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Continuous Culture Studies of Xylose-Fermenting *Zymomonas mobilis*

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ABSTRACT

The continuous cofermentation performance of xylose-fermenting *Zymomonas mobilis* at 30°C and pH 5.5 was characterized using a pure-sugar feed solution that contained 8 g/L glucose and 40 g/L xylose. Successful chemostat start up resulted in complete utilization of glucose and greater than 85% utilization of xylose, but was only reproducibly achieved using initial dilution rates at or less than 0.04/h; once initiated, cofermentation could be maintained at dilution rates of 0.04 to 0.10/h. Whereas xylose and cell-mass concentrations increased gradually with increasing dilution rate, ethanol concentrations and ethanol yields on available sugars remained approximately constant at 20–22 g/L and 80–90% of theoretical, respectively. Volumetric and specific ethanol productivities increased linearly with increasing dilution rate, rising from approx 1.0 each (g/L/h or g/g/h) at a dilution rate of 0.04/h to approx 2.0 each (g/L/h or g/g/h) at a dilution rate of 0.10/h. Similarly, specific sugar-utilization rates increased from approx 2.0 g/g/h at dilution rate 0.04/h to approx 3.5 g/g/h at dilution rate of 0.10/h. The estimated values of 0.042 g/g for the maximum *Z. mobilis* cell-mass yield on substrate and 1.13 g/g/h for the minimum specific substrate utilization rate required for cellular maintenance energy are within the range of values reported in the literature. Results are also presented which suggest that long-term adaptation in continuous culture is a powerful technique for developing strains with higher tolerance to inhibitory hemicellulose hydrolyzates.

Index Entries: Adaptation; continuous cofermentation; ethanol; xylose; *Zymomonas mobilis*.

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INTRODUCTION

Fermentation fuel ethanol is currently produced almost exclusively from either sucrose (sugarcane or beets) or starchy feedstocks (principally corn) using *Saccharomyces* yeast *(1,2)* and the largest plants operate in a continuous mode using a cascade of several fermentors in series *(3)*. Lignocellulosic biomass (energy crops) and wastes (forest, agricultural, and municipal) represent a vast potential alternative resource for ethanol production *(4)*. However, there are a number of features that make lignocellulosic feedstocks incompatible with current bioconversion process technologies. According to Lynd et al. *(5)*, it is the lack of appreciation of the enormous resource potential as well as of the differences in processing technologies that has fostered misconceptions about the potential for cellulosic ethanol and hindered the development of emerging biomass-to-ethanol process technologies.

A biomass-to-ethanol process must achieve efficient conversion of both the cellulose and hemicellulose components to be economical *(6,7)*. Unlike starch, the cellulose in woody and herbaceous biomass feedstocks is highly resistant to enzymatic hydrolysis unless the biomass is first pretreated *(8,9)*. Dilute acid-catalyzed thermochemical hemicellulose hydrolysis is an efficient and cost-effective pretreatment method *(10–12)*. The prehydrolyzate liquor (hydrolyzate) fraction is composed of a mixture of monomeric sugars, acetic acid, furfural, and a variety of lignin-derived phenolic compounds known to be inhibitory to fermentative microorganisms *(13–15)*. The sugar component is principally the pentose sugar D-xylose in the case of hydrolysis of hardwood or herbaceous hemicellulose. There have been a variety of procedures proposed for detoxifying biomass hydrolyzates (for review, *see* ref. *15*), but their efficacy is variable and their economic impact remains largely unexplored.

Yeasts presently used in sucrose and starch-based ethanol fermentations ferment only hexose sugars to ethanol. The inability of these yeasts to utilize pentose sugars is driving considerable research to develop alternative biocatalysts that exhibit performance characteristics better suited to conversion of lignocellulosic biomass feedstocks. The search for efficient xylose-utilizing ethanologens includes selection of natural isolates and various genetic manipulations of yeast and bacteria (for reviews, *see* refs. *14–16*).

Continuous fermentation systems offer several potential advantages such as increased productivity through cell-retention or cell-recycling configurations *(17)*. The engineering strategy for increasing overall productivity and reducing manufacturing costs by switching from batch to continuous operation is similar for both starch- and cellulose-based ethanol production processes (for review, *see* ref. *17*). However, the biocatalysts (both hydrolytic enzymes and fermentative microorganisms) needed to achieve high levels of conversion are quite feedstock specific *(6,18–20)*.

