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Direct Microbial Conversion

Prospects, Progress, and Obstacles

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ABSTRACT

Process development is reviewed for ethanol production from cellulosic biomass via direct microbial conversion (DMC). Experimental data addressing cellulase production and ethanol tolerance are also presented for the candidate DMC organisms *Clostridium thermocellum* and *Clostridium thermosaccharolyticum.* Two potential paths are identified for obtaining organisms for use in DMC. Path 1 involves modification of excellent ethanol producers, so that they also become good cellulase producers; Path 2 involves modification of excellent cellulase producers, so that they also become good ethanol producers. Cellulase production, ethanol tolerance, and ethanol selectivity are considered for both Path 1 and Path 2 organisms. It is concluded that *in situ* cellulase production has the potential to allow cost reductions relative to state-of-the-art process designs on the order of 50¢/gal. Based on the data available, the value of cellulase production by *C. thermocellum* corresponds to 90% of this amount. However, each process path has a strategic obstacle to be overcome: high-level cellulase expression and secretion for Path 1, and high ethanol selectivity for Path 2. Ethanol tolerance is not seen as a primary factor in choosing between DMC and other ethanol process alternatives.

Index Entries: Direct microbial conversion; ethanol tolerance; cellulase production; *Clostridium thermocellum; Clostridium thermosaccharolyticum.*

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INTRODUCTION

Potential benefits of fuel ethanol produced from biomass have frequently been presented. Realization of these benefits has been impeded by the cost of converting biomass into ethanol. Biologically mediated process steps are a particular focus for improvement, since these steps are the most expensive and least developed in current process designs (*1*).

Within the context of ethanol production processes employing enzymatic hydrolysis, four biologically mediated events can be identified: production of cellulase enzymes, substrate hydrolysis, C6 sugar fermentation, and C5 sugar fermentation. The manner in which these events are combined into process steps is the basis for differentiating bioprocessing options for ethanol production.

State-of-the-art processes for the conversion of lignocellulose to ethanol utilize simultaneous saccharification and fermentation (SSF). The term SSF is most commonly used to refer to processes wherein hydrolysis of cellulose and fermentation of hexoses to ethanol are carried out in a single vessel, and enzyme is produced in a process step separate from hydrolysis/fermentation. SSF may be coupled with pentose fermentation to improve process yields; current designs involve pentose fermentation in a third separate biological step. When compared to process designs of a decade ago, current SSF-based designs have cut ethanol production costs in half to $1.35/gal (*2*). Further price reductions, halving costs again, are thought to be necessary for biologically produced fuel ethanol to compete with petroleum on a large-scale basis at oil prices anticipated for the year 2000 (*1*).

Direct microbial conversion (DMC) processes achieve enzyme production, substrate hydrolysis, and fermentation to ethanol in a single process step. Described as early as 1933 (*3*), DMC processes may be conceived using fungi (*4,5*), bacteria (*3,6–9*), and yeasts (*10,11*). Topics relevant to DMC have been investigated by several laboratories in the last decade and are the subject of multiple reviews (*12–17*). The potential advantage of DMC lies in reduced bioconversion costs and the possibility of higher overall yields. The simplicity of DMC is also compatible with producing a low-value commodity, such as ethanol, and may allow rapid process development if key technical issues can be resolved.

No microbial systems are known that simultaneously synthesize cellulase at the required high levels and consistently produce ethanol at the required high yields. Although several approaches for developing such a system have evolved, including mixed cultures of cellulase producers and ethanol fermenters, two candidate paths are considered here. It may be feasible to modify excellent ethanol producers (e.g., *Saccharomyces cerevisiae, Zymomonas mobilis*) such that they also become good cellulase producers (Path 1). A second option is to manipulate excellent cellulase producers (fungi, bacteria) such that they also produce ethanol at high yields (Path 2).

This article considers the potential of DMC processes, encompassing both attractive features as well as barriers to utilization. Progress toward addressing such barriers is considered for both Path 1 and Path 2 approaches; new experimental results for one Path 2 option are also presented.

MATERIALS AND METHODS

Organism

Clostridium thermocellum strain ATCC 27405 was used for cellulase production experiments, and *Clostridium thermosaccharolyticum* strain HG8 was used for ethanol tolerance experiments. Both strains were kindly supplied by Arnold Demain (Massachusetts Institute of Technology). Experiments were initiated with stock cultures stored at −20°C in 50% glycerol and 50% GBG medium (*see below*). Glycerol stocks were prepared from single colonies as described previously (*18*).

Media and Culture Apparatus

Continuous culture of *C. thermosaccharolyticum* was carried out in GBG-xylose medium with preparation and composition as reported previously (*18*), except that the concentrations of $K_2HPO_4 \cdot 3H_2O$, NaH_2PO_4, and $CaCl_2 \cdot 2H_2O$ were reduced by 67% in order to prevent precipitation in ethanol-containing medium. All chemicals used were reagent grade. A detailed presentation of methods for documenting the ethanol tolerance of *C. thermosaccharolyticum* is available (*19*) and will also appear in a manuscript specifically addressing ethanol inhibition. Batch culture of *C. thermocellum* was carried out in the defined medium of Johnson et al. (*20*). Media preparation was as previously described (*18*). Culture apparatus was as described previously for *C. thermosaccharolyticum* (*18*) and *C. thermocellum* (*21*). Methods for inoculation, start-up, steady-state cultivation, and sampling were as described previously (*18*).

