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Evaluation of Recombinant Strains of *Zymomonas mobilis* for Ethanol Production from Glucose/Xylose Media

Eva Joachimsthal, Kevin D. Haggett, and Peter L. Rogers*

Department of Biotechnology, University of New South Wales, Sydney, Australia 2052, E-mail: p.rogers@unsw.edu.au

Abstract

The fermentation characteristics of two recombinant strains of *Zymomonas mobilis*, viz. CP4 (pZB5) and ZM4 (pZB5), capable of converting both glucose and xylose to ethanol, have been characterized in batch and continuous culture studies. The strain ZM4 (pZB5) was found to be capable of converting a mixture of 65 g/L glucose and 65 g/L xylose to 62 g/L ethanol in 48 h with a yield of 0.46 g/g. Higher sugar concentrations resulted in incomplete xylose utilization (80 h) presumably owing to ethanol inhibition of xylose assimilation or metabolism. The fermentation results with ZM4 (pZB5) show a significant improvement over results published previously for recombinant yeasts and other bacteria capable of glucose and xylose utilization.

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

Introduction

The development of a cost-effective fermentation process for ethanol production from lignocellulosic hydrolysates will require a microorganism that is capable of high-efficiency and high-productivity conversion of both hexose and pentose sugars to ethanol. In response to this challenge, recombinant strains of *Saccharomyces cerevisiae (1)* as well as *Escherichia coli (2)*, *Klebsiella oxytoca (3)*, and *Zymomonas mobilis (4)* have been developed, with some such strains now capable of producing ethanol at concentrations of 50–60 g/L, productivities in batch culture in excess of 1 g/L·h, and conversion efficiencies above 90% theoretical. Along with their ethanol producing abilities, other important issues need to be considered for these recombinant yeasts and bacteria, including strain stability (particularly in long-term continuous culture), resistance to inhibitors in the lignocellulosic

*Author to whom all correspondence and reprint requests should be addressed.

hydrolysates (e.g., acetate, furfural), and their potential for contamination control during extended process operation.

Of particular recent interest is the Gram-negative bacterium *Z. mobilis*, which has been shown in earlier studies *(5–7)* to be capable of higher specific rates of sugar uptake and ethanol production, and higher ethanol conversion efficiencies when compared to yeasts. Strains of *Z. mobilis* have also been shown to produce up to 125 g/L ethanol from 300 g/L glucose medium under controlled batch culture conditions (T = 30°C, pH = 5.0) *(6)*.

In the present study, recombinant strains of *Z. mobilis* developed by Zhang et al. *(4)* are further characterized in batch and continuous culture studies. This research complements that previously reported by Lawford et al. *(8)* with the recombinant strain *Z. mobilis* CP4 (pZB5), and extends it with the more ethanol-tolerant and productive strain *Z. mobilis* ZM4 (pZB5). The parent culture (viz., ZM4/ATCC 31821) of this latter strain was selected previously by our group for its enhanced characteristics *(9)* and was the subject of three patents that focused on high productivity semi-batch, continuous, and cell-recycle processes for ethanol production *(10–12)*.

Materials and Methods

Microorganisms

Recombinant *Z. mobilis* strains CP4 (pZB5) and ZM4 (pZB5), which contain the *Escherichia coli* genes for xylose assimilation (xylose isomerase, xylulokinase) and pentose metabolism (transketolase, transaldolase) on the plasmid pZB5, together with the gene for tetracycline resistance, were kindly provided by Dr. Min Zhang under a Materials Transfer Agreement (MTA) with the National Renewable Energy Laboratory (NREL), Golden, Colorado.

Media Composition and Preparation

Growth media for the recombinant *Z. mobilis* strains were as follows: glucose and xylose concentrations as specified, $(NH_4)_2SO_4$ (1 g/L in media containing 40 g/L glucose/40g/L xylose or less; 2 g/L in media containing higher sugar levels); KH_2PO_4 (2 g/L); $MgSO_4 \cdot 7H_2O$ (1 g/L); yeast extract (Oxoid, Melbourne, Victoria) (10 g/L inoculum, 5 g/L fermentation media). Media were sterilized by autoclaving at 121°C for 10 min. Media for continuous culture was filter-sterilized. 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.

