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The Effect of Acetic Acid on Fuel Ethanol Production by *Zymomonas*

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ABSTRACT

Acetic acid acts to promote uncoupling with *Zymomonas*. At pH 5, 36% of acetic acid is in the uncharged and undissociated form (HAc), which is able to permeate the plasma membrane. The transmembrane ΔpH drives the accumulation of acetic acid, which results in the acidification of the cytoplasm. The consequential increase in maintenance metabolism represents a diversion of energy that would otherwise be available for growth. At pH 5, the growth of *Z. mobilis* (ATCC 29191) was 50% inhibited with 8.3 g/L acetic acid (50 m*M* HAc) and completely inhibited by 11 g/L. Addition of 6 g/L acetic acid caused the glucose-to-ethanol conversion efficiency to decrease from 98 to 90% of theoretical maximum. The growth yield coefficient for glucose was 50% decreased by 2.3 g/L acetic acid (13.5 m*M* HAc) from 0.036 to 0.018 g cell/g glucose. However, the specific (ethanol) productivity of batch cultures was enhanced by <5 g/L acetic acid (<30 m *M* HAc). For continuous cultures, the acetic acid sensitivity depends on the growth rate (dilution rate), but an increase in specific productivity can be achieved at proportionately lower concentrations of acetic acid. At a growth rate of 0.112/h, the addition of 1.7 g/L acetic acid to the 5% glucose feed resulted in an increase in specific productivity from 2.68 to 5.87 g ethanol/g cell/h. The uncoupling effect of acetic acid could be beneficial in terms of improving the productivity in closed, continuous fermentations, such as cell recycle or immobilized cell reactors.

Index Entries: *Zymomonas;* acetic acid; fuel ethanol; energetic uncoupling; specific productivity.

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Abbreviations: D, dilution rate (h^{-1}); HAc, undissociated acetic acid; μ, specific growth rate (h^{-1}); $Y_{x/s}$, growth yield coefficient (g dry wt cells/g glucose); $Y_{p/s}$, product yield (g ethanol/g glucose); Q_p, volumetric productivity (g ethanol/L/h); q_p, specific productivity (g ethanol/g cell/h).

INTRODUCTION

In 1991, in the United States for the first time, domestic supply of fermentation ethanol exceeded demand and 100 million gallons, of the nearly 1 billion gallons produced, were exported to Brazil. Starch and sucrose comprise the major feedstocks for the fuel ethanol industry, and process modernization in the fuel alcohol industry has tended to focus on increasing productivity through the use of high-cell density, continuous-flow systems. Feedstock costs dominate the economics of fuel ethanol production, and cost-sensitivity analyses rank yield, product concentration, and productivity as the three most important technoeconomic process parameters (*1–3*). Improvements in both yield and productivity are possible with the use of alternative ethanologenic microorganisms, and the monopoly currently enjoyed by the yeast *Saccharomyces cerevisiae* is being challenged by other high-performance biocatalysts. The bacterium *Zymomonas mobilis* is generally recognized as being superior to *Saccharomyces* yeast with respect to both conversion efficiency (yield) and productivity (*4–8*). Furthermore, *Zymomonas* is just as tolerant to ethanol as yeast (*9*). Although *Zymomonas* is not currently being used industrially, both laboratory and pilot-scale operations have shown that it is capable of generating near theoretical maximum yields from several different feedstocks, including sugar cane (*10*), molasses (*11*), saccharified starch from corn (*12*), wheat (*13*), cassava, and sago (*14*), as well as an enzymatic hydrolysate of wood-derived cellulose (*15*).

Infection by lactic cultures has been reported to have a profoundly antagonistic effect on the fermentation performance of *Zymomonas* in continuous starch hydrolysate systems (*13,16*). Although the causative agent had been presumed to be lactic acid (*13*), recent observations on the effect of exogenous lactic acid with pure *Zymomonas* cultures have cast doubt on this hypothesis (*16*). Although acetic acid is only a minor metabolic by-product of lactic cultures, it has been implicated as the toxic element (*16*). Acetic acid is exploited commercially as an antimicrobial agent in the food and beverage industries (*17*). Since the undissociated acid is the active inhibitory form (*18–21*), the close proximity of the pK_a for acetic acid (4.75) and the pH optimum for *Zymomonas* support the concept that low concentrations of acetic acid could be responsible for the observed poor ethanol fermentation performance by *Zymomonas* cultures infected with lactic acid bacteria (*16*).

