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Comparative Energetics of Glucose and Xylose Metabolism in Recombinant *Zymomonas mobilis*

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Abstract

Recombinant *Zymomonas mobilis* CP4:pZB5 was grown with pH control in batch and continuous modes with either glucose or xylose as the sole carbon and energy source. In batch cultures in which the ratio of the final cell mass concentration to the amount of sugar in the medium was constant (i.e., under conditions that promote "coupled growth"), maximum specific rates of glucose and xylose consumption were 8.5 and 2.1 g/(g of cell·h), respectively; maximum specific rates of ethanol production for glucose and xylose were 4.1 and 1.0 g/(g of cell·h), respectively; and average growth yields from glucose and xylose were 0.055 and 0.034 g of dry cell mass (DCM)/g of sugar, respectively. The corresponding value of Y_{ATP} for glucose and xylose was 9.9 and 5.1 g of DCM/mol of ATP, respectively. Y_{ATP} for the wild-type culture CP4 with glucose was 10.4 g of DCM/mol of ATP. For single substrate chemostat cultures in which the growth rate was varied as the dilution rate (D), the maximum or "true" growth yield (max $Y_{x/s}$) was calculated from Pirt plots as the inverse of the slope of the best-fit linear regression for the specific sugar utilization rate as a function of D, and the "maintenance coefficient" (m) was determined as the y-axis intercept. For xylose, values of max $Y_{x/s}$ and m were 0.0417 g of DCM/g of xylose (Y_{ATP} = 6.25) and 0.04 g of xylose/(g of cell·h), respectively. However, with glucose there was an observed deviation from linearity, and the data in the Pirt plot was best fit with a second-order polynomial in D. At $D > 0.1$/h, Y_{ATP} = 8.71 and m = 2.05 g of glu/(g of cell·h) whereas at $D < 0.1$/h, Y_{ATP} = 4.9 g of DCM/mol of ATP and m = 0.04 g of glu/(g of cell·h). This observation provides evidence to question the validity of the unstructured growth model and the assumption that Pirt's maintenance coefficient is a constant that is independent of the growth rate. Collectively, these observations with individual sugars and the values assigned to various growth and fermentation parameters will be useful in the development of models to predict the behavior of rec Zm in mixed substrate fermentations of the type associated with biomass-to-ethanol processes.

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Index Entries: Recombinant *Zymomonas*; xylose; growth yield; maintenance coefficient; Y_{ATP}; ethanol; chemostat cultures.

Introduction

An understanding of the physiological characteristics of microorganisms, including factors that regulate carbon and energy metabolism during growth, can furnish useful information when engineering a bioconversion process involving different substrates *(1,2)*. Cybernetic models for predicting, with quantitative accuracy, the growth and fermentation behavior of a microorganism in both batch and continuous modes in the presence of multiple substrates have been developed from values for suitable parameters derived from independent single-substrate experiments *(3)*.

Previously we have examined the comparative energetics of glucose and xylose metabolism in ethanologenic recombinant *Escherichia coli (4)*. The homofermentative nature of *Zymomonas* and ethanologenic rec *E. coli* greatly facilitates the assessment of adenosine triphosphate (ATP) gain from substrate catabolism through well-defined metabolic pathways. The transformation of *Zymomonas* with xylose metabolism genes *(5,6)* offers the opportunity to compare the energetics of rec Zm using either glucose or xylose. Although we have studied rec Zm in both batch and continuous culture modes *(7–11)*, the chemostat experiments were with sugar mixtures and not with single substrates.

Growth yield measurements can provide useful information concerning the relationship among substrate utilization, energy production, and the synthesis of cell mass (growth) *(12–14)*. During the growth of *Zymomonas*, a small portion of the fermentable substrate is diverted to cell mass synthesis and the remainder is metabolized for the purpose of generating energy by substrate-level phosphorylations catalyzed by 3-phosphoglycerate kinase and pyruvate kinase. The energy-consuming processes (chemical, osmotic, and mechanical) can be collectively referred to as the "work" performed by the cell *(12)*. A portion of the substrate is used to produce energy to support growth, and a portion is used to support nongrowth-related functions (collectively referred to as "maintenance" functions). One of the axioms of modern microbial bioenergetics is that during growth, the consumption of energy is partly growth dependent and partly growth independent *(12–15)*. Energy that is consumed independent of growth is often referred to as "maintenance energy" (m_e) *(12–15)*, but as Thauer et al. *(12)* point out, this is a poor descriptive for this portion of the energy that is consumed independent of growth because it could potentially involve the useless consumption of energy—a process referred to by Hill et al. *(2)* as energy "wastage."