Preparation of Inocula

All inocula were prepared in stationary shake flasks incubated at 30°C. Inocula were grown under the same media conditions and xylose concentrations as for each batch fermentation, except that glucose concentrations were reduced to 5 g/L to minimize decrease in pH in shake flasks and hence

reduce initial lag phase on inoculation. Cells were grown overnight and inoculated into the fermentor to give an initial optical density (OD) (660 nm; 1 cm light path) of approximately 0.1, which corresponded to 30 mg/L dry cell weight (DCW).

Batch Fermentations

Experiments were conducted in a 2 L LH Fermentation (UK) fermentor with 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 for batch and 500 rpm for continuous culture (the latter higher value to minimize wall growth in long-term studies). Samples for sugar and ethanol determinations were collected at various times and stored at –20°C prior to analysis. Samples for dry weight and OD determinations were taken and analyzed directly.

Analytical Methods

Biomass concentrations for batch-culture experiments were determined by OD measurements (at 660 nm; 1 cm light path) and converted to DCW via a calibration curve. For continuous culture, samples were taken after steady state had been reached (5–6 generations) and direct OD and dry weight measurements taken. Several samples were taken at steady state over 24–48 h and mean and standard deviations determined.

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

Calculation of Kinetic Parameters

The maximum specific growth rate (μ_m) values were calculated from the exponential phase of growth of *Z. mobilis* on the glucose/xylose mixtures or from the phase of growth when glucose was fully utilized and only xylose remained.

The values of the specific sugar uptake rate (q_s) and the specific ethanol production rate (q_p) for combined glucose and xylose utilization were calculated over the exponential phase of growth and based on the formulae:

$$q_s = (1/x)\,(ds/dt) \text{ and } q_p = (1/x)\,(dp/dt) \quad (1)$$

where x, s and p are the concentrations of biomass, sugars, and ethanol, respectively. For the exponential phase calculations, the formulae were modified to:

$$q_s = \mu_m / Y'_{x/s} \text{ and } q_p = \mu_m \cdot Y'_{p/x} \quad (2)$$

where the yields were calculated over the exponential phase.

For the xylose utilization phase, where growth was very limited, or did not occur at all, the formulae were modified to:

$$q_s = (1/x_{av})\ (\Delta s/\Delta t) \text{ and } q_p = (1/x_{av})\ (\Delta p/\Delta t) \quad (3)$$

where Δs and Δp are the changes in the sugar and ethanol concentrations, respectively, over the time period Δt, and x_{av} is the average biomass concentration over Δt (4–6 h).

The overall yields, $Y_{x/s}$ and $Y_{p/s}$, were based on the initial and final concentrations of biomass, combined sugars and ethanol (g/L).

Results and Discussion

Kinetics of Recombinant Z. mobilis *CP4(pZB5)*

Batch Culture

The effect of varying sugar concentrations with glucose/xylose in a 1:1 ratio was evaluated for the recombinant strain *Z. mobilis* CP4 (pZB5) under controlled batch culture conditions (T = 30°C, pH = 5.0). A similar 1:1 ratio of sugars was used by Zhang et al. *(4)* in their original fermentation studies with *Z. mobilis* CP4 (pZB5). In Fig. 1, the kinetics are shown for growth on 25 g/L glucose (A), 25 g/L xylose (B), and a mixture of the two sugars (C). From the results shown in Fig. 1C, it is evident that two growth phases occurred, with the initial uptake of glucose and xylose being followed by slower growth on xylose after glucose depletion. Figure 2 shows the kinetics of growth and fermentation on 65 g/L of both glucose and xylose, and it is clear that significant uptake of xylose occurred before glucose depletion. No additional growth on xylose occurred after glucose exhaustion, although slower xylose uptake and ethanol production continued in apparent uncoupled metabolism. The relevant kinetic parameters were determined for the combined sugars in the first phase, and for xylose alone in phase two.

