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Analysis of the Variation in Ethanol Yield from Glucose or Xylose with Continuously Grown *Thermoanaerobacter ethanolicus*

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ABSTRACT

The chemostat growth of *Thermoanaerobacter ethanolicus* was limited by either glucose or xylose. Ethanol yield was dependent primarily upon length of cultivation time and secondarily upon growth rate. Low growth rates favored high ethanol yields (0.42 g/g) from xylose.

Index Entries: *Thermoanaerobacter ethanolicus;* xylose fermentation; continuous culture; growth rate; ethanol.

INTRODUCTION

The biological conversion of plant biomass to industrial ethanol is an alternative to ethanol production from petroleum feedstocks. In North America, fermentation alcohol (ethanol) is typically produced from corn starch using yeast. Extreme thermophilic (i.e., optimal growth 60–70°C) ethanologenic bacteria are being investigated as alternatives to yeast. Their high growth temperatures offer process advantages such as facilitated product recovery, and their broad substrate spectrum may allow the utilization of inexpensive carbon sources available in agricultural, forestry, and municipal wastes (*1–3*). The latter is of interest because substrate cost is a major factor determining the cost of fermentation alcohol and because yeast ferment pentoses slowly and in poor yields (*4*).

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We are currently investigating the potential of *Thermoanaerobacter ethanolicus* for fuel alcohol production. The optimal temperature for this bacterium is 68–70°C and its fermentable substrates include cellobiose, glucose, purified hemicellulose, xylan ($n < 40$) from steam exploded wood, xylose oligomers, and xylose (*5–7*). *T. ethanolicus* is reported to yield up to 1.95 mol/mol (0.50 g/g) of ethanol from glucose (*8*) and up to 1.45 mol/mol from xylose (*9*). Other nongaseous products include lactate, acetate, and under some circumstances, 1,2-propanediol (*10*); gaseous products include CO_2 and H_2. The effect of some physical and chemical parameters on the organism's ethanol production have been determined in batch culture (*5,9,11*), but the effect of growth rate itself upon the *T. ethanolicus* fermentation has not been delineated.

In the following, the growth rate of *T. ethanolicus* was manipulated by varying the dilution rate of steady-state chemostat cultures of the organism. In order to minimize sugar concentrations in the fermentor outflow, the fermentors were operated under energy-limitation. Two bacterial-substrate systems were employed for these studies: *T. ethanolicus* ATCC 31550, the wild-type strain, with glucose; *T. ethanolicus* ATCC 31938, a high-ethanol producing mutant (*12*), with xylose.

MATERIALS AND METHODS

Organisms

ATCC 31550 and ATCC 31938 were obtained as freeze-dried cultures from the American Type Culture Collection (Rockville, MD). Liquid cultures of these strains were obtained from their depositors, L. G. Ljungdahl and L. H. Carreira.

Media

Sterile, complex, mineral-enriched liquid media (*13*) with 4 g/L yeast extract (Difco) and either 4 g/L glucose or xylose (Sigma) were used for growth in stirred-tank fermentors and in test tubes. Media were prepared anaerobically as described elsewhere (*13*) with 0.32 and 0.40 g/L cysteine-HCl used, respectively, in the above two culture systems. Media were also vitamin enriched in experiments with ATCC 31938 and in some experiments trials with ATCC 31550 (the addition of vitamins did not noticeably affect the ethanol yield vs dilution rate data). Solid media with glucose (growth was unreliable with xylose solid media) were prepared in test tubes in a manner similar to liquid media for test tube cultures, except that 18 g/L agar was added, and media was freshly boiled before use.

Isolation of Pure Cultures

Freeze-dried cultures were moistened and then "pour-plated" with 14 mL of molten agar media. After solidification, plates were incubated in

Gas-Pak (BBL) anaerobic jars with H_2+CO_2 generator envelopes for three days at 60°C. Single-colony isolates were obtained by stabbing isolated colonies, inoculating into 4 g/L glucose liquid media, incubating overnight and then repeating the above procedure twice more. With both strains, two classes of colony sizes were found. The larger class ranged from 0.5 to 1.5 mm diameter, depending on the particular plating and was about twice the size of the other class. The two size classes also showed differences in their fermentation products in test tube culture, with the larger colonies producing ethanol in concentrations equal to or greater than that produced by smaller colonies, and with the larger colonies always producing a higher molar ethanol to acid ratio. That these larger colonies were true *Thermoanaerobacter ethanolicus* isolates was confirmed by comparing their whole cell extracts by SDS-PAGE with those of *T. ethanolicus* cultures obtained from L. G. Ljungdahl and L. H. Carreira, the depositors of the *T. ethanolicus* strains into the ATCC.

