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Continuous Production of Mixed Alcohols and Acids from Carbon Monoxide

A. J. GRETHLEIN,[1] R. M. WORDEN,*[,1,2] M. K. JAIN,[2] AND R. DATTA[2]

[1]*Department of Chemical Engineering, Michigan State University, East Lansing, MI 48824; and* [2]*Michigan Biotechnology Institute, 3900 Collins Road, PO Box 27609, Lansing, MI 48909*

ABSTRACT

Continuous, steady-state fermentations using carbon monoxide gas as the sole carbon and energy source have been achieved with the CO strain of *Butyribacterium methylotrophicum*. Fermentation pH was found to regulate carbon monoxide metabolism over the pH range of 6.8 to 5.0. Cell growth diminished at low pH, with washout occurring at pH 5.0. As observed previously in batch culture, lower pH values favored production of butyrate over acetate. The mechanism responsible for this trend is currently being investigated by quantification of key intracellular enzyme activities.

At low pH values, direct, steady-state fermentation of carbon monoxide to alcohols has been verified. Of major significance is the production of butanol from carbon monoxide in pure culture. This newly identified pathway provides a potential mechanism for direct bioconversion of synthesis gas to butanol.

Index Entries: Carbon monoxide; chemostat; butyrate; butanol; *Butyribacterium methylotrophicum*.

INTRODUCTION

Biological synthesis of liquid fuels from coal-derived gases has the potential for developing processes that are highly selective and efficient. Large quantities of one-carbon feedstocks, such as carbon monoxide (CO),

*Author to whom all correspondence and reprint requests should be addressed.

carbon dioxide (CO_2), methanol, and also hydrogen (H_2), can be obtained by gasifying biomass or fossil fuels, such as coal and natural gas. The major synthesis gas components, CO, H_2, and CO_2 gases, can be metabolized by a variety of microorganisms, some of which have been recently reviewed (*1–4*)).

Bacteria capable of growth on 1-carbon compounds as the sole carbon and energy source, often termed unicarbonotrophic bacteria, exhibit great potential for use in the biological conversion of synthesis gas. Several species in this classification are acid-producing and capable of acetate production from either CO or H_2/CO_2 gases. The thermophile *Clostridium thermoaceticum* exhibits doubling times of 18 h and 16 h for growth on CO or H_2/CO_2, respectively (*5*). *Peptostreptococcus productus* strain U-1, isolated from anaerobic sewage digestor sludge, has a doubling time of 1.5 h on 50% CO and 5 h on H_2/CO_2 (*6*). *Eubacterium limosum,* a sheep rumen isolate, produces acetate and minor amounts of butyrate when grown on H_2/CO_2 with a 14 h doubling time (*7*). When grown on CO, *E. limosum* produced acetate with a 7 h doubling time; however, these results were obtained using a 50% CO gas phase; doubling times increased to 18 h in a 75% CO gas phase, which was the highest percentage tested (*8*).

Butyribacterium methylotrophicum is an extremely versatile unicarbonotroph capable of biological conversion of all major synthesis gas components. Growth on H_2/CO_2 produces acetate with a 9 h doubling time (*9*). A mutant strain of this organism, the CO strain, was adapted to grow with a 12 h doubling time on 100% CO, producing acetate and minor amounts of butyrate (*10*). Recent studies in extended batch culture with the CO strain of *B. methylotrophicum* have linked fermentation pH directly to product composition with cells grown on 100% CO (*11*). In this study, acetate formation was favored over butyrate formation in a molar ratio of 32:1 at a fermentation pH of 6.8. At pH 6.0, however, equimolar production of acetate and butyrate was observed, with butyrate formation occurring only during the stationary phase of growth (*11*).

With the results obtained from batch culture identifying fermentation pH as a direct metabolic regulator of carbon monoxide metabolism in the CO strain of *B. methylotrophicum,* a primary objective of the present study was then to investigate this regulation under similar conditions in continuous culture. This involved first establishing conditions for steady-state chemostat operation and then conducting steady-state operation over a range of broth pH values.