Since a systematic physiological analysis of the effect of acetic acid on *Zymomonas* has not been reported, the purpose of this investigation was to assess quantitatively the effect of acetic acid on the neotype strain of *Zymomonas mobilis* (ATCC 29191) in pH-controlled batch and steady-state chemostat cultures, and to compare the results to the literature relating to similar studies with yeast fermentations. This article is part of our continuing series on *Zymomonas*-based ethanol fermentations (*22–29*).

MATERIALS AND METHODS

Organism

The neotype strain of *Zymomonas mobilis* ATCC 29191 (*30*) was obtained from the American Type Culture Collection (Rockville, MD). Stock cultures and inocula were prepared as described previously (*29*). Batch cultures were inoculated at a cell density of about 50 mg dry wt cells/L.

Fermentation Media and Equipment

The synthetic glucose mineral salts medium contained 1.5 g/L yeast extract (Difco) and ammonium chloride (1.6 g/L) as sources of assimilable nitrogen. The composition with respect to inorganic salts and vitamins was as previously described by Lawford and Ruggiero (*29*). Glucose was autoclaved separately. Acetic acid was added as the potassium salt. The batch and continuous fermentations were conducted in bench-top stirred-tank bioreactors with pH and temperature control, as described previously (*6*). For chemostat cultures, steady state was assumed only after a minimum of 5-vol changes (2 d).

Analytical Procedures

Growth was followed turbidometrically at 550 nm (1-cm lightpath), and culture dry weight was measured by microfiltration—washing and drying the filter (0.45 μ) to constant weight under an infrared heat lamp. Compositional analyses (glucose and ethanol) of fermentation media and cell-free spent media were determined using an HPLC equipped with an RI monitor and computer-interfaced controller/integrator (Bio-Rad Labs). Separations were performed at 65°C on an HPX-87H column (Bio-Rad); the injection vol was 20 μL.

Determination of Fermentation Parameters

In batch fermentations, the volumetric productivity (Q_p) was determined by dividing the final ethanol concentration by the time required to achieve complete glucose utilization. The Q_p^{max} was estimated as the maximum slope in plots of ethanol concentration vs elapsed fermentation time. The specific productivity (q_p) was calculated by dividing the value

estimated for Q_p by one-half the maximum biomass concentration, and the q_p^{max} was similarly calculated by substituting the value of Q_p^{max} for Q_p. In steady-state, carbon-limited continuous cultures, the Q_p is the product of the ethanol concentration and the dilution rate (D), and q_p is determined by dividing Q_p by the biomass concentration.

Terminology Relating to Acetic Acid

Acetic acid (HAc) is a weak acid with a pK_a of 4.75 and dissociates in a pH-dependent manner into two oppositely charged species, a negatively charged ion called "acetate" (Ac^-) and a proton (H^+). At a specified pH value, the relative concentrations of the dissociated and undissociated species are given by the Henderson-Hasselbalch equation:

$$pH = pK_a + \log_{10}[Ac^-]/[HAc] \quad (1)$$

For practical purposes, the acetic acid content of the fermentation medium refers to the total mass of the acid, and does not distinguish between the separate amounts of dissociated and undissociated forms. Certain ambiguity arises when the term "acetate" is also used synonymously with "acetic acid," because acetate is properly meant to refer specifically to the dissociated anion. In the context of this study, acetic acid refers to the total amount of acid, and the undissociated (protonated) form will be represented by "HAc." At pH 5, 36% of the acid is in the undissociated form.

