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Effects of pH and Acetic Acid on Glucose and Xylose Metabolism by a Genetically Engineered Ethanologenic *Escherichia coli*

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ABSTRACT

Efficient utilization of the pentosan fraction of hemicellulose from lignocellulosic feedstocks offers an opportunity to increase the yield and to reduce the cost of producing fuel ethanol. The patented, genetically engineered, ethanologen *Escherichia coli* B (pLOI297) exhibits high-performance characteristics with respect to both yield and productivity in xylose-rich lab media. In addition to producing monomer sugar residues, thermochemical processing of biomass is known to produce substances that are inhibitory to both yeast and bacteria. During prehydrolysis, acetic acid is formed as a consequence of the deacetylation of the acetylated pentosan. Our investigations have shown that the acetic acid content of hemicellulose hydrolysates from a variety of biomass/waste materials was in the range 2–10 g/L (33–166 m*M*). Increasing the reducing sugar concentration by evaporation did not alter the acetic acid concentration. Acetic acid toxicity is pH dependent. By virtue of its ability to traverse the cell membrane freely, the undissociated (protonated) form of acetic acid (HAc) acts as a membrane protonophore and causes its inhibitory effect by bringing about the acidification of the cytoplasm. With recombinant *E. coli* B, the pH range for optimal growth with glucose and xylose was 6.4–6.8. With glucose, the pH optimum for ethanol yield and volumetric productivity was 6.5 , and for xylose it was 6.0 and 6.5, respectively. However, the decrease in growth and fermentation efficiency at pH 7 is not significant. At pH 7, only 0.56% of acetic acid is undissociated, and at 10 g/L, neither the ethanol yield nor the maximum volumetric productivity, with glucose or xylose, is significantly decreased. The "uncoupling" effect of HAc is more pronounced with xylose and the potency of HAc is potentiated in a minimal salts

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medium. Controlling the pH at 7 provided an effective means of circumventing acetic acid toxicity without significant loss in fermentation performance of the recombinant biocatalyst.

Index Entries: Fuel ethanol; acetic acid; pH; xylose; recombinant *E. coli* B.

INTRODUCTION

Cost reduction is the major driving force in R&D directed toward the production of fuel alcohol from materials other than the traditional sources, namely starch and sucrose (*1–3*). The food value of these carbohydrate feedstocks seriously restricts fermentation ethanol from cost-effective competition in the alternative transportation fuels market (*2*). Lignocellulosic biomass, including short-rotation energy crops as well as agricultural, forestry, and municipal wastes, is considered an excellent alternative fermentation feedstock, because it is inexpensive, plentiful, and renewable (*4,5*). However, the cellulose component, which comprises about 50% of the dry mass, is strongly resistant to depolymerization unless it is pretreated (''prehydrolysis'') to remove the impediments to enzymic digestion that are caused by lignin and the acetylated pentosan comprising the hemicellulose fraction of biomass (*6–8*). Thermochemical depolymerization of hemicellulose is efficient and cost effective (*6,9–12*), but is accompanied by the generation of byproducts, such as acetic acid, furfural, and lignin-derived phenolics (*13*), that are toxic to pentose-utilizing ethanologenic microorganisms (*14–18*). The yeasts, currently used in starch and sucrose-based fermentations, are unable to utilize pentose sugars directly (*19*), and although various pentose-utilizing ethanologenic organisms, including yeasts, molds and bacteria, have been investigated, they all generally suffer from poor yield and productivity characteristics that are exacerbated by their sensitivity to acetic acid and other inhibitory byproducts of chemical depolymerization (*20–22*). Because the mass ratio of acetic acid to pentose sugars in hemicellulose can exceed 30% (*23*), the amount of acetic acid in lignocellulosic prehydrolysates can be high, and removal by anion-exchange resins, molecular sieves, and steam-stripping improves the rate and yield of xylose fermentations by *C. shehatae* and *P. stipitis* (*24,25*). Acetic acid toxicity of xylose fermentation by *P. stipitis* has been shown to be pH dependent, since the volumetric productivity was 50% inhibited by 0.8 and 13.8 g/L acetic acid at pH values of 5.1 and 6.5, respectively (*22*). It is worth noting that, at these different pH values, 0.8 and 13.8 g/L represent equivalent concentrations of undissociated acetic acid. However, the pH optimum for xylose-fermenting yeasts is in the range 4.5–5.5 (*26*), and most fermentations of acid-catalyzed hemicellulose hydrolysate that have been conducted at pH values above the optimum in order to circumvent acetic acid toxicity (*24, 27–30*) are suboptimal with respect to both yield and productivity (*22,26*).

Recombinant DNA technology provides an opportunity to design and engineer a specific biocatalyst capable for the efficient fermentation of xylose-rich biomass prehydrolysates (*31*). For the past two years, we have been assessing the fermentation performance of a patented (*32*), genetically engineered *Escherichia coli* B (ATCC 11303) with the "PET" plasmid pLOI297 carrying pyruvate decarboxylase and alcohol dehydrogenase genes from *Zymomonas mobilis* (*33–36*) using both synthetic lab media (*37,38*) and biomass prehydrolysates prepared by different thermochemical processes from a variety of biomass/waste feedstocks, including both hardwood (aspen) (*39,40*) and softwood (pine) (*41*), newsprint (*41*), spent sulfite liquors (*42*), and corn crop residues (*43*).

The pH-dependent nature of acetic acid toxicity makes it necessary to first establish effect of pH on the recombinant *E. coli* B (pLOI297). Previous studies on the effect of pH on the fermentation performance of this recombinant have assumed that observations made with xylose could be extrapolated to all other sugars (*35,36*). At this meeting last year, we reported results of a preliminary study in which we explored the effect of acetate on recombinant *E. coli* B (*40*); however, we did not examine either the pH-dependent nature of acetate toxicity, or the comparative effects of using different sugars in complex and minimal media.

The objective of this study was to assess quantitatively the anaerobic growth and fermentation performance characteristics of recombinant *E. coli* B (ATCC 11303 pLOI297) as a function of pH and acetic acid concentration with both glucose and xylose as sole fermentable substrates. The results of this physiological investigation are useful for the purpose of describing specific environmental conditions conducive to maximal yield, productivity, and acetic acid tolerance of this particular ethanologenic genetic construct.

Production of cloned products by genetically engineered microorganisms is central to the new biotechnology, and recent analyses of environmental conditions that limit yield and productivity have implicated the accumulation of acetate as a possible cause of not only decreased cell yield, but also genetic instability (*44*). Hence, the present study on acetic acid toxicity has broad implications in terms of process control of recombinant *E. coli* fermentations.

MATERIALS AND METHODS

Organisms

Escherichia coli B (ATCC 11303 carrying the "PET" plasmid pLOI297) (*34*) was a gift from L. O. Ingram (University of Florida, Gainsville, FL). The culture was maintained and inocula prepared as described previously (*37*).

Culture Media

The principal culture medium was Luria Broth (LB) (*45*), which was supplemented with either glucose or xylose at the concentrations specified. The composition of the defined mineral salts medium was as described previously (*37*). Potassium acetate was added at the concentrations specified as "total acid." All media were sterilized by autoclaving.

Fermentation Equipment

Batch fermentations were conducted in a vol of 1500 mL in MultiGen™ (model F2000) stirred-tank biorectors having agitation, pH (adjusted using 2N KOH), and temperature control (30°C) (New Brunswick Scientific Co., Edison, NJ).

