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Acetic Acid Production by *Clostridium thermoaceticum* in pH-Controlled Batch Fermentations at Acidic pH

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Four strains of the homofermentative, obligately anaerobic thermophile *Clostridium thermoaceticum* were compared in pH-controlled batch fermentation for their tolerance to acetic acid, efficiency of converting glucose to acetic acid and cell mass, and growth rate. At pH 6 (and pH 7) and initial acetic acid concentrations of less than 10 g/liter, the four strains had mass doubling times of 5 to 7 h and conversion efficiencies to acetic acid and cell mass of about 90% (70 to 110%) and 10%, respectively. At pH 6 and initial acetic acid concentrations of greater than 10 g/liter, only two of the strains grew, the mass doubling time increased to 18 h, and the conversion efficiencies to acetic acid and cell mass remained unchanged. Both of these strains had been selected for their ability to grow in the presence of acetate at neutral pH. The highest acetic acid concentrations reached were about 15 and 20 g/liter at pH 6 and 7, respectively. *C. thermoaceticum* is apparently more sensitive to free acetic acid than to either acetate ion or pH. It was also shown that, at pH 6 and 7, the redox potential must be at least as low as −300 and −360 mV, respectively, for growth to occur.

Clostridium thermoaceticum is an obligately anaerobic thermophile that can homoferment 1 mol of glucose to 3 mol of acetic acid, preferably at a pH of about 7 (1–5). Because of this characteristic, it provides a potentially attractive alternative to the conventional petrochemical routes to acetic acid. However, for economic viability, we have concluded that certain criteria must be met. Among them, for reasons pertaining to conventional methods of product recovery, the ability to conduct the fermentation at a pH at least as low as 4.5 is essential (4). Further, a productivity of 5 g/liter per h must be achieved. To reach the required productivity, we set as our goal a dilution rate of 0.1 h^{-1} and an acetic acid concentration of 50 g/liter.

We have reported the isolation of a strain of *C. thermoaceticum* capable of growth at pH 4.5 (4). Before isolating this strain, four other strains were compared in pH-controlled batch fermentations to determine which one should be subjected to an intensive strain improvement program aimed at reaching our predetermined goals. The following characteristics were determined at pH 6, the lowest pH at which preliminary experiments showed all four strains could grow: growth rate, tolerance to acetate, efficiency of converting glucose to acetic acid, and efficiency of converting glucose to cell mass. The results of this comparison are herein reported.

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MATERIALS AND METHODS

Organisms. *C. thermoaceticum* (Ljungdahl), a parent culture (wild type), was obtained from L. Ljungdahl, University of Georgia, Athens, Ga. *C. thermoaceticum* (Wood), a parent culture (wild type), was obtained from H. Wood, Case Western Reserve University, Cleveland, Ohio. *C. thermoaceticum* S3, an acetate-tolerant strain derived from the Wood culture after adaptation and selection on sodium acetate without mutagenesis, was isolated by and obtained from R. Gomez and D. I. Wang, Massachusetts Institute of Technology, Cambridge, Mass. *C. thermoaceticum* 1745, an acetate-tolerant strain derived from the Ljungdahl culture after ethylmethane sulfonate mutagenesis and selection on 2% sodium acetate, was isolated by and obtained from R. W. Warren, Union Carbide Corp., Tarrytown, N.Y.

Media and growth conditions. Medium 3098, growth conditions, and culture storage conditions have been previously described (4). The estimation of growth, glucose, and acetic acid have also been described (4).