RESULTS

Figure 1 illustrates the growth of *Z. mobilis* (ATCC 29191) in a synthetic glucose salts medium with the pH controlled at 5.0. Addition of 2.0 g/L potassium acetate (equivalent to 1.22 g/L "total" acetic acid) results in an accommodation period ("lag" time) of about 2 h before growth commences (Fig. 1). The maximum specific growth rate (μ_{max}) is decreased from 0.42 h^{-1} to 0.34 h^{-1} by 1.22 g/L acetic acid (Fig. 1). The growth yield coefficient ($Y_{x/s}$) is decreased from 0.036 to 0.026 g dry wt cells/g glu (Fig. 1). The effect of acetic acid on μ_{max} and $Y_{x/s}$ is shown in Figs. 2 and 3, respectively. At concentrations of acetic acid >11 g/L (at pH 5), there was no growth observed (results not shown). The dose response is not linear for either growth rate or yield (Figs. 2 and 3), and the general shape of these plots is similar to the titration of a weak acid. The dose response is relatively flat in the range 2–8 g/L acetic acid (Figs. 2 and 3). Whereas the growth rate is 50% inhibited in the presence of 8.3 g/L acetic acid (50 m*M* HAc) (Fig. 2), the growth yield is 50% inhibited with only 2.3 g/L acetic acid (13.8 m*M* HAc) (Fig. 3).

Figure 4 illustrates the effect of acetic acid on glucose catabolism by *Z. mobilis*. The fermentation profiles shown in Fig. 4 correspond to the growth experiments shown in Fig. 1. From similar plots of the production

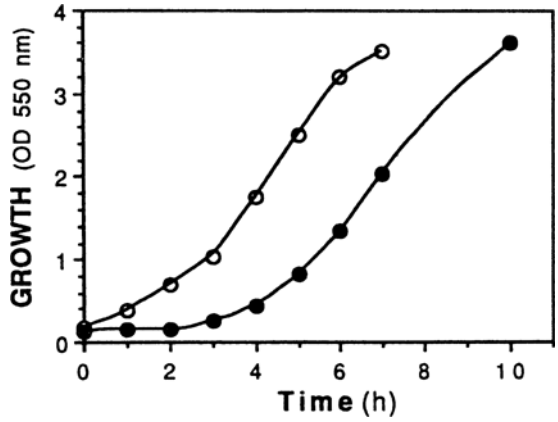

Fig. 1. Effect of acetic acid on the growth of *Z. mobilis* ATCC 29191 at pH 5. Growth was followed turbidometrically. Symbols: (○) synthetic salts medium with 35 g/L glucose; (●) 2 g/L potassium acetate added and 36 g/L glucose.

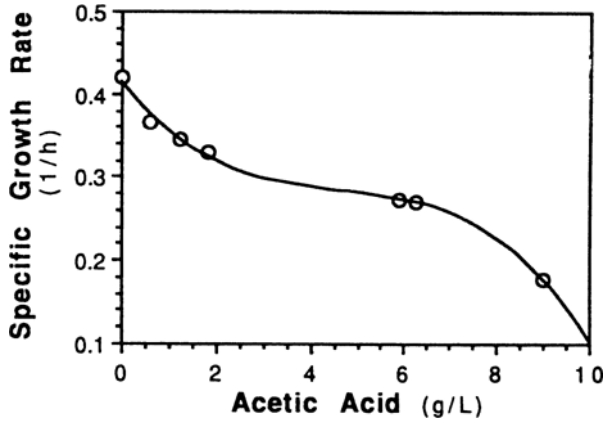

Fig. 2. Effect of acetic acid on the maximum specific growth rate of *Z. mobilis*. The synthetic salts medium contained about 35 g/L glucose, and the amount of total acetic acid is indicated. The pH of the batch culture was controlled at 5.0 by the automatic addition of 2*N* KOH.

of ethanol vs elapsed fermentation time using media containing different amounts of added acetic acid, the effect of acetic acid on volumetric productivity was determined, and the results are summarized in Fig. 5. At pH 5, the growth rate and volumetric productivity profiles are surprisingly similar with 50% inhibition of both μ_{max} and Q_p (and Q_p^{max}) by 8.3 g/L acetic acid (50 m*M* HAc) (Fig. 5). However, because of the differential effect of acetic acid on growth relative to glucose catabolism (productivity), the shape of the specific productivity (q_p) profile is quite different (Fig. 6). The values for q_p and q_p^{max} increase with increasing amounts of added acetic acid up to about 6 g/L after which there is a decline (Fig. 6).