Analytical Procedures

Growth was measured turbidometrically at 550 nm (1-cm lightpath), and culture dry wt was measured by microfiltration—washing and drying the filter to constant weight under an infrared heat lamp. Linear growth rates were determined from plots of optical density (OD) vs time using the relationship 1.0 OD_{550} = 0.34 g dry wt cells/L (*38*). Compositional analyses 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 using an HPX-87H column (Bio-Rad Labs) (injection vol = 0.02 mL).

RESULTS AND DISCUSSION

Nomenclature and Some Theoretical Considerations Concerning the Effect of Acetic Acid

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 call "acetate" (Ac^-) and a proton (H^+).

$$\begin{array}{ccccc} CH_3COOH & \longrightarrow & CH_3COO^- & + & H^+ \\ HAc & \longrightarrow & Ac^- & + & H^+ \\ \text{Acetic acid} & & \text{Acetate} & & \text{Proton} \\ \text{Undissociated} & & \text{Dissociated} & & \\ \text{(protonated} & & & & \\ \text{species)} & & & & \end{array} \qquad (1)$$

An unfortunate ambiguity in nomenclature arises from the nonspecific manner in which reference is made to "acetate." In chemical compositional analyses, reference to "acetic acid" or "acetate" generally in-

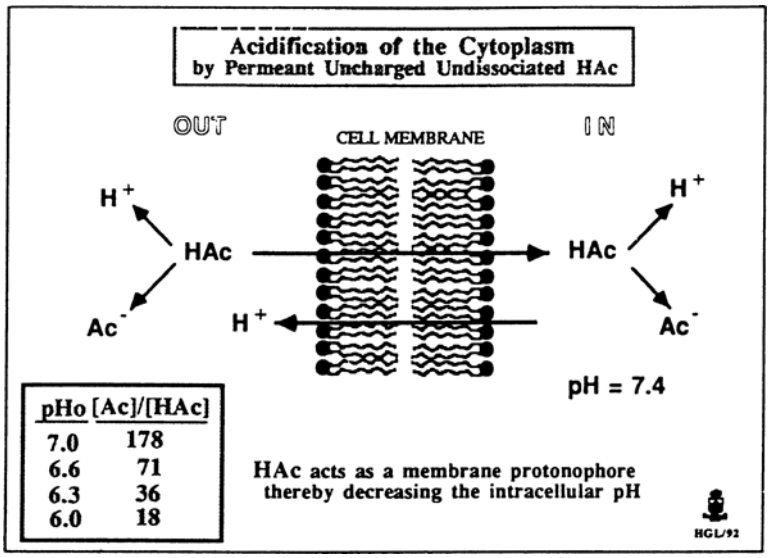

Fig. 1. The distribution of the electroneutrally permeant acetic acid between the bulk phase and the cell cytoplasm as a function of ΔpH. HAc is the undissociated, protonated form of acetic acid. The $pK_a = 4.75$, and the intracellular pH is 7.4. The Henderson-Hasselbalch equation defines the external concentration of HAc as a function of the pH of the medium.

dicates the concentration of "total acid" without intending to distinguish between the dissociated and undissociated forms of the acid. However, the biochemical mechanism relating to the antimicrobial effect of acetic acid is known to depend on the concentration of the undissociated (protonated) species (HAc) (*46–50*) which in turn is dependent on the pH. The Henderson-Hasselbalch equation defines the relative concentrations of dissociated and undissociated forms of the acid as a function of pH.

$$pH = pK_a + \log_{10}[Ac^-]/[HAc] \quad (2)$$

The uncharged form of low-molecular-weight weak acids, such as propionic and acetic acid, is soluble in the lipids of the cell membrane (*46,51*). Figure 1 illustrates that, by virtue of its ability to transverse the cell membrane freely, the protonated or undissociated form of acetic acid (HAc) acts as an electroneutral permeant species and, 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 (*50,52–56*). Although acetic acid is permeant to the membrane in the uncharged, undissociated form (*46*), the effect of the external pH (pH_o) on the extent or degree of permeability remains unknown and may well be species-specific phenomenon. For *E. coli*, the intracellular pH (pH_i) is maintained close to 7.4 (*57*), and acidification of the cytoplasm inhibits both growth and metabolism. Growth of *E. coli* is 50% inhibited when the pH_i is decreased to 6.85 (*58*).

Figure 1 also illustrates the equilibrium distribution of acetic acid between the bulk phase of the culture medium and the cell cytoplasm. If the equilibrium dissociation constant (pK_a) is the same in both these compartments, then the following relationship holds:

$$K = [H^+]_o[Ac^-]_o/[HAc]_o = [H^+]_i[Ac^-]_i/[HAc]_i \quad (3)$$

At equilibrium

$$[HAc]_o = [HAc]_i \quad (4)$$

Therefore

$$[Ac^-]_i/[Ac^-]_o = [H^+]_o/[H^+]_i \quad (5)$$

and

$$\log_{10}\{[Ac^-]_i/[Ac^-]_o\} = \log [H^+]_o - \log [H^+]_i = pH_i - pH_o \quad (6)$$

The equilibrium concentration ratio $[Ac^-]_i/[Ac^-]_o$ represents the degree to which acetic acid is accumulated by the cell cytoplasm, and the so-called "accumulation factor" (AF) can be determined as follows. Since

$$\log_{10} AF = pH_i - pH_o \quad (7)$$
$$AF = \text{antilog } \Delta pH$$

Hence, acetic acid is distributed according to ΔpH, but the above relationship is only valid at equibrium and if the amount of the undissociated acid is small compared to the total amount of acid (i.e., when the pH_o is at least 1 U higher than the pK_a or pH_o is <5.75). Distribution of the electroneutral permeant HAc will be unaffected by the membrane potential (*46*).

However, 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 exponentially 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 biocatalyst. However, the most appropriate set-point for pH control can only be properly selected with reference to the fermentation pH optimum of the particular process organism. Therefore, in attempting to define operating conditions conducive to maximal acetic acid tolerance, it is important first to establish the pH value at which the biocatalyst exhibits its highest fermentation performance in terms of yield and productivity, which have been determined from cost-sensitivity analyses to be the most important techno-economic parameters (*1,59*).

The Effect of pH on Growth and Metabolism of Recombinant *E. coli* B

Earlier studies by Ingram and his associates on the effect of pH on ethanologenic *Escherichia coli* B (pLOI297) were restricted to xylose as car-

bon source and used Luria broth buffered with 0.1–0.2*M* phosphate (*31,35,36*). It was concluded that the pH optimum for ethanol yield from xylose was 6.8 (*36*). However, the observations relating to the effect of pH on productivity were sufficiently inconsistent among different studies (*31,35,36*) as to preclude proper definition of a pH optimum (*38*). Our preliminary investigations employed pH-stat batch and chemostat cultures using glucose as substrate, and although these experiments were only exploratory in nature and in no way intended as a systematic investigation of the effect of pH on this genetic construct, the results suggested that, for glucose, the pH optimum for growth and glucose catabolism might be close to 6 (*37*). In a more recent investigation of pH-stat batch cultures and LB-xylose media, it was reported that "nearly equivalent yields and productivities were obtained from pH 6.0 to 7.6" (*60*). It was noted that, in a reproducible fashion, the xylose-to-ethanol conversion efficiency dropped from 93 to 65% (theoretical maximum) as the pH was shifted from 6.0 to 5.8 (*60*). The maximum volumetric productivity was 0.9 g/L/h at pH 6.4 and 5.8, but was about 12% lower at pH 6.0 (*60*). Nevertheless, rather surprisingly, pH 6.0 was the set point selected for all subsequent fermentation trials (for this and other genetically engineered ethanologens) (*61–63*).