Thus, whereas continuous large-scale production of ethanol from starch is a relatively mature technology, many challenges remain to achieving continuous high-yield production of ethanol from lignocellulosic feedstocks. Many researchers believe that the greatest potential for improvement of biomass-to-ethanol processes is to overcome the biological constraints associated with the conversion biocatalysts *(5,21,22)*.

Techno-economic analyses of projected commercial-scale biomass-to-ethanol processes indicate that major cost reductions can be achieved through technological advances in both process configuration and biocatalyst performance. Reflecting this, over the past decade, technical breakthroughs have led the projected cost of fuel ethanol to decrease progressively from $2.66/gal to approx $1.06–1.22/gal with current technology *(6,7,14,23)*. Provided significant efforts to advance biomass-to-ethanol process technology continue, projected production costs are expected to continue to fall to the point where fuel ethanol becomes cost competitive with petroleum-derived gasoline *(5,22)*. It is proposed that the significant additional processing cost reductions required to achieve this goal can be achieved through a combination of process consolidation *(22)*, economies of scale *(23)*, and improved energy utilization *(21,22)*. Advances in process consolidation would involve reducing the number of bioreactors in a design, for example, by moving from a process based on sequential hydrolysis and fermentation to one using simultaneous hydrolysis and fermentation *(18,19)*. Similarly, advanced designs would employ continuous rather than batch operation. The ultimate biocatalyst for an advanced process would be an ethanologenic microorganism capable of synthesizing cellulolytic enzymes in a process called direct microbial conversion or DMC *(22,24)*.

One of several biomass-to-ethanol processes currently under investigation at the National Renewable Energy Laboratory (NREL) is the simultaneous saccharification and cofermentation (SSCF) process that is based on the use of genetically engineered *Zymomonas mobilis* transformed with NREL's proprietary xylose assimilation and utilization plasmid *(25–29)*. Xylose-utilizing recombinant *Z. mobilis* has been shown to exhibit excellent cofermentation performance with respect to both yield and productivity in laboratory scale pH-controlled bioreactors using a synthetic hardwood dilute-acid hydrolyzate medium with corn-steep liquor as the sole nutritional supplement *(30)*. The results of successful batch SSCF trials with recombinant *Z. mobilis* also have been reported *(27,28)*. More recently, we reported the effect of acetic acid on the performance of recombinant *Z. mobilis* in both batch *(30)* and glucose fed-batch systems *(31)*.

This paper reports initial results from an ongoing study of continuous conversion of hardwood hemicellulose hydrolyzate to ethanol. This study focuses on the fermentation performance of the xylose-utilizing recombinant *Z. mobilis* in a pH-controlled continuous-flow bioreactor (chemostat) using both combinations of pure sugars and real hardwood hydrolyzates.

Our objective was to exploit the selective pressure provided by the continuous growth environment of a chemostat to achieve strain improvement through adaptation resulting from the long-term exposure of the recombinant microorganism to incremental increases in the level of inhibitory hydrolyzate in the feed medium. Before initiating the long-term strain adaptation effort, it was necessary to characterize the fermentation performance of the recombinant strain in chemostat culture in order to identify an appropriate dilution rate for continuous feeding of dilute-acid hydrolyzate.

MATERIALS AND METHODS

Microorganism

Zymomonas mobilis 39676:pZB4L, i.e., *Z. mobilis* host strain ATCC 39676 transformed with a derivative of the pZB5 plasmid conferring xylose assimilation and fermentation capability, as reported by Zhang et al. *(26)*. Cryovials of frozen concentrated stock culture were maintained in RM medium (10 g/L yeast extract and 2 g/L KH_2PO_4) supplemented 10 mg/L tetracycline and 10% w/w glycerol at −70°C *(30)*.