Analytical Methods

Analysis of substrate and fermentation product concentrations was performed by HPLC as described previously (*18*). Culture dry weight was determined by filtering 10-mL samples through preweighed 45-mm diameter Nucleopore filters (0.4 μm pore size), drying overnight in a 72°C oven/dessicator, and weighing the dried samples. The mass fraction of cells and residual cellulose was analyzed by quantitative saccharification as described previously (*21*). Cellulase activity is reported for cell-free culture broths prepared either by centrifugation for 10 min or filtration through 45-mm diameter Acrodisc filters (0.2-μm pore size). Activity is

reported in units (U) of μmol/L/min, calculated based on the rate of production of soluble potential glucose from 0.6 g/L Avicel (FMC Corp., Philadelphia, PA) the assay buffer developed by Johnson et al. (*20*) and the soluble potential glucose assay as reported previously (*22*). Samples were diluted until activity was proportional to the amount of culture broth added. Extracellular protein concentration was determined by the Bio-Rad (Richmond, CA) assay using bovine serum albumin as a standard.

RESULTS AND DISCUSSION

Cellulase Production

The Impact of Free Cellulase

The primary incentive for pursuing DMC is the dramatic impact of cellulase production costs on overall process economics. As discussed subsequently, cellulase production in DMC systems may involve little or no cost, depending on the basis for comparison. As a limiting case, the economic savings associated with production of free, plentiful cellulase are explored below. Thereafter, the extent to which free cellulase is or is not likely to be achieved is addressed.

The most obvious, but not the largest impact of free cellulase production is the elimination of capital and operating costs associated with cellulase production. In the most recent SSF designs available (*23,24*), these costs are about 13¢/gal.

A less obvious, but more significant cost savings associated with free cellulase is reduced bioreactor costs. Figure 1 illustrates the trade-off between enzyme and bioreactor costs for SSF using a first-order economic analysis calibrated from published data. The total bioconversion costs show a minimum cost of 60¢/gal at a 7-d hydrolysis/fermentation time, in agreement with data published by Wright (*23,24*). Overall cost reductions can be achieved by technologies that shift the cellulase cost curve left or the fermentation curve right. In the limit, when cellulase has no incremental costs, the cellulase cost curve becomes irrelevant, and the total cost curve coincides with the fermentation cost curve.

If free cellulase is available in sufficient quantity, the time of hydrolysis and fermentation can be reduced to the reactivity limit of the cellulase/substrate system. For *T. reesei* systems, the reactivity limit is approx 1-d (*25*). Available data suggests that 1/2-d may be reasonable for systems employing *C. thermocellum* (*21*), although more data, including results at high substrate concentration in particular, are needed to confirm this value. It is unclear what mechanisms, if any, may slow hydrolysis at high substrate concentrations, since rate-inhibiting product concentrations are not anticipated in a DMC context. As illustrated in Fig. 1, with free enzyme at levels sufficient to allow operation at the 1-d reactivity limit, the total bioreactor

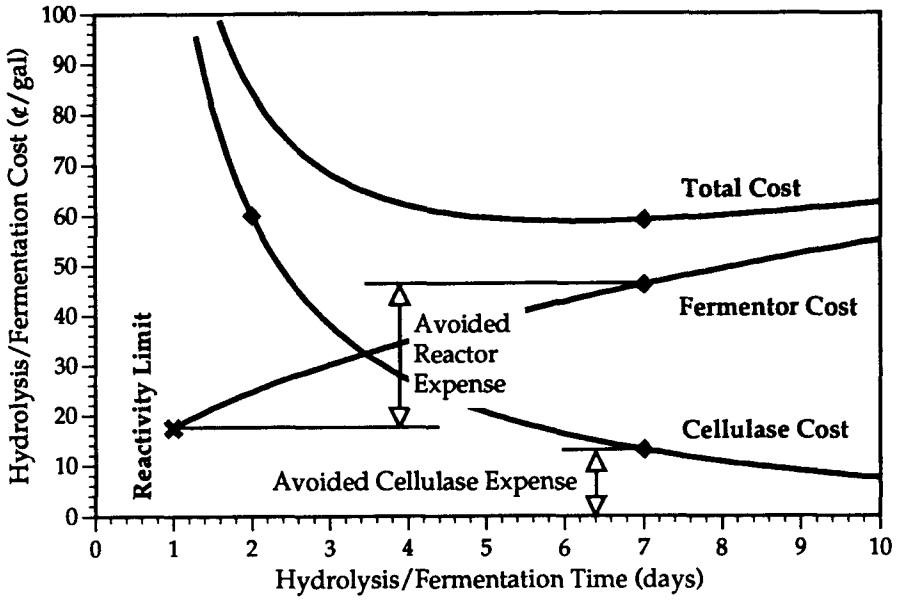

Fig. 1. Cost reductions associated with free cellulase production in the context of the fermentor/enzyme cost trade-off for SSF processes. Data points are from Wright (*24*); the cellulase/substrate reactivity limit is discussed in the text.

cost is reduced by approx 29¢/gal when compared to the SSF economic optimum.

