Effect of pH and Oxygen on Growth and Viability of *Acetivibrio cellulolyticus*

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Growth, hydrogen production and cellulose digestion by *Acetivibrio cellulolyticus* strain CD2 were considerably greater when the culture pH was maintained at 7·0 than when the pH was not controlled. Furthermore, if the pH of the growth medium was controlled the number of viable organisms was 6-fold greater after 3 d incubation and 100-fold greater after 6 d incubation compared with equivalent cultures in which the pH was not controlled. The differences were due to the combined effect of low pH values and acetic acid accumulation. The number of viable organisms was 2- to 3-fold lower after 12 h incubation in substrate-free medium containing 40 mM-acetic acid at pH 5·5 than in the same medium at pH 7·0. Addition of 90 mM-acetic acid during growth in a cellobiose-containing medium lowered the growth rate by 30% and the rate of hydrogen production by 40%. Exposure of *A. cellulolyticus* to oxygen for up to 2 h did not affect viability measurements provided that the organisms were subsequently transferred to reduced media.

INTRODUCTION

Acetivibrio cellulolyticus can grow on cellulose, cellobiose and salicin (Patel *et al.*, 1980). As growth proceeds the pH of the growth medium drops from 7·0 to 5·5 (in the absence of pH control) due to the accumulation of acetic acid; this is one of the major metabolic end-products, others being H_2, CO_2, reducing sugars and ethanol (Khan *et al.*, 1980; Patel *et al.*, 1980). The aim of this study was to determine the effects of acetic acid accumulation and decreasing pH on the growth, viability and cellulose-degrading ability of *A. cellulolyticus*. As *A. cellulolyticus* is an obligate anaerobe which requires pre-reduced media for growth (Patel *et al.*, 1980), an additional aim was to examine the effect of exposure to O_2 on the viability of *A. cellulolyticus*.

METHODS

Bacterial strain and growth conditions. Acetivibrio cellulolyticus strain CD2 (National Research Council of Canada culture collection no. 2248, ATCC 33288) was maintained in 10 ml basal medium (Patel *et al.*, 1980) containing 30 mg white facial tissue paper (Kleenex, Kimberly Clark Co., Canada), incubated at 35 °C and subcultured at weekly intervals. The tissue was present as strips (10 × 35 mm) in serum vials which were flushed with an O_2-free gas mixture of N_2/CO_2 (80:20, v/v). Unless stated otherwise, all media preparation and growth experiments were conducted using this gas mixture. The basal medium was pre-reduced by the serum bottle modification (Miller & Wolin, 1974) of the Hungate (1950) technique, using 8 ml l^{-1} of the cysteine/Na_2S reducing solution [1·25% (w/v) cysteine.HCl and 1·25% (w/v) $Na_2S.9H_2O$ in distilled water (Holdeman & Moore, 1975)]. Portions (10 ml) of the reduced basal medium were dispensed into the serum vials containing the cellulose. The vials were sealed, autoclaved at 121 °C for 15 min, and then a further 0·08 ml reducing solution was added to each vial. The initial pH was 7·0 ± 0·2.

For the experiments, culture vessels (250 ml or 500 ml Erlenmeyer flasks with side arms) contained 100 ml basal medium supplemented with 1 g facial tissue paper or D(+)-cellobiose (Eastman Kodak) and were sealed with serum stoppers. The medium was pre-reduced by the addition of 1·5 ml reducing solution per 100 ml basal medium,

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1·0 ml of which was added before autoclaving and the remainder afterwards. Cellobiose was added to the autoclaved basal medium as a 10% (w/v) filter-sterilized (Sartorius filter, 0·2 μm average pore diameter) solution which was made anaerobic by addition of 1·5% (v/v) reducing solution. To determine the effect of high acetic acid concentrations on growth and H_2 production in media with the pH controlled at 7·0, acetic acid solutions adjusted to pH 7·0 were added to the autoclaved basal medium to give initial concentrations of up to 90 mM. All cultures were incubated at 35 °C on a rotary shaker operating at 120 rev. min^{-1}. The head space in the sealed flasks was flushed daily to minimize any possible toxic effects of accumulated H_2. The inoculum consisted of 2% (v/v) of a 72 h culture grown on cellulose or a 24 h culture grown on cellobiose (with growth pH controlled at 7·0), for cellulose and cellobiose test substrates, respectively. Where indicated, the pH value was maintained at 7·0 during growth by the addition of sterile, anaerobic 2·5 M-NaOH every 4 to 6 h during the growth period; the maximum deviation from pH 7·0 in these cultures was less than 0·3.

