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The pH-Dependent Energetic Uncoupling of *Zymomonas* by Acetic Acid

Scientific Note

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

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

INTRODUCTION

Feedstock costs dominate the economics of fuel ethanol production and cost sensitivity analyses rank yield, product concentration, and productivity as the three most important techno-economic process parameters (*1–3*). Process modernization in the fuel alcohol industry has tended to focus on increasing productivity through the use of continuous, high-density, yeast fermentations (*4–6*). Although *Saccharomyces* yeast currently enjoys a monopoly as the fermentation process biocatalyst in the fuel ethanol industry (*4*), it is not the only ethanol producing microorganism. The bacterium *Zymomonas mobilis* is an ethanologen with comparable ethanol tolerance (*7*) that offers an opportunity for process improvement by virtue of its superior fermentation performance characteristics with respect to both conversion efficiency (yield) and productivity (*8–12*). Although the authors have no knowledge of *Zymomonas* currently being used in the fuel ethanol industry, both laboratory and pilot-scale operations have

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shown that it is capable of generating near theoretical maximum yields from several different feedstocks, including sugar cane (*13*), molasses (*14*), saccharified starch from corn (*15*), wheat (*16*), cassava and sago (*17*), as well as an enzymatic hydrolysate of wood-derived cellulose (*18*).

Acetic acid is normally regarded an an effective antimicrobioal agent and is used as such in both the food and beverage industries (*19*). Lignocellulosic biomass and wastes are targeted as an attractive alternative fermentation feedstock to starch and sugar for the production of fuel ethanol (*20*). The cellulose component of woody biomass is strongly resistant to depolymerization unless it is "pretreated" to remove the impediments to enzymic digestion that are caused by lignin and the acetylated pentosan comprising the hemicellulose fraction of biomass (*21,22*). Thermochemical depolymerization of the hemicellulose fraction is efficient and cost effective, but results in the production of potentially toxic byproducts such as acetic acid, furfural and lignin-derived phenolic compounds (*22,23*).

Our interest in the effect of acetic acid on *Zymomonas* grew out of studies on the effect of lactic culture contamination (*16,24*) as well as our appreciation of the problems caused by its presence in hemicellulose hydrolysates (*22*). Previously, we reported some initial observations in connection with our physiological analysis of the effect of acetic acid on *Zymomonas* at pH 5 (*25*). The toxicity of acetic acid derives from the permeability of the plasma membrane to the undissociated form of the acid (HAc) (*26*). The transmembrane ΔpH drives the accumulation of HAc, which results in the acidication of the cytoplasm (*23*). The consequential increase in maintenance metabolism that is required for homeostasis of cytoplasmic pH, represnts a diversion of energy that would otherwise be available for growth (*23,25*). Although 10 g/L of acetic acid at pH 5.0 causes complete inhibition of growth and metabolism, at lesser concentrations, there can be as much as a 100% increase in specific productivity (*25*). It was previously concluded that HAc acts in a dose-dependent manner to promote "energetic uncoupling" of *Zymomonas* (*25*). Since the dissociation constant for acetic acid (pK_a = 4.75) is close to the pH that has routinely been used in studying *Z. mobilis* fermentations, it was of particular interest to examine the pH-dependent nature of the effect of acetic acid with a view to defining the quantitative relationship between pH and the amount of acetic acid that is effective in promoting an increase in the specific productivity of *Zymomonas*.

MATERIALS AND METHODS

Organism

The neotype strain of *Zymomonas mobilis* ATCC 29191 (*27*) was obtained from the American Type Culture Collection (Rockville, MD). Stock cul-

tures and inocula were prepared as described previously (*28*). 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 glucose concentration was within the range of 35–50 g/L. The composition with respect to inorganic salts and vitamins was as previously described by Lawford et al. (*28*). Glucose was autoclaved separately. When potassium acetate (KAc) was added to the medium, the amount of "acetic acid" was calculated as the mass of KAc × 0.612. Batch fermmentations were conducted in a volume in 1500 mL in MultiGen™ (model F2000) stirred-tank bioreactors (New Brunswick Scientific Co., Edison, NJ) fitted with pH and temperature control (30°C) as described previously (*10*).

