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BIOCONVERSION OF CARBON MONOXIDE FOR PRODUCTION OF
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presented by

Andrew Jacob Grethlein

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Robert Mark Worden

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BIOCONVERSION OF CARBON MONOXIDE
FOR PRODUCTION OF
MIXED ACIDS AND ALCOHOLS

By
Andrew Jacob Grethlein

A THESIS

Submitted to
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ABSTRACT**BIOCONVERSION OF CARBON MONOXIDE
FOR PRODUCTION OF
MIXED ACIDS AND ALCOHOLS**

By

Andrew Jacob Grethlein

Direct fermentation of carbon monoxide was conducted anaerobically in both batch and continuous culture using *Butyribacterium methylotrophicum*, and the associated 1-carbon compound metabolism analyzed. Fermentation pH was determined to be a significant regulatory factor in the production of acids and alcohols, with butyric acid and butanol production observed at low pH values for both modes of operation. These results represent the first evidence for direct, pure-culture conversion of carbon monoxide to butanol. Preliminary results concerning mass transfer of carbon monoxide and the enzymatic mechanism for acid production were also obtained.

To those not as fortunate
to have had the opportunity...

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INTRODUCTION

The following work was directed towards development and operation of a continuous, steady-state fermentation system for bioconversion of coal derived synthesis gas. The obligate anaerobe *Butyribacterium methylotrophicum*, a bacteria capable of growth on several 1-carbon substrates, served as the model microorganism. Primarily conducted in laboratory scale fermenters, experiments were designed to achieve a fundamental understanding of both cellular metabolism and key factors influencing product formation rates and concentrations. To this end, anaerobic, pure culture fermentations were conducted in both batch and continuous modes, with carbon monoxide gas simulating the synthesis gas substrate.

Overall objectives included:

1. Identification in batch culture of possible regulatory factors influencing end product formation including secondary substrates, gas composition, and gas/liquid mass transfer effects.
2. Development of a continuous fermentation for *B. methylotrophicum* and operation under steady-state conditions.
3. Investigation of key intracellular enzyme activities directly involved in the conversion of carbon monoxide to reduced end products.

The significance of this work arises from its underlying process orientation. Although microbial carbon monoxide utilization is well

documented in the literature, the vast majority of reports are purely scientific investigations of both characterization and taxonomy. From an engineering standpoint this is usually without clear reference to any practical application. However, the present work has been designed around a novel process scheme for synthesis gas bioconversion. Thus it is not simply an exercise in the typical stages of fermentation development, mainly from test tube to batch fermenter to continuous culture, etc., but is specifically oriented towards a comprehensive and novel bioprocess. The research program, centered around the above objectives, was therefore designed towards achievement of specific process oriented goals rather than detailed investigations of underlying mechanisms. In this way the fundamental discoveries were not only of scientific interest but of potential industrial import.

The textual organization of this work also reflects this orientation. Chapter I introduces two topics relevant to the bioconversion process concept, the current status of industrial synthesis gas technology and the present state of understanding for 1-carbon compound metabolism in anaerobic bacteria. The chapter then continues with an outline of the specific process including a closer inspection of the literature relevant to *B. methylotrophicum*, with emphasis on known reaction stoichiometries. Finally, the chapter is concluded with a brief section on the scope of the present work through statement of the main obstacles and the directions taken to overcome them.

Chapter II describes the materials and methods utilized. Experimental results and discussion then follow in Chapter III for the batch culture studies, Chapter IV for the continuous culture work, and

Chapter V for the enzymatic analysis. The text is concluded in Chapter VI where the significant accomplishments are placed in their overall process perspective, some brief speculations are presented pertaining to the results themselves, and a few recommendations made for further development. A general description of carbon and electron balancing, a method for calculating reaction stoichiometries critical to the analysis of all the fermentation results, is presented in the Appendix.

CHAPTER I
TECHNICAL REVIEW AND PROCESS ORIENTATION

Although the current energy outlook from petroleum based resources is not as grim as in 1975, there can be no doubt that the world in general is undergoing a radical transition with respect to energy supply and demand. The new potential for "alternative" energy sources such as nuclear and solar energy as well as renewed interest in improvement of available resource utilization in the fields of natural gas and coal technology are certainly apparent from the amount of current research and development taking place today. Whatever the new designs and novel approaches currently being investigated in these fields there must always be an economic potential for industrial application. Those concepts that concern improving existing processes or which find much improved alternative uses for existing resources will most likely prove more attractive to potential investors; government, industrial, or otherwise. The reason is simply economic in terms of modification of an existing plant to meet future needs versus construction of a new facility based on new, unproven technology. However, regardless of the global incentives for development and diversification of available resources, success of future processes is difficult when compared to current albeit short-sighted dependence on dwindling resources.

The following review encompasses a brief survey of the technical setting of such a future process, in this case bioconversion of coal derived synthesis gas. Although currently a purely exploratory investigation, the fundamental principles relevant to the design of any industrial process still apply and serve as a guiding factor in process

development. Thus a familiarization with competing processes and supporting technologies as well as a thorough grounding in the current status of available biocatalysts is essential.

1. Overview of Synthesis Gas Technology

1.1. Availability of Synthesis Gas

With coal being a large domestic energy source, the size of the coal mining and coal conversion industry is understandable. As a direct energy source and as a raw material for chemical processing, coal is an attractive and existing alternative to petroleum. Coal conversion to fuels and chemicals is also a promising and current technology undergoing modification and development in all aspects. Ten years ago US coal production was about 780 million tons, and is expected to triple by the year 2000 with about 30% utilization for synthetic fuels (Exxon Co., 1979). Many routes exist for coal conversion such as direct gasification to methane or synthesis gas, direct liquefaction to liquid fuels, and conversion to hydrogen and carbon dioxide by the water gas shift reaction, as examples. Either as end or intermediate products, the major synthesis gas components (carbon monoxide, carbon dioxide, and hydrogen) can be obtained by carbon and coal gasification. This conversion is well established technically throughout the industry, although most synthesis gases produced industrially today are derived from partial oxidation of petroleum liquids or catalytic reforming of natural gas. Nevertheless, with the domestic coal resources available, coal-based processes remain an attractive alternative to existing petroleum conversion routes.

Coal gasification involves the catalytic production of high BTU gases through several different but related processes such as the Lurgi,

Koppers, and Winkler gasification processes. These processes are directed towards production of synthesis gas either for further chemical conversion or as a direct energy source. Typical exit gas compositions are shown in Table 1.

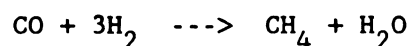
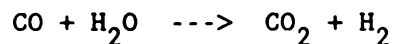
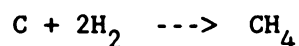
Table 1: Exit gas compositions from coal gasification processes.

<u>Process</u>	<u>Gas Composition (vol%)</u>					
	<u>CO</u>	<u>H₂</u>	<u>CO₂</u>	<u>H₂O</u>	<u>CH₄</u>	<u>H₂S</u>
BGC Lurgi ^a	55.5	28.3	5.1	0.0	6.3	3.0
Shell-Koppers ^a	65.0	32.1	0.8	0.0	0.0	1.4
Koppers-Totzek ^b	50.4	33.1	5.6	9.6	0.0	0.3

^a From Developement Status of Key Emerging Foreign Gasification Systems, TRW Report Sept. 1980.

^b From Cusumano, Dalla Betta, and Levy, 1978.

Synthesis gas is produced most commonly by reaction of coal with water, oxygen, and catalyst (steam gasification) at high temperature and pressure, which can give various amounts of several gaseous products as shown in Table 1 depending on the process reaction conditions and catalyst used. Furthermore, product composition is also influenced by reactor design, specifically catalyst bed design, whether it be fixed, moving, entrained, or some related approach (Wilson, Halow, and Ghate, 1988). Whatever the conditions, product formation is governed by four main reactions taking place in the gasifier:



Manipulation of these reactions is crucial in determining exit gas composition and thus influences all subsequent reactions. The exit gas stream is normally contaminated with some sulfur containing gases which must be removed prior to further processing.

However, regardless of the final product, whether it is synthetic liquid fuel, substitute natural gas, or even power generation from synthesis gas combustion, production of a synthesis gas stream in coal gasification processes is an integral component of coal processing operations. Thus potential sources of synthesis gas for reaction in new processes, biological or otherwise, are many and varied, existing wherever coal gasification technology is being utilized.

1.2. Present Chemical Uses of Synthesis Gas

Synthesis gas is itself a precursor to many reactions involved in coal processing. Methane production from coal to form substitute natural gas (SNG) involves catalytic coal gasification yielding methane, carbon dioxide, carbon monoxide, and hydrogen gases, followed by sulfur containing gas and carbon dioxide removal, with separation and recycling of CO/H₂ gas to the gasifier. Thus the net products are methane, carbon dioxide, and residual sulfur gases.

Many fuel hydrocarbons can be produced by direct conversion of synthesis gas. Conventional Fischer-Tropsch processes yield a variety of paraffinic, olefinic, aromatic, and oxygenated compounds, although selectivity between compounds is poor (Schriesheim and Kirshenbaum, 1981). Crude methanol can be produced catalytically, using Cu/ZnO/Cr₂O₃ catalysts, as an end product or as a precursor for longer hydrocarbon production via methanol chemistry. These indirect synthesis routes using methanol from synthesis gas include ethanol, ethylene, ethylene glycol,

and styrene production and present a host of fuel uses through direct use of methanol or conversion to gasoline enhancer compounds such as methyl tertiary butyl ether (Frank, 1982; Ehrler and Juran, 1983). Acetic anhydride and methyl acetate production is also possible and these compounds are currently being produced commercially from coal (Agreda, 1988). In general synthesis gas can potentially play an important role in C_1 chemistry development (Haggin, 1981).

Besides use as a chemical precursor, electric power generation through coal gasification is potentially a viable alternative to existing coal-fired power generation facilities (Spencer, Gluckman, and Alpert, 1982). These processes could involve direct burning of synthesis gas, cogeneration of synthesis gas and methanol for power generation, or even some combination of power generation and chemical production (Spencer, Gluckman, and Alpert, 1982).

The use of synthesis gas from coal as an industrial feedstock in the chemical industry is potentially widespread and even in some cases being performed industrially as outlined previously. The revitalization of coal and C_1 based chemistry stemming from recent developments in gasification technology will provide an increasingly attractive alternative to present petroleum based chemicals production. Yet production of coal based synthesis gas is a complicated process requiring advanced separation and purification technology. One of the major drawbacks in utilizing coal derived gases for chemical and fuel production is the required removal of catalyst poisons such as sulfur compounds (hydrogen sulfide and carbonyl sulfide) and other trace contaminants from the synthesis gas. These purification steps are energy intensive and are a significant part of the cost breakdown of the final

product, particularly when the high sulfur content of most U.S. coals is recognized (Wilson, Halow, and Ghaté, 1988). Thus there has been an increase in research and development programs in both industry and universities to explore innovative and more economical separation processes. Some of these exploratory areas include pressure-swing adsorption separation, coordinated complex separation in organic solvents, and solubility based separation. Circumvention of the entire gas separation process, achieved through microbial fermentation of the exit gasifier stream, is a biological route also under investigation.

1.3. Potential for a Bioconversion Process

Microbial bioconversion of synthesis gas as a processing step in conversion of coal to fuels and chemicals offers some key advantages over conventional and purely chemical methods but with respect to process development, the concept lacks experimental verification. A hypothetical process would involve single or multi-stage fermentation of the synthesis gas followed by product separation from the effluent broth, which is likely to be quite dilute. Pure or mixed cultures of microorganisms capable of metabolizing carbon monoxide, carbon dioxide, and hydrogen could be used.

The most attractive advantages offered by a biological scheme come from avoiding the costly separation and removal of the sulfur containing catalyst poisons contained in the synthesis gas. With highly sulfur tolerant microorganisms conversion of synthesis gas can occur without the need for purification; the H_2S and COS gases will simply be part of the overall waste stream generated by the process. Thus the direct savings will stem from the capital and operating costs associated with conventional gas separation/upgrading requirements. However, separation

of fermentation products itself will likely require some sort of energy intensive step. Similarly, requirements for strict CO/H₂ ratios in the synthesis gas are often present in chemical processing to maintain a particular product mix, which requires gas recompression and shift converting equipment. This ratio is potentially not a key factor in biological conversion with respect to product formation and therefore the extra equipment and incurred capital costs could be avoided. As with most biological systems, fermentation is conducted at relatively low pressure and temperature, at least an order of magnitude lower than in conventional gas-phase catalysis, with savings thus stemming from both operating and capital costs for compression and heating equipment. These key potential advantages have generated some small scale exploratory research activity in the synthesis gas bioprocessing area although purely in a lab scale environment.

2. Potential Microorganisms: 1-Carbon Compound Metabolism

A variety of microorganisms capable of metabolizing 1-carbon compounds such as methanol, carbon dioxide, formate, and carbon monoxide exist with great diversity in the microbial world. Some of the most promising industrially are anaerobic bacteria, specifically unicarbonotrophic anaerobic bacteria, which are bacteria capable of growth with 1-carbon compounds as the sole carbon and energy source. Anaerobic metabolism conserves the chemical energy content of the substrate very efficiently with the absence of oxygen and oxidation; anaerobic unicarbonotrophic bacteria have a metabolic efficiency greater than all other biosynthetic life forms (Zeikus, 1982). From a process standpoint the lack of oxygen, maximal energy conservation, and high

product yields associated with fermentations involving these bacteria are very desirable. A general schematic for coal gas bioconversion via 1-carbon anaerobic fermentations is shown in Figure 1. As indicated, several routes are possible for formation of fuels, chemicals, and other products by anaerobic bacteria.

The following brief survey of unicarbonotrophic anaerobic bacteria presents some key species from a substrate utilization viewpoint and is separated in terms of major end products formed.

2.1. Methanogenic Bacteria

Methane production is the keystone of methanogenic bacteria, a physiologically diverse group of microorganisms. Unicarbonotrophic metabolism links many methanogenic species, and several reviews are available (Zeikus, 1977, Balch, *et al.*, 1979, Zeikus, 1983a). Growth on hydrogen and carbon dioxide (H_2/CO_2) as the sole carbon and energy source has been well documented among many methanogens, although the physiological details of growth have been studied primarily in *Methanobacterium thermoautotrophicum* and *Methanosarcina barkeri* (Zeikus, 1983a).

M. thermoautotrophicum exhibits rapid growth on H_2/CO_2 (Zeikus and Wolfe, 1972) and has also been adapted to grow on carbon monoxide (CO) alone, albeit with substantially reduced rates compared to H_2/CO_2 and in a dilute CO atmosphere (Daniels *et al.*, 1977). Doubling times of approximately 2 hours have been documented in several studies, and even a one hour doubling time has been observed in continuous culture, making this thermophilic species the most prolific methanogen known (Zeikus, 1983a).

M. barkeri exhibits the highest degree of metabolic diversity among

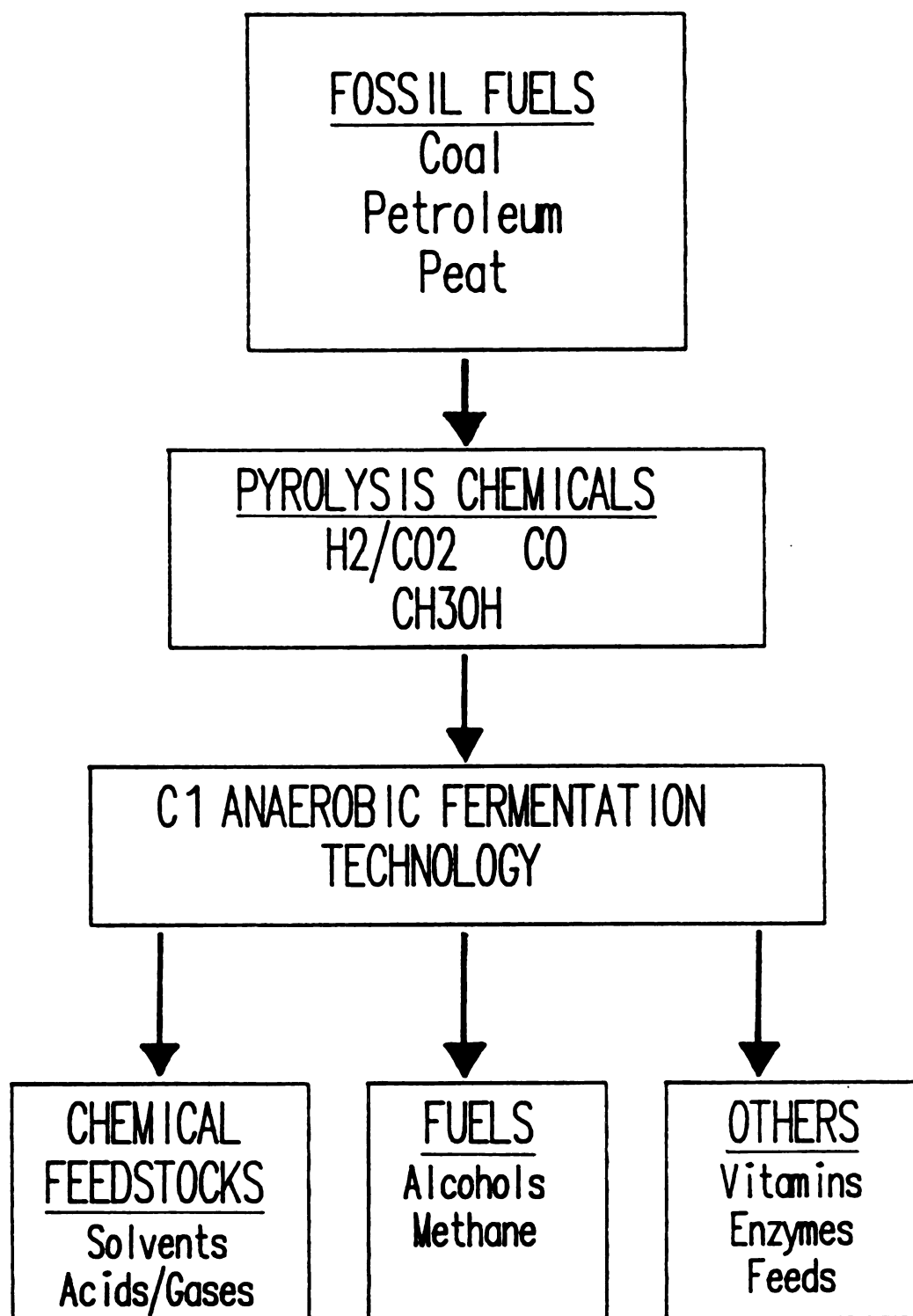


Figure 1: Potential C1 anaerobic fermentation routes.
(Adapted from Zeikus, 1980)

methanogens and is capable of unicarbonotrophic growth on H_2/CO_2 , methanol (CH_3OH), and CO (Zeikus, 1980). Mixotrophic growth is also possible with H_2/CO (Daniels *et al.*, 1977), and $H_2/CO_2/CH_3OH$ (Weimer and Zeikus, 1978). This species is also capable of growth on several multi-carbon compounds including acetate and polymethylamines (Zeikus, 1983a).

With respect to coal gasification products methanogenic bacteria could ultimately play a role in biological substitute natural gas production. Generally, methane production from coal or biomass derived synthesis gas is known as "biomethanation"; inherent in this definition is the potential for secondary product formation such as short chain organic acids. However, the basic tenet of biomethanation is in upgrading the BTU content of synthesis gas; studies with an anaerobic mixed culture of bacteria have shown CO_2 formation from CO, and methane production from H_2/CO_2 (Wise *et al.*, 1978). Enhancement of methane yields, growth rates, and improvement of rate limiting metabolic steps, as well as liquid mass transfer requirements, have been identified as areas requiring detailed study (Zeikus, 1983b), indicating that this potential technology is in the fundamental stages of development.

Economically, methane production is attractive as a waste treatment process, the attractiveness being in the consumption/treatment of organic waste materials and not in the generation of methane (Zeikus, 1983a). For application as a synthesis gas processing step, more specifically coal derived methane production, the economics will most likely require a significant increase in both the price and market demand for methane, perhaps stemming from depletion of natural gas reserves. Even in this event the chemical technology already exists for SNG production from synthesis gas, which further complicates (or

simplifies) the potential industrial niche for a methane producing bioconversion process.

2.2. Acidogenic Bacteria

Acid-producing anaerobic bacteria are widespread and numerous with many species producing a variety of compounds including acetic, propionic, and butyric acids as well as the more familiar lactic acid fermentation. When this large group of microorganisms is separated into unicarbonotrophic acidogens there still exists some diversity although there are fewer species. These unicarbonotrophes can be further differentiated with respect to acids produced, the majority existing as homoacetogens, which are acetic acid producers, with a much smaller group of homobutyrogens, which are butyric acid producing bacteria. Lesser amounts of other organic acids and ethanol are also often present as side products with the 1-carbon substrate fermentations associated with these anaerobes. The few bacteria outlined below represent the most promising species with respect to synthesis gas bioconversion based on their metabolic ability to convert hydrogen, carbon monoxide, carbon dioxide, and methanol, or combinations of these four substrates, to organic acids in pure culture; mixed culture conversion is not addressed in detail.

One thermophilic bacteria which is normally able to ferment a variety of sugars to acetate has also exhibited growth on 1-carbon compounds. *Clostridium thermoaceticum* type strain Fontaine grows both on H_2/CO_2 , with an 18 hour doubling time, and CO, with a 16 hour doubling time, but is not capable of growth on CH_3OH (Kerby and Zeikus, 1983). Growth on 100% CO was reported not to be inherent but was attained after prolonged cultural adaptation. Several key enzymes involved in 1-carbon

metabolism, including CO dehydrogenase and hydrogenase, have been isolated and characterized from this organism (Drake, et al., 1980; Drake, 1982). Stoichiometrically, approximately one mole of acetate is produced per 4 moles H_2 and 2 moles CO_2 or per 4 moles CO (Kerby and Zeikus, 1983).

