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 biomasses and 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 conversion from cellulosic biomasses to liquid fuel (ethanol) and/or soluble sugars by the cellulolytic, anaerobic organism is also within the scope of this program. Process and engineering scale-up, along with economic analyses, will be performed throughout the course of the program.

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 utilization of biomass will require the effective degradation of cellulose and hemicellulose. For this reason, studies on the xylanase activity of Clostridium thermocellum and other thermophilic anaerobes have been continued. It has been shown that the xylanase and CMCase activities of C. thermocellum during fermentation are nearly identical. Furthermore, extracellular enzymes from this organism exhibit equal xylanase and cellulase activities regardless of the type of cellulosic biomass used for growth. For example, when grown on corn stover containing cellulose and hemicellulose, or Avicel or cotton lacking hemicellulose, identical kinetics of cellulase and xylanase accumulations were observed. These results suggest that the enzyme complex is non-specific with respect to its hydrolytic actions on the substrate. However, it should be mentioned that even though C. thermocellum is capable of degrading xylans, it is not able to metabolize pentose or its oligomers. To effectively use all of the degradation products from biomass, especially xylans, we have continued our studies on an unidentified thermophilic and anaerobic (HG-2) organism that can metabolize xylose. This strain is also capable of producing extracellular xylanase as well as being environmentally compatible with C. thermocellum. Lastly, a variant of C. thermocellum has been isolated which is able to metabolize glucose. More important, this variant produces extracellular-cellulases (CMCase and filter paper activity) when it is cultivated on glucose.

The enzyme complex from C. thermocellum continues to show resistance to feedback inhibition in every assay system tested. No inhibitory effect on Avicel hydrolysis by 50 mg/l of glucose, cellobiose, or xylose was observed. Enzyme activity as measured by the hydrolysis of Remazol Brilliant Blue-bound MN300 cellulose was shown to be unaffected by 300 g/l of glucose or xylose and 100 g/l of cellobiose. A buffer system has been developed which will aid the problem of pH control in tube and shake flask cultures. It was found that MOPS buffer at 0.1 M will suitably maintain neutral pH to allow maximal cell growth and production of cellulases. Lastly, a new hyper-producing mutant has been isolated from our previous mutant AS-39. This new mutant, designated as AS-39-22, showed an increase of 40-50% in Avicel-hydrolyzing ability as well as TNP-CMCase production.

A number of different biomasses has been examined with respect to their degradation characteristics by C. thermocellum. These include thermally exploded poplar, corn cob granules, corn stover and sugar cane bagasse. C. thermocellum has been found

to be capable of degrading all of these materials, but the amount of reducing sugars accumulated was found inversely related to particle size. To increase biomass surface area, as well as biomass concentration, a packed-bed cellulose fermentor has been constructed and tested. It is possible to load up to 160 g/l of cellulose in such a reactor. C. thermocellum was shown to be adsorbed onto the bed and degrade cellulose producing reducing sugars while remained adsorbed on the cellulose bed.

The production of ethanol directly from cellulosic biomass by C. thermocellum continues to be a major effort in this program. Through serial adaptation and transfer, an isolate (S-4) has been obtained which can tolerate ethanol up to 5% (V/V). This isolate still retains its cellulolytic activity equal to the parent. Batch culture studies using cellobiose showed the production of ethanol up to 1% (V/V) with no observable inhibition. In addition, this isolate was also shown to be active in the degradation of corn stover. To further increase the tolerance of C. thermocellum to ethanol, the effect of temperature has been examined. Temperatures ranging from 45 to 65°C have been tested. An increase to ethanol tolerance was observed when a sub-optimal growth temperature of 50°C was tested. On the other hand, the resistance to acetate was found to be independent of the growth temperature.

A method for generating protoplasts from growing cultures of C. thermocellum has been developed. Maximum stabilization of the protoplasts was obtained with 20% sucrose. However, this concentration of sucrose is inhibitory to growth; thus, it precludes its use as a stabilizer of viable L-forms. Alternatively, polyethylene glycol shows promise as a stabilizer. Work on the plasmids of C. thermocellum continues to concentrate in their isolation and characterization. To date, the plasmids exhibit an anomalous sedimentation behavior in sucrose gradients. The data indicate that this is due to aggregation of the plasmids.

Studies on acrylic acid production have continued actively. Acrylic acid production by Clostridium propionicum has been further studied in order to optimize the oxidation of propionic and to acrylic acid. Optimal concentrations of resting cells and the effect of propionic acid concentration have been determined. The amount of acrylate produced has been found to increase with increasing propionate concentration, as well as with increasing cell concentration. Acrylate production is also stimulated by the presence of lactate in the reaction mixture.

Studies have been conducted on the aerobic oxidation of propionic acid to acrylic acid by resting cells and cell-free extracts prepared from Escherichia coli. Although a variety of experimental conditions have been employed, no acrylic acid was detected. Similar experiments with lactic acid also failed.

Production of lactic acid has been investigated this quarter by employing mixed cultures of Clostridium thermocellum and a thermophilic lactic acid producing bacterium. Preliminary results are very encouraging. A mixed culture fermentation lasting 140 hours was successful. It was found that lactic acid concentration in the fermentor was 5.0 gm/l at 15 hrs after inoculation of the mixed culture. Reducing sugars increased to a level of 2.5 gm/l during the same time period. From the 60th to the 120th hours of the rate of lactic acid accumulation remained constant, but at a low rate (0.05 gm/l-hr), the results demonstrate direct conversion of cellulose to lactic acid via mixed culture fermentation.

The acetone-butanol fermentation has focused on means of increasing butanol tolerance by Clostridium acetobutylicum. The effect of temperature on inoculum preparation and growth has been hypothesized to influence solvent tolerance. Inoculum and growth temperatures ranging from 30 to 45°C were tested in various combinations. It was found that when an inoculum temperature of 30°C and followed by a growth temperature of 30°C, 50% increase in the butanol tolerance can be achieved.

Acetic acid production by Clostridium thermoaceticum has continued to isolate product tolerant strains. A new isolate (S-3) has been obtained which can tolerate up to 60 g/l of sodium acetate. Detailed studies have also shown that using pH control (7.0) the acetate ion is the causative agent in the inhibition of growth. Additional results have confirmed that non-growing cells of C. thermoaceticum can produce acetic acid. These confirmation studies were performed using an antibiotic rifamcin which is able to totally inhibit growth, but the organism shows continued acetic acid production. In addition, quantitative examination of the viable cell population has also been performed and also shows the production of acetic acid in the absence of growth. Cell recovery studies have also been initiated to examine cell recycle fermentations. An inexpensive flocculant, Bentonite, has shown to be effective at 1 g/l to enhance the sedimentation rate of C. thermoaceticum by 3700-fold.

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.

TABLE OF CONTENTS

	<u>Page</u>
Scope of Program	ii
Abstract	iv
Contributors of Program	vii
Table of Contents	viii

PROGRESS REPORT

I. MICROBIOLOGY OF CELLULOSE DEGRADATION AND CELLULASE PRODUCTION	1
A. Enzyme Production by <u>Clostridium thermocellum</u> ...	1
1. Introduction	1
2. Materials and Methods	2
3. Results and Discussion	3
B. Degradation of Cellulosic Biomass to Produce Soluble Reducing Sugars by <u>Clostridium thermocellum</u> (The Packed-Bed Fermentor)	16
1. Introduction	16
2. Materials and Methods	16
3. Results and Discussion	17
4. Future Work	26
C. Production of Ethanol from Cellulose by <u>Clostridium thermocellum</u>	28
1. Isolation and Characterization of New Ethanol Tolerant Strain (S-4) of <u>C. thermocellum</u>	28
a. Introduction	28
b. Materials and Methods	28
c. Results and Discussion	31
d. Future Work	50
2. Growth Inhibition of <u>C. thermocellum</u> by Fermentation Products	51
a. Introduction	51
b. Results and Discussion	51
c. Future Work	58

	<u>Page</u>
D. Degradation of Different Cellulosic Biomasses by <u>Clostridium thermocellum</u>	59
1. Introduction	59
2. Materials and Methods	59
3. Results and Discussion	64
4. Future Work	75
E. Genetic Manipulations	76
1. Protoplast Induction and Stabilization in <u>C. thermocellum</u>	76
a. Protoplast Induction in Actively Growing Cultures	78
b. Protoplast Stabilization	78
c. Effect of Temperature on Protoplast Stabilization	79
d. Effect of Cultural Age on Protoplast Stabilization	79
e. Stabilization of Protoplast as Function of NaCl concentration and 5 of 10% sucrose	81
f. Stabilization by High Sucrose Concentrations	81
g. Stabilization by Cellobiose, Mannitol and Polyethylene glycol	84
h. Conclusions and Future Work	84
2. Isolation of Plasmids from <u>C. thermocellum</u> ..	86
F. Deregulation of Clostridial Cellulases	105
1. Resistance of <u>C. thermocellum</u> cellulases to Sugars	105
2. pH Maintenance During Flask Cellulase Fermentation	107
3. Determination of Intracellular TNP-CMCase Activity	111
4. Chloramphenicol Inhibition on Growth and Cellulase Production	111
5. Isolation of Hyperproducing Cellulase Mutant by U.V. Irradiation	115
6. Mutation and Isolation of a Cellulase Hyperproducer Using Chemical Mutagenesis	116
7. Studies on Xylan and Xylanase	117
8. Effect of Nitrogen Source on Growth and Enzyme Production	123
9. Discrepancy Between M.I.T. and Wisconsin Reports	130

	<u>Page</u>
G. Evaluation of <u>Clostridium thermocellum</u> strains ..	131
for Xylanase and Cellulase Activities	131
1. Introduction	131
2. Materials and Methods	131
3. Results and Discussion	132
4. Future Work	136
II. PRODUCTION OF CHEMICAL FEEDSTOCKS	138
A. Production of Acrylic Acid by Fermentation	138
1. Introduction	138
2. Fermentation Studies with <u>Clostridium pro-</u> <u>pionicum</u>	138
a. Introduction	138
b. Materials and Methods	139
c. Results and Discussion	139
d. Future Studies	148
3. Aerobic Oxidation of Propionic Acid to Acrylic Acid by <u>Escherichia coli</u>	150
a. Introduction	150
b. Materials and Methods	151
c. Results and Discussion	151
d. Future Work	158
4. Production of Lactic Acid	160
a. Introduction	160
b. Materials and Methods	160
c. Results and Discussion	162
d. Future Experiments	173
B. Production of Acetone and Butanol by Fermentation	176
1. Introduction	176
2. Materials and Methods	177
3. Results and Discussion	178
4. Future Work	180
C. Production of Acetic Acid by Fermentation	185
1. Introduction	185
2. Materials and Methods	186
3. Results and Discussion	187
4. Future Work	202

I. MICROBIOLOGY OF CELLULOSE DEGRADATION AND CELLULASE PRODUCTION

A. ENZYME PRODUCTION BY CLOSTRIDIUM THERMOCELLUM

1. Introduction

The economic feasibility of a microbial process for biomass utilization is largely dependent upon the price of the raw material to be hydrolyzed and further converted to products. If extensively pretreated, purified celluloses are required, the process immediately becomes less desirable compared to a process based on the use of a cheap cellulosic material. This substrate constraint has prompted the examination of C.thermocellum's behavior on materials such as corn stover. Since a significant portion of this and other "realistic" substrates is hemicellulose, or xylan, a need for information concerning the utilization and enzymatic hydrolysis of xylan by C. thermocellum arose.

Initial studies in this vein were reported last period (Dec. 1, 1977-Feb. 28, 1978). The results indicated that xylan itself was unsuitable as growth substrate, yet the extracellular enzymes produced during fermentation on Solka floc (a material which is 10% xylan) could hydrolyze xylan as well as soluble CMC. Indeed, the kinetic profiles of extracellular xylanase and CMCase accumulation were nearly identical, suggesting the possibility that the same enzyme is responsible for both activities. That xylanase and cellulase are the same enzyme, however, was not proven conclusively.

Follow-up studies to the initial work have been performed. C. thermocellum has been cultured on corn stover and the presence of xylanase has been demonstrated. The organism was also cultured on avicel and cotton, substrates which contain no hemicellulose; nonetheless, xylanase was measured. A fermentation with a mixture of Solka floc and xylan as substrate was run to determine if xylan would be utilized under these conditions, compared with its poor use when cultured on xylan alone. Sugars accumulating during this fermentation as well as corn stover fermentations were subjected to HPLC analysis to determine the products of simultaneous cellulase and xylanase hydrolysis.

2. Materials and Methods

a. Culture Conditions

C. thermocellum, ATCC 27405, was cultured in CM3 medium, as described previously, with a variety of materials as growth substrates. When the organism was cultured on avicel or cotton, it had previously undergone three successive transfers, in flasks, in CM3/avicel medium. This would effectively dilute any hemicellulose that may have remained in the original stock culture tube (CM3/Solka floc) by a factor of approximately 1×10^6 . Two different types of corn stover were used as substrates. The difference was the size of the particles, finely powdered or coarsely ground in a Wiley mill.

b. HPLC Analysis

The system is the same as described in a previous progress report (Sept. 1, 1977 - Nov. 30, 1977) with the following changes: The column used is a Whatman Partisil 10-PAC analytical carbohydrate column preceded in series by a guard column containing HC-Pellosil, a silica pellicular packing material. The solvent used is an 80:20 mixture of acetonitrile and 0.0025M KH_2PO_4 (pH 5.0 with KOH) respectively. Mixed bed ion exchange resins were no longer employed for sample clean-up. Solka floc samples were ultra filtered as described previously. Samples from corn stover cultures were passed through Pasteur pipettes containing Waters Bondapak Corasil/AX and Bondapak Porasil/C18 to remove lignins and organic acids.

Other Methods

All enzyme assays and the reducing sugar assay have been described in a previous progress report.

3. Results and Discussion

The final values of xylanase and CMCase activities, and their ratios, for flask cultures of C. thermocellum on Solka floc, corn stover, avicel and cotton, are presented in Table I.A.1.

TABLE I.A.1

<u>Substrate</u>	<u>*Xylanase Mg/ml·hr</u>	<u>*CMCase mg/ml·hr</u>	<u>Xylanase CMCase</u>
Solka floc	7.0	6.75	1.04
Corn Stover	1.95	1.9	1.03
Avicel	3.0	3.2	0.94
Cotton	4.8	5.0	0.96

*The variation in the values among the substrates reflects the time when the fermentations were ended as well as C. thermocellum's ability to grow and produce enzymes on the particular substrate.

The kinetic profiles of xylanase and CMCase during a fermentation on Solka floc were presented in our last report and were shown to be practically identical. This is true of the xylanase and CMCase profiles for avicel and corn stovers as well. (Only a final sample of the cotton culture was taken due to sampling difficulties; thus the kinetic pattern was not followed). Thus, not only are the final ratios of xylanase to CMCase approximately equal to 1 (or more importantly, constant), but also the ratios of the two activities throughout the fermentations. Because of the similarity of the kinetics of both kinds of enzyme production regardless of the substrate tested, together with the presence of xylanase in avicel and cotton cultures (substrates unlikely to induce an enzyme specific for xylan), a plausible explanation is that the same nonspecific enzyme is responsible for both cellulose and xylan hydrolysis. The significance of this inference is the following: if xylanase and CMCase activities are indeed effected by the same enzyme, then any genetic improvements in cellulase activity of C. thermocellum should be accompanied by a concomitant improvement in xylanase activity. Thus, rather than having to "hit" two gene loci for hyper-producing mutants, a single mutation may be all that is necessary to cause increases in both activities. Such a mutant would therefore have improved capabilities for degrading both the hemicellulose and cellulose present in natural substrates.

When C. thermocellum was grown in batch, no pH control, on 0.5% Solka floc plus 0.5% xylan, both the cellulose and xylan were degraded as can be seen by the residual solids curve in Figure I.A.1. Reducing sugars, measured both as xylose and as glucose, accumulated to approximately 5g/l. The maximum level of pellet protein reached, 500 µg/ml, which is roughly equivalent to 1 g/l of cells, seems too high for the amount of carbohydrate consumed. It is thought that the unusually high value of pellet protein can be attributed to interference by xylan in the Lowry protein assay. If the Lowry values are correct, then the yield of cells on the mixed substrate is around 20% which is higher than the usually observed 15% on cellulose. In Figure I.A.2, the kinetic profiles of extracellular xylanase and CMCase are shown. As in the case of the other substrates tested, the profiles and final levels reached are very similar.

In Figure I.A.1, we saw that both cellulose (Solka floc) and xylan had to have been degraded since 1.0 - 1.5 g/l of solids remained at the end of the fermentation from the initial amount of 10 g/l total. However, in order to determine whether any of the xylan had actually been utilized for cell growth (as opposed to simply accumulating in the broth) under these conditions, it was necessary to analyze the accumulated sugars by HPLC. Such an analysis would ideally indicate whether certain sugars were preferentially consumed while

FIGURE I.A.1: KINETICS OF BATCH FERMENTATION OF *C. THERMOCELLUM* ON CM 3 MEDIUM CONTAINING 0.5% SOLKA FLOC + 0.5% XYLAN

FIGURE I.A.2: CMCase AND XYLANASE ACTIVITY PROFILES FROM FERMENTATION OF C. THERMOCELLUM ON 0.5% SOLKA FLOC PLUS 0.5% XYLAN

others that were also hydrolyzed accumulated. Unfortunately, this analysis is complicated by the fact that glucose and xylobiose do not resolve. This can be seen in Figures I.A.3 and I.A.4. Figure I.A.3 is a chromatogram of a standard mixture of xylose, glucose and cellobiose. Glucose has a retention time of ~11.4 minutes. Figure I.A. 4 is a chromatogram of a xylobiose sample obtained from the Finnish Sugar Co. It is claimed to be 90% xylobiose, with 5% contamination by glucose and 5% by some trisaccharide. If the major peak in this chromatogram (peak #2) is indeed xylobiose, then it is obvious that its retention time is so similar to that of glucose that the two would be indistinguishable as a mixture. There is the possibility that peak #2 is glucose and peak #3 is xylobiose. Until a purer preparation of xylobiose can be obtained, it is impossible to make any qualitative or quantitative assessment of glucose and xylobiose accumulated in fermentations on substrates containing significant fractions of cellulose and nemicellulose. Figures I.A.5 and I.A.6 are chromatograms of broth samples from the mixed substrate fermentation and a fermentation on powdered corn stover, respectively. In Figure I.A.5, it is obvious that in this 48 hour sample the major amount of material present appears to be glucose or xylobiose or perhaps both. Significant amounts of xylose and cellobiose accumulate as well and the kinetic pattern of these two sugars is shown in I.A. 7. It should be noted that at time

FIGURE I.A.3: HPLC CHROMATOGRAM OF STANDARD SUGARS: XYLOSE, GLUCOSE AND CELLOBIOSE, EACH AT 1.0 g/l

FIGURE I.A.4: HPLC CHROMATOGRAM OF "XYLOBIOSE" SAMPLE

FIGURE I.A.5: HPLC CHROMATOGRAM OF BROTH SUPERNATANT FROM
FERMENTATION OF C. THERMOCELLUM ON SOLKA
FLOC AND XYLAN.