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 infra-red heatlamp. Compositional analyses (glucose and ethanol) of fermentation media and cell-free spent media were determined using an HPLC equipped with a RI monitor and computer-interfaced controller/integrator (Bio-Rad Labs, Richmond, CA) as described previously (*24*).

Determination of Growth and Fermentation Parameters

The maximum specific growth rate (μ_{max}) of a batch culture was calculated as 0.693 divided by the mean generation time (h). The growth yield coefficient ($Y_{x/s}$) was determined as the final cell density divided by the mass of glucose consumed (the final cell density being directly proportional the glucose concentration). The ethanol yield ($Y_{p/s}$) was calculated as the final ethanol concentration divided by the glucose concentration of the medium. The average volumetric productivity (Q_p) was determined by dividing the final ethanol concentration by the time required to achieve complete glucose utilization. The "average" specific productivity (q_p) was calculated as a function of the growth rate and growth yield, as follows (*24*):

$$_{av}\cdot q_p = \mu_{max} / Y_{x/s}\cdot \{Y_{p/s}\} \quad (1)$$

RESULTS AND DISCUSSION

Some Theoretical Considerations

Acetic acid (HAc) is a weak acid with a pK_a of 4.75 and dissociates, in a pH-dependent fashion, into an anionic species called "acetate" (Ac^-) and a proton (H^+).

$$\underset{\substack{\text{Undissociated acid}\\\text{(Protonated species)}}}{\text{ACETIC ACID (HAc)}} \longrightarrow \underset{\text{Dissociated acid}}{\text{ACETATE } (Ac^-)} + \text{PROTON } (H^+) \quad (2)$$

At a specified pH value, the relative concentrations of the dissociated and undissociated species, is given by the Henderson-Hasselbalch equation:

$$pH = pK_a + \log_{10}[Ac^-]/[HAc] \quad (3)$$

From an engineering and operational perspective, 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. The opportunity for ambiguity derives from the use of terms "acetate" and "acetic acid" as synonyms. From the perspective of describing the biochemical mechanism, the antimicrobial effect of acetic acid is known to depend on the concentration of the undissociated (protonated) species (HAc) (*23,25*). Therefore, 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."

The uncharged form of low molecular weight weak acids, such as propionic and acetic acid, are soluble in the lipids of the cell membrane (*26*). Figure 1 illustrates that, by virtue of its ability to freely traverse the cell membrane, the protonated or undissociated form of acetic acid (HAc) acts as an electroneutral permeant species. The insert in Fig. 1 shows the percent of acetic acid in the undissociated form as a function of the pH of the medium. As a membrane protonophore (H^+ transporter), HAc causes its inhibitory effect by interference with the homeostatic mechanisms related to the maintenance of a constant intracellular pH_i (*29,30*). Although the cell membrane is permeable to the undissociated form of acetic acid (*26*), the effect of the external pH_o on the extent or degree of permeability, remains unknown and may well be a species-specific phenomenon. For anerobic *Z. mobilis*, the intracellular pH_i is maintained close to 5.4 (*31*).

The Concept of "Coupled Growth" and "Energetic Uncoupling"

"Coupled growth" (or "balanced growth") refers to the situation where the rate at which energy (ATP) is required by the cell for anabolic or biosynthetic processes (growth being the sum of these processes) is