Another thermophile, *Clostridium thermoautotrophicum*, exhibits a similar metabolism. This acetogenic bacteria has an 8 hour doubling time when grown on H_2/CO_2 at 60°C with 4 moles H_2 and 2 moles CO_2 (4/2) to 1 acetate conversion stoichiometry; acetate production from CH_3OH was also observed (Wiegel et al., 1981). Growth on CO has been reported (Wiegel, 1982). The biochemical basis for these results has been elucidated enzymatically with CO dehydrogenase and hydrogenase enzyme playing an important role (Clark et al., 1982). Similarly, *Acetobacterium woodii* can grow and produce acetate from H_2/CO_2 (4/2 to 1) (Balch et al., 1977), CH_3OH (Bache and Pfennig, 1981) and also CO (Kerby et al., 1983). Enzyme isolates have included CO dehydrogenase (Ragsdale et al., 1983).

The homoacetogenic bacteria *Peptostreptococcus productus* strain U-1, isolated from anaerobic sewage digester sludge, exhibited a 1.5 hour doubling time in a 50% CO gas space, with 1 mole of acetate produced for every 4 moles of CO consumed (Lorowitz and Bryant, 1984). Also observed was growth on H_2/CO_2 producing acetate in a 4/2 to 1 ratio with a 5 hour doubling time. Several similar species have been reported to produce acetate and small amounts of butyrate and propionate from H_2/CO_2 and H_2/CO in mixed culture obtained from sewage sludge (Levy et al., 1981). Mixed cultures obtained from chicken waste grown on CO or H_2/CO_2 have produced both acetate and ethanol (2:1 molar ratio) in both batch and continuous culture (Vega et al., 1988). These mixed cultures have also

been reported to produce C_3 - C_4 alcohols and C_3 - C_4 organic acids in trace (less than 0.01 g/L) amounts (Clausen and Gaddy, 1987). However, undefined mixed bacterial cultures are relative "black boxes" with respect to specific production mechanisms and metabolic reactions and are thus much more difficult to assess and characterize; furthermore, reproducibility and steady-state maintenance of non-pure culture fermentations could be a major drawback of such systems.

A sheep rumen isolate, *Eubacterium limosum* is capable of both acetate and butyrate production depending on the substrate. When grown on H_2/CO_2 a 14 hour doubling time was observed concurrent with acetate production; minor amounts of butyrate were also detected (Sharak Genthner *et al.*, 1981). The same report indicated faster growth (7 hour doubling time) on CH_3OH with acetate/butyrate formation in a 1:1 ratio, although CO_2 and acetate were required for growth. Acetate production from CO was reported with a 7 hour doubling time, however, these results were with a 50% CO gas phase; doubling times increased to 18 hours in a 75% CO gas phase, which was the highest percentage tested (Sharak Genthner and Bryant, 1982).

Probably the most versatile of unicarbonotrophic anaerobic bacteria, *Butyribacterium methylotrophicum* is capable of both acetate and butyrate production under different growth conditions. Growth on H_2/CO_2 produced acetate in a 4/2 to 1 ratio with a 9 hour doubling time, while growth on CH_3OH , in the presence of acetate and CO_2 , produced butyrate in a 4 to 1 (methanol to butyrate) ratio, also with a 9 hour doubling time (Zeikus *et al.*, 1980; Lynd and Zeikus, 1983). Product formation and fermentation rates were further found to depend on the methanol/carbon dioxide (bicarbonate) ratio (Datta and Ogletree, 1983). A mutant strain of this

organism, *Butyribacterium methylotrophicum:CO* strain, was adapted to grow with a 12 hour doubling time on 100% CO, producing acetate and minor amounts of butyrate (Lynd et al., 1982).

If there is any potential application for bioconversion of coal derived synthesis gas it will most likely involve an acidogenic fermentation with a 1-carbon metabolism similar to one of the previously outlined anaerobic bacteria. Biological production of organic acids from synthesis gas could become practical with the right process economics but the market prices are not encouraging; acetic acid is a rather low value chemical and is currently produced industrially in large volume (Zeikus, 1983b).

However, further processing of these acids, either biologically or chemically, may result in more favorable economics. Methyl and ethyl esters of volatile organic acids could potentially be utilized as solvents, gasoline octane enhancers, and chemical intermediates; several novel processes and concepts have been proposed (Datta, 1981). Alcohols and ketones derived from acetic and butyric acid precursors are potential liquid fuel additives, industrial solvents, and intermediates for further processing to ethylene or butadiene, any or all of which could have industrial significance with a conducive market.

2.3. Sulphidogenic Bacteria

One-carbon compound metabolism in this extremely diverse group of bacteria has been documented although in general the specific physiological concepts have not been well determined. Several species, including *Desulfovibrio vulgaris*, *Thermodesulfotobacterium commune*, and *Desulfovibrio desulfuricans* have exhibited growth on H_2/CO_2 plus acetate, although growth on H_2 alone required long adaptation periods

(Zeikus, 1983a). In general these species catalyze a range of sulfate reducing reactions including sulphate, sulfur, and thiosulfate reduction. Since the production of reduced sulfur compounds is not of industrial interest in coal gasification technology, these bacteria have been included purely for emphasis of the wide range of available microorganisms that are capable of 1-carbon compound metabolism.

3. Process Outline: Biotechnology of Indirect Liquefaction

A process for bioconversion of coal derived synthesis gas must address several key issues for success industrially. In general, a well designed biological route presents some distinct advantages over the conventional chemical technology for synthesis gas processing as were previously outlined, mainly the avoidance of costly sulfur removal steps and operation at low pressures and temperatures. However, there are several disadvantages such as relatively low productivities for both cells and products and sterility requirements for microbial fermentations, as well as requirements for an efficient separation process for product recovery based on a dilute aqueous fermentation broth. Any potential bioconversion process must include at least some practical provisions for overcoming these inherent problems.

Furthermore, to justify research and development in the laboratory there must be a targeted market for the product compounds associated with the process. Certainly with favorable economic conditions, specifically a major increase in the price of available petroleum based resources, there could be a niche for biologically produced alcohols and oxygenated compounds derived from synthesis gas, a few of which were outlined in the preceding section. Research in this area may also open new avenues

for synthesis of 1-carbon derived solvents, esters, and acids through a thorough understanding of the underlying metabolic processes involved and manipulation/enhancement with proper engineering.

With support from the United States Department of Energy, the Michigan Biotechnology Institute in Lansing, Michigan is currently developing a bioconversion process for coal derived synthesis gas, of which the present work is an integral part. The goals of the contract are to establish the fundamental knowledge required for a high productivity, continuous, steady-state process converting synthesis gas to a mixture of butanol, ethanol and acetone. A significant amount of this work will include application of laboratory biochemical developments with lab-scale engineering based fermentation advances to an overall integrated process scheme. Thus not only will the fundamental knowledge base of 1-carbon fermentation metabolism be increased but also obtained will be an understanding of the bioconversion process on an engineering application level. This section is a brief review of the proposed process, including a more detailed microbiological description of the bacteria utilized in this study.

3.1. Two Stage Bioconversion of Synthesis Gas: The MBI Process

The MBI process consists of a two stage anaerobic fermentation of coal derived synthesis gas plus methanol to produce a mixed solvent broth containing primarily butanol, ethanol, and a lesser amount of acetone, followed by energy efficient distillation recovery. As outlined in Figure 2, the synthesis gas mixture enters the Stage 1 acidogenic fermentation where it is converted to a mixture of organic acids (primarily acetate and butyrate) by the versatile acidogenic unicarbonotroph, *Butyribacterium methylotrophicum* (CO Strain). The two

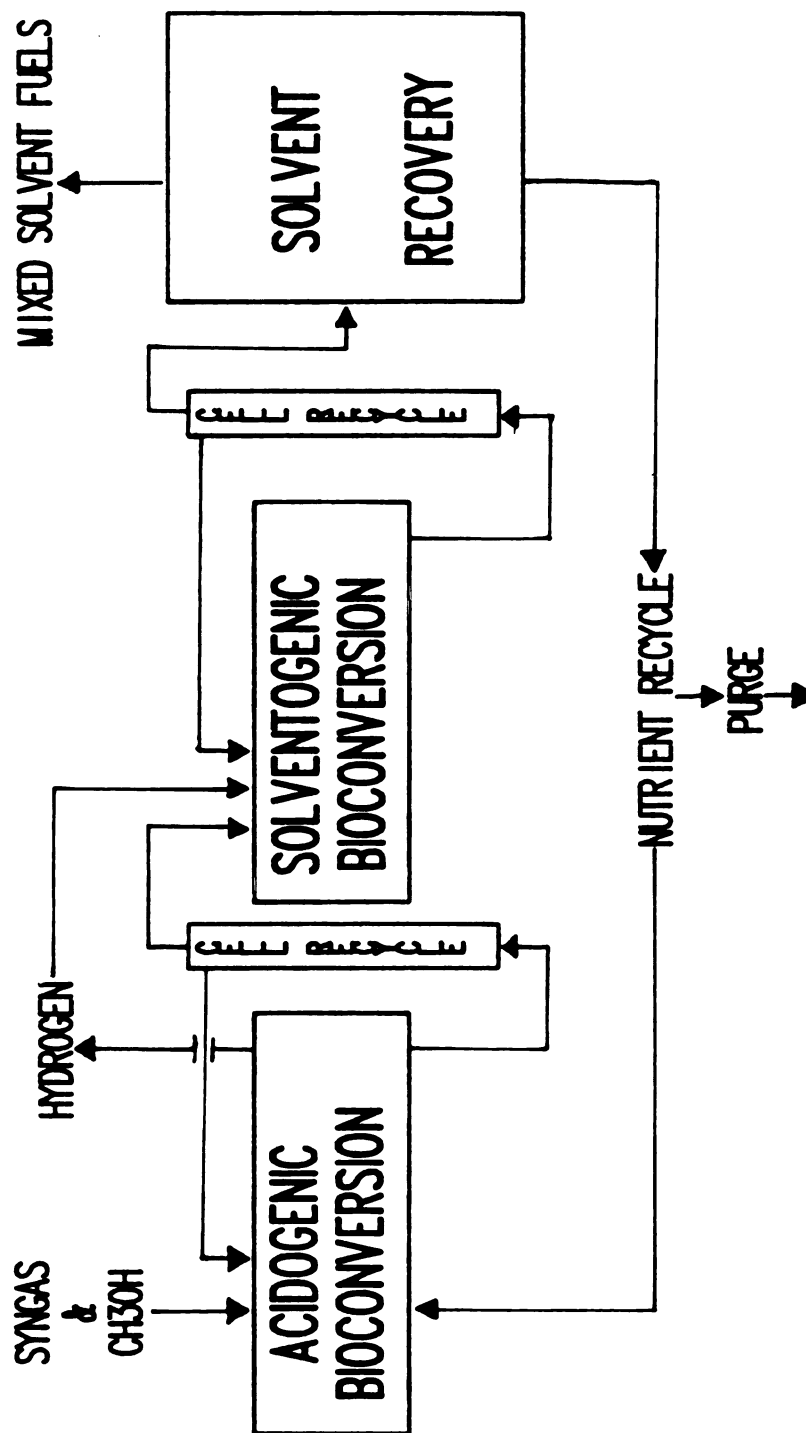
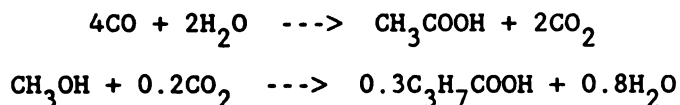


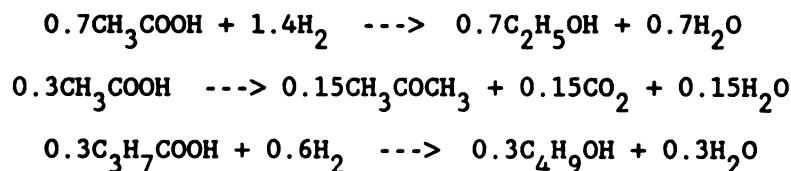
Figure 2: MBI process for bioconversion of coal derived synthesis gas.

general reactions taking place are

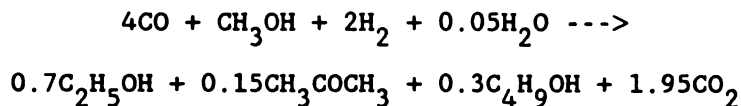


where methanol is introduced in this stage to conserve the carbon contained in the carbon dioxide produced by the first reaction by using a second butyrate-producing reaction. Thus the overall effluent stream from Stage 1 will contain hydrogen gas and a mixture of acetic and butyric acids.

Following cell removal and recycle the gaseous hydrogen stream and the aqueous broth from Stage 1 will enter the solventogenic Stage 2, where the acids will be reduced with an improved strain of *Clostridium acetobutylicum* by the general reactions



producing an aqueous solvent mixture of butanol, ethanol, and acetone. The effluent stream from Stage 2 is similarly run through a cell recycle system and the emerging dilute solvent stream subsequently recovered. The overall process stoichiometry is therefore



with reduction of carbon lost to CO_2 possible through increased methanol addition in Stage 1 and *C. acetobutylicum* strain improvement to select mutants with reduced acetone formation capability in Stage 2.

3.2. Stage 1 Biocatalyst: *Butyribacterium methylotrophicum*

The bacteria chosen for the Stage 1 acidogenic fermentation was the CO strain of *Butyribacterium methylotrophicum* outlined previously.

Besides its several 1-carbon compound metabolizing pathways, *B. methylotrophicum* generally exhibits a high sulfide tolerance and grows best at moderate temperatures (35-40°C) and pressures (1-10 atm). Compared to other acidogenic unicarbonotrophic bacteria, it is also fast-growing (12 hr doubling time on CO, 9 hr doubling time on CH₃OH). Furthermore, both acetate from CO and butyrate from CH₃OH and CO₂ are possible end products with high carbon conversion rates (Lynd et al., 1982; Lynd and Zeikus, 1983), and growth is possible on a rather simple medium (Moench and Zeikus, 1983).

The overall catabolic stoichiometries, carbon and electron recoveries, cellular yield coefficients and doubling times for *Butyribacterium methylotrophicum* are summarized in Table 2 for growth on 1-carbon substrates. It is important to note that while the carbon monoxide studies were performed with the CO strain, other 1-carbon substrate experiments were conducted with the wild-type. Generally, other than its unique carbon monoxide metabolizing capability, the CO strain exhibits the same properties as the wild-type Marburg strain when grown on substrates other than carbon monoxide (Zeikus, pers. comm.). From Table 2 the versatility and efficiency of *B. methylotrophicum* is apparent; it is also apparent from the CO reaction stoichiometry that carbon dioxide (bicarbonate) generation accounts for one half of the total carbon metabolized. This CO₂ production is the primary reason for the methanol co-substrate reaction designed into Stage 1; it can minimize, by a product forming pathway, the carbon lost to CO₂.

To date, the experiments cited for *Butyribacterium methylotrophicum* have been conducted strictly in batch culture test tube systems with product formation and kinetic analysis limited to the growth phase only.

Table 2: 1-Carbon catabolic transformations observed with Butyribacterium methylotrophicum.^a

Carbon/Energy Source	Reaction Stoichiometry ^b	Carbon Recovery (%)	Electron Recovery (%)	Doubling Time (hrs)	Yield ^c (g/mol)
H ₂ -CO ₂	1.00 H ₂ + 0.517 CO ₂ → 0.216 CH ₃ COOH + .002 C ₃ H ₇ COOH + 0.063 Cells	97	102	10	1.7
CO	1.00 CO → 0.497 CO ₂ + 0.172 CH ₃ COOH + 0.001 C ₃ H ₇ COOH + 0.129 Cells	97	97	10	3.4
CH ₃ OH-CO ₂	1.00 CH ₃ OH + 0.183 CO ₂ → 0.016 CH ₃ COOH + 0.234 C ₃ H ₇ COOH + 0.284 Cells	105	100	8	7.6
CH ₃ OH-CO	1.00 CH ₃ OH + 0.901 CO → 0.104 CO ₂ + 0.475 CH ₃ COOH + 0.097 C ₃ H ₇ COOH + 0.327 Cells	93	91	11	4.6

^a Adapted from Lynd and Zeikus, 1983; Kerby et. al., 1983; Zeikus et. al., 1985.

^b Calculated with a corresponding cell formula of [C₁H_{1.82}O_{0.50}N_{0.21}]; nitrogen balance omitted.

^c In units of grams cells produced per mole substrate consumed.

Lab scale experiments in fermenter systems were conducted in batch mode only in one report (Datta and Ogletree, 1983) but were still limited to growth phase kinetics. Thus a major goal in the MBI process development scheme is operation of a carbon monoxide fermentation in continuous mode, ie., chemostat fermentation. This and other key objectives are outlined below, for both stages in the bioconversion process.

3.3. Overall Objectives for Process Development

In determining the potential for a bioconversion process for producing liquid fuels from coal derived synthesis gas several objectives must be accomplished and their underlying principles understood. These objectives include

1. Identification of regulatory factors influencing Stage 1 end product formation including electron donor ratios and gas composition.
2. Development of a continuous fermentation system for synthesis gas conversion to mixed organic acids.
3. Obtainment of high fatty acid tolerance mutants for *Butyribacterium methylotrophicum* and mutants for *Clostridium acetobutylicum* exhibiting high productivity and solvent tolerance.
4. Design and development of efficient cell recycle systems in both stages for biocatalyst retention.
5. Integration of Stage 1 and Stage 2 fermentation systems into an overall lab scale conversion of synthesis gas and methanol to mixed solvents.

Fermentation development in both stages is an ongoing task as work proceeds towards meeting these process objectives.

4. Scope of the Present Work

The following work was concerned with objectives 1 and 2 above, mainly in studying the effect of electron donors on cellular product formation and the design and operation of a continuous carbon monoxide fermentation for *Butyribacterium methylotrophicum*. Some key problems and limitations that needed to be overcome and the general scheme for accomplishing these goals are given below.

4.1. Main Developmental Obstacles

Several critical issues needed to be addressed for the process objectives to be met, most of which either directly or indirectly concern the cellular productivity and product selectivity of the fermentation.

Although for 1-carbon metabolizing anaerobic bacteria a 10 or 12 hour doubling time is considered fast, industrially it is a very slow rate of growth, and could possibly limit the overall volumetric productivity of the fermentation. Specifically, a low growth rate means a low dilution rate in steady-state continuous culture, which results in low productivities. Moreover, the maximum obtainable cell density in normal batch culture is also rather low for this bacteria, approaching around 0.35 g/L at the onset of the stationary phase.

Observed low growth rates on carbon monoxide, especially when considering the low CO solubility in water, indicate that mass transfer of gas phase substrates thru the liquid medium to the cells may be a rate limiting factor in any CO based fermentation. The fermenter may present a completely new mass transfer environment for the cells compared to the test tube which must be controlled or at least

understood. The costs associated with impeller power requirements may limit the purely physical methods available for increasing the gas/liquid transfer.

Finally, and perhaps of most significance, is the product distribution from carbon monoxide and methanol metabolism. The CO conversion to acetate is the key reaction, with methanol acting as a co-substrate to react the generated CO₂. From the known stoichiometries this would yield acetate as the major product from Stage 1 with increasing amounts of butyrate as the concentration of methanol is increased. Several problems arise from this scheme. Addition of methanol increases the substrate cost and thus begins to neutralize the basic tenet of the process, that being the use of low cost synthesis gas for solvent production. There is likely a breakthrough point where methanol addition, butyrate production, and economics all are optimized; elucidating that point may be very difficult.

There is an additional technical difficulty with this scheme that may become a critical factor, which comes from the multi-substrate fermentation design. The concept involves a simultaneous fermentation with two separate reactions between acetate production from carbon monoxide and butyrate production from methanol, with carbon dioxide linking the two together. However, studies have shown that simultaneous growth of *B. methylotrophicum* on CO and methanol yields acetate as the predominate product with only small amounts of butyrate (see Table 2; Kerby *et al.*, 1983), thus a co-substrate fermentation may result in a product formation profile that differs significantly from the strict chemical combination of the two reactions.

Regardless of the internal cellular metabolism, simple economics

dictate that butyrate is the preferred product, since from a process viewpoint butyrate translates to butanol while acetate translates to ethanol and acetone. Therefore maximizing the butyrate producing mechanisms in conjunction with or at the expense of the acetate forming mechanisms is a key objective in the fermentation development. The slow growth rate of *B. methylotrophicum* suggests that Stage 1 may rate limit the entire bioconversion process, thus overcoming the above obstacles for butyrate formation is of critical importance.

4.2. Directions to Overcome Obstacles

The experimental plan to address some of these key issues while maintaining definite progress towards the objectives was two-fold. After initial familiarization with both the bacteria and some required analytical techniques in test tube type systems, several batch fermentations were conducted in laboratory scale fermenters to study effect of carbon source, fermentation pH, and gas mass transfer. These experiments, all conducted under similar experimental conditions, were designed to give the maximum amount of information concerning regulation of growth and product formation by the listed factors in the fewest number of experiments.

With the knowledge, experience, and insight gained from the batch culture work, efforts were then focused on the design of a continuous fermentation, followed by operation at steady-state. Further experiments were then conducted in the continuous culture system which analyzed the effect of those factors determined to be of interest in the batch fermentations. The results of the continuous culture work generated some further biochemical studies, specifically a few simple enzymatic analyses, in order to mechanistically explain the observed results. With

the completion of these experiments and an associated analysis of the most significant results, a framework was thus set for the determination of further work.

CHAPTER II
MATERIALS AND ANALYTICAL METHODS

1. Microorganism and Culture Conditions

The CO strain of *Butyribacterium methylotrophicum* was obtained from a frozen stock culture at the Michigan Biotechnology Institute (courtesy of Dr. M. Jain, M.B.I., Lansing, MI). A stock culture was maintained in 152 mL sealed serum bottles containing 50 mL of phosphate buffered medium under 100% CO 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.

2. Culture Media and Gases

A phosphate buffered basal medium, shown in Table 3, was adapted from Lynd et al., 1982, and used for culture maintenance and as liquid medium in all fermentations.

Table 3: Phosphate buffered basal medium.

<u>Component</u>	<u>Amount</u>
Double distilled water	945 mL
NaCl	0.9 g
MgCl ₂ ·2H ₂ O	0.2 g
CaCl ₂ ·2H ₂ O	0.1 g
NH ₄ Cl	1.0 g
Trace Mineral II	10 mL
0.2% Resazurin	1.0 mL

This media, consisting of mostly salts, vitamins, and minerals, contains no carbon source except for the small amount of yeast extract. Phosphate

buffer, vitamins, yeast extract, and a cysteine-sulfide reducing agent were all sterilized separately and added prior to inoculation. All inoculations with *B. methylotrophicum* were a 2% total volume addition from stock culture. Methanol, when added, was also autoclaved separately as a 50% v/v solution and added to the fermenter prior to inoculation. Gases were obtained from either Linde Division, Union Carbide Co., Warren, MI, or Airco Industrial Gases, Royal Oak, MI, in large cylinders. Gas Purity was 98.0% for the CO gas, 99.75% for the N₂ gas, and 99.998% for the CO₂ gas.