METHODS

Microorganism and Culture Conditions

A stock culture of the CO strain of *B. methylotrophicum* was maintained in our laboratory in 152 mL sealed serum bottles. These bottles contained

50 mL of phosphate-buffered media initially under a 100% CO gas headspace at 10 psig. Cultures were grown in the dark at 37°C with 100 rpm shaking and transferred to fresh bottles approximately every two weeks.

Culture Medium and Fermentation Equipment

The phosphate-buffered medium, as described previously for batch fermentation studies (*11*), contains primarily inorganic salts, nutrients, vitamins, and 0.1% yeast extract. Fermentations were initiated with the addition of 25 mL of growing *B. methylotrophicum* culture in mid-log phase, thus yielding a 2% v/v inoculum.

Continuous culture studies were performed in a round-bottomed BioFlo II fermenter (New Brunswick Scientific Co., New Brunswick, NJ) having a working volume of 1.25 L. A built-in, water-cooled mini-condenser was utilized to minimize stripping of volatile products by the effluent gas stream. The fermentation pH, temperature, liquid flow rate, and impeller speed were all automatically controlled. All chemostat fermentations were conducted at 37°C, 50 rpm, 50 mL/min 100% CO gas sparge rate, and a 0.31 mL/min liquid media flow rate; thus, the dilution rate in all fermentations was constant at 0.015 h^{-1}. Fermentation pH was controlled by the addition of 2*N* NaOH solution. CO gas flow was measured using a 50 mL buret as a bubble meter. Effluent liquid exited via an overflow port at the specified volume level. All fermentations were maintained at atmospheric pressure and operated in a chemical safety hood. Steady-state operation was defined as deviations of not more than 10% of the mean cell density value over a period of three liquid residence times, or approximately 9 d. Although rigorous, this stricture provided increased confidence in the accuracy of the data obtained. All data presented are steady-state values defined by this criteria.

Analytical Methods

Liquid samples were taken aseptically and anaerobically using nitrogen (N_2)-flushed, sterile syringes through a butyl rubber sample port. Approximately 2 mL was extracted with each sample. Cell concentration was measured by optical density at 660 nm on a Sequoia-Turner Model 340 spectrophotometer (Sequoia-Turner Corporation, Mountain View, CA). Samples with an optical density greater than 0.5 were diluted by a factor of 10. Cell mass was calculated using a previously-derived dry weight vs optical density calibration curve (*10*). Undiluted samples were observed microscopically for viability and contamination. The samples were then centrifuged in 1.5 mL Eppendorf tubes at 12,000 rpm for 2 min and the pH measured to provide external calibration for the pH controller.

Organic acid and alcohol concentrations were determined using a Hewlett-Packard 5890A gas chromatograph in tandem with a 3392A automatic sampler and 3392A integrator (Hewlett-Packard Co., Avon, PA), a 4 foot Chromosorb 101 80/100 mesh column, and a flame-ionization detec-

tor. Operating temperatures were 190, 220, and 250°C for the column oven, injection port, and detector, respectively. The N_2 carrier gas flow rate was 25 mL/min. Undiluted, centrifuged samples were acidified with 3*N* phosphoric acid (1 part acid/10 parts sample) and then transferred to sealed injection vials. All samples were automatically injected by the autosampler. All fermentation product concentrations were calibrated using 10 m*M* liquid standards prepared from stock solutions. The analysis of butanol and ethanol was confirmed by high-performance liquid chromatography using a Waters 410 differential refractometer at 35°C and a Waters 712 WISP autoinjector and Waters 600 Multi-solvent delivery system (Waters Associates, Milford, MA) with an Aminex ion exclusion HPX 87H column at 40°C. Alcohol confirmation was also obtained in an outside laboratory (Mass Spectroscopy Facility, Department of Chemistry, Michigan State University, East Lansing, MI) by gas chromatography/mass spectroscopy (Hewlett-Packard 5840 gas chromatograph and Hewlett-Packard 5895 Quadrapole Mass Spectrometer) with a Chromosorb 101 80/100 mesh column at 190°C.

