APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Feb. 1984, p. 294–298
0099-2240/84/020294-05$02.00/0

Vol. 47, No. 2

Elucidation of Growth Inhibition and Acetic Acid Production by *Clostridium thermoaceticum*

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Received 21 July 1983/Accepted 31 October 1983

The production of acetic acid by *Clostridium thermoaceticum* was studied by using batch fermentations. In a pH-controlled fermentation with sodium hydroxide (pH 6.9), this organism was able to produce 56 g of acetic acid per liter. On the other hand, when the pH was not controlled and was decreased during fermentation to 5.4, the maximum attainable acetic acid concentration was only 15.3 g/liter. To obtain a better understanding of the end product inhibition, various salts were tested to determine their effect on the growth rate of *C. thermoaceticum*. An inverse linear relationship between the growth rate and the final cell concentration to the sodium acetate concentration was found. By using different concentrations of externally added sodium salts, the relative growth inhibition caused by the anion was found to be in the order of acetate > chloride > sulfate. Various externally added cations of acetate were also examined with respect to their inhibitory effects on growth. The relative magnitude of inhibition on the growth rate was found to be ammonium > potassium > sodium. The combined results have shown that the undissociated acetic acid was much more inhibitory than the ionized acetate ion. Complete growth inhibition resulted when the undissociated acetic acid concentration was between 0.04 and 0.05 M and when the ionized acetate concentration was 0.8 M. Therefore, at low pH (below 6.0), undissociated acetic acid is responsible for growth inhibition, and at high pH (above 6.0), ionized acetate ion is responsible for growth inhibition.

In most fermentation processes for chemical production, the accumulation of end product inhibits cell growth and product formation. For homofermentative ethanol production by yeasts (1) and *Zymomonas mobilis* (4), the toxic effects of ethanol on cell growth are well documented. In the case of clostridial fermentations for chemical production, for example *Clostridium thermocellum* (3), with more than one major end product, each product could be inhibitory to cell growth. Our previous studies (6; G. Wang, Sci. D. thesis, Massachusetts Institute of Technology, Cambridge, 1982) on homofermentative acetic acid production by *Clostridium thermoaceticum* indicated that the end product, acetic acid, both in the ionized form (acetate) and in the undissociated form (acetic acid) was inhibitory to cell growth. Since then, additional studies were performed and are reported in this paper. The purpose of this research was to define the parameters which influence growth inhibition under different environmental conditions.

MATERIALS AND METHODS

Organism. *C. thermoaceticum* DSM521 was kindly furnished by H. G. Wood, Case Western Reserve University, Cleveland, Ohio.

Culture media and techniques. The solid medium for culture maintenance had the following composition (values in grams per liter): agar, 20; glucose, 5; yeast extract, 5; tryptone, 5; $(NH_4)_2SO_4$, 0.5; sodium thioglycolate, 0.5; and $MgSO_4 \cdot 7H_2O$, 0.1. The medium was prepared with distilled water. One milliliter of 0.2% resazurin was added as an indicator into Hungate tubes, heated under an overlay of CO_2 atmosphere, and sterilized for 15 min at 121°C. The sterile solidified agar tubes were then stab inoculated. Incubation of the agar tubes at 58 to 60°C for 2 to 5 days resulted in the formation of visible colonies in clusters which, when stored at 4°C, maintained their viability for periods of 6 to 12 months.

Liquid medium studies. The composition of the liquid medium is shown in Table 1. It should be mentioned that the glucose was sterilized separately, as were the sodium bicarbonate, potassium dihydrogen, and monohydrogen phosphate solutions.

Stock cultures of *C. thermoaceticum* were maintained in 10-ml Hungate tubes with the medium described in Table 1. The culture was transferred anaerobically three times per week with inoculation volumes of 0.5 to 1.0 ml into 10 ml of medium in the Hungate tubes. These tubes were incubated at 58 to 60°C. The growth of *C. thermoaceticum* in these tubes served as inoculum for shake-flask studies. Shake-flask studies were performed at 55°C in a 500-ml, anaerobic Erlenmeyer flask, containing 250 ml of medium inoculated with 20 ml of the stock culture. The flask was incubated at 60°C and overlayed with CO_2. After 24 to 36 h of incubation, these shake flasks served as inoculum for the 3-liter fermentations.

