DEGRADATION OF CELLULOSIC BIOMASS AND ITS
SUBSEQUENT UTILIZATION FOR THE PRODUCTION
OF CHEMICAL FEEDSTOCKS

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Scope of Program

This is a coordinated program to effect the microbiological degradation of cellulosic biomass and the subsequent utilization of the degradation products to produce chemical feedstocks. The microbiological aspects of cellulosic biomass degradation will focus on the use of anaerobic microorganisms which possess cellulolytic enzymes. These studies will attempt to increase the enzyme levels through genetics, mutation and strain selection. In addition, the direct use of these cellulolytic anaerobes to produce soluble products (sugars) which can then be utilized by other microorganisms to produce chemicals is also within the scope of this program. Engineering scale-up of these microbial processes is planned once the basic microbiological parameters are defined.

The second area of our major effort is devoted to the production of chemical feedstocks. In particular, three fermentations have been identified for exploration. These are: acrylic acid, acetone/butanol and acetic acid. The main efforts in these fermentations will address means for the reduction of the cost of manufacturing for these large volume chemicals.

ABSTRACT

The production of reducing sugars and other products by Clostridium thermocellum on cellulosic biomass continues to show promise. The rate of reducing sugar production using corn residue has been shown to be equal if not greater than on solka floc. In addition to being able to produce reducing sugars, ethanol and acetic acid have again been confirmed to be produced at reasonable rates by this organism on corn residue. The methodology for examination of sugar accumulation via HPLC analysis has been developed. Considerable effort was devoted to the elimination of interfering materials present in the hydrolyzates. Glucose has been shown to be the major accumulation product, along with xylose and cellobiose. Current work is being devoted towards elucidating discrepancies between reducing sugar analysis and HPLC sugar analysis in order to permit accurate material balances to be completed.

Using an enzyme preparation freed from soluble sugar by ethanol precipitation, we have shown that the chromogenic TNP-CMCase assay correlates well with CMCase activity as measured by reducing sugar liberation. Very high levels of glucose (300 g/l) and xylose (300 g/l) and cellobiose (100 g/l) showed no inhibition of TNP-CMCase activity. Methylcellulose, an inhibitor of many cellulases, did not inhibit the C. thermocellum cellulase. Attempts to isolate hyperproducing mutants of cellulase are underway.

The genetic manipulations section has concentrated in further characterizing the plasmids of C. thermocellum and in the development of protoplasts of the same microorganism. Techniques for nondestructive study of the plasmids have been developed. These techniques are used to determine the useful characteristics of these plasmids for gene cloning. Alkaline gradients have provided an estimate of the size and useful isolation procedure for one of the plasmids. In addition, a standard protocol for the production of protoplasts of C. thermocellum has been adopted.

A process and economic analysis for the production of 200 X 10⁶ pounds (90 X 10⁶ kilograms) per year of soluble reducing sugars from corn stover cellulose, using enzymes derived from Clostridium thermocellum has been designed.

The preliminary cost analysis indicates a total product cost of 8.99¢/lb (20.2¢/kg) with 62% of this cost attributable to the price of the cellulosic substrate used. The total capital investment was estimated to be \$10.0 X 10⁶.

Acrylic acid has been produced in resting cell preparation of Clostridium propionicum from both β -alanine and from propionic acid. These results are particularly exciting since this is the first time that acrylate production has been demonstrated. Using propionic acid, it is possible to achieve a 37% molar yield of acrylate at an initial rate of 0.22 gm acrylate/liter-hr; efforts to further improve yield and rate are underway using C. propionicum and some aerobic cultures.

Results from the conversion of corn stover hydrolyzates to lactic acid, a precursor to acrylic acid, have shown that up to 70% of the sugars produced are converted to lactic acid. This can be done directly in the cellulase hydrolysis water without doing any product separation prior to the lactate fermentation. Efforts are proceeding to improve the conversion yield and carry out the overall conversion of corn stover to acrylic acid in the same fermentor.

Our results on the production of acetone and butanol by Clostridium acetobutylicum has demonstrated the capability of our strain to produce mixed solvents in concentration and conversion similar to that achieved in industrial processes. Mixed solvent at 20 gm/l (butanol = 13 gm/l, acetone = 7 gm/l) has been achieved accompanied by a conversion efficiency of 0.32 gm solvent/gm glucose. Kinetic analyses show that the production of the solvents occurs through both growth association and non-growth association. This is very encouraging since the use of continuous recycle fermentation with resting cells might be employed for solvent production. Inhibition studies have shown that butanol (total inhibition at 17 gm/l) is the causative agent which prevents the accumulation of high concentration of the solvent. In situ extraction studies to overcome this problem have been initiated. A number of solvents have shown desirable extraction properties which do not cause the inhibition of growth. A process and economic analysis for the production of 26.2 X 10⁶ kg (57.6 X 10⁶ lb) of mixed solvent per year has been performed. The total capital investment was estimated to be \$20 X 10⁶. The direct manufacturing cost was

estimated to be 62.9¢/kg (28.6¢/lb) using molasses at \$53/ton as the substrate. The two main items were the raw materials which accounted for 57% and capital investment which accounted for 22% of the total manufacturing cost.

Research studies on the production of acetic acid by Clostridium thermoaceticum have focused on many areas. Detailed kinetic analyses show that low pH (H^+ ion) is the main reason for the decreased rate of growth and rate of acid production. The pH optimum was found to be about 7.0 and, at this value using pH control, high specific productivity can be attained (3 gm acetic/gm cell-Hr). From the kinetic analysis, it has been found that acetic acid formation is primarily associated with growth. Degradation products (reducing sugars) from corn residue by C. thermocellum have been used to produce acetic acid by C. thermoaceticum. Excellent conversion (0.89 gm acetic/gm reducing sugar consumed) of these products have been attained. A process and economic analysis for the production of 75.6×10^6 kg (166×10^6 lb) of acetic acid per year has been performed. A total capital investment of $\$22.9 \times 10^6$ was required. A direct manufacturing cost 10.98¢/lb (24.1¢/kg) of acetic acid was estimated which excluded the feedstock (sugar) cost. Of this total amount, 45% is associated with the utility cost primarily for product recovery, and 35% is associated with the capital investment. Process improvements for reduction of cost appear warranted.

CONTRIBUTORS OF PROGRAM

This program is under the coordination of Daniel I. C. Wang, Principal Investigator. The research and development within this program are directed by the following individuals. The percentage efforts of the Principal and Co-Principal Investigators on this project are shown along with the names of the different participants.

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This progress report acknowledges the contributions by the individuals listed above, as well as the research assistants, post doctoral research associates and technical assistants associated with this project.

I. MICROBIOLOGY OF CELLULOSE DEGRADATION AND CELLULASE PRODUCTION

A. Analysis of Soluble Sugars Derived from Cellulosic Biomass

1. Introduction

We have previously demonstrated that an accumulation of soluble reducing sugars occurs during fermentations of Clostridium thermocellum on cellulosic substrates. We have also shown that this accumulated material can be utilized by other organisms in subsequent fermentations. Until recently, our only means of measuring these soluble products has been the non-specific DNS assay for reducing sugars. We have recently begun to apply the methodology of HPLC (high pressure liquid chromatography) analysis so that we can quantitatively measure the different sugars that accumulate during the fermentation. This analysis will eventually be extended to the saccharification products resulting from post-fermentation hydrolysis of cellulose using C. thermocellum's extracellular cellulases.

Thus far, the chromatographic analysis has been hindered by the presence of unknown soluble materials derived from the original source of ligno-cellulose in our fermentation broths. These substances are detected by the differential refractometer (a very general detector used with our HPLC system)

and, because their column retention times are similar to those of the sugars, they render many of the chromatograms unreadable by virtue of poor peak resolution. Consequently, efforts have been directed toward perfecting a pretreatment clean-up procedure for the removal of interfering compounds from the broths prior to HPLC analysis. Although a completely satisfactory methodology has not yet been devised, some success has been attained through the use of mixed-bed ion exchange resins. The procedure still suffers from two drawbacks -- incomplete removal of interfering material and possible removal of some sugars. Work is in progress to further improve the clean-up. Nevertheless, preliminary analyses of sugars accumulating during fermentations on corn stovers have been performed and the results are presented here. Though the quantitation of xylose, glucose and cellobiose may be weak at this point, these early experiments do indicate that glucose is the major sugar accumulated during the fermentation.

2. Materials and Methods

a. Organism

Clostridium thermocellum ATCC 27405

b. Culture Conditions

C. thermocellum was grown at 60° in 500 ml anaerobic flasks containing 300 ml of CM3 medium (composition presented in Progress Report No. 1, 12/1/76-2/28/77) replacing MN300 cellulose with 0.9% finely powdered corn stovers. The yeast extract concentration was 0.2%. The inoculum cultures were grown on CM3 medium containing 0.9% solka floc. Throughout the growth period, samples were taken for HPLC analysis. Reducing sugars in these samples were measured using the DNS colorimetric reagent. Residual corn stovers were determined by the same method used to measure residual cellulose, described in Progress Report No. 1, page 3. Glucostat Reagent (Worthington Biochemicals) was used to measure glucose.

c. High Performance Liquid Chromatography (HPLC) Analysis

1. HPLC System

The HPLC system used consists of the following components obtained from Waters Associates, Milford, MA: (1) a model U6K universal liquid chromatograph injector, (2) a model 6000A solvent delivery system, (3) a Waters μ BONDAPAK column for carbohydrate analysis, (4) a model R401 differential refractometer, and (5) a Perkin-Elmer strip chart recorder. The

effluent used is a mixture of acetonitrile and water (80:20). The operating conditions for the chromatograms presented herein (unless otherwise noted) are: flow rate, 1.5 ml/min; operating pressure, 800 psi; attenuation, 1X; chart speed, 10 mm/min; temperature, ambient, injection volume, 25 μ l.

2. Preparation of Standards

Xylose, glucose, and cellobiose have been used to set up standard curves. Mixtures of the three sugars at concentrations between 0.25-1.5 g/l each are made with deionized water. Raffinose is included in each standard mixture (as it is in each sample) as an internal standard. The mixtures are then chromatographed under the same conditions as the unknown samples and calibration curves are constructed. The curves are linear with concentration both by peak height and area (measured manually). Concentrations in the samples are read directly from these curves, peak height being the preferred measurement. A chromatogram of a standard mixture at 1 g/l is shown in Figure I.A.1. Calibration curves are shown in Figure I.A.2 (peak height vs conc.) and Figure I.A.3 (peak area vs conc.).

3. Preparation of Samples Prior to HPLC Analysis

A variety of procedures have been tested for sample preparation; the results presented in this report use

FIGURE I.A.1: CARBOHYDRATE STANDARDS SEPARATED BY HPLC.
STANDARDS ARE ADDED AT 1 mg/ml

FIGURE 1.A.2: CALIBRATION CURVE FOR STANDARD CARBOHYDRATE RATES, PEAK
HEIGHT VS. CONCENTRATION

FIGURE I.A.3: CALIBRATION CURVE FOR STANDARD SUGAR, PEAK AREA VS. CONCENTRATION

the current best method and are derived from broth samples treated in the following manner: Whole broths are centrifuged at 12,000 rpm for 20 minutes. Two milliliters of supernatant are transferred to tubes containing 500 mg of a mixed bed ion exchange resin (Bio-Rad AG501-X8 consisting of equivalent amounts of AG50W-X8, H⁺ form cation exchanger and AG1-X8 OH⁻ form anion exchanger). To this mixture, 0.2 ml of a 11 mg/ml solution of raffinose is added such that the final concentration in the sample is 1 mg/ml. (An equal amount is added to each standard solution as mentioned in the previous section.) The raffinose serves as an internal standard whereby column retention times can be checked. It also serves as an indicator of any loss of sugars to the resin (assuming that all sugars are lost proportionally). The sample and resin are allowed to contact for 30 minutes at room temperature. The slurry is then transferred to an Amicon Centriflo membrane cone, having a molecular weight cut-off of 25,000. The membrane cone and its support are placed in a centrifuge tube and are spun at 900 xg for 30 minutes to effect ultrafiltration. This ultrafiltration step serves two purposes. It removes any large molecular weight compounds (proteins are of prime concern) that can interfere with column performance. It also produces a particulate-free ultrafiltrate that is ready for injection onto the HPLC column.

3. Results and Discussion

The pretreatment procedure described above was initially tested on one sample from a fermentation of C. thermocellum on 0.9% corn stovers taken at 43.5 hours after inoculation. The HPLC chromatograms of the sample before and after pretreatment are shown in Figure I.A.4. Since the clean-up appeared to be fairly satisfactory, samples from duplicate cultures on corn stovers (culture A and culture C) were subjected to the same pretreatment and were then chromatographed. The effectiveness of the procedure was variable, depending upon the amount of interfering substances initially present in the fermentation broths. Consequently, xylose, glucose and cellobiose could be quantitated for culture C; however, the presence of interfering material in the majority of samples from culture A made it impossible to quantitate cellobiose in all but two case. Otherwise, it was possible to quantitate xylose and glucose in this culture. Exemplary chromatograms are shown in the accompanying 3 figures, I.A.5 thru I.A.7. Figures I.A.5 and I.A.6 are chromatograms of samples from culture C taken at 32 and 56 hours respectively. Figure I.A.7 is a chromatogram of a sample from culture A taken at 43.5 hours. All samples were run under the same operating conditions (described in materials and methods) for HPLC analysis. It can be seen that xylose, glucose, and cellobiose are present in the three samples, with glucose as the major component. It should be noted that the raffinose

FIGURE I.A.4: SAMPLE 4, CULTURE C, BEFORE AND AFTER PRETREATMENT WITH AG501-X8 RESIN. HPLC ELUENT: CH_3CN : H_2O , 80:20; FLOW RATE: 1.5 ml/min; NOTE: ATTENUATION: 2X, NO INTERNAL STANDARD INCLUDED

FIGURE I.A.5: HPLC CHROMATOGRAM OF SUBMUTANT OF CULTURE V
TAKEN AT 32 HR

FIGURE I.A.6: HPLC CHROMATOGRAM OF SUPERNATANT OF CULTURE C TAKEN AT 56 HR.

FIGURE I.A.7: HPLC CHROMATOGRAM OF SUPERNATANT OF CULTURE A TAKEN AT 43.5 HR.

peak in all three chromatograms is similar in size to that shown in Figure I.A.1, the standard mixture of sugars (which was not contacted with the ion exchange resin). This indicates that the resin treatment is not causing any loss of raffinose and probably other sugar from the samples.

Using the standard curve shown in Figure I.A.2, the concentrations of xylose and glucose and, where possible, cellobiose were determined for each sample. The kinetic patterns for each culture are shown in Figure I.A.8 and I.A.9.

Shown in Figures I.A.8 and I.A.9 are the time profiles in cultures A and C respectively for total reducing sugars (measured by the DNS assay), glucose, xylose and cellobiose (as measured by HPLC analysis).

The kinetics of sugar accumulation and cellulose degradation of corn stovers is similar for each culture, though culture A had a slightly longer lag time. At the end of the fermentations, roughly 80% of the original corn stovers have been degraded. Since corn stovers are only 50% cellulose in composition, this indicates that C. thermocellum utilizes a fraction of the other materials (e.g. most likely the hemicellulose) present in the substrate. Though they will not be presented in this report, preliminary results show that C. thermocellum can grow on purified xylan, producing a measurable extracellular xylanase. Thus, it is plausible that a portion of the hemicellulose in corn stovers has been consumed by the organism.

FIGURE I.A.8: KINETICS OF SUGAR ACCUMULATION DURING GROWTH OF C. THERMOCELLUM CULTURE A ON CORN STOVERS

FIGURE I.A.9: KINETICS OF SUGAR ACCUMULATION BY CULTURE C DURING GROWTH ON CORN STOVER

The volumetric corn stover degradation rate for the period between 20 and 40 hrs for each culture is roughly 0.18 g/l-hr; this is slightly lower than the maximal degradation rate of 0.25 g/l hr seen of solka floc and MN300 cellulose.

It is obvious from these figures (Figure I.A.8 and I.A.9) that there is a large discrepancy between the measured total reducing sugars and the sum of the concentrations of glucose, xylose and cellobiose, determined by HPLC analysis (e.g. the final reducing sugar concentration for culture C is 2.5 g/l. For glucose, cellobiose and xylose, the final concentrations are 0.6 g/l, 0.45 g/l and ~0.1 g/l respectively). The rates at which the individual sugars accumulate are also different than measured for total reducing sugars.

Since there was the possibility that some of the sugars were removed by the pretreatment with AG501-X8 resin (although the internal standard would argue against this), a glucostat assay was performed in an attempt to determine the accuracy of the HPLC results for glucose. Unfortunately, it appeared that something in the samples (both before and after pretreatment) interfered with the glucostat reagent and it was impossible to quantitate glucose in this manner.

At this point, it is difficult to draw any conclusions with regard to the kinetics of a-cumulation of individual sugars during fermentations of C. thermocellum on corn stovers. The data do show that glucose is the major sugar accumulated.

This is consistent with C. thermocellum's inability to utilize glucose as a carbon source. What is particularly bothersome about the data is the discrepancy between the concentrations of total reducing sugars and the individual sugars analyzed by HPLC. The discrepancy may be attributed to (1) the presence of larger cellodextrins not analyzed by HPLC, but measured by the DNS assay, (2) the accumulation of some unknown material that is measured by the DNS assay, or (3) loss of individual sugars during the resin pretreatment, though there is no apparent loss of the added raffinose.

4. Future Work

Future work will be aimed at further development of the pretreatment procedure to obtain a reliable method for quantitative HPLC analysis of sugars. Other cellulosic substrates will be examined to determine whether the patterns of individual sugar accumulation from fermentations with C. thermocellum are similar to those observed for corn stovers. It is also planned that HPLC will be used in enzymatic hydrolysis studies. Throughout these studies, an effort will be made to clarify the discrepancy between reducing sugar assay (DNS) and chromatographic analysis.

B. Degradation of Cellulosic Biomass to Produce Soluble Sugars and Other Products

1. Introduction

The production of soluble sugars and other products from biomass by Clostridium thermocellum continues to show promise as an alternate means of cellulose degradation. Our research, using model systems such as solka floc, has shown that substantial accumulation of reducing sugars can be achieved. We have proceeded on the exploration of using more realistic biomass to see whether this approach might be useful for sugar production. In the past contract period, our primary efforts have focused on the use of corn residue as a biomass for degradation by C. thermocellum. The results from these studies will be presented.

2. Materials and Methods

The basic medium used for the growth of C. thermocellum has been reported in our previous Progress Reports (see Report No. COO-4198-1). The main difference is the cellulosic biomass which has been studied in our most recent studies. A ball-milled corn residue obtained from Dow Chemical Company (Midland, Michigan) was used for these studies.

The approximate composition of this corn residue was given to us as being 45% cellulose, 31.5% hemicellulose, and 14.4% lignins.

The ball-milled corn residue was found to be extremely small in its size distribution (20 to 80 microns). Due to the size of the particles, it was not possible to follow directly the quantitative rates of growth. Therefore, we have been using carbon dioxide evolution rate as an indirect monitor for growth of C. thermocellum on corn residue. Since all of the fermentation experiments have been conducted in 5-liter agitated fermentors, the use of an on-line, infrared CO₂ analyzer (Lira) through a purge stream of nitrogen provided the continuous assessment of carbon dioxide evolution rate. All other analytical procedures have been reported in the previous Progress Report.

3. Results and Discussion

a. CO₂ Evolution as Indicator of Growth

In order to assess whether the evolution of carbon dioxide can be used as an indicator of growth for C. thermocellum, a number of fermentations were conducted using cellobiose as the carbon source. Since this substrate is soluble, it was possible to determine the growth through optical

and gravimetric measurements. A typical fermentation profile from these studies is shown in Figure I.B.1. It can be seen from the results in this Figure that measuring the CO_2 evolution might be quite useful as a quantitative indicator of cell growth. The degree of parallelness among the three methods shown in Figure I.B.1 show identical specific growth rates for C. thermocellum when grown on cellobiose. From these results, a calibration curve was constructed to relate cell dry weight to the CO_2 evolution and is shown in Figure I.B.2.

b. Degradation of Cellulosic Biomass

Prior to our efforts toward the use of CO_2 evolution as a means of measuring growth, we had performed a number of fermentations to assess the potential of C. thermocellum in its ability to degrade corn residue. Some of these earlier studies are shown in Figure I.B.3. The results in this Figure compare the rates of degradation of solka floc and corn residue by C. thermocellum. If one examines the residual cellulose, it can be seen that the rate of degradation for solka floc is identical to that for corn residue. Furthermore, the reducing sugars accumulated during these fermentations again show comparable values. For example, from 9 gm/l of biomass, the final concentrations of reducing sugars are 4.4 and 5.0 gm/l respectively from corn residue and solka floc. Furthermore,

FIGURE I.B.1: GROWTH OF CL. THERMOCELLUM
ON CELLOBIOSE AT CONTROLLED
pH = 6.9

FIGURE I.B.2: RELATIONSHIP BETWEEN DRY CELL WEIGHT AND CO₂ EVOLUTION BY CLOSTRIDIUM THERMOCELLUM GROWN ON CELLOBIOSE (pH = 6.9)

FIGURE I.B.3: DEGRADATION OF SOLKA FLOC AND CORN RESIDUE
AND FORMATION OF REDUCING SUGARS BY C1. thermocellum

the rates of reducing sugar formation are also quite similar. High pressure liquid chromatography analyses (see Section I.A) of the reducing sugars show they are xylose, glucose and cellobiose.

In subsequent experiments, the growth of the organism was calculated through the quantitative measurements of CO₂ evolution. Some typical results are shown in Figures I.B.4 and I.B.5. The rate of growth, CMCase activity and corn residue concentration for one such fermentation are shown in Figure I.B.4. The calculated maximum specific growth rate from these results show a value of 0.3 Hour⁻¹ (doubling time = 2.3 Hours). The final extracellular CMCase is extremely high (15 gm reducing sugar/liter-hr). From an initial concentration of 9.5 g/l of corn residue, approximately 2.5 gm/l remains, representing about 75% degradation of the corn residue solids.

The products from the degradation of corn residue, in addition to cell mass and reducing sugars, acetic acid and ethanol is shown in Figure I.B.5. From 7.5 gm/l of corn residue degraded, the final concentrations of these products were: reducing sugar = 3.4 gm/l, acetic acid = 1.2 gm/l, ethanol = 0.5 gm/l and cell mass = 0.5 gm/l. These results are very encouraging since they again confirm the ability of C. thermocellum to degrade corn residue and produce products of importance.