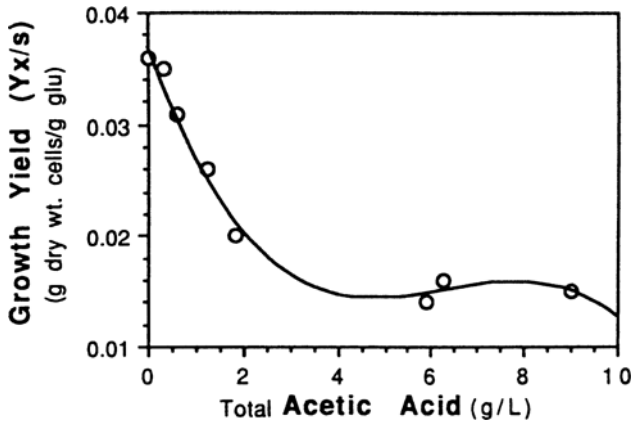

Fig. 3. Effect of acetic acid on the growth yield coefficient for glucose. Conditions were as Fig. 2. Determination of $Y_{x/s}$ is described in Materials and Methods.

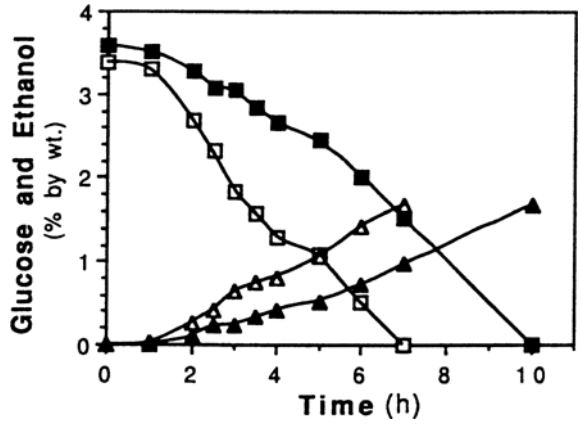

Fig. 4. Effect of acetic acid on the glucose metabolism by Z. *mobilis* at pH 5. Conditions were as described in Fig. 1. Symbols: (□) control, glucose utilization; (■) glucose utilization with 2 g/L potassium acetate added; (Δ) control, ethanol production; (▲) ethanol production with 2 g/L potassium acetate added to the medium.

The minimum inhibitory concentration (MIC) with respect to the specific productivity of *Zymomonas* (at pH 5) appears to be 8.3 g/L total acetic acid, since at lesser concentrations there is a stimulation of the specific catabolic activity (Fig. 6).

Of perhaps the most practical importance is the ability of *Zymomonas* to continue to exhibit high glucose-to-ethanol conversion efficiencies even in the presence of relatively high concentrations of acetic acid (Fig. 7—note the expanded y axis). The ethanol yield ($Y_{p/s}$) decreases from 0.49 to 0.46 g/g in the presence of 6 g/L acetic acid, but this corresponds to a decrease in conversion efficiency of only 8% of theoretical maximum (Fig. 7 and Table 1).

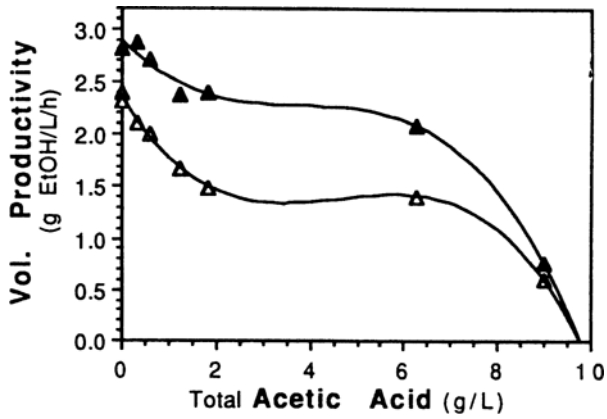

Fig. 5. Effect of acetic acid on volumetric productivity. Conditions were as for Fig. 2. Symbols: (Δ) average volumetric productivity; (▲) maximum volumetric productivity. Method for determining these parameters is described in Materials and Methods.

