivity in the absence of HAc (Table 4). At $D = 0.028$/h, the sugar-to-ethanol conversion efficiency was 89% (Table 4). Increasing the dilution rate caused a drastic reduction in process yield owing to the appearance of xylose in the effluent (results not shown). These observations on the effect of HAc are similar to those we observed previously with integrated strain C25 *(29)*. These results suggest that strain AX101 could be used for continuous fermentations at the sugar ratio and loading used in this study provided that the dilution rate does not exceed about 0.03/h. Similar observations with respect to the effect of HAc on AX101 have been reported by others *(32)*.

Conclusion

Although integrated Zm strain AX101 has the capacity to ferment arabinose, this feature was not exploited in this work because of the relatively low level of arabinose in IOGEN's hydrolysate. The reduction in the amount in byproducts represents a significant improvement in strain AX101 relative to integrated strain C25. Sensitivity to inhibition by HAc remains a major obstacle to productivity with both of the integrated strains that we have tested. Inhibition of xylose utilization by HAc is exacerbated by ethanol, and, consequently, hydrolysates with HAc and high levels of glucose are problematic. In the future, we plan to investigate a two-stage fermentation process with ethanol recovery following glucose fermentation in the first stage, a design that reduces the ethanol limitation on pentose fermentation in the second stage.

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References

1. Charley, R. C., Fein, J. E., Lavers, B. H., Lawford, H. G., and Lawford, G.R. (1983), *Biotechnol. Letts.* **5,** 164–174.
2. Stevnsborg, N. and Lawford, H. G. (1986), *Appl. Microbiol. Biotechnol.* **25,** 106–115
3. Lawford, H.G. (1988), *Appl. Biochem. Biotechnol.* **17,** 203–209
4. Lawford, H.G. (1988), *VIII International Symposium on Alcohol Fuels*, New Energy and Industrial Technology Development Organization, Sanbi Insatsu Co., Tokyo, Japan, pp. 21–27.

5. Beavan, M., Zawadzki, B., Droniuk, R., Fein, J., and Lawford, H. G. (1989), *Appl. Biochem. Biotechnol.* **20/21,** 319–326
6. 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, Canada, pp. 411–416.
7. Lawford, H. G. (1987), US patent no. 4,647,534.
8. Laword, H. G. (1989), US patent no. 4,812,410.
9. Lawford, H. G. and Rousseau, J. D. (1997), *Appl. Biochem. Biotechnol.* **63–65,** 287–304.
10. Lawford, H. G., Rousseau, J. D., Mohagheghi, A., and McMillan, J. D. (1998), *Appl. Biochem. Biotechnol.* **70–72,** 353–368.
11. Lawford, H. G. and Rousseau, J. D. (1999), *Appl. Biochem. Biotechnol.* **77–79,** 235–249.
12. Lawford, H. G., Rousseau, J. D., Mohagheghi, A., and McMillan, J. D. (1999), *Appl. Biochem Biotechnol.* **77–79,** 191–204
13. Lawford, H. G. and Rousseau, J. D. (2000), *Appl. Biochem. Biotechnol.* **84–86,** 277–294
14. Lawford, H. G., Rousseau, J. D., Mohagheghi, A., and McMillan, J.D. (2000), *Appl. Biochem. Biotechnol.* **84–86,** 295–310.
15. Hinman, N. D., Wright, J. D., Hoagland, W., and Wyman, C. E. (1989), *Appl. Biochem. Biotechnol.* **20/21,** 391–401.
16. Sprenger, G. A. (1993), *J. Bacteriol.* **27,** 225–237.
17. Feldman, S. D., Sahm, H., and Sprenger, G. A. (1992), *Appl. Microbiol.* **38,** 354–361.
18. Zhang, M., Eddy, C., Deanda, K., Finkelstein, M., and Picataggio, S. K. (1995), *Science* **267,** 240–243.
19. Picataggio, S., Zhang, M., Eddy, C. K., Deanda, K., and Finkelstein, M. (1996), US patent no. 5,514,583.
20. Picataggio, S. K., Zhang, M., Eddy, C. K., Deanda, K., and Finkelstein, M (1998), US patent no. 5,726,053
21. Deanda, K. A., Eddy, C., Zhang, M., and Picataggio, S. (1996), *Appl. Environ. Micro.* **62,** 4465–4470.
22. Zhang, M., Chou, Y. C., Lai, X. K., Milstrey, S., Danielson, N., Evans, K., Mohagheghi, A., and Finkelstein, M. (1999), Abstract no. 2–16.
23. Zhang, M., Chou, Y. C., Mohagheghi, A., Evans, K., Milstrey, S., Lai, X. K., and Finkelstein, M. (2000) Abstract no. 2–03.
24. Zhang, M., Chou, Y-C., Picataggio, S. K., and Finkelstein, M. (1995), US patent no. 5,843,760.
25. Foody, B. F. and Tolan, J. S. (2000), Abstract no. 6–07.
26. Foody, B. F. and Tolan, J. S. (2001), Abstract no. 6–05.
27. Lawford, H. G., Rousseau, J. D., and Tolan, J. S. (2001), *Appl. Biochem. Biotechnol.* **91–93,** 133–146.
28. 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.
29. Lawford, H. G. and Rousseau, J. D. (2001), *Appl. Biochem. Biotechnol.* **91–93,** 117–131.
30. Lawford, H. G. and Rousseau, J. D. (1995), *Appl. Biochem. Biotechnol.* **51/52,** 179–195.
31. Mohagheghi, A., Evans, K., Finkelstein, M., and Zhang, M. (1998), *Appl. Biochem. Biotechnol.* **70–72,** 285–299.
32. Mohagheghi, A., Evans, K., Chou, Y. C., and Zhang, M. (2002), *Appl. Biochem. Biotechnol.* **98–100,** 885–898.
33. Dennison, E. and Abbas, C. (2000), Abstract no. 2–04.
34. Lawford, H. G. and Rousseau, J. D. (1998), *Appl. Biochem. Biotechnol.* **70–72,** 161–172.
35. Joachimsthal, E. L. and Rogers, P. L. (2000), *Appl. Biochem. Biotechnol.* **84–86,** 343–356.
36. Joachimsthal, E., Haggett, K. D., and Rogers, P. L. (1999), *Appl. Biochem. Biotechnol.* **77–79,** 147–157.