Date: November 2, 1988

TITLE: Biotechnology of Indirect Liquefaction:

PIs (Authors): Rathin Datta, Mahendra Jain, Andy Grethlein, and Mark Worden

CONTRACT NO: DE-AC22-88PC79815

PERIOD: April 1, 1988 to October 1, 1988

OBJECTIVE

Biological synthesis of liquid fuels from coal derived gases and methanol has the potential for developing processes which are highly selective and efficient. Large quantities of one-carbon feedstocks such as carbon monoxide (CO), hydrogen (H_2), carbon dioxide (CO_2), and methanol can be available from fossil fuels such as coal and natural gas.

This project on indirect liquefaction of coal derived synthesis gas is focused on two stage anaerobic bioconversion. The first stage addresses bioconversion of synthesis gas (CO, H_2/CO_2) to volatile fatty acids by a CO adapted strain of Butyribacterium methylotrophicum. The second stage involves bioconversion of fatty acids to combustible solvents-acetone, butanol, and ethanol by a strain of Clostridium acetobutylicum. The acidogenic bacteria can convert CO and/or methanol directly to acetate and butyrate without consuming H_2 from syngas. The required H_2 comes from water and the shift reaction is not necessary. The hydrogen from syngas which passes through the first bioreactor can be used for conversion of fatty acids to solvents in the second bioreactor. These organisms, B. methylotrophicum and C. acetobutylicum, offer the best potential because of their demonstrated performance as reported in the literature (1-4). During this project period, our primary objectives for the acidogenic stage were to investigate factors affecting carbon and electron flow in B. methylotrophicum and development of a chemostat fermentation for this bacteria. For the solventogenic stage, the main objective was development of an improved strain for achieving increased solvent yields and productivity.

TECHNICAL APPROACH

Acidogenic conversion of carbon monoxide by B. methylotrophicum was investigated in both batch and continuous culture fermentations, with emphasis on carbon pathway manipulation to maximize butyric acid production. Previous studies without pH control had shown low levels of butyric acid production and culture pH-drop to be linked during stationary phase growth. Thus, our primary approach

was to analyze the effect of lower broth pH on the carbon monoxide fermentation. This was conducted in batch culture, with a high degree of success. Once identified as a critical parameter influencing the carbon and electron partitioning in B. methylotrophicum, fermentation pH was studied in continuous culture to determine its influence on steady state production of organic acids. Establishing conditions for steady state production was paramount for these experiments.

For conversion of the acids to solvents, wild-type sporeforming strain (ATCC 4259) of C. acetobutylicum was revived from the soil stocks. Carbohydrate fermentation by this bacteria was studied in a 2-liter fermenter (New Brunswick) to confirm its typical biphasic fermentation. The wild-type then was mutagenized to obtain improved mutant strain of this bacteria to obtain high solvent concentration, yield and productivity. Batch fermentation of improved mutant was conducted to compare the solvent concentration, yield and productivity with the parent (wildtype) strain of C. acetobutylicum.

SIGNIFICANT ACCOMPLISHMENTS

1. Batch Fermentation

An extended-batch fermentation with continuous CO sparging was conducted at a pH of 6.8. The results are shown in Figure 1. Trends in the growth and product formation curves are consistent with those obtained in both batch culture (5) and in fed-batch culture with intermittent CO addition (6). CO assimilation led to cell growth, and acetate was the primary reduced end product. No butyrate production was observed in batch culture (5), and only small amounts of butyrate were detected in fed-batch culture (6) and in the present study. The initial specific growth rate was approximately 0.05 hr^{-1}.

Carbon and electron balances were used to calculate the amount of CO consumed from product-formation data. These balances were found by Lynd et al. (5) to be within 3% for batch CO fermentations using B. methylotrophicum. The overall carbon balance determined for the data in Figure 1 is given below.