Inoculum Cultivation

For the pure-sugar study, 0.25 mL of thawed frozen stock culture was inoculated into a 125-mL culture flask containing 100 mL of sterile RM supplemented with 25 g/L xylose, 25 g/L glucose, and 10 mg/L tetracycline antibiotic. For the hydrolyzate adaptation study, the medium for seed culture production was 10% (v/v) conditioned yellow poplar hemicellulose hydrolyzate (hydrolyzate; *see* Preparation of Conditioned Hydrolyzate) supplemented with 10 g/L glucose, 15 mL/L clarified corn-steep liquor (CSL) (GPC International, Muscatine, IA), and 10 mg/L tetracycline; initial medium pH was 5.8. Inoculum cultures were incubated overnight at a temperature of 30°C and an agitation rate of 150 rpm.

Chemostat Studies

Experiments were carried out in 500-mL MultiGen fermentors (New Brunswick Scientific, Edison, NJ) using a working volume of 300 mL. Pure-sugar experiments were performed at a temperature of 30°C, an agitation rate of 150 rpm and at pH 5.5 unless stated otherwise; pH was controlled through the automatic addition of 2 *N* KOH.

Pure-Sugar Chemostat Study

Fermentation was initiated batchwise by directly inoculating at a level of 10% (v/v) with overnight grown seed culture; this corresponds to an initial cell concentration of approx 0.10 g dry cell mass per liter (g DCM/L). The batch-fermentation medium and the subsequent chemostat-feed me-

dium contained 8 g/L glucose, 40 g/L xylose, 10 mL/L clarified CSL, and 10 mg/L tetracycline. Continuous feeding was begun at a dilution rate of 0.03/h after the xylose concentration had decreased to between 15 and 20 g/L. The fermentor was sampled daily for offline analysis of cell mass and soluble metabolite concentrations, particularly for glucose, xylose, and ethanol.

Our objective was to characterize an effective operating region for performing chemostat adaptation of the culture to inhibitory hydrolyzate. We were interested in determining the approximate range of dilution rates over which effective utilization of both glucose and xylose could be achieved, i.e., conditions in which complete utilization of glucose and greater than 75% utilization of xylose occurred. Our approach was to carry out chemostat operation at a particular dilution rate until "pseudo steady-state" performance that met our criteria for effective cofermentation was achieved, i.e., sequential daily samples had approximately constant ethanol concentrations as well as constant residual xylose concentrations below 10 g/L (and constant residual glucose concentrations near zero). Once these criteria were achieved, the dilution rate was increased. The dilution rate was then maintained at this new higher value until the next steady state that met our criteria was achieved, whereupon the dilution rate was again increased. This procedure was repeated until a dilution rate of 0.10/h was reached. Efforts to operate the chemostat at a dilution rate at and above 0.11/h were ultimately abandoned because at a dilution rate of 0.11/h, the strain began to flocculate and wall growth increased dramatically; both of these factors compromised the stability of the chemostat as well as our ability to interpret the experimental results.

When chemostat operation was attempted at a dilution rate of 0.11/h, we observed increased variability in the apparent steady state with respect to culture turbidity and xylose and ethanol concentrations. We feared that wash out of the culture might occur and therefore reduced the dilution rate to 0.04/h. We also replaced the fermentor vessel with a clean vessel to reduce the possibility of wall growth confounding the results. The experiment was terminated after re-establishing a steady state at a dilution rate of 0.04/h.

Hydrolyzate-Adaptation Chemostat Study

The fermentation was started up similarly to the pure-sugar fermentation described above. In this experiment, however, the pH was controlled at 5.8 and the composition of the initial batch and feed media consisted of 10% (v/v) conditioned hydrolyzate containing 15 mL/L clarified CSL, 10 mg/L tetracycline, and supplemented with sugars to achieve final concentrations of 8 g/L glucose and 40 g/L xylose. As in the pure-sugar experiment, the chemostat was sampled daily for analysis of glucose, xylose, and ethanol concentrations; determination of cell-mass concentration was not attempted in this experiment because of the high turbidity of the hydro-

lyzate-based medium. After 7 d, the dilution rate was increased from 0.03 to 0.04/h. After 15 d, the concentration of CSL in the feed medium was raised to 20 mL/L to reduce the possibility of nutrient limitations under the stressed adaptation conditions.

