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Butyrate Production from Carbon Monoxide by *Butyribacterium methylotrophicum*

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ABSTRACT

Carbon monoxide is produced in high concentrations by gasification of coal or biomass, and is a potentially inexpensive feedstock for biological processes. A number of anaerobic microorganisms metabolize carbon monoxide, with acetate, hydrogen, or methane being the primary reduced products. The CO strain of *Butyribacterium methylotrophicum* was previously shown to grow on carbon monoxide as the sole carbon and energy source, with acetate being the primary product.

This paper demonstrates that by modifying culture conditions, the carbon and electron flow of *B. methylotrophicum* can be manipulated to yield butyrate as the major product. A butyrate concentration of 6 g/L was obtained in batch culture with continuous addition of 100% carbon monoxide. The significance of this fermentation for fuels and chemicals production from carbon monoxide is discussed.

Index Entries: Carbon monoxide; butyrate; fermentation; mass-transfer; *Butyribacterium methylotrophicum.*

INTRODUCTION

Often, the economics of bioprocesses for fuels and chemicals production are dominated by raw materials costs. For example, corn costs are approximately 60 cents per gallon of fermentation ethanol (*1*). Consequently, development of bioprocesses based on less expensive carbon and energy

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feedstocks is clearly desirable. Carbon monoxide (CO) is available in high concentration from gasification of coal or biomass, and is widely used as an industrial feedstock in the chemical industry (*2*). With the appropriate biological catalysts, CO could serve as a potentially inexpensive fermentation feedstock.

Both aerobic and anaerobic microorganisms are able to metabolize CO (*3,4*). However, the anaerobic CO fermentations would be preferable for production of relatively reduced biochemicals, since no available electrons are lost to oxygen. These fermentations typically yield CO_2, cell mass, and a relatively reduced product such as methane, hydrogen, or acetate. Among the anaerobic microorganisms able to metabolize CO, *Butyribacterium methylotrophicum* is noteworthy because of its metabolic diversity. It can grow on a variety of single-carbon substrates, either singly or simultaneously. Known substrates include CO, formate, methanol, and hydrogen/carbon dioxide (*5–7*). The CO strain of *B. methylotrophicum* (*8*) is able to grow vigorously on 100% CO as the sole carbon and energy source. The fermentation products of this strain are also diverse and vary with the substrate consumed. Acetate is produced from formate, hydrogen/carbon dioxide, CO, and mixtures of CO and methanol. Butyrate is produced from methanol in the absence of CO (*9*). This paper demonstrates that the CO strain of *B. methylotrophicum* can also convert CO to butyrate as the major product and that pH is a critical parameter in determining whether acetate or butyrate will be produced.

MATERIALS AND METHODS

Microorganism and Culture Conditions

The CO strain of *B. methylotrophicum* was developed in our laboratory (*8*) and was maintained in 152 mL sealed serum bottles containing 50 mL phosphate-buffered medium under 100% CO headspace at 10 psig. Cultures were grown in the dark at 37°C with 100 rpm shaking.

Culture Medium

The phosphate-buffered medium containing salts, vitamins, trace minerals, and 0.1% yeast extract was adapted from Lynd et al. (*8*) by making the following modifications: the cysteine sulfide content was reduced by 60%, and the phosphate buffer content was reduced by 80%. The phosphate, vitamins, yeast extract, and cysteine sulfide were sterilized separately from the other medium components and combined prior to inoculation. A 2% inoculum of growing *B. methylotrophicum* culture was used.

Fermentation Equipment

Experiments were conducted in a Multigen fermenter (New Brunswick Scientific Co., New Brunswick, NJ) using working volumes of 0.5 and 1.0 L. The pH was automatically controlled by addition of 3 *N* NaOH solution. The temperature was maintained at 37°C, and an impeller speed of 100 rpm was used. Pure carbon monoxide at 1 atm was sparged continuously at a rate of 50 mL/min.