3. Fermentation Equipment and Conditions

All fermentation systems were designed for operation under a sterile and strictly anaerobic environment. Gases were passed through a hydrogen reduced copper catalyst oven to remove any trace oxygen before entering the fermentation. Fermentation vessels and attached internal parts were autoclaved for 30 minutes minimum, and up to 90 minutes for the continuous systems with large liquid media reservoir volumes. Nitrogen gas was always introduced prior to inoculation to flush any residual oxygen from the system. Sample ports were stoppered with butyl rubber bungs and crimped with aluminium caps.

Batch experiments were conducted in Multigen fermenters (New Brunswick Scientific Co., New Brunswick, N.J.) with working volumes of 0.5 L and 1.0 L. Fermentation pH was automatically controlled by addition of either 3N or 2N NaOH solution, and base consumption measured by depletion in a graduated cylinder. Temperature was maintained at 37°C throughout all studies. Impeller speed was set at 100 rpm, and overall gas flowrate was controlled at 50 mL/min using a 50 mL buret-bubblemeter

in all batch fermentations. Methanol fermentations were conducted with the addition of a 400 mm glass condenser apparatus to the exit gas stream, with a 50% v/v mixture of ethylene glycol/water as the cooling medium, maintained at 4°C.

Continuous studies were all performed in a round bottomed 1.25 L working volume BioFlo II fermenter (New Brunswick Scientific Co., New Brunswick, N.J.), except for the gas-recycle fermentation, which used a 0.4 L working volume Multigen system. In the BioFlo II system, pH, temperature, liquid flowrate, and impeller speed were all maintained automatically by internal control. A built in water-cooled mini-condenser connected to the gas exit port was utilized. All pH-stat fermentations were conducted at 37°C, 50 rpm, 50 mL/min gas flow rate, and 0.31 mL/min liquid media flowrate; thus the dilution rate in all fermentations was constant at 0.015 hr^{-1} . Fermentation pH was controlled by addition of 2N NaOH solution. The gas recycle fermentation was operated with an overall gas flow rate through the broth of 250 mL/min, with a total gas throughput of 10 mL/min. Impeller speed was set at 200 rpm, temperature at 37°C, and pH controlled by addition of 2N NaOH. Liquid flowrate was set at 0.1 mL/min (0.015 hr^{-1} dilution rate) using a Gilson Minipuls 2 peristaltic pump (Gilson Medical Electronics, Middleton, WI) in conjunction with a GraLab Model 625 Timer/Intervalometer (Gralab Instruments Division, Dimco-Gray Company, Centerville, OH) for ontime control.

4. Fermentation Broth Analysis

Liquid samples were taken using N_2 -flushed, sterile syringes through the butyl rubber sample ports. Approximately 2 mL was extracted with

each sample. Cell density 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 0.5 were diluted by a factor of five and those with an optical density greater than 1.0 were diluted by a factor of ten. Cell mass was calculated using a previously derived dry weight vs. optical density calibration curve (Lynd *et al.*, 1982). Undiluted samples were observed microscopically for viability and contamination and subsequently centrifuged in 1.5 mL eppendorf tubes at 12,000 rpm for 2 minutes and the pH checked to provide external pH calibration for the fermentations.

5. Fermentation Product Analysis

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 detector. Operating temperatures were 190°C, 220°C, and 250°C for the column oven, injection port, and detector, respectively. Nitrogen carrier gas flow rate averaged 25 mL/min. Undiluted, centrifuged samples were acidified with 3N phosphoric acid (1 part acid/10 parts sample) and then transferred to sealed injection vials. All samples were automatically injected using the autosampler. All fermentation product concentrations were calibrated using 10 mM liquid standards from pure stock solution. Liquid samples were also analyzed periodically for added confidence on HPLC and GC-mass spectroscopy equipment in outside laboratories.

When applicable, gas headspace composition was analyzed using a 6 ft Carbosphere 80/100 mesh column in a Gow-Mac Series 580 gas chromatograph

(Gow-Mac Instrument Co., Bound Brook, NJ) with a thermal conductivity detector. Output was recorded on a Hewlett-Packard 3393A Integrator. Manual syringe injection was used. All injections were 0.4 mL volume and at atmospheric pressure. Analysis conditions were a helium carrier gas flowrate of 30 mL/min, a detector current of 200 mA, and column, detector, and injector temperatures of 150°C. Samples were calibrated with a standard curve constructed from pure gas responses. Calibrated gases included CO, CO₂, and N₂.

6. Enzymatic Analysis

Enzymatic Analyses were conducted with samples drawn from continuous culture fermentations at pH values of 6.8 and 6.0. Fermentation broth was anaerobically withdrawn by syringe with a total of 60 mL, taken after steady-state operation over a period of approximately three residence times. The samples were then centrifuged under an H₂ atmosphere at 15,000 rpm for 10 minutes using a Sorvall RC-5B Refrigerated Superspeed Centrifuge with an SS34 rotor (Biomedical Products Department, Du Pont Co., Wilmington DL), operating at 4°C. Centrifugation and subsequent treatment was performed without sterility restriction. The resulting supernatant was discarded, followed by resuspension of the liquid pellet in 2 mL TRIS buffer (Sigma Chemical Corp., St. Louis, MO), at a pH of 7.5. Resuspended cells were promptly frozen at -75°C and left frozen until extraction was performed, a period of several months.

Cell extracts were prepared from the frozen cell samples by thawing at 4°C and repeated breakage with an Aminco French Pressure Cell Press (SLM Instruments INC, American Instruments CO., Urbana, IL). Operation

was conducted at 4°C with 1100 psig pressure and under an N₂ atmosphere. Breakage was performed 2 to 3 times in this fashion. Microscopic observation confirmed breakage and derived cell extracts were flushed twice with N₂ in 25 mL anaerobic bottles and frozen at -4°C.

Analysis of both acetate kinase and butyrate kinase (ATP:acetate phosphotransferase, EC 2.7.2.1; ATP:butyrate phosphotransferase, EC 2.7.2.7) activities were measured spectrophotometrically by following the rate of NADH depletion in a 1.0 mL multi-enzyme mixture, whose composition is given in Table 4. Absorbance was measured at room temperature at 340 nm with a Gilford Response Series UV-VIS spectrophotometer (Ciba-Corning Corp., Oberlin, OH), with samples containing 5, 10, 20, and 50 µl of cell extract run concurrently.

Table 4: Sample mixture for spectrophotometric analysis of acetate kinase and butyrate kinase.^a

<u>Component</u>	<u>volume added</u>
100mM TRIS buffer, pH = 7.5	900 µl
1M Acetate(Butyrate) solution	20 µl
300mM ATP solution	20 µl
100mM Phosphoenol Pyruvate solution	10 µl
14mM NADH dissolved in TRIS buffer	10 µl
100mM MgCl ₂ ·6H ₂ O solution	10 µl
Pyruvate Kinase, 500 Units/mL	4 µl
Lactate Dehydrogenase, 330 Units/mL	4 µl

^a From Andersch, et al., 1983.

Experiments were conducted in duplicate. Blanks contained no cell extract; controls contained no reaction substrate and 10 µl extract.

Total protein content of the cell extracts was measured directly with

the Biorad protein assay (Bradford, 1976) at room temperature.

CHAPTER III

BATCH CULTURE STUDIES

With almost all previous work involving *Butyribacterium methylotrophicum* coming from test tube growth phase studies, the larger scale laboratory fermentation profile of this bacteria has remained relatively unknown. Development of this fermentation from the 20 mL test tube or the 100 mL bottle to the 1 L or 2 L fermenter is not strictly an exercise in scale-up. Laboratory fermentations can potentially present a significantly different environment for growth which can arise from impeller driven mass transfer effects, impeller shear forces, pH control, and various other sources. With the process orientation of this work, including the major goal of developing a continuous culture fermentation system for carbon monoxide bioconversion, the experimental setting from the outset was logically the fermenter and not the test tube. The implications of this approach were widespread. Equipment limitations became a deciding factor in experimental design, with replication of results not practical. Furthermore, on the engineering application level the laboratory fermenter is extremely versatile with respect to modification, integration, and mode of operation.

This chapter presents the experimental results and discussion for several batch fermentations, which were conducted in systems designed for comparison between runs as well as overall efficiency and practicality. Two main topics were investigated. Electron donor studies determined the effect on product formation of substrate mixtures of CO, CO₂, and CH₃OH relative to 100% CO. These experiments were performed to generate fermentation profiles for comparison with previous literature

reports as well as investigate stationary phase response. Carbon and electron flow studies centered on elucidating key factors regulating product formation and growth. Finally, a basic mass transfer analysis was performed to determine whether interphase CO transport was rate-limiting for the batch fermentation system.

1. Experimental Set-up and Design

Batch fermentations were conducted in 1 L and 2 L vessels constructed for long-term operation under both sterile and anaerobic conditions. Several key features of this system are depicted in Figure 3. Liquid medium volume was constant except for base addition required for pH regulation; total base volume required per fermentation was approximately 2% of the initial volume. Carbon monoxide and carbon dioxide gases were continuously sparged through the agitator and hence into the liquid broth with an exit gas stream vented from the headspace into a safety vent. For additional safety, flammable gas and carbon monoxide monitors were positioned above the fermenter to detect any hazardous gas leakage.

Continuous gas substrate addition was designed to insure the availability of carbon monoxide throughout both the growth and stationary phases, as well as to maintain a constant gas composition in the fermenter during the course of the fermentation. Gas flow rates were one or two orders of magnitude greater than normal bacterial consumption rates and thus were able to meet this objective, as detailed in a later section. With a gas phase substrate it was thus possible to operate the fermenter in a batch mode while maintaining a constant supply of substrate.

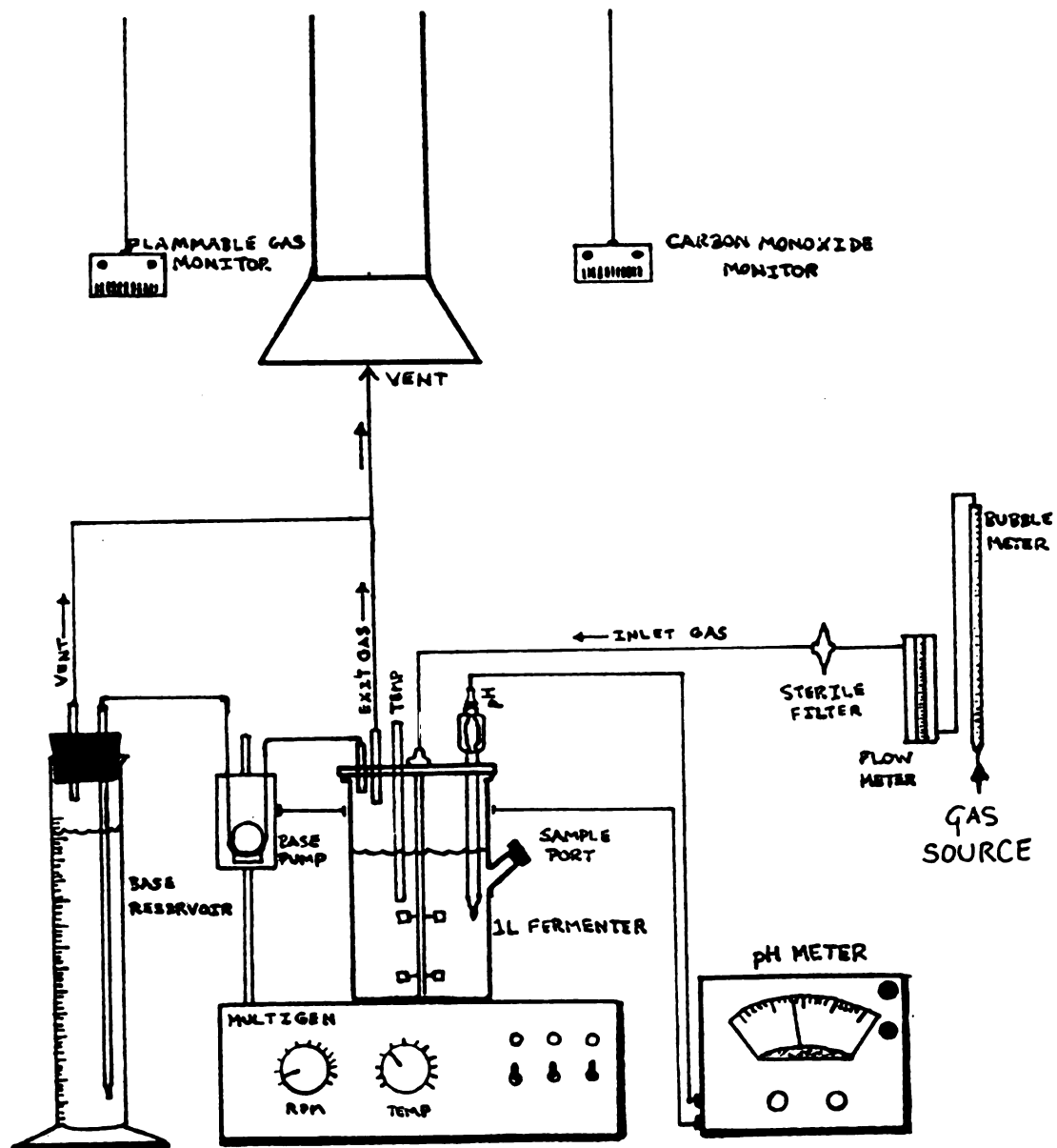


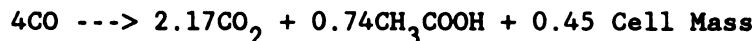
Figure 3: Batch fermentation system.

2. Electron Donor Studies: Experimental Results

Electron donor studies determined the effect of different carbon and energy sources on the growth and product formation profiles of *Butyribacterium methylotrophicum*. As outlined previously in Table 2, the versatile metabolism of this bacteria enables it to consume a variety of 1-carbon substrates either singly or concurrently. Three experiments were conducted in order to establish baseline fermenter profiles; the substrates utilized were carbon monoxide, carbon monoxide/carbon dioxide, and carbon monoxide/carbon dioxide plus methanol.

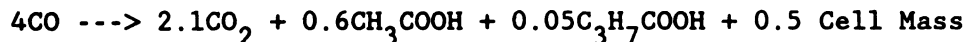
2.1. 100% Carbon Monoxide: Base Case Study

Batch growth on CO as the sole carbon and energy source has been well documented in studies using anaerobic bottles. These studies formed a basis for calculation of reaction stoichiometries, specific growth rates, and also provided a model for carbon flow in CO catabolism (Lynd *et al.*, 1982; Kerby *et al.*, 1983; Datta, 1982). Specifically, growth on CO yielded acetate, cell mass, and CO₂ as products in the following stoichiometric ratio for carbon and electrons,



(Lynd *et al.*, 1982), where the cell mass was calculated using a dry weight conversion relating grams cells (moles) to optical density (Lynd *et al.*, 1982). This determination was made from a growth phase profile with a pH change from 7.2 to 6.2 during the course of the 100 hour experiment. Similar results were obtained in a 12 day shake flask experiment with periodic CO addition and pH control between 6.5 and 7.5 in which some butyrate production was also observed during two days of stationary phase activity (Datta, 1982). The overall carbon and electron

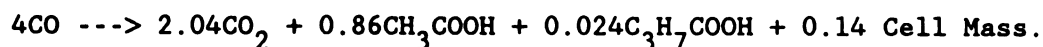
balance was



with acetate production occurring during the growth and initial stationary phase.

With the information from these experiments, an extended-batch fermentation with continuous CO sparging was conducted at a pH of 6.8 for 18 days in a 1.1 L working volume. Time course results from this fermentation are shown in Figure 4, and the corresponding molar amounts of acetate and butyrate shown in Figure 5. Two key points are clear from the data. As shown in Figure 4, growth and product formation trends are consistent with previous studies; acetate is the primary product during growth and small amounts of butyrate are produced in the stationary phase. Also evident is the continued production of acetate during stationary phase response. Figure 5 graphically depicts the molar ratio of acetate and butyrate produced during the fermentation.

Carbon and electron balances, a useful method for analyzing fermentation data detailed in the Appendix, were used to calculate the amount of CO consumed, and hence CO₂ produced, from the product formation data. These balances have been found to close within 3% for batch CO fermentations (Lynd *et al.*, 1982; Datta, 1982). The overall carbon balance determined from the data in Figure 4 is



For comparison purposes, separate fermentation stoichiometries were obtained for both the growth and stationary phases. In these calculations, the growth phase was defined as the time between the onset of growth and a significant decrease in the growth rate, approximately 72 hours in both this and previous studies (Lynd *et al.*, 1982). The

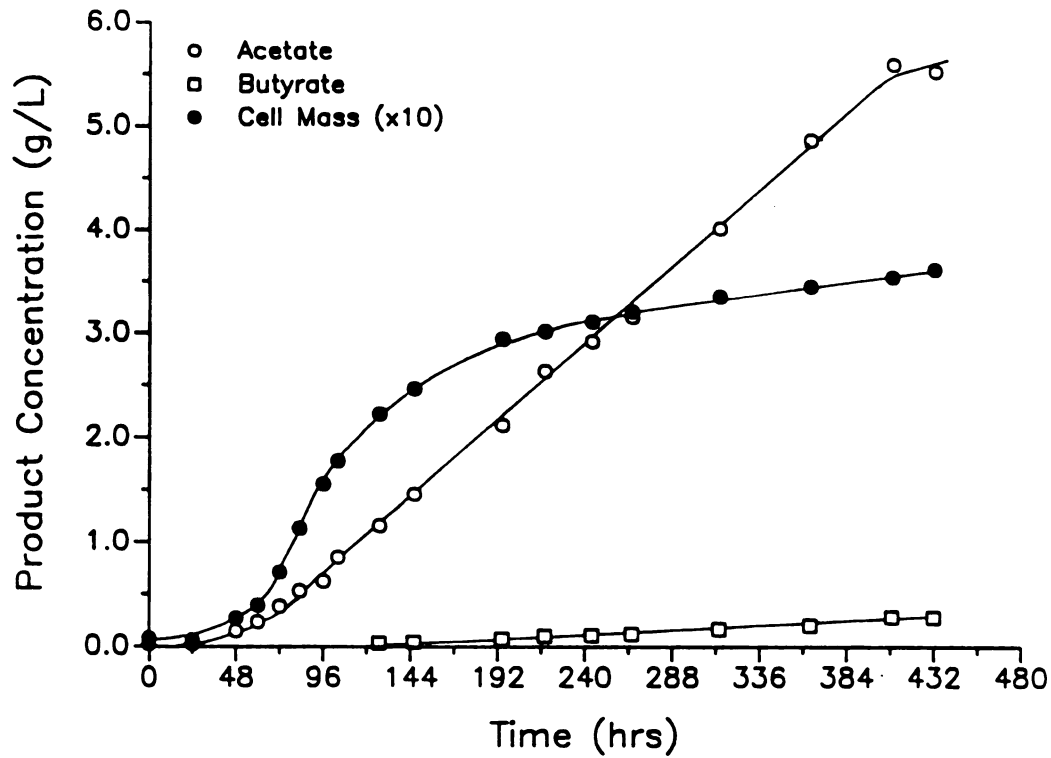


Figure 4: Batch fermentation products from 100% CO gas.

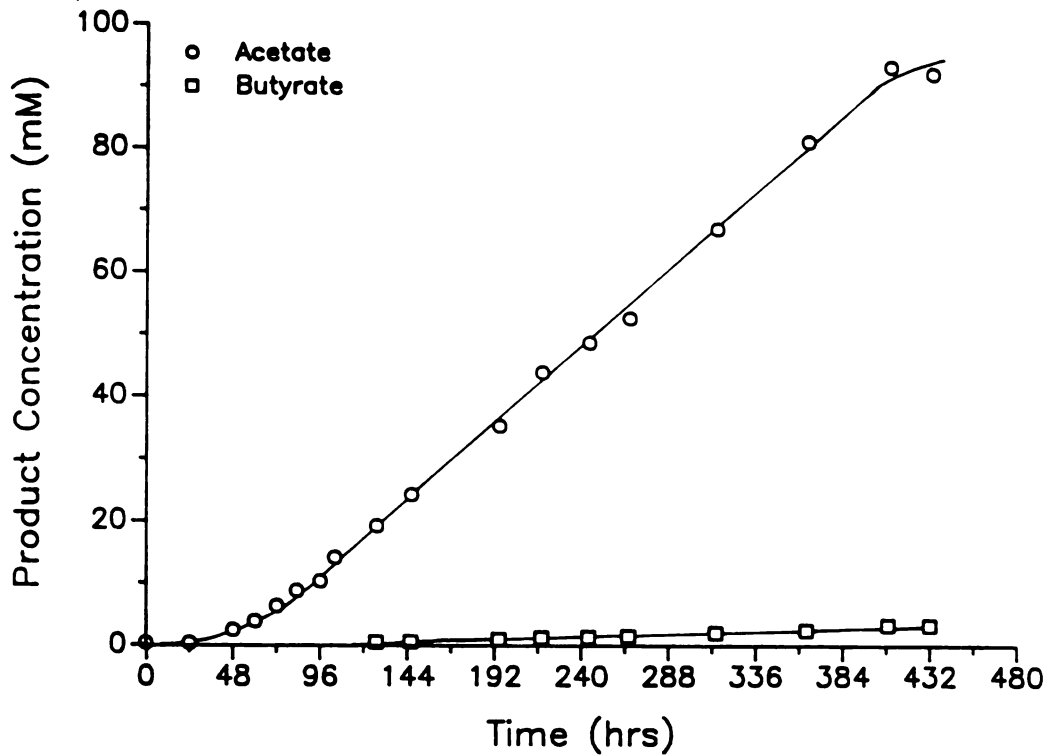


Figure 5: Molar production of acetate and butyrate from 100% CO gas.

balance during the growth phase is



and the balance during the stationary phase is



These balances confirm the results depicted in Figures 4 and 5, mainly that acetate is produced equally in both the growth and stationary phases, while butyrate production is limited to the stationary phase. Finally, the growth phase carbon balance from this fermentation is quite similar to that obtained by Lynd *et al.*, given above.