Our objective in this experiment was to determine if a chemostat fed with increasing concentrations of hydrolyzate could be used to develop a culture capable of tolerating higher levels of inhibitory hydrolyzate. The dilution rate was fixed at 0.04/h after 7 d, since the results of the pure-sugar study showed that this dilution rate enabled high-yield cofermentation of glucose and xylose. Our approach was otherwise similar to that used in the pure-sugar study. The chemostat was operated at a particular feed hydrolyzate concentration (initially 10% v/v) until sequential samples had approximately constant ethanol concentrations as well as constant residual xylose concentrations below approx 12.5 g/L (and residual glucose concentrations near zero). Once stable performance meeting these criteria was achieved, the concentration of hydrolyzate in the feed was increased by 5% v/v. Regardless of the hydrolyzate concentration, the feed hydrolyzate solution was supplemented to achieve final concentrations of glucose and xylose of 8 g/L and 40 g/L, respectively. Chemostat operation was maintained at this new higher hydrolyzate concentration until stable performance meeting our performance criteria was once again achieved, whereupon the concentration of hydrolyzate in the feed was again increased by 5% v/v. This procedure was repeated until a hydrolyzate concentration of 35% v/v was reached.

Preparation of Conditioned Hydrolyzate

Acidic-hydrolyzate liquor was obtained by dilute sulfuric acid treatment of yellow poplar sawdust in NREL's pilot scale Sunds hydrolyzer pretreatment reactor *(32)*. The pH of the as-received xylose-rich hemicellulose hydrolyzate liquor was in the range of 1.0–2.0. Conditioning was required to reduce the toxicity of the hydrolyzate to levels that enabled fermentation to occur *(33–35)*. Prior to and following conditioning, the hydrolyzate liquor was stored at 4°C. The so-called "overliming" process used to condition the hydrolyzate was as follows: while continuously agitating the solution, the pH of the as-received hydrolyzate liquor was raised to 10.0–10.5 using $Ca(OH)_2$ (solid powder). The solution was then heated to 50°C, held at this temperature for 30 min, and then cooled. Concentrated H_2SO_4 (96% w/w) was then slowly added until the solution reached pH 7.0. This overlimed hydrolyzate was sterilized by filtering through a 0.2 μm sterile filter.

Analytical Methods

Cell Concentration

Cell-mass concentration was determined by measuring turbidity (OD 600 nm) using a Milton Roy Spectronic 601 spectrophotometer (Spectronic,

Rochester, NY) zeroed with distilled water. Turbidity measurements were made using test tubes (100 mm × 13 mm) with an approximate light path length of 1.2 cm; whole broth samples were diluted into the linear range of the instrument, i.e., to achieve readings below 0.5 turbidity units. Turbidity values were converted to cell concentrations using a previously established correlation factor for this strain of 0.38 gDCM/L per turbidity unit.

Glucose, Xylose, and Ethanol Concentrations

Sample supernatants were analyzed to determine the concentrations of glucose, xylose, and ethanol (and other metabolites) using high-performance liquid chromatography (HPLC), as described previously *(30)*. Mixed-component concentration verification (CV) standards were periodically run (in duplicate) to verify calibration accuracy, and analysis was repeated if the reported concentrations of CV standards deviated from their actual values by greater than ± 2.5%.

Calculations

Ethanol yield on total available sugars, also referred to as process yield, or Y_P is the most important indicator of overall process performance. Process yields were calculated as the net grams of ethanol produced per grams of glucose and xylose available in the feed. Consumed sugar yields were calculated as the net grams of ethanol produced per grams of glucose and xylose consumed. The maximum cell-mass growth yield (i.e., corrected for maintenance), $Y_{X/S}^{max}$ (g DCM per g sugar), was determined as the inverse of the slope of the best fit linear regression for specific sugar utilization rate, q_S (g sugar per g DCM per h), as a function of dilution rate, D *(36)*. The maintenance energy coefficient, m_E (g sugar per g DCM per h), was determined as the y-axis intercept of the best fit linear regression to the q_S vs D data.

