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Comparative Ethanol Productivities of Different *Zymomonas* Recombinants Fermenting Oat Hull Hydrolysate

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Abstract

Iogen Corporation of Ottawa, Canada, has recently built a 50 t/d biomass-to-ethanol demonstration plant adjacent to its enzyme production facility. Iogen has partnered with the University of Toronto to test the C6/C5 cofermentation performance characteristics of National Renewable Energy Laboratory's metabolically engineered *Zymomonas mobilis* using its biomass hydrolysates. In this study, the biomass feedstock was an agricultural waste, namely oat hulls, which was hydrolyzed in a proprietary two-stage process involving pretreatment with dilute sulfuric acid at 200–250°C, followed by cellulase hydrolysis. The oat hull hydrolysate (OHH) contained glucose, xylose, and arabinose in a mass ratio of about 8:3:0.5. Fermentation media, prepared from diluted hydrolysate, were nutritionally amended with 2.5 mL/L of corn steep liquor (50% solids) and 1.2 g/L of diammonium phosphate. The estimated cost for large-scale ethanol production using this minimal level of nutrient supplementation was 4.4¢/gal of ethanol. This work examined the growth and fermentation performance of xylose-utilizing, tetracycline-resistant, plasmid-bearing, patented, recombinant *Z. mobilis* cultures: CP4:pZB5, ZM4:pZB5, 39676:pZB4L, and a hardwood prehydrolysate-adapted variant of 39676:pZB4L (designated as the "adapted" strain). In pH-stat batch fermentations with unconditioned OHH containing 6% (w/v) glucose, 3% xylose, and 0.75% acetic acid, rec Zm ZM4:pZB5 gave the best performance with a fermentation time of 30 h, followed by CP4:pZB5 at 48 h, with corresponding volumetric productivities of 1.4 and 0.89 g/(L·h), respectively. Based on the available glucose and xylose, the process ethanol yield for both strains was 0.47 g/g (92% conversion efficiency). At 48 h, the

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process yield for rec Zm 39676:pZB4L and the adapted strain was 0.32 and 0.34 g/g, respectively. None of the test strains was able to ferment arabinose. Acetic acid tolerance appeared to be a major determining factor in cofermentation performance.

Index Entries: Recombinant *Zymomonas*; oat hull hydrolysate; xylose; biomass hydrolysate; ethanol yield; acetic acid; productivity.

Introduction

Iogen Corporation 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.

In April 2000, Iogen started operations of a 50-t/d biomass-to-ethanol demonstration plant *(1,2)*. The plant will produce ethanol from oat hulls, corn stover, and wheat straw. The plant is located at the site of Iogen's enzyme plant, which 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.

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 contains monomers of glucose, xylose, and arabinose in a mass ratio of about 8:3:0.5, with little sugar oligomers.

Yield and productivity are the key technoeconomic parameters in the production of fuel ethanol from biomass and wastes *(3)*. The fermentation of xylose to ethanol is important in a biomass-to-ethanol process because xylose fermentation has the potential to increase the ethanol yield by up to 50% at little additional cost *(3)*. Several microbes that have been engineered to ferment xylose to ethanol (for a review, *see* refs. *4* and *5*). In a survey of industrial biocatalysts 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 *(6,7)*. For example, *Z. mobilis* offers the advantage of having generally-regarded-as-safe status; however, wild-type strains are not capable of fermenting the pentose sugars that are produced during the hydrolysis of the hemicellulose component of cellulosic feedstocks. The impediment to exploiting the superior fermentation characteristics of *Zymomonas* was removed by the construction of recombinant strains that expressed *Escherichia coli* genes for pentose metabolism *(8,9)*. At the University of Toronto, we have been conducting a systematic physiological assessment of different National Renewable Energy Laboratory (NREL) recombinant strains using synthetic hydrolysates prepared with pure chemicals, and our observations with both batch and continuous systems have been the subject of

several presentations at previous symposia *(10–17)*. In anticipation of this plant start-up, Iogen collaborated with the University of Toronto to determine the capabilities of the NREL recombinant *Zymomonas* strains to ferment xylose from Iogen's sugar stream to ethanol.

The purpose of the present study was to compare the batch fermentation performance characteristics of several different xylose-utilizing recombinants of *Z. mobilis* using appropriately diluted biomass hydrolysate that had been nutritionally amended in a cost-effective fashion. Iogen's demonstration plant is configured for both batch and continuous fermentations, but this laboratory study was carried out using batch culture. This study focused on one of Iogen's feedstocks, oat hulls, as the first that was obtained by Iogen in large quantities.