CO_2 purification system. To ensure that the CO_2 was of the highest purity attainable, the following purification and oxygen removal system was used. First, a 600-pound (ca. 272-kg) cylinder of instrument grade CO_2 (Linde) was "blown down" by opening the cylinder valve completely and venting for 90 s, closing the valve, waiting 30 min, and venting again for 30 s. This procedure was recommended by Linde to remove traces of oxygen. Next, a high-purity regulator was

installed. The gas was then passed through the following system at about 10 lb/in^2. (i) A drying filter (Plexiglas cylinder 12 in. [30.48 cm] long and 1.25 in. [3.175 cm] in diameter) was used in which the first 25% of the bed volume was packed with Drierite (W. A. Hammond Drierite Co.), the next 50% with activated carbon JXC Class 591A (Union Carbide Corp.), and the last 25% with Drierite. This filter removes moisture and organic impurities. (ii) A glass furnace tube was packed with oxidized copper turnings and inserted in a vertical gas purifying furnace operated at about 520°C (Sargent-Welch S-36517 and S-36518). This furnace oxidizes reduced compounds in the gas. The copper is regenerated (oxidized) by passing oxygen over the hot copper until it becomes dull black in appearance. (iii) Two glass furnace tubes were filled with reduced copper turnings and inserted in gas purifying furnaces heated to about 520°C. These two furnaces adsorb any oxidized compounds in the gas. Eventually, the copper becomes oxidized and is regenerated (reduced) by passing a gas mixture consisting of 97% CO_2 and 3% H_2 over the hot copper. (iv) A drying filter was packed with Drierite, 25%; molecular sieve 4A (Linde), 50%; and Drierite, 25%. The above components are connected with ⅛-in. (0.32-cm) stainless steel tubing that has been washed with methanol to remove grease and oil and passivated with 10% HNO_3 for 10 min to remove other surface impurities and inorganics. The gas thus purified is ready to use for sparging fermentors or overlaying media in Hungate tubes or serum bottles.

For fermentors, the gas flow was controlled by calibrated rotometers, and the final filter was sterile glass wool. For Hungate tubes and serum bottles, a special stainless steel gassing manifold was constructed so that CO_2 pressurization and evacuation could be controlled. To enter the tubes or bottles, the gas was passed through a Whitey quick-opening ⅛-in. tubing valve to which was attached (by tygon tubing) a sterile 0.5-ml glass syringe packed with glass wool and a 1.5-in. (3.81-cm) sterile needle.

Fermentors. The fermentors were modified jacketed glass Spinner flasks with 1-liter working volumes (Bellco model 1968). The modifications consisted of (i) two additional sidearm ports (four total); (ii) a ground glass condenser attachment port on top; and (iii) a bolt lock flange and gasket system to seal top and bottom parts. The pH was controlled with a model pH-40 controller plus pump module (New Brunswick Scientific Co.). After initial pH adjustment with HCl or acetic acid or both, control was achieved by addition of oxygen-free 50% NaOH. The redox potential (E_{meas}) was monitored with a platinum redox electrode (Ingold) and recorded in millivolts on an Orion model 701A meter. CO_2 was sparged at the rate of 0.08 V/min, and the fermentors were operated under a positive pressure of 15 to 25 mm of water. A temperature of 58°C was maintained by circulating water from a constant temperature bath through the outer fermentor jacket. Chilled water (10°C) was circulated through the condenser from a Haake model KT-33 cooling bath. A diagram of a fermentor is shown in Fig. 1.

Usually, four fermentors were operated simultaneously, three at pH 6.0 and 0, 0.5, and 1.0% initial acetic acid concentration, respectively; and one at pH 7 and 0% acetic acid initially. Samples were withdrawn with sterile CO_2-washed syringes, and the optical density at 600 nm, glucose concentration, and acetic acid concentration were measured.

Preparation of redox probes. Redox probes are routinely cleaned before use in 70 mM sodium hypochlorite solution and checked in a pH 4.0, 0.05 M potassium hydrogen phthalate buffer saturated with quinhydrone (the latter is provided in excess). Acceptable probes are stable and have a measurable output (E_{meas}) of +263 ± a few millivolts at 30°C. This corresponds to an output (E_h) of about +460 mV relative to the standard hydrogen electrode. The E_h is obtained by adding 196 mV to the measured output of the Ingold Argenthal/Pt electrode.