The growth yield ($Y_{x/s}$) is defined by the amount of cell mass produced per mass of substrate consumed. Regardless of the qualitative and quantitative description of the various components comprising "maintenance" energy, when $m_e = 0$, the growth yield becomes the maximum growth yield

(max $Y_{x/s}$) or "true" growth yield. If the substrate is expressed in terms of energy (ATP), the amount of dry cell mass (DCM) produced per mole of ATP defines Y_{ATP}. Disregarding the involvement of the portion of the conserved energy that is consumed independent of growth, the value of Y_{ATP} can be calculated from the following relationship *(13,14)*:

$$Y_{ATP} \text{ (g dry wt cells/mol ATP)} = (\text{molar } Y_{x/s}/G_{ATP}) \quad (1)$$

in which $Y_{x/s}$ represents the molar growth yield coefficient (grams of dry wt cells per mole of carbon substrate) and G_{ATP} represents the ATP gain (moles of ATP produced per mole of substrate fermented).

The metabolism of glucose and xylose by metabolically engineered *Z. mobilis* can be represented by the following mass and energy balances:

$$\text{Glucose} + \text{ADP} + P_i \longrightarrow 2 \text{ Ethanol} + 2\ CO_2 + \text{ATP} \quad (2)$$

$$3 \text{ Xylose} + 3 \text{ ADP} + 3P_i \longrightarrow 5 \text{ Ethanol} + 5\ CO_2 + 3 \text{ ATP} \quad (3)$$

According to these relationships the molar yield of ATP (G_{ATP}) from both glucose and xylose is 1.0. Hence, for rec Zm, the molar growth yield (molar $Y_{x/s}$) is equal to Y_{ATP}:

$$\text{molar } Y_{x/s} = Y_{ATP} \quad (4)$$

The concept of Y_{ATP} was introduced in 1960 by Bauchop and Elsden *(16)* and has since been the subject of several reviews *(13,17–19)*. Although, at first, Y_{ATP} was believed to be a biological constant with an average value of 10.5 g of dry wt cells/mol of ATP *(16,20)*, more extensive testing under diverse environmental conditions and involving more microorganisms has suggested that Y_{ATP} is not a constant *(12–14)*. The most likely explanation for the wide variance in values for Y_{ATP} and the discrepancy between experimental and theoretical Y_{ATP} values lies in the fact that no account is made for the energy required to perform functions that are independent of growth (i.e., "maintenance").

Utilizing the substrate-limiting conditions of a chemostat culture in which the specific growth rate (μ) can be varied by altering the dilution rate (D), Pirt *(15)* described a method for estimating the "maintenance coefficient" (m) by plotting the specific rate of substrate consumption as a function of D whereby the inverse of the slope of the predicted straight line is the value of the maximum growth yield ($m = 0$) and the y-axis intercept ($\mu = 0$) is the value of m. It is important to realize that Pirt's unstructured growth model assumes that m has a constant value that is independent of the growth rate. Performing this type of analytical exercise with chemostat cultures has proven to be a popular exercise, and there is a plethora of reports in the literature to attest to this; however, there are fewer critical assessments of the physiological significance of the value of the parameters derived from plots of q_s vs D. Such an assessment is called for when such plots do not conform to the predicted linearity. Hence, the value of m may be of limited usefulness in developing models to describe either nongrowth

related metabolism or the varying degrees of "energetic uncoupling" under different environmental conditions (McMillan, J. D., personal communication).

The overall objective of the present study was to compare growth and fermentation characteristics of xylose-utilizing recombinant *Zymomonas* in well-controlled single-substrate batch and continuous cultures using the two major sugars found in biomass hydrolysates, namely, glucose and xylose. The values assigned to various growth and fermentation parameters will be useful in developing models and control algorithms to predict the behavior of rec Zm in mixed substrate cofermentations of the type being proposed in biomass-to-ethanol processes.

Materials and Methods

Organisms

Z. mobilis CP4 (B-14023) was obtained from the US Department of Agriculture, Northern Regional Research Center (Peoria, IL). The xylose-utilizing recombinant *Z. mobilis* strain CP4 carrying the plasmid pZB5 (designated as Zm CP4:pZB5) *(5,6)* was obtained from M. Zhang (National Renewable Energy Laboratory, Golden, CO). Stock cultures were stored in glycerol at –70°C and precultures were prepared as described previously *(9)*.

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 RM medium (10 g/L of yeast extract and 2 g/L KH_2PO_4), containing about 20 g/L of xylose and 20 g/L of glucose supplemented with tetracycline (Tc) (10 mg/L), in 125-mL screw-cap flasks and grown statically overnight at 30°C in an incubator. This preseed was subcultured into inoculation flasks containing RM with 20 g/L of glucose, 20 g/L of xylose, and 10 mg/L of Tc and grown statically overnight at 30°C in an incubator. This overnight culture was used at a level of ~10% (v/v) to inoculate the batch fermentors. The initial optical density (OD) (1-cm light path at 600 nm) was in the range 0.2–0.25, corresponding to 60–75 mg of DCM/L.

Fermentation Medium

The fermentation medium (designated as ZM) was prepared with glass distilled water and contained the following ingredients: 5 g/L of Difco Yeast Extract (Difco, Detroit, MI); 3.48 g/L of KH_2PO_4; 0.8 g/L of NH_4Cl; 0.25 g/L of $MgSO_4$; 0.01 g/L of $FeSO_4 \cdot 7H_2O$; 0.21 g/L of citric acid; and 20 mg/L of tetracycline. The amount of glucose and xylose added to the medium was variable. The medium and stock sugar solutions were autoclaved separately.