Analytical Methods

Acetate and butyrate concentrations were determined using a Hewlett-Packard 5890A gas chromatograph (Hewlett-Packard Co., Avondale, PA) with a 4 foot Chromosorb 101 80/100 mesh column and a flame-ionization detector. Operating temperatures were 200, 190, and 250°C for the injector, the oven and the detector, respectively. The flow rate of the carrier gas (nitrogen) was 24 mL/min. Biomass concentration was measured by optical density at 660 nm on a Sequoia-Turner Model 340 spectrophotometer (Sequoia-Turner Corp., Mountain View, CA). Samples with an optical density greater than 0.8 were diluted by a factor of ten. Cell mass was calculated using a previously derived optical density vs dry wt calibration curve (*8*).

RESULTS

Constant-pH Fermentation

An extended-batch fermentation with continuous CO sparging was conducted at a pH of 6.8. The results are shown in Fig. 1. Trends in the growth and product formation curves are consistent with those obtained in both batch culture (*8*) and in fed-batch culture with intermittent CO addition (*10*). CO assimilation led to cell growth, and acetate was the primary reduced product. No butyrate production was observed in batch culture, (*8*) and only small amounts of butyrate were detected in fed-batch culture (*10*) and in this experiment. The initial specific growth rate was approximately 0.05 h^{-1}.

Carbon and electron balances (*11,12*) were used to calculate the amount of CO consumed from product formation data. These balances were found by Lynd et al. (*8*) to close to within 3% for batch CO fermentations using *B. methylotrophicum*. The overall carbon balance determined for the data in Fig. 1 is given by

$$4\ CO \rightleftharpoons 2.04\ CO_2 + 0.86\ Ac + 0.024\ Bu + 0.14\ C\text{-mole cells}$$

where CO_2, Ac, and Bu are the molar yields of carbon dioxide, acetate, and butyrate, respectively. One C-mole is the quantity of cell mass contain-