Materials and Methods

Microorganisms

Xylose-utilizing *Z. mobilis* recombinants 39676:pZB4L *(8)*, a hardwood hydrolysate-adapted variant of 39676:pZB4L (designated as rec Zm "adapted") *(13,15)*, CP4:pZB5 *(8)*, and ZM4:pZB5 (also known as 31821:pZB5) *(18)* were obtained from Min Zhang at NREL. Cryovials of frozen concentrated stock culture were maintained in RM medium (10 g/L of yeast extract and 2 g of KH_2PO_4) *(19)* supplemented with 10 mg/L of tetracycline (Tc) and 15% (w/w) glycerol at –70°C.

Preparation of Inoculum

A 1-mL aliquot of a glycerol preserved culture was removed from cold storage (freezer) and transferred to about 100 mL of modified RM medium (5 g/L of yeast extract and 2 g/L of KH_2PO_4) containing about 20 g/L of xylose and 20 g/L of glucose supplemented with Tc (10 mg/L) in 125-mL screw-cap flasks and grown statically overnight in a 30°C incubator. This preseed was subcultured into inoculation flasks containing modified RM with 20 g/L of glucose, 20 g/L of xylose, and 10 mg/L of Tc and grown overnight in a 30°C shaker. This overnight culture was used at a level of ~10% (v/v) to inoculate the batch fermentors. The initial optical density (1-cm light path at 600 nm) was in the range of 0.2–0.25, corresponding to 60–75 mg of dry cell mass (DCM)/L.

Oat Hull Hydrolysate

The oat hull hydrolysate (OHH) was prepared from oat hulls (Quaker Oat, Peterborough, Ontario, Canada) by the Iogen Process (Iogen, Ottawa, Ontario, Canada) using a combination of steam explosion for hemicellulose disruption and cellulase for cellulose hydrolysis. The relevant composition of the OHH (postevaporator) was 21.65% (w/v) glucose, 10.65% (w/v) xylose, and 2.7% (w/v) acetic acid (HAc).

Fermentation Media

The synthetic, pure-sugar, OHH medium contained Zymo salts (3.48 g/L of KH_2PO_4, 0.25 g/L of $MgSO_4$, 0.01 g/L of $FeSO_4 \cdot 7H_2O$, 0.21 g/L of citric acid) *(15)* and 5 g/L of Difco Yeast Extract (Difco, Detroit, MI) with varying amounts of glucose, xylose, and acetic acid. OHH media were supplemented with Zymo salts and yeast extract or, alternatively, with 0.25% (v/v) whole corn steep liquor (CSL) (approx 50% solids) (Casco, Cardinal, Ontario, Canada) and 1.2 g/L of diammonium phosphate (DAP) *(17)*. All media contained 20 mg/L of Tc. All media were sterilized by autoclaving at 121°C for 30–45 min. Stock sugar solutions were autoclaved separately. Tc was added to the sterilized medium after cooling.

Fermentation Equipment

pH-stat batch fermentations were conducted with about 1500 mL of medium in 2-L bioreactors (model F2000 MultiGen or model Bioflo 2000; New Brunswick, Edison, NJ) and fitted with agitation, pH, and temperature control (30°C). The pH was monitored using a sterilizable combination Ingold pH electrode. The standard pH control set point was either 5.75 or 6.0. In some experiments, the pH set point was adjusted at 24 h from 5.75 to 6.5. pH was maintained by automatic titration with 4 *N* KOH. Temperature was controlled at 30°C using a circulating water bath and the agitation was moderate (approx 100–150 rpm).

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 metabolic ethanol yield ($Y_{p/s}$) was calculated as the mass of ethanol produced per mass of sugar consumed. The process ethanol yield was determined by dividing the ethanol concentration by the total sugar concentration in the feed medium. The volumetric ethanol productivity was calculated by dividing the final ethanol concentration by 48 h or, alternatively, by the time taken to complete the fermentation (i.e., 100% utilization of glucose and xylose).

Results and Discussion

Since the hemicellulose component of lignocellulosic biomass consists of pentosans that are acetylated to varying degrees, all biomass hydrolysates contain acetic acid (for a review *see* ref. *20*). Because of its bacteriostatic properties, acetic acid is a well-known food preservative. The mechanism of acetic acid inhibition of bacterial growth is well understood

