THE BIOLOGICAL PRODUCTION
OF ORGANIC SOLVENTS
FROM CELLULOSIC WASTES

Progress Report

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E. K. Pye, A. E. Humphrey and J. R. Forro

University of Pennsylvania

Philadelphia, PA 19104

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Abstract

The objectives of this project are to optimize a modular process to convert cellulosic wastes to butanol and other oil-sparing chemicals. Research to date has focused on developing analytical methods, establishing a good data base and improving cellulase yields. Reliable assay methods for the Thermoactinomyces cellulase complex have been developed, measuring glucose and reducing sugar from filter paper and Avicel for total cellulase activity, viscosity change with carboxymethyl cellulose for the endoglucanase activity, and fluorescence change with methylumbelliferyl- β -D-glucopyranoside for β -glucosidase activity. Isoelectric focusing within the range pH 3.5-6.0 has proved to be a quick and useful means of determining effective cellulase complex composition. About 10 different proteins are present in the fermentation broth. Detailed procedures for U.V., and near U.V. plus 8-methoxy-psoralen mutagenesis have been developed and four mutants having 50% greater activity than the parent YX strain have been isolated. Cellulase production by Thermoactinomyces is growth related and is maximum when growth stops at 12-16 hours with 1-5% Avicel at pH 7.0-7.2 and 55°C. A multistage fermenter has been assembled for optimization of butanol versus acetone production by C1. acetobutylicum. A preliminary economic assessment, currently indicating butanol at just above 30¢/lb., is being continuously updated.

Introduction

The purpose of this project is to investigate the technical and economic constraints on a process to convert cellulosic wastes to butanol and other oil-sparing solvents and chemicals. Of the almost 1 billion dry tons of cellulose-containing wastes produced in the U.S. per year approximately 200 million dry tons are cattle feed-lot residues produced in concentrated areas primarily in the southwest U.S. (Arizona, Texas, California, N. Mexico, Colorado, etc.) Such residues have low value and in many cases are a significant pollution problem because of the run-off from the mounds. Because of these factors cattle feed-lot residues represent a convenient and economic cellulose source for bioconversion to liquid fuels and substitutes for petrochemical feedstocks. Consequently, the process we are investigating (Fig. 1) is a four module process which will a) pretreat raw cattle feed-lot residues with alkali or Na_2CO_3 , b) use a fraction of these pretreated residues to grow Thermoactinomyces for the production of a high-temperature cellulase, c) use this cellulase in a counter-current reactor to convert the bulk of the pretreated residues to a sugar syrup and d) anaerobically ferment this sugar syrup to butanol by Cl. acetobutylicum. Preliminary economic evaluation (Fig. 2) indicates that under present conditions, and with our present knowledge, butanol could be produced by this process for just over 30¢/lb. This compares with present ethylene-based butanol prices of 22-24¢/lb. which are expected to rise substantially

BIOLOGICAL PRODUCTION OF ORGANIC SOLVENTS FROM CELLULOSIC WASTES

Fig. 1

SUGAR MANUFACTURING COST ESTIMATE

¢ Charge/lb Sugar Product

Case I Case II Case III

I. COLLECTION AND PRETREATMENT

- . Feedlot Waste Collection Charge @ \$10/ton
- . Alkali Treatment 2% solution with 10% slurry
 - 50% re-use
 - 80% re-use
 - 90% re-use
- . Capital and Operating Charge; (\$25/ton Feedlot Waste)

1.4	1.4	1.4
2.0		
	0.8	
		0.4
3.3	3.3	3.3

II. ENZYME PRODUCTION (Based on estimate from (1) using T. Viride)***

- Cost of Enzymes/pound sugar produced at:
 - 10% enzyme recovery and re-use
 - 50% enzyme recovery and re-use
 - 90% enzyme recovery and re-use

4.1		
	3.0	
		2.4

III. ENZYME HYDROLYSIS AND FILTRATION COSTS (Based on estimate from (4) using T. Viride)

- Process Capital and Operating Charge - 25% conversion
- 50% conversion
- 75% conversion

4.6		
	2.3	
		1.2

IV. SOLIDS DISPOSAL

- Incineration at \$25/ton (no credit for power recovery)

<u>2.2</u>	<u>2.2</u>	<u>2.2</u>
17.6	13.0	10.9**

TOTAL

- (4) A.E. Humphrey, "Economics and Utilization of Enzymatically Hydrolyzed Cellulose", Presented at the Symposium on Enzymatic Hydrolysis of Cellulose, Aulanko, Finland, 12-14 March 1975.