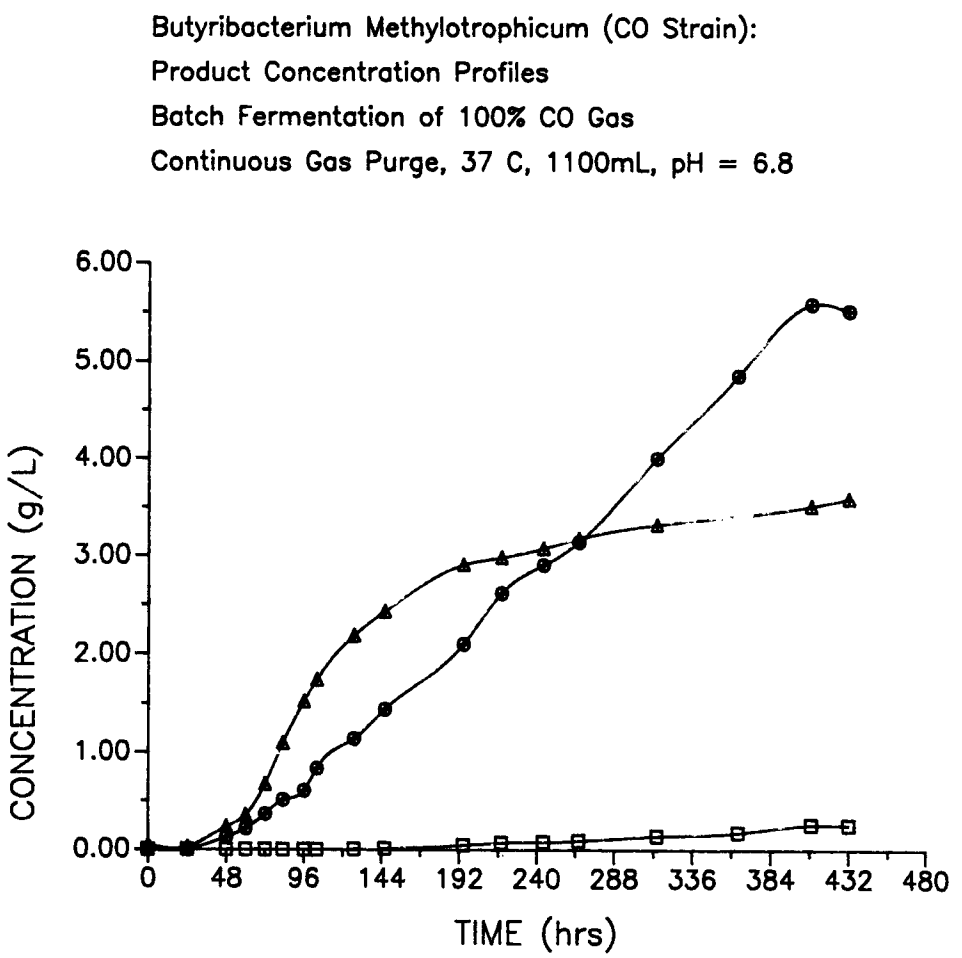

Fig. 1. Constant pH production of acetate, butyrate, and cell mass during batch fermentation with continuous carbon monoxide sparging.
Cell Mass (×10) (△——△); Acetate (○——○); Butyrate (□——□).

ing 12 g of carbon. For *B. methylotrophicum*, 1 C-mole equals 26 g (dry) cells (*8*).

Calculations were performed to estimate whether a significant fraction of the product could have been stripped from the reactor by the sparged CO. As a worst case, the effluent gas was assumed to be in equilibrium with an average liquid-phase acetic acid concentration of 3 g/L throughout the 400 h fermentation. Assuming Raoult's law holds, the gas-phase mole fraction would have been 3.4×10^{-5}, and 0.04 g of acetic acid would have been stripped. This amount corresponds to less than 1% of the total acetic acid produced. Since butyric acid is even less volatile, it can be assumed that evaporative product losses were negligible.

Acetate production occurs during both the growth and the stationary phase, and can be adequately described using the Leudeking-Piret model (*13*)