The kinetic parameters are shown in Table 1 for a range of sugar concentrations from 25/25–65/65 (g/L) glucose/xylose mixtures, and indicate declining specific rates of growth, sugar uptake, xylose uptake and ethanol production with increasing sugar concentrations presumably owing to substrate inhibition effects. The overall biomass yields are in the range 0.02–0.03 g/g, and ethanol yields 0.46–0.47 g/g.

Continuous Culture

Continuous-culture experiments have been carried out over the dilution rate range D = 0.05–0.15/h for glucose/xylose concentrations of 25/25 and 40/40 g/L. For the 25/25 sugar concentrations, substrate utilization was virtually complete (residual glucose = 0, residual xylose = 0.8–1.0 g/L) and ethanol yields in the range 0.46–0.49 g/g were achieved.

The results for 40/40 g/L are shown in Fig. 3 and it is evident that maximum uptake of glucose and xylose only occurred in the range of

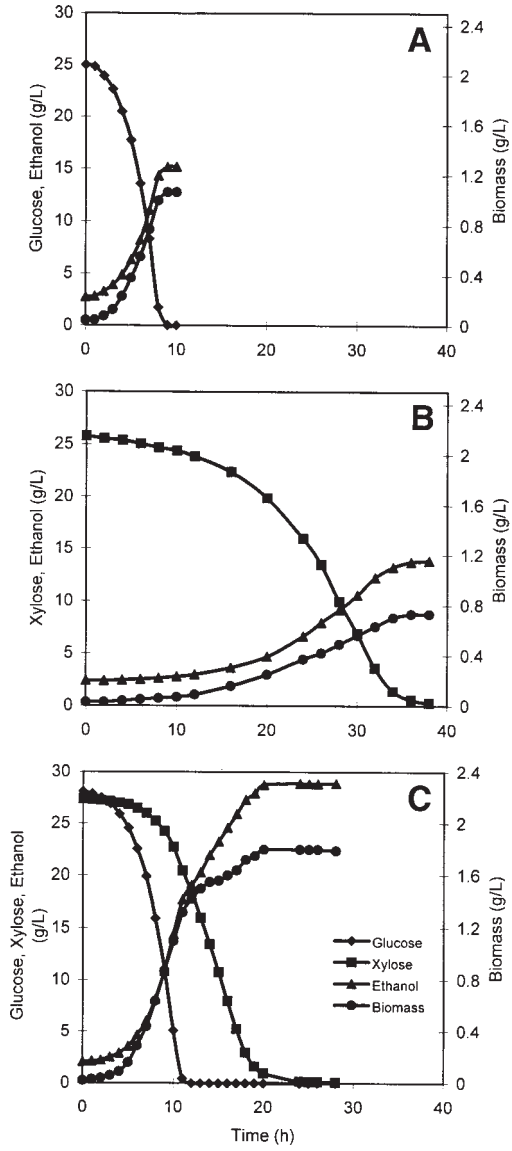

Fig. 1. Batch-fermentation data of CP4(pZB5) on 25 g/L glucose **(A)**, 25 g/L xylose **(B)**, and 25 g/L each glucose and xylose **(C)** medium.

D = 0.05–0.065/h with 2 g/L xylose still remaining at these low dilution rates. A similar observation of a low level of residual xylose (with zero glucose) was made by Lawford et al. *(13)* for chemostat culture of *Z. mobilis* 39676:pZB4L with 40 g/L xylose, 8 g/L glucose medium. In the present experiments, some decline in biomass concentrations also occurred in this low dilution-rate region and was evidence of significant uptake of substrate for maintenance energy requirements (endogenous metabo-