Since the effect of acetic acid was expected to be a pH-dependent phenomenon, it was first necessary to establish the effect of pH on the recombinant *E. coli* B (pLOI297) using both glucose and xylose as fermentation substrates. A systematic quantitative physiological assessment of the effect of pH on growth and fermentation characteristics of the recombinant *E. coli* B was conducted in pH-stat batch cultures over the pH range 5.6–7.0, and Fig. 2 is typical of the type of data collected. Figures 2A and 2B illustrate the growth of the recombinant *E. coli* B in a stirred bioreactor with the temperature controlled at 30°C and the pH controlled at 6.3. The nutrient-rich Luria broth was supplemented with either glucose (58 g/L) or xylose (42 g/L). At pH 6.3, growth in LB medium alone (i.e., without added sugar) was logarithmic with a mean generation time of 70 min (results not shown). The arrow in Figure 2A shows the final optical density (OD) achieved in the LB medium without added sugar and corresponds to a final cell density of 0.42 g dry wt cells/L. With LB medium, the final cell density is proportional to the amount of sugar added, but only to a maximum sugar concentration of about 30 g/L (glucose or xylose) (*37,38*). The maximum cell densities are 3.5 g dry wt/L and 2.4 g dry wt/L for glucose and xylose, respectively (Fig. 2). Growth of the recombinant *E. coli* B in Luria broth, supplemented with either glucose or xylose, is distinctly biphasic, whereby a short period of exponential growth (lasting for only about three generations—Fig. 2A) is followed by a more sustained period of linear growth (Fig. 2B). The nonexponential nature of the growth of this culture has been reported by others (*34,37*). The host strain, *E. coli* B (ATCC 11303), exhibits similar growth characteristics in this medium (*38*). The corresponding time-courses for glucose and xylose utilization and for ethanol

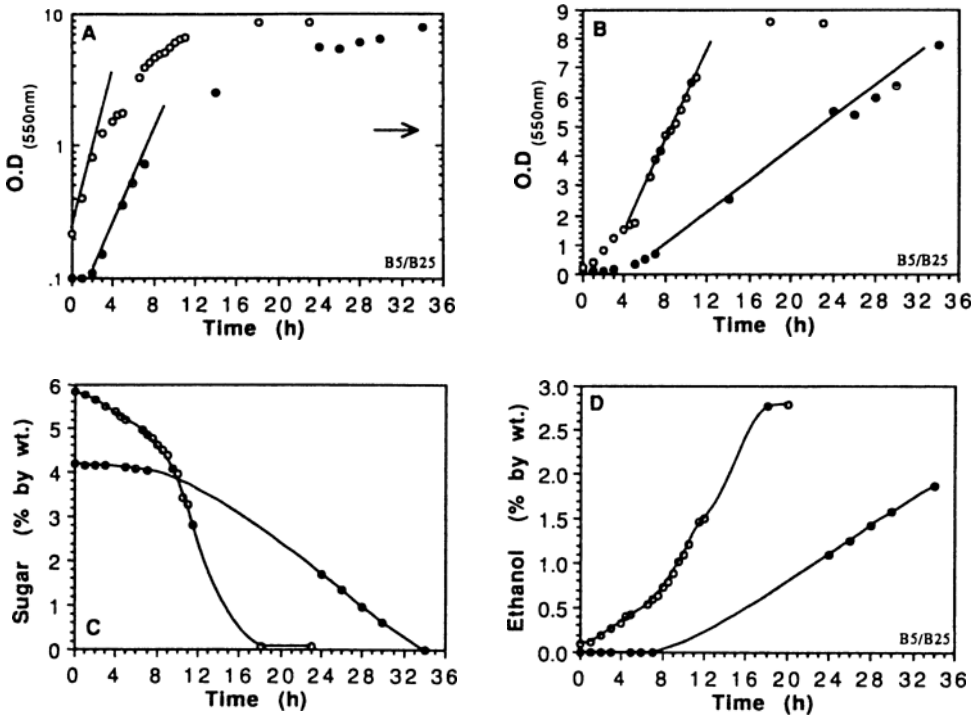

Fig. 2. Growth and metabolism of recombinant *E. coli* B (ATCC 11303 pLOI297) in Luria broth with either (O) 58 g/L glucose or (●) 42 g/L xylose. Batch fermentations were conducted in stirred-tank bioreactors (vol 1500 mL) with the pH controlled at 6.3 and temperature at 30°C. Inoculation cell density was 33 mg dry wt cells/L. Growth was followed turbidometrically as OD_{550} and plotted using either logarithmic (A) or linear scales (B). Values for the linear growth rates (Fig. 2A) were determined from the lines indicated in (B). Anaerobic metabolism (fermentation) was monitored as utilization of sugar (C) and production of ethanol (D). Tangents of max slope (D) were used to determine the max vol productivity (Fig. 6B).

production by the recombinant *E. coli* (at pH 6.3) are shown in Figs. 2C and 2D, respectively, and it is apparent that the fermentation of glucose is much faster than xylose.

From turbidometric plots of growth, similar to those shown in Fig. 2B, the linear growth rate was determined as a function of pH (Fig. 3A). The growth rate in LB with xylose is only about one-half as much as with glucose (Fig. 3A). The pH optimum for growth is 6.8 for glucose and 6.6 for xylose (Fig. 3A).

The growth yield coefficient ($Y_{x/s}$) can be determined from the mass ratio of cells (max cell density) to sugar added to the medium. Figure 3B shows the effect of pH on $Y_{X/S}$ for the recombinant growing in LB with 20 g/L added glucose or xylose. This sugar concentration is in the range where the final cell density is proportional to the amount of sugar added

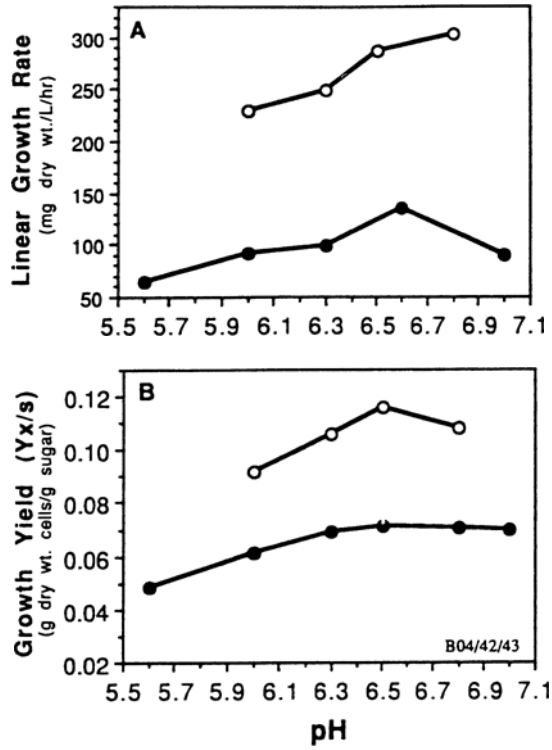

Fig. 3. Effect of pH on recombinant *E. coli* B: (A) linear growth rate and (B) growth yield coefficient. Conditions were as described in Fig. 2, except that the sugar concentration was 20 g/L glucose (O) or 20 g/L xylose (●).

to the medium. Although the growth yield coefficient for glucose had previously been shown to be unexpectedly greater than for xylose (*37*), Fig. 3B illustrates that this relationship is valid over the entire pH range tested. Whereas the pH optimum with respect to $Y_{X/S}$ for glucose (0.116 g/g) appears to be at 6.5, the value of $Y_{X/S}$ for xylose appears maximal (0.071 g/g) over the pH range 6.3 to 7.0 (Fig. 3B). Others have reported that the growth yield coefficient for this same culture is similar for both glucose and xylose. At pH 6.0, $Y_{X/S}$ for glucose and xylose was reported to be 0.048 and 0.050 g/g, respectively (*61*), whereas at pH 6.8, $Y_{X/S}$ was 0.064 g/g for both sugars (*60*).