- Basic Assumptions: (1) 90% Process Efficiency
 (2) 50% Conversion of Cellulose to Sugar
 (3) Feedlot waste: 80% cellulose after washing and available within 50 mile radius

*Case I: Worst Case; Case II: Likely Achievement; Case III: Process Goal

**The May 5, 1976, price of raw cane sugar quoted in The Wall Street Journal was 16¢/lb. N.Y. delivery.

***No credit was taken for cell biomass as a product.

Fig. 2.

as oil prices rise. It is our aim to improve the efficiency of our process to bring the price of butanol produced into a competitive position with synthetic butanol as rapidly as possible.

The modular approach has advantages of flexibility. For example, depending on economic and other factors such as supply, different cellulosic materials such as wood wastes could be used in place of feed-lot residues. Also, other cellulase producing organisms could replace Thermoactinomyces if it became advantageous to do so. In addition, the module producing solvents could be run independently on a sugar source, such as molasses, if, as is the case now, molasses are cheap and readily available. Also, the fourth (fermentation) module could be modified to produce other liquid fuels such as ethanol, or even acetate. Over the long run, however, large-scale sources of cellulose would be required to run such facilities, although it should be pointed out that only 5-10% of available feed-lot residues, converted by our process, could supply all of the current U.S. demand for butanol, ethanol, acetate and other chemicals readily produced by fermentation.

For such a process to have a national impact the price of the products must be competitive with those produced from oil and petrochemicals. For this reason we have examined the major cost-sensitive areas of the process. These include a) the cost of alkali used in the pretreatment step, b) the productivity of Thermoactinomyces for cellulase, c) the effectiveness and conversion efficiency of the cellulase for saccharification of pretreated cellulose residue and d) the conversion efficiency of the butanol fermentation. During the first year of our research we have focused on improving some of these cost sensitive areas by developing reliable and sensitive analytical systems, improving the output of Thermoactinomyces cellulase by culture and genetic modification, optimized conditions for growth of the organism, attempting to correlate cellulase activity with cellulase complex composition to predict the most effective complex for total saccharification,

and by investigating a multistage fermenter for butanol production. In addition, our preliminary economic evaluation is being continually updated as economic conditions change and as technical advances occur. It is of interest that part of this evaluation (Report COO-4070-4) indicates that aqueous acetate produced by this process may be close to economically viable at this time. However, the potential market for butanol, especially in tertiary oil recovery, is very large.

During the second year of this research we will focus on feed-lot residue pretreatment, saccharification efficiency of the cellulase on natural substrates including a standard pretreated feed-lot residue, butanol fermentation efficiency and several other technical problems and cost-sensitive areas.

The results reported in the following section are a summary of the most significant results produced to date. In addition, three reports (COO-4070-2, -3, -4) provide greater detail of our current and earlier work.

Another report, COO-4070-5, details the work performed to date by the General Electric Company under a sub-contract of the primary contract with ERDA. The results contained in this report will simply be summarized here.

Research Plan

On the basis of previous work at GE-RESO and the University of Pennsylvania it would appear that there are four main bottlenecks in the process (See Fig. 1). These are:

1. On a once through basis using a 2% alkali treatment of a 10% solid feed lot waste slurry, the alkali cost/pound of cellulose is on the order of 3¢/pound. Recycle and recovery of the alkali is essential to process economy. Also, it would be more efficient to combine the alkali treatment and sterilization processes.
2. The production of a cheap, high activity cellulase is essential to an economical process. The present strain of Thermoactinomyces has achieved conversion productivity of 2 gm sugar/liter-hr. This certainly is not optimal, for Thermoactinomyces appears to have poor cellobiase activity. Obviously, a strain selection and mutation program could be useful in developing a more efficient cellulase system and greater enzyme production.
3. Even though considerable work has been done on the Thermoactinomyces cellulase system as a whole, the exact component composition and action is not fully understood. The optimum temperature (70°C) and pH (6.0) are known. What is not yet fully understood is the role of crystalline cellulose and its potential action as a competitive inhibitor of the endo β -1,4-glucanase-cellobiosylhydrolase enzyme "complex". Also, the adsorption equilibrium isotherm for the various component enzymes of cellulases on amorphous

and crystalline cellulose are completely unknown. This information is essential to specifying, optimizing and designing a continuous enzyme reactor.