Fermentation Equipment

For flask fermentations, 100 mL of medium was added to 125-mL Erlenmyer flasks that were either sealed with screw caps and incubated statically in an incubator (closed flask) or fitted with a foam plug closure and incubated in a shaker water bath (open flask). The pH was not controlled in these flask cultures. pH-stat batch fermentations were conducted with about 1500 mL of medium in 2-L bioreactors (model F2000 MultiGen, New Brunswick Scientific, Edison, NJ) fitted with agitation (100 rpm), pH, and temperature control (30°C). Continuous fermentations were conducted with either NBS C39 BioFlo chemostats or 2-L NBS Bioflo 2000 bioreactors (New Brunswick Scientific). The working volume of these chemostats was about 350 and 1500 mL, respectively. Steady-state was assumed only after a minimum of 3 vol had exchanged and when samples taken on successive days gave similar values for cell mass, sugar, and ethanol concentrations. The pH was monitored using a sterilizable combination pH electrode (Ingold). The standard pH control set point was either 5.75 or 6.0, and the pH was kept constant by automatic titration with 4 *N* KOH. The temperature was controlled at 30°C using a circulating water bath, and the agitation was moderate (approx 100–150 rpm). The continuous fermentations were started in the batch mode using ZM medium with 25 g/L of glucose and 25 g/L of xylose. Flow was started 24 h after inoculation (preferably when the residual xylose concentration was <5 g/L).

Analytical Procedures, Growth, and Fermentation Parameters

Growth was measured turbidometrically at 600 nm (1-cm light path) (Unicam spectrophotometer, model SP1800). 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 (HPLC) as described previously *(9)*. Carbon balancing was performed by a method described previously *(4)*. The ethanol yield ($Y_{p/s}$) was calculated as the mass of ethanol produced per mass of sugar consumed. For batch cultures, the maximum specific rate of sugar utilization (max q_s, g of sugar/[g of DCM·h]) was calculated as the maximum slope in the plot of sugar concentration as a function of fermentation time divided by the average cell mass concentration during that interval. The value for maximum specific rate of ethanol production (max q_p) was determined as the product of max $q_s \cdot Y_{p/s}$. For chemostat cultures, the maximum mass growth yield (i.e., corrected for maintenance metabolism) (max $Y_{x/s}$, g of DCM/g of sugar) was determined as the inverse of the slope of the best-fit linear regression for q_s as a function of the dilution rate (D, 1/h) over a specified range of D. The maintenance coefficient (m_s, g of sugar/[g of DCM·h]) was determined as the y-axis intercept of the best-fit linear regression to the q_s vs D data *(15)*.

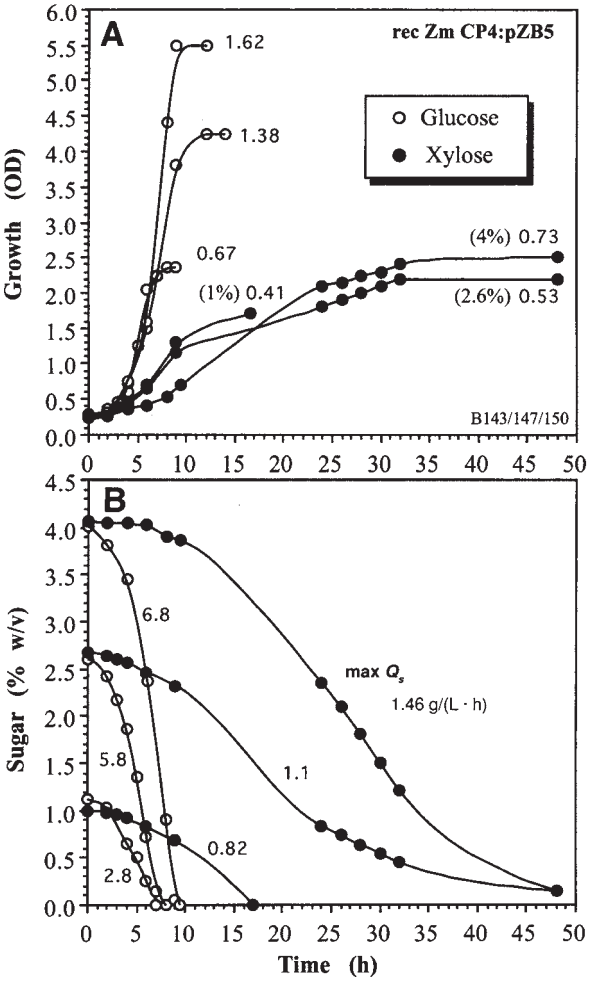

Fig. 1. Time course of pH-stat batch fermentations with rec Zm CP4:pZB5: **(A)** Growth and **(B)** sugar utilization. The numbers in (A) represent the maximum cell density (g of DCM/L) and in (B) the maximum volumetric rate of sugar utilization (max Q_s, g sugar/[L·h]). The yeast extract–based ZM medium (*see* Materials and Methods) contained either glucose (○) or xylose (●) at levels of 1, 2.6, or 4% (w/v). The pH was controlled at 6.0 and the temperature at 30°C.