$$dP / dt = \alpha \, dX / dt + \beta X \quad (1)$$

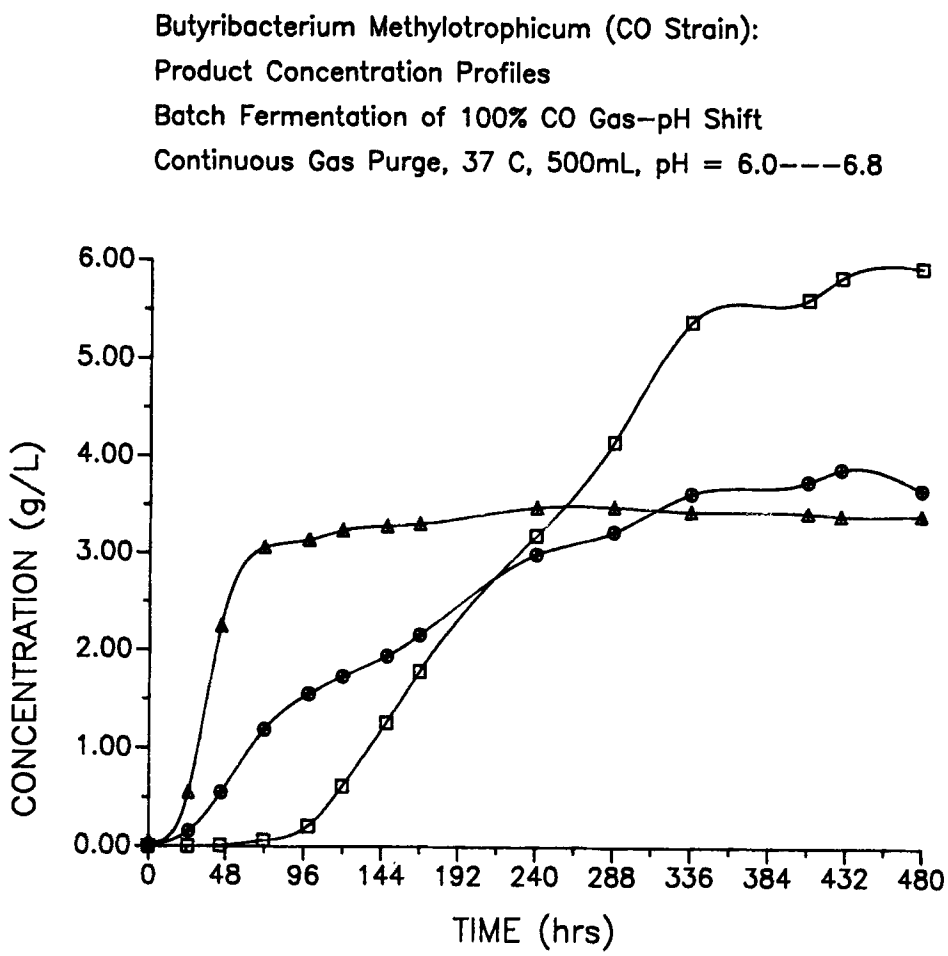

Fig. 2. pH-Shift production of acetate, butyrate, and cell mass during batch fermentation with continuous carbon monoxide sparging.
Cell Mass (×10) (△——△); Acetate (○——○); Butyrate (□——□).

where P and X are the acetate and cell concentrations, respectively, t is time, α is the rate constant for growth-associated acetate formation, and β is the rate constant for non-growth-associated acetate formation. The following values for α and β were estimated using nonlinear regression

$$\alpha = 7.2 \text{ g acetate/g cells}$$
$$\beta = 0.043 \text{ g acetate/g cells} \cdot \text{h}$$

Butyrate, on the other hand, appears to be a secondary metabolite. Its production begins as the growth rate declines, and is most rapid during the stationary phase.

pH-Shift Fermentation

Figure 2 shows the results of an extended-batch fermentation designed to investigate the effect of pH on stationary-phase butyrate production. *B. 2H methylotrophicum* cells were grown as in the first experiment until

Table 1
Percent of Available Electrons Contained in Products

Fermentation conditions	Acetate	Butyrate	Cell mass
Constant pH, growth phase	80%	0%	20%
Constant pH, stationary phase	88%	8%	4%
Constant pH, overall	86%	6%	8%
pH Shift, growth phase	71%	5%	24%
pH Shift, stationary phase	21%	78%	1%
pH Shift, overall	27%	70%	3%

the cell concentration reached a value of 0.31 g/L. At this time, the set-point of the pH controller was changed from 6.8 to 6.0. The resulting pH shift led to a dramatic increase in butyrate production relative to the constant-pH experiment. A final butyrate concentration of 6 g/L was obtained. The overall carbon balance for this experiment is

$$4\ CO \rightleftharpoons 2.30\ CO_2 + 0.26\ Ac + 0.27\ Bu + 0.055\ C\text{-mole cells}$$

The effect of the pH shift on product formation is even more pronounced when the growth and stationary phases are analyzed separately. The growth phase was taken to be the time between the onset of growth and a major decrease in the growth rate, approximately 72 h. The carbon balance during the growth phase is

$$4\ CO \rightleftharpoons 2.05\ CO_2 + 0.71\ Ac + 0.02\ Bu + 0.43\ C\text{-mole cells}$$

and the carbon balance during the stationary phase is

$$4\ CO \rightleftharpoons 2.31\ CO_2 + 0.21\ Ac + 0.31\ Bu + 0.009\ C\text{-mole cells}$$

For comparison, the constant-pH fermentation can also be divided into growth and stationary phases. The carbon balances are

$$4\ CO \rightleftharpoons 2.02\ CO_2 + 0.80\ Ac + 0.37\ C\text{-mole cells}$$

for the growth phase, and

$$4\ CO \rightleftharpoons 2.04\ CO_2 + 0.88\ Ac + 0.034\ Bu + 0.071\ C\text{-mole cells}$$

for the stationary phase.

Thus, by decreasing the pH from 6.8 to 6.0 at the onset of the stationary phase,the metabolism *B. methylotrophicum* can be shifted from acetate to butyrate production. The flow of available electrons from CO to the various products is shown in Table 1. Since heats of combustion are directly proportional to the available electron content for a wide variety of biochemicals (*14*), Table 1 can also be interpreted as the relative chemical energy content of the products.

