APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Mar. 1985, p. 522–529
0099-2240/85/030522-08$02.00/0

Vol. 49, No. 3

Modulation of Acetone-Butanol-Ethanol Fermentation by Carbon Monoxide and Organic Acids

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Received 30 July 1984/Accepted 27 November 1984

Metabolic modulation of acetone-butanol-ethanol fermentation by *Clostridium acetobutylicum* with carbon monoxide (CO) and organic acids is described. CO, which is a known inhibitor of hydrogenase, was found to be effective in the concentration range of dissolved CO corresponding to a CO partial pressure of 0.1 to 0.2 atm. Metabolic modulation by CO was particularly effective when organic acids such as acetic and butyric acids were added to the fermentation as electron sinks. The uptake of organic acids was enhanced, and increases in butyric acid uptake by 50 to 200% over control were observed. Hydrogen production could be reduced by 50% and the ratio of solvents could be controlled by CO modulation and organic acid addition. Acetone production could be eliminated if desired. Butanol yield could be increased by 10 to 15%. Total solvent yield could be increased 1 to 3% and the electron efficiency to acetone-butanol-ethanol solvents could be increased from 73 to 78% for controls to 80 to 85% for CO- and organic acid-modulated fermentations. Based on these results, the dynamic nature of electron flow in this fermentation has been elucidated and mechanisms for metabolic control have been hypothesized.

Fermentation of carbohydrates to the solvents acetone and butanol has been known since the time of Pasteur and had been developed into a commercial process during World War I (1; C. Weizmann, Br. patent 4845, 1915). *Clostridium acetobutylicum* has been the organism of choice for conducting this fermentation. Recently, a stable asporogenic strain of this organism (MTC strain, ATCC 39236) has been isolated in our laboratory and it has allowed us to operate a continuous fermentation process (C. J. Lemme and J. R. Frankiewicz, Eur. patent appl. 0 111 683, 1982).

During fermentation, *C. acetobutylicum* produces three major classes of products: (i) solvents—acetone, butanol, and ethanol; (ii) organic acids—acetic, butyric, and lactic acids; and (iii) gases—carbon dioxide (CO_2) and hydrogen (H_2). To properly understand and appreciate product yield and fermentation efficiency in a process whereby many products are formed, an electron balance of the fermentation becomes necessary. Electron balance, as elaborated by Eroshin et al. (4, 5), is based on the fact that the standard amount of reductivity (available electrons) of any organic compound has practically a constant energy value, about 27 to 28 kcal (ca. 113 to 117 kJ) per electron equivalent. This observation, in essence, allows one to equate any substrate or product on the basis of electron equivalents and conduct an electron balance, which also gives an approximation of the overall thermodynamic (combustion energy) efficiency of the fermentation. Based on data and calculations reported in the literature (7) and data obtained in our laboratory with the bacterial strain, the average product yield and electron balance for a completed acetone-butanol-ethanol (ABE) fermentation is shown in Table 1. Thus, about 75 to 78% of the electrons in the feed carbohydrate end up in the ABE solvents, and butanol accounts for 50 to 55% of the electrons. The other nonsolvent products account for about 20 to 25% of the electrons, and the major fraction of these are in the form of H_2 gas (about 11 to 15% of the electrons). Thus, an improvement in solvent yield can come from a reduction in H_2 production or a change in the yield of one solvent relative to another or both. Electron balances also show the fallacy of lumping all of the solvents together and reporting a solvent yield on this basis, unless the solvent ratio remains invariant. Thus, overall solvent yield can be increased by decreasing the production of the most reduced solvent, butanol, and increasing the production of acetone or ethanol or both.

A possible method for decreasing H_2 production during fermentation would be to specifically inhibit hydrogenases, which are the terminal enzymes in the pathway of production of H_2 gas from reduced nucleotides. Carbon monoxide is a well-known reversible inhibitor of hydrogenase. Thus, inhibition of ABE fermentation by CO would lead to an increase in the pool of reduced nucleotides in the organism, which in turn would lead to an increased production of products that require reduced nucleotides for their formation, namely, butanol and ethanol. This rationale was espoused earlier (6) when hydrogenase inhibition by CO was demonstrated in *C. acetobutylicum*. The metabolic pathway and the proposed inhibition of hydrogenase are shown in Fig. 1 where, for the sake of simplicity, NAD is used to represent a generalized electron carrier.

In this report, our findings on the effects of CO, organic acids, and moderate pressure of H_2-CO_2 (60 to 120 lb/in^2) on ABE fermentation by *C. acetobutylicum* are discussed. This research has led to a greater understanding of the metabolism and the dynamic nature of electron flow in the organism. Moreover, practical ways to improve butanol yield and control the ratios of acetone to butanol have been discovered.

MATERIALS AND METHODS

Bacterial strain. The MTC asporogenic strain (ATCC 39236) of *C. acetobutylicum* was used for all experiments. In some experiments culture inoculum was obtained from a seed culture chemostat maintained in a starch hydrolysate

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TABLE 1. ABE fermentation: product yield and electron balances[a]

mol of product	Wt yield[b]	% of electrons[b]
0.09, CH_3COOH (acetic)	0.03	3
0.024, C_3H_7COOH (butyric)	0.012	2
1.56, H_2	0.0173 (± 0.003)	13 (± 2)
0.3, CH_3-$COCH_3$ (acetone)	0.097 (± 0.01)	20 (± 2)
0.535, C_4H_9OH (butanol)	0.220 (± 0.01)	53.5 (± 2.5)
0.08, C_2H_5OH (ethanol)	0.0186 (± 0.002)	4 (± 0.4)
0.286, $CH_{1.8}O_{0.5}N_{0.2}$ (cells)	0.039	5
2.31, CO_2	0.56	0

[a] From 1 mol of $C_6H_{12}O_6$ (fermentable carbohydrate).
[b] The numbers reported are average values. Normal variations are shown in parenthesis. A positive variation in one product leads to a negative variation in another.

cornsteep liquor (CSL) medium; in other cases, fresh cultures grown from frozen stock were used.