Results and Discussion

Single-Substrate Batch Fermentations

Figure 1 shows time courses of batch fermentations with rec Zm CP4:pZB5 conducted in the controlled environment of a stirred bioreactor with the temperature at 30°C and the pH maintained at 6.0. The nutrient-rich yeast extract–salts medium contained either glucose or xylose at concentrations of 1, 2.6, or 4% (w/v). The amount of sugar in the medium was varied to test for the range of sugar concentrations for which growth (final cell mass concentration) was energy limited. Although growth was fol-

Table 1
Summary of pH-Stat Fermentation Parameters
for Recombinant *Zymomonas* CP4:pZB5[a]

	Batch fermentations			
	max q_s (g/[g cell·h])	max q_p (g/[g cell·h])	Average $Y_{x/s}$ (g/g)	molar $Y_{x/s}$ (g/mol sugar)
Glucose[b]	8.5	4.08	0.055	9.9
Xylose	2.1	1.01	0.034	5.1
	Continuous fermentations			
	max $Y_{x/s}$ (g/g)	Dilution range	m_s (g/[g cell·h])	molar max $Y_{x/s}$ (g/mol sugar)
Glucose	0.0484	(0.10–0.30/h)	2.05	8.71
	0.0272	(0.04–0.10/h)	0.20	4.90
Xylose	0.0417	(0.02–0.04/h)	0.04	6.25

[a]Fermentation conditions: pH = 6.0 and temperature = 30°C.
[b]Average $Y_{x/s}$ for wild-type CP4 = 0.058 (molar $Y_{x/s}$ = 10.4 g of DCM/mol of glu). For Zm, G_{ATP} = 1 for both glucose and xylose; therefore, molar $Y_{x/s} = Y_{ATP}$.

lowed turbidometrically (OD), Fig. 1A shows the final DCM that was determined directly by ultrafiltration. The maximum specific growth rates with glucose and xylose were 0.55/h and 0.21/h (Fig. 1A), consistent with previously reported values *(7,21)*.

The values for the maximum volumetric rate of sugar consumption (Q_s) are given in Fig. 1B, from which the value for the maximum specific rate of sugar utilization (q_s) was determined *(22)* (Table 1). For these batch experiments, the accompanying trajectories for ethanol production are not shown, but the corresponding value for maximum rate of ethanol production (q_p) is given in Table 1. The observed max q_s for rec Zm is similar to values in the literature for wild-type Zm (Table 2).

An advantage of employing relatively low sugar concentrations in these experiments is that it minimizes possible inhibitory effects owing to the accumulation of ethanol or by-products such as xylitol from xylose metabolism. Such inhibitors are inherently of concern with this type of yield determination because the final cell mass concentration reflects the average of integral effects over the entire growth (fermentation) interval, which could include end-product inhibitory effects *(1)*. Similarly, the values assigned to maximal rates could be affected by known inhibitors such as ethanol and xylitol.

In 1998, we presented a comparison of rec Zm 39676:pZB4L and a prehydrolysate-adapted derivative strain in side-by-side pH-stat batch fermentations with 4.8% glucose as the sole fermentable sugar *(11)*. From those experiments, the following parameters we determined that max q_s was 8.6 and 5.7 g of glu/(g of cell·h) and $Y_{x/s}$ was 0.04 and 0.036 g of DCM/g of glu for the parent and adapted rec Zm strains, respectively (Table 2).

Table 2
Comparative Growth and Fermentation Parameters for Batch Cultures of *Zymomonas*[a]

Strain[b]	Medium	pH	[Glu] (% w/v)	$Y_{x/s}$ (g DCM/g)	molar $Y_{x/s}$ (g DCM/mol)	max q_s (g glu/[g cell·h])	Reference
10988	Complex		<1	0.046	8.3		*16*
10988	Complex		<1	0.046	8.3	8.7	*37*
10988	Complex			0.060	10.8		*38*
29191	Complex	5.50	10	0.039	7.0	10.5	*39*
CP4	Complex	5.00	1–4	0.036	6.5	8.3	*24*
rec 39676	Complex	5.75	4.8	0.040	7.2	8.6	*11*
rec "adapted"	Complex	5.75	4.8	0.036	6.4	5.7	*11*

[a]All fermentations were at 30°C.
[b]American Type Culture Collection (ATCC) accession numbers.