Cultivation of Bacteria in Fermentors

Fermentor cultures were initiated by inoculation with 2–3 overnight 14 mL test tube cultures of *T. ethanolicus* into New Brunswick (Edison, NJ) model C30 fermentors equipped with 750 mL glass jars containing about 600 mL of anaerobic media. Cultures were grown to maximum biomass concentrations before the addition of medium, which was kept under nitrogen gas. Medium was drawn from a reservoir by a peristaltic pump and was delivered to fermentors by stainless steel tubing and at the points where flexibility was needed, silicon tubing. The rate of medium addition and thus the dilution rate of the fermentor was varied by manipulating the rate of revolution of the pump head. Under the fermentor conditions employed, the growth rate of the culture was equal to the dilution rate of the fermentor. pH control was effected with a pH controller, a combined Ag/AgCl electrode, 8 *N* KOH, and a setpoint of 6.8. Temperature was maintained at 68°C and agitation at 200 or 250 rpm. Unless otherwise indicated, nitrogen gas was introduced at the bottom of the fermentor at a rate of 25 mL/min in trials with ATCC 31550 and at a rate of 5 mL/min in trials with ATCC 31938.

Analytical Procedures

Continuous culture samples were removed after no less than 5 fermentor volumes had passed through the fermentor since the previous sampling, to allow steady-state to be reestablished between samplings. Ethanol, lactate, acetate, and xylose concentrations in cell-free fermentor broth samples were determined by high-pressure liquid chromatography with a Bio-Rad (Richmond, CA) HPX-87H column. Glucose concentrations were measured with a Yellow Springs Instruments (Yellow Springs, OH) model 27 glucose analyzer.

Regression Analysis

Only data points with < 1 m*M* sugar were used in the analysis. Other criteria were adopted in order that the analysis not be biased toward longer experiments. In the ATCC 31550-glucose system, the other criterion was that time is less than 212 h; for the ATCC 31938-xylose system, it was that the number of fermentor volumes is less than 125. Using a StatWorks program (Cricket Software Inc., Philadelphia, PA) with a Macintosh computer (Apple Computer Inc., Cupertino, CA) dependent variable data (ethanol yield and product ratios) were linearly regressed against the independent variables of growth rate, time, and the number of fermentor turnovers. Using F-ratios and corresponding probabilities from the appropriate ANOVA tables, the statistically significant ($p < 0.05$) independent variable that best described a particular dependent variable was identified. A statistical test, which has been described elsewhere (*14*), was then carried out to determine if addition of either of the other two dependent variables could significantly improve the model equation.

RESULTS

Figures 1 and 2 show that ethanol yield varied greatly during the experiments with both systems. Even for a particular growth rate, a large range of both substrate and product concentrations was seen. As a control to these variable dilution rate experiments, constant dilution rate experiments were carried out with ATCC 31938 and xylose. Conditions including the pH, agitation rate, temperature, medium composition, dilution rate, and for the most part nitrogen flowrate, were kept constant. Thus, any observed changes in ethanol yield resulted from change in the inherent metabolic characteristics of the organism being cultivated. Clearly, there is a tendency for ethanol yield to decrease with time, either in a gradual manner, as in the case of the experiment of Fig. 3C, or in a stepwise manner, as in the cases of Figs. 3A–B. The change in ethanol yield in Fig. 3A was manifested following an external perturbation of the system (decrease of nitrogen flow rate for one sampling), but in Fig. 3B it followed what was initially strictly an apparently spontaneous internal perturbation (the xylose concentration increase after 200 h).

