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Thermophilic Ethanol Production

Investigation of Ethanol Yield and Tolerance in Continuous Culture

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ABSTRACT

Ethanol yield and ethanol tolerance, the two factors that most constrain the utilization of thermophilic bacteria for ethanol production, were investigated in continuous xylose-grown cultures of *Clostridium thermosaccharolyticum*. Under xylose-limiting conditions, including varying dilution rates and feed concentrations, the ethanol selectivity (S_E, mol/mol) relative to acetic acid, lactic acid, and propane diol remained relatively constant at about 2. Product addition and removal experiments indicate that mass action effects related to the concentrations of organic fermentation products play a relatively minor role in determining the ratios of products made. Of much greater apparent importance were as yet uncharacterized regulatory mechanisms that appear to be correlated with nonlimiting concentrations of the carbon and energy-source. Substrate-plentiful transients were found to accompany S_E values >11. Such transients provide a useful model system for the study of end product control, as well as a cultivation mode with considerable applied potential. No apparent ethanol inhibition was observed, as indicated by no decrease in the maximum rate of growth allowing complete substrate utilization (0.22 h^{-1}) for endogenously-produced ethanol concentrations up to 11.4 g/L, and total endogenously-produced + exogenously-added ethanol concentrations up to 21.3 g/L. Higher concentrations of ethanol are tolerated at $\mu = 0.11\ h^{-1}$, although the onset of inhibition was not characterized at this growth rate. Results suggest that the ethanol tolerance of *C.*

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thermosaccharolyticum grown in continuous culture may be greater than that typically observed previously for thermophiles grown in batch culture.

Index Entries: Ethanol; yield; tolerance; continuous culture; thermophiles.

INTRODUCTION

Thermophilic bacteria have often been proposed for the conversion of biomass components into ethanol (*1–4*). Although many advantages and disadvantages of thermophiles as biocatalysts have been mentioned in the literature, some factors are much more important than others in the context of ethanol production. As reviewed elsewhere (*2*), the ability to ferment cellulose and pentoses are important advantages of thermophiles relative to yeast-based processes, resulting in large economic impact. Higher rates of substrate utilization, and in particular cellulose hydrolysis, may be an additional advantage of thermophilic systems relative to systems composed of mesophilic cells and enzymes.

This study was undertaken to provide additional data and understanding pertaining to the two factors thought to most constrain use of thermophilic bacteria for ethanol production (*2*): low ethanol yield, and low ethanol tolerance. Continuous fermentation of xylose by *Clostridium thermosaccharolyticum* is used as a model system. The conversion of xylose, a significant component of many biomass materials, to ethanol is somewhat problematic and is economically important (*5*). *C. thermosaccharolyticum* is representative of a group of thermophilic bacteria that ferment xylose but not cellulose (*3*).

Ethanol Yield

Factors operative at the cellular level that influence solvent yields in thermophiles are presented in Table 1. With respect to product concentrations, the data shows a strong trend toward increased solvent yields in response to H_2, and weaker trends with respect to protons and other products. Nonmetabolites with either electron-donating or inhibitory modes of action have also been found to affect solvent yields. Some cultural factors have been implicated, but no underlying theme is evident, and results are inconsistent in some cases. Recent exhaustive continuous culture studies on *C. thermosaccharolyticum* HG8 and several *Thermoanaerobacter ethanolicus* strains for which high yields have been reported encountered extensive unexplained yield variability (*31,35–37*). In these studies, fermentation parameters varied significantly over time, and apparently identical input conditions produced inconsistent results.

Table 1
Extracellular Factors Influencing End-Product Distribution
in Thermophiles and Selected Other Organisms

VARIABLE	SPECIES	SOLVENTS	COMMENTS	REFERENCE
PRODUCTS (concentration increasing)				
ethanol	C. thermocellum	-	exogenous addition	6
ethanol	T. ethanolicus	-	exogenous addition	7
acetate, lactate	C. thermocellum	+	exogenous addition	6
acetate, lactate, ethanol	C. thermosaccharolyticum	0	exogenous addition	This work
ethanol	C. thermosaccharolyticum	0	removal	This work
ethanol	C. thermohydrosulfuricum	-	removal also decreased H2 pressure	8
H2 increase	C. thermosaccharolyticum	+	decrease purge rate	9
H2 increase	C. thermocellum	+	unstirred reactor	10
H2 increase	C. acetobutyricum	+	increase butanol with low agitation	11
H2 increase	C. butyricum,E. coli,L. casei	+	inc. etoh and formate only in E.coli	12
H2 increase	C. thermohydrosulfuricum	+	unstirred reactor	8
H2 increase	T. brockii	0	increase headgas, growth inhibited	13
H2 removal via co-culture	C. cellobioparum	-	Methanobacterium rumnantium	14
H2 removal via co-culture	Ruminococcus albus	-	Vibrio succinogenes	15
H2 removal via co-culture	C. thermocellum	-	M. thermoautotrophicum	16
H^+	C. thermocellum	-	high substrate,w/o pH control	10
H^+	T. brockii	-	increase E/A as increase pH	17
H^+	C. thermosaccharolyticum	-	maximum ethanol yield at pH 7.2	18
H^+	C. thermosaccharolyticum	+	pH>5 dec.yield, continuous,strainSD105	19
H^+ (increase or decrease)	C. thermosaccharolyticum	0	in batch, no pH effect strain SD105	19
NON-METABOLITES				
CO	C. acetobutylicum	+	inhibit hydrogenase, increase butanol	20,21
CO	C. thermohydrosulfuricum	+	inhibit hydrogenase	22
Methyl viologen	C. acetobutylicum	+	increased ethanol decreased acetone	23
Benzyl viologen	C. acetobutylicum	+	increased butanol via hydrogenase inhib	24
Acetone	C. thermohydrosulfuricum	-	acetate increased, μ increased	25
Acetone	T. brockii	-	acetate decreased, μ unaffected	17
Acetone	T. brockii	-	acetate increased, increased μ	13
Fluoroacetic acid	C. thermosaccharolyticum	-	inhibited sporulation increase lactate	26
CULTURAL CONDITIONS				
Carbon source-starch/xylan	C. thermosaccharolyticum	+	glucosidic linkage appears important	19
FeSO4 increase	C. thermosaccharolyticum	+	decreased flux down lactate branch	9
FeSO4 decrease	T. ethanolicus	+	selection for increased ethanol producer	7
Cell cycle	C. thermosaccharolyticum	+	sporulating, increased ADH activity	28
Cell recycle	C. acetobutylicum	+	increase butanol and acetone	29
Growth rate decrease	C. thermohydrosulfuricum	+	if N-limit, increase μ increases lactate	30
Growth rate decrease	T. brockii	+	starch as carbon source decreases μ	13
Growth rate decrease	C. thermosaccharolyticum	+		19
Growth rate increase	C. thermosaccharolyticum	+	co-cultured w/ & w/o C. thermocellum	30
Growth rate increase	C. thermosaccharolyticum	0		This work
Growth rate increase	T. ethanolicus	0	not dependent on growth rate	31
Temperature increase	T. brockii	-	E/A 0.6 at 60°C-->0.2 at 77°C	17
Temperature decrease	C. thermosaccharolyticum	-/+	batch, w/o pH, 45°C, strain dependent	18
Presence of lignin	C. thermocellum	-	co-culture with T. ethanolicus	32
Presence of lignin	C. thermocellum	-	co-culture with C. thermosaccharolyticum	30
Presence of lignin	C. thermocellum	0		33
UNKNOWN				
Inoculum state	C. thermosaccharolyticum	~	variability in yield and tolerance	34
Inoculum state	C. thermocellum	~	variability in yield and tolerance	34
Inoculum state	T. ethanolicus	+	past history influences yield	35
(?)	C. thermosaccharolyticum	~	multiple steady states	36
(?)	T. ethanolicus	~	multiple steady states	37