FIGURE I.B.4: FERMENTATION PROFILE OF CLOSTRIDIUM THERMOCELLUM GROWN ON CORN RESIDUE
(T = 60°C, pH = 6.9)

FIGURE I.B.5: DEGRADATION PRODUCTS FROM CORN RESIDUE BY CLOSTRIDIUM THERMOCELLUM

A final analysis of the results which have been obtained from the degradation of corn residue, solka floc and cellobiose by C. thermocellum has been performed in order to compare the intrinsic capabilities of this organism for product formation. The intrinsic capabilities were calculated on a specific rate basis (gm product/gm cell-Hr) so that they can be examined and compared directly. The results from these calculations are shown in Table I.B.1. When the specific rates of reducing sugar production are compared for insoluble biomasses such as corn residue and solka floc, it can be seen that C. thermocellum has higher capacity to degrade the former than the latter. Furthermore, the maximum rate of 1.03 gm reducing sugar per gm of cell per hour indicates excellent promise from a process point of view. For example, if a continuous process using cell recycle can be developed and by maintaining a high cell concentration such as 10 gm/l, a projected volumetric productivity in excess of 10 gm of reducing sugars per liter per hour can be achieved. We believe this is possible through further research and development.

Further examination of the results in Table I.B.1 show that the specific productivities of ethanol and acetic acid are also higher using corn residue than solka floc. Surprisingly, these products are also produced by C. thermocellum at a higher intrinsic rate from an insoluble biomass (corn

TABLE I.B.1: COMPARISON OF SPECIFIC PRODUCT FORMATION RATES BY CLOSTRIDIUM THERMOCELLUM ON DIFFERENT CELLULOSIC BIOMASSES AND CELLOBIOSE

TIME OF FERMENTATION (Hours)	SPECIFIC PRODUCT FORMATION RATE		
	REDUCING SUGARS (gm Sugar/gm Cell-Hr)	ETHANOL (gm Eth/gm Cell-Hr)	ACETIC ACID (gm Acetic/gm Cell-Hr)
CORN RESIDUE			
3	0.53	0.23	0.34
4	1.03	0.20	0.32
6	0.98	0.19	0.36
8	0.96	0.23	0.38
10	0.52	0.23	0.16
SOLKA FLOC			
4	0.35	0.11	0.07
7	0.36	0.13	0.05
10	-	0.16	0.06
CELLOBIOSE			
4		0.26	0.16
6		0.27	0.20
8		0.20	0.12
10		0.12	0.10
12		0	0.11

residue) than from a soluble substrate such as cellobiose. The specific formation rates of these products also indicate the potential of this organism to produce useful products (ethanol and acetic acid) directly from cellulose.

4. Future Studies

The future studies associated in the degradation of cellulose biomasses by Clostridium thermocellum include:

- . Examine other biomasses such as corn stover, straws, and trees (if obtainable).

- . Initial continuous fermentation studies with cell recycle to increase productivity.

- . Examine the direct production of fuel (ethanol) and chemicals (acetic acid) by C. thermocellum using cellulosic biomasses.

- . Examine mutants which are hypoproducers of cellulases for biomass degradation.

- . Provide reducing sugars from biomass degradation for other studies, e.g. acetic acid - C. thermocellum and acetone/butanol - C. acetobutylicum.

C. GENETIC MANIPULATIONS

1. Plasmids: Progress Towards Their Use as Genetic Vectors.

The previous progress report confirmed the presence of plasmids in Cl. thermocellum. To utilize these plasmids as vectors requires that their properties be determined and that simple methods for their separation and handling be developed.

For the purpose of characterizing and using these plasmids, it is desirable to have a sensitive and nondestructive DNA assay technique for two reasons: first, a nondestructive assay allows the same DNA to be subjected to a battery of characterization steps in series; and later, a nondestructive assay will allow the location and recovery of vector material in quantity from a gel, gradient, or column.

To this end, the following technique was adapted to Cl. thermocellum. ^{32}P phosphate was used to label the organism's DNA. The DNA could then be counted with good efficiency without the addition of scintillation fluid, using the blue Cerenkov glow caused by the energetic beta particles emitted by ^{32}P .

A modification of the complex medium, CM4, was chosen. This medium consisted of CM4 with 5 g/l of sodium bicarbonate instead of phosphate buffer; enough phosphate for growth was provided by about 2.5 g/l of yeast extract, which was determined to be the lowest non-limiting amount of yeast extract that could be used in the medium. 0.6 millicuries of ^{32}P orthophosphoric acid was added to a 10 ml Hungate tube of the above medium, which contained 0.43 mM PO_4 as determined by the procedure of Taussky and Schorr ("A Microcolorimetric Method for the Determination of Inorganic Phosphorus" Taussky, H.H., and E. Schorr J. Biol. Chem. 202: 675-685 (1953)).

Cells labelled in this manner were harvested during active growth at a concentration of 80 mg/l, as determined by their optical density at 660 nm (see figure I.C.1). Both wild type and streptomycin resistant cultures were labelled and harvested.

Both cultures were lysed and centrifuged in cesium chloride-ethidium bromide as previously described (Progress Report 6-1-77 to 8-31-77). NOTE: The temperature of heat treatment during this step was incorrectly reported there as 80°. The temperature used for the isolation is 90°.

The results obtained from the wild type culture are shown in figure I.C.2. While this separation was carried out to isolate plasmids for their characterization, this gradient has significances in itself beyond just repeating the previous work. The earlier work was done with a streptomycin resistant mutant grown in the presence of streptomycin. This gradient confirms that the plasmid is present in the wild type and that the high copy number does not result solely from growth of the mutant in the presence of streptomycin.

Using the same cells, an attempt was made to inactivate nucleases with diethyl pyrocarbonate (DEPC) during lysis, as a gentler alternative to the heat and quench method previously described. This substitution was shown not to work.

The fractions marked in figure I.C.2 were combined and dialysed against buffer. (42% recovery of label inside the membrane; 92% of label was accounted for by sample and dialysate). A similar preparation was made from the mutant culture, as a check for differences.

I.C.1 Relation of dry cell weight to turbidity. Turbidity measured in Hungate tubes using a Turner Model 330 spectrophotometer.

I.C.2 Cesium chloride-ethidium bromide equilibrium density gradient of wild-type Cl. thermocellum. Conditions as previously reported. Arrows mark fractions recovered for further analysis.

Neutral sucrose gradients were prepared using both gamma-irradiated and unirradiated aliquots of this dialysed preparation, as well as of the original mutant preparation. These gradients are shown in figures I.C.3, I.C.4, I.C.5, and I.C.6. Nearly all of the labelled material sedimented completely to the shelf, while any linear DNA's of 50 mdal (50×10^6 daltons) or less would have sedimented no lower than fraction 40.

These results suggest the formation of large aggregates of some sort. Three treatments were tried to free homogenous populations of DNA (that is, peaks) from this aggregation: amyl alcohol extraction, pronase digestion, and alkaline sedimentation. These gradients, together with their control are shown in figures I.C.7, I.C.8, I.C.9, and I.C.10. Amyl alcohol extraction had no effect. Pronase digestion freed some DNA-containing pieces of the aggregate into the gradient, but these had a continuous size distribution, much like the control gradient. Only the alkaline gradient completely broke up the aggregation: no label sedimented completely to the bottom and two peaks were observed.

The faster of these peaks indicates a denatured covalently closed circular DNA of about 4.3 mdal (see figure I.C.11). The separated linear and circular single strands resulting from nicks in such a species of DNA should be found in adjacent fractions, forming a single broad peak that sediments 3-4 times more slowly than the CCC peak. That description fits the slower-moving peak observed in our experiment.

This plasmid species might correspond to the larger plasmid (estimated at 3.5 mdal) shown in the electron micrographs of the previous progress report. There is, however, no sign of the smaller plasmids which appeared to predominate in those micrographs.

I.C.3 Neutral sucrose velocity gradient of material recovered from cesium chloride-ethidium bromide equilibrium density gradient of ^{32}P labelled lysate from streptomycin resistant mutant. Centrifuged at 50,000 RPM for 2 hours in SW50.1 rotor. Gradient and other conditions described previously. All gradients are run over a 0.3 ml shelf of a very dense solution, which begins at about fraction 50.

I.C.4 Neutral sucrose velocity gradient of material recovered from cesium chloride-ethidium bromide equilibrium gradient of ^{32}P labelled lysate from wild-type cells (fractions marked on figure I.C.2). Conditions as in figure I.C.3.

I.C.5 Neutral sucrose gradient of same preparation as in figure I.C.4 after 35 krad of gamma-irradiation. Conditions as in figure I.C.3.

I.C.6 Calibration of neutral sucrose gradient and check on technique. Sample was the same preparation as in figure I.C.4. Conditions as in figure I.C.3. Arrows mark the positions of the linear, open circular, and covalently closed circular forms of a 5.8 mdal marker plasmid added to sample. (peaks not shown)

I.C.7 Neutral sucrose gradient of same preparation as in figure I.C.4 after amyl alcohol extraction. Conditions as in figure I.C.3.

I.C.8 Neutral sucrose gradient of same preparation as in figure I.C.4 after pronase digestion. Conditions as in figure I.C.3.

I.C.9 Alkaline sucrose gradient of the same preparation as in figure I.C.4. Open symbols mark the difference between the solid and broken lines. 1 hour, 50,000 RPM in SW50.1 rotor.

I.C.10 Control. Neutral sucrose gradient of same preparation as in figure I.C.4. Conditions as in figure I.C.3.

I.C.11 Estimation of plasmid size from sedimentation velocity of denatured covalently closed circular DNA under alkaline conditions. Open symbol is the experimental value derived from figure I.C.9. Closed symbols are data taken from the following sources:

Nucleic Acid Research 2:1459 (1975)

Eur. J. Biochem. 36:39-44 (1973)

J. Gen. Physiol. 49:103(special) (1966)

BRL catalogue p.13 (1977)

As a next step, an attempt will be made to detect and separate all the plasmid species present by applying freshly labelled and lysed cells directly to an alkaline sucrose gradient. If successful, this will be a valuable separation for preparative work.

Other future work will include mapping the restriction sites present in the separated plasmids via electrophoresis of the restriction fragments. When that is finished, we will be ready to begin isolating the cellulase genes so that they can be cloned using one of these plasmids. Whether that isolation is carried out by non-genetic or genetic means will depend on the speed with which the cell fusion work progresses (section I.C.3).

2. Curing of Plasmids

Another step of this program require that the cellulase genes be isolated. To eliminate the possibility that these genes might already be located on one of the plasmid species present, a curing experiment was carried out as follows.

Acridine orange was used as the curing agent. Concentrations of acridine orange above 25 micrograms per ml were found to be completely inhibitory to the growth of Cl. thermocellum, so a concentration of 10 micrograms per ml was chosen. The species was cultured in cellobiose-CM4 containing the curing agent. This was used to inoculate (1% inoculum) a second tube of curing medium, and this second culture was used to inoculate a third. Each growing culture was plated on cellobiose-CM4 agar. These plates were replicated after incubation to cellulose-CM4 agar and the number of colonies which failed to replicate were noted for comparison with controls which had been treated the same, except that no

curing agent was used. No loss of the ability to use cellulose was effected by this treatment.

3. Protoplasts of C. thermocellum Cells.

In the last progress report we indicated that cell fusion as a means of gene transfer was to be investigated. To that end we undertook a search for an appropriate protoplasting procedure.

Protoplasts of C. thermocellum have been formed after treatment with lysozyme in a variety of incubation media. Their osmotic and morphological characteristics are similar to those described for protoplasts of other gram negative bacteria (Lederberg, J. and St. Clair, J.; J. of Bact. 75, 143-160, 1958).

a. General protocol for protoplast production.

i. Grow cells in the presence of 0.2% glycine. This glycine concentration has been selected according to Hopwood's criteria (Nature, 286, (171-174), 1977); 0.2% glycine is clearly inhibitory for growth of C. thermocellum but still gives a reasonable yield of cells. It has been shown that such a treatment increases the protoplast formation. (Okanishi, M., Suzuki, K. and Umezawa, H., J. of Gen. Microbiol., 80, 389-400, 1974).

ii. Harvest the cells by centrifugation (10K, 10 minutes) and resuspend them in TES buffer pH 8.

iii. Centrifuge again and resuspend the cells in 4 ml of 5% sucrose in 0.05M Tris buffer pH 8.

iv. To this cell suspension, different additions are made:

- 5M NaCl to a final concentration of 2%

or - 4% MgCl₂ and 4% CaCl₂ to a final concentration of both at 0.3% each

or - 4.5 MKCl to a final concentration of 2%

Lysozyme (freshly dissolved in 0.25M Tris buffer pH 8) is also added. After the proper time for cell wall digestion, the following parameters have been observed:

- changes in O.D. at 660 nm with time.

- % of protoplasts formed (by direct microscopic counts in a Petroff-Hausser chamber, for 90% confidence, $\pm 10\%$). The % of protoplasts is defined as:

$$\% P = 100 \left(\frac{N^o \text{ of protoplasts}}{N^o \text{ of total objects}} \right)$$

- % lysis (% L) after lysozyme digestion

is defined as:

$$\% L = 100 \left(1 - \frac{\text{Total objects/ml after digestion}}{\text{Total objects/ml before digestion}} \right)$$

The resistance of protoplasts to osmotic shock has been tested by resuspension in water.

Other variables that we have studied were the temperature of incubation and the sucrose concentration.

b. Effect of temperature of incubation and sucrose concentration

The % P obtained at 45 C is higher than that produced at 37 C.

The final concentration of sucrose is 3%. In the absence of it, the %L rises up from 0-6% to 45% when protoplasts are made in a Na containing medium. If 5% NaCl is the only osmotic stabilizer the %L is 55%.

We have selected the lowest sucrose concentration which preserves the %L at a minimum (3% sucrose).

c. Effect of lysozyme concentration

The % P obtained in the Na-media, increases from 24% to 41% when lysozyme concentration is raised from 1.5 to 6.2 mg/ml. But at

the same time, the % L also becomes significantly higher (from 0% to 47%). Both %L and %P were measured after 90 minutes of incubation at 45 C.

The same effect is found in Ca-Mg media. The optimum lysozyme concentration is around 3.5mg/ml.

d. Production of protoplasts in the presence of different cations

At the same lysozyme concentration, the %P obtained in 2% NaCl is lower than that produced in Ca-Mg media; the %L is lower in the latter. More importantly seems to be the different effect of osmotic shock in both classes of protoplasts. The Na-protoplasts lyse immediately after placed in water, while the Ca-Mg -protoplasts do not and remain spherical during the next few hours. This sort of protoplast "fixation" exerted by divalent cations has been observed for other cells: (B. licheniformis Reaveley, D. A., and Rogers, H. J. (1969), Biochem. J., 113, 67-69; B. stearothermophilus, Abram, D., (1965), J. Bact. 89, 855-73; E. coli, Lederberg et al. and several streptomyces, (Okanishi, et al.).

The addition of 2% NaCl before resuspension in water, increases the susceptibility of Ca-Mg protoplasts to osmotic shock, as suggested by the drop in O.D. 660 (Table I.C.1). This fact is also confirmed by direct microscopic observation. However, the strong aggregation shown by the cells after H₂O resuspension makes impossible an accurate quantitation of the remaining protoplasts after osmotic shock. No protoplasts are seen as regular spherical bodies, after Na-protoplast are shocked, but a high fraction of them remain if Ca-Mg medium is used.

The same result was obtained in a different experiment using a lower concentration of lysozyme (2.9 mg/ml). It was found (see Table I.C.

Table I.C.1

O.D. 660 nm

Protoplasting media	*%P	*%L	Incubation time (mins)			After H ₂ O resusp	Add 2% NaCl before H ₂ O resusp	Add 0.3% Mg Cl ₂ + 0.3% CaCl ₂ before H ₂ O resusp
			0	45	90			
Mg - Ca	62%	0%	0.7	0.53	0.51	0.41	0.27	-
Na	45%	25%	0.78	0.28	0.23	0.17	-	0.23

*After 90 minutes at 45°C, in the presence of 4.2mg/ml of lysozyme.

Table I.C.2 O.D. 660

	*%P	*%L	Incubation time (mins)						time after H ₂ O resusp (mins)					Time after EDTA re-susp. (mins)				
			0	15	30	45	60	90	0	5	10	15	20	0	5	10	15	20
Na	37%	0%	0.53	0.33	0.29	0.27	0.25	0.24										
K	27%	15%	0.53	0.30	0.27	0.24	0.21	0.2										
Ca-Mg	54%	0%	0.58	0.41	0.39	0.39	0.37	0.37	0.4	0.4	0.4	0.4	0.4	0.18	0.17	0.17	0.15	0.

*After 90 minutes in the presence of 2.9 mg/ml of lysozyme at 45°C.

that the highest drop in O.D.⁶⁶⁰ is attained during the first 15 minutes of incubation. The presence of 2% KCl instead of 2% NaCl reduced the % P and increased the % L. This result was expected since K⁺ is the internal physiological ion. The 'Ca - Mg' protoplasts were resuspended, after 90 minutes at 45 C, in 0.05 MEDTA, pH 7. The O.D.⁶⁶⁰ values of this suspension were compared with those obtained after resuspension in distilled water.

The Ca - Mg protoplasts seem to lyse in the presence of 0.05M EDTA pH 7, as suggested by the O.D.⁶⁶⁰ measurements (Table I.C.2). This fact was also confirmed by direct microscopic observation although again, aggregation made impossible a good quantitation of the % P remaining.

e. Dilution of protoplasts with different diluents

After 30 minutes of incubation with lysozyme at 45 C 1:10 dilutions were made with protoplasts produced in both the Na- and (Ca - Mg) media, and counted, after 20 minutes at room temperature, in a Petroff-Hausser chamber (Table I.C.3).

f. Standard protocol

Based on the above results the following protocol has been adopted.

Exponentially growing cultures of Cl. thermocellum in CM-4 with cellobiose as the carbon source and glycine as a protoplast enhancer are subjected to lysozyme treatment in the following solution.

Lysozyme	3mg/ml
Tris buffer	0.25M pH 8.0
Sucrose	3%
CaCl ₂	0.3%
MgCl ₂	0.3%
Cysteine	0.1%

Table I.C.3

N°	Protoplasting Media	Diluted with	%P	%L
1	Ca-Mg	0.3% $MgCl_2$ + 0.3% $CaCl_2$ + 5% Sucrose pH 7.4	73%	0%
2	Ca-Mg	0.05MEDTA, 5% sucrose pH 7.4	62%	10%
3	Ca-Mg	0.5 MEDTA, pH 7.4	-	High lysis
4	Na	2% NaCl + 5% sucrose	19%	70%
5	Nā	0.1% peptone water	-	High lysis

Incubation is carried out at 45 C for 30 min.

g. Future Studies

In the next few weeks an effort will be made to establish the optimum conditions for protoplast regeneration. Once this is accomplished we will initiate cell fusion studies.

D. Deregulation of Clostridial Cellulase

1. Growth and Enzyme Production in Several Carbon Sources

Several carbon sources were tested for the growth of C. thermocellum and TNP-CMCase production in flask culture (Figure I.D.1). MN300 cellulose and cellobiose (1% each) added to CM3 medium supported about the same amount of growth. Glucose and glycerol, however, did not support growth. Addition of glucose to cellobiose or cellulose had no significant effect on growth. With respect to enzyme production, cellobiose supported higher cellulase production than did cellulose; the latter enzyme activity was determined after 2% Triton X100 treatment of whole broth.

When the amount of cellobiose added to CM3 medium was varied, cell growth was inhibited by concentrations higher than 1% (Figure I.D.2). The specific enzyme production during exponential growth (enzyme produced per unit of cell mass) was almost the same at each sugar concentration.

2. Relationship Between TNP-CMCase and CMCase Activity

It is difficult to evaluate CMCase activity in broths containing high sugar levels using the reducing sugar assay. In order to compare CMCase production to that of TNP-

CMCase, removal of sugar from the cellobiose-grown culture supernatant was carried out by ethanol precipitation (80% ethanol, 4 C overnight). The precipitate was recovered by centrifugation and dissolved in 0.05M pH 4.5 citrate buffer. CMCase activity was evaluated in the recovered protein solution and TNP-CMCase was measured both in the original broth and in the recovered fraction. The results are shown in Table I.D.1. It can be seen that a close correlation exists between CMCase activity detected by measuring reducing sugars released and TNP-CMCase activity measured by release of the chromogen. This indicates that the TNP-CMCase assay can be used to measure CMCase activity.

3. Effect of Sugars at High Concentration on TNP-CMCase

In the previous two progress reports (COO-4198-2, -3), we showed that low levels of glucose, xylose and cellobiose did not inhibit TNP-CMCase, CMCase and Avicel-hydrolyzing activity. In this report period, we tested the effect of high concentrations of sugars on TNP-CMCase which is important for the application of C. thermocellum enzyme to sugar production from cellulosic materials. As shown in Figure I.D.3, high concentration of sugars (30% glucose and xylose, 10% cellobiose) had no effect on TNP-CMCase.

4. Effect of Methylcellulose on TNP-CMCase

The effect of methylcellulose (known as an inhibitor of some cellulases) on TNP-CMCase action was examined. The addition of 3 mg/ml and 10 mg/ml methylcellulose (Pfaltz & Bauer, Inc.) to the reaction mixture containing 10 mg/ml TNP-CMC had no effect on TNP-CMCase. On the other hand, the addition of CMC (which should compete with TNP-CMC as substrate) at 10 mg/ml in the reaction mixture inhibited TNP-CMCase activity by 40%.

5. Preliminary Study on the Isolation of Hyper-producing Cellulase Mutant

C. thermocellum cells grown on CM3 medium containing 0.5% cellobiose for 24 hrs were irradiated (UV) and plated on CM3 agar medium containing 1% MN300 cellulose and 0.5% cellobiose. After 1 min of UV irradiation, a 99% kill was observed. Hyperproducing mutants showing large clear zones around the colonies are being searched for now.