Medium preparation. Maltrin M-100, a starch hydrolysate (10 dextrose equivalents), was used as a carbohydrate source. CSL (E-801) and corn gluten (PRAIRIE GOLD) were obtained from the Argo, Ill., plant. All salts, mineral acids, organic acids, and alkali added were reagent grade. Typical fermentation media would consist of 60 g of M-100 per liter with 6 to 7.5 g (dry basis) of CSL solids per liter. Organic acids were added to the requisite levels before pH adjustment (with NaOH) to the range of 5.3 to 5.6. Reagent-grade $CaCO_3$ was added (2 to 3 g/liter) where specified. Media were sterilized at 121°C for 20 min for small (<1-liter) samples and 40 min for large (>1-liter) samples.

Substrate and product analysis. Carbohydrate concentrations were determined as dextrose equivalents, using the YSI analyzer (Yellow Springs Instruments, Yellow Springs, Ohio). Samples were diluted to appropriate levels (10 to 300 mg/100 ml) in 1 M phosphate buffer, pH 5.8. Glucoamylase enzyme (20 to 30 U/ml) was added to the diluted samples and incubated at 55 to 60°C for 30 min. The samples were cooled, held at room temperature for 2 h, and then assayed for dextrose in a standardized YSI analyzer. Later, a starch analysis attachment was bought, and this allowed the direct determination of carbohydrate concentration (as dextrose equivalents) without the enzyme incubation. Carbohydrate assays were always run with known standards as well as preweighed M-100 samples. Since yield and electron balances are very sensitive to carbohydrate assay accuracy, extreme care was taken to obtain accuracy within ±1%.

Concentrations of organic acids and solvents were assayed by high-pressure liquid chromatography, using BC-X12 cation-exchange resin and eluting with 0.006 N sulfuric acid in water at 80°C.

The volumes and compositions of fermentation gases were measured by using simple liquid displacement apparatus designed in the laboratory. An Erlenmeyer filter flask was fitted with a dip tube and a tight rubber stopper. The side arm was fitted to a tight rubber septum. The flask was filled with acidified brine (to reduce CO_2 absorption). A short piece of rubber tubing, connected on both ends to truncated barrels of plastic syringes and syringe needles, served as a gas conduit. For measuring the volume of gas produced, the septum was pierced and the other needle of the tubing pierced the rubber stopper over the serum vial or tube. The

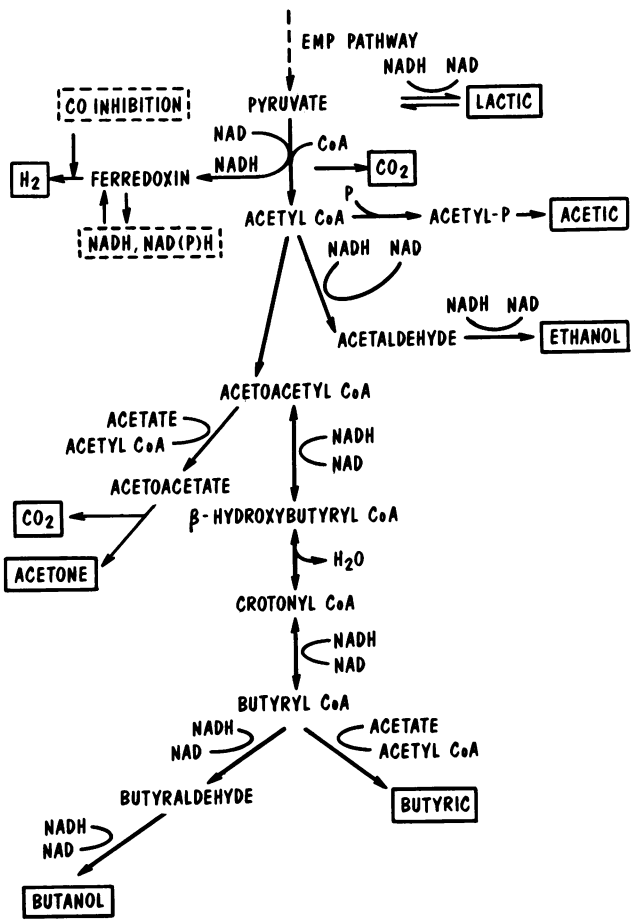

FIG. 1. Metabolic pathway and proposed inhibition of hydrogenase of *C. acetobutylicum*.

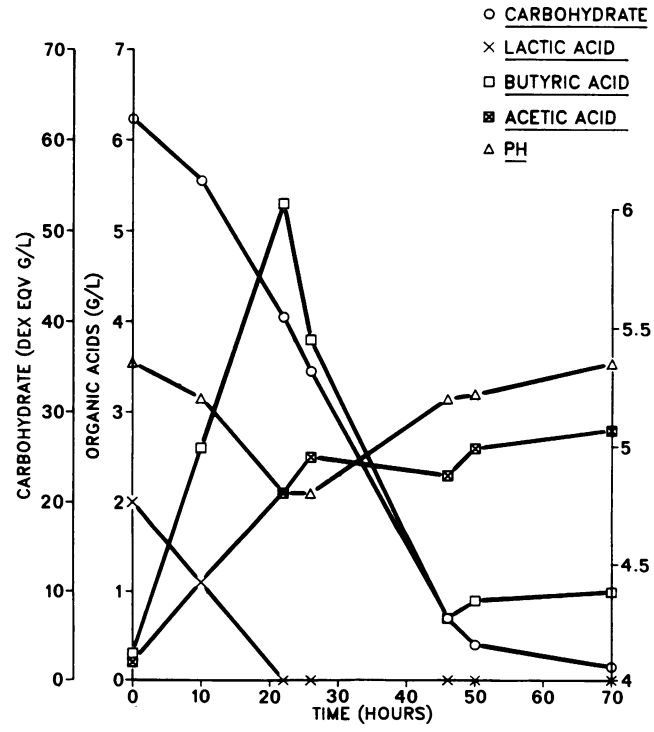

FIG. 2. Organic acid and pH profile for normal ABE fermentation (control).