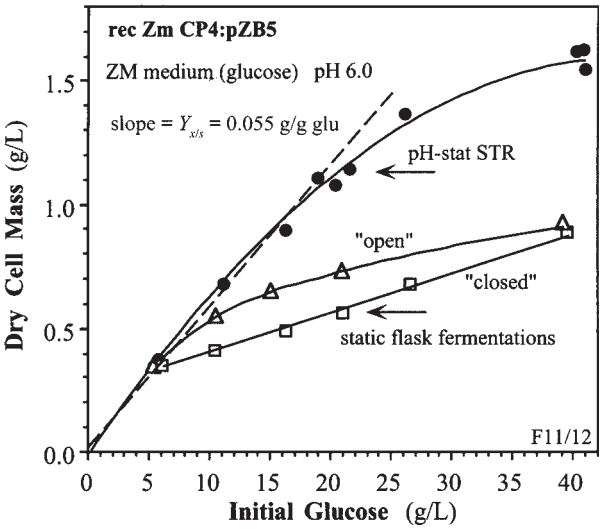

Fig. 2. Cell mass as a function of glucose in flask and pH-stat batch fermentations with rec Zm CP4:pZB5. Static flask fermentations were either in screw-capped flasks (□) or foam plugged flasks (△); the pH in these flask fermentations was not controlled. For fermentations conducted in stirred fermentors (●), the pH was controlled at 6.0. The ZM medium contained glucose in the range 0.5–4% (w/v). The temperature was 30°C.

Often physiological testing of recombinant strains is performed in flask fermentations in the absence of pH control. The results of these preliminary screening tests can be crucial in assessing the functional utility of the genetic construct, but the lack of a proper controlled environment could lead to inappropriate conclusions. Figure 2 compares the growth of rec Zm CP4:pZB5 in flask and pH-stat stirred fermentors in which the glucose concentration in the medium was varied over the range of 0.5–4% (w/v). At the higher glucose levels, there was a dramatic difference in the final cell densities of the flask and bioreactor cultures (Fig. 2). The difference between the "open" and "closed" flask cultures (*see* Materials and Methods) point to the importance not only of pH control *(22)* but also the role of dissolved CO_2 *(23)* in limiting growth (Fig. 2). Interestingly, the cell mass level (not corrected for 0.05 g of DCM/L of inoculum) at 5 g/L of initial glucose was the same in both flasks and the pH-controlled fermentor, presumably because the levels of acid and CO_2 were too low to affect growth (Fig. 2). Even with pH control, the ratio of final cell mass concentration to initial glucose concentration was lower for initial concentrations >20 g/L than for initial concentrations <20 g/L. Nutrient-rich complex medium suggests that the ratio's deviation was not caused by nutrient limitation (Fig. 2). From the slope of the tangent, the max $Y_{x/s}$ was calculated as 0.055 g of DCM/g of glu (Fig. 2). Because similar experiments with rec Zm 39676:pZB4L and its "adapted" derivative were performed with 4.8% glucose, the values for $Y_{x/s}$ determined from those experiments (Table 2) are not representative of the maximum growth yield *(11)*.

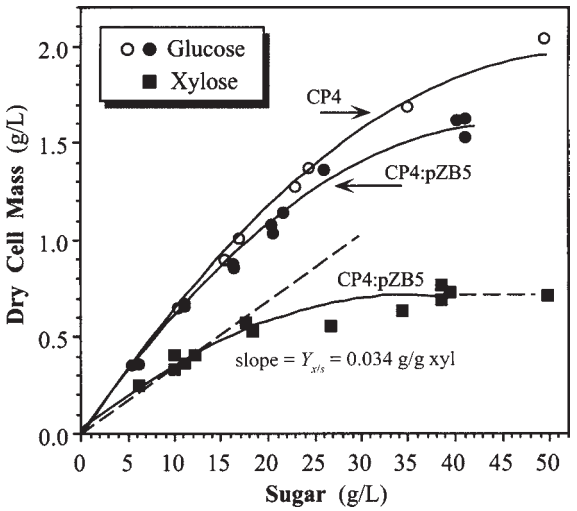

Fig. 3. Relationship between final cell mass concentration as a function of glucose and xylose in pH-stat batch fermentations. The ZM medium contained either glucose or xylose in the range 0.5–5.0% (w/v). ○, Fermentations with the parent strain CP4; ●, fermentations with the recombinant CP4:pZB5. The pH was 6.0 and the temperature was 30°C.

Batch experiments with pH-controlled fermentors showed that this phenomenon of nonlinearity with respect to the final cell mass concentration is not solely a characteristic of the recombinant since the wild-type culture CP4 behaves in a similar fashion (Fig. 3). With CP4 the max $Y_{x/s}$ was determined to be slightly higher, 0.058 g of DCM/g of glu (Fig. 3, Table 1). In another study with Zm CP4 conducted at pH 5.0 over the glucose range of 1–4%, best-fit linear regression produced a value for max $Y_{x/s}$ of 0.036 g of DCM/g of glu (Table 2), but the line did not extrapolate to zero *(24)*. The difference between the wild-type and the recombinant might be explained in terms of the so-called plasmid burden effect *(25)* whereby energy that would otherwise be available for growth is diverted for plasmid-related functions in the recombinant.