TABLE I.D.1: COMPARISON BETWEEN THE TNP-CMCase and CMCase ACTIVITY
PRODUCTION IN SUGAR FREE PROTEIN SOLUTIONS

Fermentation Time (Hr)	Growth (Klett Units)	TNP-CMCase Δ OD 344		CMCase mg sugars/h/ml Recovered Protein	CMCase TNP-CMCase
		Broth Supernatant	Recovered Protein		
0	13	0.038	0.021	-	-
17	64	0.058	0.043	1.68	39.1
25	129	0.146	0.124	2.95	23.8
40	170	0.159	0.131	4.08	31.1
50	175	0.189	0.144	4.75	33.0
64	170	0.204	0.178	5.70	32.0

FIGURE I.D.2: GROWTH AND TNP-CMCase PRODUCTION IN DIFFERENT LEVELS OF CELLOBIOSE

FIGURE I.D.1: CELL GROWTH ENZYME PRODUCTION (TNP-CMCase) AND pH OF BROTH OF CLOSTRIDIUM THERMOCCELLUM ATCC-27405

Cellobirose (●) Glucose (○) Cellulose (MN 300) (Δ) Glycerol (□)
Cellobirose-glucose (●) Cellobirose-MN 300 (▽) Glucose-MN 300 (▲)

FIGURE I.D.3: EFFECT OF SUGARS ON TNP-CMCase ACTIVITY
 o: glucose; Δ: xylose; ●: cellobiose
 EACH VALUE IS RELATIVE TO THAT WITHOUT
 ADDITION (1).

E. Process and Economic Analysis of Sugar Production from Cellulosic Biomass

1. Process Description

This process focuses on the use of cellulases derived from Clostridium thermocellum for the enzymatic hydrolysis of corn stover cellulose to fermentable sugars. Other processes have considered enzymes produced by aerobic mycelial organisms. These are complicated by the technical problems associated with mass transfer in highly viscous fermentation broths. In contrast, the anaerobic, thermophilic bacterium, Clostridium thermocellum, has obvious advantages from an engineering viewpoint. These are: (a) elimination of oxygen transfer by virtue of the anaerobiosis of the fermentation and (b) high growth temperatures (60°C) provide a favorable temperature gradient for heat transfer.

In brief, the approach taken in this design has been:

- . To minimize pretreatment steps by using a substrate (corn stovers) that requires physical size reduction only.
- . To produce cellulase enzymes in the fermentation stage.

- . To effect cellulose hydrolysis in a separate saccharification stage.
- . To recover enzymes for reuse through the use of an adsorption train.
- . To achieve partial hydrolysis in the fermentation and adsorption stages, thereby decreasing saccharification time.

The design bases for the process depicted in Figure I.E.1 are summarized in Table I.E.1. Medium components are listed in Table I.E.2.

For the continuous fermentation, the growth rate (= dilution rate at steady state) has been set at 70% of $\mu_{\max}$ observed experimentally for C. thermocellum on solka floc, i.e. 0.056 hr^{-1} . We have chosen to operate at as high a dilution rate as possible (avoiding washout) in order to take advantage of the sugar accumulating abilities of the organism which are observed to be a growth related phenomenon. At a growth rate of 0.056 hr^{-1} , the corresponding specific sugar production rate (determined experimentally) is 0.3 grams reducing sugar/g-cell-hr. We have assumed that a cell concentration of 3 g/l (five times the current experimental maximal cell concentration in batch culture) can be achieved. Using these values, the volumetric cellulose degradation rate was calculated to be 2.02 g

FIGURE I.E.1: PROCESS DIAGRAM FOR CELLULOSE HYDROLYSIS

FIGURE I.E.1: EXPLANATION OF SYMBOLS USED IN DESIGN DIAGRAM

EQUIPMENT

<u>SYMBOL</u>	<u>EQUIPMENT DESCRIPTION</u>
A 1-5	Adsorption Tanks
D 1-6	Drum Filters
H	Hydrolysis Tank
F 1-3	Fermentors
S	Storage (for corn stovers)
R	Size Reduction Equipment
M 1,2	Mixing Tanks
E	Heat Exchanger
B	Furnace
C	Sterilization Unit

FLOW STREAMS

<u>STREAM NO.</u>	<u>MATERIALS</u>	
1	Corn Stovers:	5×10^4 kg/hr
2	Ball Milled Corn Stovers:	5×10^4 kg/hr
3	Ball Milled Corn Stovers:	2.2×10^4 kg/hr
4	Ball Milled Corn Stovers:	2.8×10^4 kg/hr
5,6,7,8	Medium:	2.16×10^5 l/hr
	Corn Stovers:	2.2×10^4 kg/hr
	Temperatures:	20, 88, 130, 60°C respectively
9	Corn Stovers:	6.0×10^3 kg/hr
	Reducing Sugars:	3.46×10^3 kg/hr
10	Corn Stovers:	0.75×10^4 kg/hr
	Reducing Sugars:	1.08×10^4 kg/hr

FIGURE I.E.1: EXPLANATION OF SYMBOLS USED IN DESIGN DIAGRAM
(continued)

FLOW STREAMS (continued)

<u>STREAM NO.</u>	<u>MATERIALS</u>	
11	Residual Corn Stovers:	0.75×10^4 kg/hr to be burned
12	Corn Stovers and Adsorbed Enzyme:	2.36×10^4 kg/hr
13	Reducing Sugars (liquid stream):	1.08×10^4 kg/hr
14	Product Stream, Reducing Sugars: Concentration :	1.29×10^4 kg/hr 60 g/l

<u>ENERGY STREAM NO.</u>	<u>COOLING WATER PER UNIT</u>	<u>STEAM PER UNIT</u>	<u>HEAT REMOVED/UNIT</u>	<u>HEAT ADDED/UNIT</u>
I- III	3.3×10^5 l/hr	-	2.1×10^{10} joule/hr 2.0×10^7 BTU /hr	-
IV-VIII	2.3×10^3 l/hr	-	1.5×10^8 joule/hr 1.4×10^5 BTU /hr	-
IX	2.9×10^4 l/hr	-	2.1×10^9 joule/hr 2×10^6 BTU /hr	-
X	-	2.6×10^4 lb/hr		3.2×10^{10} joule/hr 3.0×10^7 BTU /hr

TABLE I.E.1: BASIS FOR DESIGN

OPERATING TIME PER YEAR	7,000 hr
PRODUCTION RATE	3×10^7 kg/7000 hr
PRODUCT CONCENTRATION	60 g/l
CELLULOSE CONTENT OF CORN STOVERS	50%
OVERALL CELLULOSE CONVERSION	70%
CELLULOSE FED TO FERMENTORS	2.2×10^4 kg/hr
CONVERSION OF CELLULOSE FED TO FERMENTORS	70%
PRODUCT CONCENTRATION IN FERMENTORS	16 g/l
TOTAL SUGARS PRODUCED BY FERMENTATION STAGE	27%
PRODUCT CONCENTRATION IN HYDROLYSIS TANK	50 g/l
TOTAL SUGARS PRODUCED BY HYDROLYSIS STAGE	57%
PRODUCT CONCENTRATION AFTER ADSORPTION TRAIN	60 g/l
TOTAL SUGAR PRODUCED IN ADSORPTION STAGE	16%
ENZYME ACTIVITY IN HYDROLYSIS TANK	3.25 g/l-hr (=FPA)
ENZYME ACTIVITY IN FERMENTORS	2.02 g/l-hr
ENZYME RECOVERY BY ADSORPTION	75%
CELL CONCENTRATION IN FERMENTORS	3 g/l
CELL YIELD	0.15 g cells/g cellul
GROWTH RATE	0.056 hr^{-1}
SPECIFIC SUGAR PRODUCTION RATE IN FERMENTORS	0.3 $\frac{\text{g sugar}}{\text{g cell-hr}}$

TABLE I.E.2: MEDIUM USED FOR GROWTH OF
CLOSTRIDIUM THERMOCELLUM

<u>COMPONENT</u>	<u>CONCENTRATION (g/l)</u>
Na_2HPO_4	0.4
NH_3	0.7
MgCl_2	0.06
CaCl_2	0.09
K_2SO_4	0.01
Yeast Extract	0.2
Corn Stovers	100

cellulose/l-hr. This is equated with enzyme activity based on the fact that experimental values for filter paper activity are equal to the maximum volumetric cellulose degradation rates observed in batch culture.

With a residence time of 17 hrs, 70% of the cellulose fed to the fermentors is degraded and is used for cell growth or accumulates as reducing sugars. The exit stream leaving the fermentors contains reducing sugars at 16 g/l and residual cellulose at 14 g/l (28 g/l corn stovers).

Enzymes leaving the fermentor stage and entering the hydrolysis stage are contacted with cellulose entering from the adsorption train. The filter paper activity in the hydrolysis tank was chosen to be 3.25 g reducing sugar/l-hr based on data for T. viride cellulases presented by Wilke (Cellulose as a Chemical and Energy Resource, Biotechnology and Bioengineering Symposium #5, 1975). With this filter paper activity, a residence time of 15 hours is required to bring the sugar concentration from the incoming 16 g/l (from fermentation stage) to 50 g/l. In order to achieve this filter paper activity (recall that the incoming filter paper activity is 2.02 FPA) and in order to maintain it, the liquid leaving the hydrolysis tank is contacted counter currently with corn stovers in an adsorption train. The enzyme adsorbs to the cellulose and is redeposited in the hydrolysis tank as the cellulose enters the tank. The number of adsorption units

was determined based on adsorption data (experimentally determined) indicating 40% adsorption of enzyme originally free in solution within 30 minutes contact time. Since the adsorption tanks are maintained at 60°C (as is the hydrolysis tank), the remaining hydrolysis necessary to bring the sugar concentration from 50 g/l (leaving hydrolysis tank) to 60 g/l occurs. A liquid stream containing 60 g/l of reducing sugars leaves the last drum filter in the adsorption train.

2. Cellulose Hydrolysis Plant Costing

Cost estimates for equipment were determined using "Chemical Engineering Costs" by C. E. Dryden and R. H. Furlow (1969). The listed prices have been updated for 1977 by adjusting the M & S scale of prices, based on a '270' value, by a factor of 492/270. Equipment are summarized in Table I.E.3. The total capital investment (Table I.E.4) was calculated from the purchased equipment costs by a percentage based adjustment accounting for piping, instrumentation, buildings, grounds, land, engineering and construction (Table I.E.5).

Raw material costs were based on prices appearing in the Chemical Marketing Reporter, averaged for the Month of July, 1977 (Table I.E.6). Steam, cooling water and electricity were priced at \$1.75/1000 lbs, \$0.8/1000 gallons and \$0.02/kilowatt hour respectively (Table I.E.7).

TABLE I.E.3: PURCHASED EQUIPMENT COST (PEC)

<u>EQUIPMENT</u>	<u>SIZE</u>	<u># UNITS</u>	<u>\$ UNIT PRICE</u>	<u>\$ TOTAL PRICE</u>
Fermentor	1.5×10^6 l (4.0×10^5 gal)	3	2.5×10^5	7.5×10^5
Agitator-Motor (fermentor)	300 HP	3	1.7×10^4	5.1×10^4
Seed Tank	1.5×10^5 l (4.0×10^4 gal)	1	3.6×10^4	3.6×10^4
Mixing Tank	2.6×10^6 l (6.9×10^5 gal)	2	9.6×10^4	1.9×10^5
Agitator-Motor (mixing tank)	500 HP	2	2.3×10^4	4.6×10^4
Pumps	7.2×10^4 l/hr 320 GPM	6	5.8×10^3	3.4×10^4
Pump Drives	12 HP	6	1.3×10^4	7.6×10^4
Cooling Coils	175 m ²	3	5×10^4	1.5×10^5
Hydrolysis Tank (concrete)	3.7×10^6 l (1×10^6 gal)	1	5×10^5	5×10^5
Agitator-Motor (hydrolysis tank)	500 HP	1	2.3×10^4	2.3×10^4
Adsorption Tank	5.4×10^5 l (1.4×10^5 gal)	5	5.6×10^4	2.8×10^5
Agitator-Motor (adsorption tanks)	60 HP	5	5.9×10^3	2.9×10^4
Drum Filters	63 m ²	6	1.6×10^5	9.7×10^5
Storage Tank	2.6×10^6 l (6.9×10^5 gal)	1	9.6×10^4	9.6×10^4
Roll Crusher	60 HP	1	4.14×10^4	4.14×10^4
Ball Mill	600 HP	1	1.82×10^5	1.82×10^5

TABLE I.E.3: PURCHASED EQUIPMENT COST (PEC)
(continued)

<u>EQUIPMENT</u>	<u>SIZE</u>	<u># UNITS</u>	<u>\$ UNIT PRICE</u>	<u>\$ TOTAL PRICE</u>
Conveyor Belts	10 m	2	1.8×10^4	3.6×10^4
Conveyor Belts (const. weighing)	4 m	1	6.6×10^3	6.6×10^3
Screw Conveyor	0.5 m	1	4.3×10^3	4.3×10^3
Heat Exchanger	800 m ²	1	1×10^4	1×10^4
Holding Tank (for sterilization)	1.7×10^4 l (4.6×10^3 gal)	1	1.5×10^4	1.5×10^4
Furnace	3.0×10^7 BTU/hr	1	2.7×10^5	2.7×10^5
Pumps	Assorted	8		1.5×10^5
TOTAL PEC				$\$4.0 \times 10^6$

TABLE I.E.4: TOTAL CAPITAL INVESTMENT

	<u>% PEC</u>	<u>(DOLLARS)</u>
PURCHASED EQUIPMENT COST (PEC)	100	\$ 4.0 x 10 ⁶
PROCESS PIPING	35	1.4 x 10 ⁶
INSTRUMENTATION	10	4.0 x 10 ⁵
BUILDINGS	35	1.4 x 10 ⁶
AUXILIARY FACILITIES	10	4.0 x 10 ⁵
GROUNDS	10	4.0 x 10 ⁵
LAND	5	2.0 x 10 ⁵
ENGINEERING & CONSTRUCTION	30	1.2 x 10 ⁶
CONTINGENCY	15	<u>6.0 x 10⁵</u>
TOTAL CAPITAL INVESTMENT		\$10 x 10 ⁶

TABLE I.E.5: CAPITAL RELATED COSTS

<u>ITEM</u>	<u>% TCI</u>	<u>\$</u>	<u>¢/LB PRODUCT</u>	<u>¢/kg PRODUCT</u>
Depreciation	6.67	6.7×10^5	0.3	0.7
Local Taxes	2.0	2.0×10^5	0.1	0.2
Insurance	10.0	1.0×10^6	0.5	1.0
Interest	7.0	7.0×10^5	0.4	0.8
		2.57×10^6	1.3	2.7

<u>OVERHEAD EXPENSES</u>	<u>\$/YEAR</u>	<u>¢/LB PRODUCT</u>	<u>¢/kg PRODUCT</u>
Administration (15% total labor)	4.1×10^4	0.02	0.04
Plant Overhead (70% total labor)	1.9×10^5	0.09	0.2
		0.11	0.24

TABLE I.E.6: RAW MATERIAL RELATED COSTS

<u>MATERIAL</u>	<u>AMOUNT kg/DAY</u>	<u>COST/UNIT \$/TON</u>	<u>DAILY COST \$/DAY</u>	<u>COST/LB PRODUCT ¢/SUGAR</u>	<u>COST/kg PRODUCT ¢/kg</u>
Corn Stover	9.5×10^5	30	3.1×10^4	5.6	12.5
NaOH	3.0×10^3	140	4.2×10^2	0.07	0.15
NH ₃	1.5×10^3	120	1.81×10^2	0.033	0.07
NaHPO ₄	1.6×10^3	403	6.0×10^2	0.116	0.26
MgCl ₂	2.5×10^2	268	6.8×10^1	0.012	0.03
CaCl ₂	3.6×10^2	71	2.5×10^1	0.005	0.01
K ₂ SO ₄	6.8×10^2	470	3.2×10^2	0.056	0.12
Yeast Extract	8.4×10^2	1,000	<u>8.5×10^2</u>	<u>0.15</u>	<u>0.33</u>
			3.3×10^4	5.9	13.3

TABLE I.E.7: UTILITIES AND LABOR COSTS

<u>ITEM</u>	<u>UNITS/DAY</u>	<u>UNIT COST</u>	<u>DAILY COST</u>	<u>COST ¢/Lb. PRODUCT</u>	<u>COST ¢/kg PRODUCT</u>
Steam	5×10^5 lb	\$1.75/1000 lb	$\$8.6 \times 10^2$	0.1	0.3
Cooling Water	5.1×10^6 gal	\$0.8 /1000 gal	$\$4.0 \times 10^3$	0.7	1.6
Electricity	4.7×10^4 KWH	\$0.02/KWH	$\$9.4 \times 10^2$	0.2	0.4
Labor	128 hrs	\$4 /hr	$\$5.12 \times 10^2$	0.09	0.2
Supervision	32 hrs	\$7.5 /hr	$\$2.4 \times 10^2$	0.04	0.09
Fringe Benefits		25% labor	$\$1.9 \times 10^2$	0.03	0.07
Operating Supplies		10% labor	$\$7.5 \times 10^1$	0.01	0.03
Maintenance		10% TCI	$\$2.7 \times 10^3$	<u>0.5</u>	<u>1.1</u>
TOTAL UTILITY AND LABOR				1.67¢/lb.	3.79¢/kg

A summary of the cost for conversion of cellulase to sugar are given in Table I.E.8.

3. Summary

A continuous process for the production of 2.0×10^8 pounds per year of soluble sugars hydrolyzed from cellulose has been designed.

The process is based upon the use of an anaerobic, thermophilic bacterium, Clostridium thermocellum, which produces the extracellular enzymes needed for cellulose hydrolysis.

The raw material used as the fermentation and saccharification substrate is 3.5×10^8 kg/year of corn stovers available at 3.3¢/kg (\$30/U.S.ton). Soluble reducing sugars are produced primarily in the saccharification stage, though a proportion of the total sugar is produced during the fermentation and enzyme adsorption stages. Approximately 75% of the cellulose enzymes exiting the hydrolysis (saccharification) stage are recovered and recycled through an adsorption train of counter-current mixer filters.

Production costs are estimated at 8.99¢/lb (20.2¢/kg) of reducing sugars with 62% of this cost attributable to the amount/price of the cellulosic substrate used.

It should be noted that all material balances were based on the actual cellulose fraction of corn stover, i.e. 50%.

TABLE I.E.8: SUMMARY OF DIRECT MANUFACTURING COST FOR
THE PRODUCTION OF REDUCING SUGARS FROM
CELLULOSIC BIOMASS

	COST FOR REDUCING SUGAR		
	¢/lb.	¢/kg	% OF MFG. COST
<u>Raw Materials</u>			
Corn Stover	5.6	12.5	62.30
Sodium Hydroxide	0.07	0.15	0.70
Ammonia	0.033	0.07	0.36
Sodium Phosphate	0.116	0.26	1.30
Magnesium Chloride	0.012	0.03	0.13
Calcium Chloride	0.005	0.01	0.06
Potassium Sulfate	0.056	0.12	0.6
Yeast Extract	0.015	0.33	0.17
<u>Utilities</u>			
Steam	0.1	0.3	1.1
Cooling Water	0.7	1.6	7.7
Electricity	0.2	0.4	2.2
<u>Labor Related Costs</u>			
Labor	0.09	0.2	1.0
Supervision	0.04	0.09	0.44
Fringe Benefits	0.03	0.07	0.33
Operating Supplies	0.01	0.03	0.11
<u>Maintenance</u>			
	0.5	1.1	5.6
<u>Capital Cost</u>			
Depreciation	0.3	0.7	3.3
Local Taxes	0.1	0.2	1.1
Insurance	0.5	1.0	5.6
Interest	0.4	0.8	4.4
<u>Overhead Expenses</u>			
Administration	0.02	0.04	0.22
Plant Overhead	0.09	0.2	1.0
TOTAL COST	8.99	20.2	100.00

Had they been based on the cellulose plus hemicellulose content of corn stovers (~85%), the amount of initial substrate used would be decreased to 2.0×10^8 kg/year. The net result of this decrease would be to lower the production cost by 2.3¢/lb (5.0¢/kg), thus achieving a cost of 6.7¢/lb (15.2¢/kg). The cellulosic raw material would then account for 50% of this production cost.

Since the corn stovers contribute so significantly to production costs, other cellulosic substrates may prove to be less expensive and this becomes the main area for process improvement that should be more closely examined. It should be cautioned, however, that lowering raw material costs by using a cheaper substrate (e.g. bagasse) will not necessarily insure reduced production costs if extensive pretreatment steps (and recovery of pretreatment materials) are required.

Some reduction in production costs could be achieved by increasing the specific enzyme production rate by genetic means. With a 5-fold increase in enzyme production per cell, fermentation volumes, the number of adsorption stages, and raw materials for growth would decrease, resulting in savings of up to 2.8¢/lb of product.

II. PRODUCTION OF CHEMICAL FEEDSTOCKS

A. Production of Acrylic Acid by Fermentation

1. Introduction

Previous efforts have identified several alternative pathways to permit conversion of cellulose to acrylic acid. The three anaerobic routes involve glucose, lactic acid and propionic acid as key intermediates respectively. In all of these cases, acrylic acid is formed by a dehydration of lactic acid or by an oxidation of propionic acid. Therefore, our efforts have been devoted towards maximizing the rate of lactate and propionate formation in the appropriate fermentations and in effecting the conversion of lactate and propionate to acrylate.

2. Fermentation Studies with *Clostridium propionicum*

a. Introduction

As described in previous progress reports, it was established that resting cells of *C. propionicum* could ferment a variety of substrates to propionate and acetate. Also, cell free extracts prepared from *C. propionicum* were also shown to ferment a restricted group of compounds to propionic acid

and acetic acid. In both cases, evidence supported the role of acrylic acid as an intermediate in propionic acid formation.

In this final quarter, a major effort was launched with the objective of determining quantitatively the accumulation of acrylic acid in some of the above fermentations. In addition, given the recent success of our efforts showing acrylic acid accumulation, we have proceeded to examine its production with acetone dried cell powders. This effort is directed toward development of specific inhibitors of acrylic reduction and toward elucidating possibilities of immobilized cell approaches toward acrylate production.

b. Materials and Methods

1. Cell Growth and Product Formation

Techniques for growth of *C. propionicum* and gas chromatographic analysis of product formation have been described in previous progress reports. We describe here the preparation of acetone powder of *C. propionicum*.

Cells were harvested by centrifugation, and the cell pellet suspended in tris (hydroxymethyl) amino-methane (Tris)-HCl buffer (50 mM, pH 7.5) containing 3 mM cysteine. The suspension was slowly added to at least 10 volumes

of acetone (chilled to -20°C) with vigorous stirring. Stirring was continued for 5-10 min., after which the cells were allowed to settle at -20°C . The supernatant was then decanted and residual solvent removed by vacuum filtration. The filter cake was washed with 5 volumes of acetone (-20°C) and dried by vacuum filtration. The residue was worked with a spatula to a fine powder and stored desiccated at -20°C .