As a result of *in situ* cellulase production, DMC may realize further economic advantage through improving overall process yield. In reported SSF systems, a significant feedstock portion (5–10%) is diverted from ethanol fermentation to aerobic production of seed cultures and/or cellulase. Since aerobic metabolism does not result in ethanol production, the diverted portion of feedstock is effectively lost. Similar diversion would not be necessary in DMC system operating at the reactivity limit. The overall yield improvement of a DMC system that eliminated sidestream diversion of 5–10% represents a cost savings in the range of 7–13¢/gal when compared to current SSF systems.

Other attractive features of DMC processing have been reported, including compatible fermentation and cellulase systems, higher reaction rates, and reduced cooling costs (*13*). Although in future stages of process development these features could become important, their combined impact appears to be small when compared to the savings potential of free cellulase. Ignoring these secondary features, a first-order estimate of the total potential savings associated with free cellulase production is about 50¢/gal relative to current SSF designs.

The potential savings associated with free cellulase may be effectively canceled by limitations of current DMC systems, as discussed herein. However, the analysis presented above demonstrates the very large value of the most important distinguishing feature of DMC systems: production of cellulase by the ethanol-producing fermentation system.

Evaluation of Cellulase Production Costs

Cellulase may be said to have a cost if its production either:

1. Requires fermentors or other equipment that serves no other purpose (e.g., bioreactors for cellulase synthesis by *T. reesei*);
2. Increases the size of fermentors or other equipment that do serve other purposes (e.g., an SSF reactor); or
3. Requires substrate that could otherwise be used for ethanol production.

All of these must be evaluated relative to some base case. This article uses as the base case fermentation of soluble sugars by yeast at a rate corresponding to the cellulase/substrate reactivity limit. If no costs are incurred beyond the base case for items 1–3 above, then cellulase production can reasonably be said to be free in the context of a process for ethanol production from cellulosic biomass.

Cellulase production is the major obstacle to the development of cost-effective Path 1 DMC systems. Although cellulases have been expressed in *S. cerevisiae* (*10,11,26–28*), and *Z. mobilis* (*29,30*), reported production levels are thus far impractical for DMC ethanol production. To date, research has focused on heterologous expression and secretion of single cellulase components for study and/or application to higher value products than ethanol. To our knowledge, all data reported on cellulase production by *S. cerevisiae* have been obtained under aerobic conditions with little or no production of ethanol.

Conversely, cellulase production is the most important asset of Path 2 DMC organisms. In the case of *C. thermocellum*, perhaps the bacterium most studied for use in DMC processing, approx 1/3 of the genome is thought to code for cellulase (*31*). Rates of cellulose hydrolysis are comparable for broths from *C. thermocellum* and *T. reesei* prepared under similar conditions, with specific activity much higher for the thermophilic system (*20*). In spite of these indications of high cellulase activity, few data for *C. thermocellum* are available addressing substrate partitioning to cellulase or the yield of cellulase in terms of activity. The cellulase loading, corresponding to the yield in terms of activity (U/g carbohydrate utilized), is important for evaluating cost category 2 above. Substrate partitioning is important for evaluation of cost category 3 above. Accordingly, we have recently initiated studies addressing these factors.

For Path 2 organisms, such as *C. thermocellum*, that produce ethanol and cellulase simultaneously, there are no costs associated with cost category 1, since no equipment with the sole purpose of cellulase production is required.

Data for reaction times and enzyme loading are presented in Table 1. Lynd et al. (*21*) have demonstrated high (>85% of theoretical) conversion of pretreated mixed hardwood at dilute (e.g., 5 g/L) feed concentrations in a continuous culture of *C. thermocellum* operating at a 12-h residence

Table 1
Cellulase Loading and Reaction Times for DMC Employing *C. thermocellum* and SSF Employing *S. cerevisiae*

	DMC	SSF
Reaction time for high conversion, h[a]	12	168
Loading, U produced or added/g substrate[b]	>71	7

[a]All data for dilute-acid pretreated hardwood. DMC from (*21*), for a feed with 4 g/L cellulose; SSF from Wright (*24*) for a feed with 60 g/L cellulose. High conversion denotes >85% theoretical.

[b]DMC data from this study, based on free activity only and not including cell or substrate bound cellulase; SSF data from Wright (*24*). Units of enzyme activity measured using Avicel for DMC and filter paper for SSF; data of Johnson et al. (*20*) indicate that results are similar for these two substrates.

time. The presence of excess cellulase in these experiments, together with the high affinity of *C. thermocellum* cellulase components for pretreated wood (*32*), indicates that the substrate was essentially saturated with enzyme and the system was operating near the substrate/enzyme reactivity limit. The current 7-d optimum for SSF has already been discussed in introducing Figure 1. The significant difference in these reaction times is likely to be primarily the result of the much higher cellulase loading in the thermophilic system.