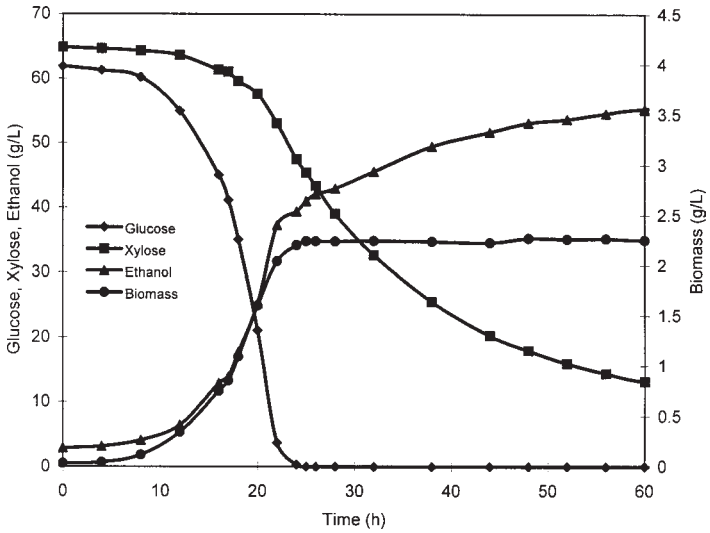

Fig. 2. Batch-fermentation data of CP4(pZB5) on 65 g/L glucose and 65 g/L xylose medium (pH 5.0, 30°C, 200 rpm).

Table 1
Kinetic Parameters for *Z. mobilis* CP4 (pZB5)
on Various Concentrations of Glucose/Xylose Media (T = 30°C, pH = 5.0)

Kinetic parameters	Glucose/xylose (g/L)			
	25/25	40/40	50/50	65/65
Glucose/xylose				
μ_m (/h)	0.42	0.39	0.28	0.27
q_s (g/g·h)	12.3	8.6	8.4	6.5
q_p (g/g·h)	4.7	3.8	3.1	3.0
Xylose				
μ_m (/h)	0.03	0.02	—	—
q_s (g/g·h)	1.2	1.1	1.1	0.6
q_p (g/g·h)	0.7	0.5	0.5	0.3
Overall				
$Y_{x/s}$ (g/g)	0.03	0.03	0.02	0.02
$Y_{p/s}$ (g/g)	0.47	0.46	0.46	0.46

lism). Ethanol yields were high at 0.49 g/g (96% theoretical) at these low-dilution rates.

Kinetics of Recombinant Z. mobilis *ZM4 (pZB5)*

Batch Culture

Batch-culture kinetic studies have been carried out on *Z. mobilis* ZM4 (pZB5) for the sugar concentrations 50/50, 65/65 and 75/75 g/L under the same media compositions and environmental conditions as for CP4 (pZB5).

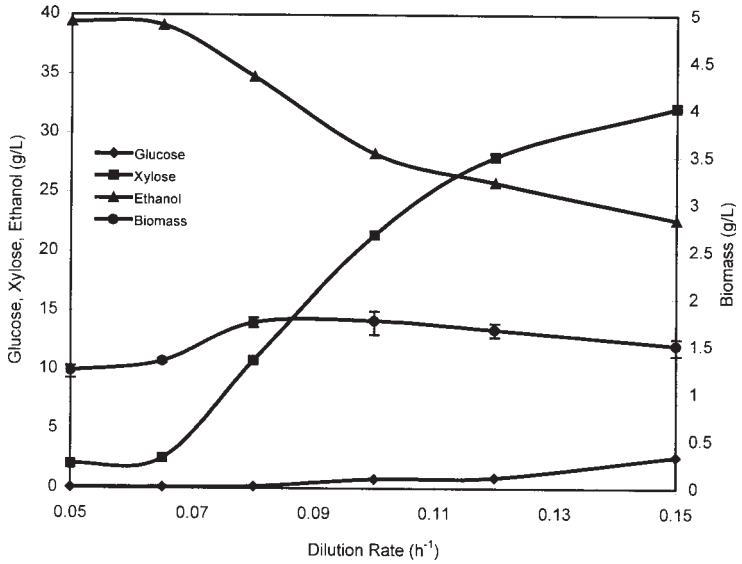

Fig. 3. Continuous culture of recombinant *Z. mobilis* CP4(pZB5) in medium containing 40 g/L glucose and 40 g/L xylose (pH 5.0, 30°C, 500 rpm).