FIGURE I.A.6: HPLC CHROMATOGRAM OF BROTH SUPERNATANT FROM
FERMENTATION OF C.THERMOCELLUM ON CORN STOVER

FIGURE I.A.7: PROFILE OF XYLOSE AND CELLOBIOSE ACCUMULATION,
AS ANALYZED BY HPLC, FROM FERMENTATION OF
C.THERMOCELLUM ON 0.5% SOLKA FLOC + 0.5% XYLAN

zero, no xylose or cellobiose were present.

Figure I.A.6 is a chromatogram of a 68 hr. sample from a fermentation on corn stover. Again the major material appears in the glucose-xylobiose region. Some cellobiose (~0.5 g/l) and a small amount of xylose (~0.2 g/l) are present. It is obvious that there are several unidentified peaks (which had not been seen when the mixed bed ion exchange resins were used for precleaning samples). It is likely that some of these compounds contribute to the total reducing sugar value and thus partially explain the discrepancy between the earlier HPLC analysis and total reducing sugars measured. None of the three peaks beyond cellobiose is cellotriose, as the latter has a retention time of ~38 minutes. These 3 peaks are present early in the fermentation and remain at relatively the same height in all samples analyzed. They are present in the broths of Solka floc cultures as well.

Until glucose and xylobiose can be resolved, little else can be said except that one or the other sugar, or possibly both are the major hydrolysis products of substrates like corn stover. As glucose is the major hydrolysis product from purer celluloses, it is speculated that it is a major product from this natural substrate as well.

B. Degradation of Cellulosic Biomass to Produce Soluble Reducing Sugars by *Clostridium thermocellum* (the Packed-Bed Fermentor)

1. Introduction

In any actual process for the production of reducing sugars and other useful products from biomass, a necessary requirement will be the ability to operate or feed cellulose at high concentrations. Concentrated cellulose slurries are highly viscous, difficult to pump and difficult to stir in conventional agitated fermentors. However, the mechanical properties of cellulose could be effectively exploited in a packed-bed fermentor where the cellulose forms the stationary phase. In this fashion, a high concentration of cellulose can be maintained and overcoming the previous problems. In this segment of the report, we will present our initial findings in the use of a packed-bed cellulose fermentor for the degradation of biomass to produce reducing sugars.

2. Materials and Methods

a. Organism

Clostridium thermocellum ATCC 27405 was used through this study.

b. Culture Conditions

The organism was grown on CM3 medium (Progress Report COO-4198-1). The Ca and Mg concentrations were reduced for this study to one-half of that previously reported to prevent precipitate formation. Inoculum was prepared by growing the organism on cellobiose (10 gm/l) in CM3 medium for 24 hours.

c. Description of Packed-Bed Fermentor

The packed-bed fermentor was made of glass containing two main parts as shown in Figure I.B.1. The packed-bed portion is a glass cylinder having 300 ml total volume. The upper portion of this fermentor contains a glass bulb which was used to separate the gas evolved during fermentation. In addition, in the upper section of the packed-bed, glass wool was packed to act as a retaining medium for the cellulose. Medium can be pumped to the packed-bed through an extended glass tube into the bottom of the bed. Liquid flows upward through the bed into the gas-liquid separator. The entire bed was submerged into a constant temperature water bath at 60°C. Although not shown in Figure I.B.1, it is also possible to circulate the fermentation broth through the packed-bed in an external loop. In this external loop, samples can be removed and pH can be controlled through base addition.

d. Analytical Techniques

The analytical techniques used to monitor the fermentations have all been reported in our previous reports. These include reducing sugars by DNS, cell growth by turbidity (Klett-Summerson), acetic acid and ethanol by gas chromatography and CMCase by DNS reagent.

3. Results and Discussion

a. Fluid Dynamic Characterization of Packed Bed

It was recognized at the onset of our packed-bed fermentor studies that liquid flow through the cellulose

FIGURE I.B.1: DIAGRAM OF PACKED-BED FERMENTOR (SCALE 1:2)

will be required. This requirement arises from the need for gas removal due to metabolism, to achieve continuous operations, and to operate with liquid recirculation for environmental control. In order to estimate the rate of liquid flow which can be achieved, pressure drop studies in the packed-bed using Solka floc were performed. The bed was loaded with 167 grams of cellulose per liter which has a total cross sectional area of 48 centimeters. Water was then circulated through and the pressure across the bed was then measured. The results from these studies are shown in Figure I.B.2. It is seen that a linear relationship between the flow rate and pressure drop is obtained. Furthermore, reasonably high flow rates can be achieved at modest pressure drops. For example, for the packed-bed fermentor as shown in Figure I.B.1, it is possible to circulate a liquid flow of 38 liters/hr. ($0.8 \text{ l/cm}^2\text{-hr}$) at a total pressure drop of 300 mm Hg ($20 \text{ mm Hg/cm} \cdot 15 \text{ cm}$). This moderate pressure drop can be easily attained in our laboratory system and the liquid flow appears to be reasonable at this time to achieve the necessary objectives.

b. Continuous Packed-Bed Fermentation Without Liquid Recirculation

The first trial in examining the performances of the packed-bed cellulose fermentor was attempted in a continuous mode of operation. Cellulose (Solka floc) was packed into the fermentor at a concentration of 50 gm/liter. The

FIGURE I.B.2: PRESSURE DROP OF PACKED-BED FERMENTOR
(CELLULOSE LOADING = 167 GM/LITER)

medium was CM3 and its composition previously presented (COO-4198-1). The start-up procedure consisted of the following. Approximately 200 ml of inoculum was pumped to the packed-bed displacing about 60% of the liquid initially in the fermentor. Medium feed and removal at 21 ml/hr, equivalent to a dilution rate of 0.07 hr^{-1} , was initiated 5 hours after inoculation. During the first 20 hours, there was little microbial action as observed by the physical appearance of the cellulose bed and the lack of gas formation. However, after 20 hours, considerable activities began to be expressed by the microorganisms. The results during this period of fermentation are shown in Figure I.B.3.

It can be seen from this Figure that the reducing sugar concentration began to increase from 2 gm/l to 5.5 gm/l. This was accompanied by a decrease in the pH indicating that acidic products (acetic) are being formed. It is also interesting to note that the cell concentration (Klett units) also began to decrease during this period. It is difficult to interpret this data, but the following explanation seems plausible. In the first 20 hours, a fraction of the inoculated cells was biologically inactive and this fraction was being continuously washed-out from the fermentor. However, the active organisms were able to adsorb onto the cellulose producing more cells which also are able to adhere onto the substrate. The net result being the formation of the end products, reducing sugars, acetic acid and ethanol.

FIGURE I.B.3: DEGRADATION OF CELLULOSE (SOLKA FLOC) BY *C. THERMOCELLUM* IN CONTINUOUS PACKED-BED FERMENTOR (DILUTION RATE = 0.07 HR^{-1})

During this active growth phase (first 4 hours of Figure I.B.3), we noted large amounts of gas formation as well as a shrinkage of the bed. Due to the utilization of the cellulose, it became necessary to remix and repack the bed at about the 4th hour as shown in Figure I.B.3. This repacking procedure is the reason for the discontinuity of the results shown in this Figure. Again, a transient period of behavior resulted and finally attaining a steady-state operation as shown from this Figure. During this steady-state period, the exit cell concentration was quite low (less than 10 Klett units corresponding to about 0.03 gm/liter) and the reducing sugar concentration was constant at about 5.5 gm/liter. At the end of the operation (20 hours), the packed-bed was disassembled and approximately 57% of the cellulose had been hydrolysed and about 0.85 gm/liter of cells was attached to the undegraded biomass.

c. Batch Packed-Bed Fermentation with Liquid Recirculation

Results from the preliminary study on the continuous operation of the packed-bed fermentor suggested that data analysis and interpretation are difficult due to the transient behaviors. It was therefore decided that the packed-bed fermentor be examined in a batch-mode where the medium is recirculated through the bed by an external loop. The bed (300 ml) was packed with Solka floc (35 gm) equivalent to a

concentration of 110 gm/liter. The recirculation loop was a vessel one liter in volume where pH (6.1) and temperature (60°C) were both controlled. The liquid circulation rate through the bed was maintained at a rate of 1.6 liter/hour. Four hundred milliliter of a 24-hour old inoculum grown on cellobiose was used. The results from this study are shown in Figure I.B.4.

Since the inoculum used was a healthy culture, immediate gas evolution was noticed. In addition, the medium exiting from the packed-up was totally clear containing no cells. The initial decrease in the reducing sugar was the result of metabolism of the organism on the residual cellobiose from the inoculum. Upon exhausting of the cellobiose in the first 12 hours, the organism began to produce reducing sugars, acetic acid and ethanol from the cellulose. It should be noted that, throughout the course of the fermentation, we did not observe significant amounts of cells exiting from the packed bed. During the subsequent 20 hours (12 to 32 hours), product formation was maximum. Unfortunately, at this time, the pH-controller malfunctioned and therefore the pH decreased immediately to about 5.6. This was accompanied by the cessation of acetic acid and ethanol formation, but reducing sugars continued to accumulate. The addition of NaOH during the portion when the pH controller operated properly, indicated a linear rate. A final reducing sugars concentration of 8 gm/liter was found at the end of the fermentation.

FIGURE I.B.4: BATCH OPERATED PACKED-BED FERMENTATION WITH pH CONTROL

The results from this fermentation are summarized in Table I.B.1. There are several interesting observations which should be noted. The degree of cellulose degradation (67%) was quite comparable to earlier studies using Solka floc. More important, the total cells which were adsorbed onto the cellulose bed were found to be nearly 14 gm/liter. This is extremely high as compared to stirred tank fermentations where cell concentrations are typically 1.0 to 2.0 gm/liter. This result indicates that the packed-bed served as an excellent collector where inherent cell recycle can be achieved. These results are extremely encouraging since they show that the use of packed-bed fermentors could be a solution in attaining high substrate and product concentrations.

4. Future Work

The future studies on this segment of the research include:

- Detail studies to obtain kinetic parameters of packed-bed fermentor.
- Examine the degradation of corn stover in packed-bed fermentor.
- Continuous operation of packed-bed fermentor with capability of cellulose addition.
- Attain high cell mass leading to high productivity and high product concentrations.

TABLE I.B.1: SUMMARY OF RESULTS FROM BATCH PACKED-BED FERMENTATION BY C. THERMOCELLUM USING SOLKA FLOC

Cellulose concentration in packed-bed	110	gm/liter
Cellulose concentration calculated over total volume (including recirculation reservoir)	38	gm/liter
Cellulose degraded	67	%
Total time	48	hours
Final cell concentration in packed-bed	14	gm/liter
% cell in solution	10	%
Final reducing sugars concentration	8	gm/liter
Final ethanol concentration	2.2	gm/liter
Final acetic acid concentration	2.4	gm/liter
Final CMCase activity	36	mg/ml-Hr

C. Production of Ethanol from Cellulose by Clostridium Thermocellum

1. Isolation and Characterization of New Ethanol Tolerant Strain (S-4) of C. Thermocellum

a. Introduction

In our last Progress Report (COO-4198-5), we reported that at 3% of ethanol or acetic acid causes nearly total inhibition of growth for C. thermocellum. To produce ethanol from biomass economically, the concentration of ethanol must be increased. In this period of our contract research, we have therefore devoted a significant effort in the isolation of an ethanol tolerant strain of C. thermocellum. The characterizations of this new strain will be presented in this report. In addition, some preliminary results of this new strain on its degradation capabilities on corn stover will also be presented.

b. Materials and Methods

The microorganism used throughout this study was Clostridium thermocellum. The parent strain, designated as ATCC 27405, was originally obtained from American Type Culture Collection, Rockville, Maryland. The new strain isolated for ethanol tolerance is designated as "S-4". The medium used in the studies is CM4 whose composition has already been reported.

An adaptation technique was used in the isolation of the ethanol tolerant strain. The procedure employed in our adaptation and selection program is shown in Figure I.C.1.1. The parent, ATCC 27405, was first serially transferred in CM4

FIGURE I.C.1.1: ADAPTATION AND ISOLATION PROCEDURE FOR ETHANOL TOLERANT STRAIN OF CLOSTRIDIUM THERMOCELLUM

medium using cellobiose as the carbon source and containing 10 g/l of ethanol. The parent was then transferred in liquid medium (cellobiose as substrate) containing stepwise increase of ethanol up to 40 g/l (5% V/V). A total of 40 transfers were performed during this adaptation process to higher concentrations of ethanol. A strain which can tolerate 5% V/V of ethanol was isolated, but was found to have weak cellulytic activity when grown on Solka floc. This strain was then passed through 15 transfers in the absence of ethanol onto medium containing Solka floc as the carbon source. The ability of this strain to grow on Solka floc equal to the parent, but tolerant to 5% (V/V) ethanol were the criteria used. Single colony isolation was then performed using solid medium containing Solka floc. This isolate is designated as "S-4" and subsequently tested for its properties.

All of the analytical procedures have already been reported and will not be repeated. The only exception is the determination of ethanol through gas chromatography. This was achieved using a Teflon column (5 ft X 1/8" OD) containing Chromosorb 101. Samples were acidified using 1 drop of 5N HCl for each milliliter of fermentation broth. The column temperature was programmed from 120°C to 160°C at a rate of 20°C/minute. The initial temperature was maintained for 2.5 minutes and the final temperature maintained for 3 minutes in order to remove other organic acids from the

column. Nitrogen was used as a carrier gas at a flow rate of 60 ml/min.

The corn stovers used in our studies were kindly furnished by Professor George T. Tsao, School of Chemical Engineering, Purdue University, West Lafayette, IN. The original corn stover contained cobs, stalks, and leaves in sizes up to 6 inches in length. It therefore necessitated some degree of size reduction. This was accomplished through ball-milling, kindly performed by the U. S. Army Natick Laboratories, Natick, MA (Drs. M. Mandel and F. Allen).

c. Results and Discussion

Using the adaptation and isolation procedures previously outlined, a strain of Clostridium thermocellum, designated as "S-4" was found to tolerate ethanol up to 5% V/V. This isolate was then tested for its growth capabilities in Hungate tubes containing cellobiose as the carbon source. The isolate was inoculated into medium containing ethanol up to 5% (V/V) and growth was measured as a percent of maximum (0% ethanol) after 40 to 50 hours of incubation. The results from these studies are shown in Figure I.C.1.2. The tolerance of the parent strain to ethanol is also shown in this Figure. It can be seen that the new isolate (S-4) exhibits a much improved tolerance to ethanol, especially between 2 to 3% V/V. For example, at 2% ethanol, the percent of maximum growth for S-4 and the parent were respectively 90% and 20%. It should be noted

FIGURE I.C.1.2: COMPARISON OF ETHANOL TOLERANCE ON GROWTH FOR PARENT (ATCC 27405)
AND ISOLATE S-4 OF CLOSTRIDIUM THERMOACETICUM

that the new strain is still inhibited by 5% ethanol to the extent of about 70%.

Since our adaptation and selection method employed cellobiose as the carbon source, the quantitative ability of the new strain to degrade cellulose is not known. To test strain S-4's cellulolytic capabilities, a fermentation study was performed using Solka floc as the substrate and the results are shown in Figure I.C.1.3. From an initial cellulose concentration of 10.5 g/l, over 80% of the substrate was degraded. This degree of degradation is quite similar to the parent strain of Clostridium thermocellum. It is seen from the result that the final reducing sugars obtained was 3.1 g/l and ethanol was 2.0 g/l. Although acetic acid production was not measured, it is reasonable to assume that its behavior will be similar to those found in the past (i.e., equal to the amount of ethanol). We conclude therefore the new isolate (S-4) is able to degrade cellulose at efficiencies similar to the parent. It is interesting to note that if we assume that acetic acid formation was equal to that of ethanol, nearly 7 g/l of products (reducing sugars, ethanol and acetic) can be derived from 8.5 g/l of cellulose degraded.

Having now the knowledge that the new isolate is able to degrade cellulose, we next asked the question whether this strain is able to degrade cellulose in the presence of ethanol. Experiments were therefore performed where the parent and

FIGURE I.C.1.3: CHARACTERIZATION OF NEW ISOLATE (S-4)
OF C. THERMOCELLUM (pH = 7.0)

S-4 were first cultivated on Solka floc as the carbon source for two days and in the absence of ethanol. These cultures served as the inoculum for subsequent testing. 24 Hungate tubes were prepared for each strain where the initial ethanol concentration was increased from 0 to 5% (V/V) in 1% increments. These tubes were then inoculated and incubated at 60°C without pH control for different periods of time. The residual sugar concentration and the reducing sugar formation were then measured during incubation. The results from these studies are shown in Tables I.C.1.1 through I.C.1.6 for ethanol concentrations of 0, 1, 2, 3, 4 and 5% (V/V), respectively. In general, the results showed that at all concentrations of ethanol tested, the new strain (S-4) is able to degrade cellulose and produce reducing sugars at a greater rate than the parent.