The yield of a given product resulting from a multiple-product fermentation is the result of biological control mechanisms that regulate the relative flux of metabolites through alternative metabolic branches. It is useful to distinguish control at the level of activity, which acts on enzymes already synthesized, and control at the level of synthesis, which acts on transcription or translation. Some data exist with respect to control mechanisms for anaerobes with solvent/acid fermentations, as presented in Table 2. These data suggest that control at the level of enzyme activity is operative in several organisms, including thermophiles, although none of the indicated relationships have been demonstrated in *C. thermosaccharolyticum*

Table 2
End-Product Control at the Level of Enzyme Activity

ENZYME	EFFECTOR	EFFECT	SPECIES	REFERENCE
lactate dehydrogenase	fructose-1,6-diphosphate	+	C. thermohydrosulfuricum	38
		+	T. brockii	39
		+	many other species	40
	NADPH	-	T. ethanolicus	41
	pyruvate	-	T. ethanolicus	41
alcohol dehydrogenase	pyruvate	+	T. ethanolicus	41
		-	C. thermocellum	39
		-	T. ethanolicus	7
glyceraldehyde 3-P dehydrogenase	NADH	-	C. thermohydrosulfuricum	22
pyruvate-ferredoxin oxidoreductase	low redox potential	+	C. pasteurianum,C. butyricum	42
hydrogenase	Acetyl CoA/CoA increase	+	C. kluyveri	43
	Carbon source for rapid growth	-	Alcaligenes eutrophus	44
	cAMP	+	A. eutrophus	44
NADH-ferredoxin oxidoreductase	low [NADH]	-	C. pasteurainum, C. butylicum	45
	low [NADH]	0	butyric Clostridia	46
	Acetyl CoA	+	butyric Clostridia	46
	CoA	-	C. kluyveri	43
Acetate kinase	ATP has a sigmoidal effect	-	C. thermoaceticum	7

per se. Huesemann and Papoutsakis (*47,48*) have demonstrated a significant role for control at the level of enzyme synthesis in *C. acetobutylicum*. Few data are available for control at this level in thermophiles to our knowledge, quite possibly owing to the lack of a suitable model system.

Consideration of metabolic control is facilitated by distinguishing between control strategies and mechanisms. Stated in general terms, a control strategy may be defined as a relationship between stimulus and response that is nonmechanistic (e.g., 'organisms reduce synthesis of cellular precursors when the precursor is available in the growth medium'). A control mechanism may be defined as a specific molecular-level description of an interaction between two or more molecules that accomplishes metabolic regulation (e.g., 'the rate of synthesis of aspartyl phosphate from asparatate is inhibited by the presence of lysine via allosteric interactions'). In their landmark review, Thauer et al. (*43*) state that fluxes in different catabolic branches are adjusted so that the ATP gain and metabolic efficiency are optimal for the respective growth conditions. Although providing a useful perspective, and consistent with approaches taken herein, this statement of a control strategy is not sufficient to specify product distributions. The range of metabolic efficiencies found in nature is very large, (Thauer et al. present values for anaerobes ranging from 27 to 80%), suggesting strongly that efficiency is not a key factor in determining the competitive fitness of a bacterium. Based on work with *C. acetobutylicum*, Papoutsakis and coworkers (*49–52*)) have put forward a refinement of the strategy proposed by Thauer et al., applying to situations

with varying ATP availability. This strategy may be stated as follows: products of metabolic branches having a higher ATP yield are preferred what ATP is scarce, and products of metabolic branches having a lower ATP yield are preferred when ATP is plentiful.

Ethanol Tolerance

As recently reviewed for bacteria by Ingram (*53*), and for yeasts by D'Amour et al. (*54*), ethanol alters the degree of polarity of both the cell membrane and cytoplasm, with consequent disruption of growth and/or fermentation-related functions at sufficiently high concentrations. Ethanol inhibition in *Clostridium thermocellum* has been studied by Herrero et al. (*55–58*). These workers found ethanol-induced changes in membrane lipid composition, such that membrane fluidity was increased in response to ethanol, and identified a blockage in glycolysis as a likely site of growth inhibition by ethanol. Lovitt et al. (*22*) concluded that ethanol inhibition in *C. thermohydrosulfuricum* is a regulatory phenomenon related to an imbalance in the pools of reduced and oxidized pyridine nucleotides.

Table 3 presents ethanol inhibition data for thermophilic bacteria, as well as yeasts and mesophilic bacteria. Whereas values are available for the maximum concentration of ethanol tolerated by yeasts and *Z. mobilis*, few such values are available for thermophiles. The values for 50% growth inhibition vary widely for thermophiles, including the same species. This variability is taken to be indicative of both the adaptability of thermophilic strains to high ethanol concentrations, as well as the tentative state of our understanding of thermophilic ethanol tolerance. All of the data in Table 3 for thermophiles were obtained in batch or fed-batch cultures, and much of the data has been reported in terms of the extent of growth (e.g., maximum OD) rather than growth rate.

With two exceptions (*61,63*), all the literature cited in Table 3 were obtained by supplementing endogenously-produced ethanol with exogenously-added ethanol, with the latter usually comprising the larger fraction of total ethanol in the medium. Greater sensitivity to endogenous ethanol has been reported for yeasts (*74–78*), and has been suggested in *C. thermosaccharolyticum* (*9*). This phenomenon has been attributed to the accumulation of endogenously-produced ethanol within the cell, resulting in intracellular ethanol concentration in excess of the extracellular concentration (*74,76*). However, the extent and importance of intracellular ethanol accumulation has been questioned on both experimental (*75,79–81*) and theoretical (*64,75*) grounds. Notably, internal and external ethanol concentrations are thought to be about equal, and endogenous and exogenous ethanol equally inhibitory, in *Zymomonas mobilis* (*64,65*).