RESULTS

An initial fermentation of 100% CO was conducted for three weeks at a pH of 6.8 with approximately 10 d of steady-state operation. As in previous batch culture studies (*11*), this system was operated with a continuous gas flow of CO much greater than the rate of consumption, thus maintaining nearly a 100% CO headspace above the liquid broth. Similar conditions in batch culture described previously favored acetate production with a corresponding acetate/butyrate ratio (molar) of 32/1. In continuous culture, the resulting molar ratio was approximately 15/1, with average steady-state concentrations of acetate and butyrate in the broth equal to 0.86 and 0.086 g/L, respectively. Ethanol production was also observed in small (< .05 g/L) quantities.

Carbon and electron balance calculations (*12,13*), based on the product composition during steady-state operation, give an overall carbon balance of

$$4CO \rightarrow 2.09CO_2 + 0.63CH_3COOH + 0.043C_3H_7COOH + 0.027C_2H_5OH + 0.43 \text{ cell mass}$$

which shows acetate and cell mass as the primary reduced products. Total consumption of CO during steady-state operation was 10.7 L at a rate of 41 mL/h or approximately 0.69 mL/min—almost two orders of magnitude less than the CO feed rate of 50 mL/min.

The overall stoichiometry allows calculation of some key fermentation parameters, such as molar yield coefficients and carbon/electron partitioning. Molar yield coefficients, defined as mol of product/mol substrate consumed, are helpful in following the carbon flow from CO to multiple

products and serve as an indication of specific pathway activity. For the above stoichiometry, the yield coefficients are

$$Y_{CO_2/CO} = 0.522 \qquad Y_{Ac/CO} = 0.158 \qquad Y_{Bu/CO} = 0.011$$

$$Y_{EtOH/CO} = 0.007 \qquad Y_{Cells/CO} = 0.108$$

which indicates both acetate and cell mass as major reduced products.

Electron partitioning, the flow of available electrons from CO to various products, can also be determined using electron balances. The electron content of a product is directly proportional to its heat of combustion (*14*), and thus represents the relative chemical energy content of that product. The electron partitioning can be represented as

$$100\%\ CO \rightarrow 63\%\ CH_3COOH + 11\%\ C_3H_7COOH + 4\%\ C_2H_5OH + 22\%\ \text{cell mass}$$

The overall carbon partitioning, or total carbon distribution from CO to products, is

$$100\%\ CO \rightarrow 52\%\ CO_2 + 32\%\ CH_3COOH + 4\%\ C_3H_7COOH + 1.5\%\ C_2H_5OH + 10.5\%\ \text{cell mass}$$

These carbon and electron balances confirm some general trends observed previously in batch culture (*11*)

1. Approximately half of the incoming carbon during CO metabolism is lost to CO_2.
2. Acetate is the major reduced product, accounting for 67% of the reduced-product carbon.

Once the conditions for continuous culture were established, steady-state experiments at fermentation pH values of 6.5, 6.0, and 5.5 were conducted. Operation at pH 5.0 resulted in cell washout. A summary of all results is presented in terms of overall stoichiometry, molar yield coefficients, electron partitioning, and carbon partitioning in Tables 1–4, respectively. Also included in Table 5 are the average steady-state broth product concentrations. Concentrations reported as "trace" indicate values less than 0.5 mmol/L.

As observed previously in batch culture (*11*), there was a significant relationship between the fermentation broth pH and the product selectivity. A marked trend exists toward more reduced product formation, specifically butyrate and alcohols, as the fermentation pH is reduced. Although the data at pH 5.5 do not support this trend entirely, the observed shift back toward less reduced product formation at this pH may be an experimental artifact. Table 5 shows a significant drop in the cell density between pH values of 6.0 and 5.5. If the dilution rate, which was maintained constant in all of the fermentations, had been lowered in order to maintain a constant cell density, the fermentation products would have