Quantitatively, the results are in agreement with the ethanol sensitivity results which were shown previously in Figure I.C.1.2. As mentioned earlier, the most dramatic range of increased tolerance by the new strain (S-4) is between 2 to 3% ethanol. To illustrate this behavior, the results from Table I.C.1.3 where the ethanol concentration was 2% (V/V) are graphically illustrated in Figures I.C.1.4 and I.C.1.5. It is seen from Figure I.C.1.4 that the degree of cellulose degradation at 2% ethanol for the new strain (S-4) is significantly improved over that of the parent. The accumulation of reducing sugars (Figure I.C.1.5) similarly show improvement of the new

TABLE I.C.1.1: COMPARISON OF CELLULOSE DEGRADATION AND SUGAR FORMATION BY PARENT AND STRAIN S-4 OF CLOSTRIDIUM THERMOCELLUM

(ETHANOL CONCENTRATION = 0% V/V)

Time (Days)	RESIDUAL CELLULOSE (Solka floc)		NET REDUCING SUGAR FORMATION	
	Parent (gm/l)	S-4 (gm/l)	Parent (gm/l)	S-4 (gm/l)
0	10.5	10.5	0	0
2	7.1	5.6	1.8	2.4
4	4.4	3.8	4.1	4.2
7	1.9	1.5	6.8	6.4
10	0.9	0.6	7.8	7.3

TABLE I.C.1.2: COMPARISON OF CELLULOSE DEGRADATION AND SUGAR
FORMATION BY PARENT AND STRAIN S-4 OF
CLOSTRIDIUM THERMOCELLUM

(ETHANOL CONCENTRATION = 1% V/V)

Time (Days)	RESIDUAL CELLULOSE (Solka floc)		NET REDUCING SUGAR FORMATION	
	Parent (gm/l)	S-4 (gm/l)	Parent (gm/l)	S-4 (gm/l)
0	10.5	10.5	0	0
2	8.6	6.0	1.6	2.2
4	8.0	4.3	2.7	4.0
7	6.2	2.1	3.4	6.1
10	2.5	1.0	6.7	7.0

TABLE I.C.1.3: COMPARISON OF CELLULOSE DEGRADATION AND SUGAR
FORMATION BY PARENT AND STRAIN S-4 OF
CLOSTRIDIUM THERMOCELLUM

(ETHANOL CONCENTRATION = 2% V/V)

Time (Days)	RESIDUAL CELLULOSE (Solka floc)		NET REDUCING SUGAR FORMATION	
	Parent (gm/l)	S-4 (gm/l)	Parent (gm/l)	S-4 (gm/l)
0	10.5	10.5	0	0
2	8.9	6.6	1.7	2.3
4	8.2	5.1	2.5	4.0
7	7.1	2.5	3.5	6.0
10	6.1	1.5	4.0	6.7

TABLE I.C.1.4: COMPARISON OF CELLULOSE DEGRADATION AND SUGAR
FORMATION BY PARENT AND STRAIN S-4 OF
CLOSTRIDIUM THERMOCELLUM

(ETHANOL CONCENTRATION = 3% V/V)

Time (Days)	RESIDUAL CELLULOSE (Solka floc)		NET REDUCING SUGAR FORMATION	
	Parent (gm/l)	S-4 (gm/l)	Parent (gm/l)	S-4 (gm/l)
0	10.5	10.5	0	0
2	9.1	7.5	1.7	2.2
4	8.4	6.1	2.2	3.5
7	7.2	3.9	3.0	5.2
10	6.3	2.9	3.7	5.9

TABLE I.C.1.5: COMPARISON OF CELLULOSE DEGRADATION AND SUGAR
FORMATION BY PARENT AND STRAIN S-4 OF
CLOSTRIDIUM THERMOCELLUM

(ETHANOL CONCENTRATION = 4% V/V)

Time (Days)	RESIDUAL CELLULOSE (Solka floc)		NET REDUCING SUGAR FORMATION	
	Parent (gm/l)	S-4 (gm/l)	Parent (gm/l)	S-4 (gm/l)
0	10.5	10.5	0	0
2	9.2	7.8	1.3	2.1
4	8.6	7.6	2.0	3.1
7	7.6	4.5	2.7	4.2
10	6.8	4.3	3.2	5.1

TABLE I.C.1.6: COMPARISON OF CELLULOSE DEGRADATION AND SUGAR
FORMATION OF PARENT AND STRAIN S-4 OF
CLOSTRIDIUM THERMOCELLUM

(ETHANOL CONCENTRATION = 5% V/V)

Time (Days)	RESIDUAL CELLULOSE (Solka floc)		NET REDUCING SUGAR FORMATION	
	Parent (gm/l)	S-4 (gm/l)	Parent (gm/l)	S-4 (gm/l)
0	10.5	10.5	0	0
2	9.3	8.7	1.2	1.8
4	9.0	8.1	1.8	2.5
7	8.1	7.0	2.3	3.3
10	7.3	6.3	2.7	3.7

FIGURE I.C.1.4: COMPARISON OF CELLULOSE (SOLKA FLOC) DEGRADATION
BY PARENT AND ISOLATE S-4 OF C. THERMOCELLUM

FIGURE I.C.1.5: COMPARISON OF REDUCING SUGAR PRODUCTION FROM CELLULOSE (SOLKA FLOC) BY PARENT AND ISOLATE S-4 OF C. THERMOCELLUM

isolate as compared to the parent. These results confirm that the new strain is able to degrade cellulose at higher concentrations of ethanol at rates greater than the parent. It also reinforces our selection technique for isolating useful strains for product formation. We feel we now have available a simple and expedient selection method for a future isolation program to obtain organisms with the desired properties.

At this point, it still remained to be answered whether the new ethanol tolerant strain is able to produce ethanol at concentrations higher than the parent. Unfortunately, we have the additional problem when high concentration of ethanol is desired. This being our inability to maintain or feed high concentration of insoluble biomass to our fermentations. For example, in our previous Report (COO-4198-5, Figure I.C.3, page 32), we resorted to batch-feeding of cellulose in order to obtain high substrate concentrations. In this previous study, about 4 g/l of ethanol and acetic acid each was obtained when a total cellulose concentration of 45 g/l was used through feedings. Even then, at these product concentrations, there was still 15 g/l of undegraded cellulose. In order to test the new strain as to its capability in producing ethanol, we decided to employ a simpler system in seeking this answer. This is the use of a soluble substrate, cellobiose, fed periodically to the fermentation. We feel that the results from such a substrate are translatable in view of our other findings. The results from this experiment are shown in Figure I.C.1.6.

FIGURE I.C.1.6: PRODUCTION OF ETHANOL BY NEW ISOLATE (S-4) OF C. THERMOCELLUM

As seen from this Figure, the initial (up to 20 hours) rate of cell growth (Klett unit) was quite rapid and exhibited typical exponential behavior. The initial ethanol formation also was exponential. Ethanol production continues in a linear fashion even though the cell growth decreased. Although acetic acid production was not measured, one would expect its concentration to be about equal to that of ethanol. A final concentration of 7.5 g/l of ethanol was obtained even though, at this time, no decrease in its rate has been observed. However, due to the dilution effect when nutrients were added, the corrected equivalent concentration of ethanol was actually 9.1 g/l. If we also assume that 9.1 g/l of acetic acid was also produced, a very impressive conversion yield had been achieved as shown below. In this experiment, a total of 45 g/l of cellobiose was added and 13 g/l remained at the end. Therefore, the sum of the product yield (ethanol = 9.1, acetic acid = 9.1, cellobiose utilized = 32) was calculated to be 0.57 grams of product per gram of substrate. These results encourage us to continue our efforts in the direct conversion of cellulosic biomass to useful products.

The final series of experiments were performed to determine the degradation capability of the new strain (S-4) on corn stovers which were recently furnished by Professor G. T. Tsao. As mentioned in the Materials and Methods Section, the only pretreatment was ball-milling of the corn stover.

Unfortunately, the dark brown color of the corn stover suspension and the suspended particles prevented us from measuring cell growth during fermentation. The fermentation was inoculated from a seed culture grown on Solka floc. pH was manually controlled at a value of about 7.0, fermentation temperature was 60°C and the initial corn stover concentration was 36 g/l. The results are shown in Figure I.C.1.7. The formation of reducing sugars (open circles), ethanol (open triangles) and acetic acid (closed squares) showed an initial rapid rate. This was followed by a decreased rate of product formation, but constant over the entire fermentation cycle. At the end of the fermentation, 8 g/l of reducing sugars accumulated and 2.2 g/l of ethanol and acetic acid each also formed. It should be mentioned that the organism did not undergo any previous adaptation to this new biomass (corn stover). We believe that serial transfer and adaptation studies should enable us to increase the degradation rate. It is sufficient to state, on the first transfer, that this organism is able to impressively metabolize a totally new substrate possessing radically different physical and chemical properties.

The degree of biomass conversion by Clostridium thermocellum is likewise quite impressive and interesting. Shown in Table I.C.1.7 are the compositions of corn stover, wheat straw and rice straw as reported by Dr. C. R. Wilke. It is seen that the sum of the carbohydrates in corn stover is only

FIGURE I.C.1.7: DEGRADATION OF CELLULOSE (CORN STOVER) BY
CLOSTRIDIUM THERMOCELLUM, STRAIN S-4
 (INITIAL CORN STOVER = 36 g/l)

TABLE I.C.1.7: COMPOSITION OF DIFFERENT BIOMASSES *

	<u>Corn Stover</u> (%)	<u>Wheat Straw</u> (%)	<u>Rice Straw</u> (%)
Glucan	35.1	32.9	36.9
Mannan	0.25	0.72	1.6
Galactan	0.75	2.16	0.4
Xylan	13.0	16.9	13.0
Arabinan	2.8	2.11	4.0
Σ Carbohydrate	51.9	54.8	55.9
Lignin	15.1	14.5	9.9
Ash	4.3	9.6	12.4
Aceotropic benzene- alcohol extractives	5.5	7.2	4.4
Protein	4	3	-
Unidentified	18	8	10
Σ	98.8	97.1	92.7

* Data from Dr. Charles R. Wilke, Lawrence Berkeley Laboratory, University of California, Berkeley, California 94720.

51.9%. In our fermentation using corn stover, an initial value of 36 g/l was employed. This is equivalent to a total carbohydrate concentration of 18.7 g/l. From this total, Clostridium thermocellum was able to produce 8 g/l of sugars and 2.2 g/l of ethanol and 2.2 g/l of acetic acid. This is equivalent to a conversion yield of 0.66 grams of product per grams of carbohydrate. We believe this yield is quite excellent and shows further encouragement for our project objectives.

d. Future Work

The future studies on this segment of the project include:

- Further increase the tolerance of C. thermocellum to ethanol.
- Incorporate the packed-bed fermentor to achieve high product concentrations.
- Increase the yield of ethanol from biomass by preventing the formation of acetic acid: this can be achieved through mutation and the use of inhibitors.
- Examine other biomasses such as bagasse, straw, Poplar, etc.

2. Growth Inhibition of C. thermocellum by Fermentation Products

a. Introduction

These studies were designed to determine the inhibition of C. thermocellum by fermentation products, as well as the effect temperature has on this inhibition.

b. Results and Discussion

1. Growth of C. thermocellum at Different Temperatures

Figure I.C.2.1 shows the growth curves of C. thermocellum within the range 45°C to 65°C. This range includes both suboptimal and superoptimal temperatures. The culture is capable of maintaining balanced growth at all temperatures. Figure I.C.2.2 indicates that the optimum temperature is around 58°C and shows that the activation energy for growth is 23 Kcal/mole. The growth rates represented in Figure I.C.2.2 are average values of several experiments.

2. Growth Inhibition of C. thermocellum by Ethanol at 60°C Experimental Protocol

An exponentially growing culture of C. thermocellum is challenged with different concentrations of ethanol by injection of 1 ml of the proper dilutions. At intervals, the optical density at 660 is measured and the inhibition index calculated according to the definition:

$$\text{Inhibition Index (II)} = 1 - \frac{A_x^t - A_x^{t_0}}{A_o^t - A_o^{t_0}}$$

where A_x^t = Adsorbance at time t, in the presence of X grs/l of ethanol

$A_x^{t_0}$ = idem, at zero time

Figure I.C.2.1: Exponential growth of *C. thermocellum* at different temperatures.

A_{O}^t = Adsorbance at time x, in the absence of ethanol

A_{O}^t = idem, at zero time

The inhibition index termed "cell yield" is the value for $t = 20$ hrs after addition.

Figure I.C.2.3. shows the results of a typical experiment. Figure I.C.2.4. shows the time dependence of the inhibition index. In order to compare results of different experiments, the II are calculated after one generation time of the control culture.

3. Growth Inhibition of *C. thermocellum* by Acetate and Ethanol at Different Temperatures

Using the same experimental protocol previously described, *C. thermocellum* cultures grown at different temperatures for 3 to 4 generations were also challenged with ethanol or with sodium acetate (pH 7.4) concentrations of 0, 2, 5, 8, 10, 13, 15, 18, 20 and 25 grs/lt; the inhibition indexes at each temperature were calculated as defined previously and the concentrations for 50% inhibition and for 25% inhibition calculated by interpolation in the corresponding II vs. ethanol concentration curve.

Figure I.C.2.5. summarized the results. While the acetate response is independent of the temperature of growth, the inhibitory effect of ethanol is clearly a function of that temperature. The maximum resistance to ethanol is attained at about 50°C in the suboptimal range. At both extreme temperatures the reduction in ethanol resistance is obvious.

Figure I.C.2.3 : Inhibition of Clostridium thermocellum by ethanol during balanced growth.

Figure I.C.2.4.: Time dependence of the inhibition index in ethanol.

- 5 grs/lt
- △ 10 grs/lt
- 15 grs/lt
- 20 grs/lt
- ◊ 25 grs/lt

Figure I.C.2.5 : Ethanol and acetate concentrations for 50% and 25% inhibitions at different temperatures.

c. Future Work

- To investigate the effect of cultural nutrition on the dependence that ethanol toxicity has on temperature.

- The inhibition exerted by other fermentation products of C. thermocellum, as well as related compounds is currently under investigation

Once this baseline data is available, we will study the lipid composition of cell membranes, as well as the possibilities of its alteration by environmental manipulations, with the specific aim of increasing the resistance to these fermentation products.

D. Degradation of Different Cellulosic Biomasses by *Clostridium thermocellum*

1. Introduction

The importance of raw material costs in the overall process of cellulose degradation stresses the need for cheap and abundantly available biomass sources. The natural choice for preliminary investigation falls on materials that are wastes or by-products of the large existing agricultural industry. The choice of substrates was also influenced by consideration of the physical nature of the materials and their suitability for continuous feeding.

In this context, studies on degradation of thermally exploded lignocellulose poplar, corn cob granules, corn stover and sugar cane bagasse by *C. thermocellum* were performed. Special consideration was given to the effect of particle size distribution on the production yields. The economic aspect of particle size reduction was also analyzed.

2. Materials and Methods

a. Poplar

The thermally exploded lignocellulose poplar used as main substrate was obtained from the Pulp and Paper Research Institute of Canada (for details, see page 15 of previous Progress Report COO-4198-5). Fermentations were run at 60°C in anaerobic shake flasks containing 300 ml of CM 3 medium. A 3% inoculum of *C. thermocellum* grown on Solka floc (SW 40,

Brown Co.) was used. The effect of the pretreatment of the substrate was studied by using as substrates the original poplar, an alkaline washed poplar, an ethanol washed poplar and a Solka floc control. The alkaline wash consisted of weighing wet poplar equivalent to 10 g of dry solids, adding 100 ml 1N NaOH and centrifuging at 3,000 xg for 10 minutes. The precipitate was suspended in 100 ml distilled water and recentrifuged. The final precipitate was resuspended in 100 ml distilled water. Ten ml samples were taken in triplicate and filtered through Whatman Paper #1 for determination of loss of solids. The remaining slurry was used for preparation of the medium. The ethanol wash was performed identically using 95% ethanol.

b. Corn Cobs

The corn cobs granules were obtained from The Andersons, Cob Division, P.O. Box 119, Maumee, OH 43537. The term GRIT-O'COBS is the trademark for material from the woody rings of the corn cob. The term LITE-R-COBS is the trademark for the combination of fine chaff, coarse chaff and pith (see Figure I.D.1). The samples are designated by numbers that refer to their particle size. Hence, GRIT-O'COBS 814 contains particles that went through screen 8 and were retained by screen 14 (U. S. standard screens). LITE-R-COBS -30 contains all particles that could go through screen 30.

Experiments were run at 60°C in anaerobic shake flasks containing 300 ml of CM 3 medium. Corn cob granules

FIGURE I.D.1: NOMENCLATURE CONCERNING CORN COBS

(From the publication "Primary Technical Properties
of Grit-O'Cobs ®, The Andersons, Cob Division)

of five different particle sizes were used as main substrates. A 3% inoculum of C. thermocellum grown in Solka floc was utilized. Samples were periodically withdrawn over the 90-hour time course of the fermentation and the pH profile was followed. Reducing sugars were measured as glucose using the DNS assay described in earlier progress reports. The reducing sugars accumulation was found by subtracting the initial glucose concentration from the final one. Residual cellulose assays were not performed since the size of the particles did not allow accurate sampling.

To overcome the problem of sampling, an experiment was run by culturing C. thermocellum in Hungate tubes containing 9 ml of CM 3 medium and 1 ml of inoculum. Fourteen different corn cob granules varying in source and particle size were used. The tubes were run in duplicate and a third tube served as blank by the replacement of the 1 ml inoculum by 1 ml of distilled water. The tubes were incubated at 60°C. After 75 hr, the contents of each tube were filtered through Whatman Paper #1. The pH was checked in the filtrate, which was then centrifuged at 20,000 x g for 15 minutes. The degraded substrate was expressed as 100 minus the percentage of undegraded corn stover in the sample to undegraded corn stover in uninoculated blank. A reducing sugars DNS assay was performed in the supernatant. The reducing sugar accumulation is expressed after correction of the reducing sugar concentration in the blanks.

c. Corn Stover and Sugar Cane Bagasse

A Wiley milled corn stover sample was obtained from Professor George Tsao, School of Chemical Engineering, Purdue University, West Lafayette, Indiana. A hammer milled sugar cane bagasse sample was obtained from Ander-Cane Inc., 1310 Carbio Center, Naples, Florida 33942. Both samples had a moisture content of 6% (dry basis).

An experiment was conducted in Hungate tubes containing 9 ml of CM 3 medium and 1 ml of C. thermocellum inoculum grown in Solka floc on 1 ml of distilled water (uninoculated control). The time course of the 60°C fermentation was followed by examining sets of tubes along 72 hr. Each set was composed of triplicates of inoculated corn stover and bagasse tubes, plus one inoculated Solka floc tube. Duplicates of uninoculated corn stover and bagasse tubes plus one inoculated Solka floc tube were also part of the sample set as controls.

Samples were filtered through Whatman Paper #1 for determination of residual substrate. The pH was checked in the filtrate, which was then centrifuged at 20,000 x g for 15 minutes. A reducing sugar DNS assay was performed on the supernatant.

Another experiment was performed to determine the particle size distribution of the corn stover and sugar cane bagasse used. Ten grams of sample were manually sifted

for 3 minutes through a set of Tyler standard screens; the mass fraction of total sample retained by each screen was determined gravimetrically.

3. Results and Discussion

a. Poplar

The washing procedures used for pretreatment of the poplar caused considerable losses of solids as can be seen from Table I.D.1.

TABLE I.D.1: EXPLODED POPLAR LOSSES DURING PRETREATMENT

Pretreatment of Poplar	% Solids Lost
Alkaline Wash	59
Ethanol Wash	48

No significant growth was detected in the flasks containing treated or untreated poplar while the Solka floc control sustained normal growth as indicated by color change and pH drop.

b. Corn Cobs

The reducing sugars accumulation and the final pH of broths obtained in shake flask experiments using different size corn cob granules are shown in Table I.D.2.

TABLE I.D.2: PERFORMANCE OF CORN GRANULES AS A CARBON SOURCE IN C. THERMOCELLUM FERMENTATIONS (SHAKE FLASK)

Carbon-Source	Reducing Sugars Accumulation (mg/ml)	Final pH
GRIT-O'COBS 814	1.34	6.55
GRIT-O'COBS 1420	1.50	6.50
GRIT-O'COBS 4060	2.93	6.30
LITE-R-COBS 1030	2.44	6.45
LITE-R-COBS -30	1.97	6.40
SOLKA FLOC	0.78	6.35

Unfortunately, a contamination has probably occurred in the Solka floc control flask and the accumulation of sugars is quite low. The highest sugar accumulation is obtained with GRIT-O'COBS particles of dimensions between 297 and 429 μm . Since the particles are not spherical, but rather needlelike, the dimensions found in the screen analysis refer to the second major dimension (in this case, the thickness of the particle).