Volumetric consumption rates for CO were calculated from the production data, in order to compare with the feed rate during the fermentation. Assuming an ideal gas at the fermentation conditions the total consumption of CO during the fermentation is

$$V = nRT/P = 12.48 \text{ L}$$

which, when divided by the total fermentation, time gives the volumetric consumption rate for CO,

$$dV/dt = 0.482 \text{ mL/min.}$$

This value is an average over both the growth and stationary phases, and is two orders of magnitude less than the feed rate of 50 mL/min.

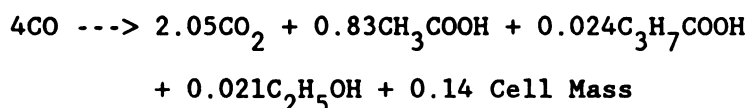
Calculations were performed to estimate whether a significant fraction of the product could have been stripped from the fermenter by the sparged CO. As a worst case, the effluent gas was assumed to be in equilibrium with an average liquid phase acetic acid concentration of 3 g/L throughout the 400 hour fermentation. Assuming Raoult's law holds, the gas phase mole fraction would have been 3.65×10^{-5} , and a total of 0.03 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.

2.2. 80%/20% Carbon Monoxide/Carbon Dioxide

With the establishment of the 100% CO fermentation as a base case profile, other fermentations could then be conducted and a logical comparison made relative to the base case. The first of these consisted of an alteration of the carbon source from 100% CO to an 80% CO/20% CO₂ mixture. This was primarily an exploratory experiment which investigated the effect of bicarbonate on CO metabolism. Since CO₂ production accounts for approximately 50% of the consumed carbon from CO, the effect of the presence of liquid phase bicarbonate in a pH controlled experiment was not expected to be drastic. Nevertheless, the possibility of direct bicarbonate regulation of CO metabolism could not be ruled out, especially in light of previous reports linking bicarbonate consumption to methanol metabolism in *B. methylotrophicum* (Datta, 1982; Lynd and Zeikus, 1983).

The same fermentation system was used as for the base case with a total gas flow rate of 50 mL/min yielding flow rates of 40 mL/min and 10 mL/min for CO and CO₂, respectively. Fermentation pH was again controlled at a pH of 6.8, and all other variables remained unchanged. The time course fermentation data from this experiment are shown in Figure 6, with the molar concentrations of acetate and butyrate shown in Figure 7. Besides the appearance of ethanol as an end product, present in minor quantities, these results are very similar to the base case. For comparison, the overall reaction stoichiometry is



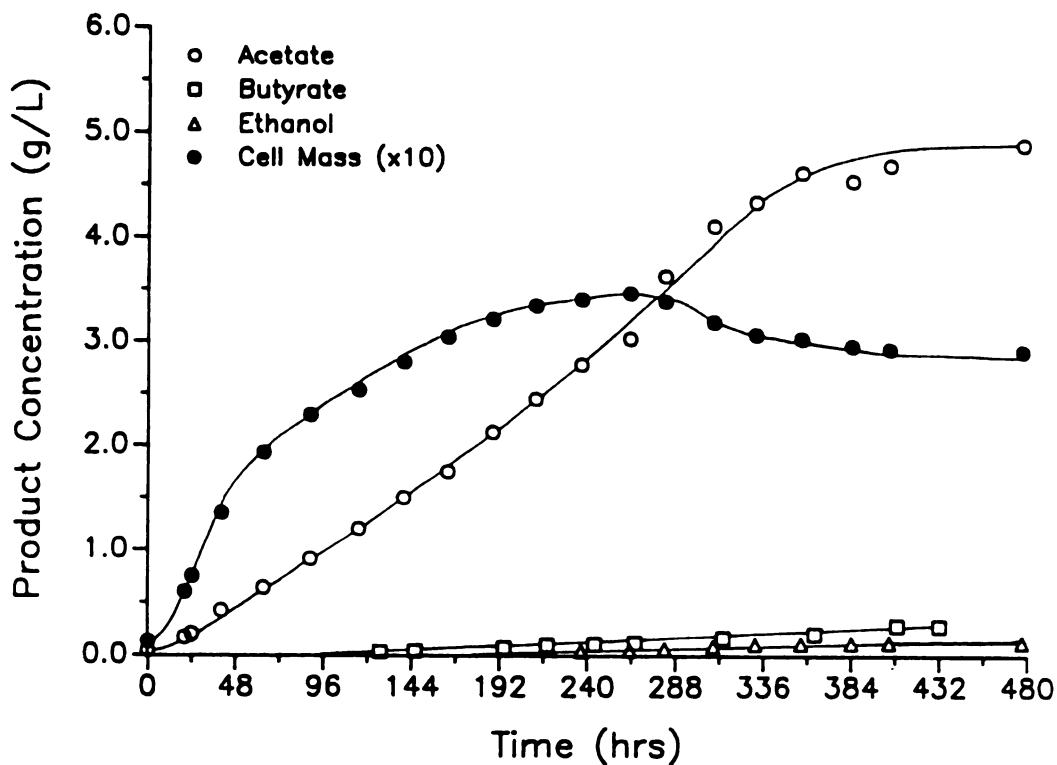


Figure 6: Batch fermentation products from 80% CO/20% CO₂ gases.

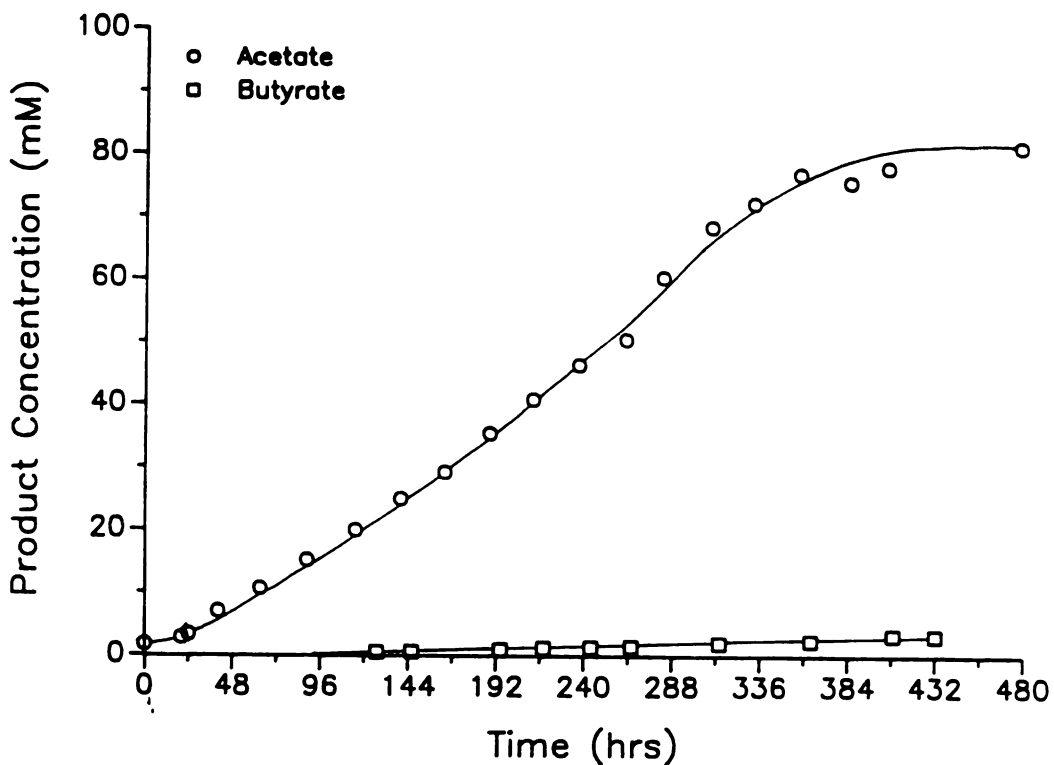
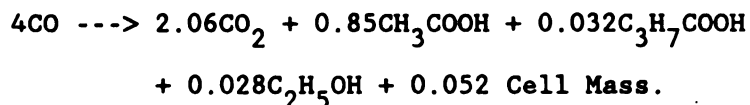


Figure 7: Molar production of acetate and butyrate from CO/CO₂ gases.

which when compared to the previous experiment shows almost no change besides the ethanol production. Interestingly, the amount of ethanol and acetate produced are equal to the total acetate production in the base case, suggesting that in this experiment minor ethanol production was induced at the expense of acetate. This trend is more pronounced when the growth and stationary phase are analyzed separately, under the same criteria as before. In this case, the growth phase balance is



and the stationary phase balance is



Differences in the cell carbon produced between the two experiments are due in part to the somewhat arbitrarily chosen time for the end of the growth phase; in the base case the determination was made at a cell density approximately 12% less than in this experiment.

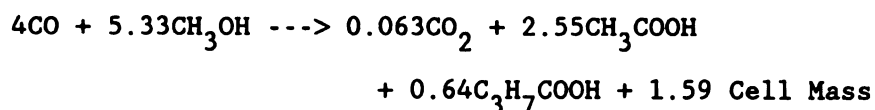
Calculations for ethanol loss by gas stripping were performed using the same worst case scenario outlined previously, with results showing an ethanol loss of slightly more than 7% of the average liquid phase concentration. Although the low concentration of ethanol in the broth and the equilibrium assumptions used to calculate this loss detract from the certainty of the value, this calculation does provide an estimate of evaporative losses for ethanol.

2.3. Carbon Monoxide/Carbon Dioxide/Methanol

The versatile 1-carbon metabolism of *B. methylotrophicum* permits use of a variety of substrate mixtures for production of organic acids. As shown previously in Table 2, production of acetate is possible from both CO (Lynd et al., 1982) and CH₃OH/CO (Kerby, et al., 1983). Butyrate can

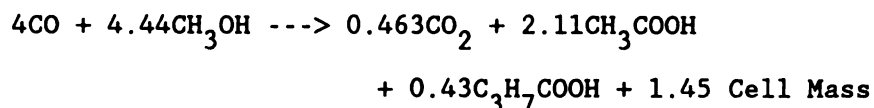
be formed from $\text{CH}_3\text{OH}/\text{CO}_2$ in the presence of acetate with net production/consumption of acetate dependent on the methanol/bicarbonate ratio (Datta and Ogletree, 1983). With this information, a tri-substrate fermentation was conducted in order to assess the possibility of simultaneous reactions involving both the CO and the CH_3OH metabolizing pathways in the presence of bicarbonate.

Continuous gassing of a 7 to 3 CO to CO_2 mixture at 50 mL/min (headspace flush only -no sparging) and an initial liquid phase CH_3OH concentration of 3.2 g/L was used. Fermentation pH was controlled at a pH of 6.8 and a glass condensing column attached to the exit gas stream operated at 4°C to prevent methanol stripping. Timecourse results are shown in Figure 8. Both acetate and butyrate were produced during growth. The results indicate an apparent death phase after consumption of all the methanol; production of acids terminates as this phase is initiated. If the end of the fermentation is taken as the point where the methanol concentration drops to zero, the corresponding stoichiometric balance is



which is a completely different profile than that of the base case.

The results from this one fermentation do not provide any conclusive insight regarding whether simultaneous metabolic pathways are operating during this experiment. Results from bottle experiments with $\text{CO}/\text{CH}_3\text{OH}$ and without pH control do show a similar stoichiometry, with a carbon balance of



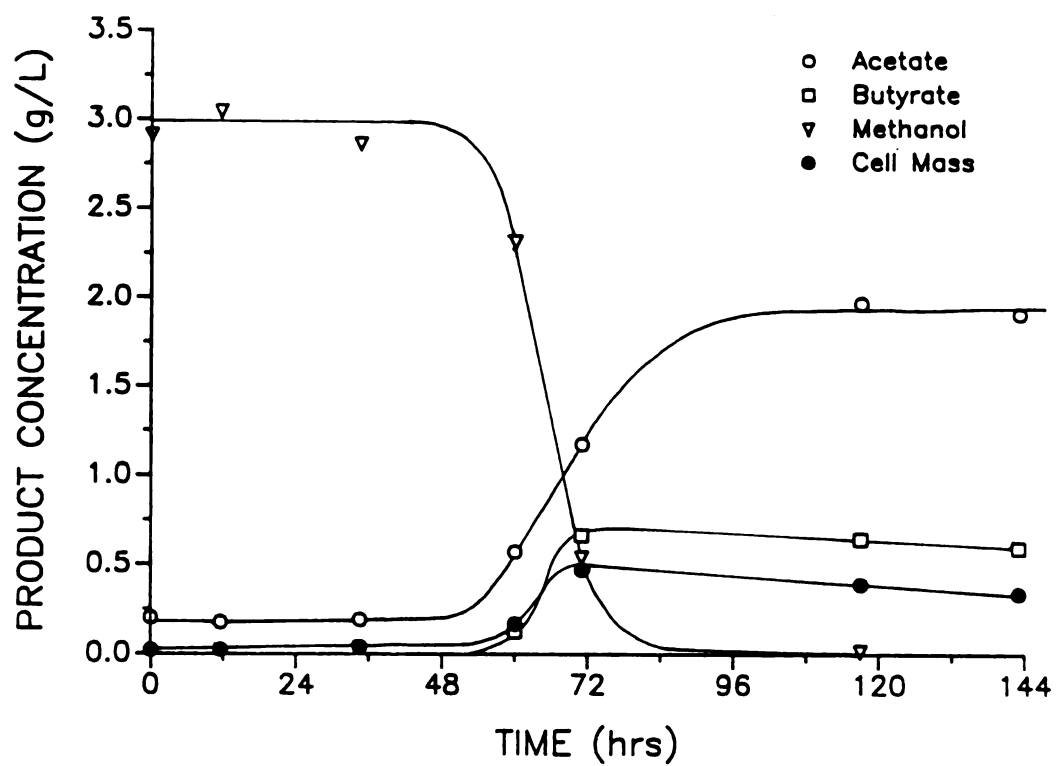
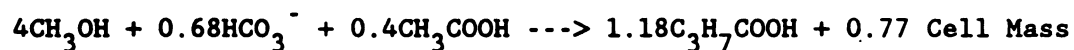


Figure 8: Batch fermentation products from CO/CO₂/CH₃OH.

which exhibits the same trend of acetate and butyrate formation with the exception of CO_2 generation (Kerby *et al.*, 1983). Possibly this difference can be explained by the additional carbon source; CO_2 was used in the present study and may have induced the $\text{CH}_3\text{OH}/\text{CO}_2$ metabolic pathway. This pathway has been observed to consume $\text{CH}_3\text{OH}/\text{CO}_2$ and produce both acetate and butyrate in the presence of excess bicarbonate by the reaction



and to produce butyrate and consume acetate in excess methanol by the reaction



which were determined in a 14 L batch fermenter (Datta and Ogletree, 1983).

The actual amount of liquid phase bicarbonate present in the tri-substrate fermentation is highly pH-dependent and can be expected to be greater than the CO_2 solubility at 37°C and a pH of 6.8 as a result of the dissociation chemistry of carbonic acid. An approximate ratio of CH_3OH to HCO_3^- , based on a rough calculation of the overall equilibrium between dissolved CO_2 and HCO_3^- , is 2:1 CH_3OH to HCO_3^- . This ratio is very significant in determining overall $\text{CH}_3\text{OH}/\text{CO}_2$ metabolism (Datta and Ogletree, 1983).

Although the specific pathway(s) in use are not clear from these results, it is evident that carbon monoxide consumption in the presence of methanol and carbon dioxide yields acetate as the primary fermentation product, with significant amounts of butyrate produced concurrently during the growth phase.

It is important to note that the carbon balance was derived from

calculations that included a constant rate of methanol stripping equal to the observed loss from the system before the onset of growth. This rate was measured to be approximately 0.008 g/L·hr which would yield an overall stripping loss of almost 20% of the original liquid phase concentration. This was most likely due to an experimental oversight which provided an oversized condenser without regard to the reflux stream required to return the condensate back to the fermenter. In essence, condensed vapor containing methanol and other organics was trapped in the condenser unit.

3. Carbon and Electron Flow Studies: Experimental Results

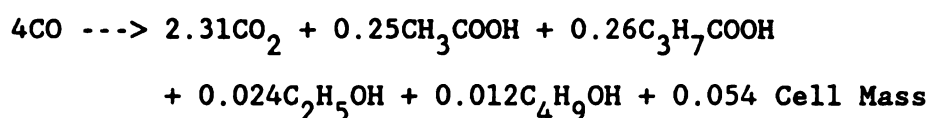
The carbon and electron flow studies in this section were designed and conducted with the objective of altering the existing carbon monoxide metabolism from acetate production towards both butyrate and ethanol synthesis. It is more desirable from an economic standpoint to produce as much butyrate as possible since ultimately butyrate is converted to butanol in Stage 2 of the proposed MBI indirect liquefaction process. Manipulation of the carbon pathway to maximize butyrate production from carbon monoxide was thus the major goal of these fermentations.

Insight towards achieving this objective came from previous batch bottle experiments without pH control which had shown low levels of butyrate production and culture pH-drop to be linked during stationary phase growth on CO (Lynd *et al.*, 1982; Datta 1982). Thus, the primary approach was to analyze the effect of lower broth pH on the carbon monoxide and CO/CO₂/CH₃OH fermentations.

3.1. 100% Carbon Monoxide: pH-Shift Studies

To investigate the effect of fermentation pH on stationary phase butyrate production, an extended batch fermentation was conducted with 100% CO in a 0.5 L working volume with all conditions unchanged from the base case. *B. methylotrophicum* cells were grown at a pH of 6.8 until the cell concentration reached a value of 0.31 g/L, which roughly corresponded to the end of the growth phase. At this time, the setpoint of the pH controller was adjusted from 6.8 to 6.0 and the fermentation continued for 2 weeks at a pH of 6.0 (the initial two days after the change were at pH values between the two setpoints). Time course results are shown in Figure 9, and the corresponding molar amounts of acetate and butyrate shown in Figure 10. As seen in Figure 9, a dramatic metabolic change resulted from the pH-shift which led to butyrate, ethanol, and butanol production relative to the base case experiment. A final butyrate concentration of 6.0 g/L was obtained, compared to 3.7 g/L of acetate, with minor quantities (> 0.25 g/L) of both ethanol and butanol also present. It must be noted that during the last 4 days of this fermentation there was a pH controller malfunction which led to a gradual increase of the pH from 6.0 to approximately 6.5 near the end of the experiment. This error most likely accounts for the decrease in production rates for all products after 336 hours of operation. Thus even greater concentrations relative to the base case are possible.

The overall carbon balance is



which shows both the linking of butyrate and CO₂ production at the expense of acetate as well as the minor amount of alcohol production.

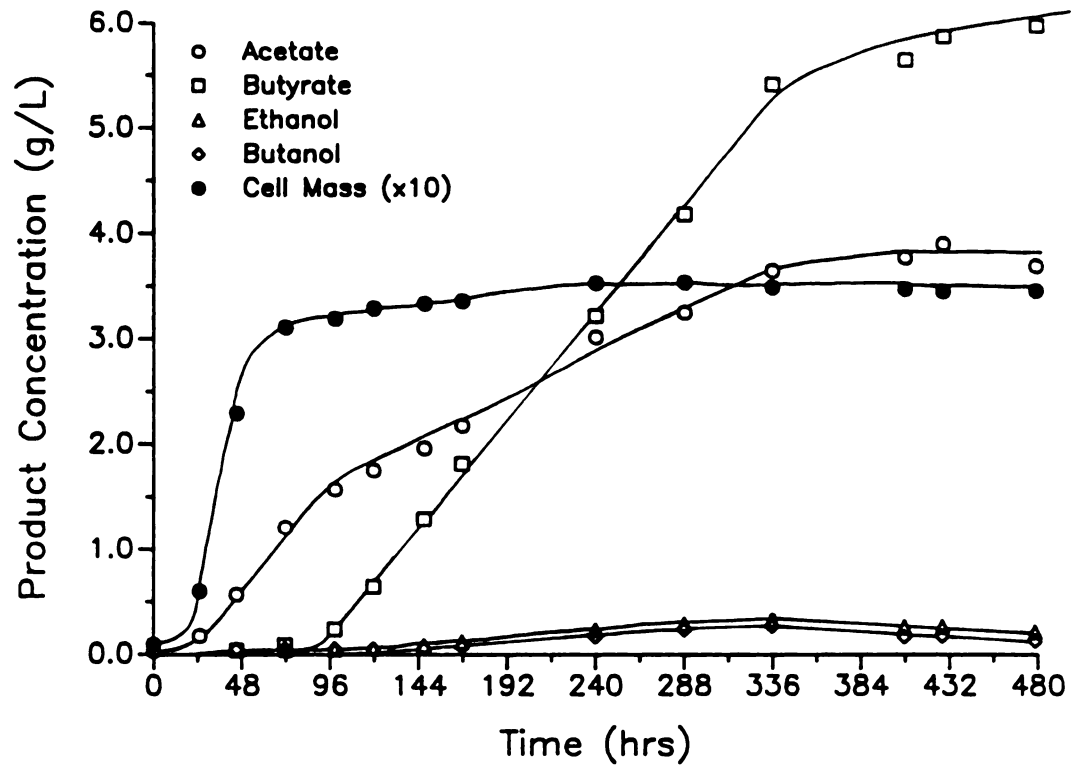


Figure 9: Batch fermentation products from 100% CO gas pH-shift #1.