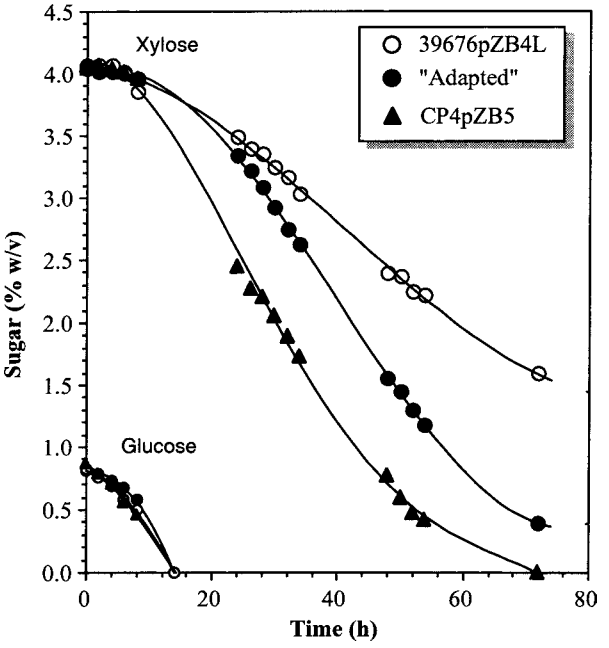

Fig. 1. Effect of 1% (w/v) acetic acid on xylose-utilizing recombinant *Z. mobilis* in a cCSL-based pure-sugar synthetic hardwood hydrolysate medium. The medium contained Zymo salts (*see* Materials and Methods) with 1% (v/v) clarified CSL (cCSL), 20 mg/L of Tc, 4% (w/v) glucose, 0.8% (w/v) xylose, and 1% (w/v) acetic acid. The pH was 6.0 and the temperature was 30°C. The values for maximum DCM concentration, ethanol yield, and productivity are given in Table 1.

(21), and its pH-dependent effect on both wild-type *(10)* and xylose-utilizing recombinant *Zymomonas* has been documented *(12)*. Recently, Joachimsthal et al. *(22)* described the isolation of an acetic acid–tolerant mutant of wild-type *Zymomonas* ZM4.

Several "conditioning" procedures have been described for reducing the toxicity of biomass hydrolysates, thereby making them less recalcitrant to fermentation *(20)*. Among these detoxification procedures, ion-exchange and liquid-liquid extraction are relatively specific for the removal of acetic acid *(23)*. However, there is an added cost associated with hydrolysate conditioning, and in this study we decided to test biocatalyst fermentation performance using unconditioned hydrolysate.

In prior separate studies, we examined the acetic acid sensitivity of different NREL xylose-utilizing recombinant *Z. mobilis* strains using a synthetic hardwood prehydrolysate medium *(10,12)*. At the time the present study was initiated in the spring of 1999, the following three NREL rec Zm strains were in our culture collection: CP4:pZB5, 39676:pZB4L, and the so-called hydrolysate-adapted variant of 39676:pZB4L (designated as adapted). Figure 1 shows the glucose and xylose consumption trajectories for a pH-stat batch fermentation of a synthetic hardwood prehydrolysate using the three rec Zm strains. The nutrient-rich medium

contained 1% (w/v) acetic acid and the pH was controlled at 6.0 (Fig. 1). Whereas all three recombinants exhibited identical ethanol yields based on sugar consumed (0.48 g/g), the process yield based on the total initial sugar concentration was 0.32 g/g for recombinant 39676:pZB4L and 0.47 g/g for CP4:pZB5, with the adapted strain exhibiting a yield that was intermediate between the others (Table 1). Under these assay conditions, only rec Zm CP4:pZB5 was able to complete the fermentation in 3 d (Fig. 1). From the results of the experiment illustrated in Fig. 1, we concluded that strain CP4:pZB5 was more tolerant than the other recombinants to acetic acid, and we proceeded to examine its growth and cofermentation performance with unconditioned OHH and pure-sugar synthetic media.

The acetic acid concentration of the evaporated OHH was 2.7% (w/v). When the OHH concentrate was diluted with water to an acetic acid level of 1% (w/v), the concentrations of glucose and xylose were 8 and 3.9%, respectively. Figure 2 compares the cofermentation performance of rec Zm CP4:pZB5 in OHH (1% acetic acid) and a pure-sugar synthetic OHH medium. Both media were supplemented with 5 g/L of yeast extract and Zymo salts (*see* Materials and Methods). The growth and fermentation parameters are summarized in Table 1. The significantly better performance of CP4:pZB5 in the synthetic medium suggests a possible role of inhibitory substances other than acetic acid in the unconditioned hydrolysate, e.g., soluble phenolic compounds derived from lignin decomposition. In addition to acetic acid, ethanol is known to inhibit xylose utilization by rec Zm with ethanol concentrations of 5.5–6% (w/v) causing complete inhibition *(16,24)*. Hence, even with the synthetic medium, ethanol could conceivably be a contributing factor in protracting or, in other experiments not shown, stalling the batch fermentation (Fig. 2).