Table I.D.3 shows the results obtained in Hungate tubes for the 14 types of corn cob granules. The final pH in uninoculated tubes was 7.2, while in the inoculated tubes it was in the range of 6.0 - 6.2. This data are plotted in

TABLE I.D.3: PERFORMANCE OF CORN COB GRANULES AS C-SOURCE IN
C. THERMOCELLUM FERMENTATIONS (HUNGATE TUBES)

C-Source	Sample	% Substrate Consumption	Reducing Sugars Production (mg/ml)
GRIT-O'COBS 814	a	8	-
	b	11	-
	1014		
	a	33	-
	b	35	1.38
	1020		
	a	38	1.58
	b	35	1.38
	1420		
	a	-	2.10
	b	34	1.58
	1440A		
	a	37	1.70
	b	39	1.53
	1440B		
	a	45	1.80
	b	43	1.60
	1440C		
	a	43	2.05
	b	40	1.78
	2040		
	a	45	2.15
	b	46	2.00
	4060		
	a	55	2.38
	b	57	2.35
	-20		
	a	49	2.58
	b	50	2.13
	-40		
	a	61	2.70
	b	62	2.75
	-60		
	a	64	2.88
	b	66	2.95
LITE-R-COBS 1030	a	44	1.85
	b	45	1.68
	-30		
	a	54	2.25
	b	55	2.30

Figure I.D.2 where substrate consumption and reducing sugar accumulation are shown as a function of particle size. The data from GRIT-O'COBS fits reasonably well a straight line and yields the following correlations:

$$\begin{aligned} X &= 1.62 - 1.86 \log D \\ \text{and} \\ Y &= 32.0 - 37.5 \log D \end{aligned}$$

where: X = reducing sugar accumulation (g/l)

Y = % corn cob consumed

D = particle size (mm)

The data from LITE-R-COBS seems to follow the same trend, but the number of samples is insufficient for any conclusion.

c. Corn Stover and Bagasse

Figures I.D.3 and I.D.4 show the time profile of the fermentation of bagasse corn stover and Solka floc. The spread of the data can be explained in terms of the difficulty in reproducing exactly the same conditions in individual Hungate tubes as opposed to shake flasks, where samples are always withdrawn from the same broth. The corn stover showed higher substrate utilization and higher reducing sugar accumulation although the final values (around 7.6 g/l residual substrate and 1 g/l reducing sugars) are relatively low as compared to the Solka floc control.

The differential screen analysis of the corn stover and bagasse samples is shown in Table I.D.4, where

FIGURE I.D.2: EFFECT OF PARTICLE SIZE OF CORN COB GRANULES (GRIT-O'COBS) ON
SUBSTRATE CONSUMPTION AND REDUCING SUGARS ACCUMULATION BY
CLOSTRIDIUM THERMOCELLUM

FIGURE I.D.3: REDUCING SUGARS ACCUMULATION AND pH DURING GROWTH OF *C. THERMOCELLUM* IN CM3 MEDIUM WITH CORN STOVER OR SUGAR CANE BAGASSE AS MAIN C-SOURCE (OPEN SYMBOLS REFER TO INOCULATED MEDIUM; CLOSED SYMBOLS REFER TO UNINOCULATED MEDIUM)

FIGURE I.D.4: RESIDUAL SUBSTRATE DURING GROWTH OF *C. THERMOCELLUM* IN CM3 MEDIUM WITH CORN STOVER OR SUGAR CANE BAGASSE AS MAIN C-SOURCE

TABLE I.D.4: DIFFERENTIAL SCREEN ANALYSIS OF CORN STOVER AND SUGAR CANE BAGASSE

MESH passed/retained	D _{p,n} (mm)	Δφ _n				D̄ _n (mm)
		Corn Stover		Sugar Cane Bagasse		
		Sample 1	Sample 2	Sample 1	Sample 2	
4/ 6	3.327	0.0042	0.0000	0.0058	0.0185	4.013
6/ 8	2.362	0.0083	0.0058	0.0132	0.0140	2.845
8/ 10	1.651	0.0423	0.0666	0.0762	0.0779	2.007
10/ 14	1.168	0.1803	0.1949	0.1733	0.1784	1.410
14/ 20	0.833	0.3075	0.2639	0.2700	0.2704	1.001
20/ 28	0.589	0.1844	0.2006	0.2151	0.2224	0.711
28/ 35	0.417	0.1319	0.1347	0.1443	0.1411	0.503
35/100	0.147	0.1270	0.1077	0.0669	0.0664	0.282
parr		0.0142	0.0260	0.0353	0.0108	0.074
A _w (x10 ² cm ² /g)		3.3	3.5	2.5	2.1	

$D_{p,n}$ is the second largest major dimension of particles retained by screen n (i.e., the mesh opening of screen n), $\Delta\phi_n$ is the mass fraction of total sample that is retained by screen n having passed through screen $n-1$ and $\bar{D}_n$ is the arithmetic mean of $D_{p,n}$ and $D_{p,n-1}$. The specific area of the samples was calculated according to the equation: (McCabe & Smith, Unit Operations of Chemical Engineering, McGraw Hill, 1976):

$$A_w = \frac{6}{\psi_s p_p} \sum_{n=1}^{n_T} \frac{\Delta\phi_n}{\bar{D}_n}$$

where: n_T = number of screens

A_w = total surface of one unit mass of sample

p_p = particle density

ψ_s = sphericity of particle

The particle density was estimated by water volume displacement and was found to be 0.42 g/ml for corn stover and 0.59 g/ml for bagasse. The sphericity was assumed to be 0.7 as suggested by McCabe & Smith for crushed materials. The calculations show that the corn stover sample had a higher specific area than the sugar cane bagasse.

d. Discussion

The use of exploded poplar as a substrate in cellulose fermentations would have the advantage of the flowability

of a slurry for the purpose of feeding continuous reactors. However, the results obtained are not encouraging since even if the growth kinetics could be improved using the pretreated poplar the losses during the washes seem to be uneconomical.

Corn cob granules appear to be a good substrate, but its use in fermentation processes would have to compete with other end-uses such as ingredients in fertilizers and animal feed.

Some plausible hypothesis to explain the dependency of sugar accumulation and substrate utilization on particle size of corn cob granules are the unfolding of tertiary and quaternary structures of cellulose and the greater specific surface area for microbial attack in smaller particles. The utilization of finer corn cob granules seems to be quite promising. Their flowing characteristics will apparently fit well in a solids feeding system for continuous fermentation processes. Evidently, the use of finer granules involves an energy intensive step of particle size reduction. The power required for crushing and grinding from large feed can be estimated by Bond law (McCabe & Smith):

$$\frac{P}{m} = \frac{K_b}{D_p}$$

where P is the power required by the grinder, m is the feed rate, D_p is the diameter of the particles produced and K_b is

a constant that depends on the type of machine and on the material being crushed.

According to this theory, a reduction in final particle size from 1 mm to 0.5 mm would require an increase of power consumption by 40%. On the other hand, according to the correlations obtained for corn cob granules, such reduction would cause an increase of 35% in the reducing sugar accumulation and in the substrate consumption. As described earlier, the raw materials represent a major portion of the costs obtained by cellulose fermentation; recent economic estimates made in our laboratory place the raw materials in the range of 50-60% of the product costs. Therefore, the increase in power required for size reduction is largely compensated by the increase in yield of product from total substrate.

The same rationale might apply to corn stover and sugar cane bagasse so the performance of these substrates in a finely divided form will be investigated. According to assays performed in the Lawrence Berkeley Laboratory, University of California, Berkeley, California 94720, the cellulose content of corn stover is around 35%. Accordingly, the substrate utilization level of 25% obtained for corn stover in these preliminary studies (with no particle size selection) is quite encouraging since the maximum level would be around 35%. In regards to the sugar cane bagasse used, its specific area is rather low (around $2 \times 10^2 \text{ cm}^2/\text{g}$) so this might partially explain the low

sugar accumulation and substrate consumption. An improvement is expected from particle size selection.

4. Future Work

- Determine the influence of particle size of corn stover and sugar cane bagasse in the fermentation pattern of cellulose degradation.

- Study by electron microscopy the spatial arrangement of substrate particles and microbial cells during the growth of C. thermocellum in order to improve growth conditions.

- Complete construction and set-up of continuous culture system using a solids feeder in order to perform preliminary runs using various natural substrates.

E. Genetic Manipulations

This phase of the project has continued to emphasize the development of gene transfer systems in C. thermocellum. Specifically, we have concentrated in characterizing the techniques for protoplast induction and stabilization, towards the goal of achieving cell fusion, as well as the development of procedures for plasmid isolation.

1. Protoplast induction and stabilization in C. thermocellum

We have developed two different methods for the formation of protoplasts from C. thermocellum. One involves the use of non-growing washed cells while in the second we induce protoplast formation in actively growing cells. The first method using resting cells was described in previous reports. The main problem of this method was the variability in cell viability in different experiments which ranged between 0.1-20%. Mg^{++} and Ca^{++} concentrations did not affect the viability (Table I.E.1.1. while the viability was decreased as the temperature of protoplast induction was increased (Table I.E.1.2.)). In the second method to induce protoplast formation from growing cells, the viability of the cells was not affected. This is probably due to the fact that this method does not involve any aerobic manipulations and the protoplast inducers and stabilizers are added anaerobically into the growing culture.

Table I.E.1.1. Viability of cells at different Ca^{++} and Mg^{++} concentrations

CaCl_2 mM	-	20	40	60	-	-	-	-
MgCl_2 mM	-	-	-	-	-	5	12.5	25
% viability	10	17	6.6	2.5	5	4	7	4

Table I.E.1.2. Viability of cells at different temperatures

viability %	0°C 20	25°C 15	37°C 0.25	44°C 0.1
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a. Protoplast induction in actively growing cultures

C. thermocellum cultures were grown in CM4-cellobiose medium, inoculated into CM4-cellobiose containing 5% sucrose and grown to $OD_{660} = 0.3$ at 60° . Lysozyme (0.5 and 100ug/ml) was added at this point and further incubated at 60° , after 3 hr incubation the samples from the cultures were observed and counted in the microscope, OD_{660} was followed throughout the incubation period. No cells were observed in any of the lysozyme treated cultures, some protoplasts were seen and a large quantity of cell debris could be seen. When higher concentrations of lysozyme were used (500 and 1000 ug/ml) the protoplasts aggregated and the OD_{660} increased with incubation time due to the accumulation of a white precipitate (presumably denatured lysozyme).

Penicillin (100, 500 and 2500 u/ml) was added as protoplast inducer with or without lysozyme. The only detectable effect of penicillin was growth inhibition. When added in addition to lysozyme, penicillin did not affect the results. In all the cultures where lysozyme was present the OD_{660} went down drastically after 5-10 min. incubation at 60° and a large quantity of cell debris was observed under the microscope. The results indicated that 50 ug/ml of lysozyme, without penicillin, was adequate to induce protoplasts in growing cells.

b. Protoplast stabilization

In order to stabilize the induced protoplasts, cultures were grown in CM4-cellobiose medium to the exponential phase. The cultures were then incubated for 30 min. at 45° and various stabilizers were added at this time before the addition of

lysozyme. Protoplast stabilization was detected by checking the drop in OD_{660} and by microscopic observation.

c. Effect of temperature on protoplast stabilization

C. thermocellum cultures were grown to the exponential phase at 60°C in CM4-cellobiose, then transferred for 30 min. at 45°C. Sucrose was added to 0, 5 and 10% final concentration and 50ug/ml lysozyme were added and the cultures incubated 10 more min. at 45°C. Finally the cultures were transferred to different temperatures and their OD_{660} followed. Fig. I.E.1.1. indicates that in the absence of sucrose an increase in temperature from 50 to 60°C has a stabilizing effect in O.D. The same stabilization obtained at 60°C without sucrose can be seen at 50°C when the cultures contain 10% sucrose. The behavior of the cultures in 5% sucrose is similar to that in the absence of sucrose. Therefore, at this point we chose 60°C and 5% sucrose as our standard conditions. This is due to the fact that C. thermocellum grows optimally at around 60°C and 5% sucrose has little or no effect on growth kinetics. However, it should be noted that in this experiment, even optimal conditions showed a 60% loss in O.D.

d. Effect of cultural age on protoplast stabilization

Cultures were grown to different stages of growth (O.D.'s ranging from 0.1 to 0.8), one-half of all the cultures were brought up to 5% sucrose and all were transferred to 45°C for 30 min. At this point lysozyme at a final concentration of

Fig. I.E.1.1. Effect of incubation temperature on the fermentation of protoplasts in actively growing cells.

Fig. I.E.1.2. Effect of growth phase on protoplast induction and stabilization

50ug/ml was added, incubation was continued at 45°C for 10 min. and at 60°C for 2 hr. The results shown in Fig. I.E.1.2. indicate that culture age has no effect on protoplast stability whether 5% sucrose is present or not. In this experiment, approximately an 80% reduction in O.D. was observed.

e. Stabilization of protoplasts as function of NaCl concentration and 5 or 10% sucrose

Cultures were grown as above and NaCl and sucrose were added as stabilizers before addition of 100ug/ml lysozyme. Fig. I.E.1.3. shows that NaCl has only a marginal stabilizing effect above that seen with 5% sucrose.

f. Stabilization by high sucrose concentrations

Cultures were grown in CM4-cellobiose to the exponential phase and transferred to 45°C for 30 min., sucrose to different final concentrations was then added followed by 50ug/ml lysozyme. Fig. I.E.1.4. shows the results obtained when the incubation was carried on at 45°C or when the cultures were transferred to 60°C after 10 min. incubation at 45°C. In Fig. I.E.4.A. it can be seen that after 45 min. incubation at 60° or 45°, 15-30% sucrose stabilizes the induced protoplasts. However, after 20 hr. incubation (Fig. I.E.1.4.A.) at 45° or 60° at sucrose concentrations of 25 and 30% the protoplast lyse while at 15-20% sucrose the majority of protoplast are still present.

Fig. I.E.1.3. Effect of NaCl concentration on the stability of protoplasts

Fig. I.E.1.4. Effect of sucrose concentration on protoplast stabilization after 45 min. (A) and 20 h (B) incubation at 45°C and 60°C.

g. Stabilization by cellobiose, mannitol and polyethylene glycol

Protoplasts were induced in the presence of either cellobiose, mannitol or polyethylene glycol at different concentrations as stabilizers (added before lysozyme). Fig. I.E.1.5. shows that polyethylene glycol and cellobiose are better stabilizers than sucrose at concentrations of 10% but not at lower concentrations, while mannitol is not a good stabilizer even at 10% concentration.

h. Conclusions and Future Work

The method of inducing protoplasts in actively growing cells seems to have several advantages over the method of protoplast induction of resting cells that was described in earlier reports. Protoplasts from actively growing cells are readily formed with low lysozyme concentrations (as low as 0.5ug/ml) leaving no detectable cells and forming mostly free protoplasts without visible cell wall attached. On the other hand, protoplasts induced from resting cells require high lysozyme concentration (4ug/ml) for their formation and appear mostly with cell wall remnants attached to them. The greatest advantage in using growing cells to induce protoplasts is that the cells do not have to be exposed to aerobic manipulations and therefore losses in cell viability are avoided.

Protoplasts induced from growing cells are well stabilized by the presence of 18% sucrose (up to 70% OD₆₆₀ remained). Ten percent polyethylene glycol and 10% cellobiose are also fair stabilizers. However, 10% sucrose inhibits growth by 50%,

Fig. I.E.1.5. Effect of Polyethylene glycol, Mannitol and Cellobiose on protoplast stabilization

therefore 18% sucrose would not be suitable for protoplast regeneration. Ten percent cellobiose inhibits growth by 70% and 10% polyethylene glycol does so by 35%. Therefore, polyethylene glycol might be a useful stabilizer for regeneration experiments since it is a good stabilizer and is a weak growth inhibitor.

We are currently trying to obtain regenerated cells from the protoplasts induced from actively growing cells and those will be used in future experiments as means for gene transfer by fusion of different strains.

2. Isolation of plasmids from C. thermocellum

Work on the bulk isolation of the plasmid DNA and characterization of the initially isolated complex is continuing. As reported previously, the sedimentation behavior of the isolated material suggested the formation of large aggregates of some sort during isolation. Since alkaline sedimentation had resulted in the partial destruction of the aggregation, sedimentation at a series of intermediate pH values was carried out in an attempt to isolate a homogenous DNA species, as well as to gain information about the aggregate.

Material recovered from an ethidium bromide-caesium chloride gradient was dialyzed and applied to 5-20% sucrose gradients of specified pH. The results at pH 7.6, 9.2 and 9.7 are shown in Figures I.E.2.1., I.E.2.2. and I.E.2.3. It can be seen that over this pH range, no change in sedimentation behavior occurs. The result at pH 10.3 is shown in Figure I.E.2.4. It can be

Figure I.E.2.1. Sedimentation velocity analyses of material recovered from CsCl-ethidium bromide gradient satellite peak. Five to 20% sucrose, 20°C, $\omega^2 t = 1.02 \times 10^{11} \text{ rad}^2/\text{sec}$, pH 7.6

Figure I.E.2.2. Sedimentation of same material as in Fig. I.E.2.1., pH 9.2.

Figure I.E.2.3. Sedimentation of same material as in Fig. I.E.2.1., pH 9.7.

Figure I.E.2.4. Sedimentation of same material as in Fig. I.E.2.1., pH 10.3.

seen that the material sediments more slowly and with a narrower distribution, but it is still broader than a homogenous DNA peak would be.

Sedimentation at pH 11.3 is shown in Figure I.E.2.5. This results in a further reduction in peak width and velocity, and sedimentation at pH 12.5, shown in Figure I.E.2.6., reduces these further still.

A composite of these results is shown in Figure I.E.2.7. for easy comparison. The behavior shown is consistent with the random breakage of an aggregate, and nonhomogenous peaks always result.

Since sedimentation at pH 10 has been reported in some cases to remove material responsible for the alkaline degradation of plasmid DNA, alkaline sedimentation of material which had been sedimented at pH 10.3 was carried out.

Figures I.E.2.8., I.E.2.9., and I.E.2.10. show the results when material from a pH 10.3 sucrose gradient was recovered and sedimented at pH 7.6, 10.3 and 12.5. The first shows that the effect of the pH treatment is not reversible, the second is a control, and the third shows that the result of sedimentation at pH 12.5 is the same, whether or not it is preceded by treatment at pH 10.3.

This alkaline gradient was also centrifuged for a longer period to determine whether more than one peak could be resolved. The results for material with pretreatment and without are shown in Figures I.E.2.11. and I.E.2.12. No separate peaks are resolved.

To determine the cause of this and to attempt another method

-92-

Figure I.E.2.5. Sedimentation of same material as in Fig. I.E.2.1., pH 11.3.

Figure I.E.2.6. Sedimentation of same material as in Fig. I.E.2.1., pH 12.5.

Figure I.E.2.7. Composite of Figures I.E.2.1. through I.E.2.6.

Figure I.E.2.8.

Figure I.E.2.9.

Figure I.E.2.10.

Figure I.E.2.11. Extended pH 12.5 sedimentation of same material as in Figures I.E.2.8. through I.E.2.10.