A 5-liter New Brunswick fermentor containing 3 liters of medium was inoculated with 250 ml of shake-flask growth of *C. thermoaceticum*. The temperature was maintained at 58°C, and the fermentor was agitated at 50 rpm and overlayed with CO_2. When the pH was controlled, 10 N NaOH was used as the neutralization agent.

Analytical procedures. The growth of *C. thermoaceticum* was measured spectrophotometrically at 660 nm with cuvettes that had a 1-cm light path. The optical density was maintained below 0.5 unit to be in the Beers law region. Cell dry weight determination was performed by using duplicate samples. Two tubes, each containing 40 ml of the fermentation broth, were centrifuged at 12,000 × *g* for 10 min. The supernatants were discarded, and the cell pellets were washed twice with 40 ml of distilled water and recentrifuged between washings. The final pellets were suspended in 5 ml of distilled water. The cell suspensions were transferred into

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TABLE 1. Medium composition for growth and production of acetic acid by *C. thermoaceticum*[a]

Component	Concn (g/liter)
Glucose	18.0 (or noted in text)
$NaHCO_3$	16.8
K_2HPO_4	7.0
K_2HPO_4	5.5
Yeast extract (Difco)	5.0
Tryptone (Difco)	5.0
$(NH_4)_2 \cdot 7H_2O$	0.25
$Co(NO_3) \cdot 6H_2O$	0.029
Sodium thioglycolate	0.5
$Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$	0.197
$NaMoO_4 \cdot 2H_2O$	0.12
$CaCl_2$	1[b]
Resazurin (0.2% solution)	1[b]

[a] Trace salts composition (in grams per liter): $MgCl_2 \cdot 4H_2O$, 5; Na_2SeO_3, 0.172; H_2BO_3, 0.1; $ZnCl_2$, 0.05; $Al_3(SO_4)_2 \cdot 12H_2O$, 0.1; $NiCl_2 \cdot 6H_2O$, 0.02; $CuCl_2 \cdot 2H_2O$, 0.01; EDTA, 5.0.

[b] In milliliters.

tared aluminum dishes and dried at 80°C for 24 h. The weights of the dried cells were then determined gravimetrically. When this procedure was used, one optical density unit was found to correspond to 0.65 g of cell dry weight per liter. The Summerson spectrophotometer with a red filter was also used to optically determine the cell concentration. The optical density reported as Klett units was found to be related to the cell dry weight as 280 Klett units equal 1 g of cell dry weight per liter.

Extracellular protein and DNA were measured by the following procedure. A total of 4 ml of the sample was centrifuged at 12,000 × *g* for 5 min, and 5 ml of cold 10% trichloroacetic acid was added to the supernatant. The macromolecular precipitate was first centrifuged and then washed twice with phosphate buffer. The DNA was extracted with 2.5 ml of hot 5% trichloroacetic acid (90°C) for 15 min. The supernatant was assayed for DNA by the diphenylamine method with calf thymus DNA as the standard. The pellet was assayed for protein by the biuret method with bovine serum albumin as the standard.

RESULTS AND DISCUSSION

A typical profile for a pH-controlled fermentation is shown in Fig. 1. The initial glucose concentration was 18 g/liter. A concentrated glucose solution (100 g/liter) was fed periodically to the growing *C. thermoaceticum* during the course of the fermentation to maintain the dissolved glucose concentration between 5 and 15 g/liter. The optical density of the cell increased exponentially for the first 30 h and decreased from then on to the end of fermentation. This decrease in cell mass was later confirmed to be due to cell lysis. Acetic acid production paralleled cell mass formation and reached a maximum concentration of 56 g/liter at the end (125 h). In a separate experiment to confirm cell lysis, the decrease of the optical density was correlated to the increase in the extracellular protein and DNA concentrations in the soluble medium during the stationary phase of growth. A total decrease of 2.4 optical density units (equivalent to 1.43 g of cell dry weight per liter) was accompanied by the recovery of 0.7 g of protein per liter and 0.06 g of DNA per liter in the medium. By using the value of 5% DNA based on cell dry weight and 50% protein based on cell dry weight as reported by Andreesen et al. (2), the equivalent calculated cell dry weights lost due to lysis were 1.4 and 1.2 g/liter, respectively, based on DNA and protein contents. These calculated values for the cell dry weight loss compare reasonably well with the decline in cell concentration as measured by the optical density. However, it is still not possible to state unequivocally whether cells in the stationary phase of growth were capable of acetic acid production. This is because it was not possible to measure quantitatively the growth rate or the lytic rate during the fermentation period when the optical density was decreasing (Fig. 1).