The combined effect of low pH (5·5) and high acetic acid concentration (40 mM) on viability was determined on exponential phase, cellobiose-grown cells. Cells were harvested from an exponentially growing culture (A_{660} 0·75) by centrifuging (16300 *g*, 15 min) and resuspended in pre-reduced basal medium (pH 7·0) at 30 times the initial concentration. Then 1 ml of the concentrated cell suspension was injected into sealed vials containing 29 ml pre-reduced basal medium adjusted to pH 7·0 or 5·5 and supplemented with 0, 25 or 40 mM-acetic acid. The vials were incubated at 35 °C. (These tests were done on harvested organisms resuspended in substrate-free medium because the organism does not grow at pH values below 6·5.)

Measurement of growth and viability. Growth in cellobiose medium was followed by measuring the turbidity at 660 nm using a Spectronic 20 spectrophotometer (Bausch & Lomb, Rochester, N.Y., U.S.A.). Viable cells were estimated by the most probable number (MPN) technique (Taras *et al.*, 1976). The sample was serially diluted into pre-reduced basal medium and each dilution was inoculated into five replicate vials of cellulose or cellobiose medium. A positive result was assessed by visual observation of tissue paper digestion, by culture turbidity and microscopy. Statistical tables were used for the calculation of the MPN of viable cells in the original sample (Taras *et al.*, 1976).

Estimation of cellulose degradation and the production of H_2 and acetic acid. The cultures were centrifuged at 16300 *g* for 20 min and the pellet of cells and cellulose was extracted with an acetic acid/nitric acid reagent (Sloneker, 1971). This extraction disrupted the cells and solubilized protein, lipid, lignin and hemicellulose (which were discarded in the supernate), leaving the cellulose fibres intact. The cellulose pellet which remained after extraction was dissolved in 75% (w/v) H_2SO_4 and carbohydrate was estimated by the anthrone reagent (Herbert *et al.*, 1971) using Whatman CF11 cellulose powder as a standard. When 10 g facial tissue paper was extracted as above, 8 g was recovered as cellulose; this was taken to be the initial cellulose concentration in Fig. 1 (*b*).

After a period of growth, the gas pressure in the sealed flasks was equilibrated to atmospheric pressure by releasing the gas into a 100 ml burette filled with water, and the H_2 content was assayed by gas chromatography (Van Huyssteen, 1967). Acetic acid in the medium was assayed as described by Ackman (1972).

Determination of oxygen tolerance. A 60 h culture in cellulose medium (100 ml) was transferred to a sterile 1 l Erlenmeyer flask plugged with sterile cotton. The culture was incubated at 25 °C and aerated by means of a magnetic stirrer (150 rev. min^{-1}). At 0, 2, 5 and 24 h, 7 ml samples were aseptically removed. Part of each sample (2 ml) was flushed with N_2/CO_2 (80:20, v/v) and serially diluted for the determination of the numbers of viable cells. Dissolved O_2 and redox potential were determined on the remaining 5 ml of each sample. The dissolved O_2 was measured with a polarographic oxygen probe (Beckman 39065) and monitored with a Beckman model 777 laboratory oxygen analyser. The oxygen probe was standardized with atmospheric O_2 and with distilled water saturated with atmospheric O_2. The redox potential (E_h) was measured using a platinum/calomel electrode (PK149, Radiometer) and a pH meter (PHM26, Radiometer).

All tests were conducted in duplicate and were repeated at least twice. Typical results are presented.

RESULTS AND DISCUSSION

A lag period of about 1 d was observed (Fig. 1 *a*, *b*) when an actively growing culture of *A. cellulolyticus* was transferred to fresh medium. This was initially thought to be due to a low cellulolytic activity because of the small inoculum size (2%, v/v). However, increasing the inoculum size to 10% (v/v) did not appreciably decrease the lag period. After 1 d incubation, H_2 production (Fig. 1 *a*) and cellulose degradation (Fig. 1 *b*) began concurrently. Between 1 and 3 d of incubation about 60% of the cellulose initially available (8 g l^{-1}) was digested. In the absence of pH control, the pH decreased from 7·0 to between 6·0 and 5·5 during this period; the lower value was at least 0·5 pH units below the minimum growth pH (6·5) for *A. cellulolyticus* (Patel *et al.*, 1980). The pH finally reached pH 5·5, a value which is close to the