For each of the *T. ethanolicus*-sugar systems, regression analysis was applied to the product data from the experiments shown in Figs. 1–3. Two measures of ethanol yield were considered—$Y_{e/s}$, the molar yield of ethanol from sugar (mol/mol) and *E/P*, the molar proportion of ethanol as product (mol/mol) (i.e., ethanol/(lactate + acetate + ethanol)). In the analyses, these two measures were treated as dependent variables and were linearly regressed against the independent variables of growth rate (μ) (which is the same here as dilution rate), time (t), and the number of fermentor turn-

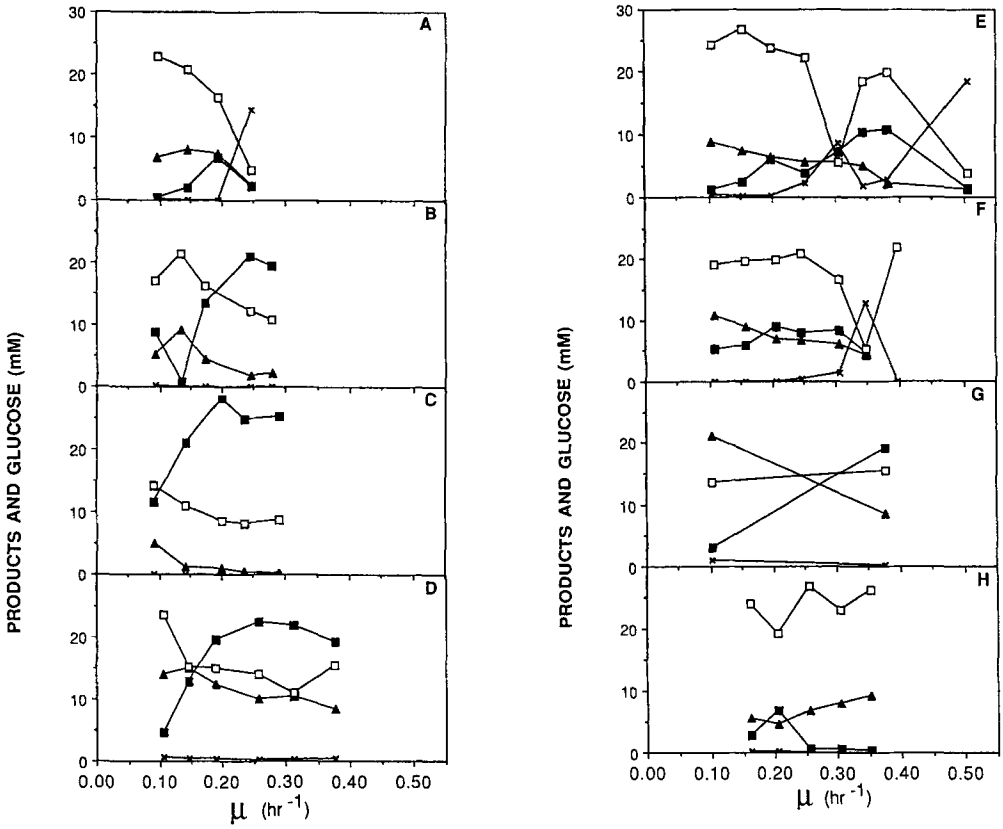

Fig. 1 A–H. Variable dilution rate experimental trials with ATCC 31550 and glucose: effect of variation in growth rate on fermentation products. The dilution rate was gradually increased in all trials except C and G, where dilution rate was gradually decreased. All figures represent independent trials except for that of G, which was actually a continuation of trial D. Since the dilution rate decrease was particularly large in trial G, 15 volumes were allowed to pass through the fermentor before sampling. Symbols: ethanol (—□—); lactate (—■—); acetate (—▲—); xylose (—x—).

overs (N) (i.e., number of fermentor volume changes). Note that N is proportional to the number of bacterial divisions that have occurred in the fermentor. Further details of the analyses are described in Materials and Methods; and some of the results are summarized in Table 1.

It was found for both systems that ethanol yields was not highly dependent on growth rate. For the ATCC 31550–glucose system, time turned out to be a better predictor than growth rate of the proportion of ethanol as product. For the data of the ATCC 31938–xylose system, the number of