The measured output of the Ingold redox probe at a pH and temperature other than 4.0 and 30°C, respectively, may be calculated from equation 3:

$$E_h = +699 - 0.198(T)(pH) \quad (1)$$

$$E_{meas} = E_h - 196 \quad (2)$$

$$E_{meas} = 503 - 0.198(T)(pH), \quad (3)$$

where +699 mV is the standard potential of the quinhydrone electrode relative to the hydrogen electrode, −196 mV is the Argenthal/Pt electrode potential relative to the hydrogen electrode, and T is the absolute temperature, °K.

RESULTS

The time courses of batch fermentations controlled at pH 6 are shown in Fig. 2 and 3 for strain S3 at initial acetic acid concentrations of 0 and 1.25%, respectively. For comparison, Fig. 4 shows a pH 7 controlled batch fermentation for the same strain. Note that, without adding acetic acid, a trace was introduced with the yeast extract and the inoculum. At pH 6, the gross acetic acid concentration reached 15 to 18 g/liter, and at pH 7, it was 20 g/liter. The most dramatic difference observed was in the mass doubling time calculated from the growth curves: about 6 h at pH 6 and 7 without added acetic acid, and about 18 h at pH 6 and an initial acetic acid concentration of 1.25%. It is of interest to note the redox potential, which decreased as the cells grew from −350 to −435 mV and then remained between −380 and −415 mV (Fig. 2). When the redox potential remained at about −280 and −300 mV, no growth occurred (Fig. 3 and 4). Upon reinoculating the fermentor, the redox potential decreased to −360 mV, and growth ensued. This was a typical observation for these fermentations. At pH 7, a redox potential of about −360 mV was needed for growth to occur; at pH 6, the redox potential had to be −300 mV. The cells themselves had the ability to reduce the redox potential, which could reach almost −500 mV at pH 7; i.e., E_h = −304 mV (Fig. 4).

From data like those shown in Fig. 2–4, mass doubling time, cell yield, and efficiency to acetic acid can be calculated. Table 1 summarizes such data for the four strains. The Ljungdahl and