Figure 3 shows that the combined inhibitory effects of acetic acid and ethanol on CP4:pZB5 cofermentation performance could be reduced by diluting the OHH concentrate such that the acetic acid concentration was <1% (w/v). One of the operational parameters that was included in our biocatalyst performance assessment criteria was that the batch fermentation be essentially completed within 2 d. At an acetic acid concentration of 0.84% (w/v), xylose utilization was incomplete after 48 h (Fig. 3), and the process yield was only 0.44 g/g (Table 1). However, at an acetic acid level of 0.75% (w/v), the fermentation was complete at 48 h (Fig. 3), with a final ethanol concentration of 4.2% (w/v) representing a volumetric productivity of 0.88 g/(L·h) and an ethanol yield of 0.47 g/g or 92% theoretical maximum conversion efficiency (Table 1). Further reduction in acetic acid level to <0.75% (w/v) resulted in an increase in volumetric productivity, but with a proportionately reduced final ethanol concentration (Fig. 3, Table 1). From the results of the experiment shown in Fig. 3, it was concluded that with rec Zm CP4:pZB5 the optimal hydrolysate composition (for complete fermentation within 48 h) with respect to sugars and acetic acid was 6% glucose, 3% xylose, and 0.75% acetic acid.

Table 1
Summary of Growth and Fermentation Parameters[a]

Figure/ experiment rec Zm	Amount of sugar (% [w/v])	Glu (% [w/v])	Xyl (% [w/v])	HAc (% [w/v])	Cell mass (g DCM/L)	Yield(p) (g/g)	Yield(m) (g/g)	Productivity (g/[L·h])	Maximum EtOH (g/L)
Figure 1									
39676pZB4L	4.8	0.8	4	1.0	0.63	0.32	0.48	0.22[b]	15.8
"Adapted"	4.8	0.8	4	1.0	0.72	0.44	0.48	0.29[b]	21.2
CP4pZB5	4.8	0.8	4	1.0	0.74	0.47	0.48	0.33[b]	23.5
Figure 2									
CP4pZB5	12 Pure sugars	8	4	1.0	1.04	0.47	0.48	1.10	58
CP4pZB5	12 OHH	8	4	1.0	1.32	0.36	0.47	0.89	43
Figure 3									
CP4pZB5	6 OHH	4	2	0.5	1.38	0.47	0.47	0.94	29
CP4pZB5	9 OHH	6	3	0.75	1.51	0.47	0.47	0.88	42
CP4pZB5	10.5 OHH	7.0	3.5	0.84	1.36	0.44	0.47	0.96	46
Figure 4									
CP4pZB5	9 Pure sugars	6	3	0.75	1.07	0.48	0.48	0.90	43
CP4pZB5	9 OHH	6	3	0.75	1.51	0.47	0.47	0.88	42
CP4pZB5	9 OHH	6	3	0.75	1.53	0.47	0.48	0.88	42
Figure 5									
CP4pZB5	9 OHH	6	3	0.75	1.53	0.47	0.48	0.89	42
39676	9 OHH	6	3	0.75	1.18	0.32	0.48	0.62	29
"Adapted"	9 OHH	6	3	0.75	1.15	0.34	0.47	0.66	31
ZM4pZB5	9 OHH	6	3	0.75	1.38	0.47	0.48	1.40	42
Figure 7									
CP4pZB5	10.5 OHH	7	3.5	0.9	0.97	0.48	0.44	0.96	46
ZM4pZB5	10.5 OHH	7	3.5	0.9	1.16	0.48	0.47	1.04	50

[a]For fermentations <48 h, the productivity was based on the time required to complete the fermentation; otherwise, the productivity was based on ethanol concentration at 48 h. DCM, dry cell mass; Yield(p), process yield based on available sugar; Yield(m), metabolic yield based on sugar utilized; HAc, acetic acid; OHH, oat hull hydrolysate.

[b]Productivity was based on a fermentation time of 72 h.