total gas generated was measured by liquid displacement, and the composition of the gas was measured by a gas chromatographic analysis of the gas contained in the Erlenmeyer collection flask. All this was performed aseptically by using sterile needles and flame sterilization of the top of the rubber stoppers. All gas samples, from collection flasks, gas lines, etc., were taken by Precision brand gas-tight syringes and injected into a gas chromatograph. A method that used an adsorbent column (Carbosive S) and a temperature program was developed to separate and measure H_2, N_2 (air), CO, and CO_2 with ±2% accuracy. Thus, measurements of carbohydrates, fermentation organic acids and solvents, and gases by these methods enabled one to determine total carbon and electron balance in a fermentation.

Fermentation. Fermentation experiments were carried out in Bellco culture tubes, Wheaton serum vials, or New Brunswick Scientific Co. Bioflo benchtop fermentors. For experiments in culture tubes and serum vials, culture media (10 ml for tubes and 20 to 50 ml for vials) were charged and sealed, and the headspaces were repeatedly evacuated and charged with anaerobic gas (90% N_2, 5% CO_2, 5% H_2) to make the media anaerobic. Then the headspaces were charged with the appropriate concentration of CO and the sealed tubes or vials were sterilized. After cooling, the media were inoculated with *C. acetobutylicum* cells (5 to 10% inoculum) and fermented at 34 to 36°C in a rotary shaker. Every set of experiments had a few control vials which were vented through a gas release tube so that no pressure built up. Vials containing CO in the headspace were laid on their sides on the shaker (100 to 150 rpm) to allow good gas-liquid mixing. The headspace gases were released and collected at regular intervals, and a calculated amount of

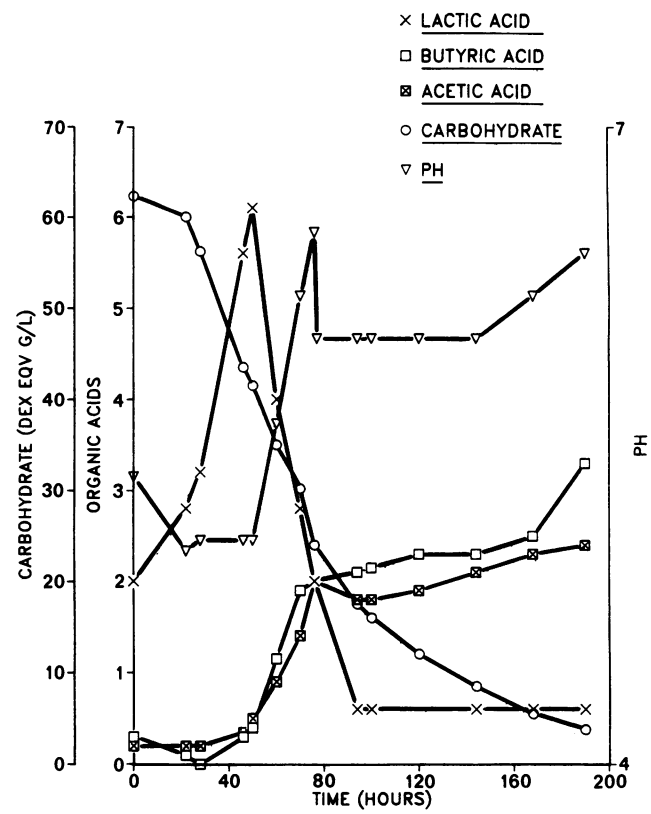

FIG. 4. Organic acid and pH profile for CO-sparged ABE fermentation (20% [vol/vol] CO).

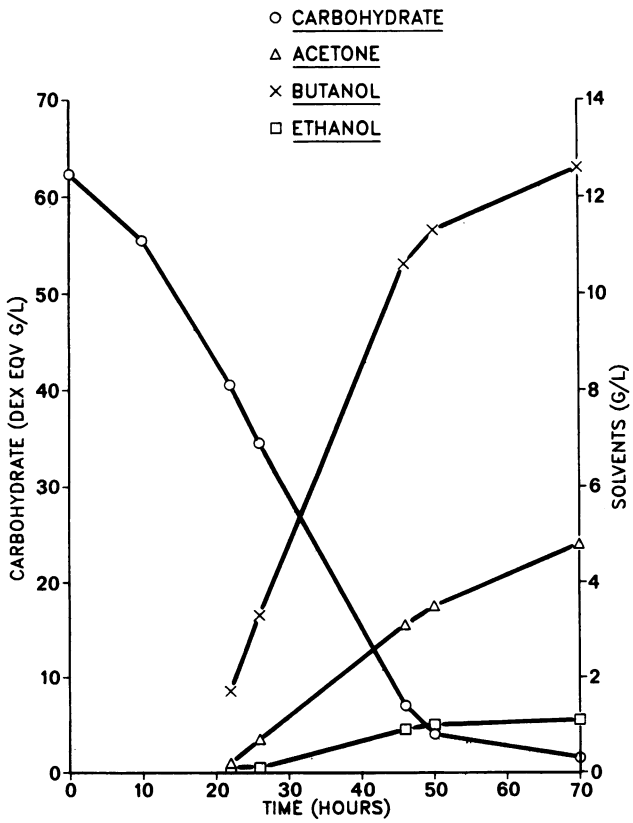

FIG. 3. Solvent profile for normal ABE fermentation (control).