From plots of the fermentation time-course (Figs. 2C and 2D), the average volumetric productivity (av Q_p, g ethanol/L/h) is calculated by dividing the final ethanol concentration by the time required for complete sugar utilization, and the maximum productivity (max Q_p) is determined as the maximum slope in a plot of ethanol concentration vs elapsed fermentation time (*see* Fig. 2D). The maximum theoretical mass yield of ethanol is 0.51 for both glucose and xylose, and the ratio of the observed product yield to the maximum theoretical yield represents the sugar-to-ethanol conversion efficiency. The effect of pH on productivity and yield

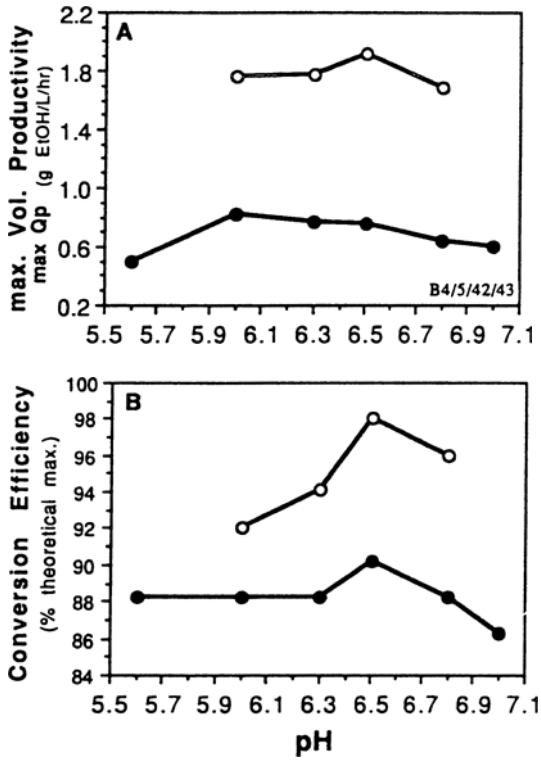

Fig. 4. Effect of pH on recombinant *E. coli* B: (A) max volumetric productivity and (B) sugar-to-ethanol conversion efficiency. Conditions were as described in Fig. 2, except that the sugar concentration was 20 g/L glucose (O) or 20 g/L xylose (●).

is illustrated in Figs. 4A and 4B, respectively. Over the entire pH range examined, both yield and productivity are higher with glucose as substrate (Fig. 4). Comparison of the max Q_p values shows that the rate of glucose metabolism is about 2.5 times faster than xylose metabolism (Fig. 4A). Whereas the max Q_p for both glucose and xylose fermentations appears relatively unaffected by pH (Fig. 4A), a pH of 6.5 appears optimal with respect to conversion efficiency for both sugars (Fig. 4B). At all pH values, the ethanol yield was higher with glucose then with xylose (Fig. 4B). In general, the values for both yield and productivity are in good agreement with the reports of others for this same recombinant strain (*36,60*).

Acetic Acid Concentration in Biomass Hydrolysates

Lignocellulosic biomass exhibits great diversity with respect to its chemical composition. The hemicellulose of deciduous (hard) woods contains a characteristically higher proportion of acetylated pentosan (prin-

Table 1
The Acetic Acid Content of Different Biomass Prehydrolysates

Biomass/waste "prehydrolysate"	HAc[a]/TRS,[b] wt%	Total acetic acid	
		g/L	m*M*
Aspen	17–18	4.0–6.2	67–103
Pine	5–11	2.8–4.9	47– 82
Hardwood SSL[c]	24–31	7.6–9.3	127–155
Softwood SSL[c]	7.6	2.7	45
Newsprint	5.2	2.0	33
Corn cobs	~8	3.0–3.6	50–60

[a]HAc = undissociated acetic acid.
[b]TRS = total reducing sugars.
[c]SSL = spent sulfite liquor.

cipally xylose) compared to coniferous (soft) woods. The amount of acetyl in woody biomass ranges from 3.8–4.4 (dry wt %) to 1.1–1.7% for hard and softwoods, respectively (*23*). When expressed in terms of the mass ratio of pentose sugars to acetic acid, the variation becomes 3–6:1 for hardwoods and 3.5–8:1 for softwoods. Herbaceous energy crops (grasses) resemble softwoods with respect to the total amount of acetyl (1.1–1.3%), but the ratio of pentose sugars to acetic acid is much higher (about 12:1) (*11*). A similarly high ratio has been observed in analyses of corn crop residues (*12,42*). Other crop residues, including wheat straw and sugar cane bagasse, exhibit mass ratios for pentose to acetic acid in the range 4:1 to 5:1, respectively (*10,28*).

Table 1 shows the acetic acid content of several different hemicellulose hydrolysates that we have investigated as potential feedstocks for fuel ethanol fermentations (*41*). Spent sulfite liquors (SSL) from pulp mills represent the only lignocellulosic hydrolysate available today in commercially exploitable quantities (*64,65*). The mass ratio of acetic acid to total reducing sugars (wt %) in all of these prehydrolysates ranged from 5 to 31% (Table 1). The concentration of acetic acid ranged from 2 to 9.3 g/L, corresponding to a molar concentration range of 33–155 m*M* (Table 1). The concentration of acetic acid was the highest in SSL produced from the sulfite pulping of mixed hardwoods, and similar concentrations have been reported by others (*25,29*). Apart from the SSL samples, all of these different biomass/waste prehydrolysates were produced at pilot scale under optimal operating conditions for maximum productivity and yield of monomeric sugars. On average, the total reducing sugar content of these prehydrolysates was about 40–50 g/L. Because of the relatively high costs associated with product recovery in fuel ethanol fermentation processes, product concentration is second only to yield in terms of its economic impact on the cost of production (*59*). For this reason, it would be

advantageous to operate at sugar concentrations >40–50 g/L; however, because of the high boiling point of acetic acid (116°C), increasing the sugar concentration by evaporation does not significantly alter the acetic acid concentration. Hence, we concluded that an amount of approx 10 g/L (166 m*M*) represented the maximal anticipated concentration of acetic acid in biomass hydrolysates, and that this amount of acetic acid therefore should be an appropriate upper concentration limit in terms of tolerance testing of the biocatalyst.