4 CO → 2.04 CO_2 + 0.86 Ac + 0.024 Bu + 0.14 C-mole cells

where CO_2, Ac, and Bu are the molar yields of carbon dioxide, acetate, and butyrate, respectively. One C-mole is the quantity of cell mass containing 12 grams of carbon. For B. methylotrophicum, 1 C-mole equals 26 g (dry) cells (5).

Acetate production occurs during both the growth and the stationary phase, and can be adequately described using the Leudeking-Piret model (7):

$$\frac{dP}{dt} = \alpha \frac{dX}{dt} + \beta X,$$

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

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

$$\beta = 1.7 \times 10^{-3} \text{ g acetate/g cells·hr}$$

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

1.1 pH-Shift Batch Fermentation

Figure 2 shows the results of an extended-batch fermentation designed to investigate the effect of pH on stationary-phase butyrate production. B. methylotrophicum cells were grown as in the first experiment until the cell concentration reached a value of 0.31 g/L. At this time, the setpoint of the pH controller was changed from 6.8 to 6.0. The resulting pH shift led to a dramatic increase in butyrate production relative to the constant-pH experiment. A final butyrate concentration of 6 g/L was obtained. The overall carbon balance is shown below.

$$4 \text{ CO} \rightarrow 2.30 \text{ CO}_2 + 0.26 \text{ Ac} + 0.27 \text{ Bu} + 0.055 \text{ C-mole cells}$$

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

$$4 \text{ CO} \rightarrow 2.05 \text{ CO}_2 + 0.71 \text{ Ac} + 0.02 \text{ Bu} + 0.43 \text{ C-mole cells},$$

and the carbon balance during the stationary phase is

$$4 \text{ CO} \rightarrow 2.31 \text{ CO}_2 + 0.21 \text{ Ac} + 0.31 \text{ Bu} + 0.009 \text{ C-mole cells}.$$

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

$$4 \text{ CO} \rightarrow 2.02 \text{ CO}_2 + 0.80 \text{ Ac} + 0.37 \text{ C-mole cells}$$

for the growth phase and

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

for the stationary phase.

Thus, by decreasing the pH from 6.8 to 6.0 at the onset of the stationary phase, the metabolism B. methylotrophicum can be shifted from acetate to butyrate production. The flow of available electrons from CO to the various products is shown in Table 1. Since heats of combustion are directly proportional to the available electron content for a wide variety of biochemicals (8), Table 1 can also be interpreted as the relative chemical energy content of the products.

These results are very important and unique because they show that by a simple shifting of the pH the metabolic regulation of the organism can be changed dramatically, and this shift enables one to produce a 4 carbon organic acid (butyric acid) directly from gaseous CO.

2. Continuous Culture Studies

With the important and unique results obtained from batch culture indicating fermentation pH as a direct regulator of the C-1 to C-4 metabolic pathway, the next goal of the project 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. Steady state operation was defined as deviations of not more than 10% of the mean cell density value over a period of three 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 valued defined by this criteria.

All continuous studies were performed strictly under the same conditions with fermentation pH as the variable parameter. Thus direct correlations can be drawn between all experiments relating pH to product mix and concentrations. Analytical techniques were performed as outlined previously. Daily microscopic observation was conducted to insure pure, non-contaminated culture conditions.

2.1 Baseline Fermentation (pH = 6.8)

An initial continuous fermentation of 100% carbon monoxide using Butyribacterium methylotrophicum was conducted for three weeks with approximately ten days of steady state operation at pH = 6.8. Conditions were continuous CO gassing at a rate of 50 mL/min, a temperature

of 37 C, an agitation rate of 50 rpm, and a dilution rate of .015 hr in a 1.25L working volume. Similar conditions in batch culture described previously favor acetate production with a corresponding acetate/butyrate ratio (molar) of 30:1. In continuous culture, these conditions result in production of acetate and butyrate at .88 and .11g/L, respectively, yielding a molar product ratio of approximately 8.3:1. Ethanol production was also observed in lower (<.05g/L) quantities. Carbon and electron balance calculations give an overall stoichiometry of