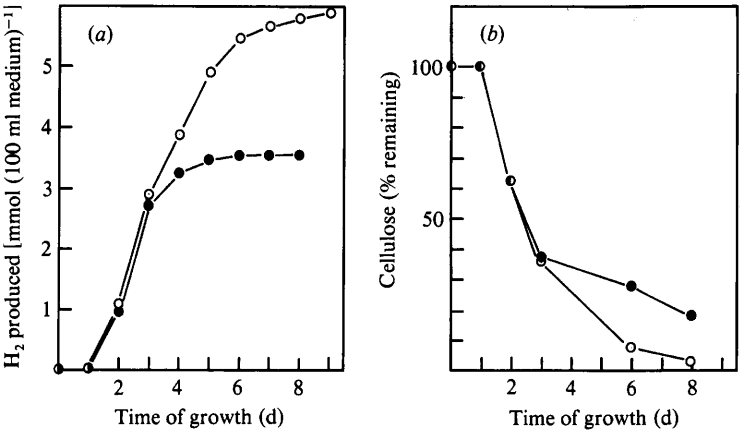

Fig. 1. Growth of *A. cellulolyticus* in medium containing cellulose with the pH maintained at 7·0 (○) and with the pH not controlled (●): (*a*) cumulative H_2 production; (*b*) percentage of cellulose remaining.

optimum for cellulase activity of this organism (Saddler & Khan, 1980). About 20% of the cellulose initially available remained undegraded after 8 d incubation in the absence of pH control. However, in cultures controlled at pH 7·0 during growth, most of the substrate was degraded after 8 d and approximately 70% more H_2 was produced (Fig. 1 *a*, *b*). These results indicated that for complete degradation of cellulose the pH must be maintained at 7·0 to maximize growth, even though this is higher than the optimum pH for cellulase activity.

Increasing the ratio of the volume of the growth medium to that of the culture vessel gas phase from 1:2 to 1:4 resulted in the production of about 15% more H_2 than that shown in Fig. 1 (*a*), probably due to the reduction of the partial pressure of H_2 in the larger volume gas phase. Cellulose degradation was not affected. High H_2 concentrations are known to inhibit H_2 production by some anaerobes (Zajic *et al.*, 1978). Our observations during cultivation (500 ml flasks) in cellobiose medium (with growth pH controlled) indicated that about 15% H_2 accumulated in the gas phase after 24 h incubation if the head space was not flushed with N_2/CO_2 (80:20, v/v) every 12 h. Although the growth (A_{660} 1·0) and cumulative H_2 produced [5 mmol (100 ml medium)$^{-1}$] after 24 h incubation were similar to those for cultures in which the head space was flushed every 12 h, the cumulative H_2 production after 36 h incubation (6·38 mmol) was 15% lower than that for cultures in which the head space was flushed. Since the other growth conditions were identical, the inhibition of H_2 production observed between 24 and 36 h of incubation was most likely due to the >15% H_2 accumulation in the gas phase.

The viability of *A. cellulolyticus* growing on cellulose (Table 1) could not be ascertained accurately before 2 d incubation since the cells could not be quantitatively dislodged from the insoluble substrate to which they were attached. The zero-time viability was calculated from the viability of the inoculum. The maximum number of viable cells was observed after 3 d incubation (Table 1). At this time the viability of pH-controlled cultures was about six times greater than that of cultures with no pH control. Irrespective of the growth conditions, there was a 15- to 25-fold decrease in the number of viable organisms between 3 and 4 d of incubation. However, for cultures with no pH control, the number of viable cells declined more rapidly after 4 d, leaving few viable organisms after 10 d. With the growth pH maintained at 7·0, no decrease in viability occurred between 5 and 7 d and, although this culture accumulated 60% more acetic acid (Table 1), there were 10^5 times more viable organisms per unit volume of culture than in the culture with no pH control. The results

Table 1. *Effect of pH on viability and acetic acid production by A. cellulolyticus during growth on cellulose*