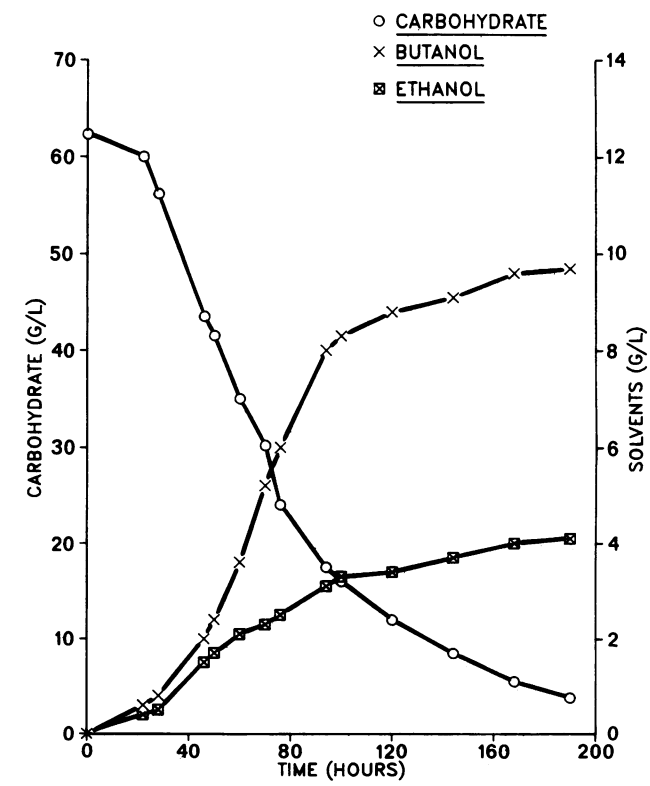

FIG. 5. Solvent profile for CO-sparged ABE fermentation (20% [vol/vol] CO).

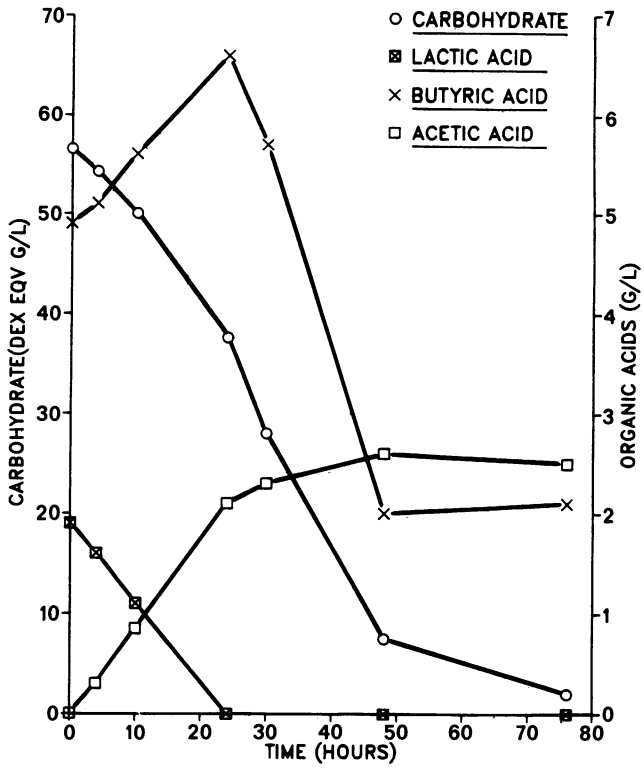

FIG. 6. Organic acid profile for ABE fermentation with butyrate addition (control).

CO was recharged to maintain the desired partial pressure of CO. In experiments to determine the effects of pressure only, the headspace gases were released at the end of the fermentation when it was measured, and the final gas pressure and composition were determined.

New Brunswick Scientific Co. Bioflo benchtop fermentors were used to scale up the fermentation and determine the time profile of a fermentation. Typically, these were carried out in a 1-liter culture volume, stirred (200 rpm), and sparged with the appropriate gas mixture. The fritted-glass gas sparger was located just below the impeller to allow good bubble dispersion. Gas mixtures, which were either purchased or mixed through appropriate flow meters, were sparged into the fermentor and vented through a cold-water condenser and a sterile filter to a gas flow-measuring burette (soap bubble type). The gas flow through the system was maintained at 60 to 80 ml/min to allow adequate mass transfer and maintain the desired gas partial pressure. Fermentor outlet gas was sampled periodically and assayed by gas chromatography. The level of dissolved CO was also measured by removing liquid samples, degassing in evacuated tubes, and measuring the CO concentration in the headspace of the tubes by gas chromatography. This double checked the dissolved gas composition in sparged fermentations.

RESULTS

Effect of sparged carbon monoxide. Initial experiments within headspace CO in enclosed serum vials showed that the organism could grow up to a CO partial pressure of 0.3 atm, but growth within any level of headspace CO would stop after 50 to 80% carbohydrate consumption and the broth pH would be very low (4.0 to 4.5). It became apparent that a proper understanding of the effect of CO on the organism's metabolism could only be obtained in a sparged fermentor where a desired concentration of dissolved CO could be maintained in the broth and the fermentation pH could be controlled. Thus, a sparged gas fermentation was conducted as described earlier. The sparge gas composition was 20% CO, 30% H_2, 48% N_2, and 2% CO_2. Before sparging was begun, a sample of the inoculated broth was removed and fermented separately without any gas sparging. This served as the control. The pH controller was set at 5.0 for the CO-sparged fermentor and at 4.75 for the control experiment. Samples from both fermentors were removed and assayed at regular intervals. Figures 2 and 3 show the time course profiles for organic acids and pH and solvents, respectively, for the control experiment. The profiles are typical of a normal ABE fermentation with the initial formation of organic acids (primarily butyric) and a concomitant pH drop, followed by a solventogenic fermentation in which butyric acid is consumed, the pH rises, and acetone, butanol, and ethanol are produced. The organic acids profile also shows a quick consumption of lactic acid which came from CSL.