4. The kinetics of the anaerobic butanol fermentations are virtually unknown. It is not clearly understood whether butanol or some other product is acting as an inhibitor and whether maintenance of high growth rates will enable yields of greater than 35% to be achieved. If an efficiency of 40% could be achieved, this would cut the process costs by nearly 25% over the previous 28-33% yield processes.

During the first year of this project our efforts are being directed primarily at the latter three areas, aiming for the establishment of reliable analytical methods and techniques, enhanced enzyme production by organism mutation and optimal growth conditions and an understanding of Thermoactinomyces cellulase characteristics. These efforts are providing the data base necessary for the translation of the unit operations to the use of natural pretreated substrates.

Specific Details

1. Kinetic and Physical Characterization of the Thermoactinomyces Cellulase

a. Research goals

The primary goal of this study was to work out an efficient separation and purification procedure for the different enzymes of the cellulase complex. As many of these enzymes are isoenzymes with small differences in molecular weight, but with distinctly different isoelectric points, preparative isoelectric focusing in a granulated gel was chosen as the main separation step. The protein concentration of the extracellular fluid of the fermentation broth was found, however, to be so low (0.25 - 2 mg/ml) that a preconcentration is necessary. This concentration step or steps should increase protein concentration but at the same time decrease salt concentrations as high salt concentrations interfere with the establishment of the pH-gradient during isoelectric focusing.

Parallel with working out separation and concentration procedures, assays for the different enzymes that constitute the cellulase enzyme system were developed.

b. Concentration of extracellular protein.

Table I shows different ways to preconcentrate and what the effects of these procedures are on the protein and the salt concentrations. By one, or by a combination of two of these procedures, it is possible to increase the protein content and decrease the salt content of a sample. Table II shows how the different concentration procedures effect the protein content of samples from the extracellular fluid from

TABLE I

Effects of Different Concentration Procedures on Protein and Salt Concentrations.

<u>Concentration procedure</u>	<u>Protein</u>	<u>Salt</u>
Ammonium sulfate	+	+
Lyophilization	+	+
Ultrafiltration	+	-
Organic solvent	+	-
Dialysis	-	-
Gel filtration	-	-

TABLE II

Effect of different concentration procedures on the protein concentration of extracellular fluids from various Thermoactinomyces fermentations.

Concentration procedure	<u>Before</u>		<u>After</u>	
	Lowry	A_{280}/A_{260}	Lowry	A_{280}/A_{260}
Ammonium sulfate	NO PRECIPITATION			
Lyophilization	0.52 mg/ml		0.49 mg/ml	
	0.26 mg/ml		0.28 mg/ml	
Ultrafiltration	4.6 mg	11.2 mg	3.3 mg	6.5 mg (conc)
			1.5 mg	4.7 mg (filtrate)
Ethanol (80%)	0.58 mg/ml	1.40 mg/ml	0.32 mg/ml	0.43 mg/ml
Acetone (75%)	0.58 mg/ml	1.40 mg/ml	0.34 mg/ml	0.75 mg/ml
Dialysis	0.26 mg/ml		0.16 mg/ml	
Gel filtration	0.52 mg		0.49 mg	
TCA (10%)	0.58 mg/ml	1.40 mg/ml	0.33 mg/ml	0.33 mg/ml

different Thermoactinomyces fermentation broths. As it has so far not been possible to work out a meaningful assay for total cellulase activity (see below) the specific activities for the differently concentrated samples are not given.

It was not possible to "salt out" the proteins by $(\text{NH}_4)_2 \text{SO}_4$, even at 95% saturation. One reason for this might be that the proteins that constitute the cellulase system are glycoproteins, which has also been found for cellulases from Trichoderma viride (1-5). The enzyme system from Thermoactinomyces has a considerably higher temperature optimum, 65°C as compared to 50°C for the Trichoderma system. The sugars on the enzymes of the Thermoactinomyces system might therefore be so extensive and protective that they even prevent the proteins from aggregation.