2. Assay for Acid Formation

Acid forming ability was assayed using the following reaction mixture (1 mM total volume):

Tris-HCl buffer, pH 7.5	50	mM
Cysteine	3	mM
Sodium Lactate or L-alanine	50	mM
Coenzyme A	0.1	mM
ATP	10	mM
MgCl ₂	10	mM
Acetone Powder	1	mg dry wt.

Prior to use, the acetone powder was suspended in Tris-HCl buffer containing cysteine (10 mg acetone powder/ml) and stirred for 30 minutes at room temperature.

Acids were analyzed by gas chromatography on Chromasorb 101 (John-Manville Co., Denver, CO) as described by J. Carlsson (Appl. Microbiol. 25: 287-289, 1973).

c. Results and Discussion

1. Detection of Acrylic Acid as a Fermentation Intermediate

Preliminary experiments with C. propionicum resting cells prepared from the standard growth medium where β -alanine was used as a main carbon source indicated that small amounts of acrylic acid could accumulate in the fermentation broth. A typical experiment summarizing the kinetics of acrylic acid production, as well as the formation of acetate and propionate, is summarized in Figure II.A.1. It should be noted that there was a rapid accumulation of acrylic acid and then a slow disappearance. The above result demonstrates the value of performing kinetic studies for, in fact, if end point analyses were conducted (as had been done in earlier work) no acrylic acid would be detected after 120 min. A second experiment was then conducted. Only this time, the fermentation was carried out under aerobic conditions. As can be seen in Figure II.A.2, more acrylic acid accumulated in this fermentation; 1.5 mM accumulated in 2 hrs. The initial rate of acrylic acid production was 3 mmole/liter hr.

The above findings are very exciting because this is the first report of acrylic acid being produced by C. propionicum. It was thought that confirmatory chemical

FIGURE II.A.1: PRODUCTION OF ACIDS FROM β -ALANINE BY
RESTING CELLS OF CL. PROPIONICUM
GROWN ON β -ALANINE

FIGURE II.A.2: AEROBIC FERMENTATION OF β -ALANINE BY β -ALANINE GROWN CELLS OF CL. PROPIONICUM

analysis of the product being called acrylic acid should be conducted. Thus, considerable time and effort was spent on studies designed to confirm that the chemical being detected on our gas chromatograms was in fact acrylic acid. For this phase of the study, combined gas-chromatography and mass spectrometry were employed.

The mass spectrum of acrylic acid was first established by injecting acrylic acid directly into the mass spectrometer. Characteristic peaks of m/e of 55 and 72 resulted. These peaks are derived from acrylic acid (m/e 72) and $\text{CH}_2 = \text{C} - \text{C} = \text{O}$ (m/e 55). Next, dilute acrylic acid standard, in the concentration range expected in the experimental samples, was injected into the gas chromatograph and the mass spectrum was taken prior to the acrylic acid peak and at the retention time of the acrylic acid peak on the gas chromatograph. Characteristic peaks at m/e of 55 and 72 were clearly observed. Pure ether then was injected into the column in order to confirm that the column is clear and no residual acrylic acid is present. No characteristic peaks at m/e 55 or 72 could be detected by the mass spectrometer after injection of the ether. Ether with a mass of 74 was readily detected.

A sample taken at 2 hrs from the aerobic fermentation of β -alanine by β -alanine grown cells (see Figure II.A.2) was then prepared. The reaction was stopped by the

addition of 2 ml of 50% H_2SO_4 and then 10 ml of ether was added to the reaction tube to extract volatile acids; the ether layer was separated after freezing of the aqueous layer at -70°C . The ether was evaporated to about 100 μl by flushing with N_2 gas. This concentrated ether solution was used for the gas chromatograph-mass spectrometer assay. Mass spectra were taken before, at and after the retention time of acrylic acid. Increases in peak m/e 55 and 72 are noted corresponding to the retention time of acrylic acid. In order to confirm that the compound was acrylic acid, the single ion monitoring technique was employed and peaks corresponding to m/e 55 and 72 were looked for. Responses for each ion level were only observed at the retention time of acrylic acid. From these results, we concluded that acrylic acid was being produced.

2. Factors Influencing Production of Acrylic Acid by *C. propionicum*

a. Effect of Electron Acceptors

Since formation of acrylic acid was confirmed, additional experiments were initiated. *C. propionicum* was grown at 37° anaerobically with the medium listed in previous progress reports with either α -alanine or β -alanine as the carbon source. The cells are harvested at early stationary

phase (about 36 hrs) and washed three times with 0.02% Na_2S solution. Cells are then resuspended with tetraethanolamine buffer (0.01 M, pH 7.5) at a concentration of 1g wet cells/ 5 ml buffer. The reaction mixture in 1 ml contains tetraethanolmine buffer (0.05 M, pH 7.5), substrate (pH 7.0, 50 mM) and resting cells (0.1 gm wet wt.). Incubation is carried out at 37°C with stirring under either anaerobic or aerobic conditions. Samples (100 μl) are drawn with a syringe and transferred to tubes which contain 20 μl of 50% H_2SO_4 in order to stop the reactions and acidify the samples at the same time. Extraction with ether was followed by gas-chromatographic analysis of volatile acids as described previously.

The first series of experiments evaluated the effect of a variety of electron acceptors on the fermentation. As can be seen in Figure II.A.3, addition of methylene blue was able to inhibit the reduction of acrylate to propionate. Note also that no acetate is detected in the presence of methylene blue. A similar result is found when 1, 2, 3, 5-triphenyltetrazolium chloride (TTC) is employed (Figure II.A.4). Therefore, it appears that exogenous electron acceptors including oxygen may be used to block the reduction of acrylic acid to propionic acid and also prevent the formation of acetate. The exact mechanism and kinetics remain to be determined.

FIGURE II.A.3: ANAEROBIC FERMENTATION OF β -ALANINE IN THE PRESENCE OF METHYLENE BLUE BY β -ALANINE GROWN CELLS OF CL. PROPIONICUM

FIGURE II.A.4: ANAEROBIC FERMENTATION OF β -ALANINE IN THE PRESENCE OF TTC BY β -ALANINE GROWN CELLS OF CL. PROPIONICUM

b. Growth Conditions

By chance, acrylic acid was first detected with cells prepared from a growth medium where β -alanine was the major substrate. The ability of α -alanine grown cells to produce acrylic acid was then investigated. When fermentation of α -alanine by α -alanine grown cells was carried out under anaerobic or aerobic conditions, no acrylic acid was detected (Figures II.A.5 and II.A.6). However, when β -alanine was employed as a substrate for the anaerobic fermentation of α -alanine grown cells, acrylic acid was formed (Figure II.A.7) and when the fermentation was carried out under aerobic conditions considerably more acrylate was produced (Figure II.A.8).

c. Use of Other Substrates

Obviously, there is not much economic incentive to produce acrylate from α -alanine or β -alanine. As our previous reports have addressed, we have shown that while C. propionicum will only grow on a few substrates (e.g. α - and β -alanine), it can ferment a variety of substrates to propionate and acetate, including lactate and pyruvate. Studies with these more attractive substrates will be the major focus of further studies.

FIGURE II.A.5: ANAEROBIC FERMENTATION OF α -ALANINE BY α -ALANINE GROWN CELLS

FIGURE II.A.6: AEROBIC FERMENTATION OF α -ALANINE BY α -ALANINE GROWN CELLS

FIGURE II.A.7: ANAEROBIC FERMENTATION OF β -ALANINE BY α -ALANINE GROWN CELLS

FIGURE II.A.8: AEROBIC FERMENTATION OF β -ALANINE BY α -ALANINE GROWN CELLS

It is important to note here that an interesting and extremely encouraging finding resulted when oxidation of propionate by C. propionicum was investigated. The objective of the experiment was to determine if one could in fact reverse the reaction of acrylate reduction and convert propionate to acrylate. As can be seen in Figure II.A.9, when propionate is the substrate, acetate appears in the fermentation broth under anaerobic conditions. When the fermentation is carried out under aerobic conditions, acrylic acid appears as an intermediate (Figure II.A.10). Of major note, the conversion yield of propionic acid to acrylic acid is 37 mole %.

3. Utilization of Acetone Powder of C. propionicum

In the previous progress report, it was indicated that the production of acrylate might be hindered by the need to uncouple acrylate from its coenzyme A (CoA) thioester. A study has been initiated to determine if chemical inhibitors could be found which would block further metabolism of acrylyl-CoA. The resulting increased cellular pool of acrylyl-CoA would favor the release of free acrylic acid by CoA transferase, especially if the substrate (lactate or propionate) was kept at a high level.

Acetone powders of C. propionicum have been prepared and assayed for acid production from lactate

FIGURE II.A.9: ANAEROBIC FERMENTATION OF PROPIONATE BY α -ALANINE GROWN CELLS

FIGURE II.A.10: AEROBIC FERMENTATION OF PROPIONATE BY α -ALANINE GROWN CELLS

and alanine. Dried cell preparations frequently have sufficiently lost the permeability properties of viable cells to serve as enzyme preparations - that is, they can be activated by coenzymes and problems of inhibitor exclusion are overcome. In addition, large quantities of cells may be conveniently processed in this manner leading to a dry stable powder.

The ability to form acetate and propionate from both lactate and alanine was retained by the acetone powder extract. Activities were comparable to those of cell-free extracts (Progress Report, June 1 - August 31, 1977).

This result indicates that acetone powders may be used to study the enzymology of acrylate formation and utilization by C. propionicum.

d. Future Studies with C. propionicum

During the next report period, we will focus on the use of whole cells for the conversion of both propionate and lactate to acrylate. Particular attention will be directed toward optimization of the conversion yield of propionate to acrylate and toward optimization of the rate and concentration of acrylate fermentation.

Having defined a working system for acrylate production, detailed studies will be initiated on the mechanisms of the formation with a focus on inhibiting enzyme

activities that lead to acrylate degradation. Some of these studies are described here:

. Examine the metabolism of acrylate by acetone powder preparations. The rate of acrylate disappearance, as well as propionate and acetate formation, will be determined by gas chromatography.

. Examine the effect of various acrylate analogs on acrylate metabolism with the aim of blocking the reduction (and/or oxidation) of acrylyl-CoA. The analogs to be tested include propionic acid, cyanoacetic acid, cyclopropane carboxylic acid, acetoacetic acid, β -hydroxypropionic acid, crotonic acid, and 4-pentonoic acid.

. Examine the effect of agents, such as hydroxylamine, mercuric chloride and p-mercuribenzoate on the production of acrylate from lactate and propionate. These agents are known to disrupt thioester linkages. The mercurials are interesting in that acetoacetyl-CoA and malonate semialdehyde-CoA are rapidly reduced to the acid and mercurothio-CoA under conditions in which acetyl-CoA is stable.

. Examine the effect of lactate dehydrogenase inhibitors (e.g. oxamate and oxalate) on the fermentation of lactate by acetone powders and resting cell suspensions.

Examine various riboflavin and FAD analogs on lactate fermentation. The role of flavoprotein electron carriers in the reduction of acrylate to propionate is well established in M. elsdenii and it is likely that C. propionicum has similar flavoproteins.

3. Fermentation Studies with Megasphaera elsdenii

a. Introduction

In our last report, we presented some preliminary data on the behaviour of M. elsdenii growing in a continuous culture. At that time, we had data only at very low dilution rates, without pH control. In this report, we present the results from M. elsdenii grown at different dilution rates and at a fixed pH of 7.0. The main objective was to maximize the molar ratio propionate/butyrate because acrylate is in the propionate pathway. Results show that this ratio goes from 0.30 at dilution rate of 0.15 hr^{-1} to 3.25 at dilution rate of 0.4 hr^{-1} .

b. Materials and Methods

M. elsdenii was grown on the same medium reported previously. Acid production was followed by gas

chromatography of the samples using an 8 ft. column packed with Chromosorb 101. The gas chromatograph used was a Hewlett Packard 5830A equipped with flame ionization detector (FID). Conditions for the G.C. analysis are:

Column temperature:	195°C
Injector temperature:	225°C
Detector temperature:	300°C
Flow Rate	: 40 ml/min
Carrier Gas	: Helium

The fermentor used in this study is a Bioflo Fermenter (New Brunswick Co.) with a liquid volume of 375 ml. The pH was controlled by an on-off solenoid valve connected to a Radiometer pH meter.

c. Results and Discussion

Table II.A.1 presents a summary of the results for six different dilution rates. It is possible to see that the residual lactate in the fermentor was near zero and that this amount increased when the specific growth rate was increased. This is consistent with lactate being the limiting nutrient.

The specific rate of lactate consumption showed a direct relationship with the specific growth rate. This is shown in Figure II.A.11.

TABLE II.A.1: SUMMARY OF RESULTS FOR ACID PRODUCTION BY M. FLSDENII

DILUTION RATE (hr ⁻¹)	CELL WT. (gm/l)	RESIDUAL LACTATE (gm/l)	ACETATE (gm/l)	PROPIONATE (gm/l)	BUTYRATE (gm/l)	VALERATE (gm/l)
0.15	0.85	0.22	0.53	0.30	1.19	0.83
0.20	0.90	0.40	0.79	0.52	1.47	1.04
0.30	0.96	0.67	1.08	0.95	0.75	0.59
0.35	0.87	1.07	1.05	1.10	0.63	0.49
0.40	0.87	1.01	1.13	1.31	0.48	0.41
0.43	0.87	0.95	1.06	1.16	0.57	0.49

FIGURE II.A.11:

SPECIFIC RATES OF ACID PRODUCTION

Using the relationship shown in Equation II.A.1, we can estimate the true growth yield and the maintenance energy.

$$\begin{aligned} \text{Total rate of Consumption} &= \text{Rate of Consumption for Growth} + \text{Rate of Consumption for Maintenance} \\ \frac{\mu X}{Y_E} &= \frac{\mu X}{Y_{EG}} + mX \end{aligned} \quad (\text{II.A.1})$$

dividing this equation by μX

$$\frac{1}{Y_E} = \frac{1}{Y_{EG}} + \frac{m}{\mu}$$

We also know that the specific rate of lactate consumption is equal to:

$$q_{LAC} = \frac{\mu}{Y_E} \quad (\text{II.A.3})$$

Substitution of equation II.A.3 into II.A.1, we got:

$$q_{LAC} = \frac{\mu}{Y_{EG}} + m \quad (\text{II.A.4})$$

According to a mass balance on cell growth at steady state, $\mu = D$, and

$$q_{LAC} = \frac{D}{Y_{EG}} + m \quad (\text{II.A.5})$$

We have plotted the data for lactate consumption in Figure II.A.12, and the resulting straight line is described by:

$$q_{LAC} = 114 D + 1.69 \quad (\text{II.A.6})$$

Therefore, the cell yield,

$$Y_{EG} = 8.78 \text{ (gm DCW/Mole lac)}$$

and the cell maintenance coefficient,

$$m = 1.69 \text{ (mmole lac/gm DCW-hr).}$$

FIGURE II.A.12:

SPECIFIC RATE OF LACTATE

CONSUMPTION

These values define the ability of M. elsdenii to utilize lactate for growth and maintenance. The maintenance term is relatively small except at low dilution rates. It would be to our advantage to increase this term since it would reduce the flow of carbon to cell synthesis and increase the specific rate of product formation. It is particularly noteworthy that, at higher dilution rates, the production of acetate and propionate is found over longer fatty acids. This is a desirable trend since acrylate lies in the pathway of propionate production. However, because of the tendency of M. elsdenii to form other propionate and acetate, work will be stopped on this organism and attention will be focused on C. propionicum.

4. The Production of Lactic Acid from Soluble Sugars

a. Introduction

The importance of lactic acid production from soluble sugars derived from cellulosic materials has been pointed out earlier. The lactic acid will be converted to acrylic acid by C. propionicum.

Lactate production involves the utilization of Lactobacillus delbrueckii NRRL-B-445, a homo-fermentative lactobacilli which converts 95% of glucose utilized into lactic

acid. Since our final objective is to couple the fermentation of cellulosic materials (i.e. corn residue) and lactic acid degradation, we are interested in the conversion of soluble sugars into lactic acid by L. delbrueckii. Also, the utilization of resting cells of L. delbrueckii were considered as a means to convert glucose to lactic acid in an attempt to increase conversion yields with lower costs since no nitrogen source or growth factors are used. In using resting cells, we are taking advantage of the fact that cells of L. delbrueckii can produce lactic acid by a non-growth associated mechanism.

Our objectives in this phase of work are:

(1) to evaluate resting cells for lactic acid production as a function of temperature; (2) to evaluate the capacity of L. delbrueckii to convert soluble sugars produced from cellulosic fermentation (i.e. corn residues); and, (3) to optimize this conversion to achieve high yields (gm lactic acid/gm soluble sugar).

b. Materials and Methods

1. Microorganism

Lactobacillus delbrueckii NRRL-B-445, a homo-fermentative lactobacilli with an optimal growing temperature at 45°C.

2. Growth Medium

The medium utilized for growth and production of lactic acid has been described in the previous Progress Report (March - May, 1977, Page 52-53).

In the production of lactic acid by resting cells at different temperatures, the following buffer-medium composition was used:

<u>INGREDIENT</u>	<u>CONCENTRATION</u> (gm/l)
Glucose	Variable
K_2HPO_4	8.8
KH_2PO_4	6.8
$(NH_4)_3$ Citrate	2.0
Tween 80	1.0 ml
Sodium Acetate	1.0 gm/l
Salt Solution	5.0 ml
Water	1000.0 ml

where: Salt solution was:

$MgSO_4 \cdot 7H_2O$	11.5 gm
$FeSO_4 \cdot 7H_2O$	0.68 gm
$MnSO_4 \cdot 2H_2O$	2.40 gm
H_2O	100.00 ml

3. Inoculum Preparation

Cultures were stored on agar slants. Inoculum was prepared by addition of 0.1 ml of a cell suspension.

from an agar slant into a Hungate tube under anaerobic conditions. These tubes were incubated at 45°C for 15 hrs and then used as inoculum for flasks or fermentors.

4. Fermentor

A 5.0 l Magnaferm bench scale (New Brunswick Co.) fermentor was used with 3 l of working volume. pH and temperature were controlled at constant values.

5. Resting Cells Experiments

All resting cells experiments were started from a batch culture which was near stationary phase. At that point, fresh glucose buffer solution was fed at a constant rate (dilution rate = 0.1 hr). Since cells were in a non-growing state, and medium has to be removed to maintain constant volume, a Biorad Ultrafiltration cell was provided to retain cell population in the fermentor.

6. Assays

Glucose was measured by the Glucostat procedure (Worthington Biochem Corp.). Lactic acid was determined by using the lactate dehydrogenase kit from (Sigma Co.). Soluble

sugars were determined by the dinitrosalysitic acid method for determining reducing sugars. Cell growth was evaluated by turbidimetry and dry cell weight.

c. Results and Discussion

1. Lactic Acid Production by Resting Cells

The results shown in Figure II.A.13 represent the time profile for production of lactic acid by resting cells of L. delbrueckii at different temperatures.

It can be seen that the production of lactic acid is a strong function of temperature. At higher temperatures (50-60°C), the capacity for lactic acid production decays rapidly. At 40 and 45°C (which are around the optimum temperature for growth), the system is fairly stable and lactic acid is produced for 40 and 90 hrs after starting the experiment.

The concentration of lactic acid obtained was around 10 gm/l when 10 gm/l glucose were fed. Looking at Table II.A.2 and Figures II.A.14 and 15, we can further analyze these data.

Table II.A.2 was prepared using the best values from each experiment. We can see that cell concentrations varied from 3 to 4.5 gm/l producing no more than 10 gm/l

FIGURE II.A.13: LACTIC ACID ACCUMULATION BY RESTING CELLS OF L. DELBRUCKII

FIGURE II.A.14: SPECIFIC PRODUCTIVITY OF LACTIC ACID PRODUCTION BY L. DELBRÜCKII

FIGURE II.A.15: RESIDUAL GLUCOSE CONCENTRATION DURING LACTIC ACID PRODUCTION BY RESTING CELLS OF L. DELBRUECKII

TABLE A. II. 2

TEMP (°C)	CELL CONC (gm/l)	FEED RATE (l/hr)	RETEN. TIME (hrs.)	INITIAL GLUCOSE CONC. (gm/l)	RESIDUAL GLUCOSE (gm/l)	LACTIC ACID (gm/l)	CONVERSION ($\frac{\text{gm lactic}}{\text{gm glucose}}$)	VOLUME PRODUCTIV. ($\frac{\text{gm lactic}}{\text{l/hr}}$)	SPECIFIC PRODUCTIV. ($\frac{\text{gm lactic}}{\text{gm cell-hr}}$)
40	4.7	0.3	10	10	0.05	9.9	0.98	0.98	0.258
40	4.7	0.3	10	10	0.00	9.9	0.99	0.99	0.211
45*	3.0	0.3	10	37	30.0	8.1	1.05	0.728	0.24
45	2.98	0.3	10	37	30.1	7.8	1.00	0.624	0.212
50	4.02	0.3	10	10	3.5	7.8	1.0	0.78	0.19
50	3.98	0.3	10	10	4.1	6.5	0.98	0.65	0.16
60	3.18	0.3	10	10	4.4	6.6	1.07	0.86	0.17
60	3.20	0.3	10	10	4.7	5.6	1.06	0.76	0.13

FOR REFERENCE: A batch culture in log phase:

Specific productivity: $2.6 \frac{\text{gm lactic}}{\text{gm cell-hr}}$

Volumetric productivity: $5.5 \frac{\text{gm lactic}}{\text{l-hr}}$

Product accumulation: $\frac{dp}{dt} = \alpha \mu x + \beta x$

Where: $\alpha = 5.78 \text{ gm lactic acid/gm cell}$

$\beta = 0.567 \text{ gm lactic acid/gm cell-hr}$

of lactic acid in all cases. Looking at the 50 and 60°C experiments, the residual glucose was constantly increasing due to the decay of specific activity with time. At 40°C, however, residue glucose not only was maintained constant but, at zero concentration, which suggests that the system was limited by substrate availability (see Figure II.A.15).

Volumetric productivities (gm lactic acid/l-hr) are comparable in all cases and are in the range of 0.7 - 1.0. Another interesting point is that conversion yields (gm lactic acid/gm glucose conversion) are 1.0, which means that 100% of glucose consumed was converted to lactic acid. The specific productivities (gm lactic acid/gm cell-hr) for all experiments are comparable in Figure II.A.14. It is important to observe that values vary from 0.20 - 0.25. If we compare these values to the specific productivity in a batch culture which is 2.6, then we have only 10% of this value for resting cells. However, a better basis for comparison is the value for the non-growth associated term from the kinetic model for lactic acid accumulation.

$$\beta X \text{ in which } \beta \text{ is } 0.56 \frac{\text{gm lactic acid}}{\text{gm cell-hr}}$$

and, from this, we can see that we have 45% of the activity associated with maintenance. The βX term is the maximum expected value for non-growing cells.