Incubation time (d)	No. of viable organisms ml^{-1}		Acetic acid produced (mM)	
	No pH control	pH control	No pH control	pH control
0	$2{\cdot}0 \times 10^6$	$2{\cdot}0 \times 10^6$	0·0	0·0
3	$4{\cdot}3 \times 10^8$	$2{\cdot}4 \times 10^9$	15·5	17·8
4	$2{\cdot}4 \times 10^7$	$9{\cdot}3 \times 10^7$	ND	ND
5	$2{\cdot}4 \times 10^6$	$2{\cdot}4 \times 10^7$	24·5	30·1
6	$2{\cdot}4 \times 10^5$	$2{\cdot}4 \times 10^7$	24·8	35·9
7	ND	$2{\cdot}4 \times 10^7$	24·9	39·9
10	$<1{\cdot}0 \times 10^1$	$2{\cdot}3 \times 10^6$	24·9	39·8

ND, Not determined.

indicated that maintaining the growth pH at 7·0 not only enabled complete substrate breakdown but also maintained a higher cell viability.

Acetic acid is toxic to some micro-organisms (Hentges, 1967; Stewart, 1975) and it accumulates in anaerobic digesters operating under non-optimal conditions (McCarty, 1964). The inhibitory effect of volatile fatty acids is due to the undissociated form (Hentges, 1967; Meynell, 1963). In the pH range 6·0 to 7·5 volatile fatty acids are almost completely dissociated (Capri & Marais, 1975). At pH 5·5 and 7·0 the amount of acetic acid (pK_a 4·75) in the undissociated form is 15·1% and 0·6%, respectively. Thus, in cultures with no pH control, the concentration of undissociated acid after 3 d incubation (pH 5·5) would be 2·3 mM compared with only 0·1 mM in cultures grown at pH 7·0. Therefore, the 10^7-fold decrease in viable organisms in the cultures without pH control, compared with the 10^3-fold decrease in cultures with pH control, was probably due to the greater accumulation of the undissociated form of acetic acid.

To substantiate the above hypothesis, the combined effect of acetic acid and low pH on the viability of cellobiose-grown organisms was studied. Exponentially growing organisms were harvested and resuspended to the same density as in the original culture in substrate-free media containing 0 to 40 mM-acetic acid at pH 5·5 or 7·0. This acetic acid range was chosen since it corresponded to the concentrations (40 and 25 mM) which accumulated in cultures with or without pH control (Table 1). The two different pH values were chosen so that the amount of undissociated acetic acid in the medium would be altered. The results showed that in the absence of acetic acid, there was little or no loss of viability after 12 h incubation at pH 7·0 or 5·5 (Table 2). However, at pH 5·5 with 25 or 40 mM-acetic acid, there was about a 4-fold decrease in viability. This decline was more pronounced after 24 h incubation with decreases of about 10^4-fold. These results indicated that at acetic acid concentrations of 25 to 40 mM, the decline in cell viability was largely due to the expected greater concentration (3·8 to 6·0 mM) of the undissociated form of acetic acid at pH 5·5 than that (0·2 mM) in 40 mM-acetic acid at pH 7·0.

The effect of acetic acid on growth and H_2 production during growth on cellobiose was investigated using initial acetic acid concentrations of 0, 20, 40 and 90 mM and maintaining the growth pH at 7·0. Acetic acid at a concentration of 20 mM did not affect H_2 production or growth. However, with 40 mM-acetic acid, H_2 production was about 20% lower (Fig. 2*a*) although the growth was similar to that in cultures with no added acetic acid (Fig. 2*b*). Both H_2 production and growth were substantially lower (40% and 30%, respectively) with 90 mM-acetic acid. With the initial acetic acid concentrations of 0, 20, 40 and 90 mM, the acetic acid concentrations after 82 h incubation had increased by 40·0, 39·4, 32·0 and 28·4 mM, respectively. Therefore, acetic acid production was inhibited by an initial acetic acid concentration which was greater than 20 mM. The results also demonstrated that at initial acetic acid concentrations above 40 mM, growth was adversely affected, even though the

Table 2. *Effect of pH and acetic acid on the viability of A. cellulolyticus cells obtained from a culture in the exponential phase of growth on cellobiose*

