Effect of pH on organic acid production by *Clostridium propionicum* in test tube and fermentor cultures

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SUMMARY

Clostridium propionicum is a chemical autotroph that metabolizes alanine to propionic acid (reduction product) and acetic acid (oxidation product). The ratio of propionate/acetate predicted by the electron balance is 2 : 1. This study reports the effect of pH on growth and organic acid production by this organism when grown in both test tube cultures initially buffered from pH 7.0 to 5.0, and in fermentors maintained at pH 7.0 and 6.5. Highest growth and organic acid production was found at pH 7.0 in both cases. HPLC analysis showed that at pH 7.0, the ratios of propionate to acetate were 0.45 : 1 (stationary tube, 24 h). The highest ratio observed was 1.8 : 1 (stationary tube, pH 6.0, 24 h). This tube produced 8.5% of the acids produced in the pH 7.0 culture tube. The identify of the major portion of the reduction products of the organism remains unknown.

INTRODUCTION

Acrylic acid and its esters are important industrial materials currently made from petroleum and natural gas. As the reserves of petroleum and natural gas diminish, alternative sources are being examined, including bacterial fermentation processes. The anaerobic acrylate biosynthetic pathway in *Clostridium propionicum* has been studied as a possible fermentation method for acrylic acid production [1,14]. This pathway, which converts alanine to acetate, propionate, CO_2, and H_2O, involves acrylyl-CoA as an intermediate.

The effect of conditions to increase acrylate production has been studied and has met with only limited success [1,14]. This paper presents data from kinetic studies on the effect of pH on formation of short chain organic acids during the active growth of *C. propionicum* under anaerobic conditions. Cultures were grown both in test tubes with varied pHs and in fermentors with constant pHs.

MATERIALS AND METHODS

Bacteria and growth conditions. C. propionicum ATCC 25522 [4] was maintained in 0.075% agar by the procedure described for aerotolerant anaerobes [6]. Vigor of the culture was maintained by daily transfers, using a 1-ml inoculum (injected into the anaerobic zone below the shallow aerobic zone at the top)/10 ml fresh media in a fermentor tube. This was repeated at least 14 days prior to use.

The basic medium contained per liter (in distilled water): 8 g alanine, 3 g peptone, 4 g yeast extract, 0.2 g cysteine HCl, 0.05 g $MgSO4 \cdot 7\ H_2O$, 0.01 g $FeSO4 \cdot 7\ H_2O$, 5 ml 1 M potassium phosphate buffer, pH 7.0, 2.5 ml saturated $CaSO_4$ solution, and 1 ml 0.2% resazurin solution (as redox indicator).

To minimize pH change during sterilization, media were heated to near boiling, cooled to room temperature, and pH adjusted to the desired level (7.0, 6.5, 6.0, 5.5, and 5.0) by the addition of 1.5 N HCl or NaOH. Solutions were dispensed into containers and sterilized. All fermentations were conducted at 30 °C.

No endospore/spore formation was observed during these experiments, although endospores could be observed in stationary cultures maintained at least 5 days.

Test tube fermentations. Test tube cultures were prepared by transferring 10.0 ml of buffered media containing agar into fermentation tubes, approximately 3/4 filled, sterilizing with loosened caps, followed by tightening while still hot. Under these conditions, only a small portion of the media became aerobic, as indicated by the narrow red (aerobic) zone at the top. Uninoculated tubes used as controls showed no appreciable O_2 uptake during this experiment. One-ml portions of 1-day cultures were used to inoculate the anaerobic portion of the media in the

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10-ml fermentation tubes, which were then incubated. Three tubes of each pH set were withdrawn periodically for analysis. The absorbance of each tube was determined and the values were averaged. The contents of the three tubes were pooled for determination of acid content. An exact volume of 10.0 ml was acidified with 0.2 ml of 18 N H_2SO_4 and frozen for later analysis by HPLC.

Continuous fermentations. The Mouse™ fermentor*, manufactured by Queue Systems, Parkersburg, WV 26102, was used for continuous fermentations. The 2.0-l flat fermentation vessel was equipped with a nitrogen sparger, stirrer, automatic temperature control, probes for monitoring pH and oxygen saturation, sample outlet, and septa for inoculation and addition of reagents (0.5 N NaOH and 0.5 N HCl). Inlet and outlet gas lines were protected by gas filters of 0.2 μm. Addition of base or acid was controlled automatically through the pH probe. After the fermenter vessel (containing 1 l of media) was assembled and sterilized, the system was sparged with N_2 to 0% O_2 saturation, and the temperature was adjusted to 30 °C. Stirring speed was 400 rpm. For inoculation of fermentation vessels, 100-ml portions of media were dispensed into screw top bottles. Fermented test tube cultures (1 day, 10 ml) were used to inoculate the bottles, which in turn were allowed to develop 1 day before being used to inoculate the fermentation vessel. After inoculation, the N_2 was shifted from sparge to overlay to prevent removal of volatile materials. Fresh media and fermented broth were added and removed at identical rates, 0.06 vol/h. Samples of 5 ml were withdrawn at 30 min intervals for growth determination by absorbance. Samples of 15 ml were withdrawn at 10 and 24 h for acid determinations; exact volumes of 10.0 ml were acidified with 0.2 ml of 18 N H_2SO_4 and frozen. The results given are from single runs of the fermentor maintained at pH 7.0 and 6.5.