2. Conversion of Soluble Sugars Produced from Corn Stover to Lactic Acid

Fermentation broths coming from corn stover degradation by C. thermocellum were evaluated for conversion to lactic acid. The results obtained are presented in Figures II.A.16, II.A.17 and II.A.18 and Table II.A.3.

The experiment was designed so complete medium for L. delbrueckii growth was added, or the broth was only diluted. From Table II.A.3, it can be seen that soluble sugars (reducing sugars) were fermented to lactic acid. However, only 40 to 60% of the sugars present were converted, depending upon whether medium was added or not. The soluble sugars are a mixture of carbohydrates where glucose, cellobiose, xylose and trisaccharides are the principal components and L. delbrueckii does not degrade xylose. Therefore, the residual sugar in Table II.A.3 is likely to be xylose and trisaccharides which are not metabolized by L. delbrueckii.

Another interesting observation is that conversion of soluble sugars to lactic acid shows values above 100%. This observation can be explained since the sugars composition in the broth is unknown and cellobiose and trisaccharides are present. Since the method of analysis is giving only % reducing ends on a weight basis, we have a higher initial molar sugar composition than shown in Table II.A.3.

FIGURE II.A.16: CONVERSION OF SOLUBLE SUGAR PRODUCED DURING GROWTH OF C. THERMOCELLUM ON CORN STOVER TO LACTIC ACID

FIGURE I.A.17: PRODUCTION OF LACTIC ACID BY *L. DELBRUECKII* FROM CORN STOVER HYDROLYZATES WITH NUTRIENTS ADDED

FIGURE II.A.18: PRODUCTION OF LACTIC ACID BY *L. DELBRUECKII* FROM CORN STOVER HYDROLYZATE WITHOUT NUTRIENTS ADDED

TABLE II.A.3: CONVERSION OF SUGAR FROM CORN STOVER TO LACTIC ACID

MEDIUM ADDED	SOLUBLE SUGAR (gm/l)	LACTIC ACID (gm/l)	RESIDUAL SUGAR (gm/l)	CONVERSION $\frac{\text{gm lactic acid}}{\text{gm sugar degr.}}$	% CONVERTED
Complete	2.1	1.5	1.0	1.22	58.0
Complete	4.2	2.35	1.89	1.01	55.0
Complete	8.4	4.65	4.70	1.22	53.6
None	2.1	1.3	0.9	1.36	47.6
None	4.2	2.3	1.79	1.27	42.6
None	8.4	5.1	3.8	1.34	45.3
None	16.8	7.4	7.3	1.01	43.45

TABLE II.A.4: CONVERSION YIELD OF SUGAR FROM CORN STOVER TO LACTIC ACID

SAMPLE	AGE (hrs)	RESIDUAL SUGARS (gm/l)	LACTIC ACID (gm/l)	CONVERSION $\frac{\text{gm lactic acid}}{\text{gm sugar}}$	SUGAR CONSUMPTION (%)
1	0	4.9	0	0	0
2	2	4.54	0.48	1.33	7.0
3	4	4.0	1.20	1.44	18.4
4	6	3.8	1.70	1.5	22.5
5	8	3.1	3.1	1.47	36.7
6	20	2.9	3.35	1.40	40.8

Looking at Figure II.A.16, the conversion of soluble sugar decreases slightly as a function of the initial concentration of sugars. All values are taken from Table II.A.3. This occurs whether or not medium was added. The results suggest a possible inhibiting or negative factor present in the C. thermocellum broth. This observation has to be examined further.

In order to complete the picture of conversion of soluble sugars to lactic acid, we ran a fermentation using a C. thermocellum broth on corn residues without cell separation or solids removal. Also, no medium components were added for growth of L. delbrueckii. The results are shown in Figure II.A.19. The sugars consumed was only 40% of the 4.9 gm/l present; this is in agreement with previous results. The conversion yield to lactic acid was again higher than 100% and about 1.4 gm lactic acid/gm sugar consumed.

From the results shown, we can conclude the following:

The conversion of soluble sugar from corn stover to lactic acid using L. delbrueckii is possible. However, at the present time, only 40-60% of the total sugar produced or converted to lactic acid depending upon whether medium for growth of L. delbrueckii is added to the C. thermocellum broth. The conversion yield (% lactic acid/gm sugar) is higher than 100 - 130%.

FIGURE II.A.19: LACTIC ACID ACCUMULATION BY L. DELBRUECKII
USING SOLUBLE SUGARS FROM CORN STOVER

d. Future Work

While encouraging, the results from conversion of corn stover to lactic acid show room for improvement. There are two major areas where work needs to be done. A more accurate analysis of the utilization of reducing sugar which reflects the initial and final composition of the sugar mixture. This is now possible since our efforts on HPLC analysis of sugar allows us to define the composition of the hydrolyzate. It appears that our apparent low utilization of sugar, i.e. 40-50%, is, to a significant extent, an accounting problem resulting from inaccuracies in sugar analysis.

Second, we need to maximize the rate of lactic acid production from the hydrolyzation. This will mean an increase in stabilization of whole cell systems and improvement in specific rates of lactate production. Effort is required in the elimination of inhibiting materials which may be present in the corn stover hydrolyzate.

5. Aerobic Oxidation of Propionic Acid to Acrylic Acid

a. Introduction

The coenzyme A activated derivative of acrylic acid is believed to be an intermediate in the metabolism of

propionic acid to lactic acid (Progress Report, June 1 through August 31, 1977). E. coli, Acinetobacter sp. and P. aeruginosa, among other aerobic organisms, can utilize propionate as the sole source of carbon and energy. Four-carbon compounds can be generated via the action of malate synthase, α hydroxy glutamate synthase, propionyl CoA carboxylase and PEP carboxylase, either singly or in combination as shown in Figure II.A.20. An attempt is being made to exploit the pathway converting propionate to acrylate. E. coli W and Acinetobacter sp. 401-N were chosen as suitable organisms for this investigation.

b. Methods and Materials

1. Conditions for gas chromatographic analysis of propionic acid, acrylic acid, acetic acid, and lactic acid, as well as conditions for the growth of soil isolates have been previously reported.

2. Escherichia coli W (ATCC 9637-2), obtained as a lyophilized culture was reactivated in nutrient broth and stored in minimal salts medium with carbon sources of specified concentrations. Cells were grown at 37°C in shaker flasks.

3. Isocitrate lyase negative mutants were searched for in ethyl methane sulfonate treated E. coli W cells,

FIGURE II.A.20: PATHWAY FOR METABOLISM AND GENERATION OF 4 CARBON ATOM COMPOUNDS FROM PROPIONATE IN E. COLI

following penicillin enrichment. Mutants were selected for their inability to use acetate, while still being able to grow on succinate. Organisms appearing as small colonies were assayed for absence of isocitrate lyase by spectrophotometric measurement of glyoxylate produced in the presence of phenyl hydrazine, by addition of isocitric acid to cell free extracts.

c. Results and Discussion

Past work focused attention on the isolation of potential acrylate producing organisms from soil. Various organisms were isolated, of which non-mycelial organisms were screened for their growth characteristics and acrylate formation. Three groups of organisms were identified on the basis of propionate and acrylate utilization.

TABLE II.A.5

<u>#</u>	<u>GROWTH ON PROPIONATE</u>	<u>GROWTH ON ACRYLATE</u>	<u>GROWTH ON PROPIONATE IN PRESENCE OF ACRYLATE</u>
1	+	+	+
2	+	-	+
3	+	-	+

None of these organisms produced detectable quantities of extracellular acrylate. Exponential phase and stationary phase cells, when resuspended in propionate, did not accumulate acrylate, acetate or lactate into the medium.

The search for acrylate production in wild type uncharacterized cells proved fruitless. It appeared that further investigation would require manipulation of a known system of propionate metabolism.

E. coli W was such an organism. The literature strongly suggested the pathway via lactate to acrylate shown in Figure II.A.20. We found that long lag periods preceeded growth of the organism on acetate and propionate. Lactate, succinate and glucose support growth readily. 1% acrylate did not inhibit a normal yield of cells on glucose. The organism required glucose or yeast extract supplemented medium to initiate growth on propionate.

Reportedly, the organism converts propionyl CoA to methyl malonyl CoA at propionate concentrations $<5\text{mM}$, whereas at higher concentrations (21 mM), propionate is metabolized - predominantly via lactate, and hence acrylate. We believe that a mutant is capable of operating the glyoxylate yet would metabolize propionate only via acrylalyl CoA at high propionate concentrations. A second mutation preventing conversion of acrylyl CoA to lactyl CoA would possibly cause the organism to accumulate acrylic acid in stoichiometric quantities (1:1).

In addition to E. coli, the oxidation of propionate by an Acinetobacter sp. strain H01-N, an aerobic gram-negative, hydrocarbon-oxidizing bacterium, is under study. This organism is related to Moraxella lavoffi which oxidizes propionate to acetate via acrylate (B. Hodgson and J. D. McGarry, Biochem. J., 107: 19, 1968). Work with this organism will also concentrate on the selection of a mutant which will grow on lactate, but not propionate or acrylate. Mutagenesis will be performed with EMS followed by antibiotic enrichment with ampicillin and cycloheximide. This procedure has been very successful in isolating a number of mutant classes, including an isocitrate dehydrogenase deficient mutant (J. A. Cruze, personal communication).

Preliminary growth experiments have shown that Acinetobacter sp. H01-N will grow readily on propionate and acrylate. The presence of 0.2 g/l p-methoxyphenol did not inhibit growth on acrylate.

d. Future Work

Isolation of the double mutant isocitrate lyase negative mutant incapable of growing on lactate.

- . Using the above mutants to examine their ability to convert propionate to acrylate in growth, in resting cell suspensions and in cell-free extracts.

- . Examination of E. coli to effect acrylate production anaerobically.

- . Examination of acinetobacter to effect the oxidation of propionate to acrylate.

B. Production of Acetone and n-Butanol by Fermentation

1. Introduction

The results from the fermentation of Clostridium acetobutylicum reported in the last Quarterly Report (COO-4198-3) indicated that the growth and product formation were inhibited by the low pH during cultivation. To increase our understanding on the kinetics of this fermentation, it is necessary to eliminate this inhibitory pH effect. Therefore, it was decided that studies of this fermentation with pH control be performed to see whether increased product concentration, as well as the rates of growth and product formation, can be achieved.

In this report, the results from these fermentations will be presented. In addition, studies on the inhibitory effects of solvents and their concentrations were also performed. Biochemical studies on finding ways to block acetone production have also been performed. Lastly, the possibility of using in situ extraction during fermentation of butanol to enhance solvent production and reducing solvent recovery cost has also been initiated. The significances of these results will be discussed.

2. Materials and Methods

a. Microorganisms

Clostridium acetobutylicum strain ATCC 824 was used (see previous progress reports).

b. Medium

Medium used was the same as that reported earlier. Slight modifications of the concentrations, yeast extract and glucose were made in some cases. These changes will be specified along with the presentations of the results.

c. Fermentor

A 5-liter fermentor with 2.5 liters working volume was used. The fermentor was equipped with a single impeller-agitation system driven by a 1/8 HP motor. Temperature of the fermentor was controlled by a heat exchanger loop in the fermentor. A pH electrode (Ingold Electrode Co.), together with a Speedomax H pH controller (Leeds and Northrup Co.) and a Masterflex pump (Cole-Palmer Co.), were used for monitoring constant pH. NaOH 2.0 N was used as the titrant. In all pH-controlled fermentations, the pH was maintained at a value of 5.0.

d. Inoculation Procedure

The culture was grown from spores after heat-shock treatment in corn mash medium (see previous reports). After incubation at 37°C for 18-24 hours, 2.5 ml of the corn mash was used to inoculate 250 ml of soluble medium in an anaerobic flask. The culture was incubated at 37°C for 12 hours. The entire content of the flask was then used for inoculation of 2.5 liter fermentor.

e. pH Controlled Fermentations

After inoculation, the cell density was monitored by turbidity measurement every four hours. One hundred ml of broth was reviewed every four hours for the turbidity measurements. Two forty-ml aliquots were centrifuged for dry cell weight determination. Five ml of the supernatant was frozen in vials for product and substrate analyses.

f. Methods of Analysis

See previous reports.

g. Growth Inhibition Study

A culture started from spores was allowed to grow in an anaerobic flask containing 300 ml soluble medium. The growth was monitored by turbidity measurement. When the culture was in the early exponential phase, 5 ml of the culture was used to inoculate each of a series of Huntgate tubes. These tubes each contained 5 ml of fresh medium and various concentration of n-butanol or acetone. The absorbance of these tubes were monitored every half hour to determine the effect of the different solvents on growth.

3. Results and Discussions

a. pH Controlled Fermentations

Four pH-controlled fermentations, Run Nos. 5, 6, 7 and 8, have been performed of which three were identical in methodology and results. In these experiments, the initial glucose concentrations were maintained in the range of 10 to 30 g/l. Glucose, yeast extract and salts (excluding sodium chloride and cysteine) were added during the course of the fermentation. These feedings were programmed by observing the rate of sodium hydroxide addition for constant pH control. The amount of sodium hydroxide required corresponds to the

amount of acid formed and thus yielded a qualitative indication as to the depletion of glucose during the course of the fermentation. The results from three fermentations were similar and a representative run (No. 8) is shown in Figure II.B.1. In this Figure, only the concentrations of the cell mass, acetone and n-butanol are shown. The arrows on the Figure indicate the time when addition of nutrients were made. The fermentation was terminated after 46 hours. Since it was found that the nutrients added a couple hours before the 46th hour did not show any further increase in the cell or product concentrations. A more detailed discussion on these fermentations will be presented later in this section of this report.

One of the problems encountered during the performance of the fermentations stated above was the lack of knowledge as to the depletion of nutrients. This is because we have no on-line analysis for determining the concentrations of glucose, butanol, acetone or butyric acid. As a consequence of this, all of three fermentations (Run Nos. 5, 6 and 8) encountered nutrient deficiency during the course of the fermentation. To overcome this problem, Run 9 was performed with an initial glucose concentration of 95 g/l and yeast extract at 15 g/l. The results of Run 9 are shown in Figure II.B.2. There was a lag time of about 30 hours after inoculation. The reason is probably due to the high initial glucose concentration which caused a shock to the inoculum. However, after the recovery

FIGURE II.B.1: PRODUCTION OF ACETONE AND BUTANOL BY *CLOSTRIDIUM ACETOBUTYLICUM* (ATCC 824) THROUGH pH CONTROL (pH=5.0) AND GLUCOSE ADDITION (RUN NO. 8)

FIGURE II.B.2: PRODUCTION OF ACETONE AND BUTANOL BY CLOSTRIDIUM ACETOBUTYLICUM(ATCC 824) THROUGH pH CONTROL (pH=5.0): RUN NO. 9

from the initial lag, the cells were able to grow at a specific growth rate of 0.39 hr^{-1} . This corresponds to the maximum specific growth rate which has been observed in other batch culture studies. The cells were able to consume over 90% of the initial glucose in about 20 hours.

From the data of these four controlled pH fermentations, detailed analyses were performed and are summarized in Tables II.B.1 and II.B.2. In Table II.B.1, the initial glucose concentration and the subsequent amounts added as well as the final glucose concentration are tabulated. Although the cell growth and product formation ceased (see Figures II.B.1 and II.B.2), it can be seen from Table II.B.1 that the depletion of glucose was not the reason for this behavior. On the other hand, if one examines the final concentrations of the solvent, one observes that n-butanol ranged between 10 to 13 gm/l and acetone ranged between 4.5 to 7 gm/l. One is tempted to hypothesize that the end product is the causative agent in the observed termination of these fermentations.

It should be mentioned, however, although these fermentations ended when the mixed solvent concentrations reached about 15 to 20 gm/l, these concentrations are quite comparable to those reported in the industrial production of acetone and butanol. Typically, the past publications on the industrial processes showed a mixed solvent concentration of about 20 gm/l at about the same ratio of acetone to butanol as our studies.

TABLE II.B.1: PRODUCTION OF ACETONE/BUTANOL BY CLOSTRIDIUM ACETOBUTYLICUM
WITH CONTROLLED pH AT 5.0

RUN NO.	INITIAL GLUCOSE CONCENTRATION	SUBSEQUENT GLUCOSE ADDED	FINAL GLUCOSE	CELL MASS	n-BUTANOL	ACETONE	ETHANOL	BUTYRIC ACID	MAXIMUM SPECIFIC GROWTH RATE
	(gm/l)	(gm/l)	(gm/l)	(gm/l)	(gm/l)	(gm/l)	(gm/l)	(gm/l)	(Hr ⁻¹)
5	20	74.7	26.5	4.1	9.7	4.5	N.D.	2.6	0.13
6	10	95.4	26.0	2.3	11.2	5.7	1.6	1.2	0.29
8	30	83.6	36.0	4.3	13.2	6.9	2.1	1.2	0.11
9	94.7	0	8.7	5.8	11.7	6.5	1.3	3.6	0.39

N.D.: not done

Another important parameter in the production of solvents by fermentation is the yield (gm of solvent/gm glucose consumed) which can be achieved as compared to the theoretical maximum. The results from the four pH-controlled fermentations are shown in Table II.B.2. The yield of butanol ranges from 0.13 to 0.21 gm of product per gm of glucose consumed whereas the yield of acetone ranges from 0.07 to 0.11. The highest mixed solvent yield was Run No. 8 where the yield was 0.32 gm of solvent per gm of glucose consumed. The theoretical maximum yield of mixed solvent was calculated (see 1st Quarterly Report, COO-4198-1) to be 0.38 gm solvent per gm of glucose. It can be seen from our best results that our strain of Clostridium acetobutylicum is capable of achieving about 84% of the theoretical maximum yield. If we compared the published results from the industrial production of acetone and butanol, typically 0.30 to 0.35 gm of mixed solvent is produced per gm of sugar consumed. We therefore conclude from the results in Tables II.B.1 and II.B.2 that we are able to produce the mixed solvents in concentrations and yields comparable to those achieved industrially. Furthermore, comparison with the theoretical yield analysis shows that there is not much room for improvement of yield as a means of cost reduction for this fermentation.

Further analyses of the results from the controlled pH fermentations were performed to elucidate the kinetics of solvent production. In particular, we were interested to find whether the production of the solvents is associated or

TABLE II.B.2: YIELD OF MIXED SOLVENT PRODUCTION BY
CLOSTRIDIUM ACETOBUTYLICUM THROUGH
pH-CONTROL FERMENTATIONS (pH=5.0)

RUN NO.	TOTAL GLUCOSE CONSUMED (gm/l)	YIELD (gm product/gm glucose)			
		n-BUTANOL	ACETONE	TOTAL MIXED SOLVENT*	CELLS
5	68.2	0.16	0.18	0.24	0.08
6	79.4	0.15	0.07	0.22	0.04
8	77.6	0.21	0.11	0.32	0.07
9	86.9	0.13	0.07	0.20	0.07

* Theoretical maximum yield of mixed solvent = 0.38 gm solvent/gm glucose

not associated with cell growth. This type of information is extremely valuable in our future research as to the plausible avenues to take with respect to the optimal process which should be employed for the production of acetone and butanol. Although four pH-controlled fermentations have been performed, we will present only the results from one such fermentation. It should also be mentioned that the fermentation (Run No. 9) which we selected for analysis in this report represents the best results of the four fermentations performed.

The data in Figure II.B.2 were analyzed in the following manner. The specific growth rates during the different times of fermentation from this batch culture were calculated through graphical differentiation. In addition, the specific productivity which is defined as the gm of solvent produced per gm of cell per hour was also obtained through graphical differentiation of the data in Figure II.B.2. The relationship between the calculated specific growth rates and specific productivities is shown in Figure II.B.3. The results show an inverse relationship between specific productivity to specific growth rate for both acetone and butanol formation. This is to say that the biosynthetic capability of the microorganism with respect to solvent production is greater when the cells are growing at lower rates. Furthermore, if one extrapolates the data to zero specific growth rate the specific productivity reaches the maximal value.

FIGURE II.B.3: RELATIONSHIP BETWEEN SPECIFIC GROWTH RATE AND SPECIFIC PRODUCTIVITY OF SOLVENT OF CLOSTRIDIUM ACETOBUTYLICUM (ATCC 824) UNDER pH CONTROL (RUN NO. 9)

The qualitative and quantitative findings of this phenomenon is extremely encouraging for the following reasons. It was proposed in an original proposal to examine the use of cell recycle in continuous culture to effect increased volumetric productivity (gm solvent/l-Hr) of the solvent. The results in Figure II.B.3 show that our rationale is sound and should be explored. For example, by the use of cell recycle, the concentration of the cells in the fermentor can be readily increased. Furthermore, if resting or non-growing cells can indeed achieve maximal product formation, this combined with the use of cell recycle should lead to an optimal system for solvent production. The application of one or both of these principles should aid in the reduction of the overall cost for solvent production.

The final analysis of the data was to determine the maximum volumetric productivity (gm solvent/l-Hr) which has been attained with the controlled pH, batch fermentation. Here again, since we were interested to know the maximum value, the analysis was performed using the best fermentation (Run No. 9 - Figure II.B.2). If one ignored the lag phase up to the 35th hour of fermentation, the volumetric productivity for butanol between 35 to 47 hours was calculated to be 1.1 gm butanol/liter-hour. Since this result is from a batch fermentation, one can conclude that significantly higher volumetric productivity can be achieved using continuous fermentation, particularly if cell recycle is employed.

b. Effect of Acetone and Butanol Concentrations on the Growth of Clostridium acetobutylicum

From the results on the controlled pH fermentations, we have observed that the concentrations of butanol and acetone never exceeded 13 gm/l and 7 gm/l respectively. We had hypothesized earlier that the product might be the causative agent in the termination of the fermentations. In order to test this hypothesis, we have performed a number of studies on the effect of acetone and butanol concentrations on the growth of Clostridium acetobutylicum. The results are summarized in Figure II.B.4.

We have employed an inhibition index in the analysis of our results. This inhibition index is defined as:

$$\text{Inhibition Index} = 1 - \mu/\mu_{\max} \quad \text{---} \quad (\text{II.B.1})$$

where: μ = specific growth rate in the presence of acetone or butanol (Hr^{-1}).

$\mu_{\max}$ = maximum specific growth rate in the absence of acetone or butanol = 0.39 Hr^{-1} .