4 CO → 2.09 CO_2 + 0.63 Ac + 0.043 Bu + 0.027 EtOH + 0.43 Cells

These values allow calculation of some key fermentation concepts--mainly electron/carbon partitioning and molar yield coefficients. Molar yield coefficients, defined as moles product produced divided by the moles substrate consumed, are helpful in following the overall conversion of substrate to multiple products. For the above products from CO the values are

$$Y_{CO2/CO} = .522 \quad Y_{AC/CO} = .158 \quad Y_{BU/CO} = .011$$

$$Y_{EtOH/CO} = .007 \quad Y_{Cell\ m/CO} = 108$$

electron partitioning, which corresponds to the distribution of the total chemical energy content of the substrate CO, is

100% CO → 63% Ac + 11% Bu + 4% EtOH + 22% Cells

Carbon partitioning, or total carbon distribution from CO to products, can be calculated as

100% CO → 52% CO_2 + 32% Ac + 4% Bu + 1.5% EtOH + 10.5% Cells

When analyzed together, these values present some interesting and valuable information for subsequent comparison at lower pH:

1. As determined in batch culture, CO_2 generation results in a loss of approximately half of the incoming carbon.

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, this pH-induced metabolic shift is expected to become more pronounced, until cell washout begins to influence the detectable product mix.

2.2. pH vs. Product Formation Fermentations

Once conditions for continuous culture were established as outlined above, experiments based on fermentation pH could be conducted and compared to the baseline pH = 6.8 fermentation. Data was collected during fermentation at steady state conditions for broth pH values of 6.5, 6.0, and 5.5. Operation was then attempted at pH = 5.0 but was discontinued due to cell washout. The results for these experiments have been combined with the baseline fermentation and presented in the form of overall stoichiometry, electron partitioning, carbon partitioning, and molar yield coefficients in Tables 2-5, respectively. Also included in Table 6 are molar (weight) product ratios, specific product weight ratios based on grams product per gram cell in Table 7, and in Table 8, the broth product concentrations in g/l.

Once again, as was observed in batch culture, there is a significant relationship between the fermentation broth pH and the product selectivity, now confirmed in continuous culture. Several important conclusions can be drawn from these data accumulated in Tables 2-8. First, and of prime importance, is the trend towards more reduced product formation, mainly butyrate and alcohols, as the pH of the fermentation is reduced. This trend parallels what has been observed in batch fermentation, and is clearly shown in all the Tables. It must be stated, however, that although the pH = 5.5 data does not support this trend entirely, we believe this to be an effect of the dilution and not directly of the pH drop. This was concluded from an examination of the data in Table 8, which shows a significant drop in the cell density (concentration) between pH = 6.0 and 5.5. If the dilution rate, which was maintained constant in all of the fermentations, was lowered in order to maintain a constant cell density, it is thus logical that all the fermentation products would be concentrated and the true metabolic stoichiometry revealed. Therefore, the small shift back towards less reduced products observed at pH = 5.5 is due not to the pH effect, but to the experimental design in which dilution rate, instead of cell density, was held constant.

Another highly significant discovery shown by these data is the production of alcohols, specifically the 4-carbon butanol, solely from the one carbon substrate CO, observed at pH = 6.0. Shown in Table 7, butanol, when normalized to the cell density, is produced by weight in greater amounts than the 2-carbon ethanol, and as seen in Table 3, contains a significant portion of the available energy content from CO. This indicates a direct metabolic pathway in B. methylotrophicum to convert gaseous carbon monoxide directly to butanol.

Also observed, as was expected, is the drop in cell production as the pH decreases below 6.0, shown in Table 8. As mentioned previously, washout was observed at pH = 5.0. Figure 1 illustrates this phenomena more clearly. Regarding CO_2

production, Tables 2, 4, and 5 indicate that carbon dioxide evolution is not significantly affected by pH, and thus reducing the carbon lost to CO_2 cannot be overcome by manipulating this parameter.