The data presented in Table 1 do not suggest that the size of bioreactors or other equipment needs to be increased for fermentation of *C. thermocellum* over that of a base case operating at the cellulase/substrate reactivity limit. Thus, costs associated with category 2 above may be zero for this and similar organisms. Evaluation of data for cost category 2 can only be preliminary at this point, because few if any data are available for DMC systems under realistic processing conditions. However, the dominant parameter determining the reaction rate, and hence the bioreactor size for a given throughput, would appear to be the yield of cellulase relative to substrate. It is unclear what, if any, physiological factors would cause this parameter to change under more realistic conditions.

Table 2 presents comparative data for substrate partitioning for *C. thermocellum* and *S. cerevisiae*. Cell yields appear to be similar for the two systems. The data in Table 2 suggest that the mass of extracellular protein produced by *C. thermocellum* is about 4% of the substrate utilized. The observed cell and enzyme yields correspond to approx 82% of the substrate carbon available for catabolic processes resulting in ethanol production for *C. thermocellum* and approx 89% of the substrate carbon available for such processes with the yeast. Corresponding theoretical ethanol yields are 0.42 and 0.45, respectively. The actual ethanol is much lower for the strain of *C. thermocellum* employed in our studies; ethanol selectivity is addressed below.

Table 2
Substrate Partitioning for *C. thermocellum* and *S. cerevisiae*

	C. thermocellum[a]	*S. cerevisiae*
Cell yield		
$Y_{X/S}$ (g cells/g substrate)	0.10–0.12	0.09–0.11[b]
Cellulase mass yield		
$Y_{P/S}$ (g protein/g substrate)	0.03–0.05	Not applicable
$Y_{X/S} + Y_{P/S}$	0.15	~0.1
Ethanol yield		
$Y_{E/S}$ (g ethanol/g substrate)		
Actual	0.24	0.42[c]
Theoretical[d]	0.42	0.45

[a]All data for *C. thermocellum* from Johnson et al. (*20*) and this study for a batch culture grown on Avicel; parameters are calculated relative to substrate expressed in soluble glucose equivalent for the purposes of comparison to yeast.
[b]Data from (*36–39*).
[c]Based on the selectivity reported in (*40*) and the theoretical yield as calculated below.
[d]Based on a carbon balance assuming that cells and protein are 46 and 56% carbon by mass, respectively (*41,42*), assuming equimolar production of ethanol and CO_2 from the mass of substrate carbon remaining allowing for cell and enzyme production.

Given the dissimilar conditions under which the parameters in Table 2 were measured, bioenergetics may provide a valuable theoretical perspective in considering substrate partitioning. Cells generate ATP via catabolism that may be used for synthesis of either cells or cellulase. The ATP requirements and thermodynamic efficiency for synthesis of cells and cellular protein are similar for many fermentative chemoheterotrophs (*33, 34*). Thus, regardless of whether cellulase is made or not, the combined yield of cells and cellulase should vary little for organisms producing ethanol with a specified ATP yield (for example, that associated with the Embden-Meyerhoff pathway). The potential ethanol yield is proportional to the total substrate metabolized less the substrate incorporated into cells and macromolecules. These considerations suggest that, in cases where the expectation of essentially constant combined yield of cells and cellulase is realized, the yield of ethanol would thus be similarly unvarying.

The substrate partitioning data presented in Table 2 are consistent with a diversion of substrate to enzyme production on the order of 4%. For economics associated with state-of-the-art processes, this substrate diversion represents a cost of 5¢/gal. The data in Table 2 imply a somewhat greater proportion of substrate material incorporated into macromolecules in *C. thermocellum* as compared to *S. cerevisiae*. This discrepancy may be owing to the greater availability of ATP accompanying acetate production relative to ethanol (*35*) and may be eliminated if high ethanol yields are achieved by *C. thermocellum*.

In summary, the limited data available are consistent with production of cellulase of *C. thermocellum* being free with respect to cost categories 1 and 2, and to involve costs on the order of 5¢/gal for category 3.