Table 2
Kinetic Parameters of *Z. mobilis* ZM4 (pZB5)
on Various Concentrations
of Glucose/Xylose Media (T = 30°C, pH = 5.0)

Kinetic parameters	Glucose/xylose (g/L)		
	50/50	65/65	75/75
Glucose/xylose			
μ_m (/h)	0.26	0.20	0.08
q_s (g/g·h)	9.5	9.0	9.3
q_p (g/g·h)	4.5	3.8	4.4
Xylose			
μ_m (/h)	0.02	0.01	—
q_s (g/g·h)	2.1	2.1	1.4
q_p (g/g·h)	1.0	0.84	0.67
Overall			
$Y_{x/s}$ (g/g)	0.025	0.025	0.01
$Y_{p/s}$ (g/g)	0.48	0.46	0.47

The results of the kinetic analysis are given in Table 2 and batch-culture data for 65/65 g/L glucose/xylose are shown in Fig. 4 for comparison with the data for *Z. mobilis* CP4 (pZB5) presented in Table 1 and Fig. 2.

Some similar trends are evident in Tables 1 and 2, although the specific rates of sugar uptake and ethanol production for ZM4 (pZB5) did not appear to be reduced by substrate inhibition, compared to the reductions

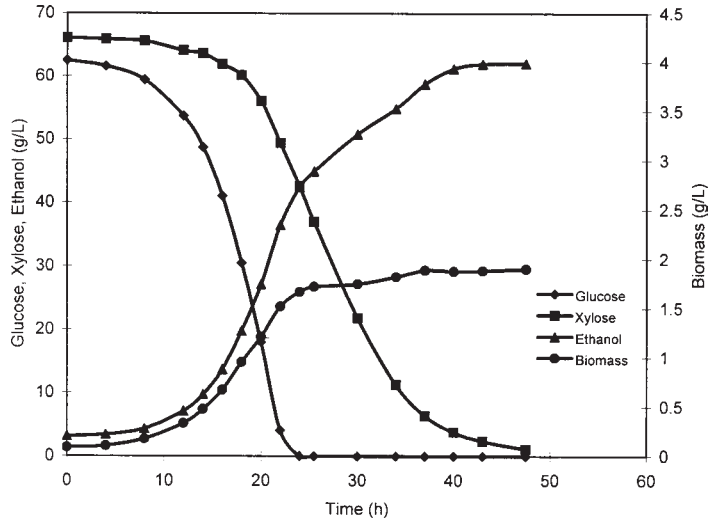

Fig. 4. Batch-fermentation data of ZM4(pZB5) on 65 g/L glucose and 65 g/L xylose medium (pH 5.0, 30°C, 200 rpm).

that occurred with CP4 (pZB5). Declining growth on xylose occurred for both recombinant strains following glucose depletion, presumably owing to ethanol inhibition.

From a comparison of batch-culture results for strains CP4 (pZB5) and ZM4 (pZB5) as shown in Figs. 2 and 4, respectively, it is evident that complete utilization of both glucose and xylose can occur within 48 h only for the latter strain. This observation is supported by the higher specific xylose-uptake rate for ZM4 (pZB5) of 2.1 g/g·h compared to 0.6 g/g·h for CP4 (pZB5) at 65/65 g/L glucose/xylose. Ethanol yields of 0.46 g/g were the same for both strains. A final ethanol concentration of 62 g/L was attained in 48 h with ZM4 (pZB5), which compares with 52 g/L after 60 h for CP4 (pZB5). This result with CP4 (pZB5) was corroborated by the recent studies of Lawford and Rousseau *(14)* who reported that this recombinant strain was completely inhibited by 55 g/L ethanol at pH = 5.0.