A problem frequently encountered in evaluating ethanol inhibition is that of differentiating between limitation by ethanol and limitation by other factors. One approach to making this differentiation would appear

Table 3
Ethanol Inhibition Data For Growth of Bacteria and Yeast

Organism/Strain	System/Substrate	Inhibition Measure[1]	[Ethanol] for Inhibition (g/L)[2] 100%	50%	Ref.
Thermophilic Bacteria					
C. thermocellum ATCC 27405	Batch/Cellobiose	Rate/Endo+Exo	12.5	6.2	59
C. thermocellum ATCC 27405	Batch/Cellobiose	Rate/Endo+Exo		4	55
C. thermocellum C9	Batch/Cellobiose	Rate/Endo+Exo		25	55
C. thermocellum ATCC 27405	Batch/Cellobiose	Extent/Endo+Exo		8	60
C. thermocellum S7	Batch/Cellobiose	Extent/Endo+Exo		48	60
C. thermosaccharolyticum HG3	Batch/Xylose	Extent/Endo+Exo		32	60
C. thermosaccharolyticum HG8	Fed Batch/Xylose	Rate/Endo		<5	61
C. thermosaccharolyticum F7-3P	Fed Batch/Xylose	Rate/Endo		<13	61
*C. thermohydrosulfuricum*39E	Batch/Glucose	Extent/Endo+Exo		10	62
*C. thermohydrosulfuricum*39EA	Batch/Glucose	Extent/Endo+Exo		50	62
T. ethanolicus JW200 mutant	Batch/Starch	Rate/Endo+Exo		100	41
Mesophilic Bacteria					
Z. mobilis ZM4	CSTR/Glucose	Rate/Endo	86	54	63
Z. mobilis ATCC 10988	CSTR/Glucose	Rate/Endo+Exo	72	50	64
Z. mobilis ATCC 10988	Batch/Glucose	Rate/Endo+Exo	70	50	65
E. coli K-12	Batch/Glycerol	Rate/Endo+Exo		37	66
C. saccharolyticum ATCC 35040	Batch/Glucose	Rate/Endo+Exo		28	67
C. saccharolyticum PNA	Batch/Glucose	Rate/Endo+Exo		52	67
A. calcoaceticus	Batch/Ethanol	Rate/Endo+Exo		25	68
Yeast					
S. cerevisiae ATCC 4126	CSTR/Glucose	Rate/Endo+Exo	94	72	69
S. cerevisiae 223	CSTR/Glucose	Rate/Endo+Exo	69	47	70
S. cerevisiae NRRL-Y-132	CSTR/Glucose	Rate/Endo+Exo	87	44	71
Baker's Yeast	CSTR/Glucose	Rate/Endo+Exo	63	43	72
P. tannophilus	Batch/Xylose	Rate/Endo+Exo		23	73

[1] "Rate" and "Extent" refer to growth, with extent typically expressed in terms of final O.D. "Endo", edogenously-produced; "Exo", exogenously-added.

[2] Obtained from kinetic models where possible, and from graphically-presented data otherwise.

to be the use of ethanol removal, and demonstration that the extent or rate of growth with ethanol removed exceeded that without ethanol removed. Continuous culture data are available for yeasts and *Z. mobilis*, however, data for bacteria other than *Z. mobilis* are from batch or fed-batch culture studies. Continuous cultures provide a valuable perspective on ethanol tolerance because: (1) results pertaining to growing cells are ensured, and (2) the culture has an opportunity to adapt to the presence of ethanol.

MATERIALS AND METHODS

Source and Maintenance of *C. thermosaccharolyticum*

Clostridium thermosaccharolyticum HG8 was kindly supplied by Arnold Demain (MIT).

Stock cultures were maintained in the GBG batch medium described under "Medium Composition and Preparation." The A Solution with the ammonium sulfate was dispensed in 10 mL amounts in crimp seal tubes (Belco Biotechnology, Vineland, NJ). B solution and xylose solution were mixed in 158 mL serum vials (Belco) at 50x concentration. All vessels were sealed, evacuated, and flushed with nitrogen, and autoclaved for 30 min at 132°C. After autoclaving, B solution and substrate were added to the A solution tubes aseptically with syringes (0.2 mL for each solution). The culture was maintained in multiple tubes and transferred aseptically with syringes to fresh medium every 3 mo. The newly inoculated tubes were incubated at 60°C for 24 to 36 h and refrigerated until needed.

Stock cultures were purified periodically by picking single colonies. GBG batch medium solution with 1.8% agar was mixed in 14 mL screw-top Hungate tubes with septum covers (Belco). B solution containing twice the normal GBG cysteine and xylose solution were prepared and autoclaved as above. The autoclaved agar tubes were melted in an 80°C water bath, and the B solution and xylose were added. Stock cultures were counted for cell density and diluted in sterilized GBG batch tubes (without xylose) to reduce the number of cells to approx 50/mL. One milliliter of the diluted stock culture was then aseptically transferred to a molten agar tube, mixed, and aseptically transferred to a sterilized petri dish that was immediately covered. The solution was allowed to solidify; it was inverted such that the agar faced down, and it was placed in a BBL GASPAK Jar (VWR, Boston, MA). Three oxygen reducing GASPAK envelopes were activated and placed in the jar. After waiting 12 h, the jar and contents were incubated at 60°C for 72 h. Using syringes, individual colonies were then picked and transferred to GBG medium in crimp seal tubes. These cultures were visually compared with other HG8 colonies, and fermentation product profiles were compared with previous stocks.

Medium Composition and Preparation

The experimental setup utilized two feed carboys, one which contained the carbon substrate (xylose), and one which served as a diluent. The relative flow rates from the diluent and xylose carboys determined the concentration of xylose, yeast extract, $(NH_4)_2SO_4$, and $FeSO_4$ fed to the reactor. The diluent carboy contained per liter of ion exchange purified water (Cole-Parmer): 2.5 g MOPS Buffer, 0.0011 g Resazurin (Kodak, Rochester, NY), 0.91 g $K_2HPO_4 \cdot 3H_2O$, 0.3 g NaH_2PO_4, 0.2 g $MgCl_2 \cdot 6H_2O$, 0.4 g L-cysteine, and 0.1 g $CaCl_2 \cdot 2H_2O$. The substrate carboy contained these components and in addition (per liter of solution): 60 g xylose (Chemical Dynamic Corp., South Plainfield, NJ), 0.05 mL Mazu df 147m (an antifoam agent, Mazer Chemical Inc., Gurnee IL), 0.1 g $FeSO_4 \cdot 7H_2O$, 1.92g $(NH_4)_2SO_4$, and 24 g Bacto yeast extract (Difco). The steady-state increasing feed concentration experiments (Fig. 3) utilized substrate carboys as above, but with higher xylose concentrations and with yeast extract, $(NH_4)_2SO_4$, and $FeSO_4 \cdot 7H_2O$ scaled proportionately. Both diluent and substrate carboys were prepared with a total volume of 18 L. All chemicals were obtained from Sigma Chemicals (St. Louis, MO) and were of reagent grade, unless otherwise noted. Nitrogen gas, prepure grade, obtained from Merriam Graves (White River Junction, VT) was used to displace oxygen in culture media and in fermentors during startup.