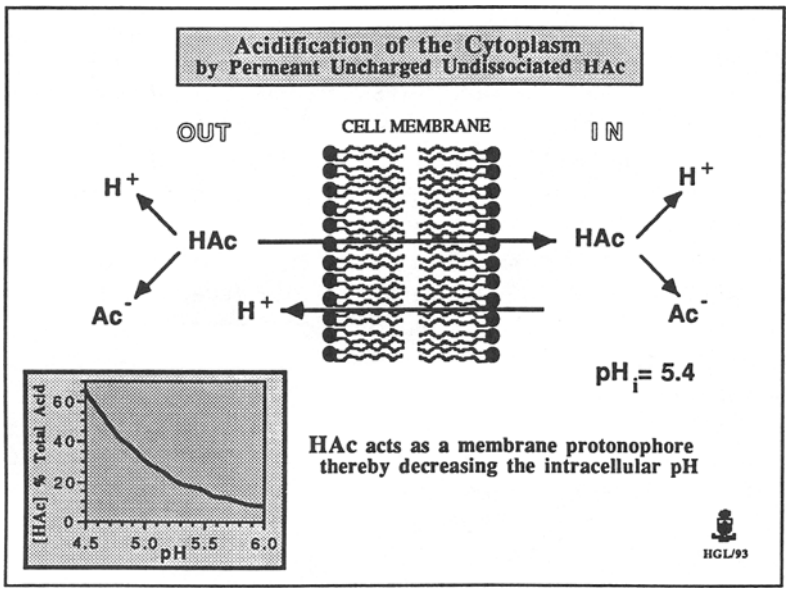

Fig. 1. The permeability of the cell plasma membrane to HAc—the undissociated form of acetic acid. The intracellular pH (pH_i) is maintained constant at about 5.4 by energy-linked membrane proton pumps which transport protons out of the cell. The Henderson-Hasselbach equation defines the concentration of HAc as a function of the pH of the medium (pH_o) (insert). The dissociation constant (pK_a) for acetic acid is 4.75.

"balanced" by the rate of energy-yielding catabolism of the carbon source. Under certain conditions, the growth yield is lower than expected on the basis of ATP yield because growth and energy production are "uncoupled." "Energetic uncoupling" occurs when energy is either wasted or used for maintenance (homeostatic) purposes rather than for growth. The maintenance energy coefficient is the amount of energy used independent of growth. Hence, in a batch culture, "energetic uncoupling" results in the decrease in growth yield independent of the rate of sugar utilization.

The Effect of Acetic Acid (9 g/L) as a Function of pH

Because of the pH dependent nature of the dissociation of acetic acid (as determined quantitatively by the Henderson-Hasselbalch equation), the concentration of the toxic species, HAc, decreases expontentially as the pH_o increases (*see* insert of Fig. 1). Consequently, pH control of the fermentation provides a facile operational approach to maximizing acetic acid tolerance of the ethanologenic biocatalyst. However, the most appropriate set-point for pH control can only be properly selected with reference to the fermentation pH optimum of the process organism (23).

Therefore, in order to define optimal operating conditions that will be compatible with maximal acetic acid tolerance, it is important to establish the pH value at which the biocatalyst exhibits its best performance with respect to both growth and metabolism (*23*). In terms of fermentation, cost sensitivity analyses have determined ethanol yield and productivity to be two of the most important technoeconomic parameters (*1,3*).

Our investigations have shown that the acetic acid concentration of lignocellulosic prehydrolysates from a variety of woody biomass resources and waste materials was in the range 2–10 g/L. Because of the high boiling point of acetic acid (116°C), its concentration does not change appreciably during evaporative concentration of the reducing sugar content of the hydrolysis liquors (*23*). It has previously been suggested that the upper concentration limit of about 10 g/L was an appropriate amount of acetic acid for tolerance testing of a potential process biocatalyst (*23*).