Effects of externally added sodium acetate on cell growth and product formation. The inhibitory effects of externally added end products on the rates of microbial growth are well documented (31). The growth inhibition is characterized by a period of growth arrest followed by exponential growth at a lower rate and eventually reaching a lower final cell concentration. Growth inhibition was determined either by comparing the maximum initial growth rates or by the final cell concentrations.

The addition of sodium acetate to the medium inhibits the growth of *C. thermoaceticum*. An inverse relationship between the initial growth rate and the concentration of sodium acetate was obtained (Fig. 2). Analytically, this relationship can be expressed as:

$$\mu = \mu_m \left[1 - \frac{p}{p_m}\right]$$

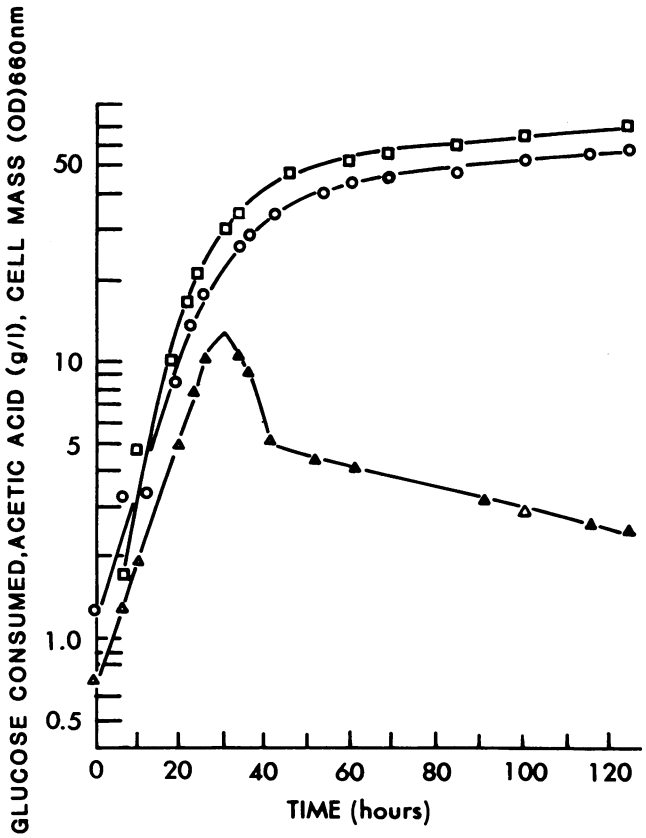

FIG. 1. Fermentation profile of a pH-controlled (pH 6.9) fermentation with *C. thermoaceticum*. Symbols: ○, acetic acid concentration (in grams per liter); □, glucose consumed; △, cell mass in optical density (OD) units.