GBG batch tubes were prepared, using the same chemicals and water source as above. The concentrations are as given above with the following exceptions (per liter of solution): 10 g MOPS, 5 g xylose, 0.01 g ferrous sulfate, 1.3 g ammonium sulfate, and 5 g yeast extract.

Substrate feed was prepared in a 20 L glass carboy combining 16 L water, MOPS, Mazu, resazurin, sodium phosphate, potassium phosphate trihydrate, and yeast extract (A Solution). The carboys were fitted with #12 three-hole rubber stoppers, holding three 1/4 in stainless tubes. One tube extended to within 1.5 in of the bottom of the carboy, and had a piece of 1/4 in tubing on the end to allow the maximum amount of solution to be withdrawn during feeding. The other two tubes extended only 2 in into the carboy. The longer tube was fitted with a tubing "T", one branch of which went to a sterilizing gas filter (Acro-50 type, 0.2 μm pore size, Gelman Scientific, Ann Arbor, MI) and the other to a 30 mL Luer-Lok glass syringe through a #5 two-hole rubber stopper. One of the shorter stainless tubes had the same setup as above except a female Luer-Lok fitting (Microgroup, Medway, MA) replaced the 30 mL syringe. The second short tube was fitted with a 3 in piece of tubing, and was used as a vent during autoclaving. All tubing was norprene (Cole Parmer) or amber latex (VWR). The stopper was held in place with a stainless steel headplate and collar with three fastening screws. In a separate 4 L carboy (VWR), 1080 g of xylose was combined with 1.5 L water (Xylose Solution). It was fitted with a #10 two-hole rubber stopper and two 1/4 in stainless tubes, one of which extended to the bottom as described above, and the other

which served as a vent through a sterilizing filter. The longer tube had the same setup as above, but the female Luer-Lok fitting was replaced by a male Luer-Lok fitting. The stopper was wired down to the top of the carboy. A third solution with 7.2 g cysteine, 3.6 g $MgCl_2 \cdot 6H_2O$, 1.8 g $CaCl_2 \cdot 2H_2O$, and 1.8 g ferrous sulfate was mixed in a 1000 mL vacuum flask with 400 mL water (B Solution). The side arm of the flask was vented hrough a 5 in piece of tubing to a gas filter. The top was fitted with a #5 one-hole stopper with a stainless tube setup extending to the bottom, as described above. The top of the stainless tube was connected to the same type of "T" setup as on the 4 L xylose carboy, and the stopper was wired down to the top of the flask. The ammonium sulfate solution was mixed in a 158 mL serum bottle with 100 mL water and sealed with a crimp seal.

Diluent was mixed in the same manner and set up as the substrate. Water (18 L) was combined with Mops, resazurin, sodium phosphate, and potassium phosphate trihydrate. A second B solution was prepared as above for addition to the diluent carboy after autoclaving.

All solutions were autoclaved for 1.5 h at 132°C. All vessels were vented during autoclaving except the ammonium sulfate solution, which was autoclaved in sealed serum vials. After autoclaving, the solutions were evacuated and flushed with nitrogen to remove oxygen. The smaller solutions were aseptically transferred to their respective 20 L carboys. The large carboys were sparged with nitrogen before and after solution addition, to remove oxygen.

Quantification of Substrates, Cells, and Fermentation Products

Culture optical density was determined in a Bausch and Lomb model 21 spectrophotometer at 660 nm in tubes having a 1.38 cm pathlength. All optical density measurements were made immediately after sampling at a 30x dilution. Quantification of xylose, ethanol, acetic acid, lactic acid, and propanediol was accomplished using HPLC, as previously described (*33*). Carbon recovery was calculated assuming one mole of CO_2 was produced per mole organic fermentation product produced and neglecting cells, using the equation:

$$\text{Carbon Recovery} = ((R_X*5) + (R_L*3) + (R_A*3) + (R_P*3) + (R_E*3)) / (R_{X_o}*5)$$

where L denotes lactic acid, E ethanol, P propanediol, A acetic acid, X_o feed xylose concentration, and X effluent xylose concentration; and R denotes the steady-state rate or production for products, and the respective rates of entry and exit relative to the system for X_o and X.

Continuous Fermentation

Continuous fermentation of xylose was carried out in a 1 L round bottomed Applikon fermentation vessel with a 700 mL working volume. The

reactor, pH probes, and all associated tubing and drip tubes were autoclaved for 30 min prior to use. All tubing connections were made with Luer-Lok fittings, and any parts that require connecting after autoclaving were wrapped in aluminum foil to preserve sterility. All connections after autoclaving were made aseptically, using flame and/or ethanol as necessary. The working volume was maintained with an adjustable stainless steel draft tube extending through the headplate of the vessel. Gas generation during fermentation provided sufficient pressure to force excess broth through the draft tube, thereby maintaining constant level. A drip tube was placed in the effluent line to prevent contamination of the culture. The broth was stirred at 600 rpm during normal operations, and at 200 rpm during startup. An Applikon motor and speed controller were used to maintain the speed of the magnetically coupled impeller. Temperature was maintained at 60°C by constantly circulating water through the reactor heat exchangers. The pH was maintained at 7.0 by addition of 25 wt% sodium hydroxide to the fermentation through a single speed (100 rpm) peristaltic pump (Cole-Parmer) controlled with an Applikon BioProcess Controller. Autoclavable pH/redox probes extended down through the headplate, and were obtained from Phoenix Electrodes, Houston, TX (model number A779792-200-DL). Redox potential was measured on the same Applikon BioProcess Controller. Media was introduced to the fermentation through the headplate. MasterFlex programmable peristaltic pumps (Cole Parmer, model number 7550-90) were used to control the substrate and diluent flow rates. Size 14 pump heads were used on feed lines. Pumps were recalibrated each time flow rates were changed or new tubing was used. Feed carboys were connected to the fermentor with Luer Lok fittings. Drip tubes on samples lines were used to prevent contamination. Feed carboys were maintained under slight positive pressure with nitrogen that had passed through a sterilizing filter.

Fermentation was started in batch mode at approx 0.5% xylose concentration, with the feed maintained at 60°C and pH 7.0. Ten milliliter of a stock culture (*see above*) was transferred aseptically via syringe from a batch tube into the reactor through a septum in the reactor headplate.