Figure I.E.2.12. Extended pH 12.5 sedimentation of same material as in Figure I.E.2.1.

of purification, material isolated from a cesium chloride-ethidium bromide gradient was subjected to different enzyme digestions followed by neutral sucrose sedimentation.

A positive control for the presence of DNA is shown in Figure I.E.2.13, when the material was treated with pancreatic DNAase. Protease K and ribonuclease, as shown in Figures I.E.2.14. and I.E.2.15. had no effect. S_1 nuclease, Figure I.E.2.16, which is specific for single stranded nucleic acid resulted in the destruction of the complex, suggesting that denatured DNA and denatured regions of the plasmid DNA are responsible for the aggregation.

In the next quarter work we will continue to attempt to elucidate the nature of the plasmides in C. thermocellum. For this purpose polyacrylamide gel electrophoresis will be employed. In addition, we are re-examining the possibility of transformation for gene transfer in C. thermocellum. A search for infecting particles (bacteriophages) from natural sources as well as from other strains of C. thermocellum has been initiated.

Figure I.E.2.13. Velocity sedimentation analysis of material recovered from CsCl-ethidium bromide gradient satellite peak, after treatment with pancreatic DNAase. 5-20% sucrose, pH 7.6, 20°C, $\omega^2 t = 1.07 \times 10^{11} \text{ rad}^2/\text{sec}$.

Figure I.E.2.14. Velocity sedimentation analysis of material recovered from CsCl-ethidium bromide gradient satellite peak, after treatment with protease K. 5-20% sucrose, pH 7.6, 20°C, $\omega^2 t = 1.07 \times 10^{11}$ rad²/sec.

Figure I.E.2.15. Velocity sedimentation analysis of material recovered from CsCl-ethidium bromide gradient satellite peak, after treatment with RNAase. 5-20% sucrose, pH 7.6, 20°C, $\omega^2 t = 1.07 \times 10^{11}$ rad²/sec.

Figure I.E.2.16. Velocity sedimentation analysis of material recovered from CsCl-ethidium bromide gradient satellite peak, after treatment with S_1 nuclease. 5-20% sucrose, pH 7.6, 20°C, $\omega^2 t = 1.07 \times 10^{11}$ rad²/sec.

F. Deregulation of Clostridial Cellulases

1. Resistance of *C. thermocellum* Cellulase to Sugars

In two previous Progress Reports (8-31-77, 11-31-77), we showed that the addition of 300g of glucose, xylose or cellobiose per liter of enzyme reaction mixture did not inhibit TNP-CMCase activity. To examine the effect of these sugars on the hydrolysis of a more crystalline substrate, a final concentration of 0.05 g/l of each sugar was added to the enzyme solution and Avicel hydrolysis was measured. Again, the sugars did not inhibit enzyme activity.

To examine the effect of high concentrations of sugars on the hydrolysis of a crystalline cellulose, we used an assay involving chromogen release from Remazol Brilliant Blue-bound MN300 cellulose (RBB-MN300). Because the activity of *C. thermocellum* cellulase measured by this assay was low, a concentrated enzyme solution was prepared by ethanol precipitation. The reaction mixture consisted of 1 ml of a 10-fold concentrated enzyme solution, 2 ml of 0.1 M citrate buffer pH 4.5, 15 mg RBB-MN300 and various concentrations of sugars. After incubation at 60°C for 20 hours, the increase in the optical density of the supernatant was measured at 595 nm. As shown in Figure I.F.1., 300 g/l glucose or xylose and 100 g/l cellobiose did not inhibit enzyme activity.

The results of the RBB-MN300 assay agree with those of Avicel hydrolysis and TNP-CMCase activity. From these data,

Figure I.F.1. Effect of sugars on RBB-MN300 cellulose hydrolyzing activity. Activity without added sugars is 1.0. Activity in the presence of sugars was expressed relative to this value.

we can conclude that, unlike most cellulases, the C. thermocellum enzyme is resistant to end product inhibition.

2. pH Maintenance During Flask Cellulase Fermentation

Preliminary experiments showed that neither phosphate buffer nor Tris.HCl buffer was an acceptable buffer for the growth medium. Morpholinopropane-sulfonic acid buffer (MOPS) did prove to be useful. Figure I.F.2. shows the effect of MOPS concentration in CM3 cellobiose medium on pH maintenance, growth, and TNP-CMCase production. In all cases, the initial pH was adjusted to 7.0. From our results, we decided that 0.1M MOPS was a suitable concentration for growth plus enzyme production.

Figure I.F.3. shows the effect of initial pH on the time course of growth and TNP-CMCase production in CM3 cellobiose medium containing 0.1M MOPS. In Figure I.F.4., the data of Figure I.F.3. on growth and TNP-CMCase production for the first 21 hours are replotted against the average pH during that time. We conclude that the optimum pH for growth and production is 6.8. However, it should be noted that the cultures begun slightly below and slightly above pH 7.0 (which showed the highest cellulase titers) showed significant cell lysis. Whether such high titers are the result of lysis or whether even higher enzyme levels would result if lysis were prevented is a question for future work. We doubt that the lysis is caused by carbon or nitrogen limitation but it could be due to formation of a toxic by-product such as ethanol and/or acetic acid.

Figure I.F.2. Effect of concentration of MOPS on pH maintenance, growth, and TNP-CMCase production.

Figure I.F.3. Effect of pH on growth and TNP-CMCase production. In the presence of 0.1M MOPS, initial pH was adjusted as shown by each symbol.

Figure I.F.4. pH vs. growth and TNP-CMCase production. Growth and TNP-CMCase production between 0 to 21 hr in Figure I.F.3. were plotted against average value of pH in the same period.

3. Determination of Intracellular TNP-CMCase Activity

Examination of C. thermocellum for intracellular TNP-CMCase activity was done. Mutant AS-39 or parent cells grown on cellobiose for 24 hours were ruptured by three methods: (1) grinding with quartz sand, (2) ultrasonication and (3) lysozyme treatment. The cell extracts were tested for TNP-CMCase activity. As shown in Table I.F.1., intracellular TNP-CMCase activity represents less than 5% of the total TNP-CMCase activity.

4. Chloramphenicol Inhibition of Growth and Cellulase Production

To determine if the presence of cellulase activity in the extracellular broth is the result of de novo protein synthesis, chloramphenicol, an inhibitor of protein synthesis, was used. A 10% inoculum of AS-39 cells was added to Hungate tubes containing CM3 cellobiose medium (0.5%) and various amounts of chloramphenicol. Growth was measured after 24 hrs incubation at 60°C. Figure I.F.5 shows the effect of chloramphenicol on growth; the minimum inhibitory concentration was 20 mg/l.

The effect of chloramphenicol on TNP-CMCase production was studied in replacement culture. AS-39 cells grown on CM3 cellobiose medium for 24 hrs were centrifuged, washed once with fresh medium, and resuspended in the same medium either with or without chloramphenicol. As a control, washed cells were also resuspended in medium with or without chloramphenicol

Table I.F.1. Ratio of intracellular and extracellular TNP-CMCase

Method of disintegration of cells	Intracellular/extracellular	
	Parent	AS-39
grinding with quartz sand*	-	0.034
Ultrasonication**	-	0.042
Lysozyme treatment***	0.0023	0.014

*30 min grinding in 0.02M citrate buffer pH 4.5

**1 min sonication. By increasing the period more than 1 min, enzyme activity in extract decreased.

***4.2mg/ml lysozyme in 0.01M Tris-HCl buffer, pH 7.2, 45 C, 3 hr. Decrease of turbidity of reaction mixture stopped after 3 hr incubation.

Figure I.F.5. Effect of chloramphenicol on growth.

Table I.F.2 Effect of chloramphenicol on TNP-CMCase production in a replacement culture

Time (hr)	Addition		Klett	pH	TNP-CMCase ($\Delta OD_{344}/\text{ml}$ culture)	
	CB*	CM**			Extracellular	Intracellular***
0			78	6.8	0.014	0.001
22	-	-	73	6.8	0.011	0.001
	+	-	167	5.7	0.128	0.007
	-	+	73	6.8	0.020	0.001
	+	+	93	6.5	0.027	0.002

*cellobiose 1%, **chloramphenicol 100 mg/l

***Washed cells with 10mM Tris-HCl buffer, pH = 7.2 were incubated with 4.2 mg/ml lysozyme for 3 hr at 45 C in the same buffer, and then TNP-CMCase activity in supernatant was measured. Activity was expressed per ml culture broth.

but without cellobiose. After 22 hours of incubation at 60°C, growth and extracellular and intracellular TNP-CMCase activities were measured (Table I.F.2). Again, the intracellular TNP-CMCase activity was quite low. The cellobiose-free control showed no change in optical density, pH, or TNP-CMCase activity both with and without chloramphenicol. Addition of 100 mg chloramphenicol/l to the cellobiose containing culture strongly inhibited both growth and the increase in extracellular TNP-CMCase activity. These results suggest that the presence of C. thermocellum cellulase in the extracellular medium is due to de novo protein synthesis and the enzyme(s) is excreted after protein synthesis.

5. Isolation of Hyperproducing Cellulase Mutants by U.V. Irradiation

In the previous report, we reported the isolation of mutant AS-39 from UV-irradiated cells of C. thermocellum ATCC 27405. This mutant produces twice the cellulase activity of the parent according to TNP-CMCase and Avicel hydrolysis assays.

Mutant AS-39 was further exposed to UV irradiation with the intention of mutating it to higher levels of cellulase overproduction. Since no new mutant was found among 30,000 colonies screened, the isolation procedure was modified as follows. The UV irradiated cells were mixed with molten CM3 agar medium containing 1% Avicel as the sole carbon source and then

poured into Petri plates. Colony formation efficiency within the agar was much better than that on the surface. Thus, the number of colonies that appeared agreed with the number we expected from microscopic counts. The probable reason is that, during inoculation and incubation, the cells within the agar are exposed to less oxygen than those on the surface.

Colonies exhibiting large clear zones were taken up into a sterile Pasteur pipet and blown by nitrogen into Hungate tubes containing CM3 medium with 0.5% Avicel as the sole carbon source. After incubation for 2 to 4 days at 60°C, tubes with actively growing cultures were measured for an increase in reducing sugars. Such a selection method should allow the isolation of cultures able to hydrolyze crystalline cellulose.

From about 350,000 survivors of mutagenesis, we tested 90 colonies in tube culture. Mutant AS-39-22 showed 50% more Avicel hydrolyzing ability than its parent AS-39 the first time tested. When this culture was transferred to CM3 Avicel and CM3 cellobiose media, it had 45% and 41% more TNP-CMCase activity than the parent, respectively.

6. Mutation and Isolation of a Cellulase Hyperproducer Using Chemical Mutagenesis

Further mutation of AS-39 has been attempted using two well known mutagens, N-methyl-N'-Nitro-N-nitrosoguanidine (NTG) and ethyl methanesulfonate (EMS). Two procedures were tested. In the first, log phase AS-39 cells, washed and re-suspended in pH 7.5 phosphate buffer, were exposed to 3% EMS

(final concentration). Survivors were plated on the surface of CM3 Avicel (2-3%) with cellobiose (0.2%) plates. After incubation at 60°C, plates were examined for large clear zones of cellulose hydrolysis. We did not isolate any overproducers by this method.

In the second procedure, log phase AS-39 cells were delivered into tubes containing molten CM3 Avicel (1-2%) with cellobiose (0.2%) agar and the agar was poured into plates. Filter paper discs (6.35 mm diam.) dipped in 5%-100% EMS (in methanol) were placed in the center of the plate's surface. NTG was also tested in this way with concentrations ranging from 1.25 to 50 µg per disc. The plates were incubated at 60°C. During this time, the material on the disc diffused into the agar forming a concentration gradient. Thus far, about 3,000 colonies from the disc technique have been examined. One has shown an increase of 20% in TNP-CMCase activity in liquid culture as compared to its parent AS-39. We plan to continue the chemical mutagenesis program with these and other modified procedures which may prove successful. The EMS has been more useful up to this point since it does show inhibition around the disk. For some unknown reason, NTG does not cause growth inhibition.

7. Studies on Xylan and Xylanase

In further attempts to find a carbon source which does not lead to cellulase production, xylan was tested as

major carbon source. Xylanase, TNP-CMCase and CMCase activities were determined using ethanol-precipitated fermentation broth. The values were compared to those of CM3-cellobiose and CM3 MN300 fermentations. Xylanase activity was measured by adding 0.6 ml of the ethanol-precipitated sample to 3 ml of a 2% xylan suspension in 0.05M citrate buffer, pH 4.5. The reaction mixture was incubated at 60°C for 1 h, centrifuged to remove xylan and 1.2 ml of the supernatant was assayed for reducing sugars (dinitrosalicylic acid method). To determine the background level of reducing sugars, 0.2 ml of the ethanol-precipitated sample was incubated in 1.0 ml of the citrate buffer without xylan under the conditions described. Xylanase activity is expressed as mg of reducing sugars produced/ml supernatant/h. Xylan alone in citrate buffer incubated as above does not release any detectable reducing sugars.

Table I.F.3 shows that the amount of the various enzyme activities detected in MN300 cellulose or xylan fermentations is low compared to the amounts detected in the CM3 cellobiose culture. This result could be explained if the enzymes were formed on MN300 or xylan, but enzyme adsorption to the polymers occurred. This possibility was tested. Into a capped vial, we added 2.5 ml of enzyme solution, 2.5 ml of 0.05M citrate buffer pH 4.5, and 0.5 g of polymer; these were incubated at 60°C for the times specified. Duplicate vials were used for each data point. The vials were removed from the

Table T.F.3.

Effect of different carbon sources on growth and enzymatic activities of C. thermocellum ATCC 27405

Fermentation Time (hr)	Carbon Source 1% (w/v)	Dry cell weight* mg/ml	TNP-CMCase $\Delta OD_{344}/ml.hr$	CMCase mg sugar/ml.hr	Xylanase mg sugar/ml.hr
0	Cellobiose	0.027	0.014	0.416	0.562
12.5		0.465	0.101	2.160	1.479
41.0		0.435	0.116	2.208	1.562
64.0		0.420	0.105	2.000	1.354
0	MN 300	0.114	0.003	0.083	0.120
12.5		0.435	0.006	0.375	0.229
41.0		0.450	0.019	0.316	0.262
64.0		0.450	0.037	0.475	0.591
0	Xylan		N.T.**	0.129	0.125
12.5			0.021	0.238	0.216
41.0			0.016	0.396	0.408
64.0			0.021	0.404	0.429
0	None***	0.024	0.012	0.088	0.116
12.5		0.057	0.018	0.258	0.229
41.0		0.036	0.023	0.316	0.200
64.0		0.036	0.024	0.266	0.196

*Calculated from Klett values. Could not be done with xylan cultures due to poor settling of polymer.

**Not tested

***Except for yeast extract.

incubator, put on ice, and their contents were centrifuged at 7500 x g for 8 minutes to separate the polymer. The percentage of the original TNP-CMCase activity remaining in the supernatant was measured. As seen in Figure I.F.6, starch, xylan and MN300 cellulose immediately adsorbed 30%, 50%, 78% of the TNP-CMCase activity respectively. If the data of Table I.F.3 are corrected for enzyme adsorbance to the polymer, enzyme production on xylan and MN300 is still considerably lower than production on cellobiose. The results suggest that xylanase and cellulase are constitutive enzymes in C. thermocellum.

Though growth of C. thermocellum on some insoluble substrates, such as cellulose MN300, can be determined by Klett measurement after 2 hr settling of the substrate by gravity, this method cannot be used to measure growth on xylan because the particle size is quite heterogeneous and some particles are small and do not settle out. Therefore, we attempted to use protein determination to measure growth. Two ml of whole broth was centrifuged at 7,500 x g, washed three times with 0.85% NaCl and subjected to the Lowry protein assay. We observed that readings of background levels (i.e., zero time) of protein in the xylan and MN300 fermentations were so high that it was not possible to measure growth with this method. The data are shown in Table I.F.4. We suspected that the polymers somehow interfered in the assay. To determine whether the alkaline treatment in the procedure was responsible, xylan and

Figure I.F.6. Adsorption of TNP-CMCase activity to sugar polymers at 60°C. 0.05g polymer was added to 2.5ml enzyme solution and 2.5ml 0.05M citrate buffer, pH 4.5.

Table I.F.4. Protein determinations on whole broths by the Lowry method

Fermentation time (hr)	Cellobiose broth		MN300 broth		Xylan broth	
	OD ₅₅₀	protein μg/ml	OD ₅₅₀	protein μg/ml	OD ₅₅₀	protein μg/ml
0	0.006	0	0.526	510	0.276	275
20	0.117	110	0.602	590	0.341	338
44	0.156	152	0.596	584	0.365	360
64	0.165	160	0.628	614	0.314	310

MN300 cellulose themselves were subjected to the Lowry procedure with and without the alkaline hydrolysis step. The results in Table I.F.5 show that both 10 mg/ml xylan and MN300 release a large amount of absorbance equivalent to 450 and 400 µg/ml protein. The absorbances found with only water treatment were much lower indicating that alkaline treatment liberated material interfering with the Lowry protein assay.

Auxanographic plate tests were also done to ascertain whether growth occurs on xylan. CM3 medium less carbohydrate was seeded with C. thermocellum and poured into petri dishes. After solidification, dry xylan was added with a spatula to one portion of the plate. After 5 d incubation, we looked for a growth zone around the xylan spot, but failed to see clear-cut growth. On other plates in which the xylan was distributed through the agar, we noted its hydrolysis by the heavy inoculum added to the plate.

We thus have failed to see any clear-cut indication of growth on xylan in our experiments. However, growth on cellobiose or MN300 does lead to xylanase production.

8. Effect of Nitrogen Source on Growth and Enzyme Production

Table I.F.6 lists the fourteen compounds that were evaluated for their effects on growth and enzyme production. All were tested at a concentration of 0.27g of nitrogen per liter. Five of them which showed either high volumetric enzyme titers or high specific enzyme values were selected for

Absorbance (OD₅₅₀)

Polymer treated for 15 min
at 100°C

	(with 0.1N NaOH)	(with water)
XYLAN	0.225, 0.223	0.061, 0.069
MN 300	0.174, 0.199	0.019, 0.006

Table I.F.5. Interference in the Lowry method for protein estimation by alkaline treatment of both cellulose and xylan. Samples contained 10mg/ml of polymer. After heat treatment the hydrolysate was diluted 1:2.