RESULTS AND DISCUSSION

Continuous-culture studies were initiated to understand what range of dilution rates would support good cofermentation performance. We were interested in maintaining selective pressure for xylose fermentation during prolonged chemostat operation, and thus wanted to maintain xylose-utilization rates that were threefold greater than those for glucose. Chemostat cultures were initiated using a feed solution containing 8 g/L glucose and 40 g/L xylose, i.e., concentrations similar to those in full-strength hydrolyzate liquors obtained by dilute acid pretreatment of hardwood feedstocks *(32)*. As a minimum level of cofermentation to indicate successful chemostat start up, we chose complete utilization of glucose and over 75% utilization of xylose. This level of conversion ensures that xylose:glucose utilization ratios remain greater than 3.5:1. The ability to

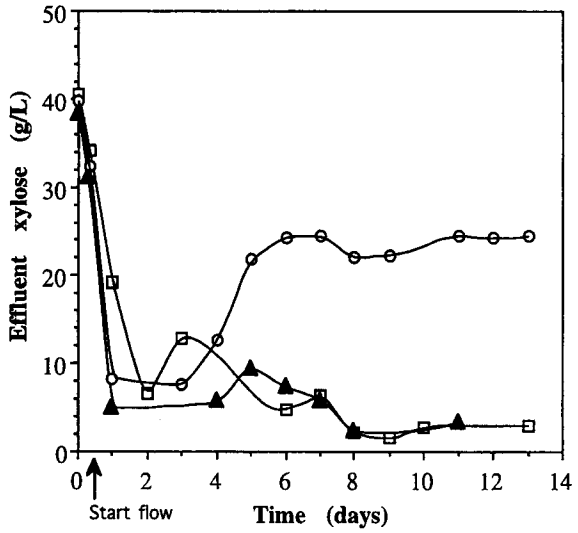

Fig. 1. Time courses during start up of recombinant Z. *mobilis* continuous cofermentations at three different initial conditions: (□), D = 0.04 h and pH = 5.5; (▲), D = 0.03 h and pH = 6.0; (○), D = 0.05 h and pH = 5.5. The medium for these experiments was RM (10 g Difco yeast extract and 2 g KH_2PO_4 per L distilled water) supplemented with 40 g/L xylose, 8 g/L glucose, and 10 mg/L tetracycline. The arrow indicates when continuous feeding was initiated.

maintain selective pressure for xylose fermentation during prolonged continuous culture should ensure that this trait is maintained and may also provide a method for long-term development of strains with improved xylose utilization capability.

Fermentations were started batchwise. Continuous feeding was initiated when the xylose concentration decreased to between 15 and 20 g/L. Successful chemostat start up resulted in complete utilization of glucose and greater than 85% utilization of xylose. Compositional analysis of successive daily samples showed stable glucose concentrations of zero and xylose concentrations below 5 g/L.

Start Up

Successful start up requires using initial feed (dilution) rates of less than or equal to 0.04/h. Figure 1 shows effluent xylose concentrations during start up of continuous cofermentations of glucose and xylose using dilution rates of 0.03, 0.04, and 0.05/h. During the week following start up, the effluent xylose concentrations generally remain low for the cultures initiated at dilution rates of 0.03 and 0.04/h. These cultures achieve stable effective cofermentation within 8 d. The steady-state xylose concentrations in these successful start ups, i.e., the xylose concentrations from 8 d onward, are below 4 g/L. In contrast, poor performance results when continuous feeding is started at a dilution rate of 0.05/h. In this case, the effluent xylose concentration remains low for the first 3 d following the initiation

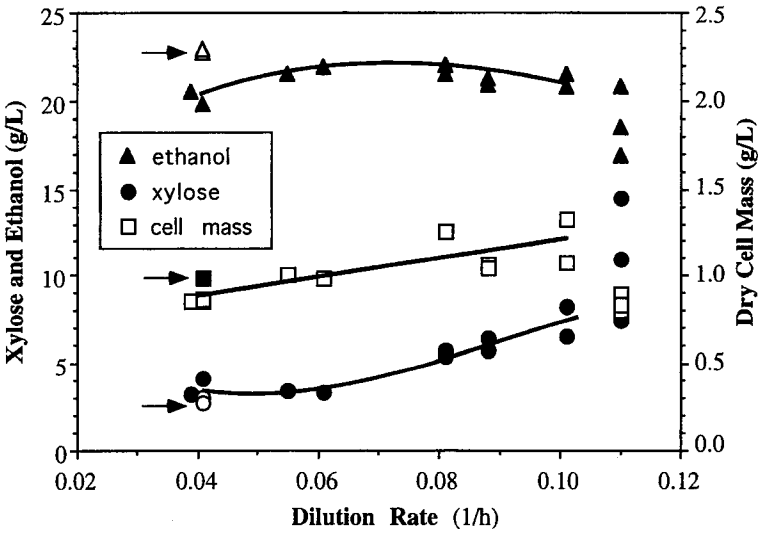

Fig. 2. Pseudo steady-state concentrations as a function of dilution rate for pure-sugar continuous cofermentation using recombinant *Z. mobilis*. The arrows and reverse symbol shading show the steady-state measurements obtained just before the experiment was terminated.

of continuous feeding, but then begins to rise. After 6 d, less than half of the feed xylose is being consumed and the xylose concentration remains above 20 g/L.