Table 1
pH Dependent Continuous Fermentation Stoichiometries for CO Consumption by *Butyribacterium methylotrophicum*

pH	Fermentation stoichiometry
6.8	$4CO \rightarrow 2.09\ CO_2 + 0.63\ CH_3COOH + 0.043\ C_3H_7COOH + 0.027\ C_2H_5OH + 0.43$ Cells
6.5	$4CO \rightarrow 2.13\ CO_2 + 0.56\ CH_3COOH + 0.082\ C_3H_7COOH + 0.026\ C_2H_5OH + 0.37$ Cells
6.0	$4CO \rightarrow 2.27\ CO_2 + 0.30\ CH_3COOH + 0.161\ C_3H_7COOH + 0.032\ C_2H_5OH + 0.029\ C_4H_9OH + 0.31$ Cells
5.5	$4CO \rightarrow 2.18\ CO_2 + 0.40\ CH_3COOH + 0.154\ C_3H_7COOH + 0.40$ Cells

Table 2
pH Dependent Molar Yield Coefficients During Continuous CO Fermentation by *Butyribacterium methylotrophicum*

	Molar yield coefficients					
pH	CO_2	CH_3COOH	C_3H_7COOH	C_2H_5OH	C_4H_9OH	Cells
6.8	.522	.158	.011	.007	ND[a]	.108
6.5	.532	.140	.020	.007	Trace[b]	.092
6.0	.568	.076	.040	.008	.007	.078
5.5	.545	.100	.038	Trace[b]	Trace[b]	.100

[a]ND denotes "not detected."
[b]"Trace" quantities indicate amounts corresponding to less than 0.5 mmol/L.

Table 3
pH Dependent Carbon Partitioning During Continuous CO Fermentation by *Butyribacterium methylotrophicum*

	% Carbon					
pH	CO_2	CH_3COOH	C_3H_7COOH	C_2H_5OH	C_4H_9OH	Cells
6.8	52.0	32.0	4.0	1.5	ND[a]	10.5
6.5	53.0	28.0	8.2	1.3	Trace[b]	9.5
6.0	56.8	15.2	16.1	1.6	2.9	7.4
5.5	54.5	20.1	15.4	Trace[b]	Trace[b]	10.0

[a]ND denotes "not detected."
[b]"Trace" quantities indicate amounts corresponding to less than 0.5 mmol/L.

Table 4
pH Dependent Electron Partitioning During Continuous CO Fermentation by *Butyribacterium methylotrophicum*

	% Electrons				
pH	CH_3COOH	C_3H_7COOH	C_2H_5OH	C_4H_9OH	Cells
6.8	63.0	11.0	4.0	ND[a]	22.0
6.5	56.0	20.5	4.0	Trace[b]	19.5
6.0	30.3	40.2	4.8	8.7	16.0
5.5	40.2	38.5	Trace[b]	Trace[b]	21.3

[a]ND denotes "not detected."
[b]"Trace" quantities indicate amounts corresponding to less than 0.5 mmol/L.

Table 5
pH Dependent Product Concentrations During Continuous CO Fermentation by *Butyribacterium methylotrophicum*

	Product concentrations, g/L				
pH	CH_3COOH	C_3H_7COOH	C_2H_5OH	C_4H_9OH	Cells
6.8	0.860	0.086	0.028	ND[a]	0.248
6.5	1.055	0.227	0.037	Trace[b]	0.284
6.0	0.689	0.536	0.056	0.081	0.286
5.5	0.475	0.266	Trace[b]	Trace[b]	0.196

[a]ND denotes "not detected."
[b]"Trace" quantities indicate amounts corresponding to less than 0.5 mmol/L.

been more concentrated and the true metabolic stoichiometry at pH 5.5 revealed. The trace amounts of butanol and ethanol at pH 5.5 excluded these compounds from the carbon and electron balances and, thus, directly influenced the calculation of the overall fermentation stoichiometry.

A highly significant result shown by these data is the continuous production of alcohols, specifically the 4-carbon butanol, solely from the 1-carbon substrate CO. Butanol is produced in greater mass concentration than the 2-carbon ethanol and, as seen in Table 3, contains a significant portion (8.7%) of the available energy content from carbon monoxide. This preliminary result indicates the potential for continuous bioconversion of gaseous carbon monoxide directly to butanol by *B. methylotrophicum*.