Ethanol Removal

The ethanol removal apparatus is shown in Fig. 1. The apparatus consists of a custom blown water jacketed stripping column (Kontes Glass, Vineland, NJ) that was maintained under reduced pressure (150 mmHg) at 60°C. Column pressure was monitored and maintained with an MKS (Burlington, MA) model 250B pressure controller, 222BA pressure transducer, and a 248 flow control valve connected to a vacuum pump. The pressure control apparatus was isolated from the system with a gas sterilizing filter. Temperature was maintained by constant recirculation of 60°C water through the column jacket. Connections were ground glass 24/40 except for connections to a custom-made graduated sampling tube with a

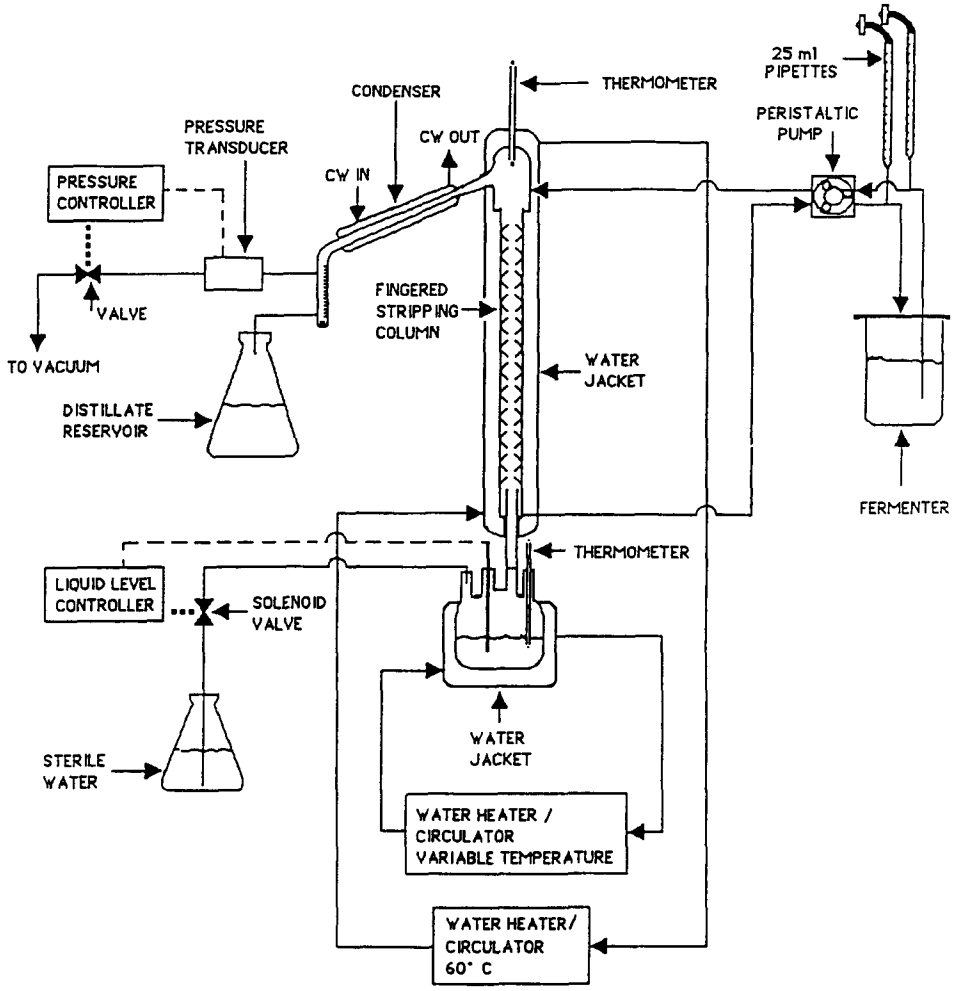

Fig. 1. Apparatus for continuous ethanol removal.

septum at one end used for sampling and flow measurements. The column was fingered, with the direction of the fingers alternating down the length of the column. Stripping steam was generated at the base of the column in a round-bottomed water-jacketed vessel. This vessel was connected to the column through a ground glass joint, and steam flow rate was varied by altering the temperature of the water jacket. Autoclaved distilled water was added to the steam generator through rubber stoppers; water level was controlled with a Cole-Parmer Dynasense model 7187 liquid level controller. A thermometer was also inserted in the steam generator through a rubber stopper. The tubing connecting the fermentor and stripping column were water-jacketed as much as possible to prevent cooling of the fermentation broth. Both feed to and return from the column were run through one peristaltic pump. The feed line was run in a size 14 pump head, and the return in a size 15. Twenty five milliliter pipettes fitted with

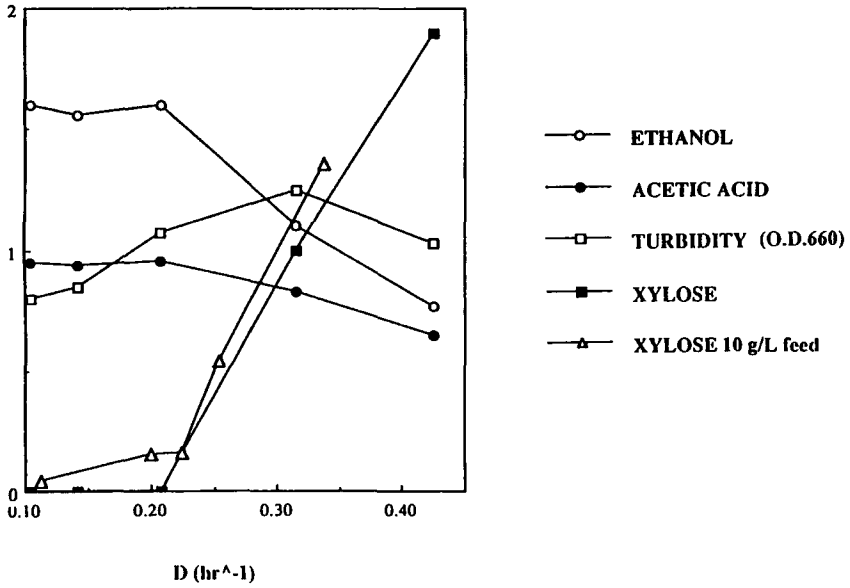

Fig. 2. Washout curve for steady state xylose fermentation by *C. thermosaccharolyticum*. All data for a 5 g/L feed except as noted.

sterilizing gas filters were attached to the feed and return lines on the reactor side of the pump with tees. Clamps were used to isolate various flow paths to allow flow measurements to and from the column using these pipette tubes. The molar ratio of liquid and vapor flows in the column was maintained at approx 2 ($\pm$0.2). The stripping column side stream flow rate was maintained equal to the reactor feed rate in all cases. All flow rates associated with the stripping column were measured each time the reactor was sampled.