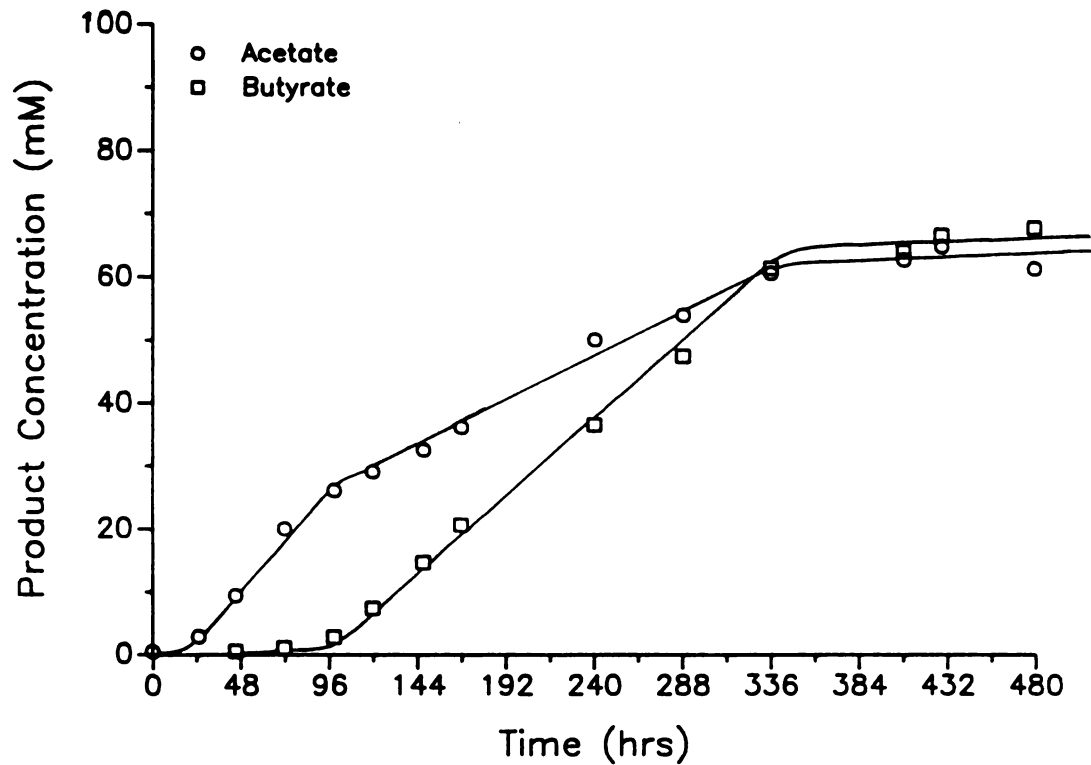
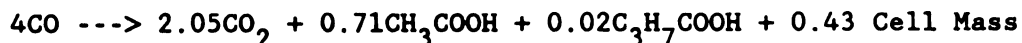
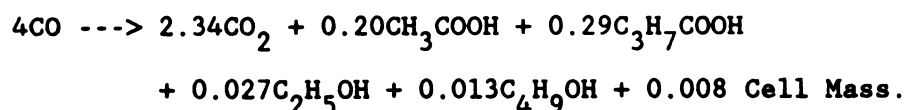


Figure 10: Molar production of acetate and butyrate from pH-shift #1.

The effect of the pH-shift on product formation is even more pronounced when the growth and stationary phase are analyzed separately, as for the base case. Using the same criteria, the carbon balance during the growth phase is



and the carbon balance during the stationary phase is



Thus, by decreasing the fermentation pH from 6.8 to 6.0 at the onset of the stationary phase, the metabolism of *B. methylotrophicum* can be shifted from acetate to butyrate production, with minor quantities of alcohols also being produced. These results are very significant and unique because they show that by a simple shifting of the pH the metabolic regulation of the bacteria can be changed dramatically, and this shift allows production of a 4-carbon organic acid (butyric acid) and 4-carbon alcohol (butanol) directly from gaseous carbon monoxide.

Equipment limitations required the use of different size fermenter vessels for the 100% CO base case and pH-shift fermentations described above. This caused a difference in the volume of gas fed to the system per unit fermentation volume, where 50 mL/min of CO gas was added in both experiments to different working volumes. The values of the volumetric CO feed rates were 45 mL/L·min for the base case and 108 mL/L·min for the pH-shift case. Since the gas was bubbled into the liquid broth, there was roughly twice as many bubbles per unit volume in the latter case, implying more surface area for interphase CO transport. Furthermore, since surface aeration can typically account for a significant portion of the overall mass transfer in laboratory scale

fermenters (Fuchs, *et al.*, 1971), and since the smaller fermenter used in the pH-shift experiment has a greater surface to volume ratio, the overall volumetric gas/liquid mass transfer rate of CO in the pH-shift fermentation was likely higher than in the base case. As described in a later section, the CO fermentations conducted during this study were expected to be mass transfer limited, and thus the different mass transfer environment must be recognized.

A critical issue then becomes whether or not the mass transfer rates have any relevance to alteration of the cellular metabolism. In the ideal case the pH effect would directly regulate the metabolism and increased mass transfer would simply provide more CO for entry into whatever pathway is under regulation. In order to clarify this point, a second pH-shift fermentation was conducted in the same system as the first only with a gas flow rate of 25 mL/min, which resulted in a volumetric CO feed rate of 50 mL/L·min: almost the same value as in the base case fermentation. Although surface effects were not addressed, this pH-shift fermentation was assumed to have a mass transfer environment approaching that of the base case.

The fermentation results are depicted in Figure 11 for the time course data and in Figure 12 for the molar amounts of acetate and butyrate. Again the effect of the pH-shift is evident with butyrate and acetate being produced in equimolar amounts at the end of the experiment, and with minor alcohol production also observed. Figure 11 shows that all products concentrations are nearly one half the amount of those observed in the first pH-shift experiment, where the volumetric gas flow rate was twice as much. The overall carbon balance for this experiment is

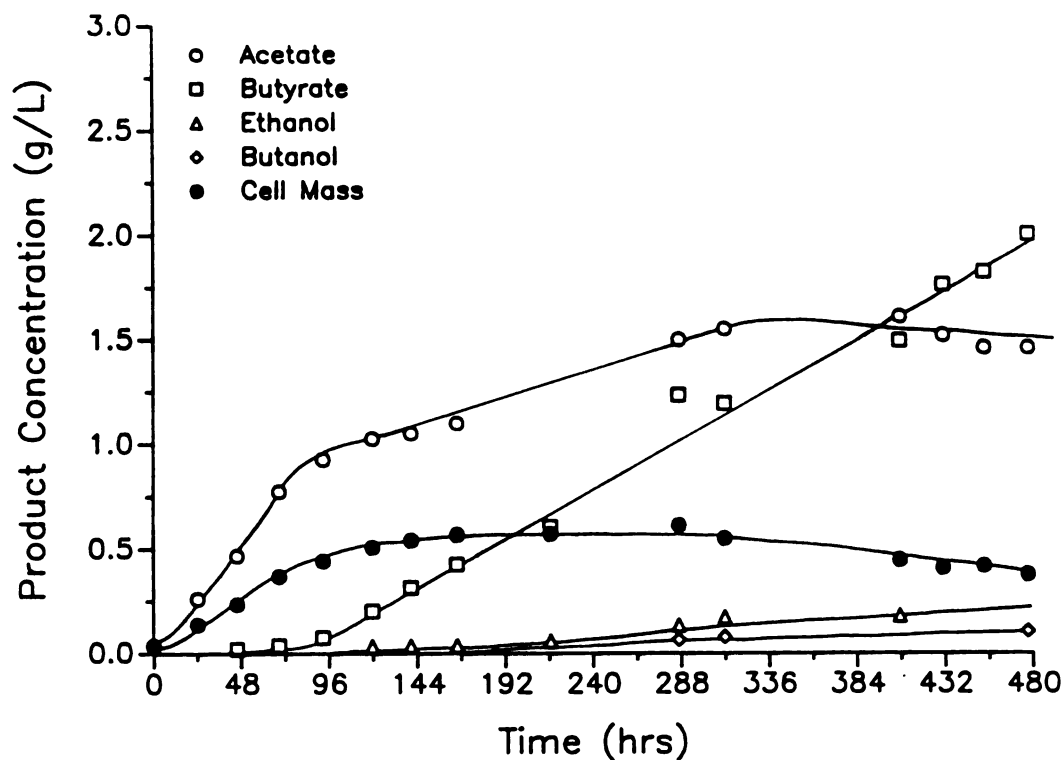


Figure 11: Batch fermentation products from 100% CO gas pH-shift #2.

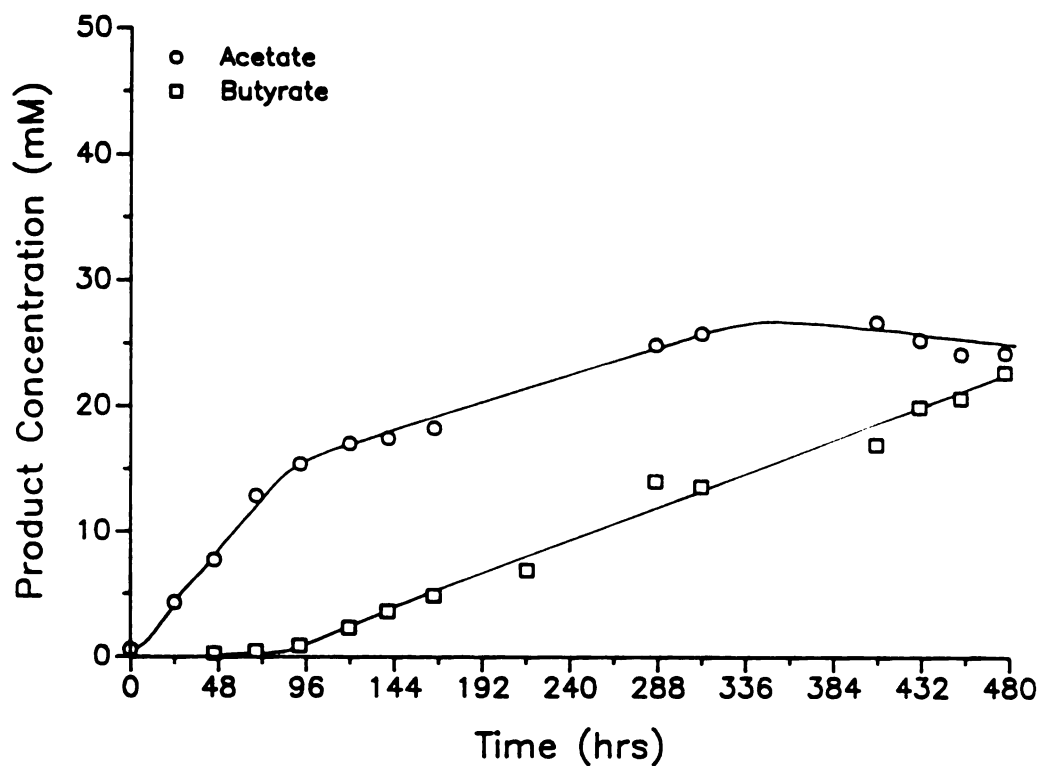
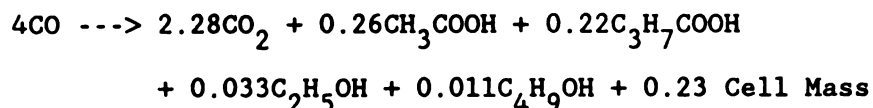
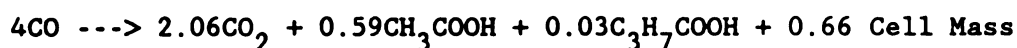


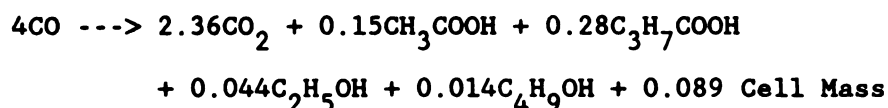
Figure 12: Molar production of acetate and butyrate from pH-shift #2.



which is equal to that of the previous experiment with the exception of the increased cell mass, an effect which could be due to a lack of product inhibition at the lower concentrations present. The carbon balance during the growth phase is



and for the stationary phase the balance is



which also is quite similar to the stoichiometries for the first pH-shift experiment. These results show that the mass transfer rate does not significantly affect the metabolic pathways for acid and alcohol production.

Further calculations, shown in Table 5, show the relationship between the gas flow rate, the working volume of the fermenter, and the overall consumption of carbon monoxide. A comparison of the base case

Table 5: Relationship between gas flow rate, liquid volume, and overall CO consumption rate.

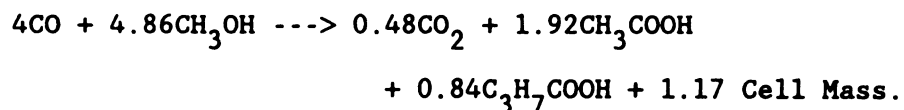
	<u>Base Case</u>	<u>Fermentation</u> <u>pH-shift #1</u>	<u>pH-shift #2</u>
Working Volume:	1.1 L	0.465 L	0.5 L
Gas Flow Rate:	50 mL/min	50 mL/min	25 mL/min
Volumetric Flow Rate:	45 mL/L·min	108 mL/L·min	50 mL/L·min
Total CO Consumed:	0.4907 mol	0.4777 mol	0.2078 mol
CO Consumption Rate (mol/L·hr):	10.3×10^{-4}	23.3×10^{-4}	9.2×10^{-4}

and the pH-shift #1 experiment shows that doubling the specific flow rate also doubles the overall CO consumption rate. Comparing the pH-shift #1 with the pH-shift #2 experiment shows that a reduction in the gas flow rate leads to a roughly proportional reduction in the CO consumption rate. These two points suggest that the fermentations are mass transfer limited, and when combined with the results of both pH-shift fermentations provide further evidence indicating that mass transfer effects are not relevant to the overall metabolic dynamics of the system, but do directly affect the actual rates of metabolism. Interphase mass transfer effects are further discussed in Section 5.

3.2. Carbon Monoxide/Carbon Dioxide/Methanol: pH Study

With fermentation pH determined to be a major factor in the metabolic regulation of *B. methylotrophicum*, the effect of pH on the tri-substrate system was investigated. The experimental system was the same in all respects as the previous CO/CO₂/CH₃OH fermentation with the exception that the pH was controlled at 6.0 instead of 6.8. Thus this was not a pH-shift fermentation as outlined previously but rather a low pH experiment. The time course results are depicted in Figure 13. As seen at a pH of 6.8, production of acetate and butyrate at a pH of 6.0 occurs during growth. These results do show, however, that the growth rate is less at the lower pH, which was expected.

An overall carbon balance can be derived under the same criteria as before and is



The effect of the lower pH is evident with the decrease in both cell and acetate production and increase in butyrate production relative to the

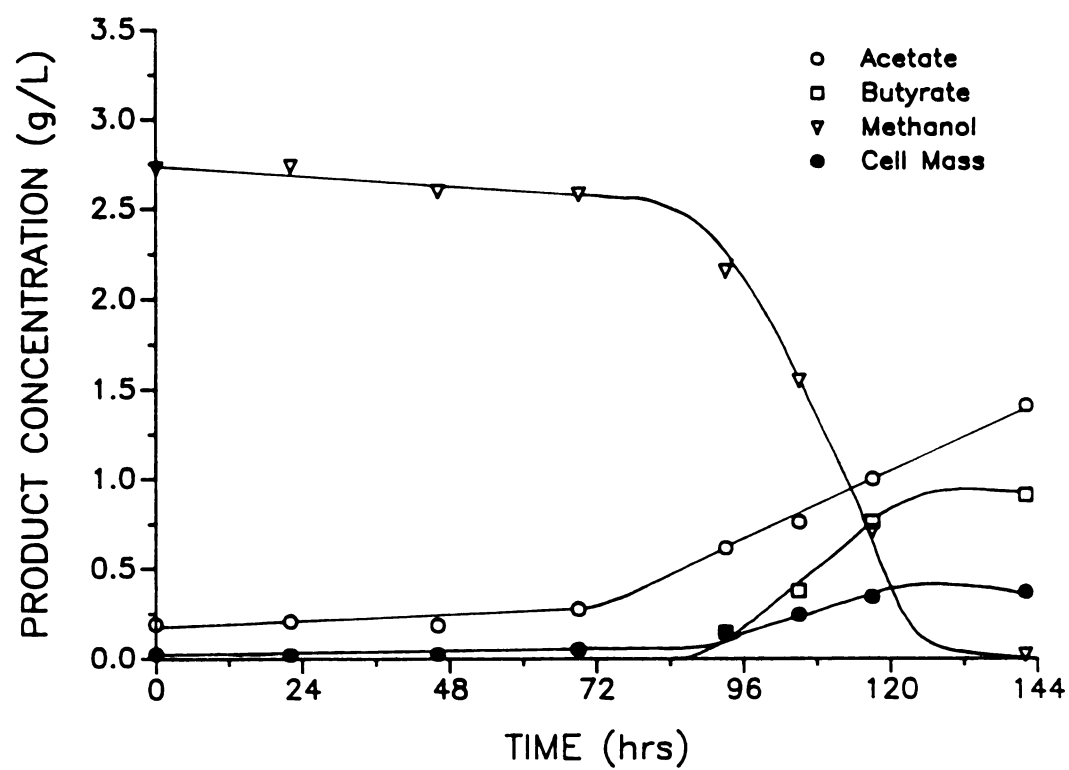


Figure 13: Batch fermentation products from $\text{CO}/\text{CO}_2/\text{CH}_3\text{OH}$: low pH.

higher pH fermentation. Accounting for previous fermentations which have shown low pH to increase butyrate formation at the expense of acetate and since the detrimental effect of low pH on cell growth is evident from this experiment, the balance is quite similar to that of Kerby *et al.*, described previously. Finally, it should be noted that at both pH values, the fermentations of $\text{CO}/\text{CO}_2/\text{CH}_3\text{OH}$ consumed almost the same total amount of CO .

4. Discussion of Batch Culture Results

The extended batch profile from the base case fermentation of 100% CO provides a distinction between growth and stationary phase product formation in *B. methylotrophicum*. With acetate being produced in both phases a simple Leudeking-Piret model (Leudeking and Piret, 1959) describes the production rate with the equation

$$dP/dt = a(dX/dt) + b(X)$$

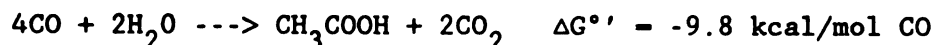
where P and X are the respective acetate and cell concentrations, t is time, a is the rate constant for growth associated acetate formation, and b is the rate constant for non-growth associated acetate formation. The base case fermentation data allows calculation of both a and b where the values are

$$a = 24 \text{ g acetate/g cells}$$

$$b = 1.7 \times 10^{-3} \text{ g acetate/g cells} \cdot \text{hr.}$$

Since the minor butyrate production is limited to the stationary phase, a growth related model for butyrate is not applicable.

The basic stoichiometric reactions for acetate and butyrate formation from CO are





where the $\Delta G^{\circ'}$ values were calculated from published data assuming combustion to aqueous bicarbonate at a pH of 7 and unit molality of all other reactants. Both reactions are sufficiently exergonic to drive ATP synthesis, although with their equivalent free energy changes neither reaction is thermodynamically favored. Butyrate is, however, the more reduced product, and formation of butyrate produces more CO_2 per mole of CO consumed than for acetate.

Little discussion is required for the 80% $\text{CO}/20\% \text{CO}_2$ experiment in relation to the base case; these two fermentations were similar as the respective carbon balances indicated. The minor amount of ethanol produced in the CO/CO_2 run is most likely due to a small shift in the partitioning metabolism of acetyl CoA, which is the likely precursor for both acetate and ethanol synthesis (see Chapter V for a discussion of the speculative enzymatic pathways involved). Whether this shift was the result of direct bicarbonate regulation is unclear from the data. Regardless of the mechanism, the effect was not pronounced.

The pH-shift experiments conclusively determined that fermentation pH is a major metabolic regulatory mechanism in this system. The alteration of carbon and electron flow away from acetate towards butyrate and alcohols induced by prudent pH adjustment is a significant achievement with respect to the indirect liquefaction process. The extent of conversion in these fermentations can be assessed in several ways. Table 6 shows the final molar and weight ratios of acetate to butyrate for the 100% CO , 80% $\text{CO}/20\% \text{CO}_2$, and the pH-shift experiments. It is evident that the low pH in the shift experiments induces production of butyrate relative to base case acetate production. The

flow of available electrons from CO to fermentation products for the base case and pH-shift #1 fermentations is shown in Table 7. Since heats of combustion are directly proportional to available electron content for a wide variety of biochemicals (Erickson *et al.*, 1978),

Table 6: Molar/weight ratios of acetate:butyrate in batch CO fermentations.

<u>Fermentation</u>	<u>Acetate:Butyrate Ratio</u>	
	<u>Molar</u>	<u>Weight</u>
100% CO Base Case	31.9	21.7
80% CO/20% CO ₂	34.7	23.6
100% CO pH-shift #1	0.98	0.65
100% CO pH-shift #2	1.16	0.81

Table 7: Percent of available electrons contained in products.

<u>Fermentation</u>	<u>Acetate</u>	<u>Butyrate</u>	<u>Cell Mass</u>
100% CO: Growth Phase	80%	0%	20%
100% CO: Stationary Phase	88%	8%	4%
100% CO: Overall	86%	6%	8%
pH-shift: Growth Phase	71%	5%	24%
pH-shift: Stationary Phase	21%	78%	1%
pH-shift: Overall	27%	70%	3%

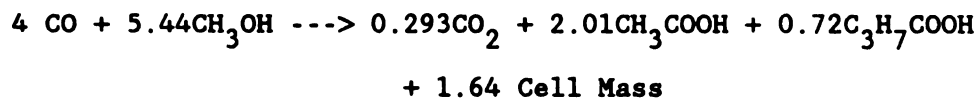
Table 7 can also be interpreted as the relative chemical energy content of the products. As shown, the pH-shift at the onset of the

stationary phase redirects the flow of carbon and electrons away from acetate and cell mass towards butyrate and minor alcohol production (not shown).

The specific mechanism(s) involved in pH regulation of *B. methylotrophicum* metabolism is not discernable from these batch culture results. Presumably, regulation occurs at key enzymatic steps involved effect is most pronounced in the stationary phase. in the reductive conversion of acetyl CoA to acetate and ultimately to butyrate. The pathway for conversion of butyryl CoA to butyrate may also be directly affected. Either direct enzymatic pH regulation is involved or else an indirect regulatory mechanism, most likely through a pH influenced mediator, is present. The data are certainly 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. Therefore, butyric acid production acidifies the medium less than acetic acid production. This chemistry would favor butyrate production over acetate production at lower pH values in order to minimize further pH reduction. This scheme is also consistent with the observed production of the corresponding 2 and 4-carbon alcohols, for which the dissociation constants are even less.