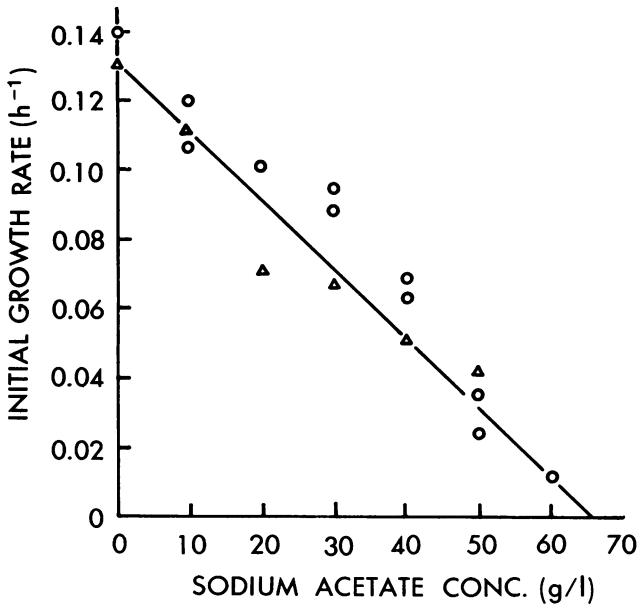

FIG. 2. Relationship between the initial growth rate of *C. thermocellum* and the externally added sodium acetate concentration (in grams per liter). Symbols: △ and ○, different sets of experiments.

where μ is the specific growth rate; μ_m is the maximum specific growth rate when p is equal to 0; p is the sodium acetate concentration (in grams per liter); and p_m is the sodium acetate concentration (in grams per liter), at which the specific growth rate is 0.

The experimental value for the maximum specific growth rate when the sodium acetate concentration is equal to 0 (μ_m) was 0.13 h^{-1}, and for the sodium acetate concentration at which the specific growth rate is 0 (p_m), it was 65 g of sodium acetate per liter in the complex medium used. The studies were performed in a 0.2 M sodium bicarbonate buffer where the initial pH was between 7.0 and 7.1. This pH was maintained during the initial growth period when the specific growth rates were measured. The decrease in the growth rate was, therefore, solely due to the sodium acetate added and not due to changes in the pH.

The final cell concentration was also measured as a function of the different sodium acetate concentrations. An inverse relationship was also found between the initial sodium acetate concentration and the final cell concentration shown in Fig. 3. Four separate experiments were performed to obtain the results on the final cell concentrations at the different sodium acetate concentrations (Fig. 3). The final pHs at the different initial sodium acetate concentrations are also shown. It can be seen that the final pH decreased as the initial sodium acetate concentration was decreased. This behavior is a result of the production of acetic acid. At low initial sodium acetate concentrations, a proportionately higher amount of acetic acid was produced due to the increased cell growth. This in turn exceeded the buffering capacity of the sodium bicarbonate, resulting in a decrease of the final pH of the medium.

To determine the cause of growth inhibition by sodium acetate, we studied and compared the inhibition pattern by other sodium salts (sulfate and chloride). If sodium ion is responsible for the growth inhibition of *C. thermoaceticum*, it would then be expected that the inhibition pattern for all sodium salts would be identical at a given sodium ion concentration. The inhibition pattern was determined by measuring the final cell concentration attained and the initial growth rate. The relationship between the final cell concentration and the different concentrations of sodium salts is shown in Fig. 4. The 50% growth inhibition for the sodium ion was found for each salt in Fig. 4 to be (in grams per liter): acetate, 10; chloride, 16; and sulfate, 23. These results show that the sodium ion per se is not the chemical species totally responsible for growth inhibition. However, since sodium acetate exhibited the greatest inhibitory effect on growth, as compared with the other sodium salts, one might conclude that the sodium ion could be a partial contributor to the inhibition.

Similar analysis with a 50% inhibitory concentration based on the total amount of solute can be calculated with the data in Fig. 4. The calculations show that the 50% inhibitory concentration based on the total amount of solutes were sodium acetate, 0.41 M; sodium chloride, 0.69 M; and sodium sulfate, 0.5 M. These different behaviors also suggest that ionic strength is probably not the cause of growth inhibition.

The relationship between the initial growth rate and the sodium concentration with sodium sulfate and sodium acetate is shown in Fig. 5. The 50% growth rate inhibition was found to be 19 g/liter for sodium when sulfate was used and 10 g/liter for sodium when acetate was used. These results further suggest that sodium in combination with acetate is responsible for growth inhibition.