It appears that there is a considerable loss in protein in all the procedures except for lyophilization and gel filtration when protein is determined by the Lowry procedure (6) with BSA as a standard. For comparison some protein determinations have also been made according to the formula $1.45 \cdot A_{280} - 0.74 \cdot A_{260} = \text{mg protein/ml}$ (7). These two protein determinations diverge widely before ethanol precipitation but after concentration they are in better agreement. There might be some aromatic pigment in the extracellular fluid of the broth that gives a large contribution to the Lowry measurements. This has been found for the broth from Trichoderma fermentations (M. Mandels, R. Brown, Jr., personal communication). Lowry and A_{280}/A_{260} determinations of TCA precipitates of extracellular protein seem to confirm that this is also the case for Thermoactinomyces fermentations. The ultrafiltration was made with a Millipore Pellicon Type PTGC,

nmwl 10,000, and large amounts of protein seemed to pass through. Experiments will be performed with an Amicon UM 10,000 filter which is used to concentrate extracellular fluid from Trichoderma (R. Brown, Jr., personal communication). In conclusion, gel filtration, organic solvent precipitation or, may be, ultrafiltration can be used to increase the protein concentration of the extracellular fluid of the fermentation broth. In the same procedures the salt concentraton decreases, which is necessary since the preparations are used for further separation by isoelectric focusing.

c. Isoelectric focusing

A number of preliminary runs have been made (Figure 3) and about 10 bands have been observed in the pH-range 3.5-6. Several samples, however, show only a few bands. The samples have been harvested at different stages of the fermentation, different substrates were used and an ultrafiltration was made before isoelectric focusing. It is therefore not possible to conclusively predict from the current data, that the composition of the enzyme system varies during the fermentation since different substrates will probably give rise to different enzyme systems and the ultrafiltration could separate certain proteins.

The high resolution and the assembly of protein bands within three pH-units imply, however, that this separation technique will be very useful in preparing and purifying the different enzymes from the cellulase system.

d. Enzyme assays

Total cellulase activity

The filter paper assay (8) was initially used to measure total cellulase activity because filter paper is considered an intermediate

Figure 3 Electrofocusing of extracellular fluid from various Thermoactinomyces fermentations in polyacrylamide gel, pH 3.5 to 9.5.

A: Grown on 1% Avicel in shake flask for 48 hours (11 μ g protein). C: Grown on 1% CMC in shake flask for 48 hours (7 μ g protein). 76: Grown on 1% Avicel in shake flask for 48 hours (27 μ g protein). W: Grown on 1% Whatman No. 1 filter paper (ballmilled) for 48 hours (9 μ g protein). M: Grown on 1% Avicel in minifermentor (10 liter) for 24 hours (16 μ g protein).

between crystalline and amorphous cellulose (8). It was not possible to obtain any linear relationship between amount of enzyme and amount of reducing sugar produced in the enzyme concentration range usually used for the cellulase system from Trichoderma (8) (See Table III). Several experiments were therefore made with different amounts of substrate (25, 100, 200 mg of FP) and decreased amounts of enzyme. The accuracy and reproducibility in those experiments were very poor (Table III) and it was considered that the FP assay might not be an entirely appropriate assay to measure total cellulase activity for the enzyme system from Thermoactinomyces.

In a second attempt, a sieved fraction of Avicel (<340 mesh) was used as substrate and the amount of reducing sugar in the fluid was measured. This means that production of mono- to small oligomers of glucose could be measured, while bonds broken by the action of endoglucanases could not be measured directly. Figures 4a and b show that the assay is linear during the first 20 minutes and up to 0.08 mg of enzyme which corresponds to about 1 mg of reducing sugar produced. As 200 mg of Avicel were used in the assay, only 0.5% becomes hydrolyzed under these conditions. Avicel always contains some amorphous material and it might very well be the 0.5% measured in this assay. This means that the way this assay was designed did not measure the total cellulase activity but rather the enzyme activity towards amorphous cellulase. Assays are currently worked out with much lower concentrations of substrate (1-2%) and they will show if Avicel still is suitable for the measurement of total cellulase activity.