3. Batch Fermentation and Mutagenesis of C. acetobutylicum

Batch fermentation of carbohydrates by the wildtype strain of solventogen, C. acetobutylicum, was conducted and the results are presented in Figure 3. A typical biphasic (acidogenic followed by solventogenic) fermentation occurred after an initial lag period. The respective butanol concentration and yield, 11.6 g/l butanol and 21.6% (from dextrose), achieved were typical for this strain.

In order to improve the culture with regard to productivity, solvent concentration and yield, the wildtype strain was mutagenized using nitrosoguanidine (NTG) and ethyl methane sulfonate (EMS). The concentration of NTG and EMS used were 100 ug/ml and 2% V/V, respectively. Mutants obtained with either NTG (Table 9) or EMS (Table 10) varied greatly in terms of substrate consumption, solvents concentration and yield. They also varied in sporulation ability. Of the mutants isolated mutant N306, N604, E604 and E610 were much better either in butanol concentration, butanol yield or both (Tables 9 and 10). Interestingly, sporulation ability in these mutants was affected. While only < 10% cells were observed sporulating (weak sporulation) in mutants N306, N604 and E604 in comparison to wildtype, no sporulation was detected in mutant E610. This indicated that sporulation character could be used to select possibly useful mutant.

Mutant E604 was further compared with wildtype. A 2-liter batch fermentation of carbohydrate (Figure 4) showed that the mutant strain had fast fermentation rate and butanol was produced in higher concentration than wildtype. The comparison data presented in Table 11 indicates that mutant E604 had substrate consumption (1.45 vs 0.79 g/l -hr) and productivity rates (0.33 vs 0.17 g/l -hr) much higher than wildtype. Mutant strain also produced a higher butanol concentration (13.0 vs 10.6 g/l) in fermentation broth and resulted in higher butanol yield (22.7 vs 21.3 wt%) when compared with wildtype.

SUMMARY AND FUTURE WORK

The batch and continuous fermentation studies conducted in our laboratories have uncovered several significant features of this first stage of the indirect liquefaction process. In batch culture it was determined that fermentation pH is a direct mediator of carbon and electron flow in B. methylotrophicum, with more reduced products being formed at lower pH. It was also observed that acetate production occurred throughout the growth phase, while butyric acid and alcohol production were linked to the stationary phase only. Steady state operation was

achieved in continuous culture, and when the effect of fermentation pH was examined it was determined that at low pH the same metabolic shift is present, and thus production of organic acids and alcohols can be achieved with one carbon compound as the sole carbon and energy source in continuous culture. Of major significance is the direct production of butanol from CO, quantified at low pH.

With the increase in reduced product formation and decrease in cell production at low pH this process is an ideal candidate for integration of a cell recycle system to maintain a high cell density in the fermenter. This will allow much higher cell concentrations, and thus overall fermenter productivities, while still retaining the benefits in terms of product yields of operation at low pH. Construction and testing of such a system is currently underway.

For the solventogenic fermentations, an improved mutant of C. acetobutylicum has been obtained. This strain will now be used for further studies with regard to organic acids uptake, solvent tolerance etc. The uptake of organic acids will be maximized, and a process with cell recycle will be developed finally for obtaining higher solvent yield.

PUBLICATIONS

1. Worden, R. M., A. J. Grethlein, J. G. Zeikus, and R. Datta. Butyrate production from carbon monoxide by Butyribacterium methylotrophicum. Proc. Tenth Symp. on Biotechnology for Fuels and Chemicals, Gatlinburg, 1988.