The Effect of Acetic Acid on Growth and Metabolism of *E. coli*

The production of acetic acid by *E. coli* has been shown to be responsible for growth inhibition and a reduction in cell yield (*66*). In addition, acetate has been linked to genetic and product instability in the production of cloned products by recombinant *E. coli* (*44*). It has been suggested that ethanol potentiates the inhibitory effect of acetic acid (*56,67*), which is particularly pertinent to the fermentation performance of recombinant *E. coli* in an acetic-acid-containing medium where ethanol is the major metabolic end product. In pH-controlled batch cultures, acetate has been shown to affect growth with a dose dependency that is logarithmic (*66*). At pH 7, anaerobic growth of *E. coli* K12(S) was 50% inhibited in the presence of 0.7 g/L acetic acid (64 μ*M* HAc) and completely inhibited by 2.2 g/L acetic acid (0.2 m*M* HAc) (*68,69*). However, aerobic growth appears to be more resistant to inhibition by acetic acid since, it required 1.4 g/L acetic acid (0.13 m*M* HAc) for 50% inhibition and 9 g/L (0.84 m*M* HAc) for complete inhibition (*68,69*). In a recent survey of several different *E. coli* cultures, Luria strain B grew at the highest rate and had the highest biomass productivity, but even more importantly in the context of the present study, it proved to be the most acetate tolerant, requiring 3.6 g/L acetic acid (0.34 m*M* HAc) for 50% inhibition of aerobic growth at pH 7 (*66*).

Although for fuel ethanol fermentations, from a practical and operational perspective, the important parameter is the amount of total acetic acid in the fermentation medium, from the perspective of the biochemical mechanism of toxicity, the concentration of the protonated species (HAc) is more important. For this reason, the effect of acetic acid on growth and metabolism was plotted as a function of the concentration of both HAc and total acid. Figures 5 and 6 show the effect of 72 m*M* total acetic acid (added to LB as 7 g/L of potassium acetate, but equivalent to 4.3 g/L of total acetic acid) on the growth and fermentation characteristics of recombinant *E. coli* B, as a function of pH, with xylose as substrate. The concentrations of HAc in these experiments were 0.40, 1.0, and 2.0 m*M* for pH values of 7.0, 6.6, and 6.3 respectively. Providing the pH was kept above 6.8, 4.3 g/L total acetic acid were not inhibitory in terms of either the rate

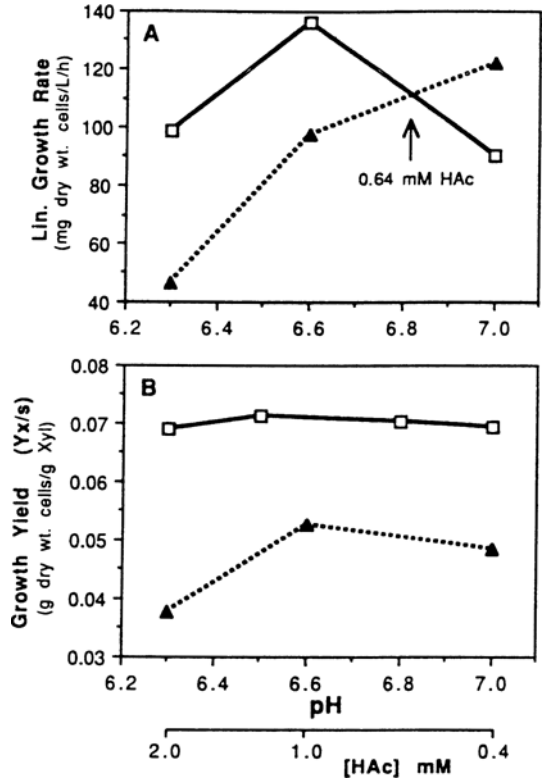

Fig. 5. Effect of acetic acid on recombinant *E. coli* B as a function of pH: (A) linear growth rate and (B) growth yield coefficient. Conditions were as described in Fig. 2, except that the sugar concentration was 20 g/L xylose. Symbols: (□) control without acetic acid (▲) with 72 m*M* potassium acetate added to the LB medium. The concentration of the protonated form of acetic acid (HAc) was calculated using the Henderson-Hasselbalch relationship ($pK_a = 4.75$).

of growth (Fig. 5A) or ethanol production (Fig. 6). The corollary to this is that both growth rate (Fig. 5A) and av Q_p were inhibited at concentrations of HAc > 64 m*M* (Fig. 6A). Figure 6B suggests that the max Q_p is not inhibited until the concentration of HAc exceeds 1.26 m*M*. This amount of acetic acid did not affect the ethanol yield ($Y_{p/s}$) from either glucose or xylose over the pH range 6.3–7.0 (results not shown). However, it did cause a significant decrease in $Y_{x/s}$(xyl) over the same pH range, from a 30% reduction at pH 7.0 (0.4 m*M* HAc) to a 50% reduction at pH 6.3 (2.0 m*M* HAc) (Fig. 5B). The results of these experiments using a fixed amount of acetic acid (4.3 g/L) confirmed that the sensitivity to acetic acid was potentiated by operating at a pH that increased the concentration of the protonated species (HAc).

Figures 7–9 show the results of experiments designed to assess the acetic acid tolerance of the recombinant *E. coli* at the more permissive pH

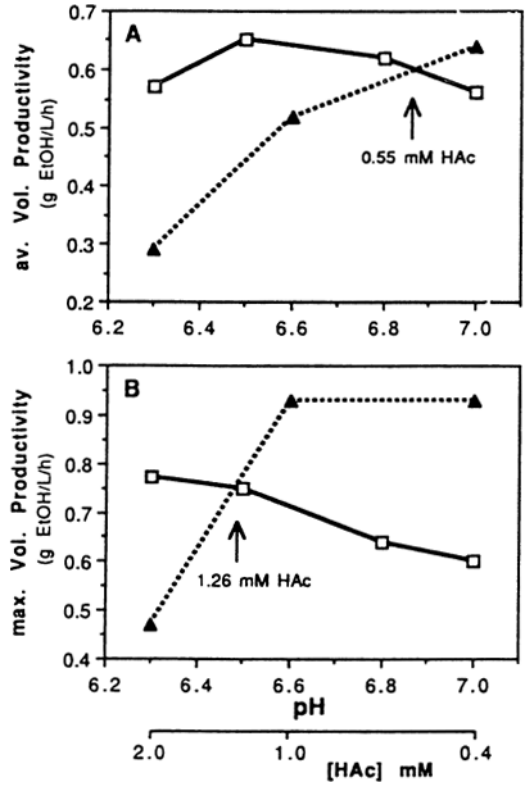

Fig. 6. Effect of acetic acid on recombinant *E. coli* B as a function of pH: (A) average volumetric productivity and (B) max volumetric productivity. Conditions were as described in Fig. 5. Symbols: (□) control without acetic acid; (▲) with 72 m*M* potassium acetate added to the LB medium.

of 7 (or 6.8 in the case of glucose). With xylose as substrate, the growth rate was inhibited at acid levels > 140 m*M* (equivalent to 8.4 g/L total acetic acid and 0.78 m*M* HAc), whereas the growth yield was inhibited at all concentrations of acetate (Fig. 7). This observation agrees well with that of Beall et al. (*60*), who noted that growth of recombinant *E. coli* B in LB-xylose was inhibited at concentrations of acetate > 80 m*M* (equivalent to 0.71 m*M* HAc at pH 6.8). The avQ_p was inhibited at acid concentrations > 110 m*M* (equivalent to 6.6 g/L total acetic acid and 0.6 m*M* HAc), but the maxQ_p was enhanced at all concentrations up 180 m*M* total acetic acid (equivalent to 10.8 g/L and 1.0 m*M* HAc) (Fig. 8). The ethanol yield from xylose (0.44 g/g) was not affected by acetic acid even at the maximum amount used (10.8 g/L—results not shown). Beall et al. (*60*) reported that concentrations of HAc > 0.9 m*M* were inhibitory with respect to both yield and productivity.