Table I.F.6. Nitrogen Sources Tested for Growth

Ammonium sulfate	Glutamate
Ammonium nitrate	Aspartate
Ammonium chloride	Lysine
Sodium nitrate	Arginine
Potassium nitrate	Leucine
Urea	Isoleucine
	Valine
	Casamino acids

further analysis. They were casamino acids, urea, valine, ammonium nitrate, and ammonium sulfate. Figure I.F.7 shows growth and enzyme production for these five nitrogen sources over a nitrogen concentration of 0.02 g/l to 0.5 g/l. Ammonium salts were the best nitrogen source for growth, but urea appeared best for TNP-CMCase production. Further examination of urea's stimulatory effect at higher concentrations was conducted. Concentrations of urea nitrogen from 0.02 g/l to 2 g/l were tested on CM3 cellobiose medium less $(\text{NH}_4)\text{SO}_4$ containing the usual 0.2% yeast extract or a higher (0.6%) yeast extract concentration. Both filter and heat-sterilized urea were examined. The results in Figure I.F.8 show that urea nitrogen concentrations higher than 0.5 g/l have no stimulatory effect when the medium contains the normal level (0.2%) of yeast extract. However, when yeast extract is increased to 0.6% (which in itself increases enzyme production), urea increases production until 1.0 g/l urea nitrogen is reached. Filter-sterilized urea behaved just like autoclaved urea.

The reason we tested yeast extract at higher levels in the above urea experiment is that we had just observed a slight stimulatory effect of raising its concentration from the usual 0.2% to 0.6% in CM3-cellobiose medium containing $(\text{NH}_4)_2\text{SO}_4$. In Figure I.F.9, it can be seen also that yeast extract can serve as sole nitrogen source, but that further supplementation with $(\text{NH}_4)_2\text{SO}_4$ stimulates growth and enzyme formation.

Fig. I.F.7. Growth and enzyme production using various nitrogen sources.

Figure I.F.8. Effect of urea at different concentrations in CM-3 cellobiose medium (0.2% Y.e.) and in the same medium with yeast extract increased to 0.6%. f.s. urea = filter-sterilized urea; otherwise the urea was autoclaved.

Fig. I.F.9. Growth and enzyme production at different yeast extract concentrations in the presence and absence of $(NH_4)_2SO_4$. Symbols on the left and right do not correspond.

We conclude that the nitrogen source combination best for volumetric TNP-CMCase production is 1 g/l of urea nitrogen (i.e., 2.14 g/l urea) plus 6 g/l yeast extract.

9. Discrepancy between M.I.T. and Wisconsin Reports

Ng, Weimer and Zeikus (Arch. Microbiol. 114, 1, 1977) of the University of Wisconsin have reported that C. thermocellum LQ8 produces cellulase on cellulose, but not on cellobiose. We have always found cellobiose to support extensive cellulase production by C. thermocellum ATCC 27405. Inoculum size was varied from 1% to 10% (v/v). In all cases, TNP-CMCase was made. Strain LQ8 produced titers of 0.17 to 0.25 $\Delta OD_{344}/\text{ml} \cdot \text{hr}$ whereas the values for ATCC 27405 were 0.18 to 0.21. Ethanol-precipitated supernatants of both strains also showed CMCase activity; the potencies were similar. At a discussion with Zeikus at the recent ASM Meeting in Las Vegas, he agreed that ATCC 27405 produces cellulase on cellobiose.

G. Evaluation of Clostridium thermocellum Strains for Xylanase and Cellulase Activities

1. Introduction

In the last Progress Report (COO-4198-5), we observed that all strains of C. thermocellum tested by us had the ability to degrade xylan. However, only the HG isolates were able to grow on xylan as the sole carbon source as measured by increases in OD⁶⁶⁰ and production of ethanol.

To quickly assay the degradation reactions, we have spotted representative C. thermocellum strains on agar plates with different carbon sources. Experiments to determine the specific cellulase and xylanase activities have also been performed.

2. Materials and Methods

a. All experiments were performed in test tube cultures with CM-4 medium containing 6 gr/liter of carbon source. Agar plates contained the same carbon source concentration.

b. Reducing sugars were determined using the DNS assay as described in earlier progress reports.

c. ATCC-2705 and LQ8 strains of C. thermocellum growing on different carbon sources were evaluated for filter paper activity (FPase) CMCase activity and xylanase activity.

Activities were measured following precipitation and concentration of extracellular proteins. Ethanol at 40% (V/V) was chosen to precipitate the proteins. Ethanol was

added to culture supernatants and the samples were maintained overnight in an ice bath. After precipitation, the samples were centrifuged, supernatants discarded and pellet resuspended in citrate buffer at different concentrations to determine enzyme activities.

Xylanase activity was measured by adding 3 ml of 2% xylan in citrate buffer (0.05 M pH - 4.8) to 0.2 ml of sample in duplicate tubes. For each sample, a control tube was included which received no xylan (this serves as a reference for background of reducing sugars in sample) and another tube which received xylan, but no sample. All samples and control tubes were incubated for 1 hour at 60°C. After the incubation, the tubes containing xylan were centrifuged at 12,000 rpm for 10 min and 1.2 ml of the supernatants were transferred to tubes for DNS assay.

CMCase and FPase were performed as described previously.

3. Results and Discussion

We had previously obtained preliminary evidence that all strains of C. thermocellum tested by us had the ability to degrade xylan. To confirm this observation, agar plates with CM4 and different carbon sources were inoculated with ATCC-27405, LQ8 and HG-2 strains. Four aliquots of culture supernatants, 20 µl each, were spotted on each plate.

As may be seen in Table I.G.1, ATCC and LQ8 strains previously grown in either Solka floc or cellobiose were able to degrade xylan. HG-2 strain previously grown in xylan, xylose or cellobiose is also able to hydrolyze xylan.

Two additional observations can be made from these data. One is the fact that HG-2 does not produce zones of clearing in Solka floc plates as expected since they are not able to grow on cellulose as the sole carbon source. Another is that ATCC and LQ8 strains when spotted on CM4-xylose agar plates showed no growth. Plates containing yeast extract and no other carbon source always supported some growth. Thus, it may be supposed that growth of HG-2 on Solka floc was due to the presence of yeast extract. In addition, it appears that xylose may be inhibitory to ATCC and LQ8 strains.

Since it was clear that all strains tested degrade xylan, we attempted to correlate growth with hydrolysis of xylan and the accumulation of reducing sugars.

Table I.G.2 shows that only HG-2 causes increases in optical density of the medium, indicating cell growth, while the medium inoculated with LQ8 strains shows a decrease in optical density, indicating xylan degradation. The optical density of these tubes was followed for 4 more days and the O.D. did not increase.

Another interesting observation was that HG-2 did not accumulate reducing sugars while growing in xylan. Confirmation of this result was obtained by paper chromatography

TABLE I.G.1: GROWTH OF DIFFERENT STRAINS ON VARIOUS CARBON SOURCES

<u>C. thermocellum</u> <u>Strains Grown In:</u>	<u>Xylan</u>	<u>Solka</u> <u>floc</u>	<u>Xylose</u>	<u>Yeast</u> <u>Extract</u>	<u>MN300</u>	<u>Microcrystalline</u> <u>Avicel</u>
ATCC-cellulose SF	GR-L	GR-L	(—)	GR	GR-L	GR-L
ATCC-cb	GR-L	GR-L	(—)	GR	GR-L	GR-L
LQ8-cellulose SF	GR-L	GR-L	(—)	GR	GR-L	GR-L
LQ8-cb	GR-L	GR-L	(—)	GR	GR-L	GR-L
HG-xylan	GR-L	GR-L	GR	GR	GR	GR
HG-xylose	GR-L	GR	GR	GR	GR	GR
HG-cm	GR-L	GR	GR	GR	GR	GR

GR = Growth on surface

(—) = No visible growth

(L) = Clearing zone indicative of substrate hydrolysis

Cb = cellobiose

TABLE I.G.2: GROWTH AND ACCUMULATION OF REDUCING SUGARS BY
C. THERMOCELLUM ISOLATES GROWING IN XYLAN

OD⁶⁶⁰

<u>Strain</u>	<u>0 hours</u>	<u>15 hours</u>	<u>25 hours</u>	<u>40 hours</u>	<u>63 hours</u>
HG-2	0.34	0.80	0.84	0.87	0.92
ATCC	0.34	0.28	0.24	0.20	0.17
LQ8	0.38	0.30	0.32	0.27	0.22

Reducing sugars: as mg xylose/ml broth

	<u>0 hours</u>	<u>15 hours</u>	<u>25 hours</u>	<u>40 hours</u>	<u>63 hours</u>
HG-2	.05	0.05	0.1	0.1	0.05
ATCC	.05	2.15	2.4	2.5	2.7
LQ8	.05	1.85	2.3	2.3	2.7

of the fermenting broth using n-butanol/acetic acid/water at < 2:1 as solvent and aniline-hydrogen phtalate as staining mixture. HG-2 strain did not produce any stainable spot of reducing sugars.

A study was conducted to determine the influence that carbon sources have on the different enzymatic activities. ATCC and LQ8 were grown on different carbon sources and as can be seen in Table I.G.3 it is clear that all activities tested were always present as long as growth occurs. It is interesting that the ATCC strain will grow in glucose and produce all extracellular enzymes. This is in contract to what has been previously reported for C. thermocellum. It should be noted, however, that growth in glucose exhibited a longer lag than in cellobiose. This may be indicative of either a phenotypic adaption to glucose or the selection of mutants with the capacity to utilize glucose.

4. Future Work

- Determine the ability of HG-2 strain to produce FPase, CMCase and xylanase. This may serve to better define strain HG-2 physiologically and to obtain more information about the mechanisms by which these enzymes are produced.
- Determine if the ability of C. thermocellum to grow on glucose as the sole carbon source is a stable mutation or a phenotypic adaption.

TABLE I.G.3: EFFECT OF CARBON SOURCE ON THE EXPRESSION OF EXTRA-CELLULAR ENZYMATIC ACTIVITIES IN C. THERMOCELLUM

Strain	Activity	Solka Floc	xylan	Cellobiose	None	Glucose	MN300
ATCC	FPase	0.010	0.014	0.042	0.002	0.030	0.005
	CMCase	1.5	0.75	4.35	0.025	1.42	1.82
	xylanase	1.2	0.57	3.45	0.020	1.25	1.05
LQ8	FPase	0.018	0.004	0.018	NM	ND	ND
	CMCase	2.5	0.27	2.05	NM	ND	ND
	xylanase	3.1	0.38	2.75	NM	ND	ND

Activity was determined at the end of the exponential phase of growth.

FPase and CMCase activity are expressed as $\frac{\text{mg Glucose}}{\text{ml-HR}}$

xylanase activity is expressed as: $\frac{\text{mg xylose}}{\text{ml-HR}}$

ND = not done

II. PRODUCTION OF CHEMICAL FEEDSTOCKS

A. Production of Acrylic Acid by Fermentation

1. Introduction

Because of the high value (~77¢/kg) and potentially high yield from glucose (~0.8 g/g), acrylic acid remains attractive as a component of an integrated process for ethanol production. Production of acrylic acid as a portable and overall process to produce ethanol could greatly improve the economic aspects of ethanol production from biomass.

During this progress report period, efforts have been directed to production of acrylic acid employing an anaerobic microorganism, Clostridium propionicum, and a facultative microorganism, Escherichia coli. In addition to research on the production of acrylic acid, research is also continuing vigorously on the production of lactic acid which potentially is an important precursor to acrylic acid.

2. Fermentation Studies with Clostridium propionicum

a. Introduction

The Progress Report (COO-4198-5) covering the period from December 1, 1977 through February 28, 1978, summarized the exciting and significant finding that acrylic acid could be produced by resting cells preparations derived from C. propionicum. These findings were also reported at the 18th Annual Meeting of the American Society for Microbiology, held May 14 - 19, 1978. The paper entitled "Production of Acrylic

Acid by Clostridium propionicum" was co-authored by M. Akedo, R. Torregrossa, C. L. Cooney and A. J. Sinskey and presented by Mr. M. Akedo. Research conducted this quarter on the production of acrylic acid by C. propionicum has had two objectives. The first objective is to investigate acrylic acid production kinetics from propionate and/or lactate. The second objective is aimed at increasing the yield of acrylic acid from the oxidation of propionate by resting cell suspensions of C. propionicum.

b. Materials and Methods

Procedures for conducting the basic experiments and analyses of products have been described previously (Progress Report COO-4198-5, Period December 1, 1977 - February 28, 1978). Cellular protein was measured using the modified Biuret procedure of Strickland (J. Microbiol. (1954) 5:698).

c. Results and Discussion

1. Effect of Propionate Concentration

The amount of acrylic acid produced by resting cell suspensions was found to be dependent on propionate concentration up to a concentration of about 200 mM (Figure II.A.1). The oxidation of propionate to acrylate is believed to require a minimum of four (4) steps:

Figure II.A.1: Dependence of acrylic acid production on the concentration of propionate and lactate. Incubation mixtures contained triethanolamine buffer (0.05M, pH 7.5), cell suspension (0.05g wet cell weight/ml) and indicated concentrations of propionate and lactate. Incubation was at 37° for 4 hrs.

The large amount of propionate required for saturation may indicate that transport of propionate into the cell is the rate limiting step in the overall conversion.

2. Effect of Lactate and Oxygen

Previous findings have demonstrated that lactate addition to a resting cell preparation oxidizing propionate to acrylic acid appears to stimulate acrylic acid production (Progress Report COO-4198-5, December 1, 1977 - February 28, 1978). To date, lactate alone has not been used as a starting substrate even though we have demonstrated that resting cells of C. propionicum can metabolize lactate producing propionate and acetate and that propionate can be converted to acrylate in the presence of oxygen. Therefore, experiments were designed in which lactate is first converted to propionate under anaerobic conditions and the resulting propionate may then be oxidized under aerobic conditions to acrylic acid.

Five different environmental conditions and combinations were therefore tested to explore the above approach. They were:

- (1) anaerobic (Figure II.A.2)
- (2) aerobic (Figure II.A.3)
- (3) anaerobic → aerobic (Figure II.A.4)
- (4) Anaerobic → aerobic → anaerobic (Figure II.A.5)
- (5) aerobic → anaerobic (Figure II.A.6)

FIGURE II.A.2: ANAEROBIC FERMENTATION OF LACTATE
BY RESTING CELLS

Each figure summarizes the results. In the presence of oxygen, propionate production was significantly decreased and the rate of propionate production also decreased. On the other hand, acetate production was stimulated in the presence of oxygen and the rate of acetate production increased. The fact that oxygen can act as an electron acceptor and thus alter the oxidation-reduction balance in the fermentation of lactate is clearly demonstrated in Figure II.A.5). Unfortunately, no acrylic acid accumulation was detected under these conditions even though other experiments demonstrated that lactate in the incubation mixture appears to stimulate acrylic acid production when exogenous propionic acid is the substrate. Therefore, it is highly unlikely that the stimulatory effect of lactate is due to lactate being converted to acrylic acid. A possible explanation of these observations is that the oxidation of lactate to pyruvate and eventually to acetate increases the cellular pool of acetyl-CoA. The excess acetyl-CoA is then readily available to activate propionic acid to propionyl-CoA and the propionyl-CoA is then oxidized. Thus, the lactate becomes a source of acetyl-CoA. The CoA moiety is then transferred to propionate via CoA transferase. When acrylic acid is released, the CoA moiety may be recycled. Acyl-CoA transferase has been extensively characterized in Peptostreptococcus (Megasphaera) elsdenii (Tung, K.K. and W.A. Wood, J. Bacteriol. (1975) 124:1462). Its characterization in C. propionicum re-

3. Optimization of Propionate Oxidation

Efforts that are concerned with the optimization of propionate oxidation reactor conditions are addressing the following variables:

- (a) concentration of propionate
- (b) concentration of lactate
- (c) cell concentration
- (d) pH
- (e) ionic strength
- (f) growth phase
- (g) oxygen tension

To date, studies have been undertaken on the first three variables--concentrations of lactate, propionate and cell mass. The propionate concentration was varied from (0 mM to 200 mM) while lactate concentration was controlled at 10 mM or 50 mM. Again, it is observed that acrylic acid production is stimulated in the presence of lactate. Increasing amounts of propionate also resulted in a higher amount of acrylate (Figure II.A.1). As cell concentration is increased, acrylic acid production also is increased (Figure II.A.7).

d. Future Studies with C. propionicum

Efforts will continue in the optimization of acrylic acid production from propionate. In addition, the ability to grow large quantities of cells and store them will be investigated. The ability to grow large quantities of cells

Figure II.A.7: Acrylic acid production as a function of cell concentration. Reaction mixtures contained triethanolamine buffer (0.05M, pH7.5), sodium propionate (0.2M) and varying amounts of cell suspension. Incubation was at 37° for 2 hrs with shaking in an aerobic atmosphere. Each point represents the average of duplicate reactions.

and store them will greatly reduce the time required for performing experiments. Preliminary experiments of a qualitative nature indicate that cells stored at -20°C retain the ability to produce acrylate.

3. Aerobic Oxidation of Propionic Acid to Acrylic Acid by *Escherichia coli*

a. Introduction

In previous progress reports, we outlined a research approach with aerobic organisms having the objective of accumulating acrylic acid via oxidation of propionic acid employing *Escherichia coli*. Our objective during this quarter was to resolve the feasibility of using *E. coli* ATCC 9637-2 for the aerobic bioconversion and accumulation of acrylate from propionate.

The research has concentrated on the isolation of the mutant as outlined in earlier reports. In addition, investigations on propionate oxidation by resting cell suspensions have been conducted. Preliminary experiments with cell-free extracts have been initiated. In the previous report, it was assumed that propionyl CoA could be carbonylated to succinyl CoA via methyl malonyl CoA. However, now in the light of the fact that *E. coli* cannot synthesize the cofactor vitamin B_{12} , this would be an unlikely pathway. Data presented on the metabolism of two, three and four carbon acids can, however, be interpreted on the basis of conversion of pyruvate to phospho(enol) pyruvate and its subsequent carboxylation to oxalacetate. The requirement for

C4 compounds could thus be satisfied by this pathway. Phospho(enol) pyruvate is known to inhibit the activity of isocitrate lyase, and this would explain the low levels of the enzyme under conditions which the cellular yield of propionate equalled that of succinate.

Inspection of our methods for the isolation of several mutants has revealed areas where selection could be made more specific. Some new techniques have been used for this purpose.

b. Materials and Methods

Culture conditions and measurement of growth have been described in earlier reports, as have been procedures for chromatographic assays and for mutagenesis.

1. Non-Volatile F.A. Assay

The gas chromatographic assay for methyl esters of non-volatile fatty acids was modified. The oven temperature was raised from 120°C to 135°.

2. Resting Cell Suspensions

20 ml of an M9 + 0.2% glucose starter culture, inoculated from a propionate slant and grown for 24 h was added to 1 liter of M9 + 0.2% propionate in a 4-liter flask. The cells were harvested in the exponential phase after 36 h. Cells were resuspended in 0.1 M phosphate buffer (pH 7.0) containing other M9 constituents, minus a nitrogen source, to an approximate concentration of 1 gm wet cell wt. per 50 ml medium. 0.2%

propionate was added unless stated otherwise. 1 ml samples were dispensed in 13 x 100 mm tubes and shaken by a 'wrist-action' shaker at 37°C. The reaction was terminated by addition of 0.1 ml 50% H₂SO₄. Samples were extracted with ether and analyzed as usual.