The time course profiles for organic acids and solvents for the CO-sparged fermentation are shown in Fig. 4 and 5. A dramatic change is observed. Lactic acid becomes the predominant organic acid in the acidogenic phase of the fermentation. The pH drops and alkali is consumed to maintain the pH at 5. Butyric acid (from the inoculum) is rapidly consumed in the initial phase of the fermentation. Later, when the pH had risen, the organism began to produce this acid. Thus, instead of a normal butyric acid peak, a lactic acid peak is observed. Solvent production begins early in the fermentation and only two solvents,

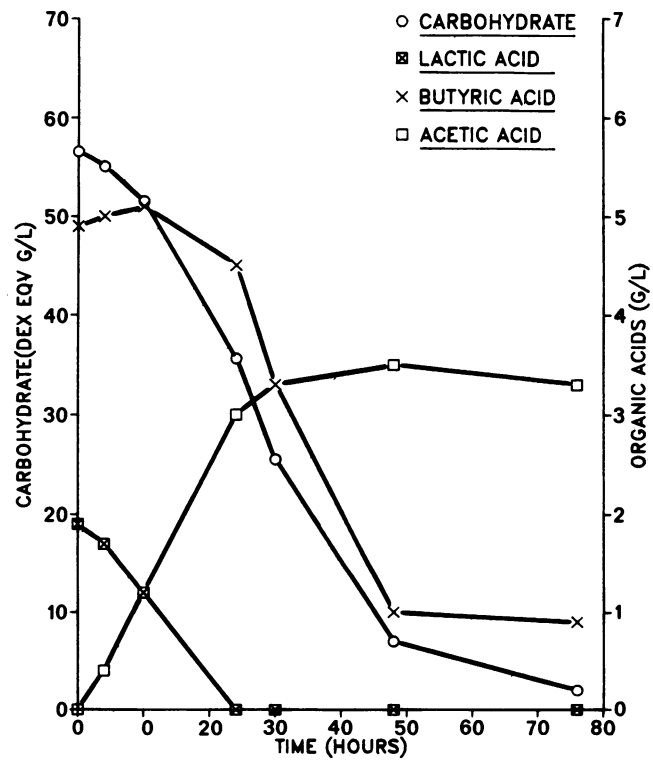

FIG. 7. Organic acid profile for CO-sparged ABE fermentation with butyrate addition (10% [vol/vol] CO).

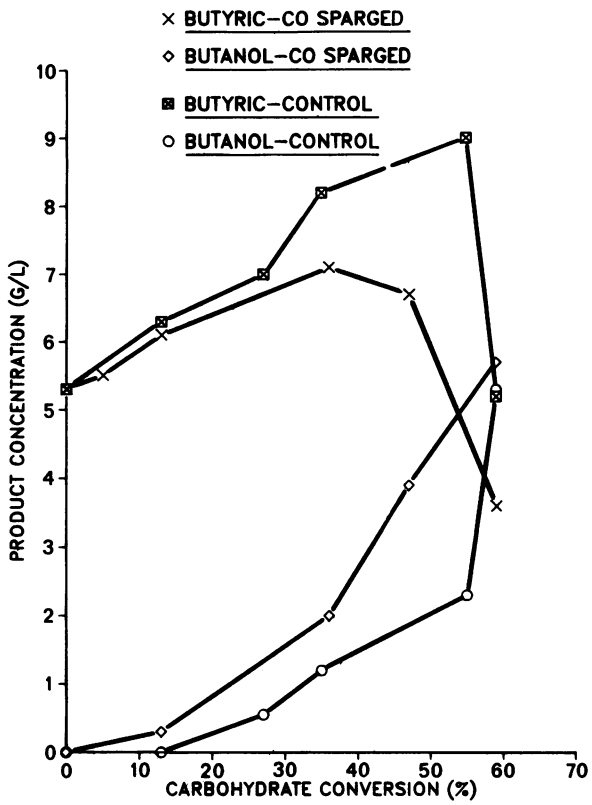

FIG. 8. Butyric acid and butanol profiles of control and organic acids plus CO-modulated ABE fermentation (10% [vol/vol] CO, 2 g of acetic per liter, 5 g of butyric per liter).

butanol and ethanol, are produced. The carbohydrate consumption rate is slower (about 2.5-fold) than that of the control. These results showed that the metabolism of the organism could be altered by CO. It was hypothesized that the flow of electrons (reducing equivalents) could be controlled by simultaneously directing away from hydrogen (inhibition of hydrogenase) into electron sinks such as organic acids.

To test this hypothesis, fermentations with butyric acid (ca. 5 g/liter) initially added to the broth were conducted. The fermentor was sparged with a gas containing ≈10% CO, ≈40% H_2, ≈40% CO_2, and ≈10% N_2, and the control was not sparged. Figures 6 and 7 show the time profiles for the two fermentations. The control (Fig. 6) shows a rise and a sharp peak of butyric acid during the early stages of fermentation. The CO-sparged experiment (Fig. 7) shows a shallow butyric acid peak and an earlier consumption of this acid. In both cases, lactic acid, initially present, is consumed. Both cases show the same rate of carbohydrate consumption. Another experiment run with an initial acids concentration of 2 g of acetic and 5 g of butyric acids per liter and the same gas sparge showed similar time profiles. The butyric acid and butanol profiles for this case are shown in Fig. 8. The abcissa is normalized to carbohydrate conversion. The butyric acid profile shows a gradual increase followed by a sharp drop for the CO-sparged experiment; the control shows a steeper rise to a higher butyric acid concentration before its consumption begins. The data also show that with CO modulation the production of butanol can be shifted to an earlier stage of the fermentation when less carbohydrate has been consumed. These experiments support the hypothesis of electron flow and modulation of ABE fermentation of our earlier paper (6).