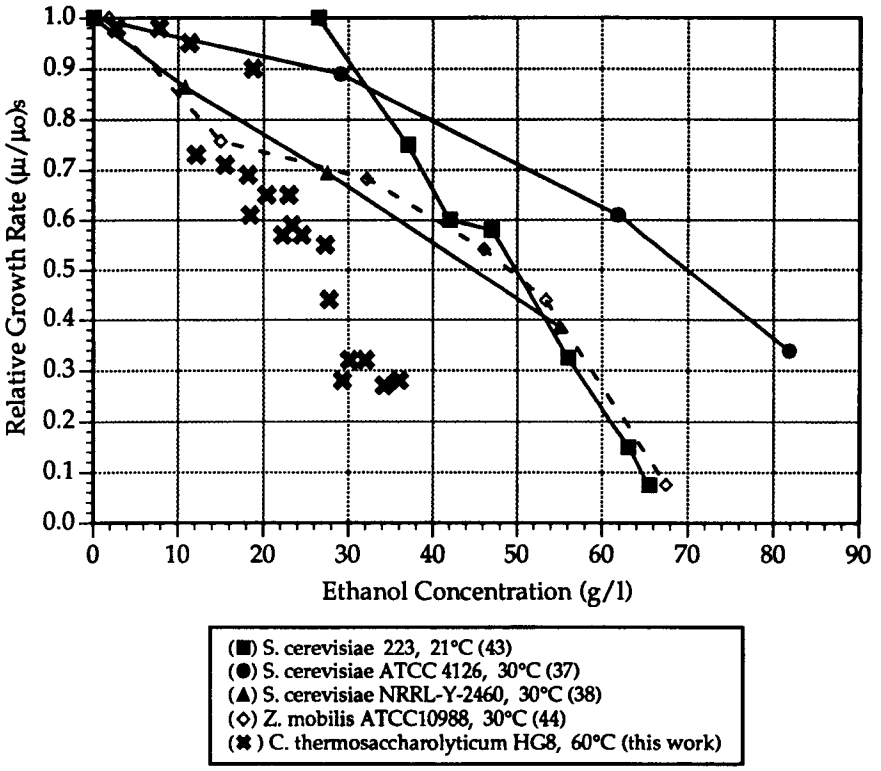

Fig. 2. Comparative ethanol tolerance. All data is for steady-state continuous cultures in the presence of predominately exogenously added ethanol.

Ethanol Tolerance

Ethanol tolerance has traditionally been considered a liability of Path 1 organisms relative to Path 2 organisms. Tolerance for the potential Path 1 organisms *S. cerevisiae* and *Z. mobilis,* considered in comparison to tolerance in a Path 2 organism below, has been extensively documented. However, description and understanding of ethanol tolerance for Path 2 organisms are much more preliminary. For example, the ethanol concentrations bringing about 50% growth rate reduction have been tabulated for several strains of thermophilic bacteria grown in batch and fed-batch culture (*18*). Values for this parameter vary from 4 to 100 g/L. This variability is taken to be indicative of the different bases for measurement (e.g, the extent or rate of growth), the ability of strains to adapt to high ethanol concentrations, and the tentative state of knowledge. With these factors in mind, we have begun a systematic study of ethanol tolerance for thermophilic bacteria and enzymes.

One measure of ethanol tolerance is the degree of ethanol-associated growth rate inhibition for utilization of soluble substrates. Figure 2 presents the ratio of the growth rate in the presence of ethanol relative to that in the absence of ethanol at constant substrate concentration $(\mu_i/\mu_o)_s$ as a

Table 3
Growth Inhibition by Ethanol
for *C. thermosaccharolyticum, S. cerevisiae,* and *Z. mobilis*

Organism	Maximum ethanol concentration for growth (g/L)			
	Topt[a]	Ref.	Topt—7°C	Ref.
C. thermosaccharolyticum	52[b]	This work	Unknown	
S. cerevisiae	55[c]	(*49*)	69–94[b,c]	(*37,38,49*)
Z. mobilis	55[c]	(*48*)	72–86[b,c]	(*44,48,53*)

[a]Topt refers to the optimum growth temperature in the absence of ethanol.
[b]Data from continuous culture.
[c]Data from batch experiments.

function of ethanol concentration. Data is presented for *C. thermosaccharolyticum,* and also for *Z. mobilis* and *S. cerevisiae,* with the incubation temperature indicated (*37,38,43,44*). All data shown were obtained in continuous culture with most of the ethanol present supplied exogenously. The specific growth rate decreases approximately linearly for *C. thermosaccharolyticum,* with 50% inhibition at 26 g/L and complete inhibition at 52 g/L. For the yeast and *Z. mobilis,* 50% inhibition is observed at an ethanol concentration of approx 50 g/L.

The growth temperature has been shown to be an important modulator of ethanol inhibition in both thermophilic (*45,46*) and mesophilic (*47–51*) organisms, with higher temperature associated with reduced ethanol tolerance in all organisms examined. Table 3 presents tolerance data at various temperatures for *S. cerevisiae, Z. mobilis,* and *C. thermosaccharolyticum.* Tolerance at the temperature optimal for growth in the absence of ethanol appears similar for all three organisms. For both *S. cerevisiae* and *Z. mobilis,* tolerance is roughly doubled at temperatures 5–7°C below the optimal growth temperature in the absence of ethanol. If a similar trend were observed for *C. thermosaccharolyticum,* the ethanol tolerance would be comparable to that in *S. cerevisiae* and *Z. mobilis.* Studies at suboptimal growth temperatures are under way in our laboratory.

Rates of soluble substrate utilization are typically three or more times faster than rates of cellulose hydrolysis by the same organism. Thus, the rate-limiting step for ethanol production from cellulosic biomass is hydrolysis. Although less widely employed than inhibition of the growth rate on soluble substrates, the degree of inhibition of cellulase activity is a second relevant measure of ethanol tolerance in the context of processes for cellulose conversion. Figure 3 presents ethanol inhibition data for *C. thermocellum* cellulase. The inhibition trend is approximately linear with 50% inhibition at 80 g/L ethanol.