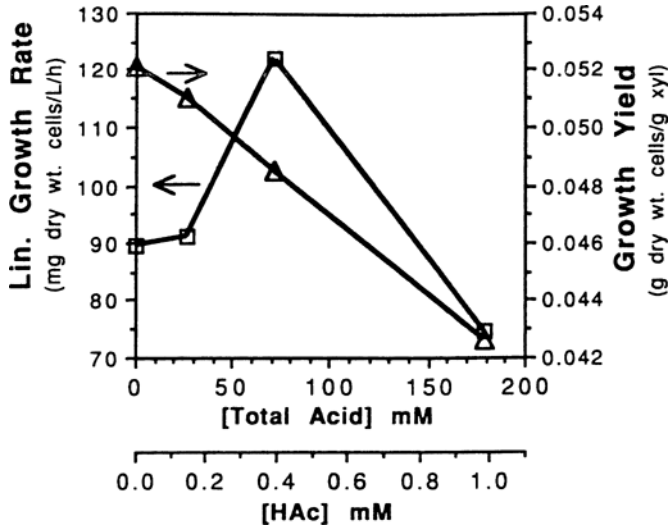

Fig. 7. Effect of acetic acid on growth of recombinant *E. coli* B with xylose at pH 7. Conditions were as described in Fig. 2, except that the sugar concentration was 40 g/L xylose. Total acetic acid concentration was calculated based on the amount of potassium acetate added to the LB medium. At pH 7, the ratio of the acetate anion to the protonated acid is 178:1.

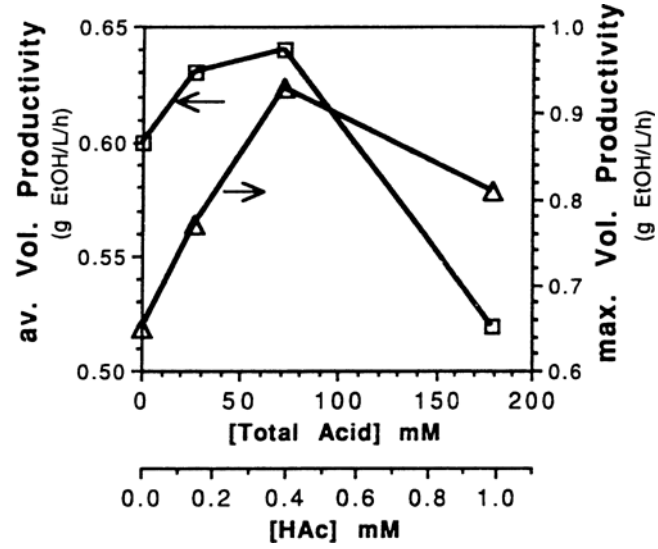

Fig. 8. Effect of acetic acid on the productivity of ethanol fermentation by recombinant *E. coli* B. Conditions were as described in Fig. 7 with xylose as carbon source.

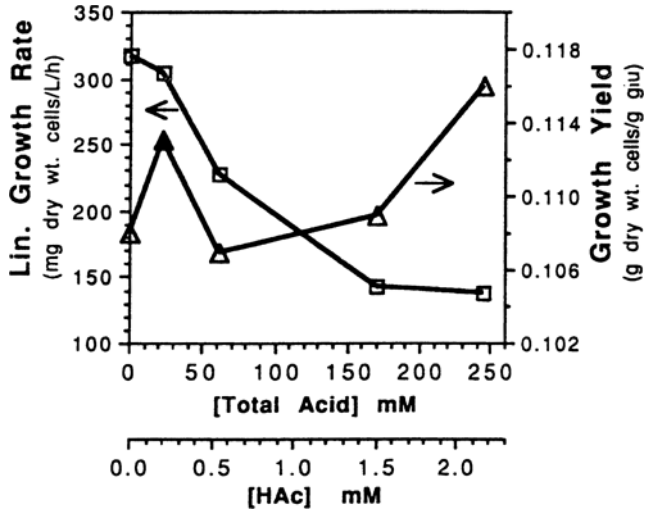

Fig. 9. Effect of acetic acid on growth of recombinant *E. coli* B with xylose at pH 6.8. Conditions were as described in Fig. 2, except that the sugar concentration was 40 g/L glucose. Total acetic acid concentration was calculated based on the amount of potassium acetate added to the LB medium. At pH 6.8, the ratio of the acetate anion to the protonated acid is 112:1.

Since the experiments with glucose as substrate were performed at pH 6.8, the same amounts of added acetate produced 1.58 times higher concentrations of HAc. With glucose, the growth rate was inhibited at all concentrations of acetic acid used and was 50% inhibited at 140 m*M* (equivalent to 8.4 g/L total acetic acid and 1.24 m*M* HAc) (Fig. 9). Since the protonated species (HAc) is the "active" inhibitory agent (*68*), comparisons with respect to the effect of acetate on the utilization of glucose and xylose are more appropriately made in terms of comparable concentrations of HAc. Growth on glucose and xylose does not appear to be equally sensitive to acetic acid, since, at 0.5 m*M* HAc, the growth rate on glucose was inhibited by 10% (Fig. 9), but with xylose, it was stimulated by 25% (Fig. 7). This is in contrast to the different and opposite effect of HAc on the growth yield coefficients for glucose and xylose. Whereas $Y_{x/s}$(xyl) was inhibited at all concentrations of HAc (Fig. 7), $Y_{x/s}$(glu) was relatively unaffected over the same concentration range and even enhanced at higher concentrations (Fig. 9). However, the effect of HAc on volumetric productivity (both the avQ_p and maxQ_p) with glucose (Fig. 10) was very similar to the pattern observed with xylose as substrate (Fig. 8). The ethanol yield from glucose (av 0.49 g/g) was not affected by acetic acid even at the maximum amount used (14.8 g/L—results not shown). Table 2 summarizes and compares the effect of HAc on growth and metabolism of the recombinant *E. coli* for both glucose and xylose.

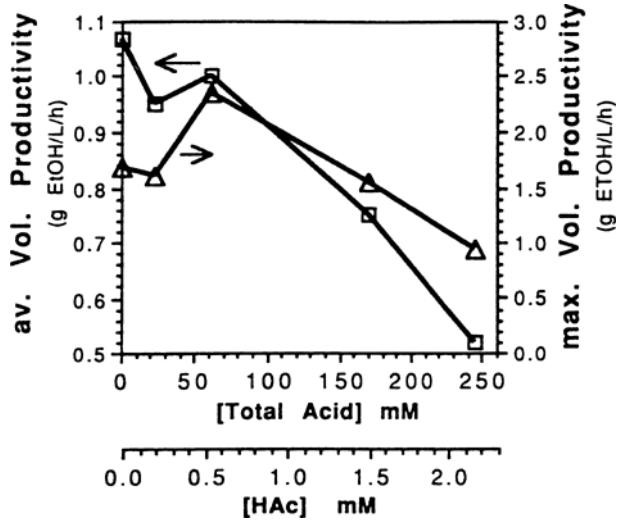

Fig. 10. Effect of acetic acid on the productivity of ethanol fermentation by recombinant *E. coli* B. Conditions were as described in Fig. 9 with glucose as carbon source.