RESULTS

Continuous Culture Under Apparently Xylose-Limited Conditions

Figure 2 presents a washout curve for steady state fermentation by *C. thermosaccharolyticum* grown with a feed xylose concentration of 5 g/L. Data for the fermentor xylose concentration are also included for a feed concentration of 10 g/L. The fermentor xylose concentration for both feed conditions remains below 0.16 g/L, corresponding to essentially complete (>97%) substrate utilization, up to a dilution rate of 0.22 h^{-1} (4.5 h residence time). This dilution rate corresponds to the maximum growth rate for complete xylose utilization, defined arbitrarily to correspond to 97%, at very low ethanol concentrations, and is denoted $\mu_{o,0.97}$. (Complete xylose utilization is defined in terms of 97% utilization in light of the fact that,

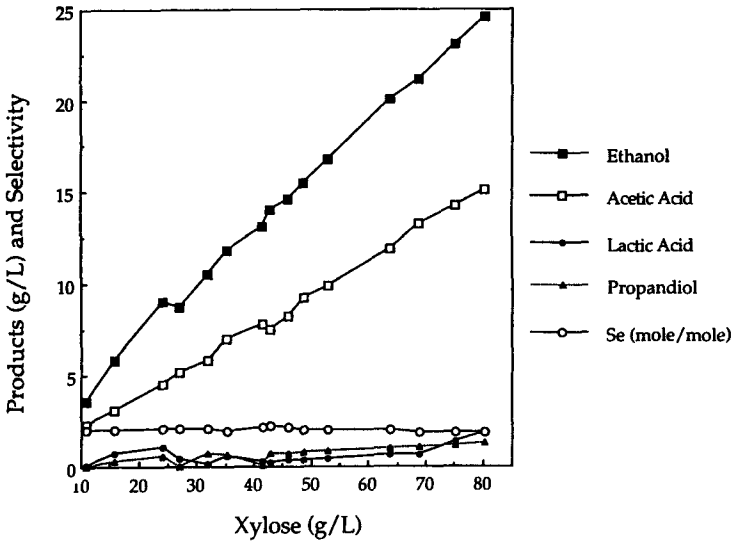

Fig. 3. Steady-state continuous xylose-limited fermentation by *C. thermosaccharolyticum* with increasing substrate concentration. Data at $D = 0.11\ h^{-1}$.

at low ethanol concentrations and dilution rates, substantially less than $\mu_{o,0.97}$, substrate utilization varied from 98 to >99% owing to apparently random phenomena, and further that this rather high value for substrate utilization is likely to be compatible with an economically feasible process.) The data are consistent with Monod-type kinetics, which predict that the fermentor substrate concentration is independent of the feed substrate concentration (*82*), and also with xylose being the rate-limiting nutrient. Little variation in product yields is observed in continuous culture at low feed concentrations as a function of dilution rate.

Steady-state fermentation of xylose at a $0.11\ h^{-1}$ dilution rate and increasing feed xylose concentration is presented in Fig. 3. Ethanol, acetic acid, and cells (indicated by optical density) are the dominant products throughout, with concentrations of both increasing in an essentially linear pattern. The ethanol selectivity (S_E, equal to the molar ethanol production rate divided by the molar rates of production of acetic acid, lactic acid, and propane diol) is essentially constant at about 2.0 for all substrate concentrations shown. Substrate utilization was essentially complete.

Control of End Product Formation

Product Addition and Removal

Addition and removal of fermentation end products was undertaken under xylose-limiting conditions to characterize the importance of mass action in controlling end product formation. Product-addition experiments also have relevance in the context of product inhibition, considered subsequently.

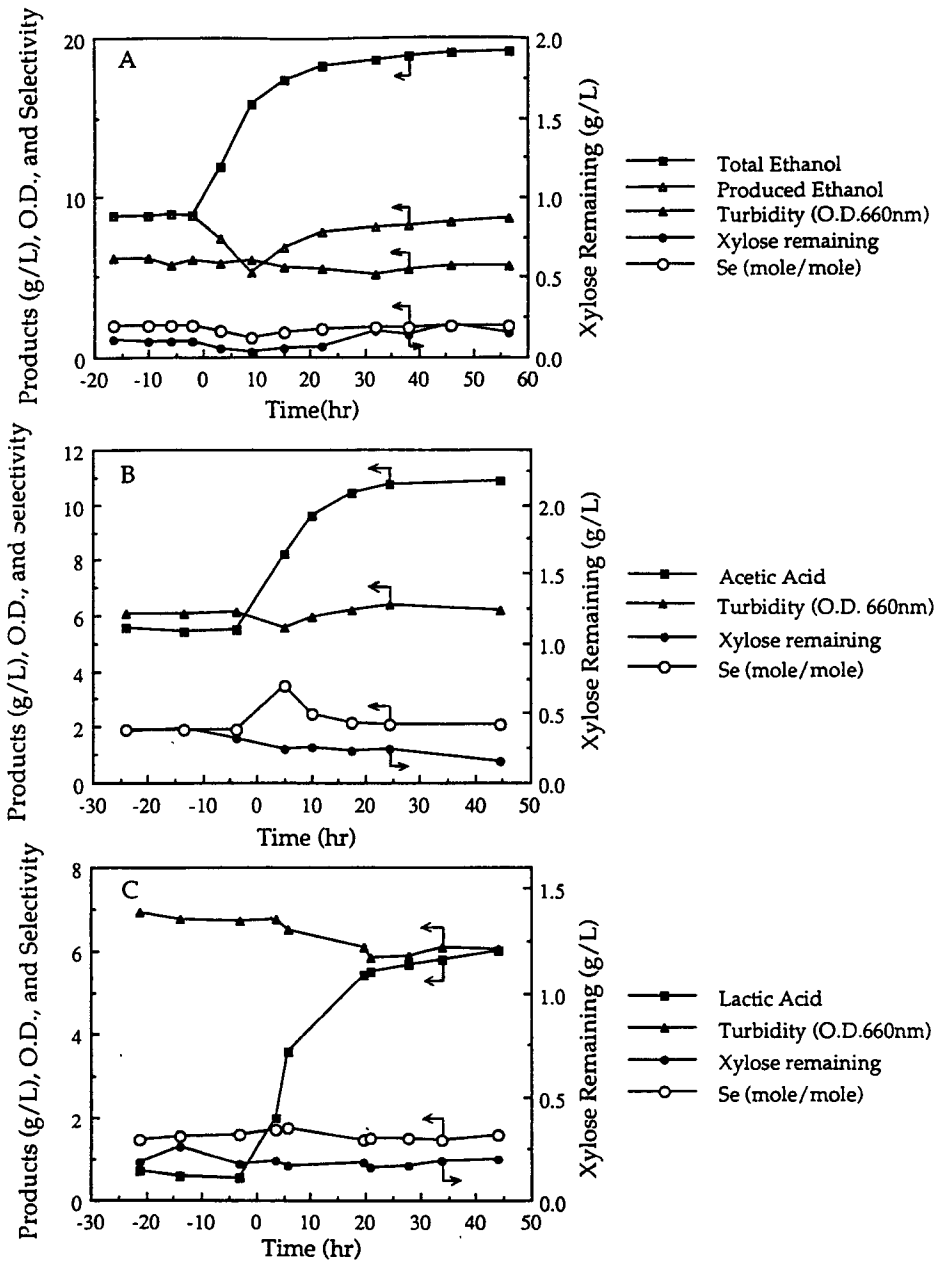

Fig. 4. Addition of fermentation products to xylose-grown continuous cultures. Feed concentration = 30 g/L. Indicated products added to the feed at time = 0. A, ethanol; B, acetic acid; C, lactic acid.