Ethanol Selectivity

High ethanol selectivity, the molar ratio of the rate of ethanol production to the sum of the rates of production of all other products, is the

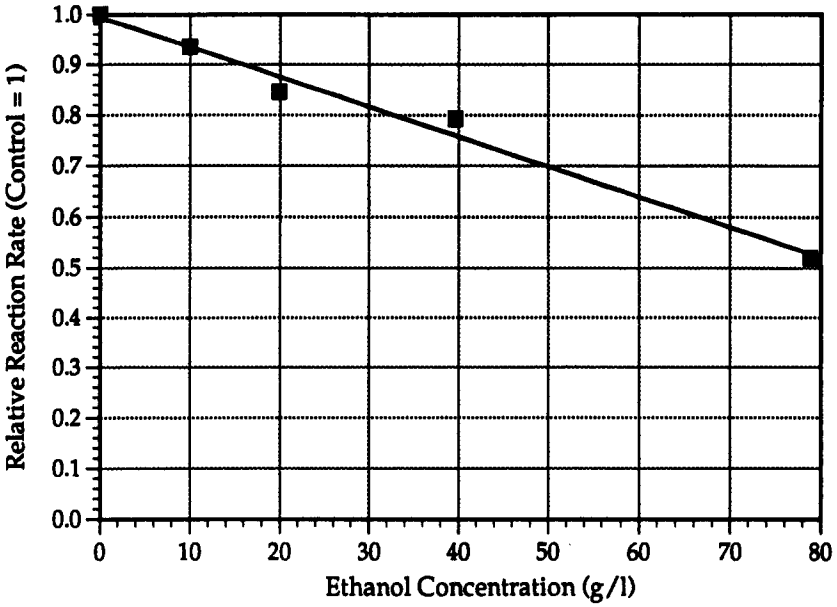

Fig. 3. Ethanol tolerance for *C. thermocellum* avicelase activity. Results are for initial rates of hydrolysis of 0.6 g/L Avicel.

primary asset of Path 1 organisms as agents of ethanol production. Based on the detailed study of byproduct formation performed by Maiorella et al. (*40*), ethanol selectivity in *S. cerevisiae* is 12.5. Data of Jobses and Roels (*44*) indicate that only minor amounts of byproducts are formed by *Z. mobilis*. Ethanol yields of 0.47–0.48 are possible with *Z. mobilis*, primarily owing to the low cell yields of this organism (*52,53*).

Obtaining consistently high ethanol selectivity under conditions of interest in a process context is the primary obstacle to development of DMC processes using Path 2 organisms. Very high selectivity has been observed in a number of thermophilic organisms (*54–57*). However, lower yields have also been observed with identical strains (*58–63*). High-yield fermentation appears to be a notoriously variable characteristic with lower yields attributable to both instability of mutant strains and dependence on culture conditions. Ethanol selectivity values of 10 or more have been reproducibly obtained by our group during transients for continuously grown cultures of *C. thermosaccharolyticum* (*18*). Studies aimed at obtaining similarly high values at steady state are under way. *Fusarium oxysporum* produces ethanol at nearly 90% of theoretical yield from cellulose at 20 g/L, but the yield has been observed to fall to 53% of theoretical with 50 g/L feeds (*5*).

Work over the past decade has offered many encouraging instances of high ethanol yields in Path 2 organisms, and progress toward an understanding of the metabolic basis of yield control has been considerable. Nevertheless, further understanding and/or strain development is clearly necessary in order to make DMC processing based on such organisms competitive with fossil fuel at prices envisioned during the next decade.

SUMMARY AND CONCLUSIONS

Of the two paths for developing DMC systems, each currently has a distinct advantage and a distinct disadvantage relative to the other. Path 1 organisms realize high selectivity, but are not as yet sufficiently good cellulase producers. Path 2 organisms include some that produce plentiful cellulase having little or no cost, but high ethanol selectivity has not been consistently achieved. Furthermore, very few studies of Path 2 organisms have been undertaken under conditions that would be realistic in a process context: high feed and product concentrations, economic growth medium composition, and the presence of possibly inhibitory products of substrate pretreatment.

For Path 1, the central challenge is the very significant task of high-level expression and excretion of cellulase. For Path 2, the selectivity issue must be resolved, and much work needs to be done at realistic process conditions. It is notable that most of the characteristics of a practical ethanol production process have been realized in Path 2 systems, but not simultaneously. These characteristics include high ethanol selectivity (*5,18*), fermentation at acceptably high substrate and ethanol concentrations (*18*), and *in situ* cellulose hydrolysis at high rates and yields (*21*).

The analysis presented herein indicates that the economic benefit associated with free cellulase production is approx 50¢/gal. The data available indicate that the value of cellulase production by *C. thermocellum* corresponds to 90% of this amount. Further studies on this and other Path 2 organisms need to be carried out to test the generality of this result. Process analyses for ethanol production from cellulose have often stressed the importance of ethanol yield improvements for reducing production costs (*23,24,64*). However, the cost reductions corresponding to improving ethanol yields of state-of-the-art processes to theoretical are of comparable magnitude to the cost reductions projected for free cellulase production.