The drop in cell production as the pH decreased below 6.0 was expected; in previous batch experiments (*11*), the percent of carbon and electrons flowing into cell mass was greatly reduced when the pH was shifted from 6.8 to 6.0. As stated previously, continuous culture at pH 5.0 resulted in cell washout. Growth was observed at this pH; however, the specific growth rate was less than 0.015 h^{-1}. Regarding CO_2 production, Tables 1–3 indicate that the percent of the carbon lost to CO_2 is not affected more than 9% by fermentation pH, and thus, carbon loss to CO_2 cannot be significantly reduced by manipulating this parameter.

DISCUSSION

Demonstration of continuous, steady-state fermentations using CO gas as the sole carbon and energy source is significant because continuous fermentations have the potential for higher reactor productivities than batch fermentations. For biological conversion of CO, and ultimately synthesis gas, to fuels and chemicals to become economically competitive, high reactor productivities will be required. In addition to demonstrating the potential for continuous bioconversion, this study has shown that the product mix of *B. methylotrophicum* can be regulated by controlling the

fermentation pH. In general, products with a higher reductance degree are preferentially formed at lower pH values.

With the exception of the experiment at pH 5.5, decreasing pH caused an increase in production of more reduced compounds, as shown in Tables 1–4. At pH 6.0, butyric acid, ethanol, and butanol were all formed. These results are consistent with the premise suggested in our previous study (*11*), relating this change in metabolism to a cellular effort to decrease further environmental acidification. Concurrent with the increased production of butyrate and alcohols was a decrease in acetate production by roughly 50% between a pH of 6.8 and 6.0. This observation coincides with the batch culture results, indicating that formation of butyrate and alcohols occurs at the expense of acetate. Furthermore, the decrease in cell production from a pH of 6.5 to 5.5 is also consistent with direct pH inhibition of cell growth at low pH.

The fermentation results at pH 5.5 are inconsistent with this trend. However, as described previously, these data may be misleading owing to the low cell density at this pH; all other data suggest that less cell formation and increased butyrate and alcohol production occur at lower pH values.

The mean cell density at pH 5.5, as shown in Table 5, was significantly lower than the higher pH experiments, presumably from low pH inhibition of cell growth. This inhibitory effect of low pH on overall cell production was confirmed at pH 5.0, where cell washout occurred. These trends define the major obstacle in development of this system: low pH values induce desirable products, but inhibit production of cell mass and, thus, reduce overall productivity. Since the highly reduced products are preferable from a commercial standpoint, these trends become even more critical. The alcohols have potential applications as fuels and solvents, and butyric acid could serve as a chemical intermediate for production of butanol, octane enhancers for gasoline, or even biodegradable polymers (*11*).

Overall, the metabolic basis for CO metabolism leading to butyrate and alcohol production, specifically from a 1-carbon compound to a 4-carbon acid and alcohol, represents a unique and potentially valuable metabolic mechanism for solvent production by *B. methylotrophicum*. Butanol production from CO represents the discovery of a new metabolic pathway previously unknown among unicarbonotrophic microorganisms. Details concerning the biochemical mechanism and its significance have been described elsewhere (*15*).

Critical research needs for development of a potential bioconversion process were also identified during this study. The low specific growth rate of this organism, as indicated by a 12 h doubling time, required a very low dilution rate. Thus, although steady-state operation was achieved, overall volumetric productivities for acids and alcohols were very low, ranging approximately between 0.001 g/L-h for alcohols and 0.015 g/L-h for organic acids. Production of acids and alcohols was achieved at concentrations of approximately 0.05–0.1% for acids and 0.005–0.01% for alcohols, levels that are industrially meaningless. Higher dilution rates

and cell concentrations are needed in order to increase product concentrations and volumetric productivities. Current research efforts designed to address these factors include identification of the enzymatic basis for alcohol production in *B. methylotrophicum* and the incorporation of a cell recycle system. Such a system could operate continuously at a low pH, attain much higher cell densities than possible with conventional chemostate fermentations and, thus, ultimately achieve much higher reactor productivities. With the application of low fermentation pH and cell recycle technology, this fermentation could potentially give rise to an economically attractive process for biological conversion of synthesis gas.

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