In pH-stat batch fermentations in which xylose was the sole sugar, the final cell mass was proportional to the initial sugar concentration only up to a xylose concentration of about 1.5% (w/v) for which the max $Y_{x/s}$ was determined to be 0.034 g of DCM/g of xyl (Fig. 3, Table 1). Joachimsthal et al. *(26)* reported a similar final cell mass concentration for this same rec Zm strain with 2.5% xylose, but with the pH controlled at 5.0. Previously we had observed that the final cell mass in xylose fermentations with rec Zm was about the same over the range of 2.5–6% *(10)* in which xylose consumption proceeded in the absence of growth (so-called uncoupled metabolism). The reason for this phenomenon remains unresolved but may be owing in part to growth inhibition by end products (ethanol and/or xylitol). Xylitol might be expected to affect the rate of xylose utilization because of its inhibition of xylulokinase *(21)*. The level of xylitol observed in our xylose

fermentations was always below the level of detection at the routine sensitivity setting of our HPLC. Because there was excellent closure of the carbon balance, we are confident that all the products have been quantified. Reports of significant amounts of xylitol being produced from xylose by rec Zm may be related to more stressful conditions *(27)*. With ZM4:pZB5 at pH 5.0 and 30°C, 2.7 g/L of xylitol was produced from an initial xylose concentration of 40 g/L. The production of xylitol was only 0.9 g/L in a medium containing initially 60 g/L of glucose and 60 g/L of xylose *(27)*. Inhibition studies on this recombinant strain showed that 1 g/L of xylitol was sufficient to reduce the growth rate to 50% of its original value *(27)*.

Since the yield of ATP from glucose and xylose catabolism in *Zymomonas* is 1 mol/mol of carbohydrate, the molar growth yield is equal to Y_{ATP} (*see* Introduction) (Table 1). In an analogous study with ethanologenic rec *E. coli* using single-substrate pH-stat batch fermentations and a defined mineral salts medium, we determined the $Y_{x/s}$ for glucose and xylose to be 0.091 g of DCM/g of glu and 0.050 g of DCM/g of xyl, respectively *(4)*. Because of recognized differences in the metabolic routes of rec *E. coli* and rec Zm, the G_{ATP} for glucose in *E. coli* is 2.0 mol of ATP/mol of glu. Because xylose enters *E. coli* in an energy-linked fashion via a proton symporter, the value for the G_{ATP} associated with xylose catabolism in *E. coli* depends on values assigned to both the stoichiometry of uptake and the proton-translocating ATPase. In our work with rec *E. coli*, it was assumed that these stoichiometries were 1 and 2, respectively; consequently, the G_{ATP} was assumed to be 1.17 mol of ATP/mol of xyl and the Y_{ATP} values for glucose and xylose were 8.2 and 6.4 g of DCM/mol of ATP, respectively *(4)*. In recent revisions of the chemiosmotic coupling theory, the stoichiometry of proton-translocating ATPase is $4H^+$/ATP *(28)*; this would reduce the value of Y_{ATP} for xylose from 6.4 to 5.3 g of DCM/mol of ATP *(4)*. These values for Y_{ATP} are quite similar to those observed with rec Zm in the present work (Table 1). Clearly, Y_{ATP} is not a biological constant *(36)*, but, rather, its value depends on the conditions of growth including the substrate (energy source).

Single-Substrate Continuous Fermentations

Our previous studies involving continuous cultures of various wild-type Zm have been at dilution rates greater than 0.08/h (Table 3). In a recent study *(29)*, we observed that the ethanol yield was affected at low growth rates owing to the production of lactic acid, but, more important, in the context of the present investigation on bioenergetics, we also noted that, at low dilution rates, the values of q_s did not conform to what might be expected from an extrapolation of the linear Pirt plot of q_s vs D at $D <$ 0.08/h. Such Pirt plots of q_s vs D *(15)* have been reported by different investigators for estimating the maintenance coefficient (Table 3).

Figure 4 is a plot of q_s (glu) vs D for rec Zm CP4. If one neglects the data at $D < 0.1$/h in Fig. 4, the best-fit line by linear regression can be extrapolated to give a y-intercept of 2.05 g of glu/(g of cell·h), and in a typical Pirt

Table 3
Comparative Growth and Fermentation Parameters for Chemostat Cultures of *Zymomonas*

Strain[a]	Temperature (°C)	Medium[b]	pH	[Glu] (% w/v)	$Y_{x/s}$ (g DCM/g glu)	molar $Y_{x/s}$ (g DCM/mol)	m_s (g glu/[g cell·h])	max q_s (g glu/[g cell·h])	Reference
10988	30	Defined	6.0	2	0.015	2.7	0.5	7.2	*32*
10988	35	Defined	6.0	2	0.015	2.7	2.3	8.8	*32*
10988	30	Defined	6.0	6	0.010	1.9	2.1	11.2	*32*
29191	30	Defined	5.5	3	0.050	9.0	1.7	9.7	*41*
29191	30	Defined*	5.5	3	0.022	3.9	1.7	9.3	*41*
29191	30	Defined	5.5	6	0.050	9.0	1.7		*40*
29191	30	Complex	5.5	10	0.060	10.8	2.9	7.2	*39*
29191	30	Complex	5.5	6	0.060	10.8	1.5		*40*
29191	30	Complex	5.0	5	0.050	9.0	3.9		*21*
29191	30	Complex	5.5	5	0.049	8.8	2.5		*21*
29191	30	Complex	6.0	5	0.049	8.8	2.0		*21*
31821	30	Complex	5.0	10	0.037	6.7	4.1	11.7	*42*
Z-1-81	30	Complex			0.024	4.3	2.5		*44*
10988	30	Complex	5.0	10	0.036	6.4	1.7		*43*
10988	30	Complex	5.0	15	0.046	8.2	3.1		*43*
10988	30	Complex	5.0	5	0.032	5.8	3.0	8.0	*31*
10988	35	Complex	5.0	5	0.025	4.5	3.1	10.5	*31*