Table 2
Effect of Gas Composition on Product Formation

Gas composition	Product concentration, g/L		
	Acetate	Butyrate	Ratio, Ac/Bu
100% CO, pH shift	3.88	5.95	0.93
100% CO, constant pH	5.54	0.26	32
80% CO, 20% CO_2, constant pH	4.87	0.21	35

DISCUSSION

Cellular Regulation of Butyrate Production

Acetate and butyrate are formed from CO by the following reactions

$$4\,CO + 2\,H_2O \rightleftharpoons Ac + 2\,CO_2 \quad (\Delta G^{\circ\prime} = -9.8\,\text{kcal/mole CO})$$

$$10\,CO + 4\,H_2O \rightleftharpoons Bu + 6\,CO_2 \quad (\Delta G^{\circ\prime} = -9.7\,\text{kcal/mole CO})$$

The $\Delta G^{\circ\prime}$ values were calculated using published Gibb's free energy of combustion data (*15*), assuming combustion to aqueous HCO_3^- and liquid water at a pH of 7, and unit molality of all other reactants. Both reactions are sufficiently exergonic to drive ATP synthesis, and their free energy changes are equivalent. Thus, neither reaction is thermodynamically favored. The reductance degrees (*11*) of butyrate and acetate are 5 and 4, respectively. Thus, butyrate is the more reduced product, and more CO_2 is produced per mole of CO consumed for butyrate production.

The mechanism by which pH affects product formation is not known. Presumably, regulation occurs at an enzymatic step involved in the reductive conversion of acetyl CoA to butyric acid. This enzyme may be affected by pH either directly (i.e., true pH inhibition) or indirectly, through a pH-influenced mediator. Bicarbonate is a possible indirect mediator. It is known to regulate growth and metabolism in a wide variety of cell types (*16*), and its concentration is strongly affected by pH via the dissociation chemistry of carbonic acid (*17*).

To test the indirect-inhibition hypothesis, an extended batch fermentation was conducted at a pH of 6.8. A gas feed of 80% CO and 20% CO_2 was used. The CO_2 was added to increase the liquid-phase bicarbonate concentration. The results are shown in Table 2. Because an increased CO_2 concentration did not significantly affect the ratio of acetate to butryate in the product, bicarbonate is probably not the primary metabolic regulator.

However, the data are consistent with direct pH regulation. For an equivalent amount of CO consumed, acetic acid synthesis yields 2.5 times

more carboxyl groups than does butyric acid synthesis. In addition, acetic acid has a larger dissociation constant than does butyric acid. Thus, butyric acid production acidifies the medium less than does acetic acid production. It follows, then, that butyrate production would be favored over acetate production at lower pH values to minimize further pH reduction. Further studies are under way to investigate the effect of pH on the metabolism of *B. methylotrophicum.*

Mass Transfer Considerations

The rate of interphase CO transport is a potential rate-limiting factor in CO fermentations. Unfortunately, efforts to characterize the simultaneous mass transfer and reaction are hindered by the lack of a convenient and reliable liquid-phase CO assay. In this study, CO consumption rates were calculated from carbon and electron balances and used to identify the rate-limiting phenomena.

Figure 3 shows the time profiles of cell concentration, specific CO uptake rate, and volumetric CO uptake rate for the constant-pH fermentation. The rate data were calculated using carbon and electron balances and numerical differentiation of the product-formation data. A three-point algorithm for nonconstant step sizes was used (*18*). Early in the growth phase, the volumetric CO uptake rate increased with cell concentration, whereas the specific CO consumption rate remained approximately constant. These trends are indicative of an adequate nutrient supply and balanced growth. However, at a cell density of approximately 0.09 g/L, the volumetric rate became constant, whereas the specific rate began to decline. As the cell concentration continued to rise, the specific rate plummeted, whereas the volumetric rate declined only gradually. These trends suggest that CO transport was rate-limiting for cell densities greater than 0.09 g/L, as discussed below.