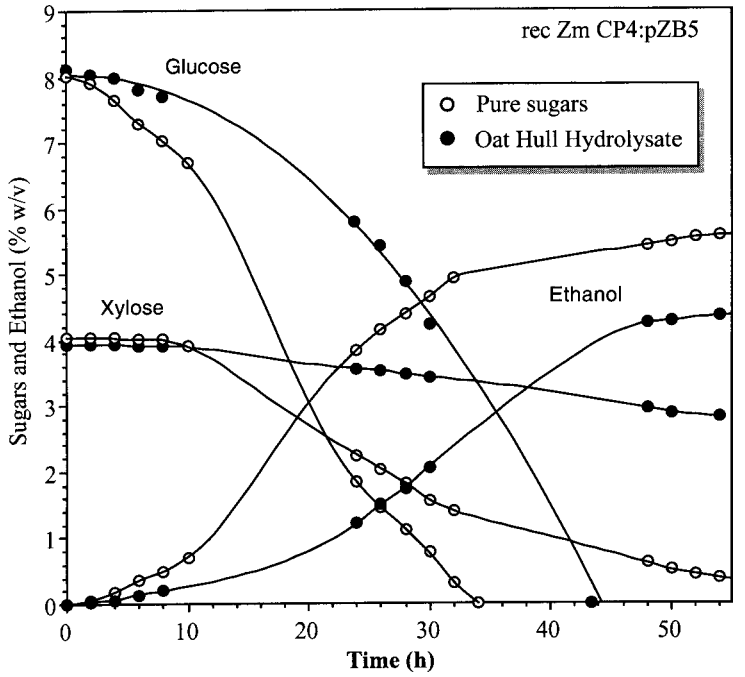

Fig. 2. Time course of pH-stat batch fermentation of OHH and a synthetic pure-sugar hydrolysate by rec Zm CP4:pZB5. The synthetic OHH medium contained Zymo salts, 5 g/L of yeast extract, 20 mg/L of Tc, 8% (w/v) glucose, 3.9% (w/v) xylose, and 1% (w/v) acetic acid. The pH was 5.75 and the temperature was 30°C. The values for maximum DCM concentration, ethanol yield, and productivity are given in Table 1.

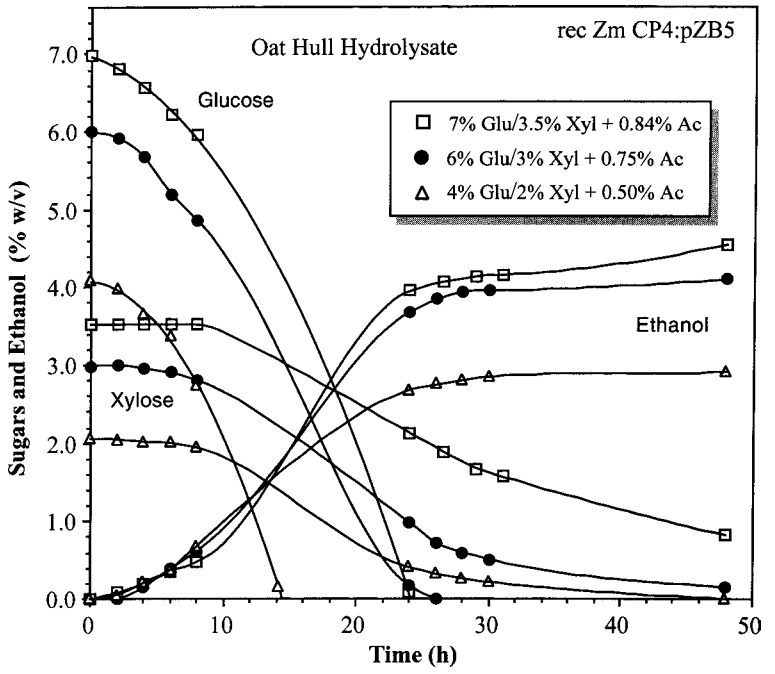

Fig. 3. Effect of dilution of the concentrated OHH on the cofermentation performance of rec Zm CP4:pZB5. The diluted OHH was supplemented with Zymo salts, 5 g/L of yeast extract, and 20 mg/L of Tc. The pH was 5.75 and the temperature was 30°C. The values for maximum DCM concentration, ethanol yield, and productivity are given in Table 1.

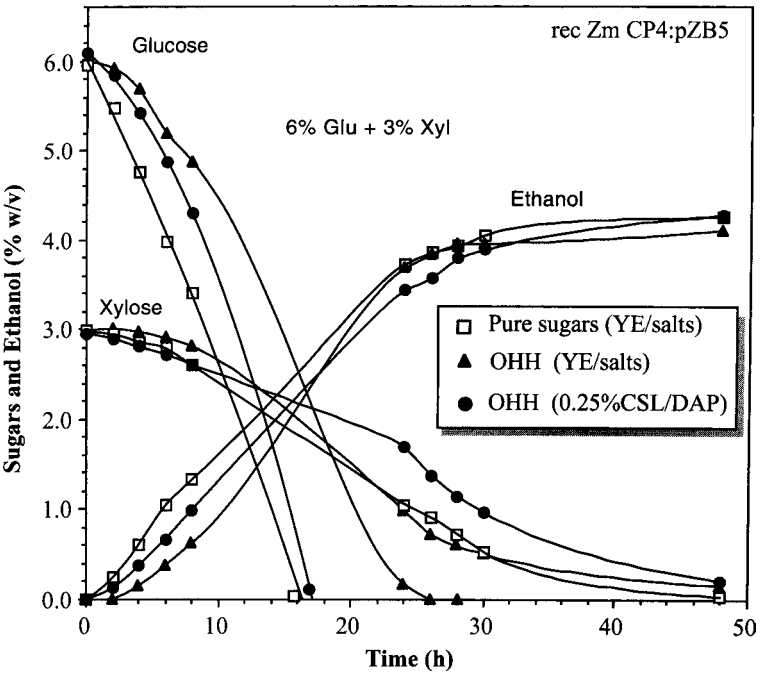

Fig. 4. Comparative cofermentation performance of rec Zm CP4:pZB5 in OHH and synthetic pure-sugar hydrolysate with 9% (w/v) sugar loading and 0.75% (w/v) acetic acid: effect of nutritional supplements. The concentration of glucose and xylose in all media was 6% (w/v) and 3% (w/v), respectively. The nutrients added were either Zymo salts and 5 g/L of yeast extract (YE) or 0.25% (v/v) CSL and 1.2 g/L of DAP. All media contained 20 mg/L of Tc. The pH was 5.75 (which was adjusted to 6.5 at 24 h) and the temperature was 30°C. The values for maximum DCM concentration, ethanol yield, and productivity are given in Table 1.