[a]ATCC accession numbers.
[b]Complex, yeast extract–based salts medium; defined, minimal mineral salts medium; defined*, N-limited.

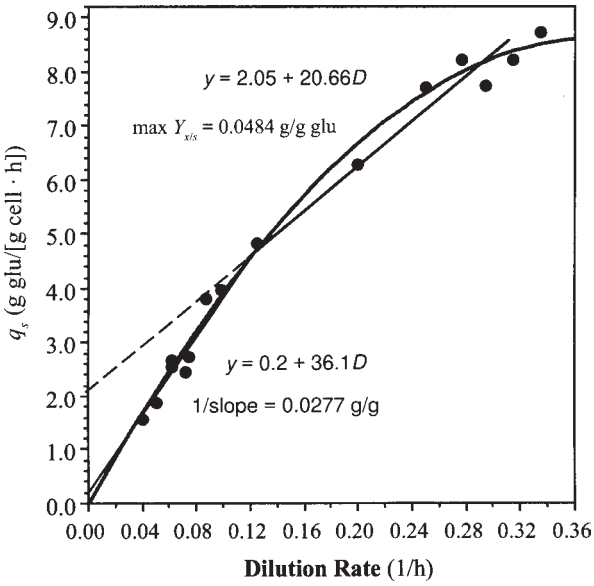

Fig. 4. Plot of specific rate of glucose utilization vs dilution rate. The carbon-limited chemostat was operated with rec Zm CP4:pZB5 in 4% glucose-ZM medium at pH 6.0 and 30°C. Sampling for determination of cell mass was made after three volume turnovers. The data were fitted by a second-order polynomial ($y = -0.13 + 45.73x - 59.76x^2$ with $R^2 = 0.99$).

plot, this would be the value assigned to the maintenance coefficient (m). However, linear regression of the q_s (glu) data in Fig. 4 at $D < 0.1$/h produced another line with a y-intercept of 0.2 g of glu/(g of cell·h). Clearly these data in Fig. 4 do not conform to the predicted linearity of the unstructured growth model. In fact, these data can be fitted well by a second-order polynomial in D that has similarities to the two-compartment structured model proposed by Jöbses et al. *(30)* to explain similar observations of a deviation from linearity for q_s (glu) with wild-type Zm at equally low dilution rates *(31)* (Table 3). Interestingly, Feischko and Humphrey *(32)* also studied the relationship between q_s and D for wild-type Zm at $D < 0.1$/h and determined the value of m to be 0.5 g of glu/(g of cell·h) (Table 3). Although our data can be fitted by a second-order polynomial *(30)*, without a structured model, the terms and constants lack any physiological significance.

The value for q_s at low dilution rates may be an underestimate of the true value because of problems with cell viability at low growth rates. Measurements of DCM do not take into account viable or metabolically active cell mass vs nonactive cell mass. Clearly, this is an area for future work, but estimates of viability are open to interpretation because of the possibility of "dormancy" in plating assays.

There are many reports in the literature in which Pirt plots have been used to deduce values for both the maximum growth yield and the maintenance coefficient. Table 3 is a compilation of some of these publications,