Regarding the CO/CO₂/CH₃OH fermentations the same trend as with the 100% CO studies was observed, mainly that of increased butyrate production and decreased acetate production at low pH. The speculative mechanism of pH regulation described above is also applicable for the tri-substrate experiments. Three possible pathways are in operation during the tri-substrate fermentation, the CO pathway, the CO/CH₃OH

pathway, and the $\text{CH}_3\text{OH}/\text{CO}_2$ metabolizing pathway, which are difficult to distinguish from the fermentation results since any one or all of the pathways could be operating and all three produce the same end products albeit in different amounts. It is likely that a strict CO metabolizing pathway is not in use since an apparent death phase occurred after depletion of the CH_3OH ; if the pathway were active, growth would be expected to continue into a stationary phase, since CO was continuously supplied to the fermentation. In terms of the metabolic mechanism, the tri-substrate fermentation results indicate that the overall product formation stoichiometry is much more similar to the $\text{CO}/\text{CH}_3\text{OH}$ data of Kerby, which may suggest that this pathway is favored. Interestingly, a combination of the $\text{CH}_3\text{OH}/\text{CO}_2$ (excess methanol) and $\text{CO}/\text{CH}_3\text{OH}$ balances in the ratio of 1:4 yields the overall balance



which is similar to the tri-substrate $\text{CO}/\text{CO}_2/\text{CH}_3\text{OH}$ high pH fermentation balance. However, this is just a mathematical contrivance from existing data; a proper thermodynamic analysis followed by a detailed enzymatic study of all 3 pathways is required before any definitive conclusions can be obtained for this complex system. Whatever the cellular mechanism, the CO and $\text{CH}_3\text{OH}/\text{CO}_2$ metabolizing pathways exhibit low activity, if any, in the presence of all three substrates. This preliminary finding could ultimately complicate the process scheme for reducing CO_2 generation by direct methanol addition to an existing CO fermentation, in that production of butyrate is not favored.

5. Mass Transfer Analysis

Interphase CO transport is a potential rate-limiting factor in CO fermentations operated at low pressure. Although experimental results with the pH-shift studies did indeed verify that the CO fermentation system was mass transfer limited, the conclusion was based on results which were purely macroscopic in nature and thus did not yield any information concerning the specific mass transfer environment. Unfortunately, characterization of simultaneous mass transfer and reaction of CO in a fermentation broth is difficult without a convenient and reliable liquid phase CO assay. Therefore, in order to assess the transport properties of the CO fermentation system, specifically the base case 100% CO fermentation, consumption rates were calculated from carbon and electron balances and used to identify the rate limiting phenomena.

Figure 14 shows the time course profiles of cell concentration, specific CO uptake rate, and volumetric CO uptake rate for the base case fermentation. The rate data were calculated using carbon and electron balances and numerical differentiation of the product formation data. A three-point algorithm for non-constant step sizes was used (Burden *et al.*, 1981). Early in the growth phase, the volumetric CO uptake rate increased with cell concentration, while the specific CO consumption rate remained approximately constant. These trends are indicative of an adequate nutrient supply and balanced cell growth. However, at a cell density of approximately 0.09 g/L. the volumetric consumption rate became constant, while the specific rate began to decline. As the cell concentration continued to increase, the specific rate dropped severely, while the volumetric rate declined only gradually. These trends strongly

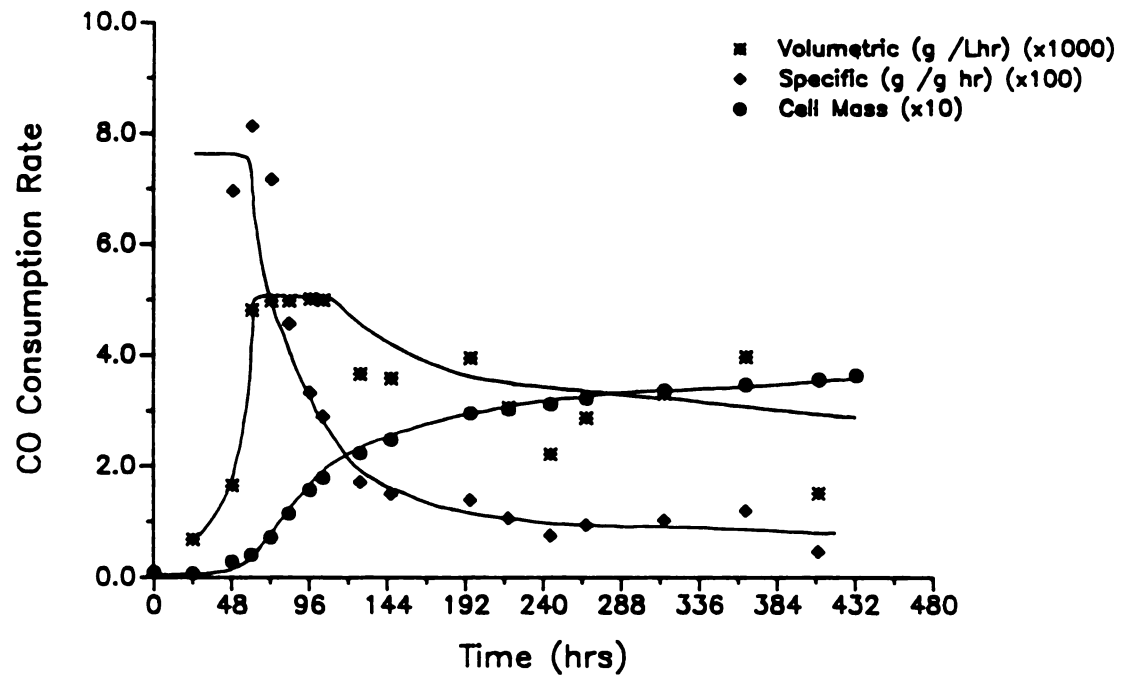


Figure 14: Volumetric and specific CO consumption rates during batch fermentation of 100% CO gas.

suggest that CO transport was rate-limiting for cell concentrations greater than 0.09 g/L, which is almost 85% of the entire fermentation time course and for which over 95% of the fermentation products were formed.

The volumetric transfer rate (Q) of a sparingly soluble, dispersed gas into a liquid phase can be described by the relation

$$Q = k_1 a (C^* - C)$$

where $k_1 a$ is the volumetric mass transfer coefficient, C^* is the liquid phase gas concentration in equilibrium with the gas phase, and C is the liquid phase gas concentration. Under mass transfer limited conditions, the liquid phase gas concentration is approximately zero, and Q becomes constant where

$$Q = k_1 a (C^*) \quad \text{at } C \approx 0.$$

Thus, for cell concentrations greater than 0.09 g/L in Figure 14, 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 from accumulating biomass; higher viscosities are known to decrease $k_1 a$ values in stirred tank fermentations (Lee and Luk, 1983).

The transport properties of gas phase substrates can vary widely and are dependent on many factors, including temperature, pressure, viscosity, and molar volume. One particularly relevant transport property is the molecular gas diffusivity, which can be calculated using the Wilke-Chang correlation (Wilke and Chang, 1955) where

$$D = 7.4 \times 10^{-8} (\psi_B \cdot M_B)^{0.5} T / (\mu \cdot V_A^{0.6})$$

with D equal to the diffusivity, ψ_B is a solvent association parameter, M_B is the molecular weight of the solvent, T is the absolute

temperature, μ is the solution viscosity, and V_A is the molar weight of the solute. Table 8 lists the solubilities and diffusivities for CO, CO₂, and O₂ at 35°C and atmospheric pressure. The diffusivity

Table 8: Transport properties of carbon monoxide, carbon dioxide, and oxygen in water at 35°C and 1 atm.

<u>Gas</u>	Solubility ^a		Diffusivity (cm ² /s)
	(mg/L)	(mmol/L)	
Carbon Monoxide	23.5	0.838	2.76 x 10 ⁻⁵
Carbon Dioxide	1162	26.4	2.60 x 10 ⁻⁵
Oxygen	34.8	1.089	3.08 x 10 ⁻⁵

^a Data from International Critical Tables, vol. III, p. 257-260, McGraw-Hill Book Company, New York, 1928.

for CO is 10% less than that of O₂. Since k_1 is proportional to the square root of the diffusivity according to surface renewal theory, $k_1 a$ values for CO should be slightly less than values measured for O₂ under similar conditions. There is a plethora of literature concerning mass transfer requirements for oxygen in aerobic fermentations, and it is helpful to compare values for CO with those of O₂, which is also sparingly soluble in water. The aqueous molar solubility of CO as shown is approximately 25% less than that of oxygen at 35°C and for CO₂ it is more than an order of magnitude higher than both O₂ and CO. Unless high pressures were used, the driving force for mass transfer in CO fermentations will be relatively low.

The results from the base case 100% CO fermentation also allow calculation of $k_1 a$, where in a mass transfer limited system

$$k_1 a = Q/C^*.$$

Input of the base case fermentation data for an average volumetric consumption rate during the stationary phase yields

$$k_1 a = (4.4 \text{ mg/L}\cdot\text{hr})/(23 \text{ mg/L}) = 0.19 \text{ hr}^{-1}$$

which is a very low value but not suprising considering the low agitation rate and the short bubble travel time in a 1 L fermenter.

6. Significance of Batch Culture Results

The batch culture fermentation results presented in this chapter have uncovered several significant features of the metabolism of *Butyribacterium methylotrophicum* and of the first stage of the indirect liquefaction process as well. Unicarbonotrophic acetate production from CO has been verified in the laboratory fermenter at a pH of 6.8 with acetate production occuring in both the growth and stationary phases. Addition of 20% carbon dioxide to the gas phase was determined not to significantly affect this pathway, although some slight alcohol production was observed during stationary phase response. The consumption characteristics for CO during these fermentation clearly indicated that CO mass transfer was rate limiting, as expected from the solubility and transport properties of CO.

Fermentation pH was found to be a direct mediator of carbon and electron flow in *B. methylotrophicum*, with more reduced products being formed at low pH. Specifically, stationary phase butyrate and acetate production was observed in equimolar quantities from CO. Minor production of alcohols was also observed concomitant with butyrate formation. The overall consequences of fermentation pH are shown in Table 9, where the pH effect on production of reduced compounds in batch

culture is summarized.

Unicarbonotrophic production of butyric acid from carbon monoxide is of major importance in that a four-carbon organic acid is produced biologically from a one-carbon inorganic substrate. The direct metabolic shift from acetate to butyrate production is most likely a result of pH

Table 9: Consequences of reduced fermentation pH on batch culture product mix.

<u>Fermentation</u>	<u>Major Product</u> ^a	<u>Minor Product</u>
100% CO Base Case	Acetate	Butyrate
100% CO pH-shift #1	Butyrate/Acetate	Ethanol/Butanol

^a Based on total moles produced.

regulated enzymatic activity in the formation of both products from their accompanying acetyl CoA and butyryl CoA precursors. The value of this shift in a process perspective is clear; butyrate is the second stage substrate for butanol formation, whereas acetate is the second stage substrate for ethanol and acetone production.

Finally, the tri-substrate fermentations of carbon monoxide, carbon dioxide, and methanol demonstrate the complexity of multi-carbon and electron donor growth. The inactivity of a distinct CO consuming pathway was observed with a large proportion of the observed metabolic activity devoted to the CO/CH₃OH pathway yielding primarily acetate. Macroscopic stoichiometric results do indicate some CH₃OH/CO₂ metabolizing activity which yields butyrate as the sole product with accompanying consumption

of acetate in excess methanol. Reduced pH, as in the 100% CO fermentations, was found to favor butyrate over acetate production with a decrease in cell growth. This result has direct impact on the process design of the acidogenic stage for synthesis gas bioconversion in that direct methanol addition in order to conserve carbon lost to CO_2 is not advantageous with respect to product formation. Thus a two-stage fermentation may be required for the acidogenic process, one for CO conversion to butyrate and acetate, and the second for methanol addition to convert CO_2 and CH_3OH to butyrate.

CHAPTER IV

CONTINUOUS CULTURE STUDIES

With the important and unique results obtained from batch culture indicating fermentation pH as a direct metabolic regulator of carbon monoxide and methanol metabolism in *Butyribacterium methylotrophicum*, the next objective was to determine the extent of this regulation under similar conditions in continuous culture. This involved first establishing conditions for steady-state operation of a chemostat and then conducting steady-state operation over a range of broth pH values.

This chapter presents the experimental results and discussion for two continuous culture systems. The first system was designed and operated as a pH-stat, where after the establishment of a baseline steady-state response, the effect of fermentation pH on CO metabolism in continuous culture was analyzed. The second system was designed for increased mass transfer and operated with the purpose of approaching a non-mass transfer limited CO fermentation.

1. Experimental Set-up and Design

Continuous fermentations were conducted in a 1.25 L working volume laboratory fermenter designed for maintenance of a strictly anaerobic and sterile environment. Key features of this system are shown in Figure 15. The liquid feed rate was kept constant by a built in peristaltic pump; a similar pump also controlled base addition for continuous pH control. Effluent liquid was not pumped but exited via an overflow port at the specified volume level. CO gas was continuously sparged thru a sparge tube into the broth and exited through an attached condenser in

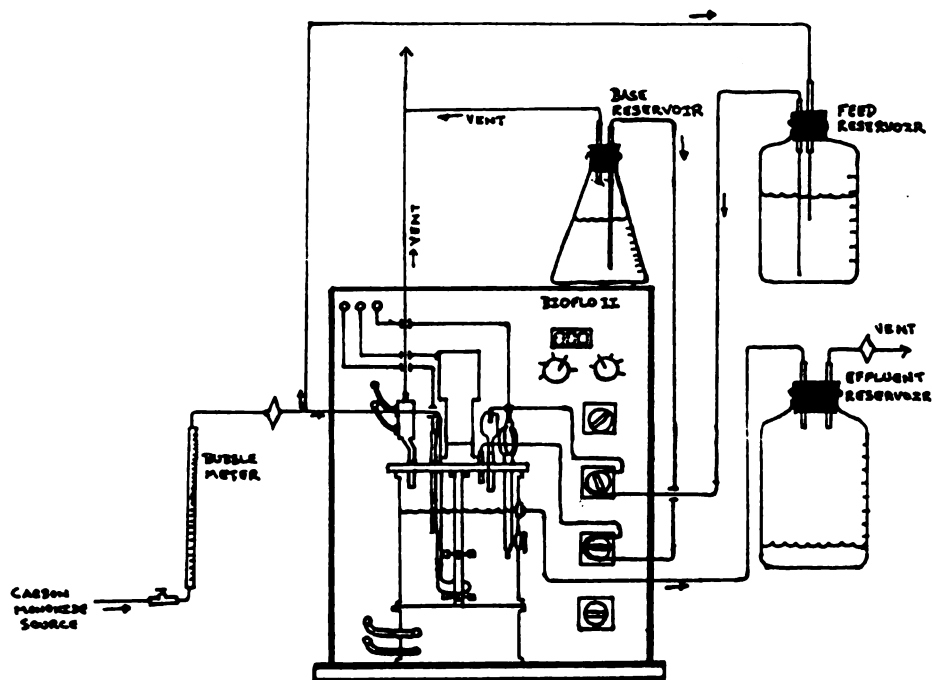


Figure 15: Continuous fermentation system for 100% CO gas.

the fermenter headplate assembly. The whole set-up was operated in a safety hood.

As in the batch culture studies, this system was operated with a continuous gas flow of CO much greater than the rate of consumption, thus maintaining a 100% CO headspace above the liquid broth. Although substrate addition was uncoupled from the liquid feed rate, the liquid stream was still used to calculate the dilution rate of the system, by definition. With the absence of any continuous culture data for *B. methylotrophicum*, a logical value for the dilution rate had to be chosen in order to achieve steady-state operation and avoid washout. A steady-state material balance on the cells in the fermenter,

$$R_G \cdot V_R = F \cdot (x - x_{in})$$

where R_G is the cell growth rate, V_R is the fermenter working volume, x is the concentration of cells in the fermenter, and x_{in} is the concentration of cells in the inlet stream, can be combined with a Monod model,

$$R_G = \mu \cdot x$$

where μ is the specific growth rate, to give

$$\mu \cdot x = (F/V_R)(x - x_{in}),$$

where if x_{in} is zero and using the definition of dilution rate gives

$$D = (1/x) \cdot R_G.$$

Insertion of previous batch data from the mid-growth phase of the base case 100% CO fermentation (Figure 4) yields

$$D = (4.47 \text{ L/g}) \cdot (.003225 \text{ g/L} \cdot \text{hr}) = 0.0144 \text{ hr}^{-1}.$$

Therefore a dilution rate of 0.015 hr^{-1} was chosen which corresponded to a liquid feed rate of approximately 0.31 mL/min. This rate was maintained constant throughout all continuous fermentation experiments.

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 nine days. Although rigorous, this stricture provided increased confidence in the accuracy of the data obtained. Henceforth, all data presented are steady-state values defined by this criteria.

2. Fermentation pH Studies: Experimental Results

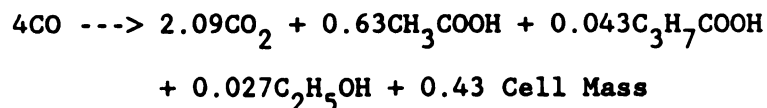
Fermentation pH studies reflect the effect of pH on the metabolic carbon and electron partitioning from CO metabolism. An initial baseline study at a pH of 6.8 was performed to test the apparatus, to form a basis for comparison with the base case batch study, and to serve as a reference profile for the other continuous culture studies. After establishment of the baseline profile, lower pH value fermentations were conducted and the response to the pH change observed during steady-state operation. These experiments were all conducted in the same fermenter system and under the same conditions with pH as the controlled variable. Thus direct correlations can be drawn between all experiments relating pH to product mix and concentrations.

2.1. Baseline Fermentation

With the calculated dilution rate, an initial baseline fermentation of 100% CO was conducted for three weeks at a pH of 6.8 with approximately ten days of steady-state operation. Similar conditions in batch culture described previously in Chapter III favored acetate production with a corresponding acetate/butyrate ratio (molar) of 32:1. In the continuous culture system, the resulting molar ratio was approximately 15:1, with average steady-state concentrations of acetate

and butyrate in the broth equal to 0.86 g/L and 0.086 g/L, respectively. Ethanol production was also observed in small (<.05 g/L) quantities.

Carbon and electron balance calculations give an overall stoichiometry of

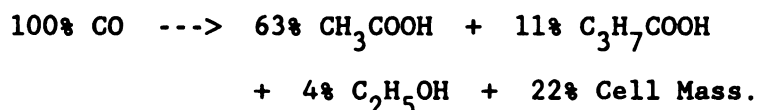


which clearly shows acetate and cell mass as the primary reduced products. Total consumption of CO during steady-state operation was 10.7 L at a rate of 41 mL/hr or approximately 0.69 mL/min; still almost two orders of magnitude less than the CO feed rate of 50 mL/min.

The overall stoichiometry allows calculation of some key fermentation parameters and concepts, mainly molar yield coefficients and carbon/electron partitioning. Molar yield coefficients, defined as moles of product divided by the moles of substrate consumed, are helpful in following the overall conversion of substrate to multiple products and serve as an indication of specific pathway activity. For the above stoichiometry the yield coefficients are

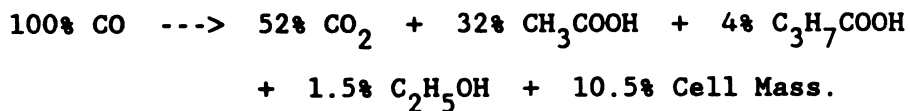
$$Y_{\text{CO}_2/\text{CO}} = 0.522 \quad Y_{\text{Ac}/\text{CO}} = 0.158 \quad Y_{\text{Bu}/\text{CO}} = 0.011 \\ Y_{\text{EtOH}/\text{CO}} = 0.007 \quad Y_{\text{Cells}/\text{CO}} = 0.108,$$

which emphasizes both acetate and cell mass as major products. Electron partitioning, which as described previously corresponds to the distribution of the total chemical energy content of the CO substrate, can be represented in a balanced equation as



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

products, is balanced as



These values present some interesting and valuable information which both confirms some general trends observed in batch culture and sets the stage for subsequent comparison at lower pH:

1. As determined in batch fermentation, approximately half of the incoming carbon is lost to CO_2 .
2. Acetate is the major reduced product, accounting for 67% of the reduced product carbon.
3. Compared to batch fermentation results at the same pH, continuous culture data indicates a small metabolic shift towards more reduced product formation based solely on the continuous environment of the system.

Therefore, as the fermentation pH in continuous culture is lowered, a pH-induced metabolic shift is expected to occur with increased production of more reduced compounds relative to acetate. This effect should continue until cell washout begins to influence the detectable product mixture.

2.2. Lower pH Fermentations

Once conditions for continuous culture were established in a baseline fermentation as outlined in the preceding section, experiments with lower fermentation pH values could be conducted and compared relative to the baseline profile. Data was collected during operation at steady-state conditions for broth pH values of 6.5, 6.0, and 5.5. Operation was then attempted at a pH of 5.0 but was discontinued due to cell washout. The results for these experiments have been combined with

the baseline fermentation results and presented in the form of overall stoichiometry, molar yield coefficients, electron partitioning, and carbon partitioning in Tables 10, 11, 12, and 13, respectively. Also included are in Table 14 the molar (weight) product ratios, specific product weight ratios based on grams product per grams cell in Table 15, and in Table 16, the average steady-state broth product concentrations. Values reported as trace indicate presence in amounts less than 0.5 mmol/L.

As observed in batch culture, there was a significant relationship between the fermentation broth pH and the product selectivity. Several important results are evident from the data. First, and of prime importance, is the trend towards more reduced product formation, specifically butyrate and alcohols, as the fermentation pH is reduced. This trend parallels what was observed in the batch culture pH-shift results, and is shown in all the tables. Although at a pH of 5.5 the data do not support this trend entirely, the deviation is most likely an effect of the dilution and of the low pH effect on cell growth. This conclusion comes from an examination of the data in Table 16, which shows a significant drop in the cell density (concentration) between pH values of 6.0 and 5.5. If the dilution rate, which was maintained constant in all of the fermentations, were lowered in order to maintain a constant cell density, all the fermentation products would be concentrated and the true metabolic stoichiometry at a pH of 5.5 revealed. Therefore, the small shift back towards less reduced product formation observed at a pH of 5.5 may be an experimental artifact.