Products were added to the medium reservoir of continuous steady-state fermentations operated at $D = 0.14\ h^{-1}$ and receiving a 30 g/L xylose feed. Product concentrations were at least doubled via exogenous addition to the feed, with specific steady-state product concentrations, including both endogenous production and exogenous addition, as follows: ethanol, 18.1 g/L; acetic acid, 10.7 g/L; lactic acid, 5.8 g/L; propanediol, 2.8 g/L. As presented in Fig. 4, no significant change in steady-state prod-

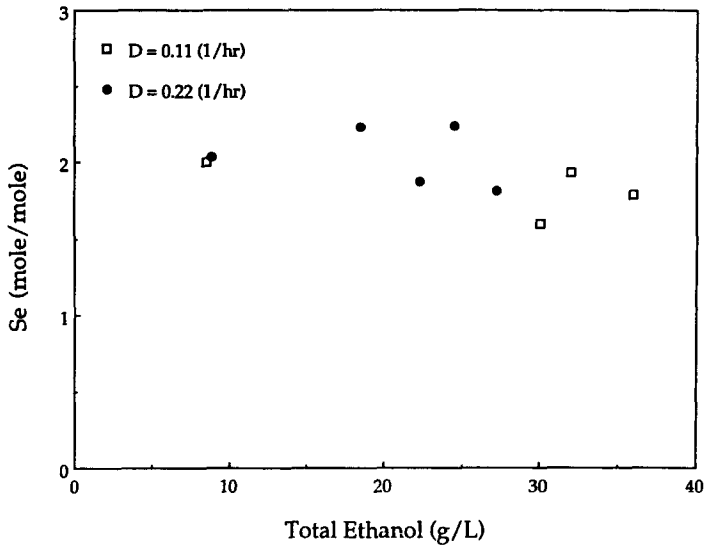

Fig. 5. Steady-state ethanol selectivity at increasing total ethanol concentration for ethanol-supplemented continuous fermentation. All data at 30 g/L xylose feed.

uct yields accompanied product addition, suggesting a small role for mass action in control of end product formation. In addition, little if any inhibition was apparent either transiently or at steady state, based on either effluent xylose or OD data. Similar data were also obtained for propane diol (data not shown).

Addition of ethanol at higher concentrations than used in the experiments depicted in Fig. 4 was undertaken in association with product inhibition studies. Figure 5 demonstrates, at most, a weak mass action effect. The maximum effect observed at $D = 0.22\ h^{-1}$ was a 15% decrease in the ethanol yield brought about by a 3.1-fold increase in the total ethanol concentration.

Ethanol removal via reduced-pressure sidestream stripping was undertaken for steady-state xylose fermentation with a 35 g/L feed. Data presented in Table 4 at both 0.22 and 0.11 h^{-1} dilution rates indicate no significant shift in product formation accompanying an approximately two-fold reduction in ethanol concentration.

Transient Fermentation

Under Nonxylose-Limiting Conditions

In the course of fermentor startup, we observed dramatically different ethanol selectivity under transient conditions with increasing substrate concentrations. When the feed concentration is increased in two steps from 10 g/L to 30 g/L xylose, S_E increases transiently to $\geq 11{:}1$ for a period typically lasting several residence times. Results, such as those presented in Fig. 6, have been carefully documented in a half-dozen independent experiments. An alternative feeding strategy, involving a linearly-increasing substrate concentration, has resulted in $S_E > 26$, but without consis-

Table 4
Continuous Xylose Fermentation With and Without Ethanol Removal[1]

Dilution Rate (hr^{-1})	Parameter	Without Removal	With Removal
0.11	Ethanol Concentration (g/L)	12.8	6.7
	O.D. (660 nm)	7.28	7.44
	Product Formation Rate (g/L*hr)		
	Ethanol	1.03	0.97
	Acetic Acid	0.57	0.57
	Lactic Acid	<0.01	<0.01
	Propane Diol	0.04	0.03
	Carbon Recovery	1.05	1.01
0.22	Ethanol Concentration (g/L)	11.4	6.4
	O.D. (660 nm)	7.95	8.36
	Product Formation Rate (g/L*hr)		
	Ethanol	1.83	1.82
	Acetic Acid	0.96	0.97
	Lactic Acid	0.11	0.09
	Propane Diol	0.13	0.10
	Carbon Recovery	0.98	0.96

[1] Feed xylose concentration: 35 g/L.

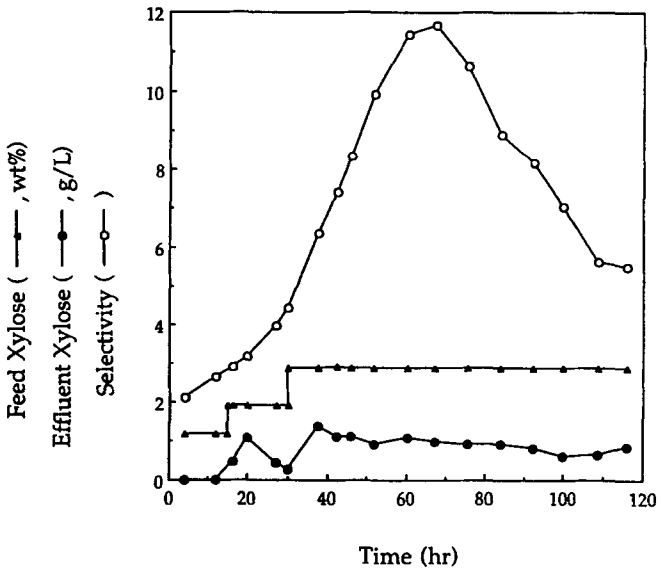

Fig. 6. Continuous xylose fermentation by *C. thermosaccharolyticum* during an increasing substrate transient. Data at $D = 0.11\ h^{-1}$.