3. Cell-Free Extracts

Cells were grown as described in Section 2. Cells (3 gms wet cell weight) suspended in 10 ml 0.1 M tris buffer (pH 7.4) containing 1 mM dithiothreitol were disrupted by sonication for 90 secs., in 15 sec. pulses followed by 1 min. intervals for cooling in ice. Suspensions were centrifuged at 15K x g for 30 min. at 4°C. The supernatant was dispensed in 1 ml quantities to prevent repeated thawing of the samples and stored at -20°C.

The standard reaction mixture in 0.5 ml consisted of 100 µl cell free extract (containing approximately 20 mg protein/ml) and 400 µl 0.1 M Tris buffer (pH 7.4) containing 0.5 mM NAD. The reduction of NAD was followed spectrophotometrically at 340 nm using a Gilford recording spectrophotometer.

As a precaution against possible action of NADH oxidase, the samples were flushed with nitrogen.

c. Results and Discussion

1. In the previous progress report, we identified aspects of our selection and enrichment procedure that required closer inspection.

(a) Enrichment of the mutants by penicillin G treatment of exponentially growing acetate positive cells resulted in enrichment of only 0.005%. Also, the growing cells were often found to be penicillin resistant. A hundred fold greater enrichment was necessary. The presence of 2 mg/ml ampicillin, either alone or with penicillin G resulted in a consistent 10^5 -fold decrease in viable cells during enrichment for 480 minutes.

(b) The method for selecting isocitrate lyase mutants was also reviewed. We had been selecting these mutants on differential media plates containing 0.01% glycerol and 0.1 or 0.2% acetate. Data on mutants actually isolated by this system has been presented earlier. Stable ace A mutants were not found. It became clear that ace A mutants plated on such media could not be distinguished from wild type cells on the basis of colony size. Mutants lacking isocitrate lyase could conceivably metabolize the roughly 1 mM glycerol and synthesize sufficient TCA intermediates to metabolize acetate normally. Our inability to isolate the mutant could result from this. The following alternatives, besides replica plating have been studied.

(1) Enriched organisms were first grown on 0.1% acetate plates for 48 to 72 hours following which the plates were sprayed with roughly 0.1 ml of a 10% solution of glycerol (corresponding to .05% in the agar). New colonies

appearing after further incubation would have the ability to utilize glycerol without being able to use acetate.

(2) This method could be further improved by spraying glyoxylate instead of glycerol and hence selecting for ace A mutants directly. To overcome possible acetate depletion in the agar, acetate is mixed with glyoxylate. Newly growing colonies would only be ace A. We have attempted the first method. Roughly 300 plates were screened without obtaining an ace A mutant. Both screening of a larger population and the second method will be undertaken.

2. Resting cell suspensions of the wild type organism were investigated. The data are presented in Tables II.A.1 and II.A.2. Propionate oxidation was almost completely inhibited in the presence of lactate. Oxamate and oxalate did not interfere significantly. Acrylate reduced the rate of oxidation some 55%. Neither acetate nor any other product of propionate metabolism was observed by the GC assay for volatile fatty acids used to assay for acrylate.

Acrylate was not metabolized by the cells when present with propionate. Propionate could not be metabolized under anaerobic conditions. The resting cell suspension experiment was repeated using lactate as a substrate (Table II.A.3). The analogs and inhibitors did not have a marked effect on the rate of lactate metabolism. Oddly, 2 peaks one at retention time of 2.33 min and another appearing as a shoulder on the internal standard (methyl oxylate) were observed to increase during

TABLE II.A.1: OXIDATION OF PROPIONATE BY RESTING CELL
SUSPENSIONS OF E. COLI

Substrate	Conc. %	Residual Propionate Conc. (gms/l)			
		0	Time (min) 120	300	540
Propionate	.2	2.0	1.61	0.45	0
	.4	4.0	2.67	1.75	1.25
	.6	6.0	-	4.77	3.87
	.8	8.0	6.29	5.84	4.34
Propionate + Glyoxylate	.2+	2.0	1.49	.710	0
Propionate Anaerobically	.2	2.0	1.83	1.97	2.26

TABLE II.A.2: OXIDATION OF PROPIONATE BY RESTING CELL SUSPENSIONS OF *E. COLI* IN THE PRESENCE OF SUBSTRATE ANALOGS AND ENZYME INHIBITORS

	Substrate	Concentration of propionate unoxidized time in mins				
		0	30	90	150	225
1	propionate 0.2% —	2.0	1.83	1.09	1.02	0.63
2	+ (NH ₄) ₂ SO ₄ 0.1%	2.0	1.99	1.05	0.55	0.47
3	+ HCO ₃ ⁻ 0.1%	2.0	-	1.04		
4	+ Lactate 0.2%	2.0	2.0	1.97	1.98	1.79
5	+ 8 hydroxy-propionate 0.1%	2.0	1.72	1.66	1.22	0.85
6	+ propionate 0.1%	2.0	-	1.76	2.01	1.78
7	+ acrylamide 0.1%	2.0	1.72	1.36	1.28	0.90
8	+ acrylate 0.1%	2.0	1.85	1.78	1.57	1.25
9	+ cyclopropane carboxylic acid 0.1%	2.0	1.84	1.72	0.92	1.05
10	+ oxalate 0.1%	2.0	1.66	1.62	1.19	0.85
11	+ oxamate 0.1%	2.0	-	1.63	1.30	1.14

TABLE II.A.3: OXIDATION OF LACTATE BY RESTING CELL SUSPENSIONS

Substrate		Metabolized Lactate gms/l			
		time (min)			
		0	120	300	540
1	Lactate 0.5%	5.0	3.44	1.84	0.98
2	+ Propionate 0.2%	5.0	3.42	2.27	1.52
3	+ oxamate 0.2%	5.0	3.72	2.12	1.35
4	+ acrylate 0.1%	5.0	3.07	2.48	2.15
5	+ arsenite	5.0	3.36	2.54	1.72

the time course (Figure II.A.8). The peaks were not free carboxylic acids, nor pyruvate or β hydroxy propionate. Although they appear at RT's where unmethylated propionate and acrylate appear, it is not clear how they could escape methylation. We are attempting to identify these products of lactate metabolism.

3. Cell free extracts are being studied for their ability to oxidise propionate in the presence of ATP, NAD, and CoA or Acetyl CoA. Very high endogenous rates of NAD reduction interfere with analysis. Dialysed extracts are being prepared.

d. Future Work

- Cell free extracts capable of oxidising propionate in the presence of ATP, CoA and NAD will be prepared. They will be used to aid in the identification of an acrylate intermediate and in characterizing mutants deficient in propionate metabolism.

- Identify the products of lactate metabolism by resting cell suspensions.

- Isolate propionate negative, lactate positive mutants, irrespective of their ability to metabolize acetate as the sole carbon source.

FIGURE II.A.8: LACTATE UPTAKE AND APPEARANCE OF UNKNOWN PEAK AT RT 2.36 AND SHOULDER ON PEAK RT 2.94

RT = 1.87 = Lactic Acid - methyl ester.

RT = 2.94 = Internal Standard Oxalic Acid - methyl ester

4. Production of Lactic Acid

a. Introduction

In this section of the report, production of lactic acid, which is a key intermediate in the acrylic acid conversion pathway, is being reported on.

Since our final objective is the conversion of cellulose into useful chemicals, it is especially important to evaluate the integration of lactic acid production with the degradation of cellulose.

Basically, the approach is to grow C. thermocellum with the lactic acid producer, "Thermolactic bacterium", as a mixed culture. Cellulose is to be the only added carbon source. A commensalistic type of interaction is expected to take place, where C. thermocellum growing on cellulose will provide enough soluble sugars to support growth of Thermolactic bacterium and, therefore, result in accumulation of lactic acid.

This report covers the results on the characterization of "Thermolactic bacterium" isolated and investigated as a lactic acid producer at 60°C. This bacterium was tested as a mixed culture with C. thermocellum for lactic acid accumulation using Solka floc as the only carbon source. Data of physiology of Lactobacillus planterium is also presented.

b. Materials and Methods

1. Microorganisms

Lactobacillus plantarum NRRL-B-477 is a homo-fermentative lactobacilli, when grown on Glucose. It accumulates

lactic acid plus acetic acid when grown on Xylose. The optimal growth temperature is 37°C. "Thermolactic bacterium" is a homo-fermentative, thermophilic lactic acid producer obtained from DuPont Corp. Its optimal temperature for growth is 60°C. Clostridium thermocellum ATCC-27405 (see Report COO-4198-1, pages 2-3).

2. Growth Medium

The medium utilized for growth and production of lactic acid has been described in previous progress report (COO-4198-2, March - May, 1977, pages 52-53). This medium was used for the cultivation of L. plantarum and "Thermolactic bacterium".

Preservation of "Thermolactic bacterium" was done in the same medium; however, the glucose concentration was 2.5 gm/l, and the buffer capacity of the medium was doubled.

3. Inoculum Preparation

The standard procedure for inoculum preparation was followed (see Report COO-4198-4, pages 108-109). Handling and growth of C. thermocellum was done following the methodology and growth conditions described in previous reports (see Report COO-4198-1, pages 2-3).

4. Fermentor

A 5 liter Magnaform bench scale (New Brunswick Co.) Fermentor was used at 3 liter working volume. pH and temperature were controlled at constant values.

5. Assays

During the fermentation, kinetics and concentrations of the following parameters were determined.

- (a) cell concentration
- (b) lactic acid
- (c) acetic acid
- (d) reducing sugars
- (e) ethanol

In all cases, we used the standard methodology described in previous reports for each parameter.

c. Results and Discussion

1. Conversion of Pentose Sugars into Lactic Acid by *L. plantarum*

In order to improve reducing sugar utilization during cellulose degradation, we investigated removal of pentoses by using *L. plantarum* NRRL-B473. The initial characterization of the strain was done with respect to carbon sources utilization. The results obtained are shown in Figure II.A.9. It was found that *L. plantarum* can utilize glucose, cellobiose and xylose as carbon sources with accumulation of lactic acid. However, for the case where xylose is metabolized, there is accumulation of acetic acid together with lactic acid. This is due to the fact that the cell splits 5 carbon sugars into 3 and 2 carbon products. The typical numbers in this experiment are shown in Table II.A.4. Conversion values of 0.9 gm lactic

FIGURE II.A.9: CARBON SOURCES METABOLIZED BY LACTOBACILLUS PLANTARUM NRRL-B473 UNDER NO pH CONTROL

TABLE II.A.4: CARBON SOURCE UTILIZATION BY
L. PLANTARUM NRRL-B473

Carbon Source	Conc (gm/l)	Cell Conc (gm/l)	Lactic Acid (gm/l)	Acetic Acid (gm/l)	Conversion (gm Lactic/gm Sugar)
Glucose	5	0.83	4.9	-	0.98
	10	1.30	8.7	-	0.92
Cellobiose	5	1.05	4.9	-	0.98
	10	2.41	7.6	-	0.89
Xylose	5	0.55	2.5	1.9	0.38
	10	1.03	5.0	3.8	0.38

acid/gm sugar consumed were obtained for both glucose and cellobiose. However, only 50% of xylose was converted to lactic acid.

Figure II.A.10 shows the kinetics for xylose metabolism by L. plantarum under controlled pH. This experiment was done to evaluate the kinetic constants of the fermentation, and also to quantify the capacity of the microorganism to accumulate lactic acid under optimal conditions. A specific growth rate value of 0.21 hr^{-1} was found for the logarithmic phase. The complete set of data analysis is shown in Table II.A.5.

2. Production of Lactic Acid at High Temperatures (60°C) by A Thermophilic Bacteria, "Thermolactic bacterium"

One of the limitations to design of the original mixed culture system was meeting the need for a thermophilic bacteria which were able to grow and produce lactic acid at 60°C. Such bacteria was found and then characterized with respect to carbon sources utilized. The results obtained are shown in Figure II.A.11. "Thermolactic bacterium" was able to grow on glucose and fructose as carbon sources, with the consequent accumulation of lactic acid. It appears "T. bacterium" was able to grow on xylose, but further experiments are required to confirm this result.

The results shown in Figure II.A.12 demonstrate the performance of "T. bacterium" under pH controlled conditions. The kinetics of the experiment showed that it is

FIGURE II.A.10: KINETICS OF GROWTH OF LACTOBACILLUS
PLANTARUM NRRL-B-443 ON XYLOSE
pH CONSTANT = 6.0; TEMP. = 37°C

TABLE II.A.5: KINETICS OF GROWTH AND PRODUCT ACCUMULATION OF
LACTOBACILLUS PLANTARUM NRRL-B473 ON XYLOSE

Sample N°	Age (Hrs)	Cell Conc (gm/l)	Lactic Conc (gm/l)	Acetic Acid (gm/l)	Specific Growth Rate (1/Hr)	Productivity ($\frac{\text{gm lactic ac}}{\text{g/l}}$)	Conversion ($\frac{\text{gm lactic ac}}{\text{gm xylose}}$)
1	3	0.307					
2	4	0.40	1.8	1.0	0.26		0.56
3	6	0.61	3.9	2.1	0.21	1.05	0.59
4	8	0.93	7.0	4.6	0.21	1.55	0.58
5	10	1.29	9.9	6.5	0.16	1.45	0.53
6	12	1.29	13.1	8.3	0.0	1.60	0.62

pH Controlled at: 6.0

Temperature : 37°C

Initial Sugar : 25 gm/l

FIGURE II.A.11: CARBON SOURCES FOR THERMOLACTIC BACTERIA

FIGURE II.A.12: KINETICS OF GROWTH AND PRODUCT ACCUMULATION
FOR THERMOLACTIC BACTERIUM (CRD) ON GLUCOSE
pH CONTROLLED = 6.5
TEMPERATURE = 60°C

able to metabolize glucose and, with the production of lactic acid, the specific growth rate was shown to be a very strong function of the lactic acid concentration. The specific growth rate decays very rapidly with increasing concentrations of lactic acid. Typical conversion values of 0.8 - 0.9 gm lactic acid/gm glucose consumed were found (Table II.A.6).

These results demonstrate the capacity of T. bacterium to accumulate l-lactic acid at 60°C when growing on glucose, and therefore provide a basis for its utilization in mixed culture experiments with C. thermocellum.

3. Lactic Acid Accumulation in a Mixed Culture Population

A mixed culture population of C. thermocellum and T. bacterium using Solka floc as the only carbon source was conducted. The results of such an experiment are shown in Figure II.A.13. This figure represents the time profile for growth and product accumulation, for the mixed culture experiment. The culture was started with C. thermocellum only until the 42nd hour, then "T. bacterium" was inoculated. Lactic acid and reducing sugar were followed during the fermentation. It was found that lactic acid accumulated up to 5.0 gm/l after 15 hrs during the mixed culture fermentation. Reducing sugars increased up to 2.5 gm/l during the same period of time. From the 60th to 120th hours, the rate of lactic acid accumulation was constant at a very low value (0.05 gm/l-hr). Reducing

FIGURE II.A.6: KINETICS OF GROWTH AND PRODUCT ACCUMULATION OF THERMOLACTIC BACTERIUM

Sample	Age	Cell Growth	Residual Glucose	Lactic Ac	Sp Growth Rate	Productivity <u>gm Lactic Ac.</u>	Conversion <u>gm Lactic Ac.</u>
N	(Hrs)	(gm/l)	(gm/l)	(gm/l)	(1/Hr)	1-Hr	mg Glucose
1	8	0.46	19.34	2.79	0.62		
2	10	1.01	17.29	7.16	0.35	2.31	0.71
3	12	1.23	9.10	13.26	0.17	2.96	0.92
4	14	1.63	6.46	18.10	0.07	2.41	0.83
5	16	1.63	3.0	19.6	0.03	1.50	0.89
6	18	1.69	1.5	21.6	0.03	2.0	0.91
7	20	1.66	0.0	23.7	0.0	2.0	0.94

pH controlled at 6.5

Temperature 60 C

FIGURE II.A.13: KINETICS OF PRODUCT ACCUMULATION FOR A MIXED CULTURE POPULATION OF C. THERMOCELLUM AND T. BACTERIUM

sugar concentration remained constant below 2.0 gm/l. Acetic acid and ethanol accumulated in the normal way (up to 1.0 gm/l). The results in Table II.A.7 show that after 60 hrs, 75% of the cellulose was consumed, and 58% of it went into lactic acid during the fermentation. Further information on this experiment is given in Table II.A.7 and Figure II.A.14.

Although there is still much room for improvement, the results obtained so far are encouraging and demonstrate the feasibility of a mixed culture system for the production of lactic acid directly from cellulose.

4. Future Experiments

Future experiments will concentrate on the biochemical engineering aspects of the mixed fermentation. Optimization studies conducted in the future should lead to a methodology and process for the direct conversion of cellulose to lactic acid and other useful products.

TABLE II.A.7: KINETICS OF PRODUCT ACCUMULATION FOR A MIXED POPULATION OF
C. THERMOCELLUM AND T. BACTERIUM

Sample (N°)	Age (Hrs)	Cell Growth (K.V.)	Lactic Acid (gm/l)	Acetic Acid (gm/l)	Ethanol (gm/l)	Red. Sugars (gm/l)	Res. Cellulose (gm/l)
1	40	175	0.93	0.41	0.40	0.76	6.93
2	42	180	1.13	0.45	0.44	0.79	6.52
3	44	145	1.60	0.48	0.43	1.0	5.90
4	46	148	2.30	0.49	0.45	1.35	4.85
5	48	149	3.2	0.51	0.48	1.81	3.9
6	50	150	4.75	0.55	0.55	2.50	2.56
7	55	150	4.80	0.57	0.59	2.30	2.61
8	60	150	4.90	0.58	0.65	2.1	2.23

pH CONTROLLED AT: 6.8
TEMPERATURE : 60°C

FIGURE II.A.14: KINETICS OF PRODUCT ACCUMULATION FOR MIXED POPULATION OF C. THERMOCELLUM AND T. BACTERIA

B. Production of Acetone and Butanol by Fermentation

1. Introduction

One of the problems encountered in the acetone/butanol fermentation is the low volumetric productivity of the mixed solvent (g/l-hr). This is caused mainly by the toxicity of n-butanol on Clostridium acetobutylicum resulting in a low final concentration of the mixed solvents. The concentration of n-butanol that causes total growth inhibition for C. acetobutylicum was determined to be 17 g/l (Progress Report COO-4198-4). The toxic mode of action of n-butanol towards C. acetobutylicum has not been established. However, based on previous reports on the toxicity of n-butanol towards other organisms and the physical properties of n-butanol as an organic solvent, n-butanol probably disrupts the structure of the membrane by partitioning into the lipid bi-layer.

It has been well established that the fatty acid composition of the bacterial membrane affects the permeability, transport properties and fragility of bacteria, such as E. coli (Haest et al., 1972; Akamatsu, 1974). Techniques for manipulating the composition of the membrane fatty acids have been reported and have been applied to both yeast and bacteria. The synthesis of fatty acid is either inhibited by using an antibiotic, cerulenin (Omura, 1976), or impaired by mutation (Silbert, 1975). Then, extraneous fatty acids were fed to these microorganisms to obtain membranes with a different fatty

acid profile. However, these techniques are quite involved, and therefore we decided to approach this problem in a different manner.