CO-modulated fermentation. (i) Yield and product distribution. The experiments with CO sparge showed that metabolism and flow of electrons in the fermentation could be altered by CO modulation. It was difficult, however, to obtain accurate material balances in these experiments because of the difficulties in condensing all vapors, loss of solvents in sparge gases, and inability to measure net H_2 production. Consequently, the effects of CO modulation on solvent yields and product distributions were evaluated in experiments in serum vials.

The effect of CO modulation on net H_2 production is shown in Table 2, where the results of two fermentations run simultaneously are compared. The experiments were run in sealed serum vials. The CO-modulated fermentation showed about 40% less net production of H_2 and a higher production of butanol and ethanol. Acetic (4.3 to 4.8 g/liter) and butyric (1.8 g/liter) acids were used as buffering agents and electron sinks.

Reduced H_2 production by CO modulation has been substantiated by other experiments and by electron balances of products. On the average, electrons in net H_2 production can be reduced from the average of 13 ± 2% for a normal fermentation to about 8 ± 1% for a CO-modulated fermentation. These and other serum vial experiments were conducted, and it was established that, from the viewpoint of fermentation stability and productivity, the optimum concentration ranges are as follows: for CO, 0.1 to 0.15 atm; for butyric acid, 4 to 6 g/liter; and for acetic acid, 2 to 5 g/liter.

A series of experiments within various levels of CO (varying from an initial concentration of 0.1 atm to 0.15 atm) and an initial butyric acid concentration of about 5 g/liter were run. Controls with and without butyric acid (sets 2 and 1) and with butyric acid and pressure (set 3) were also run simultaneously. The data on substrate conversion and product concentrations are shown in Table 3, and electron balances are shown in Table 4. The presence of butyric acid increases solvent yield when compared with the control without butyric acid (cf. sets 1 and 2). Increased pressure (up to ~5 atm) by fermentation gases does not change solvent yield or distribution or butyric acid uptake (cf. sets 2 and 3). Headspace CO concentrations in the range of 0.1 to 0.15 atm increases butyric acid uptake as well as butanol yield. Acetone yield is decreased and ethanol yield is increased. Electron balances show that butanol yield can be increased by about 10% and electron efficiency to total solvents can be increased 2 to 3% by CO modulation.

TABLE 2. CO modulation and net H_2 production

Run no.	CO headspace at t = 0 (atm)	Carbohydrate concn (g/liter as dextrose)		Organic acids and solvents (g/liter)							H_2 produced (g/liter)
				Acetic		Butyric		Acetone (t = 48 h)	BuOH (t = 48 h)	EtOH (t = 48 h)	
		t = 0	t = 48 h	t = 0	t = 48 h	t = 0	t = 48 h				
C1	0 (control)	47.3	0.4	4.8	4.0	1.5	1.8	6.2	11.7	0.9	1.0
B2	0.1	47.3	1.8	4.3	3.2	1.8	0.4	4.3	12.8	2.5	0.62

TABLE 3. CO modulation and butyrate uptake: substrate and product concentrations

Set no.	Carbohydrate (g/liter as dextrose)		Organic acids (g/liter)				Butyric acid uptake (g/liter)	Solvent concn (g/liter)		
			Acetic		Butyric					
	t = 0	t = 72 h	t = 0	t = 72 h	t = 0	t = 72 h		Acetone	Butanol	Ethanol
1 (control without butyric, vented)	51.4	0.3	0.1	2.0	0	0.6		4.2	11.5	1.1
2 (control with butyric, vented)	51.4	0.3	0.1	2.6	4.8	1.4	3.4	5.1	13.3	0.8
3 (control with butyric, pressure)	51.4	0.3	0.1	2.4	4.8	1.5	3.3	5.1	13.4	0.8
4 (0.1 atm of CO, 0 to 72 h)	51.4	0.4	0.1	2.4	4.8	0.8	4.0	3.8	14.7	1.6
5 (0.15 atm of CO, 0 to 24 h; 0.1 atm of CO, 24 to 48 h; 0.05 atm of CO, 48 to 72 h)	51.4	0.3	0.1	2.9	4.8	1.0	3.8	2.8	14.7	1.3
6 (0.1 atm of CO, 0 to 24 h; 0.15 atm of CO, 24 to 72 h)	51.4	0.3	0.1	2.3	4.8	0.6	4.2	2.9	15.0	2.0

TABLE 4. CO modulation and butyrate uptake: electron balances

Set no.[a]	Total electron equivalents from converted substrates (carbohydrate + organic acids)	% of electron equivalents in products				% of electrons in ABE solvents
		Acetic	Acetone	Butanol	Ethanol	
1	7.01	3.6	16.3	52.7	4.1	73.1
2	7.8	4.4	17.9	55.5	2.7	76.1
3	7.8	3.8	18.2	55.6	2.7	76.5
4	7.81	3.9	12.9	61.1	5.4	79.4
5	7.88	5.0	10.0	62.6	4.4	77.0
6	7.82	3.8	10.2	62.4	6.8	79.4

[a] See Table 3 for the fermentation conditions for each set.

Another series of experiments studied the effect of simultaneous addition of acetic and butyric acids to fermentations with and without CO. The optimum initial concentrations were found to be about 2 g of acetic acid and 5 g of butyric acid per liter. The data from these experiments are presented in Tables 5 and 6. Control 1 had no added organic acids or CO; control 2, set 7, and set 8 had acetic and butyric acids with 0, 0.1, and 0.15 atm of CO, respectively; and set 4 was conducted earlier with 5 g of butyric acid per liter and 0.1 atm of CO. The data show that addition of about 2 g of acetic acid per liter with 5 g of butyric acid per liter increases the production of acetone in the CO-modulated fermentations. The acetone/butanol ratio can be about 1:2.7 for this case versus 1:2 for control and about 1:4 for set 4, where only butyric acid was added. Butanol and solvent yield are also increased by CO modulation (Table 7).

