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Characterization of a High-Productivity Recombinant Strain of *Zymomonas mobilis* for Ethanol Production from Glucose/Xylose Mixtures

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Abstract

The fermentation characteristics of a recombinant strain of *Zymomonas mobilis* ZM4(pZB5) capable of converting both glucose and xylose to ethanol have been further investigated. Previous studies have shown that the strain ZM4(pZB5) was capable of converting a mixture of 65 g/L of glucose and 65 g/L of xylose to 62 g/L of ethanol in 48 h with an overall yield of 0.46 g/g. Higher sugar concentrations (e.g., 75/75 g/L) resulted in incomplete xylose utilization (80 h). In the present study, further kinetic evaluations at high sugar levels are reported. Acetate inhibition studies and evaluation of temperature and pH effects indicated increased maximum specific uptake rates of glucose and xylose under stressed conditions with increased metabolic uncoupling. A high-productivity system was developed that involved a membrane bioreactor with cell recycling. At sugar concentrations of approx 50/50 g/L of glucose/xylose, an ethanol concentration of 50 g/L, an ethanol productivity of approx 5 g/(L·h), and a yield ($Y_{P/S}$) of 0.50 g/g were achieved. Decreases in cell viability were found in this system after attainment of an initial steady state (40–60 h); a slow bleed of concentrated cells may be required to overcome this problem.

Index Entries: Recombinant *Zymomonas mobilis*; xylose fermentation; lignocellulosic hydrolysates; inhibition; ethanol.

Introduction

Genetic engineering with the ethanologenic *Zymomonas mobilis* has achieved a recombinant strain able to ferment glucose and xylose *(1)*. Previous batch studies by our group on *Z. mobilis* ZM4(pZB5) at high sugar concentrations *(2)* found that an ethanol concentration of 60–65 g/L of ethanol could be achieved within 48 h with ethanol yields 92–94% theoretical.

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The use of a substrate mixture of 75 g/L of glucose and 75 g/L of xylose resulted in significant declines in biomass yield and specific growth rate *(2)*. Related kinetic studies have been reported recently on other xylose-utilizing recombinants of *Z. mobilis (3)*. In the present investigation, additional studies at high concentrations of these sugars were carried out in order to gain a better understanding of the uptake of glucose and xylose by the recombinant strain ZM4(pZB5).

Ethanol, produced from a renewable resource such as lignocellulose, has the potential to be an efficient and environmentally sustainable fuel. During pretreatment processes, substances that are inhibitory to microorganisms are released from lignocellulosics. These inhibitory compounds include acetic acid, other organic acids, phenolics, and other degradation products (such as furfural and 5-hydroxymethyl furfural). Acetic acid and furfural have been reported to be highly toxic to *Z. mobilis* at levels similar to those found in lignocellulosic hydrolysates *(4)*. It is important that an organism used for lignocellulosic fermentation has resistance to the effects of these substances. The effects of acetate on the parent ZM4 strain and an acetate-resistant mutant ZM4/Ac® have been reported previously *(5)*, and the present investigation extends this evaluation to the recombinant strain ZM4(pZB5).

In the pretreatment of lignocellulosic material for fermentation, relatively high temperatures and acidic conditions are used. Previous studies on *Z. mobilis* have reported mutants capable of fermentation at 42°C *(6)*, and the usual pH conditions for *Z. mobilis* are 5.0–5.5. The effects of variation in the fermentation temperature (30–37°C) and pH (4.5–5.0) are now reported for the recombinant strain.

Cell recycling offers the possibility of highly productive systems owing to the accumulation of biomass in high concentrations. A laboratory-scale membrane bioreactor with cell recycle is now characterized for cofermentation of glucose and xylose by recombinant *Z. mobilis* ZM4(pZB5).

Materials and Methods

Microorganism

The recombinant *Z. mobilis* strain ZM4(pZB5) containing the *E. coli* genes for xylose assimilation (xylose isomerase, xylulokinase) and pentose metabolism (transketolase, transaldolase) on the plasmid pZB5, together with the genes for tetracycline resistance, was kindly provided by Dr. Min Zhang under a Materials Transfer Agreement with the National Renewable Energy Laboratory, Golden, CO.

Composition and Preparation of Media

Growth media for the recombinant *Z. mobilis* were as follows: glucose and xylose concentrations as specified; yeast extract (Oxoid) (10 g/L inoculum, 5 g/L fermentation media); KH_2PO_4 (2 g/L); $MgSO_4 \cdot 7H_2O$ (1 g/L); and $(NH_4)_2SO_4$ (1 g/L in media containing <40 g/L of glucose and 40 g/L of xylose; 2 g/L in media containing higher sugar levels). Media were steril-

ized by autoclaving at 121°C for 10 min for batch culture studies and filter sterilized for continuous culture studies. Tetracycline was added aseptically to the sterile media at room temperature, at a concentration of 10 mg/L, as a selection pressure for plasmid (pZB5) maintenance.