Figure 2 represents the results of a series of batch fermentations with *Z. mobilis* using a synthetic glucose salts medium and shows the effect of pH on growth rate (Fig. 2A), growth yield (Fig. 2B), and specific productivity (Fig. 2C). Whereas the pH optimum for growth was 5.5 (Fig. 2A,B), the calculated value for specific productivity (*see* Materials and Methods for details) was maximal at pH 4.5 (Fig. 2C). It should be pointed out that this value for q_p is not the maximum specific productivity because it does not take into account the contribution from nongrowth associated metabolism (i.e., the equivalent maintanence energy coefficient in terms of ethanol production) (*25,32*). However, it is known that maintenance metabolism increases with decreasing pH over the range tested (*28*). The specific rate of ethanol production associated with maintenance metabolism at pH 4.5, 5.0, 5.5, and 6.0 has been estimated to be 1.78, 1.53, 1.29, and 1.05 g EtOH/g cell/h, respectively (*28*).

Using a medium containing 9 g/L acetic acid, the pH optimum for growth was shifted to 6.0, and at lesser pH values, there was a marked decrease in both specific growth rate (Fig. 2A) and growth yield (Fig. 2B). Even at pH 6.0, $Y_{x/s}$ was decreased from 0.047 to 0.030 g cells/g glu by 9 g/L acetic acid (equivalent to 8 mM HAc) (Fig. 2B). The decrease in $Y_{x/s}$ is presumably owing to the diversion of energy away from biosynthetic processes (growth) to satisfy the increasing energy requirement for the maintenance of a constant intracellular pH with cytoplasmic acidification resulting from the entry of HAc (*see* Fig. 1) (*25*). In the presence of 9 g/L acetic acid, growth of *Z. mobilis* was completely inhibited at pH 4.5 (Fig. 2A).

Energetic uncoupling results in a decrease in growth rate and yield that is independent of the rate of catabolism (*25*). With 9 g/L acetic acid, the specific productivity was stimulated at pH values greater than 5.0 with a sharp optimum appearing at pH 5.5 (Fig. 2C). Since the effect of acetic acid on non-growth associated (i.e., "maintenance") metabolism as a function of pH, is not known, the contribution of maintenance