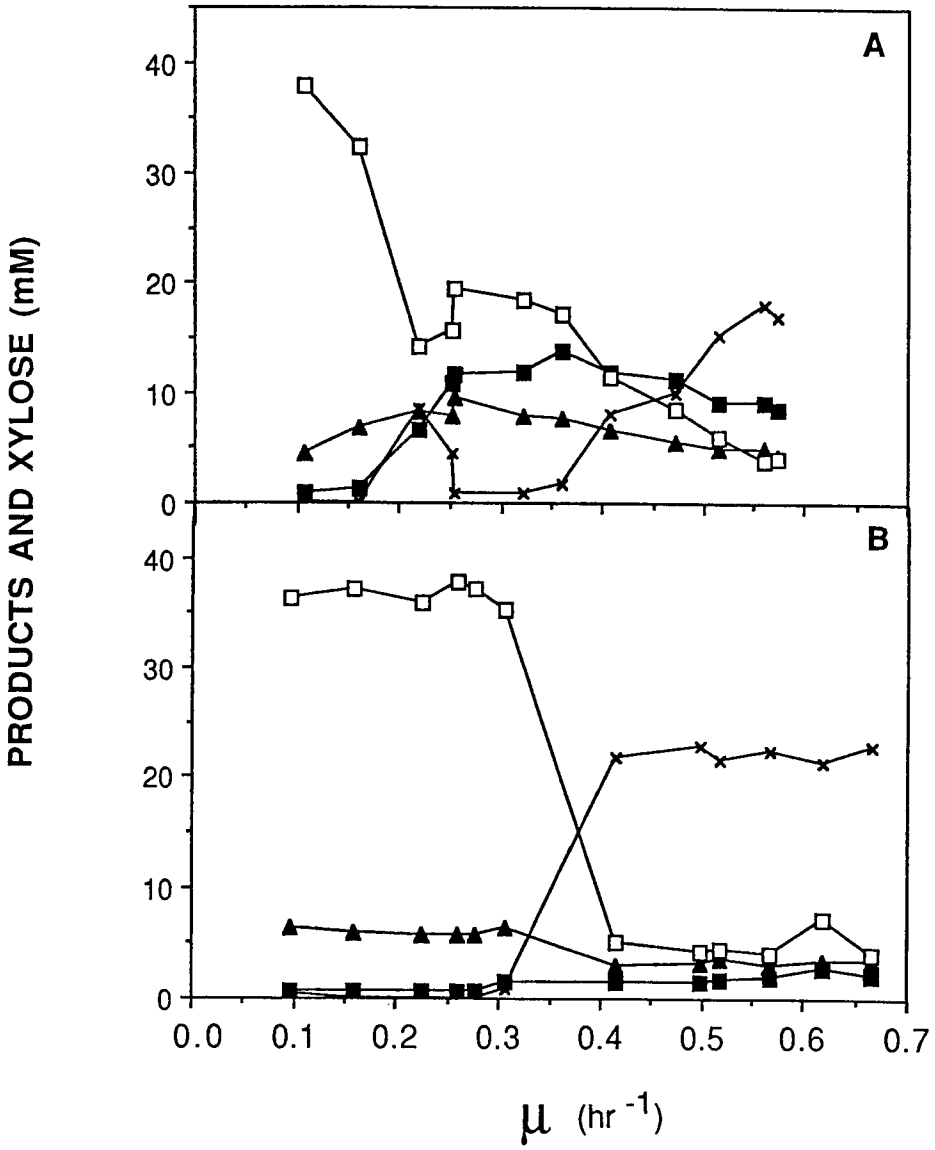

Fig. 2 A-C. Variable dilution rate experimental trials with ATCC 31938 and xylose: effect of variation in growth rate effects on fermentation products. Dilution rate was gradually increased in both trials. Symbols: ethanol (—□—); lactate (—■—); acetate (—▲—); xylose (—x—).

fermentor turnovers emerged as the better predictor of ethanol yield and ethanol product fractions. Growth rate was found to have some (negative) effect on the ethanol yield of the latter system, because a significantly better prediction of yield could be achieved if both growth rate and the number of turnovers were treated as dependent variables as opposed to the number of turnovers alone. On the other hand, growth rate made no significant improvement to prediction by time of the ethanol data of the ATCC 31550-glucose system.