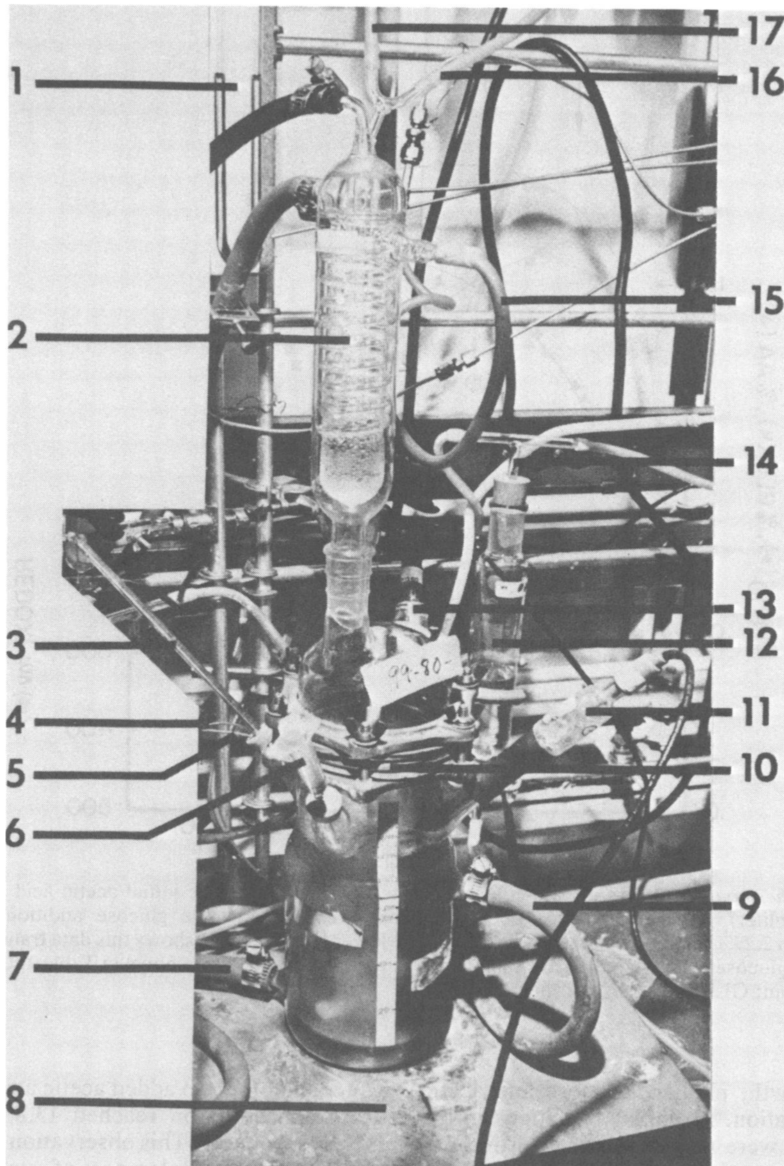

FIG. 1. Fermentor used for pH-controlled batch fermentation. 1, Water-filled U-tube manometer; 2, off-gas condenser; 3, thermometer; 4, NaOH feed line from pH controller; 5, CO_2 sparge; 6, sampling syringe; 7, heating water-in; 8, magnetic stirrer; 9, heating water-out; 10, flange and gasket system; 11, redox probe; 12, NaOH reservoir to pH controller; 13, pH probe to controller; 14, nitrogen overlay for NaOH reservoir; 15, exhaust gas from condenser; 16, exhaust gas to final filter; 17, exhaust gas to U-tube manometer.

Wood strains were the first examined in our multiple fermentor system. With the possible exception of fermentors operating at initial acetic acid concentrations of 1%, fermentor failures were traced to gross mechanical failure, the introduction of oxygen, or the inability to reach the required redox potential. When strain 1745 was tested, a new procedure was introduced to distinguish between mechanical failures and the inability of the strain to grow under the imposed conditions. Immediately preceding fermentor inoculation, a medium sample was aseptically withdrawn from the fermentor, transferred to a sterile Hungate tube, evacuated and overlaid with CO_2 twice, inoculated with the same culture (and in the same proportion) used to inoculate the fermentor, and incubated at 58 to 60°C. This Hungate tube control was examined period-