The preceding results comparing the growth inhibitory effects of different sodium salts show that sodium ion is not totally responsible for growth inhibition and imply that the acetate ion also contributes to growth inhibition. Clearly, the obvious experiment for confirming acetate as the cause of growth inhibition is a comparison of the growth inhibitory effects of different acetate salts. The results from these experiments with sodium, potassium, and ammonium acetate are shown in Fig. 6. Unfortunately, both potassium and ammonium ions were found to be more toxic than either

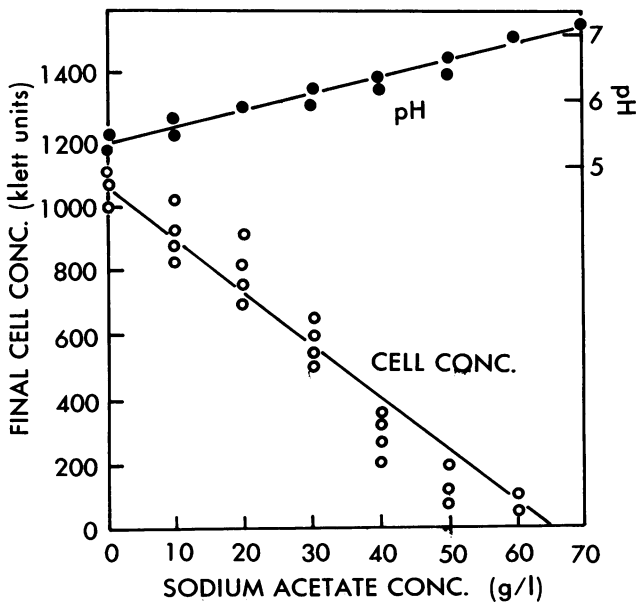

FIG. 3. Relationship between the final cell concentration and final pH and externally added sodium acetate concentration (in grams per liter). Symbols: ○, final cell concentration; ●, final pH.

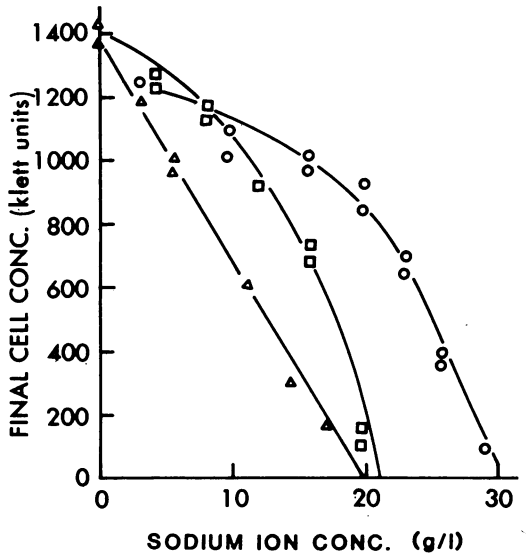

FIG. 4. Effects of sodium ion as different salts on the final cell concentration (in grams per liter) of *C. thermoaceticum*. Symbols: △, sodium acetate; □, sodium chloride; ○, sodium sulfate.

sodium or acetate ions. These results are supported by separate observations that complete growth inhibition by ammonium and potassium chloride occurred at 5 and 25 g/liter, respectively. The relative magnitude of inhibition by the cations on the growth rate was found to be $NH_4^+ > K^+ > Na^+$. At neutral pH with sodium hydroxide as the neutralizing agent, acetate ion was the dominant ion responsible for growth inhibition. The results also agree with other pH-controlled fermentations (6); the highest final product concentration was achieved with sodium hydroxide rather than potassium or ammonium hydroxide as the neutralizing agent.

Growth inhibition at low pH. Thus far, all of the inhibition studies were performed at pH 7.0. The results also suggest that the acetate ion exerts the inhibitory effect on growth. However, it is our belief that the cause for inhibition of cell growth and acetic acid formation below pH 7.0 is different than the cause for inhibition above this pH. The evidence leading to this conclusion can best be seen in Table 2.