From the above considerations it seems likely there is no quick, reliable and meaningful way of assaying total cellulase activity. The

TABLE III

Reducing sugar (R.S.) produced when amounts of enzyme (prot) and filter paper (F.P.) are varied.^{x)}

<u>25 mg</u>		<u>50 mg</u>		<u>100 mg</u>		<u>200 mg</u>	
mg prot	mg R.S.	mg prot	mg R.S.	mg prot	mg R.S.	mg prot	mg R.S.
-	-	0.005	0.010-0.023	0.005	0.065	0.005	0.055-0.115
-	-	0.01	0.035-0.053	0.01	0.130-0.150	0.01	0.255-0.365
0.025	0.065-0.075	0.025	0.083-0.182	0.025	0.138-0.258	0.025	0.160-0.445
-	-	0.038	0.196	-	-	-	-
0.050	0.060-0.090	0.050	0.147-0.370	0.050	0.225-0.325	0.050	0.355-0.537
-	-	0.092	0.659	-	-	-	-
-	-	-	-	0.125	0.865-1.1	-	-
-	-	0.46	1.275	-	-	-	-

^{x)} Assay: Whatman No. 1 filter paper, 1 x 6 cm = 50 mg.

0.5 ml enzyme solution.

1.5 ml 0.05 M phosphate buffer, pH = 5.9

60 minutes incubation at 65°C.

3 ml DNS reagent (16) added.

5 minutes incubation in boiling water.

15 ml water added.

A₅₅₀ measured.

Reducing sugar determined from standard curve with glucose and same amount of filter paper.

Figure 4a. Reducing sugar produced from Avicel as a function of amount of enzyme (protein).

Assay: 1 ml 20% Avicel (<325 mesh), 1 ml of enzyme in 0.05 M phosphate buffer, pH = 5.9. Sixty minute incubation in shaking water bath at 65°C. Reaction stopped on ice bath; Avicel allowed to settle for 1/2 hour. 0.5 ml of supernatant added to 0.75 ml DNS-reagent (16). Five minutes incubation in boiling water bath. A₆₄₀ measured.

Figure 4b. Reducing sugar produced from Avicel as a function of time. 0.004 mg of enzyme (protein).

Assay: See text to Figure 2a.

most meaningful way would be to incubate the extracellular fluid with cellulose substrates of varying crystallinity and measure the amount of glucose produced. Such experiments will be performed in the coming year.

Endo and exo- β -glucanase activities

When carboxymethyl cellulose (CMC, 4M6SF, Hercules) is the substrate, the decrease in viscosity is a measure of the hydrolytic action of endoglucanases (2,4,5,9,10). It has been shown that ΔF (the difference in fluidity of the sample and the blank) is linear with time and amount of enzyme for the cellulase system from Trichoderma viride (R. Brown, Jr., personal communication). This is also valid for the cellulase system from Thermoactinomyces (Figure 5a and b). From the ΔF values the specific endoglucanase activity for one crude preparation has been calculated to be 3 compared to 10 for a crude preparation from Trichoderma viride (R. Brown, Jr., personal communication).

The amount of reducing sugar released from CMC measures the total amount of β -glycosidic linkages hydrolyzed by all enzyme preparations.

Figure 6 shows that the initial velocity and specific activity is three times higher for a crude enzyme preparation from a broth grown on CMC as compared to enzyme preparations from broths grown on either Avicel or Whatman No. 1 filter paper. Thus, different enzyme systems are produced with different substrates and this same substrate has the highest susceptibility against that particular enzyme system. Similar effects have been noticed for Trichoderma viride grown on different substrates (11).

The amount of glucose produced when the crude enzyme system uses a cellulose substrate, e.g. CMC, is a measure of how far the

Figure 5a. Change in fluidity (ΔF) of carboxymethylcellulose (CMC) as a function of time with various amounts of enzyme (protein).

Assay: 10 ml 0.5% CMC (4M6SF, Hercules) in 0.05 M phosphate buffer, pH = 5.9, 5-50 μ l enzyme. Efflux-time measured in Cannon-Fenske viscometer 150 at 65°C.

Figure 5b. Change in fluidity (ΔF) of CMC as a function of amount of enzyme (protein) at 10 minutes.

Assay: See text to Figure 3a.

Figure 6. Reducing sugar produced from CMC as a function of time. Enzyme from fermentations on 1% Avicel, CMC and ballmilled Whatman No. 1 filter paper.