From the results in Figure II.B.4, it can be seen onset of growth inhibition by butanol occurs at about 13 gm/l. At 17 gm/l

FIGURE II.B.4: EFFECT OF ACETONE AND BUTANOL ON THE INHIBITION OF GROWTH OF CLOSTRIDIUM ACETO-BUTYLICUM (ATCC 824)

of butanol total growth inhibition results. On the other hand, at 35 gm/l of acetone (highest value tested), there is no inhibition of growth. From these results, we conclude that the butanol is the toxic agent which was responsible for the termination of the fermentation. Furthermore, it is our conclusion that this is the reason why the published results on the industrial production of acetone/butanol were never able to exceed butanol concentration greater than 15 gm/l. Research to overcome this problem must be performed in order to overcome this bottleneck.

c. Effect of Acetopyruvate on Acetone-Butanol Fermentation

In the Third Quarterly Progress Report (COO-4198-3, Page 91), we reported on investigations which were designed to block acetone production. It was thought that eliminating diversion of carbon substrate to end products other than butanol (i.e. acetone) would result in increased productivity of butanol. Initial experiments were conducted and it was found that acetopyruvate, an analog of acetoacetate, known to inhibit acetoacetate decarboxylase in vitro was found to inhibit growth (Table II.B.4 of Report COO-4198-3). These results suggested that some aspect of metabolism other than acetone production was affected. It was postulated that acetopyruvate was

able to block butyric acid formation and thereby prevent the cell from maintaining an electron charge balance. For this reason, it was desirable to test for the reversal of acetopyruvate inhibition by addition of butyric acid to the initial fermentation medium.

In the initial experiments, addition of butyric acid to soluble medium containing 4 mM acetopyruvate did not reverse acetopyruvate inhibition. This was probably due to the fact that addition of butyric acid lowered the pH of the fermentation broth and prevented growth.

The experiment was then repeated using sodium butyrate and at concentrations above 20 mM were found to reverse growth inhibition by acetopyruvate. The effect of 20 mM butyric acid and higher concentrations on the fermentation with and without acetopyruvate are summarized in Table II.B.3. Acetone production in the presence of 4 mM acetopyruvate was not inhibited. Therefore, there appears to be little value in using acetopyruvate in increasing the butanol yields from glucose.

It should be noted, however, that addition of sodium butyrate does significantly raise the final concentration of butanol in the fermentation broth. Although the results are best described as preliminary, it is likely that development of high butyric acid concentration early in the fermentation may increase the rate of butanol fermentation.

TABLE II.B.3

Effect of Butyric Acid on Inhibition of Growth by Acetopyruvate with Cl. acetobutylicum

Acetopyruvate Addition	Butyric Addition g/l	Final OD ₆₆₀	Initial pH Adjusted in NaOH	Final pH	Acetone g/l (final conc.)	Butyric g/l (final conc.)	Butanol g/l (final conc.)
+4mM	-	-	4.61	4.78	-	-	-
-4mM	-	1.01	4.61	3.79	.06	1.6	.7
+4mM	1.8	.92	5.01	4.4	.3	1.7	2.2
-4mM	1.8	.68	5.01	4.3	.14	1.6	1.6
+4mM	3.5	.67	4.98	4.8	.3	2.1	2.1
-4mM	3.5	.68	4.98	4.6	.4	1.1	4.2
+4mM	6.7	1.1	5.14	5.10	.53	1.9	5.0
-4mM	6.7	1.1	5.14	5.14	.4	1.9	4.5

To soluble medium butyric acid is added, pH is adjusted with 1N NaOH and ~4mM acetopyruvate is added; a 0.2ml inoculum from an overnight culture of Cl. acetobutylicum strain ATCC 824 is added and the cultures are incubated for 48 hours at 37°C.

d. Extraction of Butanol to Overcome Toxicity

1. Introduction

Production of acetone and n-butanol by Clostridium acetobutylicum takes place at the end of the logarithmic growth phase. We have shown that the butyric acid production follows closely with cell growth. When butyric acid has accumulated, the conversion of the acid to butanol occurs and allows butanol to accumulate. We have also shown that butanol is toxic to growth. Inhibition of growth begins at concentration of 13 gm/l butanol. On the other hand, similar and higher concentrations of acetone were not inhibitory. The inhibitory effect of butanol could explain the observations that, in most of our pH-controlled batch fermentations, the maximum concentration of butanol detained was never greater than 14-16 g/l although glucose was fed at a sufficient rate.

In order to overcome product inhibition, two approaches could be envisaged: (1) removal of the toxic product from the broth; or (2) development of high tolerance strains. The first approach will be discussed in this part of the report.

The removal of butanol continuously from a fermentation broth could be achieved through solvent extraction. Solvent extraction is attractive since butanol is

sparingly soluble in water (9.1 ml in 100 ml H_2O at $25^{\circ}C$), and highly soluble in lipophilic solvents. Extraction by lipophilic solvents at neutral pH has the advantage that acetic acid and butyric acid are charged and therefore likely to remain in the broth. The availability of these acids to the microorganism is essential for the production of acetone and butanol. Another advantage of solvent extraction is in product recovery. Recovery from broth involves the fractionation of an azeotrope (b.p. of butanol is $117.7^{\circ}C$). A high boiling point solvent will enable separation by a simple distillation process, and will save on energy as well as on the equipment required.

The following criteria were used for choosing an organic solvent:

- . High extraction of butanol from the broth versus minimal solubility in the broth.
- . Minimal toxicity to the microorganism and to the enzyme systems responsible for the conversion of acid to solvents.
- . Easily recovered for reuse and low cost.
- . Density different than the broth and ability to form two phases upon standing.

2. Materials and Methods

Microorganism used was C. acetobutylicum ATCC 824. Media, inoculation procedures, and methods of analysis has been already reported (see previous quarterly reports COO-4198-1, -2).

"Simulated broth" was used for measuring the apparent distribution. Its composition was:

0.75 g	KH_2PO_4
0.75 g	KH_2HPO_4
1.0 g	NaCl
10 g	Glucose - H_2O
13.5 g	Butanol
7.0 g	Acetone
2.0 g	Butyric Acid
1.0 g	Acetic Acid

water to 1 liter, pH = 6.0 adjusted with NaOH. The "simulated broth" reflects the main constituents in the actual broth at the end of a pH-controlled fermentation (see previous reports).

Determination of apparent distribution was accomplished in the following fashion. One-ml of the organic solvent and 1 ml of the "simulated broth" were carefully pipetted into a 5 ml glass vial. The glass vial was tightly stoppered with a Teflon lined cap. The vials were agitated for 10 min at room temperature, and set aside for complete separation of phases for additional 20-30 min. One-half ml of the

water phase was removed for analysis.. For each separate solvent, duplicate extraction vials were analyzed. Control vials containing "simulated broth" in the absence of solvent were similarly treated. The disappearance of butanol, acetone and butyric acid from the water phase of the extracted samples was compared to the control. "Apparent distribution" was calculated for acetone and butanol. "Apparent distribution" was defined as the ratio between the concentration of product in the organic phase to the concentration of the product in the water phase. These values have no relation to the distribution coefficient used in the literature and they are used here for the sake of comparison among solvents.

The effect of solvents on growth was determined in the following manner. The effect on growth was determined while growing the culture in Hungate tubes.

Tubes containing 5 ml sterile liquid medium were inoculated with an actively growing culture (10% inoculum). Absorbance at 660 nm was used to follow growth. The entire sealed tube was placed in a Turner photometer, absorbance read against similar tube containing water. Two hours after inoculation, 2 ml of non-sterile solvent were injected through the septum. The tubes were mixed occasionally and absorbance was monitored until cessation of growth. When fermentation was terminated, the organic phase was removed and cells spun down. The pH of the clear broth, as well as butyric acid, butanol and acetone production were determined.

The effect of solvent on production of acetone and butanol was performed in Hungate tubes. A large inoculum (2 ml) of a 24-hour old culture was transferred to a series of Hungate tubes having 5 ml of sterile medium at the following compositions:

Medium A: Normal liquid medium, described before.

Medium B: Medium A + 2.07 g/l butyric acid,
pH = 4.4

Medium C: 0.75 g/l KH_2PO_4
0.75 g/l K_2HPO_4
1 g/l NaCl
0.50 g/l Cysteine
20 g/l Glucose
pH = 6.0

Medium D: Medium C + 2.5 g/l butyric acid,
pH = 6.0

Three hours after inoculation, 3 ml of solvent were added to all the tubes except to the control. Tubes were occasionally mixed and incubated for 60 hours at 37°C. Upon termination of the fermentation, the organic phase was separated, absorbance was monitored and then cells were spun down and the clear broth was monitored for final pH and for concentration of products.

3. Results and Discussion

a. "Apparent distribution" of various Solvents

The properties of a large number of solvents were compared by extraction of "simulated broth". The equilibrium concentrations of butanol and acetone in the water phase and in the organic phase were determined.

The "apparent distributions" for butanol and acetone were calculated and presented in Table II.B.4. The results for butyric acid were not included since it was found that for all solvents tested and at neutral pH 95% or more of the acid remained in the water phase. Acetic acid could not be determined accurately by our methods. It is assumed that its distribution is similar to the butyric acid.

From our results in Table II.B.4, it is evident that, in order to extract butanol efficiently, it is required that the solvent molecule will have a group available for the formation of hydrogen bonds.

It is clear that alcohols and esters are better solvents for extraction than paraffins and aromatic compounds. Those results could be anticipated from basic organic chemistry principles.

As the carbon backbone of the ester or alcohol is increased, the extraction power diminishes. A

TABLE II.B.4: SOLVENTS TESTED (AT 1:1 VOLUME RATIO)
FOR BUTANOL EXTRACTION

<u>Solvent</u>	<u>APPARENT DISTRIBUTION</u> <u>= C Solvent/C Water</u>	
	<u>Acetone</u>	<u>Butanol</u>
Cyclohexane	0.22	0.27
Benzene	1.19	1.07
CCl ₄	0.52	0.49
C ₁₄ -C ₁₅ Parafin	0.112	0.110
Kerosene	0.197	0.190
Ligroin	0.177	0.206
Silicone Oil	0.177	0.228
n-Butyl Ether	0.332	1.19
1-Octanol	0.51	7.68
1-Decanol	0.34	4.12
Ethyl Acetate	2.0	4.02
Ethyl Hexanoate	0.56	2.12
Ethyl Heptanoate	0.47	1.81
Ethyl Octanoate	0.52	1.81
Ethyl Decanoate	0.43	1.59
Ethyl Palmitate	0.27	0.82
1,3, Heptandial Dipropionate	0.58	2.41
1,3, Nonandiol Dipropionate	0.49	1.71

plausible explanation to this behavior is that, in larger molecules, the groups responsible for hydrogen bonding are less available, reflecting also the fact that larger molecules have lower solubility in water.

b. Effect of Various Solvents on Growth

The toxicity of various solvents and the effect on growth was determined (Figure II.B.5). It was found that the paraffins and the silicone oil do not effect growth (Table II.B.5).

A growth curve similar to the control was found for very long esters and diesters. However, alcohols up to decanol are highly toxic to growth (higher alcohols are solids and not useful for liquid extraction).

Among the esters, it is found that the longer the hydrocarbon chain, the lower is the toxicity.

The final values for the production of acetone, butanol and butyric acid are given in Table II.B.6 for several solvents. Since this experiment was terminated before the production of butanol took place, the concentration of butyric acid reflects the conditions of the culture. Benzene, e.g., which is toxic, stops growth and very little production of acid is measured. Paraffin which is not toxic allows production of acid similar to the control.

TABLE II.B.5: EFFECTS OF VARIOUS SOLVENTS ON GROWTH OF
CL. ACETOBUTYLICUM

SOLVENT USED	EFFECT ON GROWTH (relative scale)*
Carbon Tetrachloride	(-)
Benzene	(-)
n-Butyl Ether	(-)
1-Octanol	(-)
1-Decanol	(-)
C ₁₄ -C ₁₅ Paraffin	(++)
Silicone Oil	(++)
n-Butyl Acetate	(-)
n-Hexyl Acetate	(-)
Ethyl Hexanoate	(-)
Ethyl Heptanoate	(+-)
Ethyl Palmitate	(++)
1,3 Heptandiol dipropionate	(+)
1,3 Nonandiol dipropionate	(++)

* Explanation for the relative scale used:

(++) = max. growth similar to control

(-) = no growth at all

(+-) = some growth

Refer also to Figure II.B.5 for comparison

FIGURE II.B.5: GROWTH OF CLOSTRIDIUM ACETOBUTYLICUM IN PRESENCE OF DIFFERENT SOLVENTS

TABLE II.B.6: PRODUCTION OF ACETONE, BUTANOL AND BUTYRIC ACID
IN THE PRESENCE OF SOLVENTS AND AT THE END OF
THE LOGARITHMIC GROWTH

SOLVENT USED	CONCENTRATION OF PRODUCT AFTER 24Hr		
	ACETONE (gm/l)	BUTANOL (gm/l)	BUTYRIC ACID (gm/l)
Ethyl Heptanoate*	0.043	0.076	0.692
Benzene	0.020	0.060	0.464
C ₁₄ -C ₁₅ Paraffin*	0.036	0.286	2.249
Control*	0.055	0.449	2.324

* Average of two experiments

c. Effect of Various Solvents on The
Production of Acetone and Butanol

The effects of various solvents on a growing culture could be entirely different from the effects of the same solvents on resting cells. Since the production of butanol occurs in the resting culture, it was necessary to evaluate the effect of various solvents on the culture at that stage. In this experiment, a large inoculum (30%) of a 24-hr old culture was used. It was anticipated that the culture would reach stationary phase in a short time and solvent addition will take place after termination of growth, and before the production of much of the butanol. We also attempted to evaluate the role of butyric acid and butyrate in inducing the enzymes necessary for butanol production. The same inoculum was introduced therefore, to four different media. The results for the production of butanol are presented in Table II.B.7. The concentrations of acetone are not presented for simplicity, but they reflect the same behavior exhibited by the butanol. Concerning the production of butanol, the following conclusions may be drawn:

The effects of solvent on the growth are similar to the effects on the production, e.g. a solvent that inhibits growth will lower the production of butanol.

TABLE II.B.7: BUTANOL PRODUCTION IN THE PRESENCE OF SOLVENT PHASE

SOLVENT USED	BUTANOL CONCENTRATION IN THE BROTH (g/l)				CALCULATED AMOUNT OF BUTANOL (mg) *	
	MEDIUM A	MEDIUM B	MEDIUM C	MEDIUM D	MEDIUM A	MEDIUM D
1,3 Heptandiol dipropionate	0.45	0.19	0.17	0.94	5.3	13.4
1,3 Nonandiol dipropionate	0.37	0.32	0.22	1.82	3.2	15.9
Ethyl Heptanoate	0.23	0.22	0.19	0.19	2.0	1.7
Ethyl Octanoate	0.20	0.16	0.15	0.69	1.8	6.1
Ethyl Decanoate	0.36	0.31	0.22	0.95	3.1	8.2
Ethyl Palmitate	0.47	-	0.30	2.33	3.7	18.2
Control 1	0.38	0.39	0.17	2.68	2.7	18.8
Control 2	1.00	1.14	0.41	4.33	7.0	30.3

* Calculation based on "apparent distribution" (see Table II.B.4)

. The longer the carbon chain, the higher is the production. In terms of toxicity to the micro-organism, ethyl palmitate is a better solvent than ethyl decanoate and in turn ethyl deconoate is better solvent than ethyl octanoate.

. In order to get maximum production, the presence of butyrate ion and not butyric acid is essential. In medium B, the production was similar to medium A, although butyric acid was included in the medium. Medium D was the best, since it include glucose and butyrate at almost neutral pH. Very little production is found in medium C which includes only sugar and salts.

The right side of Table II.B.7 includes the calculated amounts of butanol produced based on the "apparent distribution" measured before. The values obtained here are for comparison only. It is evident that ethyl palmitate and 1,3 Nonandiol dipropionate are the solvents of choice. However, both solvents are expensive and their extractive power is very low. It remains to be proved whether those solvents increase the productivity and eliminate the inhibition imposed by butanol.

Economical analysis of the use of those solvents is necessary. If it is found that the use of those solvents is not practical and it is desired to use less expensive

solvents, problems of toxicity will arise. In that case, we might try to perform the extraction outside the fermentor. As suggested before, we intend to perform future fermentations in a continuous mode with cell recycle. The addition of a continuous extractor in such a setup is relatively easy. The solvent of choice in this case might be 1-octanol which is less expensive (40¢/lb) and has a high "apparent distribution" (equal to 6.3 see Table II.B.4). The economical analysis of the extraction process and the above mentioned experimental protocol will be performed in the future.

4. Future Studies

From the results of our past studies, there are a number of avenues worthy of exploration for the acetone/butanol fermentation. In the upcoming contract period, we plan to perform the following studies.

- . Increase productivity through continuous fermentation.
- . Examine the kinetic behavior of this fermentation using continuous culture data.
- . Initial cell recovery through flocculation to achieve cell recycle.
- . Continuation of butanol extraction to remove toxicity and reduce recovery cost.
- . Examine the production of acetone without butanol formation.

C. Process and Economic Analysis of Acetone and Butanol Production

1. Introduction

The production of acetone/butanol by Clostridium acetobutylicum has been analyzed with respect to the equipment requirement and manufacturing cost. The objective of this analysis was to pin-point the areas of cost sensitivities. These cost identifications represent what we believe are reasonable technically at this time. They, therefore, offer areas for research and development exploration where cost reduction might be achieved.

2. Basis for Design

The process information for solvent production for this analysis was obtained primarily from Beesch (1952), Peterson and Fred (1932) and Dyr, et al. (1958). Product recovery was based on the data of Othmer (1936). Equipment costs were determined using the publications of Dryden and Furlow (1969) and Peters and Timmanhaus (1968). To account for inflation of the equipment, the Marshall and Stevens indices were employed: M & S = 270 (1968) M & S = 492 (1977). The cost for raw materials were obtained from Chemical Marketing Reporter (January, 1977) and the Commodity Year Book (1976).

The quantitative process information used for this analysis is summarized in Table II.C.1.

3. Process Flow

The process consists of three parts: medium preparation, fermentation and product recovery. The flow sheet of the process is shown in Figure II.C.1. Supply of sugar is mixed with required mineral salts and is sterilized through a continuous plate-type heat exchanger. The flow rate is 1.82×10^5 liters/hour. The sterile feed is passed into two of the four fermentors (only one is shown on the figure). These fermentors are operated in rotation of two at a time. The rotation allowed the continuous operation of the process. The volume of each of the fermentors is 4.55×10^6 liters. After the fermentation is completed (50 hours), the contents of the fermentors are emptied into the holding tank for settling. The broth is then fed into the beer still of the recovery system. The concentrated beer is then separated by the vapor re-use process (Othmer, 1936). 2.5 kg of steam per kg of solvent are required for the recovery of the solvents. The total solvent production rate is 2.99×10^3 kg/hr or 2.62×10^7 kg/year. 69.9% is n-butanol, 29.8% acetone and 0.3% ethanol. From the fermentor, 3.88×10^2 kg of hydrogen and 1.44×10^4 kg of carbon dioxide are produced hourly. The hydrogen is used as a burning fuel.

TABLE II.C.1: BASIS OF DESIGN FOR THE PRODUCTION OF ACETONE/BUTANOL

Annual Production	26.2 X 10 ⁶ kg mixed solvent		
Organism	<u>Clostridium acetobutylicum</u>		
Carbon Source	6% solution from cellulose hydrolysis		
Concentration of Solvents	<u>Butanol</u> 12 gm/l	<u>Acetone</u> 5.2 gm/l	<u>Ethanol</u> 0.5 gm/l
Yield of Solvent (gm/gm)	0.20	0.086	0.009
Type of Process	Batch		
Fermentation Time	50 Hours		
Turnaround Time	50 Hours		

FIGURE II.C.1: PROCESS FLOW DIAGRAM FOR THE PRODUCTION OF ACETONE AND BUTANOL

The production of the solvents requires 1.67×10^8 kg of steam and 1.90×10^{13} joules of electricity per year. The utilization of hydrogen produced as fuel will return 1.85×10^{14} joules of energy per year.

4. Purchased Equipment Cost and Total Capital Investment

The purchased equipment costs for the production of acetone and butanol are shown in Table II.C.2. The different items, their sizes and costs are shown in this Table. The factor method was employed to estimate the total capital investment. The factors employed for the different items, as well as the actual cost based on 1977 are shown in Table II.C.3. From our analysis, the production of 26.2×10^6 kg of mixed solvent per year will require a total capital investment of 20.0×10^6 dollars.

5. Direct Manufacturing Costs

The direct manufacturing cost for the production of acetone/butanol is summarized in Table II.C.4. The unit costs for the utilities are shown in this Table. The plant was assumed to have zero salvage value when depreciated on a straight-line basis over a period of 15 years.