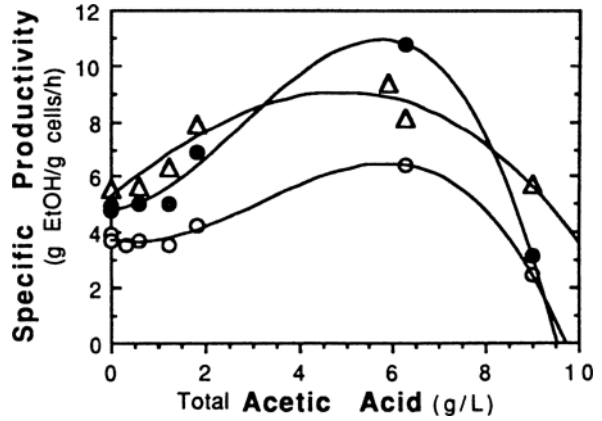

Fig. 6. Effect of acetic acid on specific productivity. Conditions were as for Fig. 2. Symbols: (○) average specific productivity; (●) maximum specific productivity. Methods for determining these parameters is described in Materials and Methods. (Δ) alternative method used to calculate q_p^{max} involving μ_{max}, $Y_{x/s}$, and $Y_{p/s}$ (*see text* for details).

The effect of adding acetic acid to the medium feed reservoir of a glucose-limited chemostat culture of *Z. mobilis* (pH 5) is summarized in Table 1. The dilution rate was 0.112 h^{-1} (about 25% μ_{max}), and the glucose concentration in the feed was 50 g/L. This dilution rate was chosen, because it was similar to the growth rate at which infection by lactic acid bacteria had been observed to cause continuous culture instability that was manifested by a decrease in yield and productivity (*16*). Experiments with model systems and exogenous lactic acid indicated that lactic acid *per se* was not the causative agent, but did not rule out the possible involvement of acetic acid, which is a minor metabolic byproduct of lactic acid bacteria.

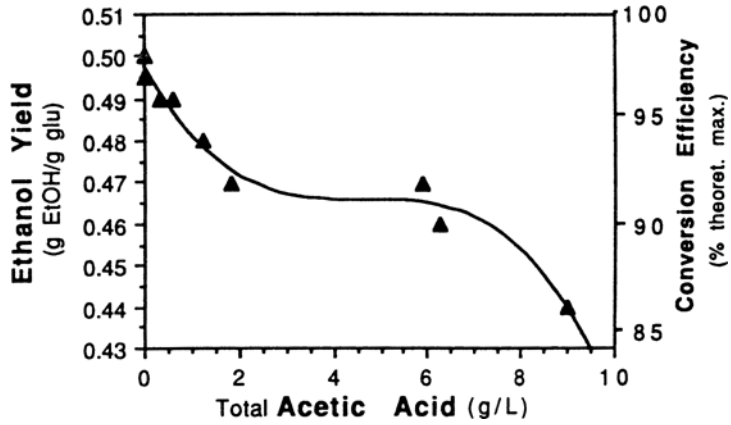

Fig. 7. Effect of acetic acid on ethanol yield. Conditions were as for Fig. 2.

Table 1
Comparative Effect of Acetic Acid
on *Saccharomyces cerevisiae* and *Zymomonas mobilis*

Acetic acid (total acid) g/L	Undissociated, HAc, m*M*	$Y_{x/s}$ g cell/g glu	$Y_{p/s}$ g EtOH/g glu	Conversion efficiency, %	Specific productivity, g EtOH/g cell/h
S. cerevisiae[a] (pH 4)					
Continuous culture (D = 0.15/h)					
0	0	0.120	0.390	77	0.49
3	42.3	0.050	0.490	96	1.47
6	84.6	0.045	0.385	76	1.28
Z. mobilis (pH 5)					
Batch culture					
0	0	0.036	0.49	98	4.0
6	36	0.016	0.46	90	6.4
Continuous culture (D = 0.112/h)					
0	0	0.020	0.48	95	2.68
1.7	10.2	0.009	0.48	94	5.87

[a]Data for *S. cerevisiae* taken from Maiorella et al. (1983, [45]) (*see also* Vega et al., 1987 [46]).

At a concentration of 1.7 g/L acetic acid, the growth yield of *Z. mobilis* (μ = 0.112/h) decreases about 50%, and because the utilization of glucose continues to be complete, the specific productivity of the culture doubles in value from 2.68 to 5.87 g ethanol/L/h (Table 1). The product yield (0.48 g/g) is not affected by this amount of acetic acid (Table 1).