Growth measurements. Optical scattering of the cell suspension at 695 nm in a Beckman DU-6 UV-Visible spectrophotometer was used for estimation of growth rate. No interference was observed from the pink oxidized form of resazurin.

HPLC acid assay. A modification of the procedure of Guerrant [10] was used for organic acid determinations. Frozen acidified samples (10.2 ml) were thawed and extracted as described. HPLC separations were carried out using an Aminex-HPX-37H-87H ion exclusion column, 300 × 7.8 mm (Bio-Rad Laboratories, Richmond, CA), with a mobile phase of 5% (v/v) acetonitrile/0.07 N H_2SO_4, a flow rate of 0.5 ml/min, and ambient (20 °C) temperature. Samples used for HPLC analysis contained 1% of the volume of the final extract. Detection was by UV absorbance at 210 nm. Identities of the acids were established by comparison of retention times with known acids, and confirmed by co-chromatography with standard solutions. The purest available grades of the organic acids were obtained from Aldrich Chemical Co., Metuchen, NJ, and used to prepare standard solutions with the HPLC liquid phase as solvent. Extracts of uninoculated media used as controls gave small peaks in the positions of formic and 3-phenylpropionic acids. Corrections were made for these factors.

RESULTS

Growth characteristics

The original *C. propionicum* ATCC 25522 culture grew slowly. Activity of the culture was increased by daily transfers for several weeks; growth rate dropped sharply upon cessation of the daily transfers. The change in growth rate appears to be reversible as vigor could be restored by a new sequence of transfers. However, the growth rate of this activated strain is low when compared to the rates obtained with *C. sporogenes* 3121 [11].

Growth in test tube fermentations

The initial pH has a marked effect on both the specific growth rate and final cell density (Fig. 1, Table 1). Test tube cultures with initial pH 7.0 and 6.5 had both the highest initial growth rates and greatest final cell density. The maximum specific growth rates observed, 0.1 Δ absorbance (695 Mm h^{-1}), were at pH 7.0 and 6.5 and lasted 3 h before diminishing. Comparable final cell con-

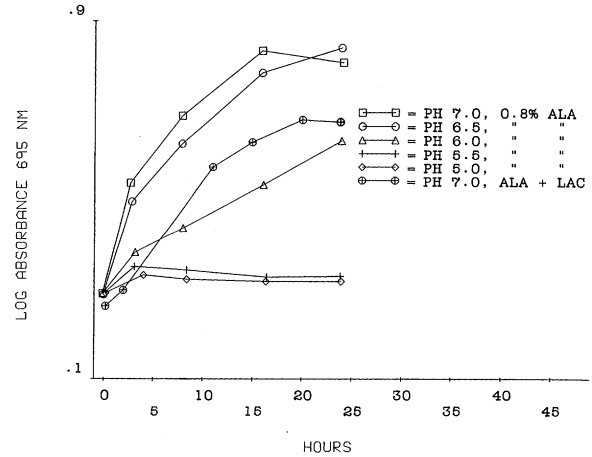

Fig. 1. Growth of vigorous strain of *C. propionicum* ATCC 255[illegible] in a 0.8% alanine medium at different initial pHs.

* Reference to brand or firm name does not constitute endorsement by the U.S. Department Agriculture over others of a similar nature not mentioned.

TABLE 1

Effect of initial pH on organic acids by *C. propionicum* ATCC 25522 test tube cultures