Characterization of Continuous Cofermentation

Figure 2 shows steady-state xylose, cell mass (dry cell mass), and ethanol concentrations as a function of dilution rate; no glucose was detected at any of the steady states. There is some scatter in the data but good cofermentation performance was achieved up to a dilution rate of 0.10/h. As discussed in the subheading MATERIALS AND METHODS, the system became unstable at a dilution rate of 0.11/h; the data obtained at a dilution rate of 0.11/h is shown in Fig. 2 for comparative purposes but is not considered a reliable indicator of true steady-state performance. As Fig. 2 illustrates, with the exception of the data obtained at a dilution rate of 0.11/h, the xylose concentration increased in an approximately linear fashion with increasing dilution rate, increasing from approx 3 g/L at a dilution rate of 0.04/h to approx 7 g/L at a dilution rate of 0.10/h. Similarly, the cell-mass concentration increased with increasing dilution rate, but only very slightly, rising from approx 0.9 g DCM/L at a dilution rate of 0.04/h to approx 1.1 g DCM/L at a dilution rate of 0.10/h. In contrast, the ethanol concentration remained at 21–22 g/L independent of dilution rate between dilution rates of 0.04 and 0.10/h.

Volumetric productivity increased linearly with dilution rate, rising from approx 0.8 g/L/h at a dilution rate of 0.04/h to approx 2.1 g/L/h at a dilu-

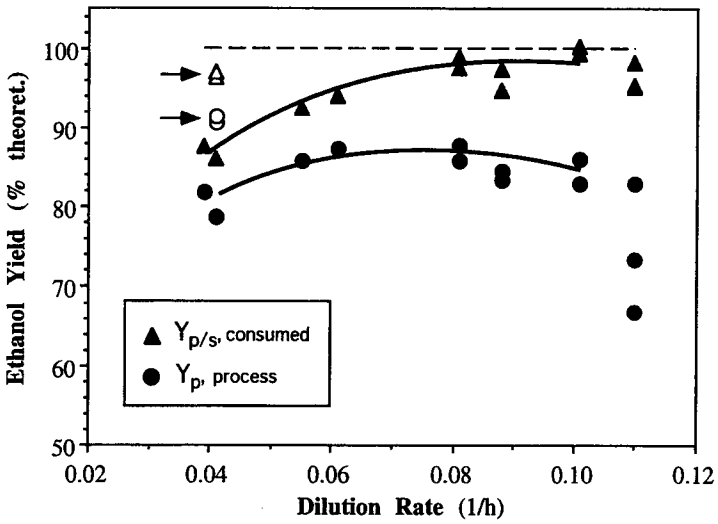

Fig. 3. Ethanol yield as a function of dilution rate. The arrows and reverse symbol shading indicate the steady-state measurements obtained just before the experiment was terminated.

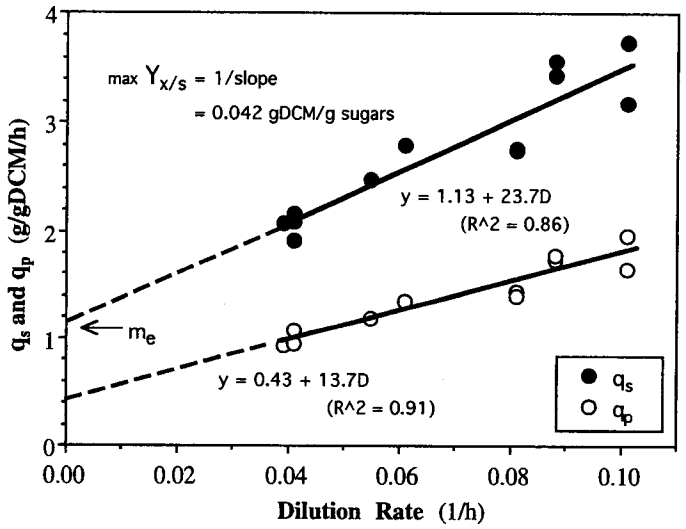

Fig. 4. Specific sugar-utilization rate and specific productivity as a function of dilution rate.