Assay: 0.005 mg enzyme (protein) in 1 ml of 0.05 M phosphate buffer, pH = 5.9, 1 ml 1% CMC. Incubated at 65°C. Reaction stopped by addition of 3 ml DNS-reagent. Incubated in boiling water bath for 5 minutes. A₆₄₀ measured.

saccharification has proceeded due to the combined actions of endoglucanases, exoglucanases and β -glucosidases. Figure 7 shows that only 30-40% of the amount of reducing sugar recorded is actually glucose. The remainder must be associated with oligosaccharides.

At present the HPLC (high pressure liquid chromatographic) technique is under development. This technique allows the detection of hydrolysis products such as monomers up to hexamers of glucose (12, R. Brown, Jr., personal communication) and thus gives the possibility to determine the specific action of the different enzymes after they have been purified.

β -glucosidase (cellobiase) activity

This enzyme splits cellobiose into two molecules of glucose during the hydrolysis of cellulose (10,13,14). The enzyme uses, however, several other substrates one of which is methylumbelliferyl β -D-glucopyranoside (15, Figure 8). When using the crude enzyme preparation the reaction is linear over 30 minutes and with enzyme concentrations up to 0.03 mg of enzyme. The assay was worked out with this substrate in the first place because the product is fluorescent and therefore the assay is the most sensitive. The enzyme assay will also be worked out with cellobiose and p-nitrophenyl- β -D-glucoside as substrates.

Conclusions

The proteins from the extracellular fluid of the fermentation broth can be concentrated and thereby it is possible to perform analytical and preparative isoelectric focusing. About 10 different proteins have been detected with analytical isoelectric focusing. The different assays have been worked out for the crude enzyme

Figure 7 Reducing sugar and glucose produced from CMC as a function of amount of enzyme (protein). Reducing sugar assay: See text to Figure 6. Incubation time 1 hour. Glucose assay: 1 ml of enzyme in 0.05 M phosphate buffer, pH 5.9, 1 ml 1% CMC. Incubated for 1 hour in 65°C. Reaction stopped by boiling 10 minutes. 0.2 ml reaction mixture added to 2.5 ml glucostat (Worthington Biochemical Corp., New Jersey). Incubated for 1 hour at 37°C. A_{400} measured.

Figure 8 Methylumbelliferone produced from methylumbelliferyl β -D-glucopyranoside as a function of time.

Assay: 0.1 ml 10 mM methylumbelliferyl β -D-glucopyranoside + enzyme in 0.05 M phosphate buffer, pH = 5.9 to 1 ml. Incubation at 65°C. Reaction stopped with 3 ml 0.2 M glycine buffer, pH = 10.3. Methylumbelliferone determined by fluorescence (excitation: 365 nm; emission: 450 nm).

preparations and some alterations might be needed to adapt them to the purified enzymes. The purified enzyme will be characterized viscosimetrically with CMC as a substrate, the hydrolysis products from CMC will be identified by HPLC and the β -glucosidase activity will be assayed with cellobiose, methylumbelliferyl β -D-glucoside and p-nitrophenyl β -D-glucoside. With these three assays it will be possible to characterize all the components (endoglucanases, exoglucanases and β -glucosidases) of the cellulase system from Thermoactinomyces.

2. Optimization of Cellulase Production

a. Research goals

The main research goal of this portion of the investigation was to determine the conditions for the optimum cellulase production by a Thermoactinomyces sp. In attempting to achieve these goals the research has been developing along two basic lines

- development of accurate and reliable analytical laboratory techniques which will enable the determination of the three main fermentation variables, i.e., enzyme activity, cell biomass and protein levels.
- identification of the important fermentation parameters, based on which an optimization strategy may be built in order to attempt total cellulose digestion.

b. Development of analytical techniques

Efforts in this area have been focused on the establishment of a cellulase assay which is fast and meaningful and on identifying the best assays for cell mass and protein concentration measurements.

Filter paper assay has proved to be satisfactory for our needs in this study and is now performed on a routine basis. As far as cell biomass and protein levels are concerned, protein material balances have been established which take into account the cellular protein, the supernatant protein and the protein adsorbed on the solid cellulose. These balances are given in Table IV. In order to measure all these different concentrations a Kjeldahl nitrogen determination and a Lowry protein analysis are performed on the centrifuged solids and on the liquid supernatant. Several problems have been encountered along the way which have or are being solved. We are hopeful these techniques will enable us to better estimate activity and production.