Table 2
Effect of Undissociated Acetic Acid
on Recombinant *E. coli* B

	MIC[a] for HAc,[b], m*M*	
Parameter	Glucose	Xylose
Growth rate	>0	0.8
Growth yield ($Y_{x/s}$)	+[d]>0	>0
Product yield ($Y_{p/s}$)	NA[c]	NA[c]
Vol productivity		
Average Q_p	0.5	0.6
Maximum Q_p	1.4	+[d]<1.0

[a]MIC = minimal inhibitory concentration (m*M*).
[b]HAc = undissociated form of acetic acid.
[c]NA = not affected (over range tested).
[d] + = stimulated.

Experiments with a Defined Mineral Salts Medium

Although Ingram's physiological investigations with this recombinant ethanologen have focused exclusively on fermentation performance assessment using nutrient-rich Luria broth (*34,36,60*), our investigations have also involved the use of a nutritionally lean, defined mineral salts

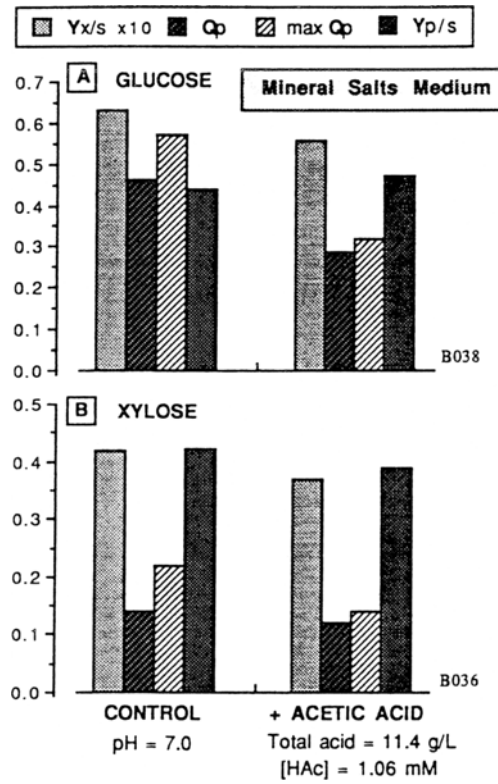

Fig. 11. Effect of acetic acid on growth and sugar catabolism by recombinant *E. coli* B in a defined mineral salts medium. The pH was controlled at 7.0. The composition of the defined salts medium is described in Materials and Methods, and contained either (A) glucose, 21.3 g/L; or (B) xylose, 18.9 g/L. The total acetic acid concentration was 11.4 g/L (equivalent to 1.06 m*M* HAc).

medium, with either xylose or glucose as carbon source (*37,38*). In this investigation, we examined the effect of adding a relatively large amount of acetic acid (11.4 g/L) to this medium. With the pH controlled at 7, the concentration of HAc is 1.06 m*M*. The product yield is not decreased with glucose as substrate (Fig. 10A), but with xylose, $Y_{p/s}$ is decreased by 6% (Fig. 11B). However, the growth yield coefficients ($Y_{x/s}$) for glucose and xylose are both decreased by 11% (Fig. 11). The same concentration of HAc did not appear to have any inhibitory effect on $Y_{x/s}$(glu) in the nutrient-rich, Luria broth (Fig. 7). The effect of this concentration of HAc on maximal productivity is also different in nutrient-rich and minimal media. Whereas maxQ_p appears to be relatively unaffected by 1 m*M* HAc in Luria broth (Figs. 8 and 10), in the minimal medium, the value of max-Q_p is reduced 44% with glucose (Fig. 11A) and 37% with xylose (Fig. 11B). These observations with the defined salts medium suggest that the toxicity of acetic acid can be affected by the nutritional status of the medium.

CONCLUSIONS

It is concluded that because the toxic effect of acetic acid is pH dependent and because the pH optimum for the recombinant *E. coli* B (pLOI297) is broad (over the range 6–7), operation of the bioreactor at pH 7 achieves sufficient acetic acid tolerance without significant loss in fermentation performance (in terms of both yield and productivity) to accommodate the amount of acetic acid that can be anticipated in biomass/waste hydrolysates. Judging from the differential effect of HAc on growth and metabolism (ethanol productivity) summarized in Table 2, it appears that acetic acid acts as an "uncoupler" by disassociating these two processes. Energy derived from catabolism that is normally directed to growth is diverted for the purpose of maintaining a constant value of cytoplasmic pH. HAc seems to be a more potent "uncoupler" in the case of xylose compared to glucose. The minimum inhibitory concentration (MIC) for HAc for volumetric productivity (for both glucose and xylose) is about 0.55 m*M* (equivalent to 6 g/L total acetic acid at pH 7) (Table 2). Unfortunately, the short-term nature of batch fermentations precludes an adequate assessment of the possible negative effect on culture (genetic) stability that has been recently attributed to acetate (*44*).

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REFERENCES

1. Wright, J. D. (1988), *Chem. Eng. Progress* **84,** 62–68.
2. Wyman, C. E. and Hinman, N. D. (1990), *Appl. Biochem. Biotechnol.* **24/25,** 735–753.
3. Bull, S. R. (1990), *Energy from Biomass & Wastes XIV,* Klass, D. L., ed., Institute of Gas Technology, Chicago, IL, pp. 1–14.
4. Lynd, L. R. (1990), *Appl. Biochem. Biotechnol* **24/25,** 695–719.
5. Lynd, L. R., Cushman, J. H., Nicholas, R. J., and Wyman, C. E. (1991), *Science* **251,** 1318–1323.
6. Grethlein, H. E. (1985), *Bio/Technology* **3,** 155–160.
7. Grohmann, K., Himmel, M., Rivard, C., Tucker, M., Baker, J. Torget, R., and Graboski, M. (1984), *Biotech. Bioeng. Symp.* **14,** 139–157.
8. Kong, F., Engler, C. R., and Soltes, E. (1992), *Appl. Biochem. Biotechnol.* **34/35,** 23–35.