At the higher sugar concentration of 75/75 g/L glucose/xylose (*see* Fig. 5), although the glucose was fully utilized at 36 h, the uptake of xylose was incomplete at 80 h with residual xylose of 12 g/L. A final ethanol concentration of 67 g/L was attained at this time. One significant difference between these results and those at 65/65 g/L was the major reduction in final biomass concentration in the latter case (which would influence the overall xylose-uptake rate during the latter part of fermentation). For the lower sugar level, 1.7 g/L biomass was produced (overall $Y_{x/s} = 0.025$ g/g), whereas at the higher sugar level, only 0.9 g/L biomass was produced ($Y_{x/s} = 0.011$ g/g) with no further growth occurring after glucose depletion. The experiment at 75/75 g/L has been carried out twice and similar results obtained in both cases.

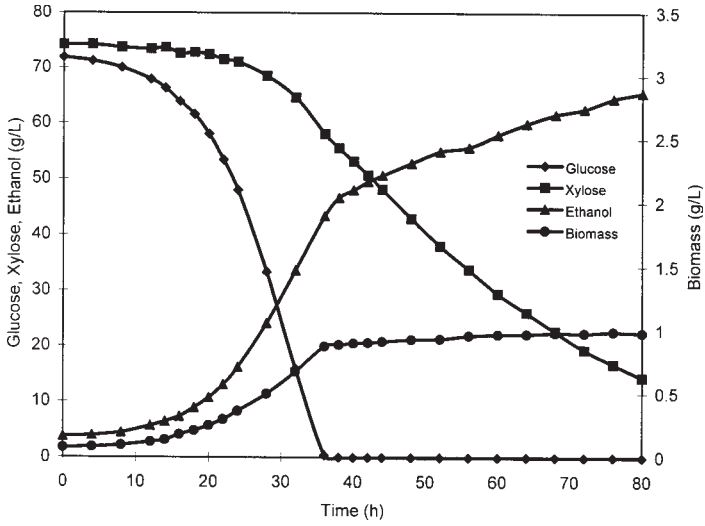

Fig. 5. Batch-fermentation data of ZM4(pZB5) on 75 g/L glucose and 75 g/L xylose medium (pH 5.0, 30°C, 200 rpm).

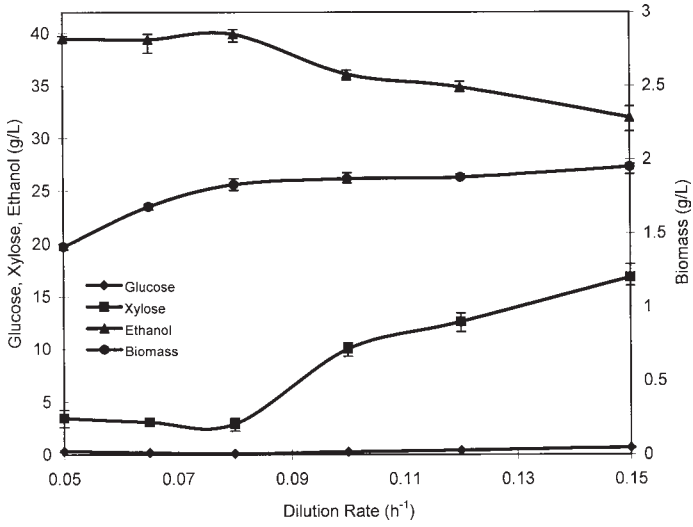

Fig. 6. Continuous-fermentation profiles of ZM4(pZB5) on 40 g/L glucose and 40 g/L xylose medium at various dilution rates (pH 5.0, 30°C, 500 rpm).

Continuous Culture

Continuous-culture data for *Z. mobilis* ZM4 (pZB5) for medium containing 40 g/L glucose and 40 g/L xylose are shown in Fig. 6, over the dilution-rate range D = 0.05–0.15/h. For the low-dilution rates D = 0.05–0.08/h, the glucose was fully utilized and the residual xylose was 2–3 g/L. As the dilution rate was increased above D = 0.08/h, the residual xylose concentration increased although the residual glucose concentration