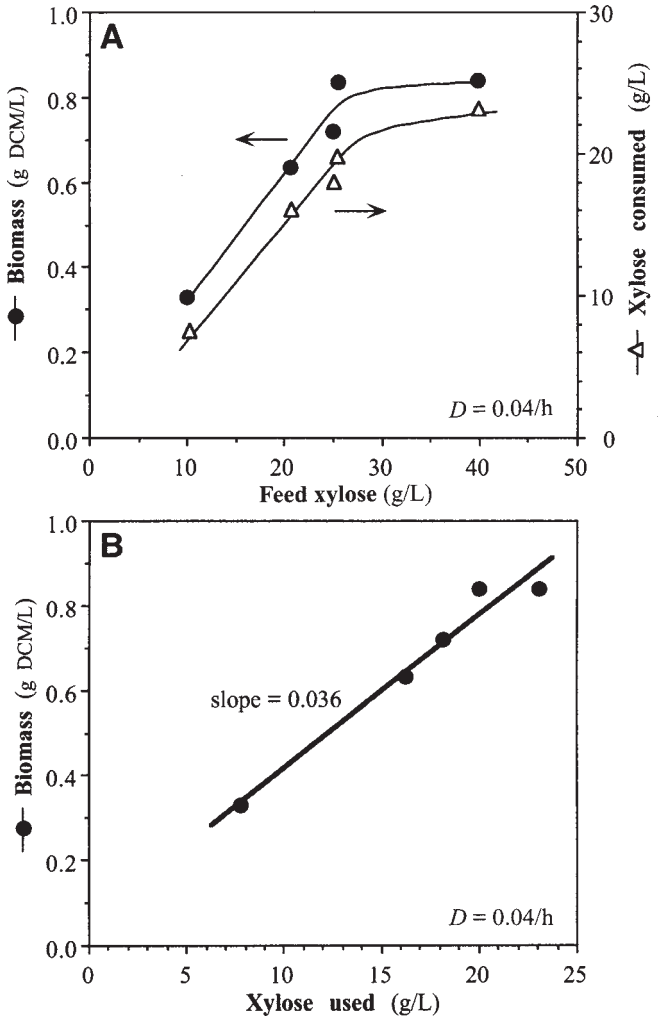

Fig. 5. Steady-state cell mass and residual xylose concentrations in continuous fermentations with rec Zm CP4:pZB5 as a function of entering xylose. The chemostat was operated with ZM medium over the range 0.038–0.042/h. The pH was 5.75 and the temperature was 30°C. **(A)** Cell mass as a function of xylose in the feed medium. **(B)** Cell mass as a function of xylose consumed.

and although not exhaustive, it is intended to convey the variation reported for the values of these parameters. The variation can be explained partly by the wide range of different assay conditions including Zm strain, medium composition, glucose concentration, pH, temperature, and range of dilution rates.

Prior to this study, continuous fermentations with xylose-utilizing rec Zm involved mixtures of glucose and xylose *(8,11,26,27,33)* in which the chemostat was operated at $D < 0.12$/h and xylose was never completely utilized. Figure 5A shows the relationship between cell mass and the amount of xylose consumed as a function of xylose in the feed for rec Zm

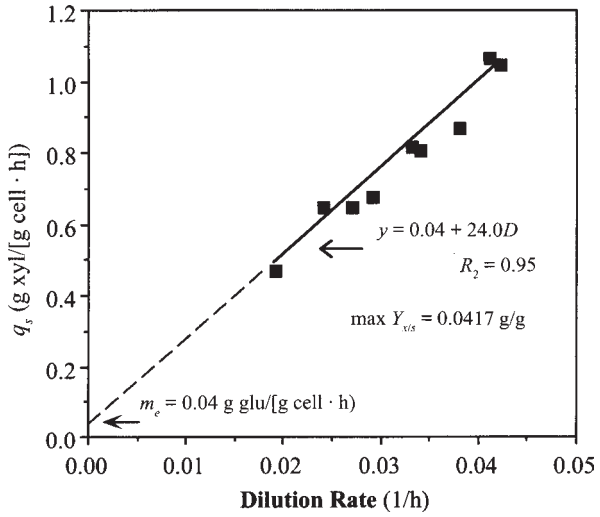

Fig. 6. Plot of specific rate of xylose utilization vs dilution rate. The chemostat was operated with rec Zm CP4:pZB5 in ZM medium at pH 5.75 and 30°C. The medium contained xylose in the range 1.0–2.5% (w/v). In all cases, xylose was present in the effluent (*see* Fig. 5). Sampling for determination of cell mass was made after a minimum of three volume turnovers.

CP4 at a relatively constant dilution rate of about 0.04/h. The pH was 5.75 and xylose was the only sugar in the chemostat feed medium. As was observed in batch cultures with xylose (Fig. 3), the cell density in the chemostat reached a plateau value of 0.8 g of DCM/L at input xylose concentrations higher than 2.5% (Fig. 5A). For comparison, it is interesting to note that at D = 0.04/h with 4% glucose in the feed (instead of xylose), the cell mass was 1.0 g of DCM/L with no glucose detected in the effluent; under these conditions q_s was 1.6 g of glu/(g of cell·h) and the growth yield was 0.025 g of DCM/g of glu (Fig. 4). For the xylose chemostat, the average growth yield (based on xylose consumed) was 0.036 g of DCM/g of xyl (Fig. 5B).

Figure 6 shows that over the narrow dilution range of 0.02–0.04/h with 1–2.5% xylose in the feed at pH 5.75 and 30°C, the values for q_s can be linearized by regression analysis. The inverse of the slope gives a value for the maximum growth yield of 0.042 g of DCM/g of xyl, and the y-axis intercept gives a value for m of 0.04 g of xyl/(g of cell·h) (Fig. 6, Table 1).

From single-substrate chemostat experiments, the values assigned to Y_{ATP} for glucose and xylose are similar to those observed in single-substrate batch fermentations (Table 1). In our xylose chemostat experiments, xylose was always present in the effluent. Although this may be owing to the low affinity of the shared sugar transporter (*glf*) for xylose *(34,35)*, it could be argued that the culture was not energy limited and hence the value for Y_{ATP} may not be valid.

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