TABLE II.C.2: PURCHASED EQUIPMENT COSTS FOR THE PRODUCTION OF ACETONE/BUTANOL

ITEM and DESCRIPTION	SIZE (liter)	QUANTITY	COST/UNIT (Dollars)	TOTAL COST (Dollars)	% of PEC
Surge Tank, Carbon Steel	3.41×10^6	2	4.55×10^5	3.10×10^5	20.5
Main Fermentor, Carbon Steel	5.68×10^6	4	2.01×10^5	8.02×10^5	
Holding Tank, Carbon Steel	9.08×10^6	1	2.55×10^5	2.55×10^5	
Inoculation Fermentor, Carbon Steel	1.44×10^5	6	2.85×10^4	1.71×10^5	
Inoculation Fermentor, Carbon Steel	4.92×10^3	12	4.70×10^3	5.65×10^4	
Storage, Acetone	5.30×10^4	1	1.70×10^4	1.70×10^4	
Storage, Butanol	1.25×10^5	1	2.73×10^4	2.73×10^4	
Storage, Ethanol	5.68×10^2	1	1.53×10^3	1.53×10^3	
Cooling Tower		1	1.82×10^5	1.82×10^5	2.3
Pumps			2.57×10^5	2.57×10^5	3.2
Recovery Unit			3.64×10^6	3.64×10^6	45.4
Sterilizer			7.84×10^4	7.84×10^4	1.0
Cooling Pipes			2.19×10^6	2.19×10^6	27.3
Insulations			2.19×10^4	2.19×10^4	0.3
TOTAL				$\$8.01 \times 10^6$	100.0

TABLE II.C.3: TOTAL CAPITAL INVESTMENT FOR THE
PRODUCTION OF ACETONE/BUTANOL

<u>ITEM</u>	<u>COST \$</u>	<u>% OF TCI</u>
PEC	8.01×10^6	40
Process Piping	2.80×10^6	14
Instrumentation	8.01×10^5	4
Building Preparation	2.80×10^6	14
Auxiliary Facilities	8.01×10^5	4
Ground and Yard	8.01×10^5	4
Land	4.01×10^5	2
Engineering & Construction	2.40×10^2	12
Contingency	<u>1.2×10^6</u>	<u>6</u>
 TOTAL CAPITAL INVESTMENT (TCI)	 $\$20.0 \times 10^6$	 100%

TABLE II.C.4: DIRECT MANUFACTURING COST FOR THE PRODUCTION OF ACETONE/BUTANOL

<u>DIRECT MANUFACTURING COST</u>	<u>COST (¢/kg OF SOLVENT)</u>	<u>% of TOTAL MANU- FACTURING COST</u>
<u>Raw Material</u>		
NH ₄ OH, 29%	0.40	0.64
Mg SO ₄ 7H ₂ O	0.07	0.11
Na ₂ HPO ₄	0.29	0.46
KCe	0.18	0.29
*Molasses	36.08	57.34
<u>Utilities</u>		
Electricity (2¢/kw-hr.)	0.40	0.64
Steam (\$1.75/1000 lb)	2.46	3.91
Gas Credit (\$1.50/10 ⁶ BTU)	- 1.00	- 1.59
Cooling Water (\$0.80/1000 Gal)	0.18	0.29
<u>Labor and Related Costs</u>		
Labor	1.34	2.13
Supervision	0.25	0.40
Fringe Benefits (25% Labor)	0.40	0.64
Operating Supplies	0.13	0.21
<u>Maintenance</u>	7.65	12.16
<u>FIXED CHARGES</u>		
Depreciation (6.67% TCI/Yr)	5.10	8.11
Local Taxes (2% TCI)	1.53	2.43
Insurance (1% TCI/Yr)	0.76	1.21
Plant Overhead (70% Labor)	1.11	1.76
<u>GENERAL EXPENSES</u>		
Administration (15% Labor)	0.24	0.38
Interest (7% TCI)	5.35	8.50
TOTAL	62.92¢/kg (28.6 ¢/lb)	100

* Molasses cost \$53/ton with sugar content of 56%, i.e. sugar cost = \$0.104/kg

The direct manufacturing cost per kilogram of solvent is estimated to be 62.92 cents (28.6¢/lb). Of this total, about 57% was for sugars for solvent production. In our estimate, we have used molasses at \$53/ton as the sugar for fermentation. We, therefore, conclude that an inexpensive source of sugar can reduce the direct manufacturing cost significantly.

D. Production of Acetic Acid by Fermentation

1. Introduction

The research on acetic acid production by Clostridium thermoaceticum has been studied in this present contract period through a number of different approaches. The objectives for these different studies were:

- . Kinetic analyses of growth and acetic acid production.
- . Medium composition studies for acetic acid production to reduce medium cost.
- . Production of acetic acid by Cl. thermoaceticum using soluble sugars from cellulose degradation by Clostridium thermocellum.
- . Mutation and selection for isolates of Cl. thermoaceticum having higher tolerance for acetate.

Each of these studies will be presented individually.

2. Kinetic Analyses of Growth and Acetic Acid Production

a. Introduction

In the previous progress reports (see COO-4198-1 and COO-4198-3), we have reported a number of fermentations on the production of acetic acid by Clostridium thermoaceticum in batch culture with and without pH control. We have accumulated sufficient information at this time to allow us to perform some detailed kinetic analyses on this fermentation. The objective of these analyses is to clearly define the pertinent kinetic parameters of this fermentation which will allow us to pursue future relevant research.

b. Materials and Methods

The materials and methods for the results to be presented in this section have already been reported in our previous progress reports (see COO-4198-1, -2, and -3). Therefore, they will not be repeated at this time.

c. Results and Discussion

We have reported previously that the growth of Clostridium thermoaceticum appears to be critically influenced

by the pH during fermentation. During batch cultivation of this organism without pH control, the change in pH is the result of the production of acetic acid. We have analyzed a large number of these experiments where the pH during fermentation was continually changing. Through graphical techniques, it was possible to calculate the specific growth rate of this organism as a function of the pH. The results from these calculations are shown in Figure II.D.1. It can be seen that below a pH of 6.7, the growth of this organism is totally inhibited. Between pH values of 6.7 to 7.1, there is a precipitous increase in the organism's growth rate with increasing pH reaching a maximal at about pH 7.1. As the pH further increases to 8.0, again a decrease in the organism's growth rate results. However, in this pH range (7.0 to 8.0), the decreased growth rate is not too drastic attaining a lowest value of about 70% of the maximal. It, therefore, appears that there is a region of pH (7.0 to 8.0) where respective growth of Clostridium thermoaceticum can be achieved.

In our second kinetic analysis, we were interested to know the effect of the culture pH on the specific product formation (gm acetic/gm cell-hr) of this fermentation. Shown in Figure II.D.2 are the results from such an analysis. Here again, pH exerts a significant influence on the ability of Clostridium thermoaceticum to produce acetic acid. There again is an optimum pH about 7.1 where the specific product

FIGURE II.D.1: SPECIFIC GROWTH RATE OF CLOSTRIDIUM THERMOACETICUM
AS A FUNCTION OF pH (T = 60°C)

FIGURE II.D.2: SPECIFIC PRODUCT FORMATION BY CLOSTRIDIUM THERMOACETICUM
AS A FUNCTION OF pH AT 60°C

formation is maximum. When the results from Figures II.D.1 and II.D.2 are combined, the relationship between the specific growth rate to the specific production is graphically shown in Figure II.D.3. There is a great deal of data scattering in this figure since the pH variations were quite substantial from which these results were obtained. Nevertheless, there does appear to be a qualitative trend to show that substantial production of acetic acid is associated with the growth of the organism. This is to say as the growth rate increases the specific product formation also increases. It is difficult to conclude whether the production of acetic acid can be expected from non-growing cells (i.e. at specific growth rate = 0) due to the data scatter at low growth rates.

As mentioned previously that the data presented in Figures II.D.1, II.D.2 and II.D.3 were from many batch fermentations where pH was not controlled. There was, however, one fermentation out of all of these experiments where the data scatter was significantly less. When the results from this fermentation were examined, the relationship between specific growth rate and specific production formation, as shown in Figure II.D.4, was obtained. The results in this Figure show that the acetic acid is produced primarily through growth associated as indicated by the positive slope in this Figure. Furthermore, at zero growth rate, the specific product formation also approaches zero, showing that little or no production of acetic acid can be expected from non-growing cells.

FIGURE II.D.3: SPECIFIC PRODUCT FORMATION AS A FUNCTION GROWTH RATE
FOR C. THERMOACETICUM (NOTE: pH WAS NOT CONTROLLED)

FIGURE II.D.4: SPECIFIC PRODUCT FORMATION AS A FUNCTION OF
GROWTH RATE FOR C. THERMOACETICUM
(pH NOT CONTROLLED)

In order to increase the concentration of acetic acid produced by Clostridium thermoaceticum, it was reasoned that though pH control at the optimal value might be beneficial. Since our previous results showed that the pH optimal for growth is about 7.0, pH control at this value using NaOH was examined. A large number of fermentations have been performed where the pH during fermentation was controlled. However, we have had a great deal of problems associated with the results reproducibility. Specifically, when fermentations were performed under identical conditions, different results were obtained. Even though the reproducibility was not ideal, the results were, nevertheless, informative.

Shown in Figure II.D.5 is the relationship of the specific growth rate of Clostridium thermoaceticum as a function of the acetic acid concentration (corrected for sodium acetate) for two different experiments. These two experiments are shown to illustrate the problem of reproducibility. The upper curve in this Figure shows that, under pH control (7.0), extremely high specific growth rates can be attained. For example, the maximum specific growth rate of 0.3 Hour^{-1} is nearly three times greater than the maximum value which has been achieved when the pH was uncontrolled. Furthermore, the upper curve in the Figure shows that about 20 gm/l of acetic acid can be produced without any impairment to the growth rate of this organism.

FIGURE II.D.5: DEPENDANCE OF GROWTH RATE UPON ACETIC ACID
CONC. FOR *C. THERMOACETICUM*
($T = 60^{\circ}\text{C}$, $\text{pH} = 7.0$)

On the other hand, the lower curve in Figure II.D.5 did not exhibit the high growth rate and high acetic acid concentration previously shown. The only explanation for these different behaviors is the manner in which the inoculum was prepared. It is our belief that high specific growth rate resulted from organisms which were acclimatized through serial inoculum transfer. We are actively searching for the exact reason(s) for these different behaviors.

When these two experiments are analyzed with respect to the specific product formation (gm acetic/gm cell-Hr) to the acetic acid concentration, the results in Figure II.D.6 were obtained. Here again, one observes the same qualitative relationship as that shown in Figure II.D.5. It should be mentioned that the specific product formation under controlled pH is significantly higher than those obtained when pH was not controlled. For example, the upper curve in Figure II.D.6 exhibited a specific product formation of about 3 gm of acetic acid per gm of cell per hour. This is greater than three times the maximum value obtained when pH was uncontrolled. These high values of specific product formation are extremely encouraging since they indicate that the organism is inherently capable of producing acetic acid at extremely high rates. Thus, one can anticipate high volumetric productivities which ultimately should result in reduced cost for manufacturing.

When the results from Figures II.D.5 and II.D.6 are combined, the relationship between specific growth rate and

FIGURE II,D.6: DEPENDENCE OF SPECIFIC PRODUCT FORMATION
AS A FUNCTION OF ACETIC ACID CONC. FOR
C. THERMOACETICUM

specific product formation was obtained and shown in Figure II.D.7. Here again, the data indicate that the acetic acid is produced primarily through growth association. This is unfortunate since the concept of using resting cells or immobilized cells will not be beneficial in the production of acetic acid by Clostridium thermoaceticum.

3. Medium Composition Studies for Acetic Acid Production to Reduce Medium Cost

a. Introduction

As will be shown in Section II.E of this report on the economic assessment of acetic acid production, we have assumed that an economic medium will be developed for the growth of Clostridium thermoaceticum. There are a number of approaches where research in medium development could reduce the cost for acetic acid production. We have started two programs in seeking answers to this problem. The first approach is to examine an inexpensive complex nutrient, corn steep liquor, as the nitrogen source, as well as supplying other growth promoting compounds for the growth and acetic acid production by Cl. thermoaceticum. The second is to examine the use of a defined medium in order to elucidate the exact nutritional needs of Clostridium thermoaceticum for growth and acetic

FIGURE II.D.7: SPECIFIC PRODUCT FORMATION VERSUS SPECIFIC GROWTH RATE FOR *C. THERMOACETICUM*
(pH controlled = 7.0)

acid production. Our preliminary findings on these studies will not be presented.

b. Materials and Methods

All of the studies were performed in tube cultures incubated at 60°C for three days. All analyses were end points since the tube culture volumes did not permit kinetic analysis. Corn steep liquor was obtained from A. E. Staley Co. The amount of corn steep liquor added to each tube was calculated on a dry solids basis. The growth of the organism in corn steep liquor could not be measured spectrophotometrically because of the suspended solids in this medium. Glucose was analyzed using the Glucostat (calbiochem) and the acetic acid by gas chromatography.

When grown on a defined medium, the growth of the organism can be measured spectrophotometrically. Using our calibration curve through gravimetric and spectrophotometric analyses, the cell dry weight can then be calculated. The composition of the defined medium for growth and acetic acid production by Cl. thermoaceticum is shown in Table II.D.1.

TABLE II.D.1: DEFINED MEDIUM COMPOSITION FOR GROWTH
AND ACETIC ACID PRODUCTION BY
CLOSTRIDIUM THERMOACETICUM

<u>COMPONENT</u>	<u>CONCENTRATION (gm/l)</u>
Glucose	18
NaHCO ₃	16.8
K ₂ HPO ₄	7
KH ₂ PO ₄	5.4
(NH ₄) ₂ SO ₄	1.32
MgSO ₄	0.12
CaCl ₂	0.01
Na-thioglycolate	0.57
Lysine	0.07
Methionine	0.25
Biotin	50 µg
Nicotinic Acid	1 µg
Trace Salt:	
Fe ⁺⁺	10 ⁻⁴ M
Co ⁺⁺	10 ⁻⁶ M
Mo ⁶⁺	10 ⁻⁷ M
W ⁶⁺	10 ⁻⁷ M
Se ⁴⁺	10 ⁻⁷ M

c. Results and Discussion

The ability of Clostridium thermoaceticum to grow and produce acetic acid using corn steep liquor as the nitrogen source and other growth promoting factors is shown in Figure II.D.8. It can be seen from this Figure that the amount of acetic acid produced increases with the corn steep liquor solids. However, the results in this Figure do not show a linear dependence of acetic acid produced to the corn steep liquor solids. This is because when acetic acid is produced at higher concentrations, the pH decrease is also higher. For example, the pH of the final fermentation broth ranged from 5.8 to 6.8 for corn steep liquor solids in the range of 3.7 to 25 gm/l. If one examines the conversion of glucose to acetic acid, it can be seen that the efficiency (0.8 gm acetic/gm glucose) is quite comparable to that found previously. These constant conversion efficiencies confirm that the asymptotic approach of acetic acid production at high corn steep liquor solids is a result of decreases in the pH of the fermentation broth. These results are quite encouraging since the cost of corn steep liquor is only about 10 to 15¢ per pound on a dry basis. We hope to continue our studies in this area to determine the minimum requirement of corn steep liquor for maximum acetic acid production.

The studies on the defined medium for growth and acetic acid production by Clostridium thermoaceticum were

FIGURE II.D.8: PRODUCTION OF ACETIC ACID BY
CLOSTRIDIUM THERMOACETICUM
USING CORN STEEP LIQUOR

performed with the following objectives in mind. The first objective was to determine the minimal nutritional need of this organism. The findings from these studies should allow us first to determine whether a defined medium might be used for the production of acetic acid in an economic fashion. The second objective was to use this information to design and to explore the use of other complex nutrients (e.g. soy bean meal, cotton seed meal, etc.) as an alternate means to obtain an economical production medium. Since we have only performed some preliminary studies, it is not possible to answer these questions in a definitive at this time.

Our preliminary results on the growth and acetic acid production using the defined medium (see Table II.D.1) are shown in Table II.D.2. The results are encouraging since the amount of acetic acid produced (7 gm/l) is quite high compared to the amount of cell mass produced (0.46 gm/l). The average specific growth rate was only 0.03 hour^{-1} which is substantially lower than those obtained using complex medium. These results are sufficiently encouraging that further exploration in this area will be continued.

TABLE II.D.2: GROWTH AND ACETIC ACID PRODUCTION BY
CLOSTRIDIUM THERMOACETICUM
ON DEFINED MEDIUM

TIME OF FERMENTATION	AVERAGE SPECIFIC GROWTH RATE	FINAL CELL CONCENTRATION	FINAL ACETIC ACID CONCENTRATION
(HOURS)	(HOUR^{-1})	(gm/l)	(gm/l)
72	0.03	0.46	7

4. Production of Acetic Acid by *Clostridium Thermoaceticum* using Soluble Reducing Sugars from the Degradation of Corn Residue by *Cl. thermocellum*

a. Introduction

The ultimate objective of this contract is to explore the potential of cellulosic biomass as the raw material for the production of petroleum sparing chemical feedstocks. In the degradation of cellulosic biomass by *Clostridium thermocellum*, we have shown that this organism can accumulate significant amounts of reducing sugars from cellulose such as solka floc and corn residue. We have also shown that the soluble reducing sugars produced by this organism are mainly xylose, glucose and cellobiose. Since *Cl. thermoaceticum* is known to be able to convert xylose and glucose to acetic acid, the soluble reducing sugars from cellulose degradation should be well suited for this fermentation. For these reasons, studies were performed using *Cl. thermoaceticum* to see its capability of using these types of raw materials for acetic acid production.

b. Materials and Methods

The fermentation broth from the degradation of corn residue (see Section I.B.) by *Clostridium thermocellum* was collected and concentrated 10-fold by rotary vacuum evaporation.

Different reducing sugar concentrations were made from the concentrate and added to the fermentation medium (see Report COO-4198-1) for acetic acid production by Cl. thermoaceticum. Tube cultures were inoculated and incubated at 60°C for 3 days and analyzed for the acetic acid formed and the reducing sugars remained. High pressure liquid chromatography analyses of the broth before and after fermentation were also performed. The methods for these analyses have been described in Section I.A of this report.

c. Results and Discussion

The production of acetic acid by Clostridium thermoaceticum using reducing sugars from corn residue produced by Cl. thermocellum is shown graphically in Figure II.D.9. It can be seen that there is a linear relationship between the acetic acid produced to the reducing sugar consumed. The conversion efficiency based on reducing sugar is higher (0.89 gm acetic/gm reducing sugar) than that achieved from glucose. However, this conversion efficiency could be misleading since our calibration for reducing sugars is based on glucose. We know that the reducing sugars from corn residue contain xylose and its reducing capability must be properly corrected with relationship to glucose. The actual conversion efficiency can best be estimated using HPLC. At this time, our HPLC system

FIGURE II.D.9: PRODUCTION OF ACETIC ACID BY CLOSTRIDIUM THERMOACETICUM USING REDUCING SUGARS PRODUCED FROM CORN RESIDUE BY CL. THERMOCELLUM

still requires further modifications to assess accurately sugars from complex fermentation broths.

We have, however, performed some preliminary HPLC analyses of the fermentation broths before and after fermentation. Due to the presence of contaminating products in the broth (e.g. amino acids, salts, lignin, etc.), the chromatograms are not as ideal as one would desire. Shown in Figure II.D.10 are the HPLC chromatograms before and after fermentation. It can be seen that the broth before fermentation contains mainly xylose, glucose and cellobiose. However, after fermentation by Cl. thermoaceticum, xylose appears to be totally utilized and only a small amount of glucose remained. On the other hand, Cl. thermoaceticum does not appear to utilize cellobiose for acetic acid production. These results are quite encouraging since they indicate an approach to cellulosic biomass degradation and acetic acid production are technically sound. We will continue our efforts in this area.

5. Mutation and Selection for Isolates of Clostridium thermoaceticum Having Higher Tolerance for Acetic Acid

a. Introduction

One of the present problems associated with the production of acetic acid is the inability of Cl. thermoaceticum to tolerate low pH and high acetate concentration.

FIGURE II.D.10: HIGH PRESSURE LIQUID CHROMATOGRAMS OF CORN
RESIDUE REDUCING SUGARS BEFORE AND AFTER
FERMENTATION BY CLOSTRIDIUM THERMOACETICUM

BEFORE FERMENTATION

AFTER FERMENTATION

Overcoming these problems will ultimately reduce both fermentation and recovery costs. We have embarked research programs to overcome both of these problems. It should be mentioned that our approaches are in two different directions. The first of these is to isolate strains of Cl. thermoaceticum which have higher tolerance for sodium acetate. These isolates can then be tested under pH controlled fermentations to increase the acetate concentration. The second approach is to select isolates which have higher tolerance to hydrogen ion concentration (low pH). These isolates can then be tested to see their ability to produce acetic acid without pH control.

b. Materials and Methods

The strategy used to select isolates which have higher tolerance to sodium acetate concentration employs a natural selection procedure. Serial transfers of Clostridium thermoaceticum are made into progressively higher concentrations of sodium acetate at constant pH (7.0) in liquid media. The growth of those isolates capable to tolerating high acetate concentration is then measured. The second approach is to mutagenize the organism with different mutagenic agents. The survivals are then tested for growth at low pH, as well as high acetate concentration. We have yet progressed sufficiently in the latter approach as to assess our success.

c. Results and Discussion

Using the serial transfer technique into successive higher concentrations of sodium acetate, we have been able to select isolates which appear to have higher tolerance for sodium acetate. The results are shown in Figure II.D.11. In this Figure, the specific growth rate as compared to the maximum value (zero acetate) for different sodium acetate concentrations of the parent (wild type) and a selected strain is shown. It can be seen that the increase in acetate tolerance is not too great, but encouraging. For example, the parent exhibited no growth at 42 gm/l of acetate whereas the new isolate is still able to grow at a rate of 40% of the maximum at this concentration. We hope to continue using this approach as well as through mutation in addition to selection.

6. Future Studies

The future studies in the production of acetic acid by Clostridium thermoaceticum include:

- . Continuous culture under pH control to obtain reproducible kinetics
- . Examine means for cell recovery to achieve cell recycle leading to increased productivity

FIGURE II.D.11: COMPARISON OF GROWTH RATES OF ACETATE-TOLERANT STRAINS OF CLOSTRIDIUM THERMOACETICUM

- . Examine more economical means of product recovery
- . Examine inexpensive media for acetic acid production
- . Continue efforts in using reducing sugars from cellulosic biomass for acetic acid production
- . Mutation and selection programs to increase concentration of acetic acid and/or acetate concentration

E. Process and Economic Analysis of Acetic Acid Production

1. Introduction

The production of acetic acid by Clostridium thermoaceticum has progressed to the point that a process analysis, along with economical considerations, were felt to be warranted. This analysis was performed with two functions in mind. The first was to examine the overall process and production cost as they reflect the present state of research. The second was to identify those cost sensitive areas where further research and development can be directed.

It should also be mentioned that most of the data related to the fermentation aspects of this process were those obtained in our laboratory. Unfortunately, our contract does not deal with the recovery of products and, therefore, the designs were based on the available results in the published literature.

2. Description of the Process

a. Kinetics and Rate of Acetic Acid Production

The plant is assumed to be built in the vicinity of a plant where hydrolysis product from cellulosic biomass is

available. The fermentable sugar will be delivered continuously as a 6% solution. The annual sugar delivery will be 90.7×10^6 kg/yr. This corresponds to an annual production of 75.6×10^6 kg of acetic acid.

C. thermoaceticum ferments xylose, fructose and glucose with acetic acid as the only major product. The conversion of glucose to acetate is almost stoichiometric. Under normal growth conditions, approximately 2.5 moles of acetate are formed for each mole of glucose consumed. In the design of this process, 90% of cells leaving the fermentor are recovered (as non-viable cells) and are recycled as nutrient feed for the growing of cells in the fermentor. It is assumed that the cell yield under these conditions will be 0.15 g cell/gr glucose consumed. According to experimental data obtained from our laboratory, the acetic acid production can be considered as being essentially growth associated. The specific growth rate (μ) is, however, quite sensitive to the acetate concentration. This relationship is shown in Figure II.E.1. The acetate will be produced in four separate one-stage continuous stirred fermentors. The productivity of the system is shown in Figure II.E.2 as a function of the acetate concentration. Because of the advantages of higher acetate concentrations, the fermentors will be operated at a concentration of 42 g/l sodium acetate which represents productivity of 1.41 g/l-Hr of acetic acid.