Initial pH	Final pH	From alanine			Metabolic products[a] From other amino acids					Formate	Total	Ratio pro/ac
		ac	prop	(total)	*i*-but	*n*-but	*i*-val	*n*-val	(total)			
		Conc (μmol/ml)										
pH 7.0												
10 h	6.28	9.4	6.9	(16.3)	0.6	1.3	2.8	0.9	(5.6)	1.7	23.6	0.73
24 h	5.95	13.9	6.3	(20.2)	0.6	1.6	1.0	–	(3.2)	1.4	24.8	0.45
pH 6.5												
10 h	6.18	5.5	4.4	(9.9)	0.6	0.5	2.4	0.5	(4)	1.2	15.1	0.8
24 h	5.87	12.5	1.8	(14.3)	0.3	1.1	0.4	0.2	(2)	1.5	17.8	0.14
pH 6.0												
10 h	5.95	2.1	1.7	(3.8)	0.2	0.2	0.8	0.1	(1.3)	0.8	5.9	0.81
24 h	5.82	0.5	0.9	(1.4)	0.2	–	0.4	–	(0.6)	0.1	2.1	1.8
pH 5.5												
10 h	5.74	2.0	2.1	(4.1)	0.2	0.2	0.8	0.2	(1.4)	0.8	6.3	1.05
24 h	5.62	1.2	1.4	(2.6)	0.1	0.2	0.3	0.3	(0.9)	0.5	4	1.17
pH 5.0												
10 h	5.33	2.3	1.5	(3.8)	0.2	0.2	0.2	–	(0.6)	0.8	4.4	0.65
24 h	5.30	0.7	0.6	(1.3)	0.1	0.2	0.2	–	(0.5)	0.8	1.8	0.86

[a] ac, acetate; prop, propionate; i-but, *i*-butyrate; n-but, *n*-butyrate; i-val, *i*-valerate; n-val, *n*-valerate.

centrations (as measured by optical density) were observed at pH 7.0 and 6.5. Growth was distinctly slower at pH 6.0. At pH 5.5 and 5.0, small increases in absorbance lasted for 3 h, suggesting a period of initial growth. This was followed by a stationary period lasting 24 h during which slight decreases in absorbance occurred. Over a 24-period at 30 °C, both the pH 7.0 and pH 6.5 test tube cultures grown on 0.8% alanine medium had a specific growth rate of ca. 0.03 h^{-1}. This compares favorably with a previously observed growth rate, 0.024 h^{-1}, of the same organism grown on 0.3–0.9% alanine at 37 °C [1].

Growth in continuous fermentations

Fermentor experiments were conducted at pH 7.0 and 6.5 (Fig. 2, Table 2). Both experiments had a turnover rate of 0.06 vol/h. Both fermentors, after 2-h lag periods, had

TABLE 2

Organic acids produced by *C. propionicum* ATCC 25522 in fermentor cultures

	From alanine			Metabolic products[a] From other amino acids					Formate	Total	Ratio pro/ac
	ac	prop	(total)	*i*-but	*n*-but	*i*-val	*n*-val	(total)			
	Conc. (μmol/ml)										
Maintained at pH 7											
10 h	13.9	1.4	(15.3)	0.5	1.4	1.5	0.7	(4.1)	1.4	20.8	0.1
24 h	20.1	1.3	(21.4)	0.4	0.7	1.5	0.4	(3.0)	0.8	25.2	0.07
Maintained at pH 6.5											
10 h	10.7	1.6	(12.3)	0.2	1.4	0.4	–	(2.0)	0.9	15.2	0.15
4 h	10.1	0.5	(11.6)	0.4	0.4	0.2	0.4	(1.4)	0.4	13.4	0.05

, acetate; prop, propionate; i-but, *i*-butyrate; n-but, *n*-buyrate; i-val, *i*-valerate; n-val, *n*-valerate.

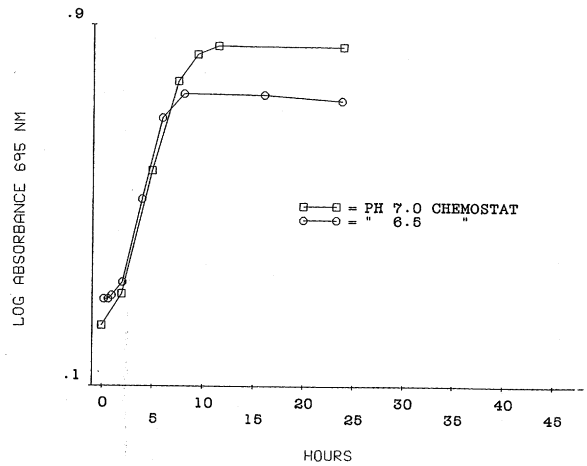

Fig. 2. Growth of a vigorous strain of *C. propionicum* ATCC 25522 in 0.8% alanine medium in fermentors maintained at 7.0 and 6.5, with a constant flow rate of 0.06 vol/h.

maximum specific growth rates of 0.09 Δ absorbance (695 Mm) h^{-1}. The pH 7.0 fermentor attained a higher absorbance 695 nm (0.85) than the pH 6.5 fermentor (absorbance 695 nm = 0.74). After reaching these maxima, the level of cell material in each fermentor (measured by absorbance) remained constant for the duration of the experiment (24 h).

