BIOCONVERSION OF PLANT BIOMASS TO ETHANOL

Final Report
for the Period

MASTER

76 December 15 — 78 December 31

Ronald E. Brooks, Tah-Mun Su, Michael J. Brennan, Jr.,
Joanne Frick, and Marie Lynch

July 1979

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GENERAL ELECTRIC COMPANY
Corporate Research and Development
P.O. Box 8
Schenectady, N.Y. 12301

Prepared for the

U.S. DEPARTMENT OF ENERGY
Solar Energy Research Institute
Golden, CO 80401

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ABSTRACT

Two approaches to ethanol production via thermophilic mixed culture fermentation of pretreated wood were investigated. The initial studies of wood biodelignification by Chrysosporium prunosum and subsequent mixed culture fermentation to ethanol using a cellulolytic strain of sporocytophaga and a strain of Bacillus stearothermophilus proved to be premature for a development effort. Studies of the fermentation of SO₂/steam-treated poplar by a mixed culture of C. thermocellum and C. thermosaccharolyticum were, however, technically and economically promising.

Wood pretreatment to enhance microbial utilization, the microbiology and biochemistry of pure and mixed culture fermentation of cellulose by C. thermocellum and C. thermocellum and C. thermosaccharolyticum, and techniques for improving ethanol tolerance and yield were investigated. Considerable progress in overcoming the technical barriers to efficient ethanol production from wood have been demonstrated; however, additional studies and development work are required before technical feasibility can be established.

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I. INTRODUCTION

Plant biomass is increasingly becoming an attractive renewable source of energy and chemicals as a result of various environmental, supply, and cost concerns about fossil and nuclear energy. The technical feasibility of biomass utilization depends upon the development of high-yield crops and an appropriate conversion technology for producing useful products. Crop yield is particularly important because, in most biomass utilization processes, the cost of biomass and the acreage required to produce the biomass are significant factors. Ethyl alcohol is an attractive product, not only because of its fuel value and convenience of storage, but because it may also serve as a chemical feedstock. Yeast fermentation technology for converting sugar into ethanol is an old and proven process which is not directly applicable to the more plentiful and less expensive lignocellulosic substrates like wood and agricultural residues. The direct thermophilic fermentation of such lignocellulosics to ethanol is a straightforward and flexible approach to biomass utilization.

Commercial development of a biomass conversion process depends upon the price and availability of biomass as well as plant size and engineering complexity and the sustained demand for ethanol. Many of the administrative, institutional, and marketing problems associated with ethanol production and use are minimized if the farmer (dairy, cattle, and grain) is both the producer and primary consumer of ethanol for use as a liquid fuel. Surplus ethanol could be sold in local or regional markets to supplement farm income. This recommendation is based upon the favorable balance between fossil energy input and potential biomass energy output, which is characteristic of most agricultural operations. This approach has the primary advantage of encouraging stable biomass prices and increased supply while reducing raw material transport costs. The primary impediment to small-scale, decentralized plants arises from the possible loss of normal economies of scale as they relate to the cost of ethanol production. However, if the economic penalty

for small-scale decentralized operation is not too great, the overall concept should make sound economic and social sense.

Cost-competitive ethanol from biomass requires (1) an efficient means of pretreating plant biomass to enhance its susceptibility to microbial attack, (2) rapid and efficient fermentation of cellulose and hemicellulose at high substrate concentrations, and (3) an ethanol concentration high enough to minimize the recovery cost (in Btu and dollars).

The research effort described in this report addresses most of the technical and economic barriers to the commercial development of an ethanol-from-wood process. The objective of this research is the laboratory demonstration of the technical feasibility of a mixed culture, direct fermentation of pretreated wood to ethanol. Earlier reports have documented the experimental protocols and the pertinent literature references. Accordingly, the emphasis in this final report will be upon a summary of the experimental results and a discussion of research findings.

A. TECHNICAL APPROACH

Initially, we proposed an all-biological process for converting wood to ethanol. The process was based upon biodelignification of wood with Chrysosporium pruinsum in a high-solids reactor and subsequent direct thermophilic mixed culture fermentation to ethanol. A cellulolytic strain of sporocytophaga and a strain of B. stearrowthermophilus were used to convert the enzymatically produced soluble sugars to ethanol. Experimental difficulties were encountered in reproducibly culturing C. pruinsum on solid media and in determining the growth requirements of sporocytophaga. The latter organism was selected because of its cellulolytic activity. At that time, the ability of C. thermocellum to produce ethanol from cellulose was not widely recognized; however, comparative studies using a C. thermocellum strain (LQ8), provided by Professor Zeikus, indicated that the ethanol production of LQ8 was comparable to that obtained from the mixed culture. Strain LQ8 was subsequently demonstrated to be contaminated with a strain of C. thermosaccharolyticum

which fermented both xylose and glucose to ethanol as did B. stearothermophilus. The latter, however, was not compatible with C. thermocellum. C. thermosaccharolyticum, which formed a very stable mixed culture, was therefore substituted to ensure conversion of hemicellulose to ethanol. Subsequent studies were focused on characterizing and improving the C. thermocellum - C. thermosaccharolyticum mixed culture cellulose fermentation.