tion rate of 0.10/h (not shown). Figure 3 shows that process ethanol yields (ethanol yield based on available glucose and xylose) were approx 85% of theoretical over the entire range of dilution rates tested. Calculated metabolic yields (ethanol yield based on consumed sugars) were above 90% of theoretical and trended higher with increasing dilution rate (Fig. 3).

Figure 4 shows specific ethanol productivity and specific sugar utilization rate (i.e., rates on a per-gram DCM basis) as a function of dilution

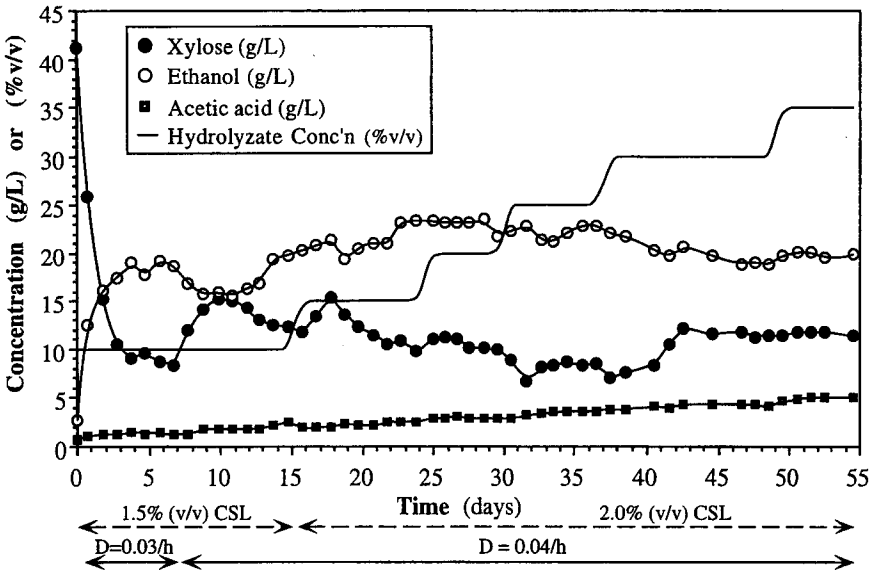

Fig. 5. Adaptation to increasing concentrations of overlimed biomass hydrolyzate.

rate. The specific rate of ethanol production increased linearly with dilution rate, increasing from a low of approx 1.0 g/g/h at dilution rate 0.04/h to a high of approx 1.8 at dilution rate 0.10/h. The specific rate of sugar utilization increased in a similar fashion, rising from a low of 2.0 g/g/h at dilution rate 0.04/h to a high of approx 3.5 g/g/h at dilution rate 0.10/h.

Adaptation to Inhibitory Hydrolyzate

The pure-sugar study results indicate that excellent xylose utilization is achieved if cofermentation is carried out at a dilution rate of 0.04/h. For this reason, this dilution rate was chosen for running the long-term adaptation experiment. Figure 5 shows the time course of this experiment, which was begun using a hydrolyzate concentration of 10% v/v. This initial hydrolyzate level was chosen because batch studies showed that 10% v/v hydrolyzate is not inhibitory (not shown); the hydrolyzate significantly inhibits batch fermentation performance by xylose-fermenting *Zymomonas mobilis* at levels of 30% v/v or higher *(35)*.

As in the pure-sugar study, cofermentation was initiated batchwise, with continuous feeding of 10% v/v hydrolyzate (supplemented with pure sugars) starting after the effluent xylose concentration fell to 15–20 g/L. When good cofermentation performance was achieved at a given hydrolyzate level, i.e., when no residual glucose was detected and the residual xylose concentration was below 10 g/L (or near 10 g/L and trending downward), the concentration of hydrolyzate in the feed was increased by 5% v/v. Thus, the feed concentration was increased from 10 to 15% v/v at day 15, and then increased from 15 to 20% v/v at day 24; and so on. As Fig. 5

shows, after 48 d of progressive adaptation, the culture achieved good cofermentation in the presence of 35% v/v hydrolyzate. This hydrolyzate level corresponds to an acetic acid level of approx 5 g/L, a level of acetic acid inhibitory to batch cofermentation performance *(30)*.