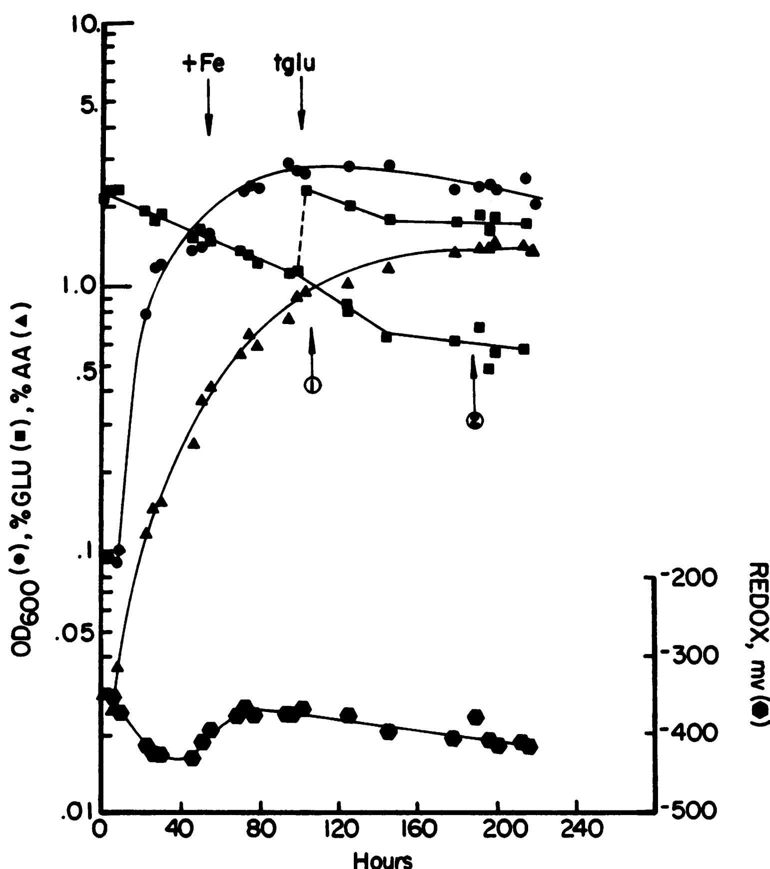

FIG. 2. *C. thermoaceticum* S3 pH 6.0-controlled batch fermentation. The initial acetic acid concentration was 0 (~0.3 g/liter). Where indicated, Fe and glucose were added. The glucose addition brought the concentration to 2.29%, as shown in the upper curve. The lower glucose curve shows this data transposed to give the cumulative glucose loss. (1) and (2) are sample points used for calculations shown in Table 2. OD_{600}, Optical density at 600 nm; GLU, glucose; AA, acetic acid.

ically for growth, pH, and glucose and acetic acid concentration. As initial conditions in the Hungate tube were nearly identical to those in the fermentor, positive results in the Hungate tube and negative results in the fermentor indicated the ability of the strain to grow under the imposed conditions and mechanical failure in the fermentor. As the pH was not controlled in the Hungate tube, when growth occurred, the final pH was always lower than the initial pH.

As seen in Table 1, sample point 1, in six of nine experiments, biomass production had stopped when the gross acetic acid concentration (i.e., initial acetic acid concentration plus net acetic acid production) reached about 10 g/liter (9.3 to 12.5 g/liter). The exceptions were strain S3, at pH 6 and an initial acetic acid concentration of 12.5 g/liter, where the gross concentration reached 18 g/liter; and strain 1745, pH 6 and initial acetic acid concentration of 5.3 g/liter and pH 7, no added acetic acid, where the gross concentration reached 13.8 and 14.0 g/liter, respectively. This observation supports the reported acetate tolerance of strains S3 and 1745. Acetic acid production continued after net growth stopped, reaching about 15 g/liter at pH 6 and about 20 g/liter at pH 7 (sample point 2).

Theoretically, the ratio of moles of acetic acid produced to moles of glucose consumed is 3:1. In practice, about 10% of the glucose was expected to be incorporated in the biomass. Therefore, the mole ratio was expected to be less than 3:1 and the efficiency to acetic acid to be about 90%, i.e., grams of acetic acid produced/grams of glucose consumed × 100. For the most part, the mole ratios and efficiencies observed were those expected (within our experimental error) and were similar for the four strains. The calculated substrate yield coefficients (Y_S), based on the biomass produced shown in Table 1, were