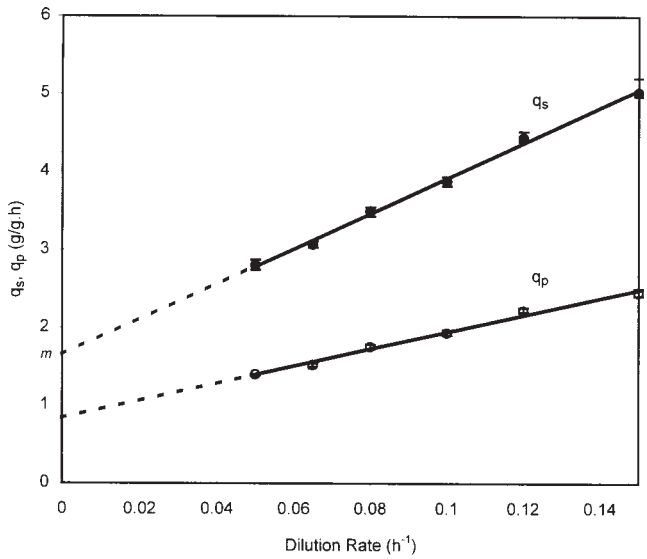

Fig. 7. Estimation of maintenance energy coefficient (m) from continuous culture data for *Z. mobilis* ZM4(pZB5) on 40 g/L glucose and 40 g/L xylose medium (pH 5.0, 30°C, 500 rpm).

remained close to zero. It is interesting to compare these results with those reported for CP4 (pZB5) with the same medium (Fig. 3), where it was found that the residual xylose concentration increased to 10 g/L at a dilution rate of 0.08/h. This indicates a higher specific xylose-uptake rate (q_s) for strain ZM4 (pZB5), a result that is consistent with the batch-culture data comparison between the two strains.

Further analyses of the continuous-culture data are shown in Fig. 7, where the overall specific sugar-uptake rates for glucose plus xylose (q_s) and the specific ethanol-production rates (q_p) are plotted as functions of the dilution rate (D). Linear relationships were evident for both q_s and q_p vs D, and the maintenance energy coefficient (m) was estimated by extrapolation of the q_s linear plot to D = 0. As shown in Fig. 7, a maintenance energy coefficient (m) was estimated by regression analysis from the intercept on the Y-axis as 1.65 g/g·h and a true yield ($Y_{x/s}$) of 0.044 g/g estimated from the reciprocal slope of the line. Analysis of the data in Fig. 6 gave an ethanol yield ($Y_{p/s}$) based on total sugars utilized of 0.49–0.50 g/g.

It is interesting to note that previous studies on the determination of the maintenance energy coefficient for *Z. mobilis* ZM4 (parent strain) have reported an averaged value of 1.6 g/g·h based on a 100 g/L glucose medium and using a cell-recycle, continuous-culture system for analysis *(15)*. The present results with ZM4 (pZB5) indicate that the incorporation of the 14.30 kb plasmid, encoding genes for xylose assimilation and pentose metabolism *(4)*, has had no evident effect on the maintenance energy requirements of *Z. mobilis* ZM4.

Conclusions

An improved recombinant strain of *Z. mobilis* ZM4 (pZB5), developed at NREL, has been characterized in batch- and continuous-culture kinetic evaluations and compared with the recombinant strain CP4 (pZB5) studied by other authors *(4,14)*. This former strain was capable of fully utilizing a 65/65 g/L glucose/xylose substrate at pH = 5.0 in 48 h and producing 62 g/L ethanol with a yield based on total sugars ($Y_{p/s}$) = 0.46 g/g. At increased sugar levels of 75 g/L glucose/75 g/L xylose, the overall fermentation rate was considerably reduced and a final ethanol level of 67 g/L achieved. Comparisons of previously reported data on glucose/xylose fermentations for recombinant strains *of S. cerevisiae (1)*, *E. coli (2)*, and *K. oxytoca (3)* have shown that *Z. mobilis* CP4 (pZB5) can achieve comparable (if not better) results based on maximum ethanol concentrations, ethanol yields, and batch-fermentation productivities *(16)*. The present data with ZM4 (pZB5) shows a further enhancement compared to CP4 (pZB5) and underscores the potential of a *Zymomonas*-based lignocellulosic fermentation process.

Acknowledgment

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