DISCUSSION

Acetic acid is used in the food industry as a preservative (*17*), and its efficacy is based on its antimicrobial activity. The toxicity of acetic acid for both yeast and bacteria is pH dependent (*19,21,31–33*). By virtue of its ability to traverse the cell membrane freely, the protonated species (i.e., undissociated acid) acts as a protonophore and causes its inhibitory effect by bringing about the acidification of the cytoplasm, thereby collapsing the transmembrane pH gradient and destroying the homeostasis with respect to the intracellular pH (*18–20,34*). The intracellular pH for *Zymomonas* has been estimated at 5.4 (*35*). Hence, with the external pH controlled at 5.0, there is a transmembrane pH differential (ΔpH) of 0.4 U, and the proton gradient (higher concentration outside) drives the passive diffusion of protons into the cytoplasm. The rate of proton entry into the cell is a function not only of the transmembrane ΔpH, but also the proton permeability of the plasma membrane (*21,36*). However, the influx of protons into the cytoplasm is assisted by the permeant undissociated HAc, which accumulates in the more alkaline compartment (cytoplasm) in proportion to ΔpH (*34*). Therefore, to prevent acidification of the cytoplasm, the cell must divert more energy to maintenance and less to growth. In this way, the undissociated form of acetic acid acts to "uncouple" anabolism (growth) from catabolism (generation of ATP from glucose metabolism). Energetic uncoupling is manifested by a decrease in growth rate and yield that is dependent of the rate of catabolism (*25*). However, from the foregoing, it is apparent that the degree of "uncoupling" caused by HAc depends on a rather complex interaction between several different variables:

1. Membrane permeability;
2. ΔpH; and
3. The concentration of extracellular HAc.

The uncoupling effect of acetic acid has been observed with yeast (*36,37*) and bacteria (*38*). At pH 7, anaerobic growth of *E. coli* (strain K12S), with glucose as carbon source, was 50% inhibited by 0.7 g/L acetic acid (*19,39*) whereas an ethanologenic recombinant *E. coli* B exhibited increased acetic acid tolerance, with 50% inhibition of growth (linear) requiring about 10 times more acetic acid (*21*). Since growth of *Zymomonas* was 50% inhibited by about the same amount of acetic acid (8 g/L), it appears that these two different ethanologens have similar tolerances to acetic acid at their respective pH values of 7 and 5. Although the active inhibitory agent is the protonated, undissociated form of acetic acid, comparisons made in terms of the HAc concentration can be misleading because of differences in the optimal pH for growth, as well as differences in the intracellular pH. This

is exemplified by comparing the effect of acetic acid on the anaerobic growth of *E. coli* K12S (*39*) and *Z. mobilis*, in which 50% inhibition was achieved with 0.064 and 50 m*M*, respectively.

The maximum specific rate of glucose utilization (q_s^{max}) is related to the specific growth rate (μ_{max}) and the growth yield coefficient ($Y_{x/s}$) by the equation shown below:

$$q_s^{max} = \mu_{max}/Y_{x/s} + m_e \quad (2)$$

where m_e represents the maintenance energy coefficient (*40*). At pH 5, the value of m_e for *Z. mobilis* (ATCC 29191) has been reported to be 3.16 g glu/g cell/h (equivalent to 1.52 g EtOH/g cell/h for $Y_{p/s}=0.48$ g/g) (*29*). In this study, an estimate for the value for m_e (in terms of product formed rather than substrate utilized) was extrapolated as the y-axis value in a plot of q_p vs D and, by linear regression analysis, found to have a value of 1.4 g EtOH/g cell/h (results not shown). However, the effect of HAc on maintenance metabolism is not known, but remains as part of our ongoing investigation. For this reason, we have chosen to neglect the contribution of m_e in our calculation of q_p^{max}. Consequently, if the contribution from the maintenance energy metabolism is neglected, then the value for the maximum specific productivity can be calculated as a function of the growth rate and growth yield, as follows:

$$q_p^{max} = Y_{p/s}\,(\mu_{max}/Y_{x/s}) \quad (3)$$

where $Y_{p/s}$ is the product (ethanol) yield. When the q_p^{max} was calculated according to this relationship, the values were in good agreement with the estimates made for the specific productivity based on the volumetric productivity and the average biomass concentration (as 50% final concentration) (Fig. 6). By this method of calculating the specific productivity (data fit by a third-order polynomial), the maximal value of 9 g EtOH/g cell/h is achieved with 5 g/L acetic acid (Fig. 6).