We also have developed methods for monitoring the sugar profile during the cellulose fermentation; glucose is measured using an Enzymax Glucose Analyzer, while the dinitrosalicylic acid (DNS) method is used for measuring the total reducing sugars in the fermentation broth.

c. Study of the Thermoactinomyces fermentation

We have cultivated Thermoactinomyces on glucose, cellobiose, AVICEL and SOLKA-FLOC media (See Figures 9 through 13). The glucose experiments have shown that the protein content of the cells is reasonably constant during the run. These experiments have indicated that it is possible to obtain high cell densities using a fed-batch technique monitored through the culture fluorescence; however, an oxygen-limited situation is eventually reached in high concentration media during which the cell yield sharply decreases and an intermediate begins accumulating in the media.

TABLE IV
CALCULATION OF BIOMASS AND
ADSORBED ENZYME CONCENTRATIONS

Lowry Protein of Solids = Lowry Protein + Lowry Protein
of cells of Adsorbed Enzyme

$$P_s = aX + bA$$

where: P_s = total measured solids by Lowry protein

a = Lowry protein content of cells = 0.45

X = cell mass

b = Lowry protein of adsorbed enzyme = 1

A = adsorbed enzyme

Kjeldahl Nitrogen of Solids = Kjeldahl Nitrogen + Kjeldahl Nitrogen
of cells of Adsorbed Enzyme

$$N_s = N_x X + N_A A$$

where: N_s = total measured solids by Kjeldahl nitrogen

N_x = Kjeldahl nitrogen content of cells = 0.095

N_A = Kjeldahl nitrogen content of adsorbed enzyme = 0.128

Then:

$$X = \frac{N_s - 0.128 P_s}{0.0374}$$

$$A = P_s - 0.45X$$

FIGURE 9
GROWTH ON 1% AVICEL

FIGURE 10
GROWTH ON 1% SOLKA FLOC

FIGURE 11

GROWTH OF THERMOACTINOMYCES ON 1% CELLOBIOSE
(55°C, 500 ML SHAKE FLASK)

FIGURE 12

GROWTH OF THERMOACTINOMYCES ON 1% GLUCOSE
(55°C, 14 LITER FERMENTOR)

FIGURE 13
FILTER PAPER ACTIVITIES FOR GROWTH ON 1% AVICEL

The AVICEL and SOLKA-FLOC experiments have shown that it is possible to obtain about 70% cellulose utilization in reasonably short periods, i.e., less than 24 hours. Optimal extracellular filter paper activity is obtained after 16 hours into the fermentation (See Figures 9-13). Cellulose activity is much lower with SOLKA-FLOC as substrate than with AVICEL.

3. Kinetics of Cellulose Hydrolysis

a. Research goals

The research goals for this portion of the study are to investigate the kinetics of cellulose hydrolysis by the cellulase system derived from a sp. of Thermoactinomyces. During the initial phases, pure cellulose (AVICEL) has been used as the substrate. Ultimately this system will be used to degrade the cellulose in feed-lot wastes.

b. Studies of enzyme activity

Initial work has centered primarily on the development of quantitative analytical techniques for monitoring sugar production and enzymatic activity during the hydrolysis reaction. The major effort has been concentrated on establishing and verifying assays for the cellulase complex of Thermoactinomyces using samples from various fermentations carried out within our laboratory group. It is felt that there are two major types of assays applicable: 1) those monitoring overall cellulolytic activity, allowing comparison between intra- and inter-group experimentation and 2) those monitoring activity of a specific component of the cellulase system.

The literature indicates that the filter paper activity measurement developed by Mandels, et al., is the best overall activity indicator of cellulase activity. Since most other groups have used it, we

felt it had an advantage over other methods since it would allow for comparison of our work with that of others. Therefore, filter paper assay was one of the assays adopted to monitor our fermentations, but was not felt to be sufficiently precise for specific enzymology (See part 1 of this report).

c. Hydrolysis Reactor

An eight stage counter-current hydrolysis reactor has been designed and is being constructed by the shop. When this is completed work will begin on obtaining continuous steady-state kinetic data for the hydrolysis reactions.