Fermentation Studies

Batch experiments were conducted in a 2-L LH fermentor (LH Engineering, Maidenhead, Berks, UK) using a working volume of 1 L. Environmental conditions were controlled at a temperature of 30°C, pH 5.0 (by addition of 3 *M* NaOH), and an agitation rate of 200 rpm. Cell-recycle experiments had a working volume of 700 mL and an agitation rate of 350 rpm to minimize any wall attachment of cells. Samples for sugar and ethanol determinations were collected at various times following centrifugation at 5000*g* and stored at –20°C prior to analysis. Samples for dry wt and optical density (OD) determinations were taken and analyzed directly.

Analytical Methods

Biomass concentrations for batch culture experiments were determined by OD measurements (at 660 nm) and converted to dry cell weights via a calibration curve *(2)*. For cell-recycle studies, direct OD and dry wt measurements were taken.

Glucose, xylose, and ethanol concentrations were determined from sample supernatants using high-performance liquid chromatography with an Aminex HPX-87H column (Bio-Rad, Hercules, CA) with 5 m*M* H_2SO_4 (at 65°C, 0.6 mL/min) as the mobile phase. Standards containing mixed components were periodically run to verify calibration accuracy.

Kinetic parameters were calculated with MATLAB software (MathWorks, Inc., published by Prentice Hall, 1995, Englewood Cliffs, NJ) using least-squares polynomial fitting. Unless otherwise specified, specific rates were calculated as maximum values determined during the exponential phase of growth. Full details of the calculation method are provided in an earlier publication *(2)*.

Results

Batch Culture

Effect of High Sugar Concentrations

The effect of high sugar concentrations on ZM4(pZB5) was further evaluated by varying the ratio of glucose to xylose (T = 30°C, pH 5.0). This complements studies carried out at 50/50, 65/65, and 75/75 g/L of glucose/xylose mixtures as reported previously *(2)*.

As shown in Fig. 1A, for a mixture of 100 g/L of glucose and 50 g/L of xylose, the fermentation was incomplete after 80 h, with an ethanol concentration of 68 g/L. Biomass yield was low at 0.007 g/g, based on total sugars utilized, and ethanol yield was 0.48 g/g on a similar basis. For comparison, the kinetics on 100 g/L of glucose medium are shown (Fig. 1B).

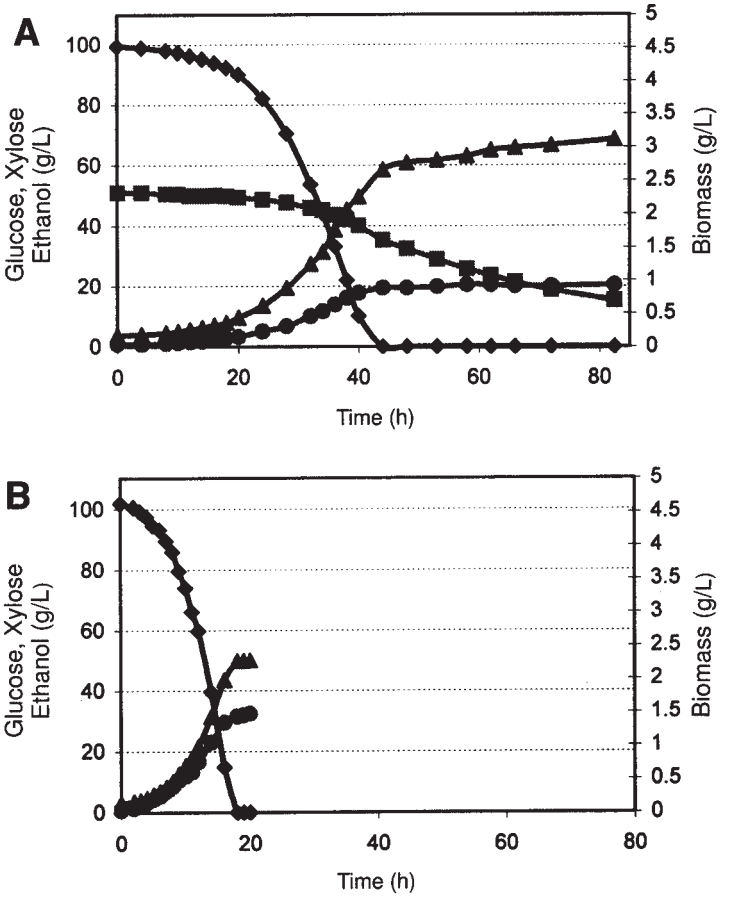

Fig. 1. Fermentation profile of ZM4(pZB5) on **(A)** 100 g/L of glucose and 50 g/L of xylose; and **(B)** 100 g/L of glucose media. ◆, Glucose; ■, xylose; ▲, ethanol; ●, biomass.