The membrane of living organisms has to be maintained in a liquid-crystalline structure to carry out the essential functions. Since saturated fatty acids usually have higher melting points than unsaturated fatty acids, cells growing at higher temperatures could have higher content of saturated fatty acids. This, in turn, could impart liquid-crystalline structures to the membrane.

Thus, we hypothesized that C. acetobutylicum growing at temperatures other than 37°C might have a significant change in the fatty acid content in the membrane. Such change might possibly cause a different resistance to n-butanol.

2. Materials and Methods

a. Inoculum Preparations

A culture of C. acetobutylicum ATCC 824 started from spores was allowed to grow in an anaerobic flask containing 250 ml soluble medium at 37°C. The culture was used to inoculate another 5 anaerobic flasks, each containing 250 ml soluble medium. These flasks were incubated at five different temperatures: 25, 30, 37, 45 and 50°C. From the mid-log phases of growth, these cultures were then used for the inhibition study.

b. Growth Inhibition Study

Since only cultures incubated at 30, 37, and 45°C were able to grow, only these three temperatures were studied. Nine series of Hungate tubes were used where each series consisted of tubes containing 5 ml soluble medium with n-butanol ranging from 0 to 25 g/l. These tubes were inoculated with 5 ml of appropriate inoculum and incubated at one of the three temperatures. The experimental design of these studies is tabulated in Table II.B.1. The turbidities of these tubes were followed by using a Turner Spectrophotometer set at 660 nm. For cultures that showed growth, at least one doubling of cell mass was monitored.

3. Results and Discussion

The growth rates of each culture at the different temperatures and different concentration of n-butanol were calculated from the turbidity measurements. The results are presented in terms of the growth inhibition index which is defined as:

$$\text{Growth Inhibition Index} = 1 - \mu/\mu_0. \quad (1)$$

where μ = specific growth rate in presence of n-butanol
(hr⁻¹)

μ_0 = specific growth rate without n-butanol added
(hr⁻¹)

The results from this inhibition study when the growth temperature was at 37 and 45°C are shown in Figure

TABLE II.B.1: EXPERIMENTAL DESIGN ON THE EFFECT OF N-BUTANOL
ON GROWTH OF C. ACETOBUTYLICUM AT DIFFERENT
TEMPERATURES

Inoculum Temperature (°C)	Growth Temperature (°C)		
	30	37	45
30	A	D	G
37	B	E	H
45	C	F	I

NOTE: Each letter represents a series of 10 tubes.

II.B.1 and Figure II.B.2 respectively. The effects of n-butanol on these cultures are similar and confirmed the results we previously reported (Progress Report COO-4198-4). The onset of butanol inhibition on growth is at about 10 g/l. Fifty percent inhibition on growth occurs at about 14 g/l. Total inhibition of growth was observed at 17 g/l. When the growth temperature was at 30°C, very different results were obtained. These results are shown in Figure II.B.3. The cultures originated from inoculum grown at 37 or 45°C (not shown) responded similarly as the others (Figure II.B.1 and II.B.2). However, the culture that was started from inoculum grown at 30°C has a higher resistance to n-butanol. The 50% inhibition concentration of n-butanol is 21 g/l. This is about 1.5 times of the concentrations obtained at the other temperatures. Total inhibition of growth occurs when butanol concentration reaches 25 g/l.

This study does not, however, confirm the hypothesis initially set forth. However, a marked difference of resistance to butanol for the microorganisms was observed when the growth temperature was varied. The possibility of increasing the tolerance of C. acetobutylicum towards n-butanol by lowering the temperature was revealed. This would be helpful in process design to optimize the volumetric productivity of mixed solvent.

4. Future Work

- Continue study of operating conditions for higher productivities.

FIGURE II.B.1. EFFECTS OF N-BUTANOL ON GROWTH OF C. ACETO-
BUTYLICUM ATCC 824 AT 37°C

FIGURE II.B.2: EFFECTS OF N-BUTANOL ON GROWTH OF C. ACETO-
BUTYLICUM ATCC 824 GROWING AT 45°C

FIGURE II.B.3: EFFECTS OF N-BUTANOL ON GROWTH OF C. ACETO-
BUTYLICUM ATCC 824 GROWING AT 30°C

- Continue study of activities of resting cells.
- In situ extraction using resting cells or immunized cells.

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C. Production of Acetic Acid by Fermentation

1. Introduction

In a previous Progress Report (COO-4198-4), we had reported the isolation of a strain (S-2) of Clostridium thermoaceticum which was able to tolerate higher concentrations of sodium acetate than the parent. This isolate has undergone further adaptation and selection to increase its tolerance to acetate. Some of the properties of this new isolate (S-3) will be presented in this report.

In the last Progress Report (COO-4198-5), the kinetic analysis on cell growth and acetic acid production suggested both growth and non-growth associated product formation are possible. The possibility of using non-growing cells for acetic acid production might be quite important in the development of an industrial process. In view of the previous findings, a number of supplemental studies have been performed to directly assess whether non-growing cells can indeed produce acetic acid. The results from these studies will be helpful in the conceptualization as to the type of fermentation system most appropriate for acetic acid production.

There is little doubt that the best desirable method in the production of acetic acid will be a continuous process. It is not certain, however, at this time the exact type of fermentation system which might be used. Some possible considerations include: continuous culture with and without

cell recycle, resting cells in packed columns or immobilized, or in situ product removal fermentors. We have therefore initiated studies in all of these areas in order to assess the problems which might be encountered in each of these systems. These studies, along with those others, should allow us to conclude the most viable production scheme in the design of a process.

2. Materials and Methods

Most of the analytical methods used in our studies have already been presented in previous progress reports and, therefore, will not be repeated. The only new experimental method which has not been reported is the studies on cell flocculation. These experiments were performed using cells from exponential phase of growth in shake flasks and mixed with different concentrations of the flocculant (Bentonite, mineral hydrocolloid, American colloids Co., Skokie, Illinois). The cell suspension containing Bentonite was mixed for about one minute and then transferred to the sedimentation column. The sedimentation column was made of a 2.5 cm diameter glass tube and 30.5 cm in length. Anadnoted divisions on the tube allowed the measurement of the sedimented cell-liquid interface for calculating the sedimentation rates. Samples from the top and bottom in the column were removed after 30 minutes of sedimentation for measuring the respective cell concentrations.

3. Results and Discussion

a. Properties of New Acetate Tolerant Strain (S-3)

A newly isolated strain (S-3) of Clostridium thermoaceticum has been under examination with respect to its acetate tolerance properties. Shown in Figure II.C.1 is the relationship between sodium acetate concentration to the specific growth rate of this isolate. The new strain is able to tolerate acetate up to 60 gm/liter before total growth inhibition results. In comparison to the parent and the previous isolate (S-2), total inhibition resulted at sodium acetate concentrations of 43 and 50 gm/liter, respectively. These results again confirm that the use of the adaptation technique offers an expedient method for strain selection.

The question was also asked as to which ion (cation or anion) exerts the toxic effect on the growth of C. thermoaceticum. Although it has been assumed that the anion (acetate) is the causative agent, this has never been tested in a definitive fashion. A series of experiments were therefore performed and the results shown in Table I.C.1. Sodium acetate alone and in combination with sodium chloride was tested to examine their effect on the growth of C. thermocellum (S-3). It is seen from Table II.C.1 that, as the sodium acetate was increased from 40 to 60 gm/liter, the specific growth rate decreased from 0.038 hr^{-1} to zero. However, when sodium acetate was tested in combination with sodium chloride, the results

FIGURE II.C.1: RELATIONSHIP FOR NEW STRAIN (S-3) OF
CLOSTRIDIUM THERMOACETICUM BETWEEN
SODIUM ACETATE CONCENTRATION AND
GROWTH

TABLE II.C.1: EFFECT OF SODIUM AND ACETATE ION CONCENTRATIONS
ON THE GROWTH OF CLOSTRIDIUM THERMOACETICUM

Sodium Acetate (gm/l)	Sodium Chloride (gm/l)	Sodium Ion (gm/l)	Acetate Ion (gm/l)	Specific Growth Rate (Hour ⁻¹)
40	0	11.2	28.8	0.038
50	0	14.0	36.0	0.025
60	0	16.8	43.0	0
40	10	15.1	28.8	0.032
30	10	13.3	21.6	0.055

showed that the increase in the concentration of the sodium ion did influence the specific growth rate, but to a much lesser extent than the acetate ion. For example, at a constant acetate concentration of 28.8 gm/liter, when the sodium ion was increased from 11.2 gm/liter (NaAC = 40 gm/liter) to 15.1 gm/liter (NaAC = 40 and NaCl = 10 gm/liter), a small decrease in the specific growth rate was observed (0.038 to 0.032 hr^{-1}). A linear interpretation of the data at 54 gm/liter (between 50 and 60 gm/liter) of sodium acetate was made which is equivalent to a sodium ion concentration of 15.1 gm/liter and acetate concentration of 38.9 gm/liter. At this condition, the interpolated specific growth rate was calculated to be 0.015 hour^{-1} . On the other hand, at the same sodium ion concentration (15.1 gm/l) but lower acetate concentration (28.8 gm/l), a significantly higher specific growth rate (0.032 hr^{-1}) was obtained. These results show that the acetate ion is the main causative agent in exerting a toxic effect to the growth of C. thermoaceticum.

b. Production of Acetic Acid by Non-Growing Cells

Since all of our previous results have indicated the possibility of non-growing cells to produce acetic acid, a number of independent experiments were performed to address this question more definitively. To test the capability of non-growing cells to produce acetic acid, the following experiments were performed. Three shake flasks were inoculated

and allowed to grow and produce acetic acid until cessation of both occurred. To one of the cultures, additional glucose and bicarbonate buffer were added and the second, with no addition, served as control. Since the antibiotic rifamcin has been found to totally inhibit growth, 5 mg/liter of this antibiotic was also added to the third culture along with glucose and bicarbonate buffer. All three flasks were then allowed to incubate at 60°C for 36 additional hours. The results from this study are shown in Table II.C.2. It is seen that, when glucose and bicarbonate were added, in the absence and presence of rifamcin, acetic acid continues to be produced. Since the culture containing the antibiotic cannot possibly achieve further growth, we conclude that resting cells are indeed capable of producing acetic acid.

In order to further elucidate this phenomenon, a different type of experiment was also performed. Concentrated suspension of C. thermoaceticum was first prepared through centrifugation. This concentrated suspension was then inoculated into the complete medium, but containing 10 gm/liter of glucose and 50 gm/liter of acetic acid (actually added equivalent to 70 gm/liter sodium acetate). The culture was then incubated at 60°C and the acetic acid, optical density and viable cell count were determined. The results are shown in Figure II.C.2. Acetic acid was observed to continue to be produced from 50 to 56 gm/liter. However, the optical density of the

TABLE II.C.2: THE EXAMINATION OF NON-GROWING CELLS OF C. THER-
MOACETICUM ON THEIR ABILITY TO PRODUCE ACETIC
ACID (INCUBATION AT 59°C FOR 36 HOURS)

Description	Initial Acetic Acid (gm/l)	Final Acetic Acid (gm/l)
Control (no glucose)	15	15
Glucose + Bicarbonate	15	20
Glucose + Bicarbonate + 5 mg/l Rifamcin	15	22

FIGURE II.C.2: ACETIC ACID PRODUCTION BY CLOSTRIDIUM THERMOACETICUM AT HIGH PRODUCT CONCENTRATION: BEHAVIOR OF CELL LYSIS AND VIABILITY

culture showed a continual decrease to approximately 50% of the initial value. The decrease in optical density would indicate cell lysis. The viable cell count during the same period decreased from about 5.8×10^6 per ml to 2×10^2 per ml. One can reasonably conclude that, during this period of acetic acid production, no net cell growth had occurred. These results further substantiate the ability of resting cells to produce acetic acid and encourage our efforts in further examining this concept for product formation.

c. Continuous Culture, Cell and Product Recovery

It is envisioned that ultimately a continuous process will be employed for the production of acetic acid. So far, all our studies have been performed using batch cultivation of Clostridium thermoaceticum. Therefore, we have initiated continuous culture studies to compare these results with our previous findings. Although only one continuous culture experiment has been completed, the results will be presented since they serve as a useful guide for future experimental programs.

The continuous culture study was performed in a 1-liter total volume fermentor using pH control (7.2), CO_2 overlay and 60°C . An inlet glucose concentration of 10 gm/liter and a dilution rate of 0.07 hour^{-1} were maintained. The results from 40 hours of steady-state operation are shown in Figure II.C.3. It is seen that the acetic acid concentration was 7.5 gm/liter and the cell concentration was 2.5 gm/liter. The

FIGURE II.C.3: CONTINUOUS CULTURE OF CLOSTRIDIUM THERMOACETICUM

($T = 60^{\circ}\text{C}$, $\text{pH} = 7.2$, $D = 0.07 \text{ Hr}^{-1}$)

volumetric productivity and specific product formation were calculated to be respectively 0.53 gram acetic per liter per hour and 0.21 gm acetic acid per gram of cell per hour. The specific product formation is slightly lower than that found from batch culture studies. For example, from our previous Progress Report (COO-4198-5, Figure II.C.5) at the same specific growth rate of 0.07 hr^{-1} , the specific product formation rate was found to be 0.28 gm acetic/gm cell-hr. Our explanation for the differences is the following. The data from the batch culture studies were obtained when the acetic acid concentration (15 gm/l) was higher than that in the continuous culture (7.5 gm/l). Since the production of acetic acid is both growth and non-growth associated, one would expect that more energy will be required to maintain the intracellular integrity as the external acetic acid concentration is increased. This increase in the energy utilization, often called "cell maintenance", is achieved through the degradation of glucose to acetic acid where ATP is derived. We therefore hypothesize that, due to a higher acetic acid, more energy was expended corresponding to a higher non-growth associated acid formation. It is the difference in this non-growth associated acetic acid formation rate which reflected the lower specific product formation of our continuous culture study. We therefore predict that the specific product formation rate will be increased when the acetic acid concentration is increased.

It is also envisioned that the continuous process for acetic acid production will use either cell recycle or immobilized resting cells. In both cases, the recovery of cells in an economical way will be required. Since Clostridium thermoaceticum physically is quite small (about one micron in diameter), conventional cell recovery methods (e.g. centrifugation or filtration) will probably be impractical. We have therefore initiated studies on finding flocculating agents which will enhance our cell recovery technique. Our experiments have focused on the use of a mineral hydrocolloid, Bentonite, to flocculate C. thermoaceticum. This agent is selected in view of its low cost (about 2¢/lb) and its known effectiveness to flocculate microorganisms.

The results of our flocculation experiments are shown in Figure II.C.4. Bentonite concentrations up to 3 gm/liter were tested. At approximately 1 to 1.5 gm/liter of Bentonite, the sedimented cell concentration was 22 gm/liter from an initial cell concentration of 5 gm/liter. The supernatants at these flocculant concentrations were essentially void of cells and our calculations show a 99% recovery efficiency. The sedimentation velocity of the flocculated cells at 1 to 1.5 gm/liter of Bentonite was found to be 1.2 cm/minute. The sedimentation velocity of C. thermoaceticum in the absence of flocculation was calculated to be 0.00033 cm/min. These results show a sedimentation enhancement factor of 3700 fold when Bentonite

FIGURE II.C.4: FLOCCULATION OF CLOSTRIDIUM THERMOACETICUM USING BENTONITE (INITIAL CELL CONC.= 5 GM/L)

is used. We conclude, therefore, from these impressive sedimentation rates that Bentonite will be an excellent agent for the recovery of C. thermoaceticum. We have also examined the potential adverse effect of Bentonite on the growth and acetic acid production by C. thermoaceticum. Bentonite concentrations of 0.5 to 1.0 gm/liter were tested and showed no effect on growth and product formation. We might add that the cells grown in the presence of Bentonite exhibited the same flocculation characteristics upon removal from the fermentor.

The last segment of the past research efforts was devoted to in situ product recovery. Although we have a modest effort in isolating acetate tolerant strains, there does exist a more attractive alternate. This being the production of the free acid and the in situ removal of the product leading to a more economic process. We have focused our efforts on the use of liquid ion exchangers.

We envision that a liquid ion exchanger in the hydrocarbon phase could be incorporated and thus allowing the extraction of acetate and changing the partition coefficient so that the hydrocarbon phase would be favored. From a process point of view, the following is envisioned. Acetic acid is extracted into the hydrocarbon phase with the ion exchanger. The hydrocarbon phase is removed and the acetic acid is recovered by cracking the ionic bond between the acid and the exchanger with heat. For this purpose, a tertiary amine would be required

as the ion exchanger so that the formation of the amide with acetic acid is avoided. The ion exchanger is recombined with hydrocarbon and added back to the fermentor.

The use of such a system requires compatibility with the organism used to produce acetic acid and that the ionic bond can be broken without excessive amounts of heat.

In the first phase of experimentation, a suitable hydrocarbon phase was selected by evaluating its effect on the growth of C. thermoaceticum. The solvents tested are shown in Table II.C.3. Two things are apparent. Only the long chain alkanes are suitable and the cyclic compounds are better suited than their straight-chained counterparts.

Tridodecylamine (laurylamine) used as the ion exchanger in the first experiments was found to be particularly effective at dissolving the cell walls. Detergency was expected to decrease with the length of the hydrocarbon chain. However, some compromise must be reached because the small chain triamines are very water soluble. Conceivably, trihexylamine or tricyclohexylamine would be suitable for this purpose. As neither of these compounds were available for use, particularly the latter, the compound tribenzylamine was examined. Here, cells in contact with tribenzylamine in either decane or hexadecane were found to grow well.

The next phase involved determining the temperature at which the ionic bond between acetic acid and the

TABLE II.C.3: EFFECT OF ORGANIC SOLVENTS ON THE CELL GROWTH OF C. THERMOCELLUM

<u>Solvent</u>		<u>Cell Growth</u>
Hexane		-
Ligroin	C ₅ -C ₈	-
Octane		-
Cyclooctane		±
Decane		+
Hexadecene		++
o-Xylene		-

amine would thermally crack, to allow the recovery of the acid. With the triamines, the pKa is 10.5. Therefore, tridodecylamine was used for this experiment. The acetic acid was recovered between a temperature of 170°C to 185°C. Subsequent experimentation with pKa reductions to 9.5, in this case quinoline, the acetic acid was recovered at a reduced temperature of 155°C to 170°C.

Although quinoline itself is not a useful compound for the recovery of acetic acid, derivatives of pyridine might be useful since the pKa of pyridine is about 5.0. One potentially useful compound would be 2,4,6 tri-n-hexylpyridine. Furthermore, the compound tricyclohexylamine may also be a useful compound because its extremely hindered steric configuration would show a much lower pKa than the trialkylamines.

4. Future Work

- Evaluation of kinetics using continuous culture.
- Design gravitational sedimentation unit to be used with continuous culture with cell recycle.
- Studies with resting cells, determination of their stability and an examination of the use of polyacrylamine gel entrapment to increase that stability.
- Acetate removal using ion-exchange.