FIGURE II.E.1: SPECIFIC GROWTH RATE OF *C. THERMOACETICUM* AS A FUNCTION OF SODIUM ACETATE CONCENTRATION (T = 60°C, pH = 7.2)

FIGURE II.E.2: PRODUCTIVITY OF SODIUM ACETATE BY C. THERMOACETICUM
AS A FUNCTION OF ACETATE CONCENTRATION

b. Cell Recovery

Due to the present lack of specific information, the cells are assumed to flocculate under conditions similar to those for cell recovery in ICI's SCP-process (Pseudomonas spp. on methanol). This flocculation method (see Figure II.E.3) involves a short pretreatment (30 min.) at high pH (pH = 10) and high temperature (85°C). The pretreated cells will flocculate and settle by addition of acid to pH = 4. The yield is reported to be higher than 90%. In our acetic acid process, the flocculated cells will be separated from the broth by the use of a drum-filter, washed briefly on the filter and recycled to the fermentor as a cell-slurry (15% DCW). It is assumed that 90% of the cell material can be recycled in this manner.

c. Recovery of Acetic Acid

The acetic acid stream from the fermentors will be concentrated in vacuum evaporators (pH = 7) to an acetate concentration of 416 g/l, as shown in Figure II.E.4. The concentrated stream is acidified with sulfuric acid, and the water/acetic acid phase is fed to a distillation tower as a vapor. Butyl acetate is introduced as an entrainer in the upper part of the tower to aid in the separation process. The bottom fraction (97% acetic acid) will be redistilled to give a high quality product (> 99% acetic acid).

FIGURE II.E.3: CELL RECOVERY PROCESS

FIGURE II.E.4: ACETIC ACID RECOVERY PROCESS

d. Recovery of Sodium Sulfate

For each mole acetic acid produced, half a mole sodium sulfate is produced and will be recovered as by-product. This is accomplished by cycling the hot and nearly saturated solution from the evaporator through a cooled crystallizer (see Figure II.E.5). The precipitated salt will be washed and the combined wash water and mother liquor will be recycled to the evaporator.

e. Plant Location, Utilities

The plant will be located at the Gulf coast and is assumed to have access to cooling water, steam and electricity at prices of \$0.80/1000 gallons, \$1.75/1000 lbs. and 2¢/KWhr respectively. The steam and cooling water pressures are both assumed to be greater than 60 psig. The temperature of steam is available at greater than 143°C and cooling water at temperature of 20°C.

f. Mass and Energy Balances: Equipment Specifications

A schematic flow diagram on the overall process is shown in Figure II.E.6. The process flow streams have been

FIGURE II.E.5: SODIUM RECOVERY PROCESS

FIGURE II.E.6: PROCESS FLOW STREAMS AND EQUIPMENT DESIGNATIONS

given number designations. The definitions of these streams, along with their respective rates, are shown in Table II.E.1. Each piece of equipment in Figure II.E.6 has also been assigned a letter and number designation. The descriptions on this equipment, as well as the sizes and costs, are shown in Table II.E.2.

Shown in Figure II.E.7 is a schematic flow diagram with respect to the energy balances for the production of acetic acid. This is the same diagram as shown previously in Figure II.E.6. However, the heat balances have been added in this diagram and shown symbolically by the letter "Q" throughout the process. The quantitative heat or cooling requirements of these streams have been calculated and tabulated in Table II.E.3. Also shown in this Table are the costs for heating and cooling associated with the production of acetic acid.

g. Basis of Cost Estimates

The process equipment required for the production of acetic acid were mainly estimated from Dryden and Furlow (1969). However, the equipment costs in this publication were for 1968. In order to estimate the 1977 costs, the Marshall and Steven (M&S) indices were employed. The M & S indices for 1968 and 1977 were 270 and 492 respectively. The "factor method" was then used on the total purchased equipment cost (PEC) to account for other related costs for plant construction. The percentages used for the factor method are shown in Table II.E.3.

FIGURE II.E.7: HEAT BALANCES AND FLOWS

TABLE II.E.1: DEFINITION OF FLOW STREAMS AND RATES FOR
ACETIC ACID PRODUCTION

1.	6% SUGAR	173 X 10 ³	ℓ/Hr
2.	COMPLETE MEDIA & CONDENSED STEAM	305 X 10 ³	ℓ/Hr
3.	MEDIA	281 X 10 ³	ℓ/Hr
4.	4.3% ACETATE	281 X 10 ³	ℓ/Hr
5.	5.8% ACETATE	194 X 10 ³	ℓ/Hr
6.	10.2% ACETATE	107 X 10 ³	ℓ/Hr
7.	41.6% ACETATE	20 X 10 ³	ℓ/Hr
8.	pH ¹⁰ FERMENTATION BROTH	20.2 X 10 ³	ℓ/Hr
9.	pH ⁴ FERMENTATION BROTH	24.1 X 10 ³	ℓ/Hr
9a	WASH WATER	1.2 X 10 ³	ℓ/Hr
10.	CELL SLOP 15% DCW +H ₂ O	100 X 10 ³	ℓ/Hr
11.	50% SODIUM HYDROXIDE	234	H ₂ O/Hr
12.	50% SODIUM HYDROXIDE	8.24 X 10 ³	ℓ/Hr
13.	97% SULFURIC ACID	4.06 X 10 ³	ℓ/Hr
14.	LIQUID AMMONIA	18.9	kg/Hr
15.	30% SUPPLEMENTAL SALTS	100	ℓ/Hr
16.	FERMENTATION SUPPLEMENT	100 X 10 ³	ℓ/Hr
17.	CARBON DIOXIDE PRODUCTION	79	M ³ /Hr
18.	FERMENTATION FILTRATE	25.3 X 10 ³	ℓ/Hr
19.	COLUMN FEED VAPOR 25.4% ACETIC ACID	25.3 X 10 ³	H ₂ O/Hr.
20.	SODIUM SULFATE FEED	261 X 10 ³	kg/Hr
21.	CRYSTALYZER RETURN	242 X 10 ³	kg/Hr
22.	SODIUM SULFATE HEPTAHYDRATE	19.3 X 10 ³	kg/Hr
23.	97% ACETIC ACID	8.89 X 10 ³	kg/Hr
24.	99% ACETIC ACID	34.5 X 10 ³	kg/Hr
25.	99% ACETIC ACID REFLUX	25.9 X 10 ³	kg/Hr
26.	99% ACETIC ACID (GLACIAL) PRODUCT	8.63 X 10 ³	kg/Hr
27.	72% BUTYL ACETATE 28% WATER	90.0 X 10 ³	kg/Hr
28.	BUTYL ACETATE ENTRAINER FEED	65.0 X 10 ³	kg/Hr

TABLE II.E.1 (Cont'd)

29.	BUTYL ACETATE RECOVERED	253	kg/Hr
30.	WATER BUTYL ACETATE	65.2×10^3	kg/Hr
31.	WATER & 1% BUTYL ACETATE	25.5×10^3	kg/Hr
32.	WASTE WATER (100°C)	25.3×10^3	kg/Hr
33.	STEAM FEED TO STERILIZER (140°C)	32.0×10^3	kg/Hr
34.	STEAM REMOVED FROM STERILIZER (100°C)	23.8×10^3	kg/Hr.

TABLE II.E.2: DESCRIPTION AND COST OF EQUIPMENT FOR ACETIC ACID PRODUCTION

	<u>EQUIPMENT DESCRIPTION</u>	<u>MATERIAL</u>	<u>SIZE</u>	<u>NET COST</u> (\$1,000)
A1	SINGLE STAGE RECIPROCATING COMPRESSOR		500 CFM	270. M&S 13.7
A2	CENTRIFUGAL COMPRESSOR		24 X 10 ³ CFM	270.
B1	CARTRIDGE AIR FILTER		500 CFM	5.5
C1	CONVENTIONAL FORCED CIRCULATION CRYSTALLIZER		530 Ton/Day	200.
D1	AZEOTROPIC DISTILLATION TOWER BUBBLE TRAY	COPPER	30 Tray X 115"	135.
D2	ACETIC ACID DISTILLATION TOWER BUBBLE TRAY	COPPER	10 Tray X 54"	38.
D3	STRIPPING TOWER-BUBBLE TRAY	STAINLESS	10 Tray X 48"	14.
E1-3	THREE STAGE LONG TUBE VERTICAL EVAPORATOR	STEEL	50,000 Ft ² Each	1,410.
E4	LONG TUBE VERTICAL EVAPORATOR	STAINLESS	5,315 Ft ²	330.
F1-4	PRESSURIZED FERMENTATION TANKS	STEEL	4 X 606 X 10 ³ Gal	1,360.
H1	SHELL AND TUBE CONDENSOR	COPPER	682 Ft ²	7.8
H2	ADMIRALTY FINTUBE HEAT EXCHANGER		8,050 Ft ²	27.0
R1-3	ROTARY DRUM FILTER	STAINLESS	500 Ft ²	316.

TABLE II.E.2 (Cont'd)

<u>EQUIPMENT DESCRIPTION</u>		<u>MATERIAL</u>	<u>SIZE</u>	<u>NET COST</u> (\$1,000)
M1-4	ELECTRIC MOTOR AND IMPELLER		500 Hp	180.
M5	ELECTRIC MOTOR AND IMPELLER		.5 Hp	2.
S1-4	STEAM INJECTED, CONTINUOUS STERILIZER	STEEL	134 M each	9.5
T1	SUGAR SOLUTION SURGE TANK	STAINLESS	460 X 10 ³ Gal	10.0
T2	PRESSURIZED AMMONIA STORAGE TANK	STAINLESS	1.3 X 10 ³ Gal	12.0
T3	SALT SOLUTION STORAGE TANK	STAINLESS	4.4 X 10 ³ Gal.	10.0
T4	SODIUM HYDROXIDE STORAGE	STAINLESS	370 X 10 ³ Gal.	50.0
T5	SULFURIC ACID STORAGE	LEAD LINED	182 X 10 ³ Gal.	70.2
T6	HOLDING TANK	STEEL	2.4 X 10 ³ Gal.	8.5
T7	HOLDING TANK	STAINLESS	7 X 10 ³ Gal.	36.5
T8	HOLDING TANK	STAINLESS	67 X 10 ³ Gal.	40.0
T9	SODIUM SULFATE STORAGE TANK	STEEL	2.28 X 10 ⁵ Gal.	47.0
T10	ACETIC ACID STORAGE TANK	STAINLESS	4.04 X 10 ⁵ Gal.	100.0
T11	DECANTING TANK	STAINLESS	12 X 10 ³ Gal.	35.0
P1	MAJOR PUMPS		10 X 760 GPM	40.0

TABLE II.E.2 (Cont'd)

	<u>DESCRIPTION</u>	<u>EXCHANGE AREA</u>	<u>MATERIAL</u>	<u>COST (M&S 270)</u>
H1	CONDENSER	1,470 Ft ² m ²	Stainless	14,200
H2	CONDENSER	8,050 136.8	Stainless	77,800
H3	CRYSTALYZER COOLER	9,056 842	Stainless	84,200
H4	CRYSTALYZER HEATER	2,870 267	Stainless	26,700
H5	FERMENTOR COOLING	5,260 489	Steel	18,500
H6	MEDIA COOLING	6,133 570	Steel	21,500
H7	SUGAR PREHEAT	4,902 450.6	Steel	17,200
H8	D1 HEATER	1,765 169	Stainless	16,414
H9	D2 HEATER	1,240 115	Stainless	11,500
H10	D3 HEATER	246	Steel	<u>600</u>

TOTAL COST = \$5.02 x 10⁶

TABLE II.E.3: HEATING AND COOLING REQUIREMENTS
FOR ACETIC ACID PRODUCTION

	Joules/hr Heat	Joules/hr Cool	kg/hr Steam	liters/hr Water	\$/day Steam	\$/day Water	¢/lb. Product for Steam	¢/lb. Product for Water
Q1								
Q2	68.5×10^9		31.98×10^3		2.96×10^3		0.65	
Q3	50.98×10^9		-23.8×10^3		-2.20×10^3		0.40	
Q4		46.9×10^9		320.91×10^3		1.63×10^3		0.36
Q5		2.40×10^9		163×10^3		$.83 \times 10^2$		0.18
Q6	18.5×10^9		87×10^3		8.06×10^3		1.8	
Q9	60.5×10^9		28.3×10^3		2.62×10^3		0.57	
Q10		5.5×10^9		466×10^3		2.36×10^3		0.52
Q11	51×10^9		23.7×10^3		2.19×10^3		0.48	
Q12	20.1×10^9		9.4×10^3		$.87 \times 10^3$		0.19	
Q13	14.1×10^9		6.6×10^3		$.61 \times 10^3$		0.13	
Q14		14.1×10^9		48.21×10^3		$.24 \times 10^3$		0.05
Q15		77×10^9		263×10^3		1.33×10^3		0.29
Q16	$.3 \times 10^9$		$.15 \times 10^3$		$.014 \times 10^3$		0.0003	
TOTAL	364×10^9	217×10^9	163×10^3	126×10^6	15.1×10^3	6.5×10^3	3.31	1.40

The utility costs have already been described in Section II.E.2.e. Raw materials costs were obtained from the July issues, 1977, of the Chemical Marketing Reporter.

3. Direct Manufacturing Cost for Production of Acetic Acid

From the previous sections, it is now possible to calculate the direct manufacturing cost for the production of acetic acid by Clostridium thermoaceticum. There are three major sections in this overall cost analysis: capital investment, raw materials, and utilities and labor. Shown in Table II.E.3 is a summary of the total capital investment (TCI). It can be seen that, for the production of 76.5×10^6 pounds per year of acetic acid, a total capital investment of $\$22.88 \times 10^6$ will be required.

Shown in Table II.E.5 is the summary which the raw material cost was derived. In this table, the units/day used, cost per unit, daily operating cost and the cost per pound of acetic acid produced are tabulated. It should be stated that, in the calculated raw material cost, we did not include the cost of the feedstock for acetic acid production. Furthermore, a by-product credit for the sodium sulfate was assumed. It can be seen that, if a by-product credit for sodium sulfate can be assumed, the raw material cost will be 1.87¢/lb acetic acid produced. On the other hand, if one neglects the credit for sodium sulfate, the cost for raw materials then becomes 5.6¢/lb. of acetic acid.

TABLE II.E.4: TOTAL CAPITAL INVESTMENT (1977)

<u>ITEM</u>	<u>COST</u> (Millions of Dollars)	
PURCHASED EQUIPMENT COST (PEC)		\$ 9.15
PROCESS PIPING	35% PEC	3.20
INSTRUMENTATION	10% PEC	.92
BUILDINGS (INDOORS & OUTDOORS)	35% PEC	3.20
AUXILIARY FACILITIES	10% PEC	.92
GROUNDS AND YARDS	10% PEC	.92
LAND	5% PEC	.46
ENGINEERING AND CONSTRUCTION	30% PEC	2.74
CONTINGENCY	15% PEC	<u>1.37</u>
TOTAL CAPITAL INVESTMENT (TCI)		<u>\$22.88</u>

TABLE II.E.5: COST OF RAW MATERIALS FOR ACETIC ACID PRODUCTION

MATERIAL	UNITS/DAY	COST/UNIT	DAILY COST	PRODUCT COST/LB.
NaOH	108 tons	\$ 158	\$ 17,000	3.7 ¢
H ₂ SO ₄	173 tons	48	8,225	1.8
NH ₃	0.50 tons	130	65	0.014
H ₃ PO ₄	766 lbs.	0.145	111	0.024
MgSO ₄ ·7H ₂ O	887 lbs.	0.085	75	0.016
TRACE ELEMENTS				0.05
Na ₂ SO ₄ ·7H ₂ O	285 tons	-60	-17,000	-3.73
TOTAL RAW MATERIAL COSTS	(Credit for Na ₂ SO ₄)			1.87¢
	(No Credit for Na ₂ SO ₄)			5.60¢

Shown in Table II.E.6 are the utility and labor costs for the production of acetic acid. It can be seen that these costs amount to 6.64¢/lb. of acetic acid produced.

A summary, including all components for the direct manufacturing cost for the production of acetic acid, is shown in Table II.E.7. In the calculation of the fixed charges, the plant was depreciated over a period of 15 years on a straight-line basis. The local taxes and insurance were assumed to be 2% and 10% respectively of the total capital investment. The interest rate for borrowing capital was assumed to be 7%. Lastly, the plant maintenance was assumed to be 10% of the total capital investment.

From the results in Table II.E.7, it is our estimate that the direct manufacturing cost for acetic acid will be 10.98¢/lb. This cost, however, excludes the feedstock (sugar) cost for the acid production. Of this total, the major fraction (44.99%) of the production cost is for the utilities, primarily those associated with product recovery. It, therefore, becomes apparent that research innovations in this area should be emphasized if improved process economics are desired. The second major cost is in the fixed charges. In this case, reduction in production cost can be achieved through process improvement, such as higher productivity of acetic acid production. It appears from this analysis that reduction in cost will be necessary if acetic acid is to be produced through fermentation.

TABLE II.E.6: UTILITIES AND LABOR COSTS
FOR ACETIC ACID PRODUCTION

ITEM	UNITS/DAY	UNIT COST	DAILY COST	COST/LB. PRODUCT
STEAM	3.91×10^6 kg	\$3.86/1000 kg	\$15,100	3.31 ¢
COOLING WATER	30.2×10^6 l	0.21/1000 l	6,400	1.40 ¢
ELECTRICITY	50,000 Kw-Hr	.02/Kw-Hr	1,000	0.23 ¢
LABOR	200 Hrs	.04/Hr	800	0.17 ¢
SUPERVISION	24 Hrs	7.5 /Hr	180	0.04 ¢
FRINGE BENEFITS		25% Labor	245	0.05 ¢
OPERATING SUPPLIES		10% Labor	98	0.002¢
MAINTENANCE		10% TCI	6,270	1.37 ¢
ADMINISTRATION		15% Labor	215	0.044¢
PLANT OVERHEAD		70% Labor	988	0.21 ¢
TOTAL UTILITY AND LABOR COST				6.64 ¢

TABLE II.E.7: DIRECT MANUFACTURING COSTS FOR
THE PRODUCTION OF ACETIC ACID
(EXCLUDING COST OF SUGAR)

	MANUFACTURING COST (¢/LB.)	PERCENT OF TOTAL (%)
Raw Materials:		
NaOH	3.7	
H ₂ SO ₄	1.8	
Other Nutrients	0.1	
Na ₂ SO ₄	(-3.73)	
Subtotal	1.87	17.03
Utilities:		
Steam	3.31	
Water	1.40	
Electricity	0.23	
Subtotal	4.94	44.99
Labor:		
Operators	0.17	
Supervisor	0.04	
Fringe Benefits	0.05	
Operating Supplies	0.002	
Plant Overhead	0.21	
Administration	0.044	
Subtotal	0.52	4.74
Fixed Charges:		
Depreciation	0.92	
Local Taxes	0.28	
Insurance	0.14	
Interest	0.94	
Subtotal	2.28	20.76
Maintenance	1.37	12.48
TOTAL	10.98¢/LB.	100.0%

4. Outcomes of Process and Economic Analysis Leading to Future Research

It has been assumed in our analysis that the cell material can be recycled as nutrient feed. This has not yet been demonstrated. The desire to use the cells as nutrients is because materials such as yeast extract and tryptone are prohibitively expensive. Further pretreatment of the cell mass may be necessary to ensure proper growth if they are to be used as proposed.

The cell recovery method is based on the process used by ICI in their single cell protein plant. Here, cells pretreated with alkaline are flocculated by acidifying the broth. It is conceivable that this process may not work as well with our culture of C. thermoaceticum. However, it should be noted that bentonite clays have been observed to cause flocculation of C. thermoaceticum and could be used in this scheme with further research and development.

It should be noted that the cost of utilities is the largest (45%) item in the total production cost. Most of this is spent on concentrating the stream from the fermentor to the distillation towers. Any increase in product concentration which can be realized without major sacrifice in productivity can appreciably reduce this production cost.

Lastly, our analysis shows this process is quite capital intensive in that 36% of the production costs are related

to equipment cost. Furthermore, 50% of that cost is for the fermentors and the 3-stage evaporators. Thus, an increase in productivity (without a decrease in acetate conc.) would decrease the fermentor volume and, consequently, the equipment related costs.

The three-stage evaporator turns out to be very economical method to concentrate the product stream from the fermentor; saving substantially on the steam requirements in the pre-evaporation to the azeotropic distillation tower. However, alternate technology, such as electrodialysis, might be considered to achieve this concentration. Electrodialysis may provide an even more economic concentration of product, as is shown in the analysis on the following pages.

CALCULATIONS

$$1 \text{ Lb. Acetic Acid} = \frac{454}{60} \quad 7.57 \text{ moles}$$

$$1 \text{ moles} = 6.023 \times 10^{23}$$

$$1 \text{ coulb} = 6.2419 \times 10^{18}$$

$$1 \text{ mole electrons} = 96.5 \times 10^3 \text{ coulb}$$

$$1 \text{ volt-coulb} = \text{watt-sec}$$

$$7.57 \text{ Moles} = 730.17 \times 10^3 \text{ coulb}$$

$$\text{Voltage across plate} = 1.6 \text{ volts (a typical value in such systems)}$$

$$\text{Energy} = 1.1683 \times 10^6 \text{ watt-sec}$$

$$= 1.168 \times 10^3 \text{ Kw-sec}$$

$$= .324 \text{ Kw Hr/Lb. Acid}$$

$$@ 2\text{¢/Kw Hr} = .65\text{¢/Lb.}$$

Assume unit is 50% eff => 1.3¢/Lb.
(many electrodialysis units are 85% eff on current)

Giving Cost of about 7.6¢/Lb.

(Note that at 25% eff, cost = 8.9¢/Lb.)

POTENTIAL OF ELECTRODIALYSIS
(rough estimate)

Savings in PEC ~ 20%

→ Maintenance

+ Fixed Charges

+ General Expenses ≈ 3.13¢/Lb.

Reduction in Utilities Cost
(not including cost of dialysis)

4.94¢/Lb. → ~ 2.64¢/Lb.

Reduction in Raw Materials

5.60 → ~ . 3¢/Lb.

Labor Costs (unchanged) .26¢/Lb.

Cost of Electrodialysis 1. 3¢/Lb.

7.63¢/Lb.

We, therefore, recommend that alternate product recovery schemes be explored. In particular, electrodialysis should be analyzed in the initial assessment.