Table 1 provides a kinetic analysis of the fermentation data, and for comparison a similar analysis is provided for 50/50, 65/65, and 75/75 g/L of glucose/xylose mixtures as well as for ZM4(pZB5) growing solely on glucose medium (100 g/L). Calculations of specific rates of sugar uptake and ethanol production are maximum values determined for the exponential phase and for the secondary fermentation phase following glucose depletion.

Several observations can be made from this analysis. The first is that the maximum specific growth rate was influenced by increasing total sugar concentrations and decreased from approx 0.25 1/h at 100 g/L of total sugars to 0.10 1/h at 150 g/L of total sugars. A similar trend owing to increasing substrate inhibition has been reported previously *(7)* for the host strain ZM4 over the glucose range of 100–300 g/L; however, the trend with ZM4(pZB5) with glucose/xylose mixtures was evident at lower sugar concentrations.

Table 1
Kinetics of ZM4(pZB5) on Glucose/Xylose Mixtures

Kinetic parameter	Glucose/xylose concentration (g/L)				
	50/50	65/65	75/75	100/50	100/0
Glucose/xylose					
μ_{max} (1/h)	0.26	0.17	0.10	0.09	0.25
$q_{Smax,glucose}$ (g/[g·h])	8.3	7.1	8.9	10.2	11.9
$q_{Smax,xylose}$ (g/[g·h])	1.4	1.3	0.9	1.1	N/A[a]
q_{Pmax} (g/[g·h])	4.6	3.9	4.9	5.3	5.5
Xylose					
q_{Smax} (g/[g·h])	2.1	2.1	1.5	0.8	N/A
q_{Pmax} (g/[g·h])	1.0	0.8	0.7	0.3	N/A
Overall					
$Y_{X/S}$ (g/g)	0.019	0.014	0.007	0.007	0.014
$Y_{P/S}$ (g/g)	0.48	0.46	0.47	0.48	0.46

[a]N/A is not applicable.

From Table 1, the maximum specific glucose uptake rate ($q_{smax,glucose}$) for ZM4(pZB5) was within the range of 7.1–10.2 g/(g·h) for all glucose/xylose mixtures. A related observation was true for the host strain ZM4 with $q_{smax,glucose}$ being in the range of 9–11 g/(g·h) for the glucose range of 100–300 g/L *(7)*.

The maximum specific xylose uptake rate ($q_{smax,xylose}$) was in the range of 0.9–1.4 g/(g·h) during the phase of growth/fermentation on the combined sugars and was approx 10–15% of the maximum specific glucose uptake rate. The maximum specific uptake rate of xylose during the second phase of xylose utilization (mainly fermentation) decreased with increasing total sugar concentrations. This presumably resulted from increasing ethanol inhibition effects at the higher sugar levels.

The overall yield of biomass ($Y_{X/S}$) was significantly reduced with the increasing sugar levels owing to the metabolic uncoupling that occurred at the higher ethanol levels. This was evident also for the host strain ZM4, with growth being fully inhibited at 86 g/L of ethanol, whereas ethanol production continued up to 127 g/L at T = 30°C, pH 5.0 *(7)*. The effect was more pronounced for ZM4(pZB5), with ethanol concentrations of 50–60 g/L being sufficient to inhibit further growth on xylose; however, xylose uptake and ethanol production continued at much reduced rates.

Significantly less biomass was produced at the higher total sugar concentrations, and this resulted from the lower overall growth rates in the second phase of growth/fermentation. For example, with 65/65 g/L of glucose/xylose, a biomass concentration of 1.9 g/L was produced, whereas for 75/75 or 100/50 g/L of glucose/xylose, the biomass concentration was approx 1.0 g/L.

Ethanol yields remained high ($Y_{P/S}$ = 0.46–0.48 g/g), based on total sugars utilized. These high yields seemed to be largely unaffected by the initial total sugar concentrations or different sugar ratios.

Table 2
Energetics of Recombinant *Z. mobilis*

- Glucose metabolism:
  glucose + ADP + P_i —→ 2 ethanol + 2 CO2 + ATP
- Xylose metabolism:
  3 xylose + 3 ADP + 3 P_i —→ 5 ethanol + 5 CO2 + 3 ATP

Theoretical yield

- Biomass:[a] $Y_{X/S}$ (glucose) = 10.5/180 = 0.058 g/g
  $Y_{X/S}$ (xylose) = 10.5/150 = 0.073 g/g
- Ethanol: $Y_{P/S}$ (glucose, xylose) = 0.51 g/g

[a]Based on assumption of Y_{ATP} = 10.5 g biomass/mol ATP.

Table 2 summarizes the energetics of ZM4(pZB5) and the theoretical yields that are predicted in the absence of uncoupled metabolism or additional energy requirements. It is clear from this analysis that biomass yields on either glucose or glucose/xylose mixtures are significantly less than theoretical projections.