Cost-Effective Nutritional Amendment of Hydrolysate

The requirement for nutritional supplementation of fermentation media has a significant economic impact on large-scale production of fuel ethanol. Complex supplements such as yeast extract are very costly, and we have previously shown that CSL is a cost-effective substitute for yeast extract *(17)*. At low-level amendment of hydrolysate by CSL, there is a requirement for added nitrogen, which can be supplied in the form of inorganic nitrogen as ammonium salts *(11)*. Figure 4 shows that rec Zm CP4:pZB5 performs comparably when the hydrolysate is amended either with a combination of 0.25% (v/v) whole CSL (50% solids) and 1.2 g/L of DAP or with 5 g/L of yeast extract and Zymo salts (*see* Materials and Methods). Recently, it has been estimated that the economic impact of this nutritional supplementation on large-scale production of ethanol is 4.4¢/US gal of ethanol *(17)*.

Comparative Cofermentation Performance Assessment Using OHH

In September 1999, we received another xylose-utilizing recombinant Zm strain from NREL: ZM4:pZB5 (also known as 31821:pZB5). This strain

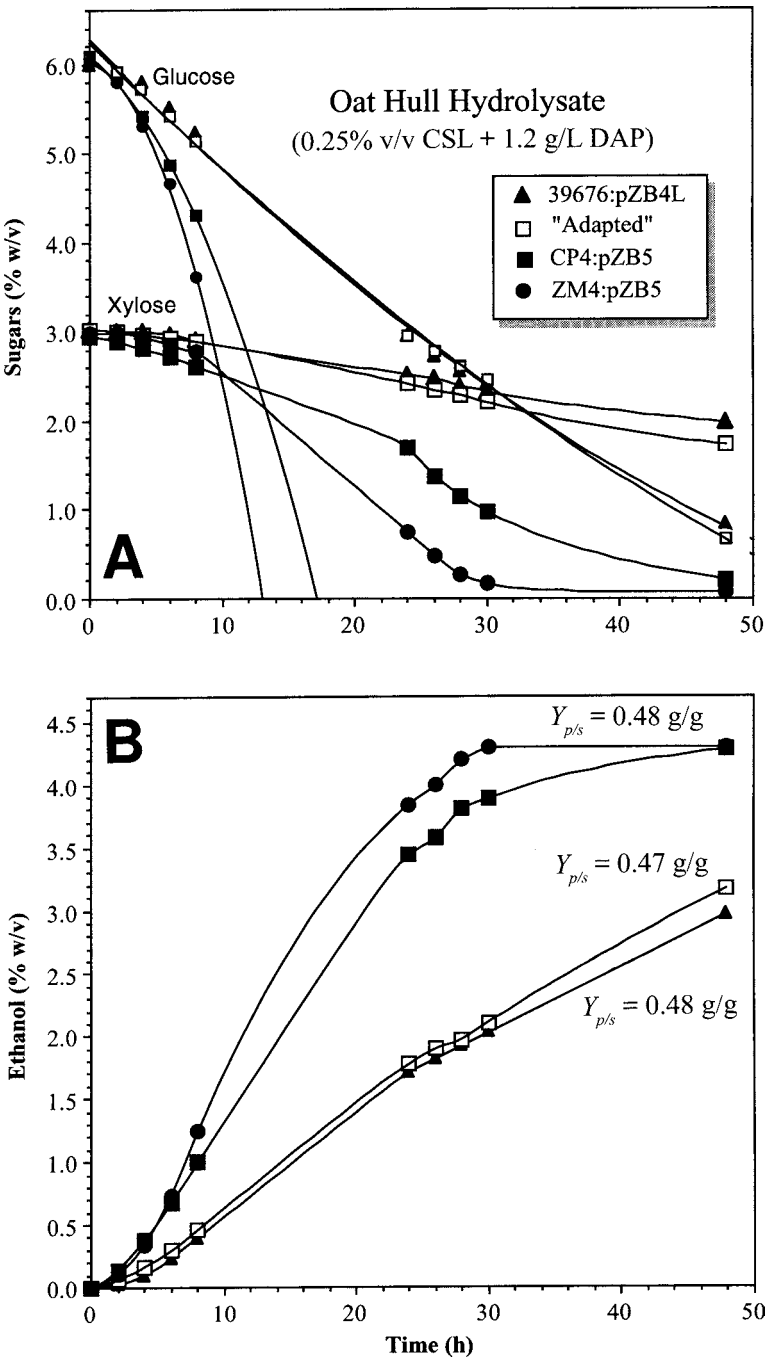

Fig. 5. Comparative cofermentation performance of four different *Z. mobilis* recombinants in oat hull hydrolysate (0.75% [w/v] HAc) with cost-effective minimal nutrient supplementation. **(A)** Sugar utilization; **(B)** ethanol production. The concentration of glucose and xylose in the oat hull hydrolysate was 6% (w/v) and 3% (w/v), respectively. Tc (20 mg/L) was added to the medium. The level of acetic acid was 0.75% (w/v). The nutrients added were 0.25% (v/v) CSL and 1.2 g/L of DAP. The pH was 5.75 (which was adjusted to 6.5 at 24 h), and the temperature was 30°C. The values for maximum DCM concentration, ethanol yield, and productivity are given in Table 1.