The volumetric transfer rate (Q) of a sparingly soluble, dispersed gas into a liquid phase is described by

$$Q = k_1a\,(C^* - C) \tag{2}$$

where k_1a is the volumetric mass transfer coefficient, C^* is the liquid-phase concentration in equilibrium with the gas phase, and C is the liquid-phase concentration. Under mass-transfer limited conditions, $C \approx O$, and Q becomes constant. Thus, for cell concentrations greater than 0.09 g/L in Fig. 3, CO transport appears to have been the rate-limiting factor. The gradual decrease in Q observed in the latter stages of the fermentation may have been due to the increasing broth viscosity visually observed during this time. Higher viscosities are known to decrease k_1a values in stirred-tank fermentations (*19*).

The transport properties of CO and oxygen are compared in Table 3. The aqueous molar solubility of CO is approximately 25% less than that of oxygen at 35°C. Consequently, mass-transfer driving forces in CO fermen-

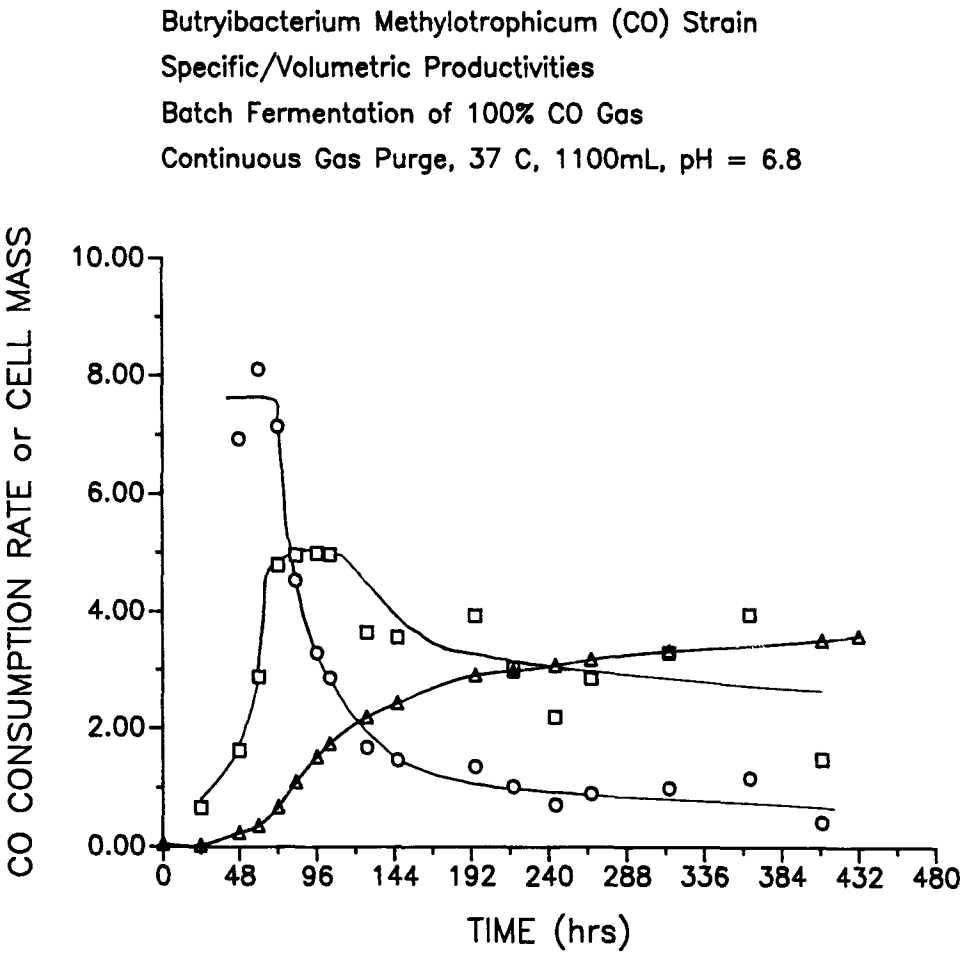

Fig. 3. Cell mass production and carbon monoxide consumption rates during constant pH fermentation.

Cell Mass (g/L x 10) (△——△); Volumetric CO Consumption Rate (g/L · h × 1000) (□——□); Specific CO Consumption Rate (g/g cells · h × 100) (○——○).