Inhibition of Acetate on Glucose and Xylose Fermentation

The kinetics on 25 g/L of glucose and 25 g/L of xylose media of ZM4(pZB5) were evaluated in the presence of sodium acetate concentrations over the range of 0–12 g/L. As shown in Fig. 2, the addition of acetate significantly affected glucose and xylose uptake for the recombinant strain. The addition of 4 g/L of sodium acetate (equivalent to 2.9 g/L of acetic acid) had the effect of increasing the fermentation time for xylose uptake from 18 h to 40 h. Table 3 provides detailed kinetic analysis.

The specific glucose uptake rate increased with the addition of 4, 8 g/L of sodium acetate, and the specific xylose uptake rate showed a similar trend. Xylose uptake following glucose depletion was reduced in the presence of 4 g/L of sodium acetate. Using 12 g/L of sodium acetate, only about 5 g/L of xylose was utilized in 36 h. The specific glucose uptake rate increased until 12 g/L of sodium acetate was used. At this level of acetate, the parent strain ZM4 also demonstrated major inhibition on glucose *(5)*. The significant decrease in biomass yield appears to result from increased metabolic uncoupling in the presence of acetate and possible reduced availability of adenosine triphosphate (ATP) *(8)*.

Effect of Temperature and pH on Glucose and Xylose Fermentation

Figure 3 presents the results of varying the temperature from 30 to 33, to 37°C, and the pH between 4.5 and 5.0 with 25 g/L of glucose and 25 g/L of xylose media on the kinetics of ZM4(pZB5). Table 4 presents a detailed analysis of these fermentations. It is evident from the results that a decrease in the maximum specific growth rate occurred at the less optimal conditions. This was accompanied by a decrease in the biomass yield. The specific glucose and xylose uptake and ethanol production rates were relatively unchanged except at the most extreme conditions of 37°C, pH 4.5. The overall ethanol yield remained unchanged, except for this latter condition.