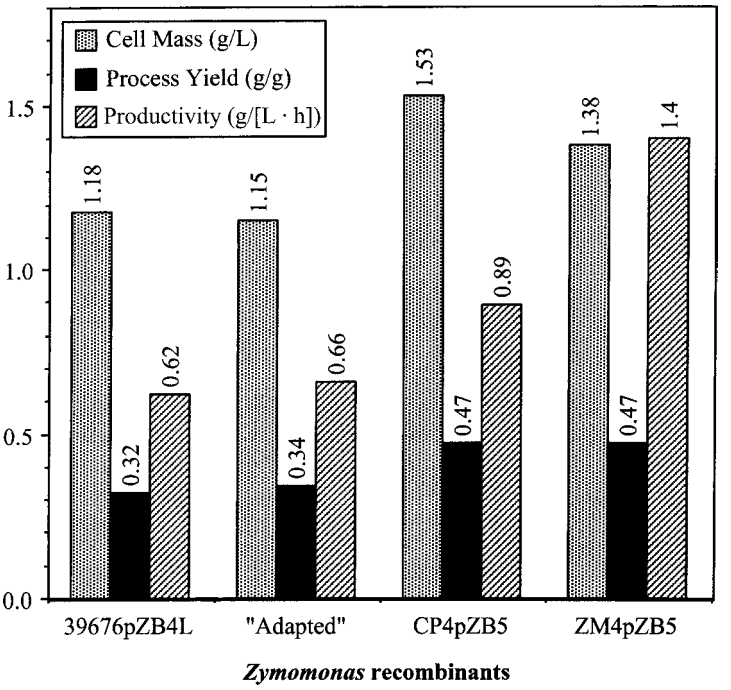

Fig. 6. Comparative growth and fermentation performance profiles for four different *Z. mobilis* recombinants using oat hull hydrolysate. Cell (dry) mass, process ethanol yield, and volumetric ethanol productivity data are taken from the experiment illustrated in Fig. 5 (*see* also Table 1). Ethanol productivity was based on a 48-h batch fermentation except for rec Zm ZM4:pZB5, of which the fermentation was complete in 30 h.

was purported to be superior to CP4:pZB5 in both batch and continuous fermentations *(18)*. Side-by-side pH-stat batch fermentations were conducted with all four xylose-utilizing rec Zm strains using OHH (6% glucose, 3% xylose, and 0.75% acetic acid) supplemented with 0.25% (v/v) CSL and 1.2 g/L of DAP (Fig. 5). The pH was controlled initially at 5.75, but the control set point was adjusted at 24 h to 6.5. Under these test conditions, neither the recombinant 39676:pZB4L nor the adapted variant performed very well (Fig. 5). The improved performance exhibited by CP4:pZB5 under this test condition was consistent with the results of the comparative performance assessment using synthetic hardwood prehydrolysate with 1% acetic acid (Fig. 1). Recombinant CP4:pZB5 completed the fermentation of the unconditioned OHH in 48 h with a yield of 0.47 g/g and a productivity of 0.89 g/(L·h) (Fig. 5, Table 1). However, ZM4:pZB5 outperformed CP4:pZB5 by completing the fermentation in 30 h with a yield of 0.47 g/g and a productivity of 1.4 g/(L·h) (Fig. 5, Table 1). The growth and fermentation parameters for all four recombinants are compared graphically in Fig. 6. The superior performance exhibited by rec Zm ZM4:pZB5 substantiates earlier claims made by Joachimsthal and Rogers *(18)*.