Table 3
Transport Properties of Carbon Monoxide and Oxygen in 35°C Water

Gas	Solubility,[a] mg/L	Solubility,[a] mmole/L	Diffusivity,[b] in water, cm^2/s
Carbon monoxide, CO	23.4	0.84	2.76×10^{-5}
Oxygen, O_2	33.4	1.09	3.08×10^{-5}
Ratio, CO/O_2	0.70	0.77	0.90

[a] Source: International Critical Tables (*20*).
[b] Calculated using the Wilke-Chang Correlation (*21*).

tations would be relatively low, unless high pressures were used. The diffusivity of CO is 10% less than that of oxygen. Since k_1a is proportional to the square root of the diffusivity, according to Surface Renewal theory (*22*), k_1a values for CO should be slightly less than values measured for oxygen under similar hydrodynamic conditions. Further studies are underway to characterize the influence of CO transport on fermentation productivities.

Significance of Butyrate for Fuels and Chemicals Production

The bioconversion of CO to butyrate by *B. methylotrophicum* is significant in that it demonstrates the biological synthesis of a four-carbon hydrocarbon from an inorganic, single-carbon substrate. CO is a major component of synthesis gas, which can be obtained from a wide variety of sources, such as natural gas and gasification of coal, biomass, or petroleum. Because the US has large reserves of coal and biomass, CO utilization would not require the import of raw materials.

The economics of CO (or synthesis gas, a mixture of primarily CO and H_2) as a potential fermentation feedstock are difficult to evaluate, because of the scarcity of available data. However, the Tennessee Eastman Company is known to use synthesis gas at a rate of 900 T/d for production of acetic anhydride (*23*). The use of coal as a feedstock and the performance of the Texaco gasifier have been identified as key factors contributing to the favorable process economics. A rough comparison of the cost per available electron equivalent can be made between synthesis gas and corn-derived sugars. The cost of corn-derived glucose was \$0.59/kg in 1980 (*24*). This cost translates into a cost of \$0.005 per available electron equivalent. The cost of synthesis gas was estimated by Marlatt and Datta (*25*) to be \$0.003 per standard cubic foot, or \$0.0012 per electron equivalent. Thus, preliminary estimates of the relative cost of synthesis gas are encouraging. More accurate data are needed to provide a more detailed feasibility analysis.

There are several potential commercial applications of butyrate. It can be esterified using methanol or ethanol to produce methyl butyrate or ethyl butyrate. These esters have potential applications as solvents, gasoline octane-enhancers, and precursors for ketone synthesis. Conceptual processes for esterification of dilute aqueous streams of butyrate and subsequent ester recovery have been proposed (*25*).

Butyrate can also be reduced to butanol, which has existing markets as a solvent for surface coatings and as a chemical intermediate. This reaction is possible via fermentation. *Clostridium acetobutylicum* is able to convert butyrate to butanol using hydrogen or small quantities of glucose to supply the reducing equivalents. Meyer et al. (*26*) have demonstrated that acetate and butyrate can be rapidly converted to ethanol and butanol

by sparging a continuous, glucose-limited culture of *C. acetobutylicum* with CO. Cell growth was found to continue during alcohol production, but at a reduced rate.

A third potential application of butyrate is in the production of biomedical polymers and biodegradable thermoset plastics. Bacteria such as *Rhodobacter sphaeroides* are known to convert mixed fatty acids, such as butyrate and valerate, to biodegradable copolymers of 4 and 5 carbon hydroxyacids and terpolymers of 4, 5, and 7 carbon monomers. The physical properties of these polymers are now being studied to evaluate their commercial potential (27).

CONCLUSIONS

B. methylotrophicum can convert CO, as the sole carbon and energy source, to either acetate or butyrate. Acetate is produced in both the growth and stationary phases, whereas butyrate is produced primarily in the stationary phase. The ratio of acetate to butyrate produced during the stationary phase can be controlled by pH adjustment. Further research is needed to verify the biochemical basis for this regulation and to optimize butyrate yields.

Butyrate has potential commercial value as a precursor of butyryl esters, butanol, and biodegradable polymers. However, volumetric reaction rates and final product concentrations need to be increased. The low solubility of CO in aqueous solutions makes interphase CO transport a likely rate-limiting factor in CO fermentations.

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