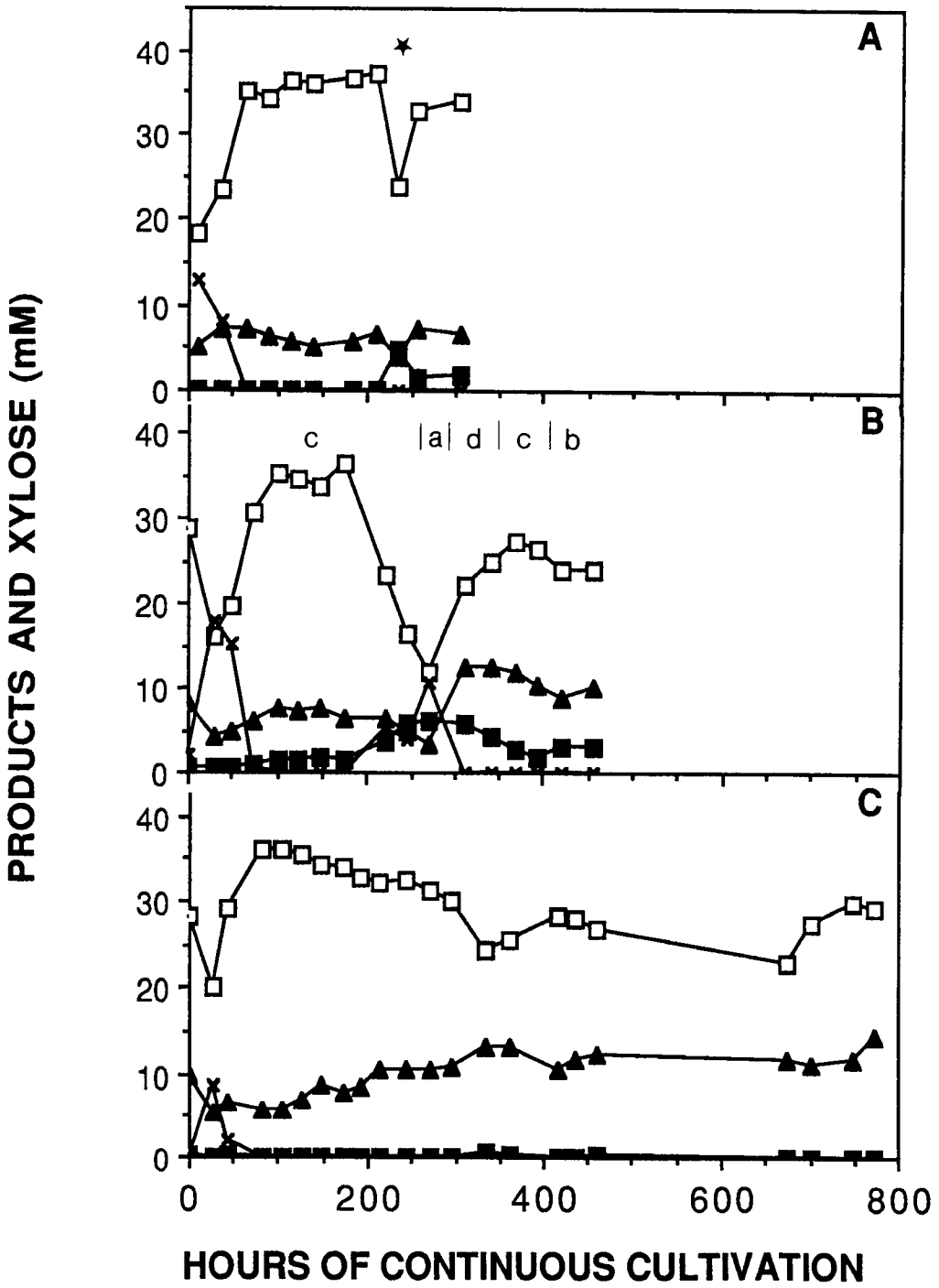

Fig. 3 A-C. Constant dilution rate experimental trials with ATCC 31938 and xylose: change in fermentation products over time. A: Dilution rate of 0.16 h^{-1}; nitrogen flow rate of 5 mL/min, except over the asterisked time period, where the nitrogen flowrate was 0 mL/min. B: Dilution rate of 0.20–0.21 h^{-1}; nitrogen flowrate of 0, 6, 10–12, and 16 mL/min, respectively, where a, b, c, and d are indicated. C: Dilution rate of 0.16–0.18 h^{-1}; nitrogen flowrate of 10–12 mL/min. Symbols: ethanol (—□—); lactate (—■—); acetate (—▲—); xylose (—x—).

Table 1
Summary of Regression Analyses of Chemostat Data: Coefficients of Correlation (r) Between Ethanol Yield Measures and Independent Variables

Dependent variable	Independent variables				
	Simple linear regression			Multiple linear regression	
	μ	t	N	N, μ	N, t
System 1: ATCC 31550-glucose					
$Y_{e/s}$	n.s.[a]	n.s.	n.s.	—	—
E/P	n.s.	0.43	n.s.	—	—
System 2: ATCC 31938-xylose					
$Y_{e/s}$	0.44	0.56	0.67	0.73	n.s.
E/P	0.48	0.54	0.64	0.72	n.s.