The results in Table 2 were obtained when different concentrations of sodium acetate, sodium sulfate, and sodium propionate were added initially to the fermentation medium containing 18 g of glucose per liter. In the presence of the added salts, acetic acid was produced resulting in a decrease in pH. In Table 2 the initial salt concentrations and the amount of acetic acid produced are shown. For sodium acetate, the final acetic acid concentration represents what was added plus what was produced. Also shown in Table 2 are the final pH values from these fermentations.

From the final pH and the final acetic acid concentration, the undissociated acetic acid concentration can be calculated by the Henderson-Hasselbalch equation. The undissociated acetic acid concentration was found to be constant. At all sodium sulfate concentrations, the final pHs and the final acetic acid concentrations were approximately constant at 5.4 and 15 g/liter, respectively. The calculated undissociated acetic acid concentration for all salts tested was essentially between 0.04 and 0.05 M. The values of the final pH and the final acetic acid concentration for the added propionate salt were quite similar compared with those for the added acetate salt. The calculated total undissociated acid concentration

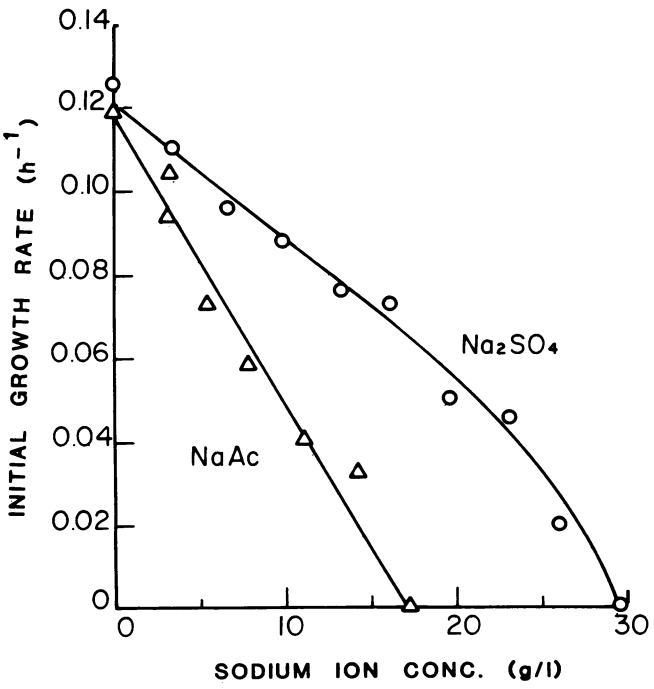

FIG. 5. Comparison of the initial growth rates of *C. thermoaceticum* at different sodium ion concentrations (in grams per liter). Symbols: △, sodium acetate; ○, sodium sulfate.

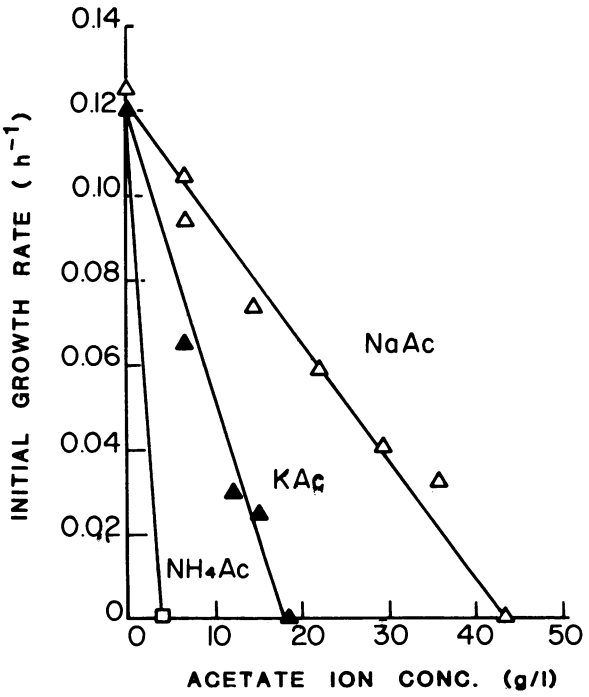

FIG. 6. Comparison of the initial growth rates of *C. thermoaceticum* at different acetate ion concentrations (in grams per liter). Symbols: □, ammonium acetate; ▲, potassium acetate; △, sodium acetate.