Experimental results for ethanol tolerance of *C. thermosaccharolyticum* indicate that tolerance may be greater than has generally been assumed. Experimentation in continuous culture, which allows repeated adaptation over time periods spanning several days (*19*), may be one factor in explaining the relatively high tolerance reported here. As with cellulase production, further work is necessary at other conditions (e.g., growth temperatures) and with other organisms. In addition to these preliminary data, a number of factors indicate that ethanol tolerance is not likely to be a primary factor in choosing between DMC and other ethanol process alternatives. Such factors include the availability of energy-efficient separation technologies (*64*), the favorable process energy balance made possible by burning process residues (*1*), and the relatively modest ethanol concentrations in current wood-based process designs (*23,24*).

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REFERENCES

1. Lynd, L. R., Cushman, J. H., Nichols, R. J., and Wyman, C. E. (1991), *Science* **251,** 1318–1323.
2. Hinman, N. D., Wright, J. D., Hoagland, W., and Wyman, C. E. (1989), *Appl. Biochem. Biotechnol.* **20/21,** 391–401.
3. Veldhuis, M. K., Christensen, L. M., and Fulmer, E. I. (1933), *Ind. Eng. Chem.* **28,** 430.
4. Gong, G.-S., Maun, C. M., and Tsao, G. T. (1981), *Biotechnol. Lett.* **3(2),** 77–82.
5. Christakopoulos, P., Macris, B. J., and Ketos, D. (1989), *Enz. and Microbial Technol.* **11,** 236–239.
6. Avgerinos, G. C: and Wang, D. I. C. (1980), *Ann. Rep. Ferm. Processes* **4,** 165–191.
7. Murray, W. D., Wemyss, K. B., and Kahn, A. W. (1983), *Eur. J. Appl. Microbiol. Biotechnol.* **18,** 71–74.
8. Ng, T. K., Weimer, P. J., and Zeikus, J. G. (1977), *Arch. Microbiol.* **114,** 1–7.
9. Wang, D. I. C., Cooney, C. L., Wang, S.-D., Gordon, J. and Wang, G. Y. (1978), *Proceedings Second Annual Symposium on Fuels from Biomass,* Shuster, W. W. ed., Rensselaer Polytechnic Institute, Troy, NY, pp. 537–570.
10. Shoemaker, S., Schweickart, V., Ladner, M., Gelfand, D., Kwok, S., Myambo, K., and Innis, M. (1983), *Bio/Technol.* **1,** 691–696.
11. Skipper, N., Sutherland, M., Davies, R. W., Kilburn, D., Miller, R. C., Warren, A., and Wong, R. (1985), *Science* **230,** 958–960.
12. Rogers, P. (1986), *Advances in Applied Microbiology,* vol. 31, Laskin, A. I. ed., Academic, New York, pp. 1–59.
13. Lynd, L. R. (1989), *Advances in Biochemical Engineering/Biotechnology,* vol. 38, Fiechter, A. ed., Springer-Verlag, New York, pp. 1–52.
14. Slapack, G. E., Russell, I., and Stewart, G. G. (1987), *Thermophilic Bacteria and Thermotolerant Yeasts for Ethanol Production,* CRC, Boca Raton, FL.
15. Wang, D. I. C., Avgerinos, G. C., Biocic, I., Fang, S. D., and Fang, H. Y. (1983), *Philos. Trans. R. Soc. London* **B300,** 323–333.
16. Wiegel, J. and Ljundahl, L. G. (1986), *CRC Crit. Rev. Biotechnol.* **3(1),** 39–108.
17. Bisaria, V. S. and Mishra, S. (1989), *CRC Crit. Rev. Biotechnol.* **9(2),** 61–103.
18. Lynd, L. R., Ahn, H.-J., Anderson, G., Hill, P., Kersey, D. S., and Klapatch, T. (1991), *Appl. Biochem. Biotechnol.* **28/29,** 549–570.
19. Ahn, H.-J. (1991), M.S. Thesis, Dartmouth College, Hanover, NH.
20. Johnson, E. A., Sakajoh, M., Halliwell, G., Madia, A., and Demain, A. L. (1982), *Appl. Env. Microbiol.* **43,** 1125–1132.