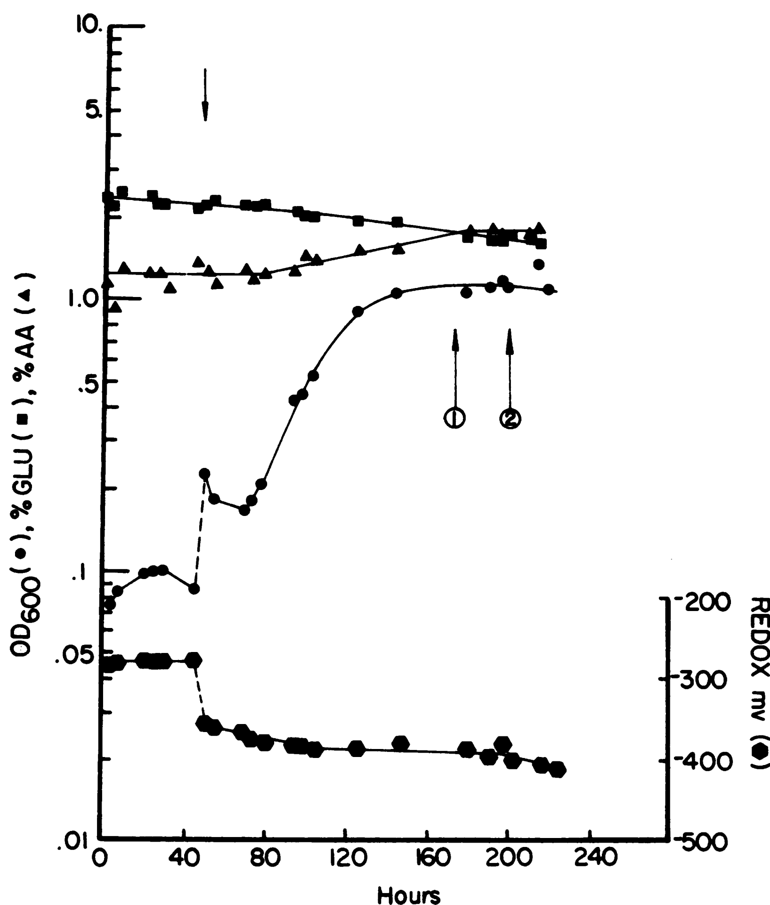

FIG. 3. *C. thermoaceticum* S3 pH 6.0-controlled batch fermentation. The initial acetic acid concentration was 1.25%. ↓, Fermenter re-inoculated. (1) and (2) are sample points used for calculations shown in Table 2. OD_{600}, Optical density at 600 nm; GLU, glucose; AA, acetic acid.

generally lower than the predicted 0.1 g of cells per g of glucose. This was not unexpected, as net growth had ceased at sample points 1 and 2, i.e., cells in stationary phase. During exponential growth, the calculated Y_S more closely approached the 0.1 expected (Fig. 2–4).

The mass doubling times for the four strains were about the same under similar conditions. At pH 6 and 7 and up to an initial acetic acid concentration of 7 g/liter, the mass doubling time was 5 to 7 h. Again, note the dramatic effect of a higher acetic acid concentration as seen for strain S3 at pH 6 and an initial acetic acid concentration of 12.5 g/liter, at which the mass doubling time was 18 h. This clearly reflected the sensitivity of these strains to acetic acid.

Although the fermentor failed, the Hungate tube control for strain 1745 did grow at pH 6 and an initial acetic acid concentration of 11.1 g/liter. Only strains S3 and 1745 grew at initial acetic acid concentrations of greater than 10 g/liter. This again reflected the reported acetate tolerance of these strains.

DISCUSSION

Four strains of *C. thermoaceticum* were compared in controlled pH batch fermentations and in the presence of different initial acetic acid concentrations. The experiments were conducted at pH 6, the lowest pH at which all four strains could grow, and pH 7 for comparison. The initial acetic acid concentrations varied between 0 and 12.5 g/liter. Since as the cells grew they produced acetic acid and subsequently lowered the pH, controlling the pH enabled us to distinguish between inhibition caused by acetic acid and inhibition caused by low pH.

One of the more interesting discoveries was the effect of redox potential on growth. At pH 7, a redox potential of about −360 mV was needed