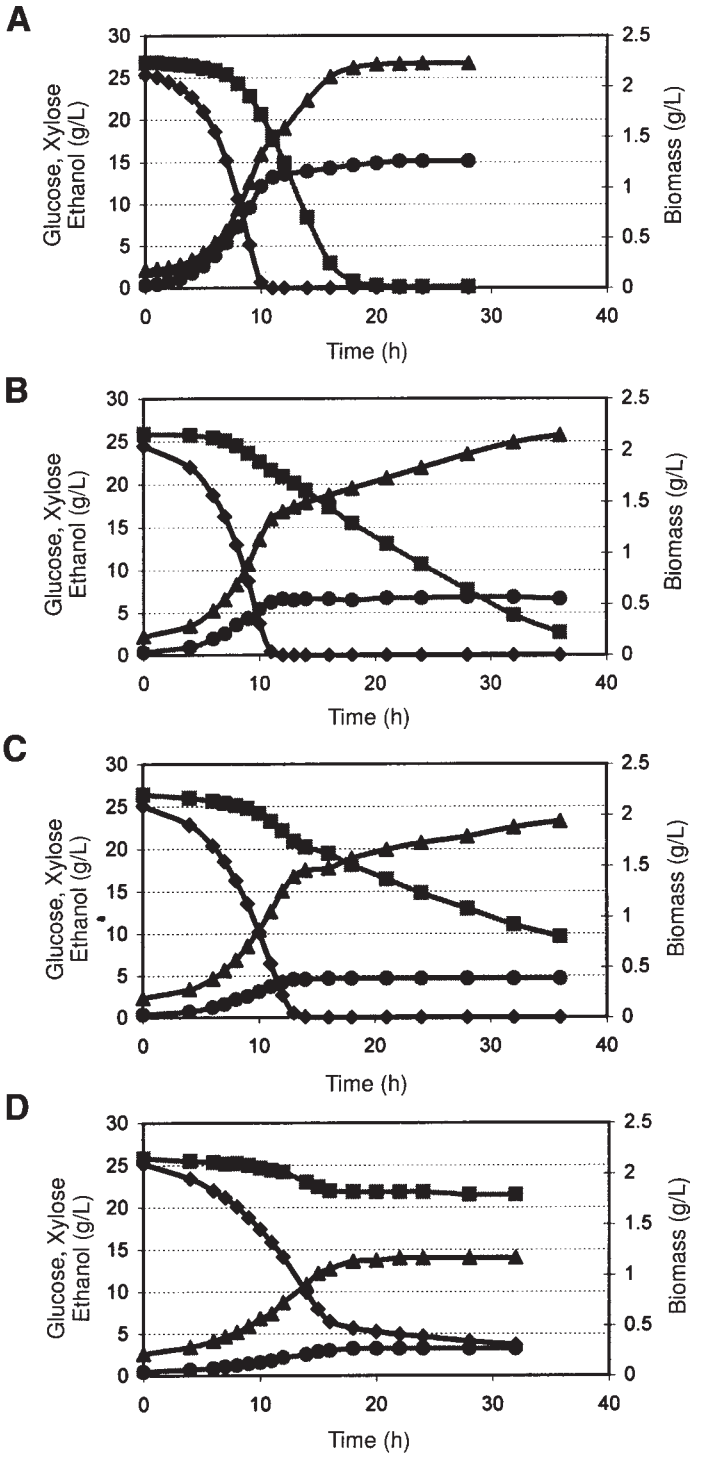

Fig. 2. Fermentation profiles of ZM4(pZB5) on 25 g/L of glucose and 25 g/L of xylose media with **(A)** 0 g/L; **(B)** 4 g/L; **(C)** 8 g/L; and **(D)** 12 g/L of sodium acetate. ◆, Glucose; ■, xylose; ▲, ethanol; ●, biomass.

Table 3
Kinetic Parameters of ZM4(pZB5) on 25 g/L of Glucose and 25 g/L of Xylose at Various Concentrations of Sodium Acetate

	Concentration of sodium acetate (g/L)			
Kinetic parameter	0	4	8	12
Glucose/xylose				
μ_{max} (1/h)	0.43	0.26	0.24	0.15
$q_{Smax,glucose}$ (g/[g·h])	9.2	14.5	18.1	11.4
$q_{Smax,xylose}$ (g/[g·h])	2.6	2.7	4.1	2.6
q_{Pmax} (g/[g·h])	4.7	8.6	8.4	6.5
Xylose				
q_{Smax} (g/[g·h])	3.0	1.8	1.8	—
q_{Pmax} (g/[g·h])	1.5	0.7	0.6	—
Overall				
$Y_{X/S}$ (g/g)	0.024	0.011	0.009	0.009
$Y_{P/S}$ (g/g)	0.47	0.49	0.50	0.46

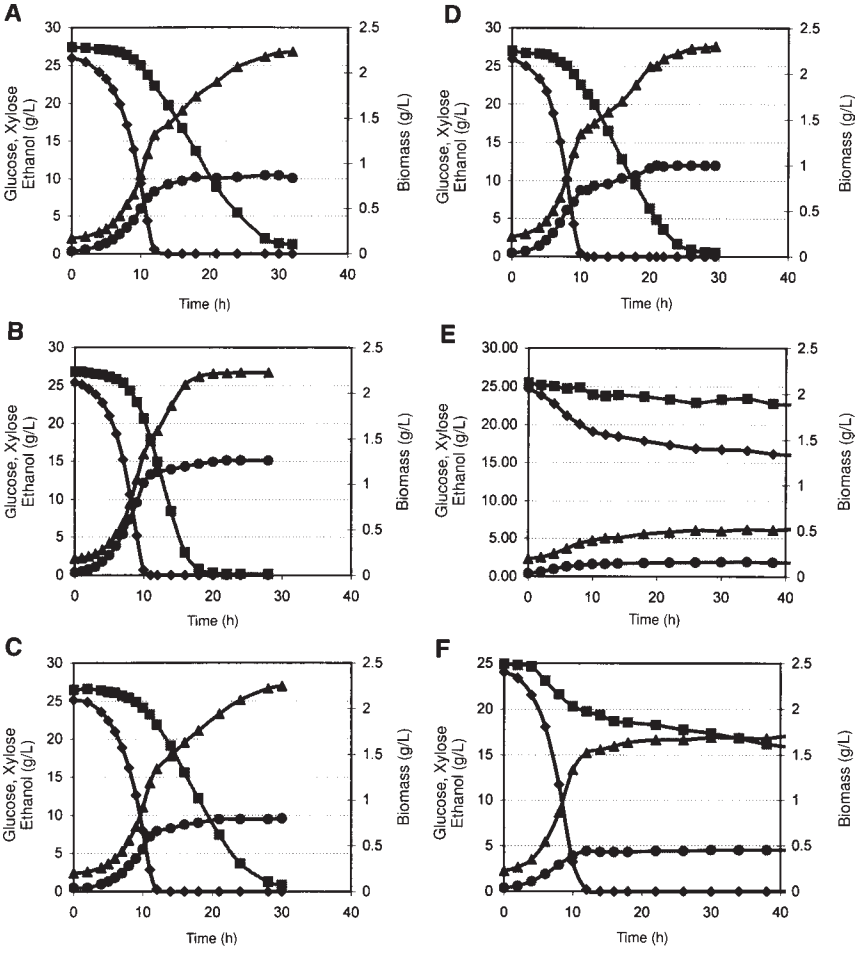

Fig. 3. Fermentation profiles of ZM4(pZB5) on 25 g/L of glucose and 25 g/L of xylose media at various conditions: **(A)** pH 4.5, 30°C; **(B)** pH 5.0, 30°C; **(C)** pH 4.5, 33°C; **(D)** pH 5.0, 33°C; **(E)** pH 4.5, 37°C; and **(F)** pH 5.0, 37°C. ◆, Glucose; ■, xylose; ▲, ethanol; ●, biomass.