A second highly significant result shown by these data is the continuous production of alcohols, specifically the 4-carbon butanol,

Table 10: pH-Dependent continuous fermentation stoichiometry for CO consumption by Butyribacterium methylotrophicum.

pH	Fermentation Stoichiometry	
6.8	$4 \text{ CO} \rightarrow$	$2.09 \text{ CO}_2 + 0.63 \text{ CH}_3\text{COOH} + 0.043 \text{ C}_3\text{H}_7\text{COOH} + 0.027 \text{ C}_2\text{H}_5\text{OH} + 0.43 \text{ Cells}$
6.5	$4 \text{ CO} \rightarrow$	$2.13 \text{ CO}_2 + 0.56 \text{ CH}_3\text{COOH} + 0.082 \text{ C}_3\text{H}_7\text{COOH} + 0.026 \text{ C}_2\text{H}_5\text{OH} + 0.37 \text{ Cells}$
6.0	$4 \text{ CO} \rightarrow$	$2.27 \text{ CO}_2 + 0.30 \text{ CH}_3\text{COOH} + 0.161 \text{ C}_3\text{H}_7\text{COOH} + 0.032 \text{ C}_2\text{H}_5\text{OH} + 0.029 \text{ C}_4\text{H}_9\text{OH} + 0.31 \text{ Cells}$
5.5	$4 \text{ CO} \rightarrow$	$2.18 \text{ CO}_2 + 0.40 \text{ CH}_3\text{COOH} + 0.154 \text{ C}_3\text{H}_7\text{COOH} + 0.40 \text{ Cells}$

Table 11: pH-Dependent molar yield coefficients for CO consumption by Butyribacterium methylotrophicum.

pH	CO ₂	CH ₃ COOH	C ₃ H ₇ COOH	Molar Yield Coefficients		C ₄ H ₉ COOH	Cells
6.8	.522	.158	.011	.007	--		.108
6.5	.532	.140	.020	.007	Trace		.092
6.0	.568	.076	.040	.008	.007		.078
5.5	.545	.100	.038	Trace	Trace		.100

Table 12: pH-Dependent electron partitioning for CO consumption by Butyribacterium methylotrophicum.

pH	CH ₃ COOH	C ₃ H ₇ COOH	% Electrons		C ₄ H ₉ OH	Cells
			C ₂ H ₅ OH			
6.8	63.0	11.0	4.0	--		22.0
6.5	56.0	20.5	4.0	Trace		19.5
6.0	30.3	40.2	4.8	8.7		16.0
5.5	40.2	38.5	Trace	Trace		21.3

Table 13: pH-Dependent carbon partitioning for CO consumption by Butyribacterium methylotrophicum.

pH	CO ₂	CH ₃ COOH	% Carbon		C ₄ H ₉ OH	Cells
			C ₃ H ₇ COOH	C ₂ H ₅ OH		
6.8	52.0	32.0	4.0	1.5	--	10.5
6.5	53.0	28.0	8.2	1.3	Trace	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	Trace	10.0

Table 14: pH-Dependent product ratios for CO consumption by Butyribacterium methylotrophicum.

pH	Ac/Bu	Product Ratio ^a	
		Ac/EtOH	Ac/BuOH
6.8	14.7 (10)	23.4 (30.7)	--
6.5	6.8 (4.6)	22.0 (28.5)	Trace
6.0	1.9 (1.3)	9.5 (12.3)	10.5 (8.5)
5.5	2.6 (1.8)	Trace	Trace

^a Values given are weight (molar) ratios.

Table 15: pH-Dependent specific product weight ratios for CO consumption by Butyribacterium methylotrophicum.

pH	CH ₃ COOH	Specific Ratio ^a		
		C ₃ H ₇ COOH	C ₂ H ₅ OH	C ₄ H ₉ OH
6.8	3.47	0.35	0.11	--
6.5	3.72	0.80	0.13	Trace
6.0	2.41	1.87	0.20	0.28
5.5	2.42	1.36	Trace	Trace

^a Units in grams product per grams cell mass.

Table 16: pH-Dependent product concentrations for CO consumption by Butyribacterium methylotrophicum.

pH	Product Concentrations (g/L)				Cells
	CH ₃ COOH	C ₃ H ₇ COOH	C ₂ H ₅ OH	C ₄ H ₉ OH	
6.8	0.860	0.086	0.028	--	0.248
6.5	1.055	0.227	0.037	Trace	0.284
6.0	0.689	0.536	0.056	0.081	0.286
5.5	0.475	0.266	Trace	Trace	0.196

solely from the 1-carbon substrate CO, observed at a pH of 6.0. Shown in Table 15, butanol, when normalized to cell density, is produced by weight in greater amounts than the 2-carbon ethanol, and as seen in Table 12, contains a significant portion 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*.

Also observed, as expected, was the drop in cell production as the pH decreased below 6.0, shown in Table 16. As stated previously, cell washout was observed at a pH of 5.0. Regarding CO₂ production, Tables 10, 11, and 13 indicate that carbon dioxide evolution is not significantly affected by fermentation pH, and thus reducing the carbon lost to CO₂ cannot be achieved by manipulating this parameter.

3. Gas Recycle Fermentation Study: Experimental Results

With a mass transfer limited system the sparging rate for gas addition becomes a controlling factor in the overall consumption rate of carbon monoxide. Another important parameter which influences CO transport from the gas to the liquid phase is the agitation rate for the impeller shaft. In order to conserve gas and to ensure minimal cell damage from shear effects, the gas flow rate and the agitation rate of the batch and continuous culture studies were set at values which were relatively low. To address the issue of a possible non-mass transfer limited fermentation, or to at least increase the production of reduced products by increasing the mass transfer rate, a gas recycle system was designed and operated in a continuous culture fermentation at a low pH.

3.1. Experimental Set-up and Expectations

The principle of the gas recycle system was to uncouple the overall gas throughput of the fermentation from the rate of gas sparging into the liquid broth. A 0.4 L laboratory fermenter with automatic pH control and liquid feed addition, similar to the system for the continuous pH studies, was used. However, the gas exit stream from the fermenter headspace was not vented directly but instead was rate controlled with a peristaltic pump. A separate recycle pump was also positioned to draw gas from the vessel headspace, mix it with incoming fresh gas, and return the resulting stream thru a sterile filter to the fermenter. Entry was by a sparge tube located in the liquid broth. This gas stream was fitted with a water trap to preserve filter integrity. With the expected small consumption rates of CO, the exit pump therefore controlled the rate of gas throughput, and the internal recycle pump determined the gas flow rate into the liquid.

The experimental design was an exploratory effort conducted in order to observe CO metabolism in a much different transport environment. A low pH value was used to reconfirm the pH effect and to hopefully establish large productivities for both butyrate and butanol. Several possible outcomes were foreseen:

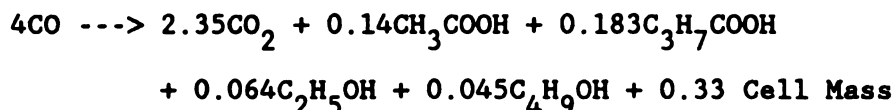
1. The resulting increase in CO transport would provide more substrate per unit time and thus increase both concentration and productivity for all products in a mass transfer limited system.
2. Mass transfer limitations would disappear, causing the liquid phase CO concentration to approach the solubility limit; the system would thus become

reaction rate limited.

3. Possible substrate inhibition would arise due to dissolved CO present in the liquid phase.
4. Product inhibition due to increased liquid phase concentrations of alcohols could occur.

3.2. Operation at a pH of 6.0

The system was operated with an internal gas recycle rate of 250 mL/min, an overall gas flow rate of 10 mL/min, and an agitation rate of 200 rpm at a pH of 6.0. Compared to the continuous pH studies this corresponded to an increase in the specific gas flow rate to the liquid by a factor of approximately 16 and a four-fold increase in the agitation rate. The calculated reaction stoichiometry is



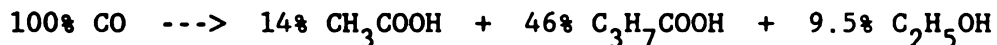
which shows butyrate as the primary fermentation product. Alcohol production is also significant in this balance. This balance was obtained from total steady-state product concentrations of

$$[\text{CH}_3\text{COOH}] = 0.246 \text{ g/L} \quad [\text{C}_3\text{H}_7\text{COOH}] = 0.468 \text{ g/L} \quad [\text{C}_2\text{H}_5\text{OH}] = 0.085 \text{ g/L} \\ [\text{C}_4\text{H}_9\text{OH}] = 0.097 \text{ g/L} \quad [\text{Cell Mass}] = 0.234 \text{ g/L}$$

The total substrate consumed, calculated assuming no stripping losses, was 3.8 L of CO gas at a rate of approximately 0.3 mL/min. The molar yield coefficients arising from this stoichiometry are

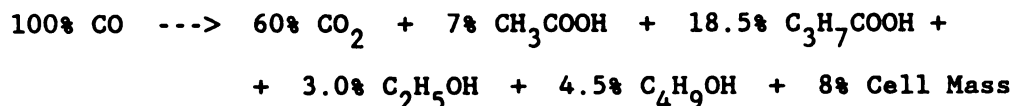
$$Y_{\text{CO}_2/\text{CO}} = 0.588 \quad Y_{\text{Ac}/\text{CO}} = 0.035 \quad Y_{\text{Bu}/\text{CO}} = 0.046 \\ Y_{\text{EtOH}/\text{CO}} = 0.016 \quad Y_{\text{BuOH}/\text{CO}} = 0.011 \quad Y_{\text{Cells}/\text{CO}} = 0.082$$

Electron partitioning shows almost 50% of the chemical energy flow towards butyrate, where





The carbon partitioning shows a similar trend for the reduced products, where



indicating the typical loss of carbon by CO_2 generation.

4. Discussion of Continuous Culture Results

Several advances towards achieving the objectives of the first stage for the indirect liquefaction process were made in the development and operation of a continuous culture fermentation for *Butyribacterium methylotrophicum*. Foremost of these advances was the described baseline fermentation for which steady-state production was maintained for over a period of 10 days. Once established as a workable system, several other experiments were conducted relating fermentation pH to production of reduced compounds, all operated for several residence times under steady-state conditions. These studies were successful both in establishing the mode of operation as well as in the production of desired acids and alcohols. Perhaps even more importantly, the limitations set by the CO substrate and low growth rate of *B. methylotrophicum*, as expressed through the continuous culture results presented in this chapter, indicate the key areas in which future fermentation research must be emphasized, as highlighted briefly in the following discussion and with more detail in Chapter VI.

The baseline fermentation results at a pH of 6.8 represent the first continuous fermentation for pure culture conversion of carbon monoxide, with primary production of acetate and cell mass. The occurrence of both

butyrate and ethanol in low quantities with respect to the fermentation pH are in accordance with the general metabolic trend observed in batch culture, only with increased expression of the more reduced products. The batch studies showed butyrate and ethanol production to be non-growth associated, which suggests that the cells in continuous culture at a pH of 6.8 are predominantly in growth phase metabolism with a small proportion of the total cell population at any one time in the stationary phase. Although this observation/conclusion is perhaps technically inaccurate, modelling the cell population as a discrete system with proportions in all phases of the growth cycle does provide a descriptive explanation of the population dynamics, particularly in light of the pH studies.

With regard to the pH effect on fermentation stoichiometry the results tabulated in Tables 10-13 are quite conclusive. With the exception of the experiment at a pH of 5.5, decreasing pH caused increased production of more reduced compounds, with butyric acid, ethanol, and butanol all being formed at a pH of 6.0. These results are consistent with the premise put forth in Chapter III, relating this change in metabolism to a cellular effort to decrease further environmental acidification. The complexity of the specific mechanisms involved is potentially overwhelming. Concurrent with the increased production of butyrate and alcohols is a decreased production of acetate 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 6.8 to 6.0 is also consistent with direct pH inhibition of cell growth.

The results of the fermentation at a pH of 5.5, as mentioned previously, are probably a function of the controlled variable (dilution rate), since all previous results would suggest less cell formation and increased butyrate/alcohol production at lower pH values. The apparent metabolic shift back towards acetate and cell production, indicating a higher proportion of cells in the growth phase, is explainable when the mean cell density is compared to the higher pH experiments as shown in Table 16. At a pH of 5.5 the cell density is significantly lower, presumably from the pH effect on cell growth. With a lower cell density, more CO is available per cell and thus substrate limited induction of stationary phase response may not be as prevalent compared to higher cell densities. This results in a metabolic shift with respect to the total population in the opposite direction. There is evidently a competitive situation between fermentation pH regulation of cell growth and substrate availability from the standpoint of overall control of fermentation product formation; the two factors are antagonistic as the pH drops below 6.0. With cell washout occurring at a pH of 5.0, the pH effect is likely the controlling factor at such low values. This result encompasses the major obstacle in development of this system: low pH values induce favorable products but inhibit production of cell mass and thus lower overall productivity. Addressing this conflict is the foundation for the recommended future work discussed in Chapter VI.

For the gas recycle fermentation at a pH of 6.0, the results show slight stoichiometric increases in butyrate, ethanol, and butanol formation compared to the same pH fermentation conducted in the pH studies. Cell production is also slightly increased. However, acetate formation is over 50% less in the gas recycle system. Thus a strict

increase in the carbon and electron flow due to increased CO transfer is not a likely mechanism. Since the drop in acetate is not accompanied by a drop in cell mass, a metabolic shift away from growth phase metabolism is also an unlikely mechanism. The alternative explanations have little experimental support. Direct CO inhibition of the acetate forming pathway would require a non-mass transfer limited condition in the liquid phase. The specificity of such inhibition is improbable relative to the complex product mixture associated with the CO fermentation. However, the overall volumetric consumption of CO, and thus the overall volumetric productivities, were less for the gas recycle case as shown in Table 17, which shows a 23% reduction in the overall consumption rate

Table 17: Volumetric production and consumption rates for the pH-stat and gas recycle fermentations at a pH of 6.0.

<u>Volumetric Rate</u>	<u>Fermentation</u>	
	<u>pH-Stat</u>	<u>Gas Recycle</u>
CO Consumption: (mol/L·hr)	.0022	.0017
Production (g/L·hr)		
Acetate:	.0103	.0037
Butyrate:	.0080	.0070
Ethanol:	.0008	.0013
Butanol:	.0012	.0015

of CO due to the gas recycle system, resulting in reduced productivities for the major fermentation products. Thus although the carbon and electron partitioning was increased in favor of more highly reduced products, the overall formation of all products was decreased. One

possible mechanism is CO inhibition due to an increased liquid phase CO concentration. This would be the result of a non-mass transfer limited environment. The reduction in acetate formation is not clear from such a hypothesis, but could be a possible outcome of a feedback inhibition mechanism from the increased alcohol concentrations present in the gas recycle fermentation. In general, no conclusive explanations can be made without a mass transfer analysis accompanied by some specific enzymatic studies.

5. Significance of Continuous Culture Results

Steady-state, continuous culture of *Butyribacterium methylotrophicum* was established as set forth by the process objectives for the conversion of carbon monoxide to organic acids. The low rate of growth, as indicated by a 12 hour doubling time, required a very low dilution rate for the fermentation system. Thus, although steady-state operation was achieved in all experiments, overall productivities were very low; roughly 4 L of effluent broth was obtained after 10 days of operation in each case. Production of acids and alcohols was achieved at concentration levels of approximately .05% to .1% for the acids and .005% to .01% for the alcohols, levels that are industrially meaningless. These aspects of the fermentation require improvement.

The significance of the continuous studies, besides the general achievement of such systems, is the metabolic basis for continuous culture of CO and its regulation through pH change. The trend of CO consumption 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 in *Butyribacterium*

methylophilum. With the identification and expression of this mechanism comes also some critical information concerning development of the process. Achieving higher dilution rates is desirable for increased productivities. Obtaining higher cell densities is also an area requiring much improvement. The continuous culture studies have shown cell production, reduced product formation, and fermentation pH to all be intimately linked; under most circumstances they are antagonistic to each other. These findings have identified the crucial factors involved and dictate the direction required for improvement in the critical aspects of CO bioconversion.

CHAPTER V

ENZYMATIC STUDIES

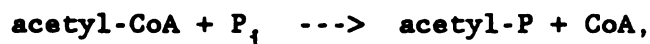
The cellular mechanism(s) which constitute the observed pH effect in both batch and continuous culture are likely involved with regulation of internal cell pH. Although the biochemical basis for this effect is a complex system of enzymes, electron carriers, and membrane bound proteins, the overall macroscopic consequences are readily observable: lower external pH values induce production of more highly reduced compounds. With increased production of butyrate at a pH of 6.0 in batch and continuous modes of operation, production increases resulting directly from increased carbon and electron flows, it follows that there is some enzymatic mechanism which is enhanced to allow the higher processing rates. Moreover, the specificity of such a mechanism requires it to be "downstream" of pathways that lead to products not affected by the pH change. Thus even with the complexity of the entire C_1 metabolic cycle, investigation of some key individual reactions from the whole pathway can, in theory, lend insight to the overall mechanism.

This chapter consists of the results and discussion for an attempt at such investigation in *Butyribacterium methylotrophicum*, accompanied by presentation of a speculative biochemical pathway for unicarbonotrophic metabolism in anaerobes. The experimental hypothesis and procedure were of a basic, exploratory nature and by no means all-encompassing or even conclusive. However, the principle of the enzymatic investigation was sound and if conducted as detailed in the discussion, could reveal a vast amount of scientific information concerning pH controlled enzymatic regulation of 1-carbon metabolism.

1. Experimental Hypothesis: Direct pH Regulation of Enzyme

Activities for Butyrate and Acetate Production

Production of butyrate and acetate from CO by *B. methylotrophicum* ultimately results from the ATP synthesizing conversions of their respective CoA precursors. Both acetyl CoA and butyryl CoA are metabolic pathway branch points in anaerobic bacteria (Thauer, et al., 1977). Organic acid production from these precursors is a two step enzymatic conversion involving first a transferase activity to replace the CoA group with a phosphate group. The second step is a phosphorylation exchange in which ATP is produced along with the accompanying acid. The specific reactions are

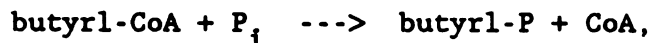


catalyzed by phosphotransacetylase (acetyl-CoA:ortho-phosphate acetyltransferase) and

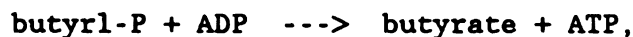


which is catalyzed by acetate kinase (ATP:acetate phospho-transferase).

For butyrate formation the analogous reactions are



mediated by phosphotransbutyrylase (butyryl-CoA:ortho-phosphate butyryltransferase) and



catalyzed by butyrate kinase (ATP:butyrate phospho-transferase). These reactions are all generally known to be reversible.

The experimental hypothesis was therefore to correlate changes in acetate and butyrate production rates to the overall activities of these four enzymes. The observed pH effect of increased butyrate production at

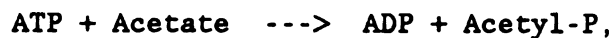
lower pH values should thus be reflected in increased enzyme activities for butyrate formation at those same values. The principle equally applies to all fermentation products and their catalyzing enzymes. The specific mechanisms for enzyme activation was not at issue; the focus was the overall change in activity as a function of the external fermentation pH.

Experimental and practical limitations confined the enzymatic analysis to the final enzymes in each pathway, acetate and butyrate kinase. Thus the results presented below cannot be conclusive since the first step of each reaction pathway was not analyzed. However, the basic tenet of the experiments remained unchanged.

2. Experimental Procedure

Analysis of acetate kinase and butyrate kinase enzyme activity was based on cellular samples taken from the continuous fermentations conducted at pH values of 6.8 and 6.0. Samples extracted from operation at steady-state were lysed to obtain cell extracts which contained varying amounts of free protein and enzyme. These extracts were the enzyme source for all experiments.

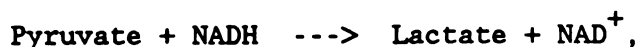
The enzyme assay a spectrophotometric analysis which followed NADH depletion in a complex reaction mixture, as detailed in Chapter II, section 6. The overall reaction occurring in the experiment was a three step conversion of acetate or butyrate to lactate, where the first reaction was



catalyzed by acetate kinase, the second reaction was



catalyzed by the enzyme pyruvate kinase, and the last reaction was



catalyzed by lactate dehydrogenase. The same reaction mixture was used for both enzymes with either acetate or butyrate as the first reaction substrate. The series of reactions was initiated by addition of cell extract containing the acetate kinase and butyrate kinase enzymes.

The cell extracts also contained acetate and butyrate from the fermentation broths which were present in amounts much less than the concentration of organic acid substrate used in the reaction mixture, due to the dilution factor. Thus cross-reaction from either substrate present in the extracts was assumed to be negligible. Similarly, the dilutions of crude extract minimized any endogenous NADH oxidase activity.

3. Results and Discussion

Duplicate runs were conducted with the extracts from the continuous fermentations at pH values of 6.8 and 6.0 for both acetate and butyrate kinase. Total protein content of the extracts was also determined using a standard BioRad protein assay, including a simultaneously run calibration curve. The absorbance data was obtained over a 10 minute time period and a linear fit obtained in all cases over the first 2.7 to 3.3 minutes. Linearity between additions of 25 μL and 100 μL of extract was also obtained for each case. These rates of absorbance depletion, as well as the protein content of the extracts used, are summarized in Table 18. The absorbance data indicates overall rate increases for both enzymes as the pH decreases from 6.8 to 6.0. This effect is due in part to the increase in enzyme concentration between the respective extracts,

as reflected in the protein assays.

Table 18: Absorbance rates for acetate kinase and butyrate kinase at pH values of 6.8 and 6.0.

	Absorbance Rate (dA/dt) (<u>min⁻¹</u>)	Protein Content (<u>mg/mL</u>)
Acetate Kinase (50 μ L sample)		
pH = 6.8:	0.0124	0.45
pH = 6.0:	0.0272	0.88
Butyrate Kinase (100 μ L sample)		
pH = 6.8:	0.0236	0.45
pH = 6.0:	0.0563	0.88

Since absorbance is an arbitrary variable, the data were converted to the form of U/mg, which is units of enzyme activity per mg total protein, or specific activity. Activity units are defined as the amount of enzyme that catalyzes the conversion of 1 μ mol of substrate per minute. This conversion was achieved using Beer's Law,

$$dA = dC/E \cdot L$$

where dA is the change in absorbance, dC is the concentration change of substrate, E is the extinction coefficient for the substrate, and L is the path length for the light source. Proper unit conversion and division by the extract protein content then ultimately yields the specific activity, presented in Table 19. The specific activities of both enzymes are increased at lower pH values, with the effect almost doubled for butyrate kinase compared to acetate kinase.