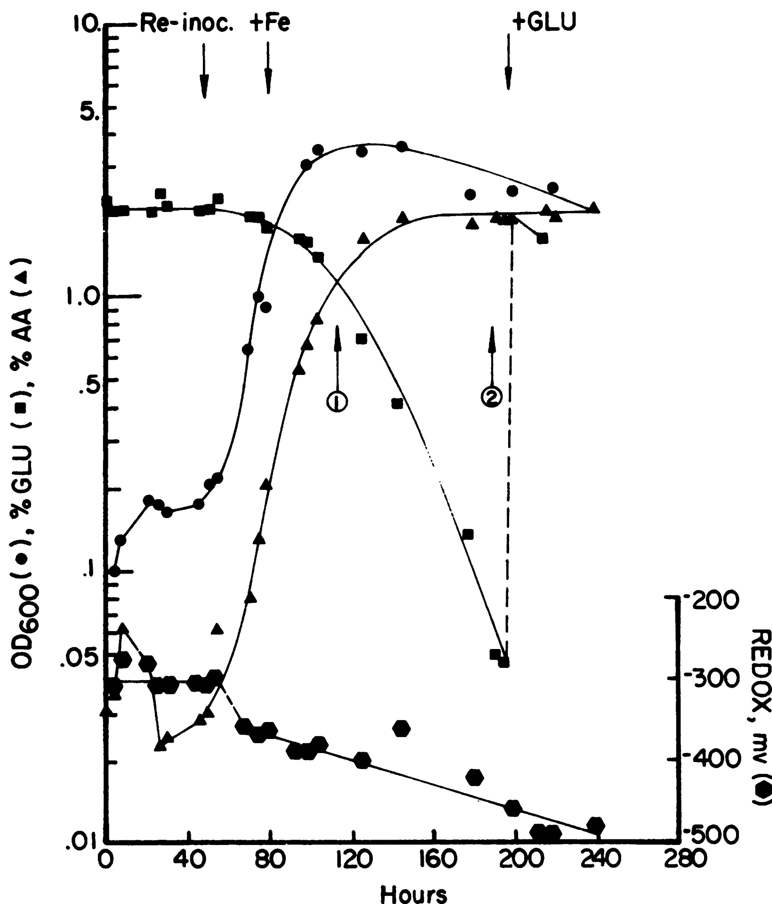

FIG. 4. *C. thermoaceticum* S3 pH 7.0-controlled batch fermentation. The initial acetic acid concentration was 0 (~0.3 g/liter). Where indicated, the fermenter was re-inoculated, and Fe and glucose were added. (1) and (2) are sample points used for calculations shown in Table 2. OD_{600}, Optical density at 600 nm; GLU, glucose; AA, acetic acid.

before growth could occur. At pH 6, the potential had to be about −300 mV. It could be shown that, for every decrease in pH of one unit, the equivalent redox potential required for growth increased by about 60 mV; i.e., at pH 5, the redox had to be at least −240 mV, etc. The cells themselves had the ability to reduce the redox potential, which could reach almost −500 mV at pH 7. The nature of the reducing agent is unknown. In a previous report, the ability to achieve the required redox potential was shown to be a key parameter in selecting for strains capable of growth at an even lower pH, i.e., 4.5 (4).

The four strains were compared for tolerance to acetic acid, conversion efficiency to acetic acid, conversion efficiency to cell mass, and growth rate. Only strains S3 and 1745 grew at pH 6 and an initial acetic acid concentration of greater than 10 g/liter, reflecting their tolerance to acetate. The gross acetic acid concentrations reached at pH 6 and 7 were 15 and 20 g/liter, respectively, and were similar for the four strains. The higher concentration observed at pH 7 may reflect a greater sensitivity of the strains to acetic acid as opposed to acetate ion. This conclusion was also supported by the observation that, at pH 6 and 7 and initial acetic acid concentrations of less than 10 g/liter, the mass doubling time for the four strains was 5 to 7 h; i.e., the growth rate was not affected, only the final product yield. The sensitivity to acetic acid was also reflected in the dramatic increase in mass doubling time to 18 h for strain S3 at pH 6 and an initial acetic acid concentration of 12.5 g/liter. Further support for the free acid being more inhibitory than either acetate ion or pH is shown in Table 2. At pH 6 and 15 g/liter total

TABLE 1. *C. thermoaceticum* strain comparison, pH-controlled batch fermentation

Strain	pH	Initial AA[a] concn (g/liter)	Sample point[b]	Glucose consumed (g/liter)	Net AA produced (g/liter)[c]	Biomass produced (g/liter)	AA/glucose molar ratio	EFF[d] to AA (%)	Mass doubling time (h)
Ljungdahl	5.8 ± 0.1	7.0	1	6.0	4.0	0.47	2.00	67	7
			2	13.0	8.0	0.38	1.85	62	
Wood	6.1 ± 0.1	0 (0.3)	1	13.0	9.0	1.0	2.08	69	5
			2	18.0	15.0	1.13	2.50	83	
S3	6.0 ± 0.1	0 (0.3)	1	12.0	10.0	0.70	2.49	83	5
			2	16.0	14.0	0.60	2.62	88	
	6.0 ± 0.1	5.2	1	10.5	7.3	0.48	2.12	70	6
			2	12.0	9.8	0.38	2.44	82	
	6.0 ± 0.1	12.5	1	5.5	5.5	0.25	3.00	100	18
			2	6.5	5.5	0.25	2.54	85	
	7.0 ± 0.1	0 (0.3)	1	10.0	11.0	0.88	3.30	110	7
			2	20.5	20.0	0.69	2.93	98	
1745	6.0 ± 0.1	0 (0.4)	1	13.5	10.5	0.58	2.35	78	5
			2[e]						
	6.0 ± 0.1	5.3	1	9.8	8.5	0.53	2.60	87	5
			2	15.3	9.8	0.30	1.92	64	
	6.0 ± 0.1	11.1[f]							
	7.0 ± 0.1[g]	0 (0.7)	1	15.0	13.3	1.2	2.61	89	5
			2	30.3	20.6	0.55	2.07	66	