DISCUSSION

The major organic acids detected in this study were acetic and propionic acids, the expected metabolites from alanine [14]. Other organic acids derived from amino acids were produced in much smaller quantities. The higher saturated acids found in this study, *i*-butyric, *n*-butyric, *i*-valeric, and *n*-valeric are believed to be metabolic products of longer chain amino acids contained in the peptone in the medium. *C. propionicum* has been reported to convert threonine to propionate and butyrate. This organism also converts valine to isobutyrate, while leucine and isoleucine are converted to isovalerate [7]. Phenylalanine and tyrosine have been reported to form small amounts of phenylacetic and *p*-hydroxyphenylacetic acids, but these were not detected [9]. Formation of *n*-valeric acid, not previously reported from *C. propionicum*, is reported here on the basis of retention time and co-chromatography. Formic acid, identified on the same basis, has not been previously reported with *C. propionicum*; it has, however, been reported as a metabolite from *C. sporogenes* [11].

Acrylic acid, involved as the acrylyl-CoA intermediate in the anaerobic acrylate biosynthetic pathway of *C. propionicum* [1,14], was not detected as a free acid. Previous studies also found no effective method for producing acrylic acid, which could theoretically be formed by shunting acrylate off from the anaerobic pathway [1]. Anaerobic metabolism produced trace amounts of acrylate under exceptional circumstances. Small quantities of acrylate accumulated after incubation of whole cells of *C. propionicum* with lactate in the presence of 3-pentynyl-CoA (an inhibitor of acyl dehydrogenase) [2]. Acrylate was the product of dehydration of (R)-lactate catalyzed by cell-free extracts of *C. propionicum* [13].

C. propionicum under aerobic circumstances produced acrylate by oxidation of propionic acid when whole cells are exposed to O_2. This was most effective in the presence of a synthetic electron acceptor, methylene blue [1]. However, these conditions were toxic to the cell, and the acrylate production was transient.

All test tube fermentations (Table 1) showed a considerable drift from the initial pH. The final pH ranged from 6.28 to 5.30. Acid production was highest in the test tube fermentations adjusted to an initial pH 7.0, with acid production decreasing with lower initial pH (Table 1). Acid production was much lower in the tubes initially buffered to pH 6.5, 5.5, and 5.0. Both acetate and propionate concentrations decreased after 10 h. The highest propionate : acetate ratio observed was 1.8 : 1, the value obtained with tubes initially buffered at pH 6.0. At the lowest pHs, a brief period of growth was followed by a long period in which no additional growth was observed, although changes in acid composition indicated that the metabolism of these resting cells was still active.

Cell growth in the fermentor cultures was considerably more rapid than cell growth in the test tube cultures, reaching maximum growth in 6–10 h. There was no essential difference in growth between the fermentor cultures at the two pHs examined, pH 7.0 and 6.5. The test tube cultures required 15–20 h, with greatest growth in tubes at initial pH 7.0. Growth decreased in tubes buffered to lower initial pHs.

The general metabolic inactivity and low acid production at low pH agrees with observations regarding the uncoupling effect of acetate at low pH [3]. A pH differential of 0.4–0.7 pH units between the interior and exterior of the cell is apparently essential for substrate accumulation and other transport processes. The differential is maintained by ATPase driven extrusion of protons from the cells [12]. However, acetic acid, in its un-ionized form, acts to uncouple this process by diffusing passively across the cytoplasmic membrane, effectively rendering the membrane permeable to protons [3]. Thus, as the pH of the medium approaches the p*K*a of acetic acid (pH 4.8) where it is less ionized, there is effective destruction of the pH gradient and associated metabolic processes (including acid production).

Clostridium propionicum is considered an anaerobic chemophore, obtaining its energy from coupled oxidation/reduction of alanine to form acetate as the oxidation product and propionate as the reduction product. The balanced redox reaction predicts a 2 : 1 propionate : acetate ratio [1,14]. This ratio was not found in our studies. We found a preponderance of acetate, with the level of propionate only 5–15% of the acetate. Only under the conditions of slow growth and minimal acid production in the test tube cultures adjusted to pH 6.0, 5.5, and 5.0, was there slightly more propionic acid, and even here the composition did not attain the expected 2 : 1 ratio of propionate to acetate. We interpreted these results to indicate that the metabolism of this rapid-growing culture of *C. propionicum* affords additional outlets for reduction than the formation of propionate.

Proteolytic *Clostridia* (including *C. propionicum*) contain long chain fatty acids [8]. Formation of cells in a rapidly growing culture would require biosynthesis of considerable quantities of the cellular fatty acids. This presents an outlet for reducing power, [H·], either as NADH or ferrodixin-coupled reduction. We consider this was the most probable outlet for the remainder of the reducing power. Other possible outlets for surplus [H·] such as hydrogen evolution [5] or formation of formic acid [15] would not appear to be sufficient to account for the missing propionate. Further studies with complete fermentation balances are needed for verifying the importance of these possible outlets.

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