The enzymatic studies provide an interesting but by no means

conclusive indication of the effect of fermentation pH on two key product forming enzymes. The experimental hypothesis was partially verified in that at pH 6.0 there was increased butyrate kinase activity

Table 19: Specific activities of acetate kinase and butyrate kinase at pH values of 6.8 and 6.0.

	Specific Activity (U/mg)		
	<u>pH = 6.8</u>	<u>pH = 6.0</u>	<u>% Change</u>
Acetate Kinase:	0.0885	0.0993	12.2
Butyrate Kinase:	0.0842	0.1028	22.1

and hence increased butyrate production. However, by the same simple logic a decrease would be expected in the specific activity of acetate kinase, a result not observed in this study. Since the enzymes were not isolated, the actual amount of each enzyme in both extracts was not quantified. One possible explanation for this discrepancy stems from the reversible nature of the acid-forming enzymes, where at low pH there is an increase in the specific activity of acetate kinase because this enzyme is actively consuming acetate in conjunction with some fermentation broth deacidification mechanism. Since this enzyme catalyzes the final cellular processing step for acetate production, it would logically be the rate controlling step for acid consumption.

From the above results, the question of whether or not the kinase enzymes are "key" regulatory enzymes cannot be answered conclusively. Several studies linking enzyme activity to high pH acetate and butyrate production and low pH ethanol and butanol production in *Clostridium acetobutylicum* have shown butyrate kinase and phosphotransacetylase to

be much more sensitive to pH than their counterparts phosphotransbutyrlase and acetate kinase (Andersch *et al.*, 1983; Hartmanis and Gatenbeck, 1984). If also true in *B. methylotrophicum*, the results for butyrate kinase would assume more significance relative to those of acetate kinase.

From several reports on CO metabolism (Lynd *et al.*, 1982; Kerby *et al.*, 1983; Zeikus *et al.*, 1985;) and on organic acid and alcohol production by *C. acetobutylicum* (Kim *et al.*, 1984; Datta and Zeikus, 1985) a general metabolic pathway for unicarbonotrophic production of acids and alcohols can be constructed, as seen in Figure 16. As a general mechanism, lower pH values are thought to be directly linked to inhibition of NADH processing enzymes present in the metabolic cycle upstream of acetyl CoA, thus releasing these molecules for production of butyrate and alcohols downstream of acetyl CoA, where many of the pathways require reducing power (G-J Shen, pers. comm.).

Regarding the energetic basis for the observable pH effect, the most likely explanation, as briefly described in Chapter III, involves cellular maintenance of internal pH. The complexity of this mechanism prohibits inclusion of a detailed literature survey, but briefly stated, the proton motive force and electrostatic potential across the cell membrane are outcomes of cellular energy expenditure to maintain a constant internal environment. Low external pH creates a large driving force for proton transfer across the membrane and requires the cell to expend energy to transport protons back across. One consequence of a lowering of the intracellular pH is that acids produced by the normal metabolic cycle remain in an undissociated form and cannot be transported out of the cell (Thauer, *et al.*, 1977), thus causing

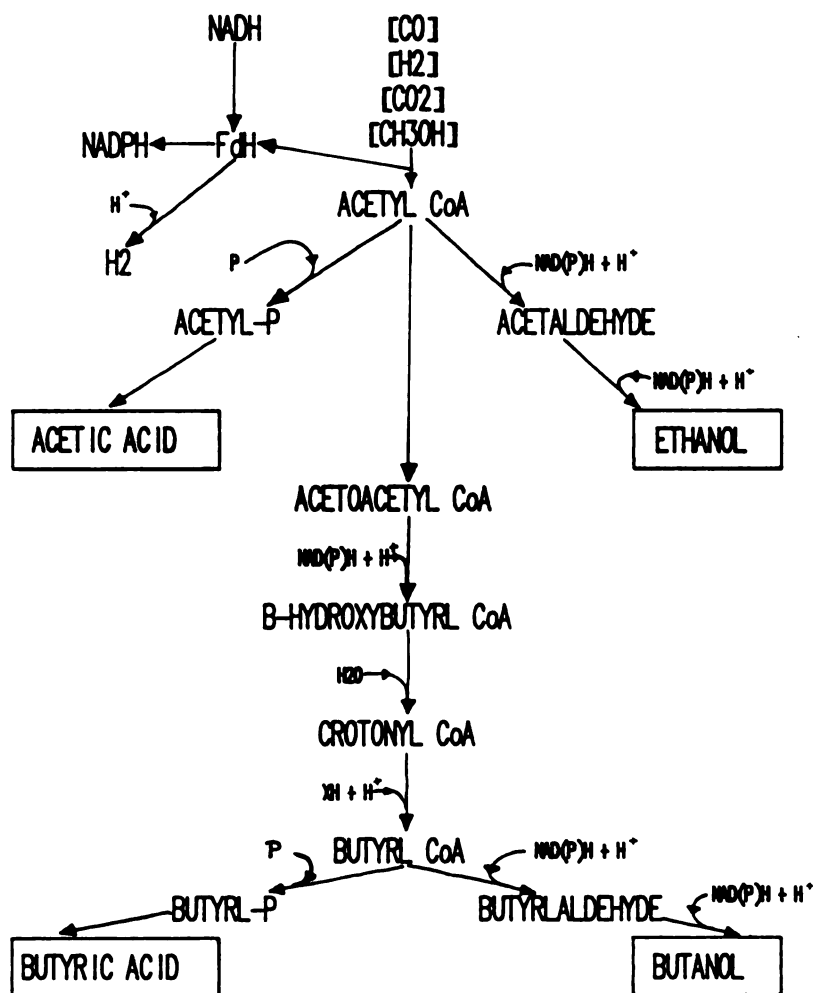


Figure 16: Speculative biochemical pathway for C1 metabolism in *Butyribacterium methylotrophicum*.

increased internal acidification. To counteract the potential consequences of a lowering of the internal pH from a high external driving force, production of neutral alcohols and weakly dissociating acids is initiated to circumvent a deleterious decrease in the intracellular pH. This effect has been investigated and characterized most notably in *C. acetobutylicum* (Huang, *et al.*, 1985; Huang, *et al.*, 1986) where production of neutral solvents is orders of magnitude greater than that of *B. methylotrophicum*. Nevertheless, the principle applies equally in both systems.

4. Significance of Enzymatic Results

The limited nature of the intracellular enzyme studies precludes any conclusive statements concerning enzymatic pH regulation of 1-carbon metabolism in *Butyribacterium methylotrophicum* except that an enzyme directly involved in the butyrate synthesizing pathway was found to exhibit increased activity at low pH. A detailed analysis of the acid kinases, phosphotransferases, and perhaps an NADH linked oxidoreductase during a batch fermentation time course without pH control would most likely provide the same type of information but with a much clearer picture of the pH effect on enzyme activity. The results presented above indicate that fermentation pH is definitely a factor in the metabolic dynamics of acid production. The question remains as to how great and under what circumstances the activity is regulated. Since the low productivities of both acetate and butyrate and the even lower rates of alcohol formation are two critical factors requiring improvement, a detailed biochemical analysis is probably not warranted with the present state of the bioconversion technology.

CHAPTER VI

CONCLUSIONS AND RECOMMENDATIONS

The potential for carbon monoxide bioconversion by *Butyribacterium methylotrophicum* was investigated in three parts in this study, with the overall setting of the work based on the MBI indirect liquefaction process for coal derived synthesis gas. Batch fermentations, development and operation of a several continuous fermentations, and some enzymatic studies were all conducted with identification of key metabolic and process variables as the primary goal. Within this framework of experiments, a logical determination for further work can be established.

Several of the process objectives set forth in the technical review were addressed in detail during this study, and a few of the key developmental obstacles overcome. Batch culture carbon and electron flow was successfully manipulated, fundamental fermentation stoichiometries were established in batch for several combinations of 1-carbon substrates, and a first order mass transfer analysis was performed. A long-term, continuous fermentation was operated at steady-state under varying conditions affecting product mix and cell growth. Cell samples from this system were analyzed and two key acid kinase enzymes found to be directly affected by fermentation pH. Overall, the experimental plan was successful in studying both the basic cellular response of CO metabolism, as well as identifying some critical factors concerning the process scheme.

1. Overall Significance of the Study

The value of butyric acid production compared to acetic acid production from CO, in both the specific process setting and from a general economic standpoint, is the focal significance of this work. Long term batch fermentations showed conclusively that fermentation pH regulation of CO metabolism in *B. methylotrophicum* results in a dramatic shift from acetate to butyrate production. Although the mechanism is not fully understood, low pH induced formation of more reduced compounds is not a novel mechanism and has been observed in other anaerobic processes, most notably the acetone, butanol, and ethanol (ABE) fermentation catalyzed by *Clostridium acetobutylicum*. Minor production of alcohols is also consistent with this scheme. Furthermore, confirmation of the pH effect in continuous culture provides the first glimpse for a direct and continuous butyric acid producing fermentation with CO as the sole carbon and energy source. However, the metabolic nature of stationary phase butyrate production limits the overall productivity of such a process, with stationary phase cell growth at low pH being the critical factor. Such results indicate a crucial need for incorporation of a cell retention system to minimize loss of cell mass.

Production of alcohols directly from CO by *B. methylotrophicum* in both batch and continuous culture systems is scientifically and perhaps even industrially significant. Specifically, pure culture butanol production from carbon monoxide represents the first experimental evidence for the existence of a direct pathway for biological production of a 4-carbon alcohol from a 1-carbon inorganic substrate. At low pH, butanol and ethanol production in continuous culture were shown to contain a significant portion of the overall incoming energy. The

theoretical mechanism for the pH effect suggests that alcohol production should surpass acid production as the fermentation pH is lowered; thus, with proper engineering at pH values even lower than those examined in this study, a primarily solventogenic CO fermentation could potentially be realized. Achievement of such a system would have a direct impact on the Stage 2 of the indirect liquefaction process, perhaps leading to its exclusion.

The fundamental stoichiometric results from the CO/CO₂/CH₃OH fermentations indicate a more complicated system than was initially supposed. The linking of CO metabolism to CH₃OH metabolism thru CO₂ was conceptually sound but was experimentally determined not to be favored. Instead, acetate production was favored even at low pH from the combination of substrates. These results are, however, preliminary and not at all exclusive with respect to the overall concept.

The first order analysis of CO mass transfer in the laboratory fermentation systems indicated gas/liquid mass transfer limitations under the operating conditions. This result was anticipated; the gas flow and agitation rates chosen were quite low. Optimization of mass transfer rates beyond simple increases in these parameters is not a critical factor in the development of the process and therefore should not be emphasized. The magnitude and significance of the metabolic discoveries preempts such work.

The most remarkable aspect of the pH effect is its relative simplicity. In the improvement of any process those modifications that least complicate the system are preferable. Optimization and control of fermentation pH is technically simple, requires no extra equipment or processing steps, and is relatively inexpensive. This effect is very

advantageous to the process. Furthermore, the inhibitory effect of low pH on cell growth can be overcome with insightful engineering, as described in the pH-shift experiments. The batch, continuous, and enzymatic studies all show conclusively that this basic fermentation variable is of major significance in the metabolic regulation of the CO bioconversion process.

2. Recommended Future Work

The results of the fermentation studies reveal two critical areas of CO bioconversion requiring further research and development. As stipulated in the overall process objectives, and now supported experimentally, an efficient cell retention system will be essential during continuous operation for maintenance of high cell densities in the fermenter. Design and application of such a system is a major engineering task requiring both innovative technology and specialized equipment. The other area of importance is the conversion of CO/CH₃OH for reduction of overall carbon lost to CO₂. This scheme must be reevaluated and developed in light of the present findings. Both of these areas are briefly addressed below with respect to the experimental results obtained during this study.

2.1 Incorporation of a Cell Recycle System

The fermenter productivities for the continuous studies were very low as a result of the low dilution rate and also the low product concentrations. With the pH effect of increased reduced product formation and decreased cell mass formation, optimization of productivities for both cells and favorable fermentation products is counterproductive at low pH values. The overall fermenter productivity

depends on dilution rate, growth rate, production rates, and mass transfer rates for CO₂, all of which are related to the cell metabolism. The low cell densities obtained in continuous culture, particularly at low pH, directly contributed to the low productivity of the system. One method of circumventing this obstacle is through the application of a membrane-type cell recycle system.

In principle, such a cell recycle system operates as a porous membrane barrier thru which only certain sized molecules may pass. The effluent fermentation broth may thus be screened with the large cells and biomass particles retained on one side and a permeate containing the fermentation products on the other, as depicted in Figure 17. The retentate stream containing the cells is then recycled back to the fermenter. Overall dilution rates are based on the magnitude of the permeate stream. The effect is thus an uncoupling of the liquid throughput from the loss of cell mass. This effect will both increase the fermenter cell density as well as create conditions conducive for stationary phase growth.

Several obstacles exist towards development of such a system, although the outlook is promising. Blockage or fouling of the membrane is a key concern. At the high cell densities obtainable, a large proportion of the cells are not viable and thus aggravate the fouling problem during cell decomposition. Furthermore, typical microfiltration membranes require high tangential liquid flow rates to keep the membrane relatively clear of biomass; operation at these rates is limited by the growth rate of the system, which is quite low in this case.

Nevertheless, the potential of cell recycle technology is evident in

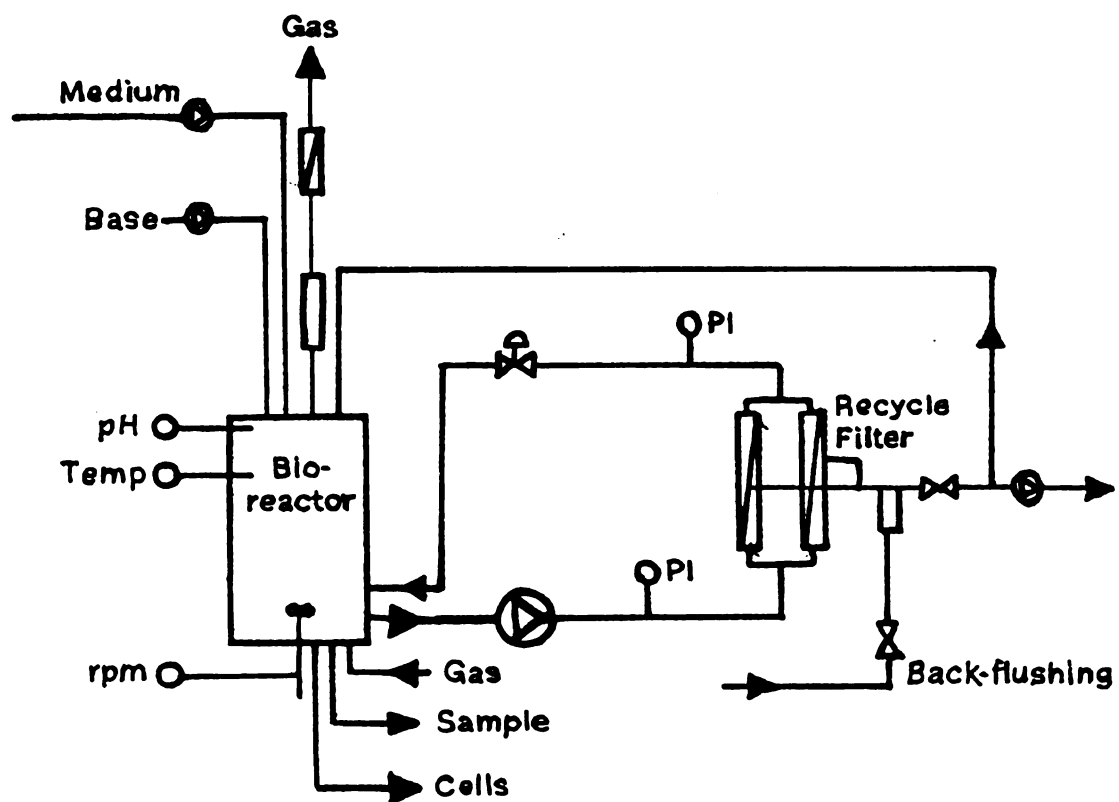


Figure 17: General schematic for proposed cell recycle system.

both the literature and in some preliminary tests performed with *B. methylotrophicum*. Fermentations with the wild type strain grown on glucose and using a relatively impractical plate and frame recycle system have attained cell densities as high as 4-5 g/L, with an approximately 50% viable population (J. S. Shieh, pers. comm.). Productivities were also increased proportionately. Tenfold increases in biomass and products have been observed with a more conventional cartridge-type assembly, using the anaerobic thermophile *Clostridium sulfurogenes*, grown also on glucose (Nipkow, unpublished results).

Incorporation of a cell recycle system into the CO bioconversion process will likely present unforeseen obstacles and results due to the very nature of the fermentation. However, the principles of the technology are proven and equipment is presently available for immediate testing and application.

2.2 Design of a Two-Stage CO/CH₃OH Fermentation

The use of both carbon monoxide and methanol as substrates for production of butyrate will require some further designing of the first stage of the indirect liquefaction process. Specifically, the preliminary results shown in batch culture indicate that co-consumption of CO and CH₃OH is not favorable for butyrate production, and should not be conducted in the same fermenter. The logical and simplest solution is to run two fermenters in series with CO as the substrate in the first and CH₃OH/CO₂ as the substrate in the second, with the CO₂ ultimately coming from the CO metabolism in the first fermenter. Molar consumption rates for both species indicate that the CO reaction will be rate-limiting in any two stage fermentation. Although simple in concept, application in continuous culture and with steady-state operation will

be a significant objective. Furthermore, in theory such a system would require complete CO consumption in order to achieve a relatively pure CO₂ stream leaving the first fermenter, a rather unlikely prospect considering the present system.

2.3. Preliminary Economic Analysis

Guidelines for any potential process should be based on at least a tenable economic foundation. Although bioconversion of coal derived synthesis gas is exploratory and not presently industrially relevant, a preliminary economic analysis can reveal critical aspects of the process that are not apparent in the laboratory. With the available data, a first-order analysis of CO bioconversion will reveal where improvements must be made, whether in cell productivity, rate of CO consumption, concentration of volatile products, or some other key area. A simple economic model based on available data and sound engineering judgement will prove invaluable both in answering pertinent questions related to potential application as well as guiding the direction of future work.

3. Summary Statement

Production of mixed organic acids and alcohols has been achieved directly from CO in both batch and continuous culture with *Butyribacterium methylotrophicum*. These results address several of the process goals for indirect liquefaction of coal-derived synthesis gas, including production of butyrate as a primary product from CO metabolism, continuous production of butanol during steady-state operation, and identification of fermentation pH as a controlling variable in 1-carbon metabolism. Preliminary results concerning the mass transfer environment for CO utilization and the enzymatic basis for the

pH effect were also obtained. With the experimental results and appropriate analysis, several key determinations regarding the direction of future work were made, in light of the overall process objectives.

APPENDIX

APPENDIX

1. Review of the Carbon and Electron Balancing Method

The method of carbon and electron balancing for determination of biological reaction stoichiometries is a powerful tool when data for consumption/production of one or two species is unavailable. These balances also serve to calculate percent carbon and electron recovery when data for all species is available. Carbon balancing, as detailed below, is simply a material balance on the reaction based on the moles of carbon atoms reacted. Electron balancing is a balance on the available electron equivalents and is analogous to an energy balance on the biological reaction.

Specifically, electron balancing is derived from molecular energy changes associated with changes in oxidation number due to electron transfer between species. Atomic oxidation number is characteristic of the valance electron(s) of the atom which stoichiometrically guide the reaction and for carbon containing compounds can assume several values. The energy content of carbon molecules is related to the oxidation number, with highly reduced molecules being energy rich. Changes in oxidation number arise from electron transfer between species, often coupled to redox reactions involving electron carriers such as NADH.

Within this setting, the reductance degree of a compound is defined as the number of equivalents of electrons available for transfer in oxidative catabolism, and is established on a basis of one gram atom carbon. For atoms, several reductance degrees are



and thus for several compounds can be calculated as



and *ad infinitum*. Reductance degree therefore forms a basis for energy balancing biological reactions where the total electrons supplied by the reactants (substrates) must equal the total electrons obtained by the products. The material balance for carbon is also achieved in this fashion.

As a specific example related to calculations made during this study, the carbon and electron balances for a 100% CO batch fermentation will be illustrated. Fermentation data for such an experiment include concentrations of acetic acid and butyric acid but no data is obtained for CO consumed and CO₂ produced. For this anaerobic reaction, a likely stoichiometry is of the form



where the lower case letters represent total moles of each species with cell mass expressed with a molecular formula based on one gram mole carbon. If this general reaction is balanced with respect to available electrons, with the Cell Mass term having a value of 4.21, the resulting equation is

$$a(2) + b(0) + c(0) \rightarrow d(0) + e(8) + f(20) + g(4.21)$$

which yields

$$a(2) \rightarrow e(8) + f(20) + g(4.21).$$

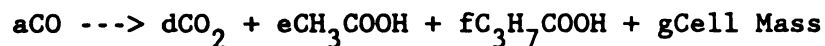
The data for total acetate, butyrate, and cell mass is now converted to total moles and inserted in this equation,

$$a(2) \rightarrow 0.10(8) + 0.0031(20) + 0.015(4.21)$$

and the value for *a*, the total moles CO consumed, calculated. This value is

$$a = 0.4624 \text{ moles CO consumed.}$$

If the reaction is now balanced with respect to carbon, the equation is of the form



which upon insertion of the total carbon atoms in each molecular formula yields

$$a(1) \rightarrow d(1) + e(2) + f(4) + g(1).$$

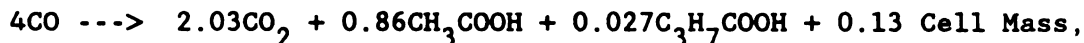
Again the product data, including the calculated value for moles CO consumed, is inserted and now the value of b, the total moles CO₂ produced, calculated from

$$.4624(1) \rightarrow d(1) + 0.10(2) + 0.0031(4) + 0.015(1)$$

which gives

$$b = .2350 \text{ moles CO}_2 \text{ produced.}$$

The overall carbon and electron balance for this fermentation can now be normalized to an arbitrary amount of substrate, in this case 4 moles of CO was chosen. The resulting final balance is thus



which is a representative balance derived for a 100% CO batch fermentation.

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