Table 4
Kinetic Parameters of Fermentation of 25 g/L of Glucose and 25 g/L of Xylose for ZM4(pZB5) at Various Temperature and pH Combinations

Kinetic parameter	pH 4.5/30°C	pH 4.5/33°C	pH 4.5/37°C	pH 5.0/30°C	pH 5.0/33°C	pH 5.0/37°C
Glucose/xylose						
μ_{max} (1/h)	0.29	0.29	0.24	0.43	0.36	0.26
$q_{Smax,glucose}$ (g/[g·h])	10.6	12.0	11.5	9.2	12.4	13.0
$q_{Smax,xylose}$ (g/[g·h])	2.4	2.2	2.8	2.6	2.2	4.0
q_{Pmax} (g/[g·h])	5.3	6.1	3.9	4.7	6.1	6.8
Xylose						
q_{Smax} (g/[g·h])	1.9	2.4	—	3.0	2.2	0.3
q_{Pmax} (g/[g·h])	1.1	1.3	—	1.5	1.1	0.5
Overall						
$Y_{P/S}$ (g/g)	0.47	0.48	0.33	0.47	0.45	0.45
$Y_{X/S}$ (g/g)	0.016	0.015	0.011	0.024	0.018	0.012
Q_P (g/[L·h])	0.77	0.82	0.07	1.02	0.68	0.29

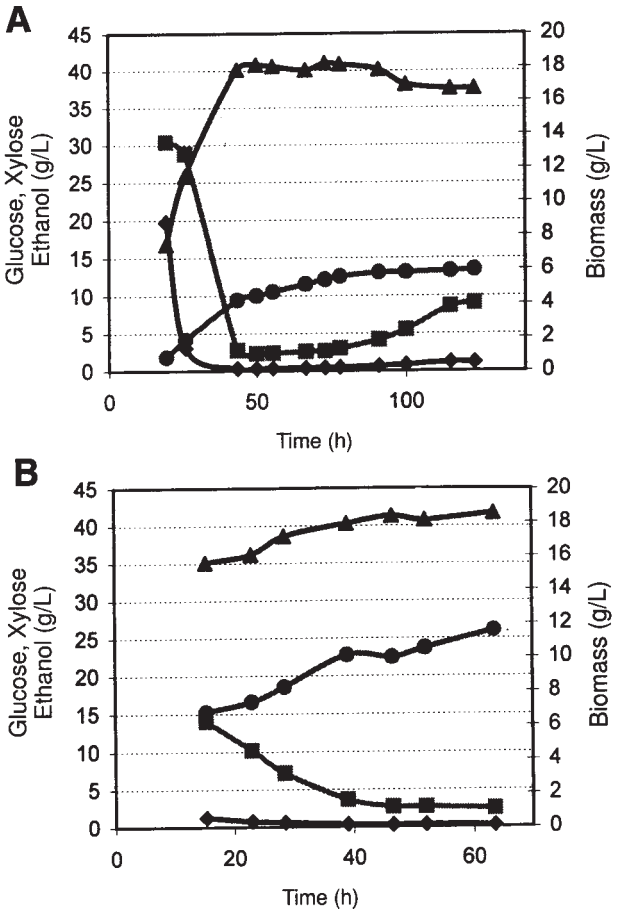

Fig. 4. Fermentation profile of the continuous cell recycle of ZM4(pZB5) on 40 g/L of glucose and 40 g/L of xylose medium at **(A)** D = 0.1 1/h; and **(B)** D = 0.15 1/h. ◆, Glucose; ■, xylose; ▲, ethanol; ●, biomass.

Cell Recycling

Laboratory-scale, continuous culture cell-recycle studies were carried out using a Filtron Ultrasette membrane unit (Pall Gelman, Ann Arbor, MI, cat. no. OS994C72). This cross-flow filtration module involves an open channel design with modified polyethersulfone membranes (700-cm^2 effective filtration area, 0.16-μm pore size). A variable-speed peristaltic pump (Watson-Marlow 604S, Falmouth, Cornwell, England) was used to circulate the culture though the membrane unit.