TABLE 2. Calculation of undissociated acetic acid concentration in the fermentation broth of *C. thermoaceticum* with different sodium salts

Initial concn of the following added salts: (g/liter)	Final acetic acid concn (g/liter)	Final pH	Undissociated acid concn (M)
Sodium acetate			
0	15.3	5.39	0.049
0	15.3	5.40	0.048
10	22.3	5.60	0.048
20	29.5	5.71	0.050
30	35.6	5.80	0.051
Sodium sulfate			
10	14.5	5.40	0.044
20	15.1	5.46	0.041
30	15.2	5.40	0.048
40	14.5	5.40	0.044
Sodium propionate			
10	13.4	5.66	0.040[a]
20	13.4	5.79	0.040[a]
30	12.5	5.99	0.039[a]

[a] Total amount of acetic acid and propionic acid.

(acetic acid plus propionic acid) was 0.04 M when the propionated salt was added. This value again is quite similar to the calculated undissociated acid concentration of 0.05 M when the acetate salts were added. These results suggest that cell growth and product formation of *C. thermoaceticum* will stop once the undissociated acetic acid concentration reaches a value between 0.04 and 0.05 M.

From the results presented thus far, we conclude that both acetate ion and undissociated acetic acid can exert inhibitory effects on the growth of *C. thermoaceticum*. Quantitatively, total growth inhibition by undissociated acetic acid occurs when its concentration is between 0.04 and 0.05 M (~2.79 g/liter). By using these criteria for growth inhibition, it is now possible to calculate the concentrations of the respective species by the Henderson-Hasselbalch equation as a function of pH. These calculations are presented in Table 3.

In the calculations for the different chemical species, it was assumed that the maximum acetate achievable in the acetate ion concentration would be 48 g/liter (0.8 M). From this assumption, the respective undissociated acetic acid concentration and the total acetic acid concentration (undissociated acetic acid plus acetate ion) can be calculated at different pHs. From the results in Table 3, it can be seen that below pH 6.0, 2.7 g of undissociated acetic acid per liter is reached before attaining an acetate ion concentration of 48 g/liter. Above pH 6.0, the inhibitory concentration of acetate ion would be reached before attaining the maximum inhibitory undissociated acetic acid concentration of 2.7 g/liter.

TABLE 3. Calculation of acetic acid concentration and undissociated acetic acid concentration at different pHs for 48 g of acetate ion per liter

pH	Undissociated acetic acid concn (g/liter)	Total acetic acid concn (g/liter)
5.0	37.3	85.3
5.2	17.0	65.0
5.5	8.5	56.5
5.7	5.4	53.4
6.0	2.7	50.7
6.2	1.1	49.1
6.4	1.1	49.1
6.8	0.43	48.4
7.0	0.27	48

Qualitatively, the results from this study are in agreement with those published previously (5) on the inhibitory effects of acetate ion and undissociated acetic acid on *C. thermocellum*. Specifically, the previous findings (5) have shown that the organism is far more sensitive to the free acid than the acetate ion. The major difference between the results in this paper and those previously published (5) is the absolute concentrations of the acetate ion and the free acid which *C. thermoaceticum* can produce and tolerate. For example, in the previous report (5), the maximum acetic acid concentration obtained was 20 g/liter at pH 7.0. On the other hand, the maximum acetic acid concentration obtained in the present study was 56 g/liter at pH 6.9. Therefore, the inhibitory levels of the free acid and the acetate ion on *C. thermocellum* differed between the two studies. The equilibrium calculations in this study (Table 3) were presented by the previous authors (5). The conclusions from our study are identical to those previously presented (5).

These results are important if mutation and selection programs are to be considered to obtain overproducers. The type of chemical species and its concentration must be considered with care when the selection process is used for finding overproducer mutants of this organism.

ACKNOWLEDGMENTS

We wish to acknowledge the financial support of the U.S. Department of Energy (contract no. EG-077-S-02-4178), the Union Carbide Corp. and the Monsanto Chemical Co.

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