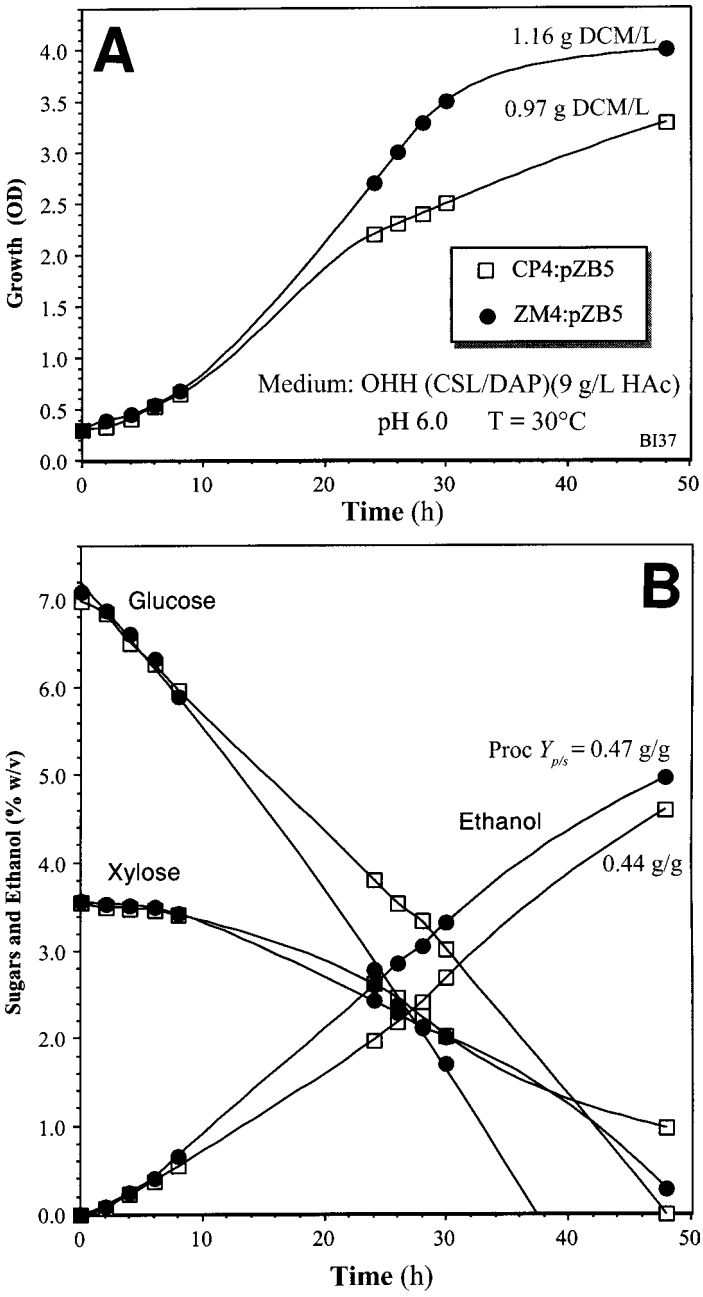

Fig. 7. Comparative cofermentation performance of rec Zm CP4:pZB5 and ZM4:pZB5 in oat hull hydrolysate (OHH) and synthetic pure-sugar hydrolysate with 10% (w/v) sugar loading and 0.9% (w/v) acetic acid. **(A)** Growth; **(B)** sugar utilization and ethanol production. The concentration of glucose and xylose was 7% (w/v) and 3.5% (w/v), respectively. The nutrients added were 0.25% (v/v) CSL and 1.2 g/L of DAP. Tc (20 mg/L) was added to the medium. The pH was 6.0 and the temperature was 30°C. The values for maximum DCM concentration, ethanol yield, and productivity are given in Table 1.

The excellent performance of ZM4:pZB5 prompted us to test its capacity to ferment a higher level of hydrolysate with proportionately high concentrations of glucose, xylose, and acetic acid. Figure 7 shows that ZM4:pZB5 still outperforms CP4:pZB5 at an acetic acid level of 0.9% (w/v). In fact, the CP4pZB5 fermentation appears to be "stuck" at 48 h, and the process yield falls from 0.47 to 0.44 g/g (Fig. 7). For ZM4:pZB5, productivity falls from 1.4 to 1.0 g/(L·h) (Table 1).

From a regulatory perspective, all of the rec Zm strains tested in this study are disadvantaged because they all carry the Tc-resistance gene, which was incorporated into the plasmid carrying the xylose utilization genes for convenience of selection as part of the process of genetic engineering. Also, that all these recombinants are xylose utilizing by virtue of the expression of plasmid-borne genes makes them all susceptible to instability in long-term operations such as either series batch fermentations (draw-and-fill) or continuous fermentations. Finally, none of these recombinants had the ability to utilize arabinose. These issues have been addressed by NREL, and in the future we anticipate testing the new genome integrated C5-utilizing Zm strains developed at NREL that are both devoid of the Tc resistance gene and able to utilize xylose and arabinose *(25)*.

Acknowledgments

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