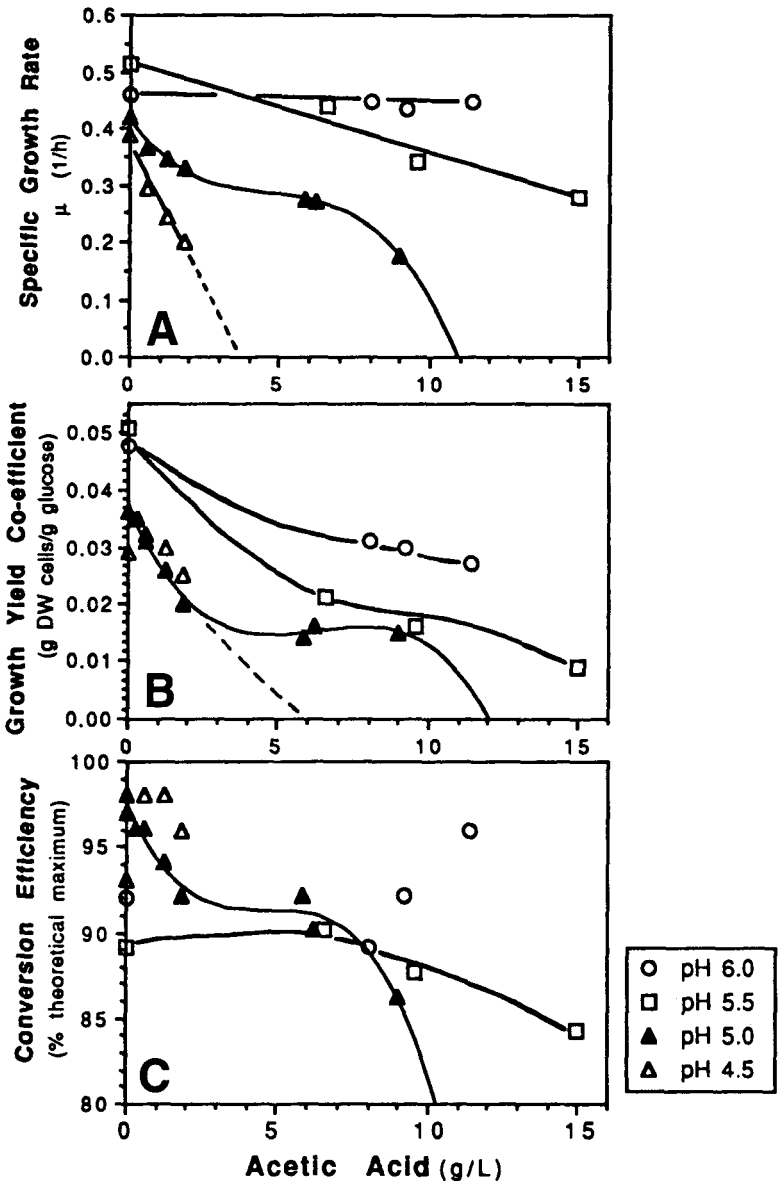

Fig. 2. The effect of pH and acetic acid on growth and ethanol production by *Z. mobilis*. Symbols: (open circles), control, no acetic acid; (filled circles), 14.72 g/L potassium acetate added to the medium (equivalent to 9 g/L total "acetic acid"). (**A**) specific growth rate, (**B**) growth yield coefficient, (**C**) specific productivity (*see* Materials and Methods for equation used to calculate this parameter).

metabolism in the presence of acetic acid was not included as a separate entity in the calculation of q_p. Previously, it had been shown that, at pH 5, q_p was enhanced at concentrations of acetic acid less than 5 g/L (equivalent to < 30 30 m*M* HAc) (25). The results of this study suggest that stimulation of q_p occurs at concentrations of HAc less than about 50 m*M* (Fig. 2C).

The Effect of Acetic Acid as a Function of Concentration and pH

In a series of experiments involving over 20 pH-stat batch fermentations, the effect of varying concentraitons of acetic acid on *Zymomonas* growth and metabolism was examined at 4 different pH values over the range 4.5–6.0. Previously, only the effect at pH 5.0 had been investigated and at acetic acid concentrations in excess of about 10 g/L, *Z. mobilis* did not grow (*25*). The data illustrated in Fig. 3A shows that, as expected, the toxicity of acetic acid is potentiated at pH 4.5, with growth being 50% inhibited at about 2 g/L acetic acid and complete inhibition projected to be at 4 g/L (Fig. 3A); however, concentrations of acetic acid greater than 10 g/L are tolerated if the pH increased above 5.0. At pH 6.0, 12 g/L acetic acid appears to have no appreciable effect on either the growth rate (Fig. 3A) or product yield (Fig. 3C), although the growth yield is decreased about 40% (Fig. 3B). As discussed previously (*25*), the effect on $Y_{x/s}$ is well described by a third-order polynomial such that there is a rapid decline at lower concentrations followed by a plateau at intermediate concentrations (Fig. 3B). It is difficult to assess the effect of acetic acid on $Y_{p/s}$ and rather than any specific trend the data probably more accurately reflects variation and scatter (Fig. 3C). However, for practical purposes it is important to note that when the pH was controlled at 5.0 or above, the efficiency of glucose-to-ethanol conversion was consistently high, averaging greater than 90% (Fig. 3C).

Figure 4 illustrates the effect of acetic acid in terms of productivity. Whereas the average volumetric productivity is affected by the growth "lag time" (*25*) caused by acetic acid (Fig. 4A), the calculated value for the specific productivity reflects a combination of three parameters; the specific growth rate, the growth yield, and the product yield (Fig. 4B). The contribution from maintenance metabolism was not known and therefore was not included in this calculation of q_p. For this type of calculation, the highest degree of energetic uncoupling corresponds to the maximum value for q_p. Previously, it had been demonstrated that, at pH 5.0, this occurs with an acetic acid concentration of about 5–6 g/L (*25*). This amount of acetic acid at pH 5.0 corresponds to 30–36 m*M* undissociated acid (HAc). For experiments conducted at pH 6.0, the highest concentration of HAc was only 10.6 m*M* (12 g/L acetic acid), and consequently a maximal value of q_p was not anticipated (Fig. 4B). At pH 4.5, energetic uncoupling was not evident, since even at the lowest amount of acetic acid tested, 0.5 g/L (equivalent to 5.3 m*M* HAc), q_p was decreased about 30% (Fig. 4B). The observations represented in Fig. 2C resulted from a single concentration of acetic acid (9 g/L) tested at different pH values and suggest that maximal energetic uncoupling occurs at about 23 m*M* HAc. The results shown in Fig. 4B indicate that the maximum value for q_p occurs at 15 g/L acetic acid at pH 5.5, which corresponds to 38 m*M* HAc.