Figure 4 presents data from experiments at two dilution rates, 0.1 and 0.15 1/h, using 40 g/L of glucose and 40 g/L of xylose medium (T = 30°C, pH 5.0). Steady-state conditions were achieved after 50–60 h with essentially zero residual glucose and 2–5 g/L of residual xylose. The biomass concentration increased to approx 6 g/L at 0.1 1/h, with an ethanol concentration close to 40 g/L. At 0.15 1/h, a biomass concentration of 10 to 11 g/L,

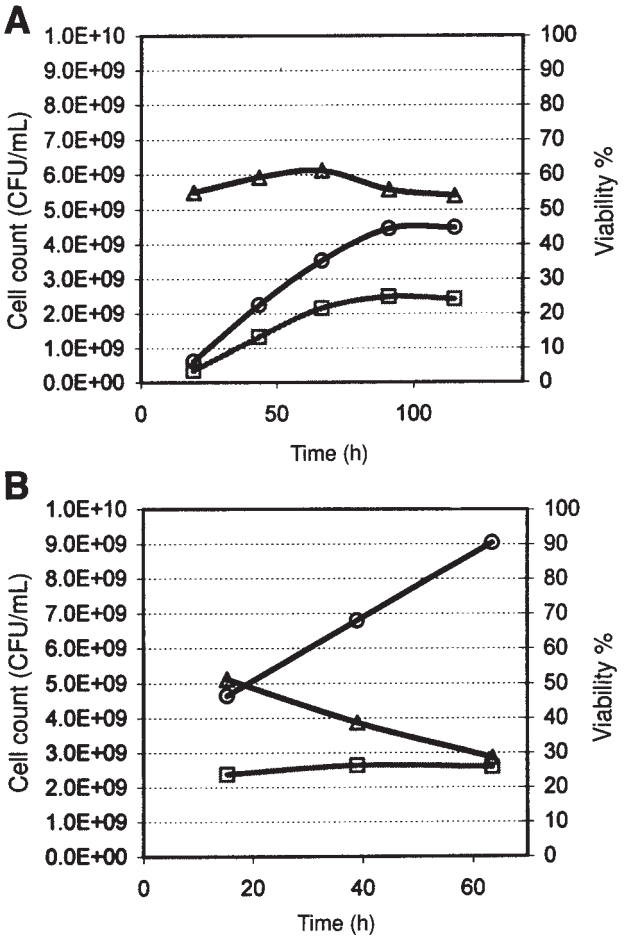

Fig. 5. Viability of cells in continuous cell culture on 40 g/L of glucose and 40 g/L of xylose medium at **(A)** $D = 0.1$ 1/h; and **(B)** $D = 0.15$ 1/h. ○, Total cell count; □, viable cell count; △, percentage of viability.

an ethanol concentration of 41 g/L, and an ethanol productivity of 6.2 g/(L·h) were achieved. Cell viability was monitored (Fig. 5) and found to reach a constant level of 55–60% at the lower dilution rate. However, at the higher dilution rate, as the total cell concentration increased, the percentage of viable cells was found to decrease continuously.

Another cell-recycle study was performed using 50 g/L of glucose and 50 g/L of xylose medium at a dilution rate of 0.1 1/h (Fig. 6). A biomass concentration of approx 6.2 g/L with an ethanol concentration of 50 g/L and an ethanol productivity of 5.0 g/(L·h) were obtained after 50–60 h. However, the viability of cells was found to decrease after 50 h of operation, indicating possible shear effects on the cells in the membrane-recycle system at 5 to 6 g/L of cell concentrations or the negative effect on cell viability of sustained uncoupled metabolism with essentially zero cell growth rates.