21. Lynd, L. R., Grethlein, H. E., and Wolkin, R. W. (1989), *Appl. Env. Microbiol.* **55,** 3131–3139.
22. Lynd, L. R. and Grethlein, H. E. (1987), *Biotechnol. Bioeng.* **29,** 92–100.
23. Wright, J. D. (1988), *Energy Prog.* **8(2),** 71–78.
24. Wright, J. D. (1988), *Chem. Eng. Prog.* **84(8),** 62–74.
25. Grethlein, H.E., Allen, D. C., and Converse, A. O. (1984), *Biotechnol. Bioeng.* **26,** 1498–1505.
26. Beguin, P. (1990), *Annu. Rev. Microbiol.* **44,** 219–248.
27. Penttila, M. E., Lehtovaara, P., Bailey, M., Teeri, T. T., and Knowles, J. K. C. (1988), *Gene* **63,** 103–112.
28. Zurbriggen, B., Bailey, M. J., Penttila, M. E., Poutanen, K., and Linko, M. (1990), *J. Biotechnol.* **13,** 267–278.
29. Brestic-Goachet, N., Gunasekaran, P., Cami, B., and Baratti, J.-C. (1989), *J. Gen. Microbiol.* **135,** 893–902.
30. Lejuene, A., Eveleigh, D. E., and Colson, C. (1988), *FEMS Microbiol. Lett.* **49,** 363–366.
31. Lamed, R. and Bayer, E. A. (1988), *Adv. Appl. Microbiol.* **33,** 2–41.
32. Bernardez, T. D. (1990), Master of Engineering Thesis, Dartmouth College, Hanover, NH.
33. Stouthamer, A.H. (1979), *Microbial Biochemistry,* Vol. 21, Quayle, J. R. ed., University Park Press, Baltimore, pp. 1–47.
34. Ho, K. P. and Payne, W. J. (1979), *Biotechnol. Bioeng.* **21,** 787–802.
35. Thauer, R. K., Jungermann, K., and Decker, K. (1977), *Bact. Rev.* **41(1),** 100.
36. Aiba, S., Shoda, M., and Nagatani, J. (1968), *Biotechnol. Bioeng.* **10,** 845–864.
37. Bazua, C. D. and Wilke, C. R. (1977), *Biotechnol. Bioeng. Symp.* **7,** 105–118.
38. Ghose, T. K. and Tyagi, R. D. (1979), *Biotechnol. Bioeng.* **21,** 1401–1420.
39. Peringer, P., Blachere, H. Corrieu, G., and Lane, A. G. (1974), *Biotechnol. Bioeng.* **16,** 431–454.
40. Maiorella, B., Blanch, H. W., and Wilke, C. R. (1983), *Biotechnol. Bioeng.* **25,** 103–121.
41. Papoutsakis, E. T. (1984), *Biotechnol. Bioeng.* **26,** 174–187.
42. Erickson, L. E. (1980), *Biotechnol. Bioeng.* **22,** 451–456.
43. Holzberg, I., Finn, R. K., and Steinkraus, K. H. (1967), *Biotechnol. Bioeng.* **9,** 413–427.
44. Jobses, I. M. L. and Roels, J. A. (1986), *Biotechnol. Bioeng.* **28,** 554–563.
45. Herrero, A. A. and Gomez, R. F. (1980), *Appl. Environ. Microbiol.* **40(3),** 571–577.
46. Lovitt, R. W., Longin, R., and Zeikus, J. G. (1984), *Appl. Environ. Microbiol.* **48(1),** 171–177.
47. Fieschko, J. and Humphrey, A. E. (1983), *Biotechnol. Bioeng.* **25,** 1655–1660.
48. Huang, S.-Y., and Chen, J.-C. (1988), *Enz. Microb. Technol.* **20,** 431–439.
49. Sa-Correia, I. and Van Uden, N. (1983), *Biotechnol. Bioeng.* **25,** 1665–1667.
50. Van Uden, N. (1984), *Adv. Microb. Physiol.* **25,** 195–251.
51. Van Uden, N. (1985), *Annual Reports on Fermentation Processes* **8,** 11.
52. Nipkow, A., Sonnleitner, B., and Fiechter, A. (1986), *J. Biotechnol.* **4,** 35–47.
53. Rogers, P. L., Lee, K. J., Skotnicki, M. L., and Tribe, D. E. (1982), *Advances in Biochemical Engineering,* vol. 23, Fiechter, A., ed., Springer-Verlag, New York, pp. 37–84.

54. Avgerinos, G. C. and Wang, D. I. C. (1983), *Biotechnol. Bioeng.* **25,** 67–83.
55. Carreira, L. H., Weigel, J., and Ljungdahl, L. G. (1983), *Biotechnol. Bioeng. Symp.* **13,** 183–191.
56. Wang, D. I. C. and Dalal, R. (1986), Patent no. 4,568,644.
57. Zeikus, J. G., Ben-Bassat, A., and Hegge, P. J. (1980), *J. Bacteriol.* **243,** 432–444.
58. Hon-Nami, K., Coughlan, M. P., Hon-Nami, H., Carriera, L. H., and Ljungdahl, L. G. (1985), *Biotechnol. Bioeng. Symp.* **15,** 191–205.
59. Lacis, L. S. and Lawford, H. G. (1988), *Arch. Microbiol.* **150,** 48–55.
60. Lacis, L. S. and Lawford, H. G. (1989), *Biotechnol. Lett.* **10(8),** 603–608.
61. Mistry, F. and Cooney, C. L. (1989), *Biotechnol. Bioeng.* **34,** 1295–1304.
62. Slaff, G. F. and Humphrey, A. E. (1981), *Presentation at the 182nd Meeting of the ACS.*
63. Ward, P. J. and Mutharasan, R. (1986), *Presentation at the 192nd Meeting of the ACS.*
64. Lynd, L. R. (1990), *Appl. Biochem. Microbiol.* **24/25,** 695–719.