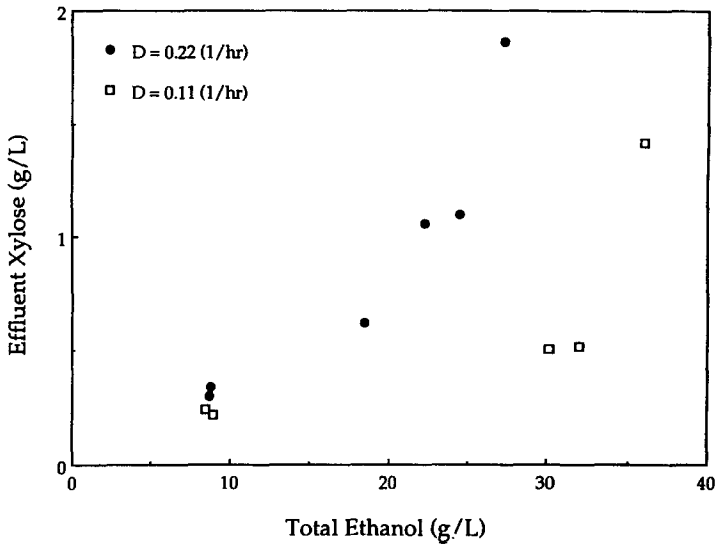

Fig. 7. Steady-state effluent xylose concentration with increasing total ethanol concentrations for ethanol-supplemented continuous fermentation. Data with 30 g/L xylose feed.

tency. In all cases where high S_E have been observed, the fermentor xylose concentration exceeded steady-state levels, although the amount of the increase is somewhat variable. Furthermore, the return to steady-state xylose concentrations is accompanied by return to steady-state S_E values. Thus, xylose-plentiful conditions appear to be correlated with elevated production of ethanol in lieu of acetate.

Ethanol Tolerance

Ethanol was added to continuous cultures in order to determine the tolerance to ethanol present in the growth medium. Experiments were carried out at $D = 0.22\ h^{-1}$ and also $D = 0.11\ h^{-1}$. The former corresponds to $\mu_{o,0.97}$, the latter to $1/2\ \mu_{o,0.97}$.

Steady-state fermentor substrate concentration data is plotted vs total ethanol concentration (endogenous + exogenous) in Fig. 7. Complete xylose utilization was obtained for total ethanol concentrations up to 21.3 g/L (obtained by linear interpolation between data at 18.5 g/L and 24.5 g/L) at $D = \mu_{o,0.97}$, indicating no apparent ethanol inhibition below this value. For a given product concentration, lower effluent xylose concentrations accompany fermentation at $D = 0.11\ h^{-1}$, as compared to $D = 0.22\ h^{-1}$.

As may be seen from the transient data presented in Fig. 8, data obtained at $D = 0.22\ h^{-1}$ and total ethanol concentrations ≥ 22 g/L exhibit a transient increase in fermentor xylose concentration and decrease in optical density, followed by somewhat decreased xylose concentration and increased optical density. These data are consistent with an adaptation by the culture to higher ethanol concentrations.

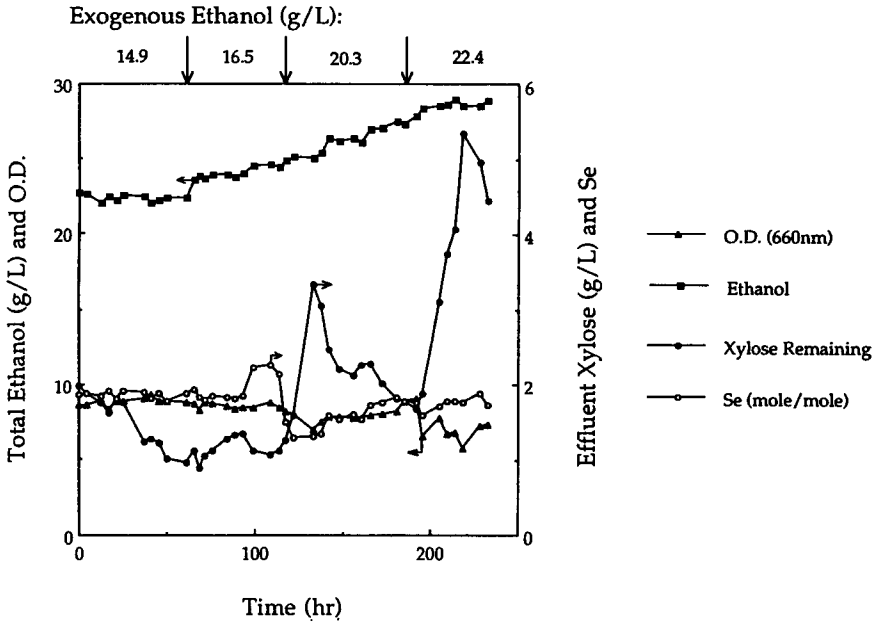

Fig. 8. Continuous fermentation with successive addition of ethanol. Date at $D = 0.22\ h^{-1}$, 30 g/L feed. Values between the arrows indicate concentrations of ethanol added.

DISCUSSION

Under xylose-limiting conditions, ethanol selectivity was found to be remarkably constant. Product addition and removal experiments indicate that mass action effects in response to the concentration of organic end products are relatively minor factors in determining the ratio of fermentation products formed by *C. thermosaccharolyticum*. Of much greater apparent importance are as yet uncharacterized regulatory phenomena that appear to be correlated with nonlimiting concentrations of the carbon and energy source. Our data and observations are consistent with an energetically based end product control strategy, such as has been demonstrated by Papoutsakis and coworkers in the mesophile *C. acetobutylicum* (*49–52*). Of particular note, Huesemann and Papoutsakis (*48*) demonstrated elevated solvent yields in response to transients involving increased substrate concentration. In light of the dramatic effect of substrate availability, it seems plausible that substrate and/or ATP-related effects may explain some of the variable ethanol yields that have been reported in the literature (*2,31,35–37*). Further characterization of the role of ATP availability under conditions with varying product yields and fermentor operating schemes to take advantage of this phenomenon to produce high solvent yields over prolonged periods are under investigation in our laboratory.

Kinetic models for growth with product inhibition typically include the product of a substrate-dependent term, as a result of which the growth rate increases with increasing substrate concentration, and a product-dependent term, as a result of which rate decreases with increasing product concentration (*63,69–72,77*). When this functionality applies, addition of product at a concentration sufficiently high to cause inhibition results in an increased fermentor substrate concentration under constant growth rate conditions. Furthermore, when increased substrate concentration does not accompany product addition under constant growth rate conditions, the concentration of added product may be inferred to be subinhibitory. This approach to the study of ethanol inhibition has been employed previously by Aiba et al. (*72*).

We observed no apparent inhibition in the rate of growth, as indicated by complete substrate utilization at $D=\mu_{o,0.97}$, for endogenously-produced ethanol concentrations up to 11.4 g/L, and total endogenous + exogenous concentrations of up to 21.3 g/L. Still higher ethanol concentrations are tolerated at lower dilution rates. This degree of ethanol tolerance is greater than that indicated by most of the studies presented in Table 3 for *C. thermosaccharolytiucum* and for other thermophiles. One possible explanation for the difference between the tolerance we have observed and that previously reported for thermophilic species is that our results are in continuous culture, whereas previous studies have been in batch or fed-batch culture. The data presented herein (Fig. 8 especially) strongly indicates that adaptation to ethanol is operative. It is possible that ethanol tolerance is inducible in thermophiles, and more nearly constitutive in yeasts.

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