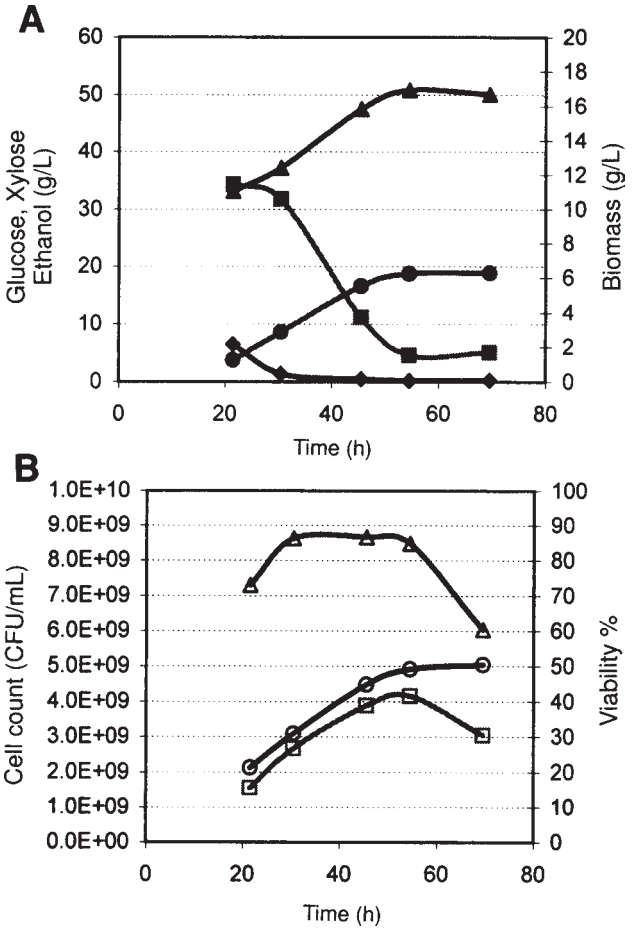

Fig. 6. Continuous cell culture on 50 g/L of glucose and 50 g/L of xylose medium at $D = 0.1$ 1/h. **(A)** Fermentation profile: ◆, glucose; ■, xylose; ▲, ethanol; ●, biomass. **(B)** Viability of cells: ○, Total cell count; □, viable cell count; △, percentage of viability.

Discussion

From the detailed kinetic analysis of varying sugar concentrations, it was evident that the main influence of the increasing sugar concentrations for glucose/xylose mixtures was to reduce the maximum specific growth rate, increase the extent of uncoupled metabolism, and consequently reduce overall biomass yields (Table 1). Maximum specific sugar uptake rates during the major growth and fermentation phase, and overall ethanol yields (90–94% theoretical), were relatively constant. From a theoretical analysis of the cellular energetics, and assuming 10.5 g of biomass/mol of ATP, it was apparent that biomass yields were significantly lower than theoretical projections. This trend was even more evident at the higher sugar concentrations. This may be indicative of lower levels of ATP production than predicted theoretically, significant uncoupled metabolism

with no cell growth, or additional ATP requirements of the recombinant strain to maintain the plasmid (pZB5) under conditions of increasing sugar and, subsequently, ethanol concentrations. Evidence of depleted levels of nucleoside triphosphates (mainly ATP) for ZM4(pZB5) growing on xylose, when compared to those for growth on glucose, comes from our recent ^{31}P nuclear magnetic resonance (NMR) studies *(8)*. Clearly, more detailed studies of the energetics of the recombinant strain would be beneficial.

Other studies have reported on a common transporter (glucose-facilitated diffusion transporter, Glf) for glucose and xylose uptake by *Z. mobilis (9)*. The present kinetic studies with different sugar concentrations would seem to indicate that the activity of the common transporter, as evidenced by the maximum specific uptakes of both glucose and xylose, was largely unaffected by increasing sugar concentrations, at least until ethanol concentrations began to reach inhibiting levels. However, the inhibition studies with sodium acetate (and increasing temperatures above 30°C) indicated increased maximum specific uptake rates of glucose and xylose under these higher stress conditions. It is possible that the common transporter can respond to these conditions and thus facilitate enhanced sugar uptake in attempting to mediate these stress conditions.

From the investigation into the effects of fermentation conditions on the cofermentation of glucose and xylose, it appears that one or more of the cloned enzymes involved in xylose uptake and assimilation in ZM4(pZB5) is more sensitive to the effect of increasing temperature and/or the inhibitory effect of ethanol (at about 50–60 g/L) than enzymes of the Entner-Doudoroff pathway *(7,10)*. This would also appear to be true for cloned enzymes involved in the uptake of arabinose by a recombinant strain of *Z. mobilis*, in which further uptake of arabinose ceases once the ethanol concentration reaches 20 g/L *(11)*. Inhibition may also result from the accumulation of xylitol phosphate in the recombinant strain, because this has been reported to increase with increasing temperature for some organisms *(12)*. Xylitol accumulating in the form of xylitol phosphate has been reported previously to have inhibitory effects on *Z. mobilis (13)*. Other investigators using ^{13}C-NMR flux analysis have identified a metabolic bottleneck in a recombinant xylose-fermenting *Z. mobilis* at the level of the heterologous xylulokinase *(14)*.

Cell recycling has led to a significant improvement in productivity. At the higher dilution rate of D = 0.15 1/h, an ethanol productivity in excess of 6 g/(L·h) was maintained for at least 20 h. Although the percentage of viable cells decreased, a stable ethanol concentration of 40 g/L with 97% sugar utilization was achieved. At 50/50 g/L of glucose/xylose, an ethanol concentration of 50 g/L was sustained at D = 0.1 1/h. However, the increasing load of nonviable cells, with the associated increase in biomass concentration, may necessitate a slow bleed of concentrated cells to provide for a low cell growth rate and maintain long-term stable operation.

Appendix: Nomenclature

Q_P = volumetric ethanol productivity (g/[L·h])
$q_{P\max}$ = overall maximum specific ethanol production rate (g/[g·h])
$q_{S\max}$ = overall maximum specific substrate utilization rate (g/[g·h])
$Y_{P/S}$ = overall product yield on substrate (g/g)
$Y_{X/S}$ = overall biomass yield on substrate (g/g)
$\mu_{\max}$ = maximum overall specific growth rate (1/h)

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