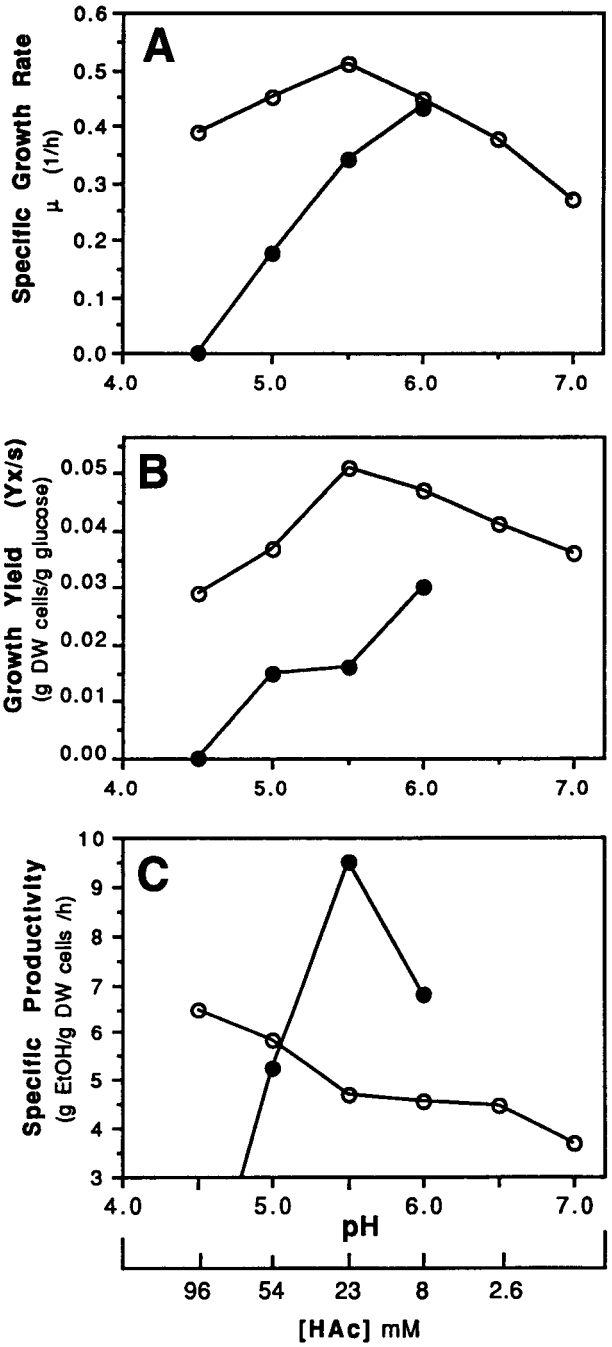

Fig. 3. The effect of acetic acid on Z. *mobilis* as a function of concentration and pH. (**A**) specific growth rate, (**B**) growth yield coefficient, (**C**) glucose-to-ethanol conversion efficiency as percentage of theoretical maximum.