These results suggest that there is tremendous potential for strain improvement through continuous adaptation to higher levels of hydrolyzate. Further adaptation work clearly needs to be performed, however, to develop a strain that can tolerate full-strength, or near full-strength, hydrolyzates. The results nonetheless support the widely held view that the selective pressure of a controlled-chemostat growth environment provides a powerful technique for strain adaptation. For this reason, this chemostat adaptation work is continuing with the hope that strains exhibiting greater tolerance to acetic acid and other inhibitory components found in pretreatment hydrolyzates will be developed.

DISCUSSION

In the pure sugar study, the lowest metabolic ethanol yields occurred at the beginning of the experiment, i.e., the yield based on consumed sugars was only approx 85% when the first steady state was obtained at a dilution rate of 0.04/h (Fig. 3). In contrast, just before terminating the experiment, the measured metabolic ethanol yield at dilution rate 0.04/h was approx 95% (data points indicated by arrows shown in Fig. 3). The yields on consumed sugars actually remained above 95% after the dilution rate was raised to 0.08/h (at day 30) to the point the experiment was terminated (at day 50). The roughly 10% apparent increase in measured metabolic efficiency of the culture at dilution rate 0.04/h from the beginning to the end of the experiment possibly demonstrates an improvement in the strain's performance in continuous culture. From our perspective, the potential opportunities for developing a superior culture through long-term chemostat adaptation are real.

Regression analysis of the specific sugar-utilization-rate data gives values of 0.042 g/g and 1.13 g/g/h for $Y_{X/S}^{max}$ and m_E, respectively. However, there is scatter in the data and the value for the correlation coefficient is relatively low ($R^2 = 0.86$). It is therefore unwarranted to assign too much confidence to the accuracy of these parameter estimates. This said, Table 1 shows that these values are in the range of those reported by other investigators for nonrecombinant (i.e., glucose-fermenting) strains of *Z. mobilis*. In addition to being strain specific, these physiological and bioenergetic parameters are influenced by many other factors such as medium composition, pH, and temperature. It is thus difficult to draw significant conclusions from a comparison of reported literature values, i.e., the absence of uniformity with respect to strains and environmental growth conditions confounds clear interpretation of such comparisons *(40)*. Moreover,

Table 1
Maximum Cell-Growth Yields and Maintenance Coefficients Reported for *Zymomonas*

Z. mobilis strain	Sugar Substrate	$Y_{X/S}^{max}$ (gDCM/g substrate)	m_E (g substrate/g DCM-h)	Ref.
29191	glucose	0.037-0.060	1.0-2.5	37-40
31821	glucose	0.035	2.2	41
Z-1-81	glucose	0.024	2.5	42
10988	glucose		0.5	43
39676(pZB4L)	glucose-xylose	0.042	1.1	this work

plots of qs vs D are often observed to be biphasic *(37,43,44)*, which further complicates the interpretation of such data as originally proposed by Pirt *(36)*.

It is generally postulated that the requirement for maintenance metabolism is because of a combination of growth-dependent energy-requiring processes and energy-wasting processes (e.g., ATPase activity or its energetic equivalent) *(36)*. *Z. mobilis* is relatively unique among ethanologenic microorganisms in using the Entner-Doudoroff (ED) pathway to convert glucose to pyruvate, thus achieving a net yield of only 1 mole ATP per mole glucose fermented to ethanol *(45)*. In this context, it is interesting to note that the reported ATPase activity of crude homogenates of *Z. mobilis* is 7.5 mmol ATP/gDCM/h, which is equivalent to 1.35 g glucose/gDCM/h assuming a net ATP yield per mole glucose of 1.

Our results suggest that the maintenance requirement of the xylose-fermenting strain may be a bit lower than that of the typical glucose-fermenting strain, but additional measurements need to be made to confirm this. Additional chemostat studies are planned to assess more thoroughly how $Y_{X/S}^{max}$ and m_E vary as a function of key operating variables, such as temperature, pH, and feed-sugar concentrations.

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