[a]n.s. indicates that the equation with this/these variable(s) is not significantly better ($p<0.05$) at predicting the ethanol yield measure (dependent variable), than is the corresponding equation with one less variable (e.g., an equation with N and t as independent variables does not predict $Y_{e/s}$ in system 2 significantly better than does N alone; an equation with μ as an independent variable does not predict $Y_{e/s}$ in system 1 significantly better than does a constant). r^2, the square of the correlation coefficient (r) gives the fraction of variation of the dependent variable that can be described by the independent variables.

DISCUSSION

Growth rate did not emerge as a strong determinant of ethanol productivity. Growth rate could make no significant contribution to the prediction of the ethanol yield data of the ATCC 31550-glucose system, but it was able to do so for the ATCC 31938-xylose system. It was found in this latter case that higher growth rates were unfavorable to high ethanol concentrations. There are no other reports known to the authors' knowledge of the effect of growth rate in continuous culture on yield of ethanol by *T. ethanolicus*.

The growth rate in batch culture has been varied in an ATCC 31550-glucose system (*11*) and in an ATCC 31938-xylose system (*13*) by varying the initial concentration of the sugar. In the former study, which used a closed system with no pH control, it was found that the ratio for the ethanol/glucose consumed was greater for an initial glucose concentration of 20 g/L compared to one of 10 g/L. This was the case whether points of ethanol maxima or whether points of equal glucose consumption were compared. In the latter study, which used a nitrogen gas sparged fermentor with pH control, it was found that the ratio of ethanol/xylose consumed was greater with an initial xylose concentration of 4 g/L compared to one of 12 or 31 g/L. This was the case whether points of maximum biomass concentration or whether points of equal xylose consumption were compared.

No consistent pattern of growth rate effects on ethanol yields have been found with other thermophilic ethanologens. The following various effects of growth rate on ethanol yield have been found: a large negative effect for glucose-limited *C. thermohydrosulfuricum* (*15*); a slight negative effect for xylose-limited *C. thermohydrosulfuricum* (*15*); no effect for a glucose-limited *Thermoanaerobium brockii* variant (*16*); and a positive effect for glucose-limited *T. brockii* DSM 1457 (*16*). As well, when *C. thermocellum* was continuously cultivated on Avicel or pretreated mixed hardwood, presumably under conditions of energy limitation, no growth rate effect was seen (*17*). Growth rate effects have also varied when the energy source was in excess. A large negative growth rate effect was observed with *C. thermohydrosulfuricum* under conditions of nitrogen limitation with either glucose or xylose as the energy source (*15*). Such an effect was also observed with data from a chemostat with *T. brockii* DSM 1457 operated at high dilution rates (*16*). (Though glucose-limited at low dilution rates, the type of limitation is uncertain at higher dilution rates where there are elevated glucose concentrations). On the other hand, little effect was seen in a preliminary study of apparently product-limited *C. thermosaccharolyticum* HG-8 and xylose (*18*).

From the present authors' experience with the variability of *T. ethanolicus* in continuous culture, the question arises as to the reproducibility of some of the above observations. Notably, Mistry and Cooney (*18*) also reported difficulty in repeating results for a particular set of environmental conditions after cultivation at a different set of conditions. In contrast to the observations of the above two groups, Sonnleitner et al. reported the ability to reproduce results at low dilution rates in an experiment in which the dilution rate of a chemostat was intermittently decreased to low values during a sequence of growth conditions wherein the dilution rate was otherwise gradually increased (*16*).

Because ethanol concentration is so strongly dependent on time or the number of fermentor turnovers, it seems that a primary focus of future continuous culture studies should be the elucidation of the mechanism that brings about a decrease in fermentor ethanol concentration over time. The changes in ethanol yields in the above constant dilution rate experiments evidence a genetic change in the cultured organism over the course of the experiments. During these experiments and in the variable dilution rate experiments, fermentor samples were taken in order to make SDS extracts for subsequent SDS-PAGE analysis and to subinoculate into test tubes with media for subsequent incubation and product analysis. Results of these analyses (presented in part in (*10*)) corroborated the genetic change evidenced above. A mutation-selection process towards improved growth characteristics with respect to the substrate or to an inhibitor is believed to be taking place in such a way that the evolved organisms produce less ethanol. The theoretical considerations (*19–21*) and experimental observations of our own and of others (*16*) that have led to this belief have been previously discussed (*10*). The rate of culture

change seems to be enhanced if the system is pertubed in some way, such as by dilution rate changes or by temporary xylose concentration increases. Clearly, if this organism is to be considered for continuous fermentor operation, the driving forces for culture change and their mechanisms of acting must be further identified. This will provide the background for the development of either a set of suitable operating conditions or of a genetically modified organism.