9. Grethlein, H. E., Allen, D. C., and Converse, A. O. (1984), *Biotech. Bioeng.* **26,** 1498–1505.
10. Grohmann, K., Torget, R., and Himmel, M. (1986), *Biotechnol. Bioeng. Symp.* **17,** 135–151.
11. Torget, R., Werdene, P., Himmel, M., and Grohmann, K. (1990), *Appl. Biochem. Biotechnol.* **24/25,** 115–126.
12. Torget, R., Walter, P., Himmel, M., and Grohmann, K. (1991), *Appl. Biochem. Biotechnol.* **28/29,** 75–86.
13. Stanek, D. A. (1958), *Tappi J.* **41,** 601–609.
14. Beck, M. J. (1986), *Biotechnol. Bioeng. Symp.* **17,** 617–627.
15. Fein, J. E., Tallim, S. R., and Lawford, G. R. (1984), *Can. J. Microbiol.* **30,** 682–690.
16. Frazer, F. R. and McCaskey, T. A. (1989), *Biomass* **18,** 31–42.
17. Nishikawa, N. K., Sutcliffe, R., and Saddler, J. N. (1988), *Appl. Microbiol. Biotechnol.* **27,** 549–552.
18. Ando, S., Arai, I., Kiyoto, K., and Hanai, S. (1986), *J. Ferment. Technol.* **64,** 567–570.
19. Jefferies, T. W. (1981), *Biotechnol. Bioeng. Symp.* **11,** 315–324.
20. Lynd, L. R. (1989), *Adv. Biochem. Eng. Biotechnol.* **38,** 1–52.
21. Skoog, K. and Hahn-Hägerdal, B. (1988), *Enzyme Microbiol. Technol.* **10,** 66–88.
22. Prior, B. A., Kilian, S. G., and du Preez, J. C. (1989), *Process Biochemistry* **24,** 21–32.
23. Timell, T. E. (1967), *Wood Science and Technology* **1,** 45–70.
24. Tran, A. V. and Chambers, R. P. (1986), *Enzyme Microbiol. Technol* **8,** 439–444.
25. Parekh, S. R., Parekh, R. S., and Wayman, M. (1987), *Process Biochemistry* **22,** 85–91.
26. du Preez, J. C., Bosch, M. and Prior, B. A. (1986), *Enzyme Microb. Technol.* **8,** 360–364.
27. Lee, Y. Y. and McCaskey, T. A. (1983), *Tappi J.* **66,** 102–107.
28. van Zyl, C., Prior, B. A., and du Preez, J. C. (1988), *Appl. Biochem. Biotechnol.* **17,** 357–369.
29. Wilson, J. J., Nishikawa, N. N., Deschatelets, L., and Nguyen, Q. (1990), Vol. I and II. Final Report of DSS Contract File #051SZ.23283-8-6103. Alternative Energy Division; Energy, Mines and Resources Canada, Ottawa.
30. Björling, T. and Lindman, B. (1989), *Enzyme Microbiol. Technol.* **11,** 240–246.
31. Ingram, L. O., Alterthum, F., Ohta, K., and Beall, D. S. (1990), in *Developments in Industrial Microbiology,* Pierce, G. E., ed., vol. 31, Elsevier, New York, pp. 21–30.
32. Ingram, L. O., Conway, T., and Alterthum, F. (1991), United States Patent 5,000,000.
33. Ingram, L. O. and Conway, T. (1988), *Appl. Environ. Microbiol.* **54,** 397–404.
34. Alterthum, F. and Ingram, L. O. (1989), *Appl. Environ. Microbiol.* **55,** 1943–1948.
35. Ingram, L. O., (1990), in *Energy from Biomass & Wastes XIV,* Klass, D. L., ed., Institute of Gas Technology, Chicago, IL, pp. 1105–1126.

36. Ohta, K., Alterthum, F., and Ingram, L. O. (1990), *Appl. Environ. Microbiol.* **56,** 463–465.
37. Lawford, H. G. and Rousseau, J. D. (1991), *Appl. Biochem. Biotechnol* **28/29,** 221–236.
38. Lawford, H. G. and Rousseau, J. D. (1991), in *Energy from Biomass & Wastes XV,* Klass, D. L., ed., Institute of Gas Technology, Chicago, IL, pp. 583–622.
39. Lawford, H. G. and Rousseau, J. D. (1991), *Biotechnol. Letts.* **13,** 191–196.
40. Lawford, H. G. and Rousseau, J. D. (1992), *Appl. Biochem. Biotechnol.* **34/35,** 185–204.
41. Lawford, H. G. and Rousseau, J. D. (1992), in *Energy from Biomass & Wastes XVI,* Klass, D. L., ed., Institute of Gas Technology, Chicago, IL (in press).
42. Lawford, H. G. and Rousseau, J. D. (1993), *Appl. Biochem. Biotechnol.,* this vol.
43. Lawford, H. G. and Rousseau, J. D. (1992), *Biotechnol. Letts.* **14,** 421–426.
44. Zabriskie, D. W. and Arcuri, E. J. (1986), *Enzyme Microb. Technol.* **8,** 706–717.
45. Luria, S. E. and Delbruck, M. (1943), *Genetics* **28,** 491–511.
46. Nicholls, D. G. (1982), *Bioenergetics—an Introduction to the Chemiosmotic Theory,* Academic, Toronto, pp. 56–58.
47. Postma, E., Verduyn, C., Scheffers, W. A., and van Dijken, J. P. (1989), *Appl. Environ. Micobiol.* **55,** 468–477.
48. Pampulha, M. E. and Louriero, V. (1989), *Biotechnol. Letts.* **11,** 269–274.
49. Verduyn, C., Postma, E., Scheffers, A., and van Dijken, J. P. (1990), *J. Gen. Microbiol.* **136,** 395–403.
50. Repaske, D. R. and Adler, J. (1981), *J. Bacteriol.* **145,** 321–325.
51. Conway, E. J. and Downey, M. (1950), *Biochem. J.* **47,** 347.
52. Mitchell, P. (1973), *J. Bioenergetics* **4,** 63–91.
53. Padan, E. D., Zilberstein, D., and Schuldiner, S. (1982), *Biochim. Biophys. Acta.* **650,** 131–156.
54. Booth, I. R. (1985), *Microbiol. Rev.* **49,** 359–378.
55. Verduyn, C., Postma, E., Scheffers, A., and van Dijken, J. P. (1990), *J. Gen. Microbiol.* **136,** 405–412.
56. Pampulha, M. E. and Loureiro-Dias, M. C. (1989), *Appl. Microbiol. Biotechnol.* **31,** 547–550.
57. Padan, E., Zilberstein, D., and Schuldiner, S. (1981), *Biochim. Biophys. Acta* **650,** 131–156.
58. Salmond, C. V., Kroll, R. G., and Booth, I. R. (1984), *J. Gen. Microbiol.* **130,** 2845–2850.
59. Hinman, N. D., Wright, J. D., Hoagland, W., and Wyman, C. E. (1989), *Appl. Biochem. Biotechnol.* **20/21,** 391–401.
60. Beall, D. S., Ohta, K., and Ingram, L. O. (1991), *Biotechnol. Bioeng.* **38,** 296–303.
61. Ohta, K., Beall, D. S., Mejia, J. P., Shanmugan, K. T., and Ingram, L. O. (1991), *Appl. Environ. Microbiol.* **57,** 893–900.
62. Ohta, K., Beall, D. S., Mejia, J. P., Shanmugan, K. T., and Ingram, L. O. (1991), *Appl. Environ. Microbiol.* **57,** 2810–2815.
63. Barbosa, M., de F. S., Beck, M. J., Fein, J. E., Potts, D., and Ingram, L. O. (1992), *Appl. Environ. Microbiol.* **58,** 1382–1384.

64. Safi, B. F., Rouleau, D., Mayer, R. C., and Desrochers, M. (1986), *Biotechnol. Bioeng.* **28,** 944–951.
65. Mueller, J. C. (1970), *Pulp and Paper Magazine Canada* **72,** 72–76.
66. Luli, G. W. and Strohl, W. R. (1990), *Appl. Environ. Microbiol.* **56,** 1004–1011.
67. Booth, I. R. and Kroll, R. G. (1983), *Biochem. Soc. Trans.* **11,** 70–73.
68. Smirnova, G. V. and Oktybr'skii, O. N. (1985), *Microbiology (USSR)* **54,** 205–209.
69. Smirnova, G. V. and Oktyabr'skii, O. N. (1988), *Microbiology (USSR)* **57,** 446–451.