(ii) Increased butanol concentration. The previous experiments with CO modulation were conducted within 45- to 55-g/liter initial carbohydrate concentrations, and balances were obtained at the end of the fermentation when 98 to 100% of the carbohydrate was consumed. Our data showed that, under CO modulation, butanol could be produced at an early stage of the fermentation. Thus, a CO-modulated fermentation with butyrate could, in theory, proceed to a higher level of butanol if the same degree of carbohydrate conversion could be achieved. This hypothesis was tested by running CO-modulated fermentations with high (90 to 100 g/liter) initial carbohydrate concentration and high initial nutrient concentrations (12.5 g of CSL solids and 15 g of corn gluten per liter). The data presented in Table 8 show that the CO-modulated fermentation has a higher uptake of butyric acid than the control and also proceeds to a higher concentration of butanol (16.1 versus 14.2 g/liter). Addition of gluten increases the butanol concentration further to about 18.5 g/liter and acetone production.

Summary of results. The results from CO modulation of ABE fermentation can be summarized as follows.

(i) CO is an effective metabolic modulator for ABE fermentation. The concentration range for dissolved CO should correspond to 0.1 to 0.2 atm of partial pressure and optimally between 0.1 and 0.15 atm.

(ii) Metabolic modulation by CO is particularly effective when a sink for electron is available to the organism. Organic acids such as butyric acid are particularly effective electron sinks.

TABLE 5. CO modulation with acetic and butyric acids: carbohydrate and acids concentrations

No.	Carbohydrate (g/liter as dextrose)		Acetic acid concn (g/liter)		Net acetic acid production (g/liter)	Butyric acid concn (g/liter)		Butyric acid uptake (g/liter)
	t = 0	t = end	t = 0	t = end		t = 0	t = end	
Control 1 (no acids)	49.5	0.4	0	3.6	3.6	0	2.1	
Control 2 (2 g of acetic and 5 g of butyric per liter)	47.7	0.5	1.4	3.3	1.9	5.0	2.4	2.6
Set 7 (2 g of acetic and 5 g of butyric per liter + 0.1 atm of CO)	47.7	0.5	1.4	3.7	2.3	5.0	1.2	3.8
Set 8 (2 g of acetic and 5 g of butyric per liter + 0.15 atm of CO)	47.7	0.5	1.4	3.8	2.4	5.0	1.2	3.8
Set 4 (5 g of butyric per liter + 0.1 atm of CO)	51.4	0.3	0.1	2.4	2.3	4.8	0.9	3.9

TABLE 6. CO modulation with organic acids: solvent production profile

No.	Solvent products (g/liter)			Ratio		
	Acetone	Butanol	Ethanol	Acetone	Butanol	Ethanol
Control 1 (none added)	5.3	10.2	0.7	1	1.88	0.13
Control 2 (acetic + butyric)	5.6	11.0	0.4	1	1.96	0.07
Set 4 (butyric + CO)	3.8	14.7	1.6	1	3.83	0.42
Set 7 (acetic + butyric + CO)	5.2	13.7	1.2	1	2.65	0.23
Set 8 (acetic + butyric + CO)	5.2	14.2	1.1	1	2.74	0.21

(iii) The uptake of organic acids is enhanced by CO modulation. Increases of butyric acid uptake of 50 to 200% over the control have been observed under various fermentation conditions.

(iv) CO modulation within organic acid uptake induces solventogenic fermentation at an earlier stage of the fermentation compared with controls.

(v) CO modulation decreases the production of acetone and increases the production of butanol and ethanol. Acetone production can be reduced to zero.

(vi) The ratio of the solvents acetone and butanol can be controlled by CO modulation and organic acid addition. The acetone/butanol ratio can be varied from 1:4 to 1:2.5 without curtailing productivity.

(vii) Butanol yield can be increased by 10 to 15% (compared with control) within CO modulation and organic acids uptake.

(viii) Total solvent yield can be increased 1 to 3% and the electron efficiency to ABE solvents can be increased from 73 to 78% for control to 80 to 83% for CO-modulated fermentation. This increase in efficiency is primarily derived from a reduction in H_2 production.

(ix) When using a medium with excess carbohydrate and nutrients, CO modulation with butyric acid uptake can lead to a higher final concentration of butanol than of control.

DISCUSSION

Most heterofermentative anaerobic bacteria produce hydrogen during growth on carbohydrates. The principal reasons for H_2 production are maintenance of metabolic balance and pH. In clostridia, hydrogenase is the terminal enzyme in H_2 production, and it catalyzes the reduction of protons to molecular hydrogen with reduced ferredoxin as the physiological electron donor. Hydrogenases are inhibited by carbon monoxide. The inhibition is reversible and light sensitive. Recent studies have shown that the iron in the active site of the enzyme is attacked by CO and that iron in the low oxidation state is involved in the Fe-CO complex formation (10).

The proposed metabolic pathway and increase of reduced nucleotides (NAD representing a generalized electron carrier) by inhibition of hydrogenase is shown in Fig. 1. An increase in the pool of reduced nucleotides should, in theory, increase the products that require them, namely, the alcohols. However, the organism has another mechanism for channeling these electrons by reduction of pyruvate to lactate. This was observed by Simon (9), who showed that *C. acetobutylicum* produced lactic acid when sparged with a stream of CO. Our observations show that when ca. 0.2 atm of CO (0.8 atm of other gases) is used and the fermentation pH is controlled, the organism can switch from a lactic acid fermentation to a solventogenic fermentation. During the solventogenic stage, lactic acid is metabolized by the organism. No acetone is produced in such a fermentation. These observations lead us to hypothesize the following pathways and control mechanisms in this organism.