The continuous culture results obtained thus far allow some assessment of the potential of *T. ethanolicus* for fuel alcohol production. The highest yield of ethanol from glucose seen in the above results was only 1.2 mol/mol (0.31 g/g), even though initial substrate concentrations were only 4 g/L. When the glucose concentration in the reservoir was increased, the yield was even lower, with a yield of 0.51 mol/mol (0.13 g/g) from 20 g/L glucose ($D = 0.22\ h^{-1}$). By manipulating redox potential with chemical agents, Ward and Mutharasan attained a yield of 10 mol/mol (0.26 g/g) with medium of the same glucose concentration ($D = 0.045\ h^{-1}$) (*22*). However, both yields are poor in comparison with yields obtained with 100 g/L medium in continuous culture of 1.6 mol/mol (0.41 g/g) by *Saccharomyces cerevisiae* (*23*) or of 1.8 mol/mol (0.47 g/g) by *Zymomonas mobilis* (*24*).

T. ethanolicus looks more promising in its fermentation of xylose. The present study is the first known to the authors that demonstrates the possibility of continuous cultivation of this organism with xylose as an energy source. Its most impressive feature to date is its high yield of up to 1.4 mol/mol (0.44 g/g) from xylose, which was obtained from 4 g/L xylose medium with complete utilization of the xylose. It has been possible to achieve this yield at dilution rates up to $0.31\ h^{-1}$ and to maintain such yields for periods up to 150 h (at a dilution rate of $0.16\ h^{-1}$). This yield is significantly higher than that of 1.0 mol/mol (0.32 g/g), which has been reported for *C. thermosaccharolyticum* HG-8 (*18*) and for *Pachysolen tannophilus* (*25*), though the media employed in these cases were 18 and 52 g/L, respectively. The highest reported ethanol concentrations in published continuous culture experiments are those of 12 g/L by *P. tannophilus*, with a yield of 0.32 g/g (*25*), and of 13 g/L by *Candida shehatae* (*26*), with an unknown yield. However, Lynd relates in a review of thermophilic bacteria (*27*) that he and collaborators have produced 17 g/L ethanol with *C. thermosaccharolyticum*. The maximum ethanol concentration thus far found in continuous culture with *T. ethanolicus* has been 4.7 g/L, which was obtained with a 31 g/L xylose medium (unpublished results). Since 13 g/L residual xylose was present, this represents a yield of only 0.85 mol/mol (0.26 g/g). It is anticipated that the yield from this medium will be improved by adjusting components so that xylose-limitation can be achieved. With the 4 g/L xylose medium, the dilution rate can be increased to 0.31 h^{-1}, without residual xylose appearing. Thus, a volumetric productivity of 0.50 g/g h can be achieved. A productivity of 0.55 g/g h has been achieved with the 31 g/L medium, but is expected to also be improved by manipula-

tion of medium components. These values compare favorably with values of 0.55 and 0.65, which have been achieved in systems with *C. thermosaccharolyticum* HG-8 (*18*) and *C. shehatae* (*26*). Cell recycle could presumably increase the volumetric productivity of a *T. ethanolicus* system as it has with a *C. thermosaccharolyticum* system (*18*).

In summary, results were shown in the above of the glucose- or xylose-limited growth of *Thermoanaerobacter ethanolicus* in fermentors. Although a high degree of inter-experimental trial variation was found, it was determined that ethanol yield was primarily dependent on the length of time of cultivation. Evidence suggests that this is a result of the cultured bacterium mutating to a strain that produced less ethanol. Growth rate made a secondary contribution to the prediction of ethanol yields from xylose (lower yields resulted from higher growth rates) but not to those from glucose. The most impressive result observed in the above experiments is the yield from xylose of 0.42 g/g from 4 g/L xylose achieved at 0.31 h^{-1}.

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