4. Kinetics of the Butanol Fermentation

a. Research goals

The objective of this portion of the study is to maximize the production of n-butanol from a sugar syrup derived from cellulosic waste. This requires the

- gaining a better understanding of the pathway by which glucose is converted to n-butanol cells during growth.
- determining the kinetic constants for growth and for solvent production initially utilizing a synthetic glucose medium and then later on using a sugar syrup derived from hydrolysis of feed-lot wastes.
- minimizing the maintenance requirement of cells by growing at a high rate and recycling cells.
- genetic blocking or inhibiting the formation of solvents (acetone and alcohol) other than n-butanol.

b. Multistage fermentation unit

It was decided to study the kinetics of the system in a four-stage continuous reactor. This allows the separation of the growth stage from the reducing stages where the various solvents are produced. This also minimizes the inhibitory effects of the solvents on growth. The first stage is being designed to operate at conditions of maximum growth rate in order to minimize the glucose required for maintenance.

Five glass continuously stirred tank reactors (CSTR's) have been purchased from Belco Glass, Inc. of Vineland, New Jersey. A 250 ml CSTR and a 500 ml CSTR will be interchanged for the growth stage. Three 500 ml CSTR's will be used for the solvent production stages. Each reactor is jacketed for temperature control. The pH will also be controlled in each individual stage.

c. Fermentation mechanism

The literature (See attached diagram) shows the possibility of production of several compounds using Clostridia acetobutylicum including ethanol, acetone, isopropanol, n-butanol, acetomethylcarbinol, acetic acid, and n-butyric acid. A chromatographic analysis is being developed to quantitatively follow these compounds in the presence of glucose. In addition to these compounds which are in the liquid phase, CO₂ and H₂ are also produced. These can be analyzed chromatographically on a gas chromatograph with a thermal conductivity detector, which we presently are trying to obtain. An additional expense that very likely will occur is the modification of our existing Beckman GC55 for back-flushing glucose off the columns.

The bacteria which are being studied come from two strains of Clostridium acetobutylicum purchased from ATCC. At present, we

are having difficulty growing the bacteria satisfactorily, on synthetic medium.

The important variables in the separation of physiological states of the bacterial will be the pH and the temperature. Batch runs from the literature indicate the buildup of acetic and n-butyric acids during the growth stage, with minimal amount of solvent production. Concurrently, H_2 and CO_2 are produced. The pH was not controlled in these batch runs. It may be this variable which results in the switch from acid production to the neutral solvent production. This will be investigated.

Clostridia may possess the ability to use molecular hydrogen in the reduction of acetic and butyric acids to alcohols. If this is true, the H_2 produced in the growth stage reactor must be circulated to the subsequent stages. This could reduce the loss in yield as acetic acid and butyric acid. A continuous run by Dyr showed an 19% yield on n-butanol. If it is assumed that the acids are reduced quantitatively to butanol, a yield of 25% is obtained. This agrees with batch runs from the literature. We hope, however, by controlling temperature, pH, and recycling the H_2 to achieve butanol yields between 30 and 40%. These values must be obtained for this process to be economically attractive. To do this it is essential that the production of acetone be minimized. This might be accomplished by finding a strain of Clostridium that does not produce acetone, but the success of this effort is not likely in a short period of time. An alternative approach will be to feed acetone to the solvent production stages in an effort to block the formation of acetone by the bacteria. If successful, an acetone recovery and recycle system would be necessary in later development.

The first experimental effort here will be to run the fermentation in a batch system. The intermediates, pH and products will be followed closely. This first step is necessary to determine the exact products formed by the organism being used. The batch data will aid in the planning of the continuous runs. Batch runs were begun during the first week in April. Data are now being obtained from these runs.

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Contract Compliance

It is the opinion of the Principal Investigator that the results reported have, together with the work anticipated to be performed during the remainder of the current contract year, will see this project in essentially complete compliance with the original contract requirements. We do not, at this time, expect any failure to comply with these requirements.

The following percentages of effort are estimated:

	<u>Up to present</u>	<u>Up to end of contract year</u>
Dr. E. Kendall Pye, Principal Investigator	35%	50%
Dr. Arthur E. Humphrey, Co-Principal Investigator	15%	25%