(i) The organism has an active pathway for lactate metabolism. It can produce or consume lactate. Lactate is probably converted to acetate and butyrate and then to the alcohols. Conversion of lactate to acetate and butyrate has been observed in *Butyribacterium methylotrophicum*.

(ii) The concentrations of reduced nucleotide pool and coenzyme A (CoA) play a crucial role in metabolic regulation and product formation. The metabolic pathway shows that acetone production offers the organism a mechanism for recycling CoA without using reduced nucleotides or generating protons. Thus, when the reduced nucleotide pool is increased in proportion to CoA, the organism switches away from the acetone pathway.

(iii) The switchover from acidogenesis to solventogenesis need not be triggered by concentrations of butyrate or acetate, and a sharp acid peak is not necessary for the onset of solventogenesis. Perhaps the concentrations and concentration ratios of reduced and oxidized nucleotides, CoA, and ATP are the important parameters for the switch.

These studies have also revealed the dynamic nature of electron flow in the organism. Thus, channeling electrons away from hydrogen can lead to production of lactate and the alcohols. However, when a sink for electrons, such as butyric acid, is added to the fermentation, the organism can channel the electrons towards acid reduction to alcohol. This modulation, or control of flow of electrons from a source to a sink, stabilizes the fermentation and increases productivity and the uptake of organic acids.

From a practical viewpoint, CO modulation of ABE fermentation can be commercially significant. Increased butanol yield reduces the cost of required fermentables. The acetone/butanol ratio can be manipulated and this allows process flexibility to market conditions. The increased uptake of organic acids (especially butyric acid) is very significant because butyric acid can be produced from cellulosic materials and also corn fibers by direct fermentation with many anaerobic bacteria (2). Methanol can also be converted to butyric acid with high yield (~80%) by anaerobic bacteria (3, 11). Thus, butyric acid can be produced by fermentation of these potentially inexpensive sources. Under normal ABE fermentation, the uptake and conversion of

TABLE 7. CO modulation with organic acids: yield and electron balances

No.	Total electron equivalents consumed from all substrates	% of electrons in ABE solvents			Electron efficiency	Yield[a]	
		Acetone	BuOH	EtOH		Solvent	Butanol
Control 1	6.76	21.6	48.8	2.7	73.1	0.33	0.21
Control 2	7.10	22.9	52.7	1.5	77.1	0.34	0.22
Set 4	7.81	12.9	61.1	5.4	79.4	0.37	0.268
Set 7	7.37	19.5	60.2	4.4	84.1	0.39	0.269
Set 8	7.29	19.8	63.1	4.0	86.9	0.40	0.278

[a] Yield = grams of product/(grams of dextrose equivalents consumed + grams of butyric acid consumed).

TABLE 8. Increased butyrate uptake and butanol concentration in CO-modulated fermentation

No.	CO headspace (atm)	Carbohydrate concn (g/liter as dextrose)		Initial gluten concn (g/liter)	Butyric acid (g/liter)		Butyric acid uptake (g/liter)	Solvent concn (g/liter)		
		t = 0	t = end		t = 0	t = end		Acetone	Butanol	Ethanol
Control 9, 1	0	89.5	26.7	0	7.0	5.2	1.8	6.8	14.1	0.4
Control 9, 2	0	89.5	27.1	0	7.0	5.2	1.8	6.7	14.2	0.4
Set 9, 1	0.1	86.6	33.0	0	7.0	2.0	5.0	3.7	16.3	0.9
Set 9, 2	0.1	86.6	21.3	0	7.0	2.2	4.8	3.8	15.9	0.8
Set 9, 3	0.1	103.7	21.3	15	4.8	1.7	3.1	7.4	18.7	1.2
Set 9, 4	0.1	103.7	21.1	15	5.8	1.8	4.0	7.0	18.2	1.1

organic acids are small. Recent efforts in France have attempted to produce butanol from cellulose by using mixed cultures of a cellulolytic *Clostridium* sp. and *C. acetobutylicum* (8). The results are not promising due to poor uptake and conversion of organic acids. Carbon monoxide modulation could aid this process and make it feasible.

Conclusion. Carbon monoxide has been found to be an effective metabolic modulator for ABE fermentation by *C. acetobutylicum*. Metabolic modulation by CO is particularly effective when organic acids such as butyric and acetic acids are added to the fermentations and dissolved CO concentrations are maintained in the partial pressure range of 0.1 to 0.15 atm. Under these conditions the organic acids, especially butyric acid, act as an electron sink and are reduced to butanol. The uptake or organic acids is enhanced and the butanol yield is increased by 10 to 15% over control. The ratio of acetone/butanol can be controlled by CO partial pressure and addition of various levels of organic acids. Fermentation with no production of acetone has been demonstrated. CO-modulated fermentation with butyric acid uptake can also lead to a higher final butanol concentration than control.

These results also reveal that the metabolic pathways for lactic acid can be amplified and, also, lactic acid can be converted to solvents. The trigger for the onset of solventogenesis is not necessarily a sharp acid peak or proton concentration, but rather the concentrations or concentration ratios of reduced and oxidized nucleotides, CoA, or ATP. Moreover, the nature of the flow of electrons is dynamic and electrons can be channeled to preferred directions by maintaining sources and sinks.

ACKNOWLEDGMENTS

We are grateful to C. J. Lemme, S. A. Lemmel, and J. R. Frankiewicz for valuable technical assistance and suggestions.

We are grateful to Moffett Technical Center, CPC International, for permission to publish this paper and for a research grant to J. G. Zeikus, University of Wisconsin.

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