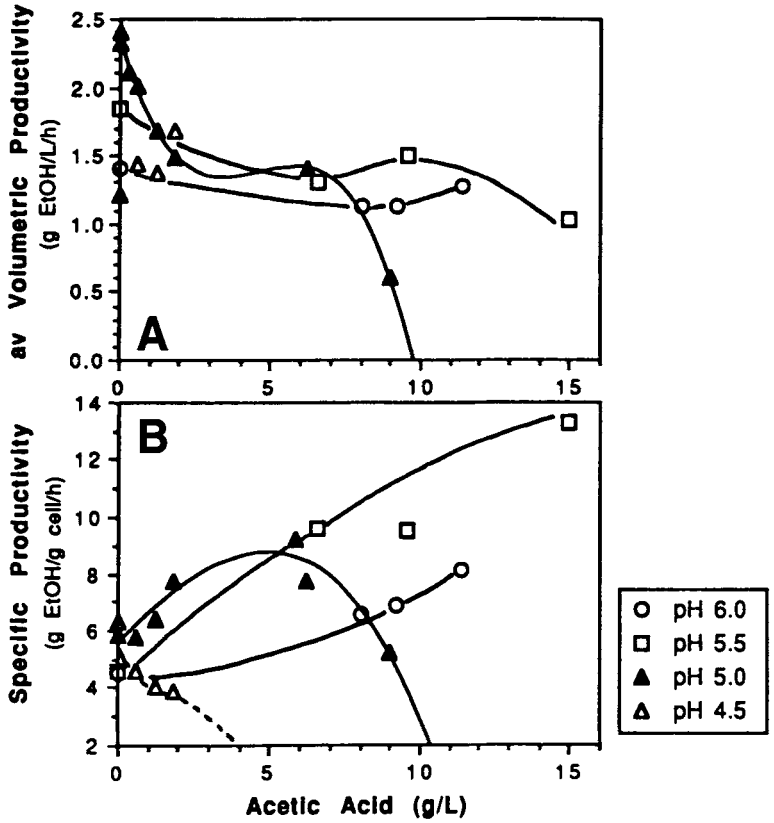

Fig. 4. The effect of acetic acid on *Z. mobilis* as a function of concentration and pH. (**A**) average volumetric productivity, (**B**) specific productivity (*see* Materials and Methods for equation used to calculate this parameter).

Our observations with *Zymomonas* and acetic acid are very similar to those made by others with yeast (*33,34*). The pH optimum for *Saccharomyces* yeast and *Zymomonas* is similar (*11,35*) and, for comparative purposes, it is interesting to note that for yeast at pH 4, an acetic acid concentration of 3 g/L (42 m*M* HAc) caused a threefold increase in specific productivity and an increase in conversion efficiency from 77 to 96% (*33*). These observations with yeast have been explained in terms of the energetic uncoupling effect of acetic acid whereby the maintenance energy is increased causing the growth yield to decrease (*33,34*). Even at lower cell densities the batch fermentations with media containing the acetic acid were completed in a shorter period of time (*34*). The consequence is an increase in specific productivity and with less carbon diverted for growth, the product yield also increases.

CONCLUSIONS

pH-dependent energetic uncoupling of *Zymomonas* by acetic acid occurs by virtue of the permeability of the plasma membrane to the undissociated form of acetic acid (HAc) and the acidification of the cytoplasm resulting from the uptake of HAc and the consequential diversion of energy away from biosynthetic processes (growth) in order to maintain constant intracellular pH. Energetic uncoupling is manifested by an increase in specific productivity. The degree of uncoupling caused by HAc depends on a rather complex interaction between several different variables including membrane permeability, the transmembrane ΔpH and the concentraiton of undissociated form of acetic acid in the medium. Within the pH range of 5.0–5.5, maximal energic uncoupling is produced by 30–38 m*M* HAc. For practical purposes, in terms of the concentration of acetic acid, this corresponds to about 5 and 15 g/L at pH 5.0 and 5.5, respectively. Assuming any upper limit concentration of acetic acid in hydrolysate fermentation media of about 12 g/L, inhibition of *Z. mobilis* in terms of both ethanol yield and productivity is avoided by controlling the pH in the range of 5.5–6.0.

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