Using a microcalorimetric technique, the q_s^{max} for uncoupled *Z. mobilis* has been estimated to be 0.99 mmol glu/g cell/min (equivalent to 10.7 g glu/g cell/h) (*41,42*). If the $Y_{p/s}$ is 0.48 g/g, then this corresponds to a q_p^{max} of 5.14 g EtOH/g cell/h. However, a patented strain of *Z. mobilis* ZM4 (ATCC 31823) is claimed to have a specific productivity of "at least" 5.4 g EtOH/g cell/h (*7,43*) although an independent study failed to corroborate the claims regarding this culture's superior productivity characteristics (*26*). Cromie and Doelle (*44*) observed q_p^{max} values for *Zymomonas* as high as 15 g EtOH/g cell/h. Energetic uncoupling of *Zymomonas* has been accomplished through various nutritional limitations (including nitrogen, phosphate, and potassium) with about a 1.6-fold increase in specific productivity (*4,27*).

Maiorella et al. (*45*) have studied the effect of acetic acid on continuous ethanol fermentations using *Saccharomyces cerevisiae*, and for comparative purposes, the results are summarized in Table 1. At pH 4 and the dilution rate fixed at 0.15/h, a concentration of 3 g/L acetic acid caused

about a 60% reduction in growth yield and a three-fold increase in specific productivity, but from the perspective of economic importance to fuel ethanol production, the dramatic improvement in ethanol yield is more impressive, from 77 to 96% of the theoretical maximum glucose-to-ethanol conversion efficiency (Table 1). With twice the concentration of acetic acid (6 g/L), the conversion efficiency reverts to the lesser value of 76%, which is characteristic of the control culture without acetic acid (Table 1). It has been suggested that this uncoupling effect of acetic acid on yeast could be commercially exploited in "closed" continuous ethanol fermentations (e.g., cell-recycle system or immobilized cell reactor), because it has been shown that the addition of 3 g/L acetic acid to the feed results in a 50% improvement in productivity and a 12% increase in yield (*46*).

The results of our preliminary investigation on the uncoupling effect of acetic acid on a steady-state glucose-limited continuous culture of *Z. mobilis* are similar to those reported for yeast by Maiorella et al. (*45*) (Table 1). At a specific growth rate of 0.112/h (25% μ_{max}), the growth yield of the *Z. mobilis* chemostat culture is 50% inhibited by the addition of 1.7 g/L acetic acid to the fermentor feed, and this amount of acetic acid is about 25% of that required to produce the same effect in a batch culture operating at μ_{max} (Table 1). Although acetic acid toxicity was assessed at a pH value near the growth optimum for both *S. cerevisiae* and *Z. mobilis*, the differences in sensitivity to acetic acid can be explained, at least in part, by the different external pH values. Because acetic acid is 85% undissociated at pH 4 compared to 36% at pH 5, its toxicity can be expected to be more potent at the more acid pH value.

From this study, it can be concluded that acetic acid acts to promote energetic uncoupling with *Zymomonas*. At pH 5, growth was completely inhibited by acetic acid at 11 g/L; however, the specific productivity of batch cultures is enhanced by HAc concentrations <30mM (<5 g/L acetic acid). For continuous cultures, the acetic acid sensitivity depends on the growth rate (dilution rate), but an increase in specific productivity can be achieved at proportionally lower concentrations of acetic acid. It was estimated that the concentration of acetic acid produced by a contaminating lactic culture was in the range 0.3 to 0.6 g/L (*16*). The present results indicate that *Zymomonas* ($\mu=0.112$/h) can withstand concentrations of acetic acid (at pH 5) that are about threefold higher without the continuous flow bioreactor suffering "wash out," and it seems therefore unlikely that acetic acid is the sole causative agent of the perturbation observed following infection by lactic acid bacteria (*13,16*).

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