REFERENCES

1. Zeikus, J. G., R. Kerby, and J. A. Krzycki. Science 227:1167, 1985.

2. Datta, R and J. G. Zeikus. Appl. Environ. Microbiol. 49:522, 1985.

3. Kim, B. H. and J. G. Zeikus. Develop. Indus. Microbiol. 43:549, 1985.

4. Lovitt, R. W., B. H. Kim, G. J. Shen and J. G. Zeikus. Crit. Rev. Biotechnol. 7:107, 1988.

5. Lynd, L., R. Kerby, and J. G. Zeikus. J. Bacteriol. 149:255, 1982.

6. Datta, R.. Biotechnol. and Bioeng. Symp. Ser. 12:177, 1982.

7. Laudeking, R. and E. L. Piret. J. Biochem. Microbiol. Technol. Eng. 1:393, 1959.

8. Erickson, L. E., I. G. Minkevich, and V. K. Eroshin. Biotechnol. and Bioeng. 20:1595, 1978.

Table 1. Percent of Available Electrons Contained in Products

Fermentation Conditions	Acetate	Butyrate	Cell Mass
Constant pH, Growth Phase	80	0	20
Constant pH, Stationary Phase	88	8	4
Constant pH, Overall	86	6	8
pH Shift, Growth Phase	71	5	24
pH Shift, Stationary Phase	21	78	1
pH Shift, Overall	27	70	3

Table 2. Fermentation Stoichiometry of CO in B. methylotrophicum in relation to pH

pH	Fermentation Stoichiometry
6.8	$4CO \rightarrow 2.09CO_2 + .63Ac + .043Bu + .027EtOH + .43Cells$
6.5	$4CO \rightarrow 2.13CO_2 + .56Ac + .082Bu + .026EtOH + .37Cells$
6.0	$4CO \rightarrow 2.27CO_2 + .303Ac + .161Bu + .032EtOH + .029BuOH + .31Cells$
5.5	$4CO \rightarrow 2.18CO_2 + .402Ac + .154Bu + .40Cells$

Table 3: Electron Partitioning during pH-Dependent CO Fermentation by B. methylotrophicum

pH	% Electrons				
pH	Acetate	Butyrate	EtOH	BuOH	Cells
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 4: Carbon Partitioning During pH-Dependent CO Fermentation by *B. methylotrophicum*

pH	% Carbon					
	CO_2	Acetate	Butyrate	EtOH	BuOH	Cells
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 5: Molar Yield Coefficients During pH-Dependent CO Fermentation by *B. methylotrophicum*

pH	CO_2	Acetate	Butyrate	EtOH	BuOH	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 6: Product Ratios During pH-Dependent CO Fermentation by *B. methylotrophicum*

pH	Ac/Bu	Ac/EtOH	Ac/BuOH
6.8	14.7(10)*	23.4(30.7)	-
6.5	6.83(4.65)	22.0(28.5)	Trace
6.0	1.88(1.28)	9.5(12.3)	10.5(8.51)
5.5	2.61(1.79)	Trace	Trace

*Product ratios on molar (weight basis)

Table 7: Specific Product Weight Ratios During pH-Dependent CO Fermentation by B. methylotrophicum

pH	Acetate	Butyrate	EtOH	BuOH
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

Table 8: Product Concentrations in Fermentation Broth During pH Dependent Co Fermentations by B. methylotrophicum

pH	Acetate	Butyrate	EtoH	BuOH	Cells
6.8	.860	.086	.028	--	.248
6.5	1.055	.227	.037	Trace	.284
6.0	.689	.536	.056	.081	.286
5.5	.475	.266	Trace	Trace	.196

Table 9: Analysis of Mutants of Clostridium acetobutylicum Obtained After NTG Mutagenesis