Incubation conditions		No. of viable organisms ml^{-1}		
		Incubation time:		
pH	Acetic acid concn (mM)	0 h	12 h	24 h
7·0	0·0	$1·30 \times 10^9$	$1·09 \times 10^9$	$5·40 \times 10^7$
5·5	0·0	$1·30 \times 10^9$	$1·30 \times 10^9$	$1·60 \times 10^7$
5·5	25·0	$1·30 \times 10^9$	$0·35 \times 10^9$	$6·30 \times 10^5$
7·0	40·0	$1·30 \times 10^9$	$1·09 \times 10^9$	$3·48 \times 10^7$
5·5	40·0	$1·30 \times 10^9$	$0·35 \times 10^9$	$3·30 \times 10^5$

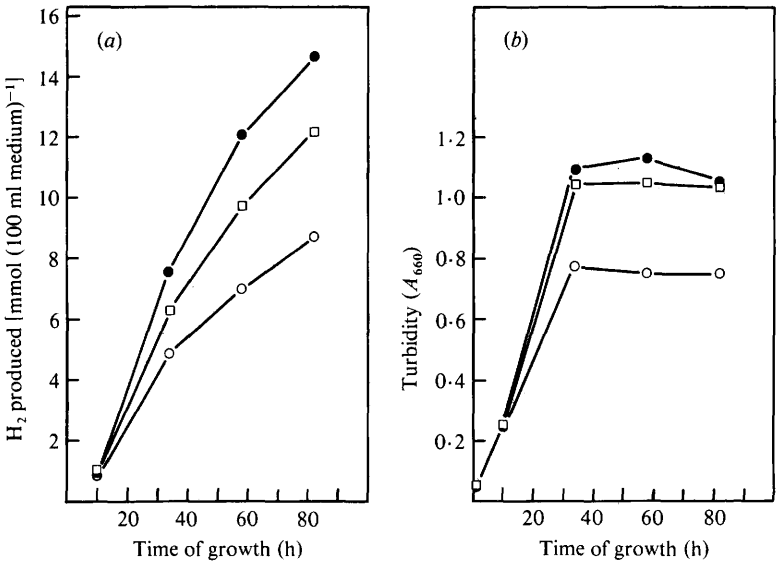

Fig. 2. Growth of *A. cellulolyticus* on medium containing cellobiose in the presence of acetic acid at initial concentrations of 0 (●), 40 mM (□) and 90 mM (○) with the pH of the medium maintained at 7·0: (*a*) cumulative H_2 production; (*b*) growth.

Table 3. *Viability of A. cellulolyticus culture after exposure to oxygen*

Time of exposure (h)	E_h of medium (mV)	Partial pressure of O_2 (mmHg)	No. of viable organisms ml^{-1}
0	−300	—	$2·40 \times 10^8$
2	+220	110	$2·40 \times 10^8$
5	+230	110	$4·30 \times 10^7$
24	+290	130	$1·50 \times 10^6$

growth pH was maintained at 7·0. Under normal cultivation conditions the maximum acetic acid accumulation during growth of *A. cellulolyticus* in 1% (w/v) carbohydrate media did not exceed 40 mM. Therefore, the inhibition of growth due to acetic acid accumulation is less likely to occur under normal circumstances, as long as the growth pH is maintained at 7·0.

The effect of O_2 on the viability of *A. cellulolyticus* cells (Table 3) obtained from an exponentially growing culture indicated that the cells could tolerate up to 2 h exposure to O_2

without measurable loss in viability—provided that they were subsequently transferred to pre-reduced media. After 5 h contact with O_2, only 18% of the cells were able to grow. Although *A. cellulolyticus* is an obligate anaerobe which requires pre-reduced media for growth, the viability appeared to be relatively unaffected by short periods of exposure to O_2. In this respect *A. cellulolyticus* did not differ from some other anaerobic bacteria belonging to the genera *Bacteroides, Peptostreptococcus* and *Clostridium* (Carlsson *et al.*, 1977; Tally *et al.*, 1975). The tolerance to O_2 could, as demonstrated for other anaerobes, be due to the capacity of the cells to reduce O_2 and/or due to detoxification of O_2 through activities of enzymes like peroxidase and superoxide dismutase (Morris, 1976; Rolfe *et al.*, 1978; Wimpenny & Samah, 1978). In the present study, no attempt was made to determine the presence of specific defence mechanisms of *A. cellulolyticus* against O_2 toxicity. The results indicated that it is possible to harvest the cells aerobically and resuspend them in anaerobic conditions without loss of viability.

The results of the present study indicate that acetic acid accumulation in conjunction with low pH values lowers cell viability and that *A. cellulolyticus* has a high tolerance to exposure to O_2.

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