[a] AA, Acetic acid.
[b] At sample point 1, the glucose consumption curve, acetic acid production curve cross, and net biomass production had stopped. At sample point 2, glucose consumption and acetic production had essentially stopped (see Fig. 2–4).
[c] Gross acetic acid concentration = initial acetic acid concentration + net acetic acid production.
[d] EFF, Conversion efficiency.
[e] Not done; run terminated.
[f] Fermenter did not grow, although Hungate tube control did grow.
[g] This was a fed batch experiment.

product concentration, the free acid concentration had reached 1.4 g/liter or 4.8 times that at pH 7 at the same total product concentration. The acetate ion concentration at pH 6 was actually lower than at pH 7. Data at pH 5 for low pH-tolerant strains obtained by intensive development (4) indicated that a total product concentration of about 3.6 g/liter is inhibitory. The calculated free acid concentration at pH 5 and 3.6 g/liter total product concentration was also 1.4 g/liter.

At pH 7, the acetate ion may have become inhibitory at 20 g/liter, before the free acid reached about 1.4 g/liter. The cultures did not appear to be nutrient limited, at least at pH 7, because excess glucose was present, and it has been shown that the culture will deplete the glucose before the other nutrients. However,

TABLE 2. Equilibrium values for free acid and acetate ion concentration[a]

pH	Equilibrium value at following total product (ion + free acid) concn (g/liter):[b]									
	3.6		10.0		12.5		15.0		20.0	
	Ac^-	HAc	Ac^-	HAc	Ac^-	HAc	Ac^-	HAc	Ac^-	HAc
7.0	3.52	0.072	9.80	0.20	12.25	0.25	14.7	0.30	19.6	0.40
6.0	3.25	0.342	9.05	0.95	11.31	1.19	13.6	1.43	18.1	1.90
5.0	2.14	1.40	6.10	3.90	7.63	4.88	9.15	5.85	12.2	7.80

[a] Calculated at pH 5, 6, and 7 for several total product concentrations at 60°C and the initial salt content of medium 3098.
[b] Ac^-, Acetate; HAc, free (un-ionized) acetic acid.

there may be different requirements at pH 6. Alternatively, Wang et al., using a different medium, reported reaching an acetic acid concentration of 37 g/liter at pH 7 (5). (The ratio of free acid to acetate ion was not given.) Therefore, our inability to reach acetic acid concentrations of greater than 20 g/liter at pH 7 remains difficult to explain.

In the early stationary phase (sample point 1), the calculated efficiency to acetic acid varied from 67 to 110%, was similar for the four strains, and was in reasonable accord with a predicted efficiency of 90%. Conversion efficiency to cell mass was also about what was expected and was similar for the four strains.

The mass doubling time at pH 6 of 5 to 7 h corresponds to a specific growth rate of 0.10 to 0.14. If this growth rate can be maintained at pH 4.5, our criterion of a dilution rate of 0.1 h^{-1} will have been met without requiring cell recycle (4).

The only discernible difference among the four strains was the apparent increase in tolerance to acetic acid by strains S3 and 1745. Using an experimental design similar to that used here, i.e., multiple controlled pH batch fermentation, strain 1745 was subjected to an intensive strain development program aimed at reaching our predetermined goals of pH 4.5 and a productivity of 5 g/liter per h. The results of this development program were reported (4).

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