Strain	Sporulation	Starch Utilization (g/l)	Acid Acetic (g/l)	Acid Butyric (g/l)	Acetone	Solvent Ethanol (g/l)	Butanol	Butanol Yield (%)
WT	S	33.0	1.7	0.0	2.4	t	7.3	22.1
N303	S	38.6	1.4	0.0	2.1	t	7.5	19.4
N306	WS	42.2	1.0	0.0	2.4	0.9	9.1	21.6
N308	WS	26.3	2.0	0.7	2.5	t	7.5	20.7
N310	S	32.9	2.1	0.8	2.3	t	6.9	21.0
N604	WS	40.5	1.1	0.0	2.6	0.8	8.8	21.7
N607	S	32.3	2.0	1.2	2.1	t	6.3	19.5

Starch as dextrose; N301 - NTG, 3 for 30' treatment, 01 as mutant number
S = spore former; WS = weak spore former; NX = non-spore former

Table 10: Analysis of Mutants of Clostridium acetobutylicum Obtained After EMS Mutagenesis

Strain	Sporulation	Starch Utilization (g/l)	Acid Acetic (g/l)	Acid Butyric (g/l)	Acetone	Solvent Ethanol (g/l)	Butanol	Butanol Yield (%)
WT	S	33.0	1.7	0.0	2.4	t	7.3	22.1
E301	S	2.3	0.4	0.7	0.0	0.0	0.0	--
E303	S	3.9	0.4	0.8	0.0	0.0	0.0	--
E305	S		0.4	0.7	0.0	0.0	0.0	--
E306	S		0.4	0.7	0.0	0.0	0.0	--
E308	S	2.4	0.3	0.7	0.0	0.0	0.0	—
E309	S	39.9	1.4	0.0	2.1	0.7	7.0	17.5
E601	S	34.3	1.5	0.4	2.0	t	6.4	18.7
E602	WS	38.0	1.1	0.0	2.3	t	7.8	20.5
E604	WS	34.2	1.9	0.4	2.6	t	8.1	23.7
E606	S	43.3	0.8	0.0	2.4	1.0	7.9	18.2
E610	NS	46.2	1.2	0.0	2.6	0.8	9.2	19.9

Table 11: Comparison of Wild Type and Mutant of *C. acetobutylicum* (Batch Fermentation)

Strain	Substrate Consumption Rate	Actual Final Butanol g/l	Butanol Productivity g/l-hr	Butanol Yield (wtT%)
Wildtype	0.75	10.6	0.17	21.30
E604	1.45	13.0	0.33	22.70

AUTHORS INDEX

L

M

N

P

R

★ U.S. GOVERNMENT PRINTING OFFICE: 1990-0-11-259

Reproduced by NTIS

National Technical Information Service
Springfield, VA 22161

This report was printed specifically for your order from nearly 3 million titles available in our collection.

For economy and efficiency, NTIS does not maintain stock of its vast collection of technical reports. Rather, most documents are printed for each order. Documents that are not in electronic format are reproduced from master archival copies and are the best possible reproductions available. If you have any questions concerning this document or any order you have placed with NTIS, please call our Customer Service Department at (703) 605-6050.

About NTIS

NTIS collects scientific, technical, engineering, and business related information — then organizes, maintains, and disseminates that information in a variety of formats — from microfiche to online services. The NTIS collection of nearly 3 million titles includes reports describing research conducted or sponsored by federal agencies and their contractors; statistical and business information; U.S. military publications; multimedia/training products; computer software and electronic databases developed by federal agencies; training tools; and technical reports prepared by research organizations worldwide. Approximately 100,000 *new* titles are added and indexed into the NTIS collection annually.

For more information about NTIS products and services, call NTIS at 1-800-553-NTIS (6847) or (703) 605-6000 and request the free *NTIS Products Catalog,* PR-827LPG, or visit the NTIS Web site **http://www.ntis.gov**.

NTIS
Your indispensable resource for government-sponsored information—U.S. and worldwide

U.S. DEPARTMENT OF COMMERCE
Technology Administration
National Technical Information Service
Springfield, VA 22161 (703) 605-6000

PE99009039

BX

BIN: M35 08-1*-99
INVOICE: 79651*
SHIPTO: 1*509337
PAYMENT: NONE