Alternative pretreatment schemes were also analyzed, and chemically augmented low-pressure hardwood steaming was selected as the most attractive method of enhancing the microbial digestibility of wood. A new process scheme, shown in Figure 1 and described below, was developed to reflect the revised approach to obtaining ethanol from wood.

B. DESCRIPTION OF GENERAL ELECTRIC CORPORATE RESEARCH AND DEVELOPMENT PROCESS

Relatively coarse hardwood (and/or softwood, if amenable) chips are steamed at low pressure in the presence of supplemental amounts of sulfur dioxide (or equivalent reagent) for a brief time and are rapidly decompressed. The partially defibrated wood is neutralized with ammonia gas and fed directly into a fermentor, which operates at a temperature of approximately 60 °C. A mixed culture of Clostridium thermocellum and thermosaccharolyticum is employed to ferment the readily digestible substrate to ethanol. C. thermocellum is used to solubilize cellulose and to convert cellobiose to ethanol. C. thermosaccharolyticum is employed to ferment the pentose sugars produced during pretreatment but not utilized by C. thermocellum to ethanol. Product recovery and cell recycling are accomplished by continuous withdrawal of the broth to a vacuum distillation chamber (modified vacuform) and subsequent distillation to produce 95% ethanol. After ethanol separation, the cell mass is returned to the fermentor to maintain cell density while lignin is discharged, partially dried, and used to fuel the pretreatment steam boiler. If practical, spent stillage is recovered and used as a fertilizer.

Figure 1. GE/CRD Direct Ethanol Fermentation Process (30×10^6 gallons/year, 95 vol. %, #/day unit)

II. MATERIALS AND METHODS

A. ORGANISMS

C. thermocellum LQ8 was provided by J.G. Zeikus, Department of Bacteriology, University of Wisconsin, Madison, Wisconsin. C. thermocellum Q was provided by L.Y. Quim, Department of Bacteriology, Iowa State University, Ames, Iowa. C. thermosaccharolyticum was isolated as a contaminant of LQ8.

Culturing Conditions

The anaerobic culture technique method of Hungate (1), as modified by Miller and Wolin (2), was used for most of this work. Individual colonies were also isolated from pour and spread plates in an anaerobic growth chamber. The composition of one liter of the medium was essentially that published by Weimer and Zeikus (3) with some modification: K_2HPO_4 , 2.9 g; KH_2PO_4 , 1.5 g; $(NH_4)_2SO_4$, 1.3 g; $MgCl_2$, 1.0 g; $CaCl_2$, 1.5 g; yeast extract, 2.0 g; cysteine hydrochloride 1.0 g; $FeSO_4$ 0.00125 mg; and the desired carbon source.

Chemicals

Microcrystalline cellulose PH 105 Avicel was purchased from the FMC Corporation. β -D(+) Cellobiose was obtained from the Sigma Chemical Company. Poplar (Populus tristis) was provided by R. Freitas and C.R. Wulke, Lawrence-Berkeley Laboratory, University of California, Berkeley, CA.

B. ANALYSIS

Soluble sugars were analyzed initially by the anthrone method (4) and later by the dinitrosalicylic acid procedure (5). The anthrone method proved unsatisfactory for wood sugar mixtures containing glucose and xylose. Attempts to quantify the component sugars by HPLC on μ -Bondapak carbohydrate column (Waters Associates) were unsuccessful because of poor resolution. Quantitative sugar analysis was performed by gas-liquid chromatography (Hewlett-Packard Model 5830) of trimethylsilyl derivatives as recommended

by C.R. Wilke (6). Multiple peaks were observed for each sugar because of the mutarotation which occurred during the silylation reaction. An alternative derivatization procedure based on reaction with hydroxylamine and N-trimethylsilylimidazole in pyridine (7) gave single peaks and proved less time consuming.

Volatile acids and ethanol were analyzed by gas chromatography, with the use of a glass column packed with the porous polymer Chromosorb 101.

Cellulase activity was determined by the published procedure of Su and Paulavicius (8).

β -Glucosidase activity was measured by liberation of p-nitrophenol according to G. Okada (9) with the following modifications. Enzyme solution (0.4 ml) was added to a reaction mixture of 0.0136 M p-nitrophenyl β -D-glucoside (0.2 ml) and 0.1 M phosphate buffer (0.2 ml) at pH 7.0. After incubation for 30 min at 50 °C, 0.4 ml aliquots were removed and added to 5.0 ml of 0.1 M sodium carbonate. The concentration of p-nitrophenol was measured by determining the optical density at 420 nm.

Cellulose, hemicellulose, and lignin were analyzed according to C.R. Wilke *et al.* (6). Weighed dry samples were mixed with 10 ml of 72% sulfuric acid, allowed to stand for three hours, with hourly mixing for a few seconds. The samples were then diluted to 50 ml with distilled water and allowed to stand overnight. Individual samples were then vacuum-filtered through a tared 0.4 μ m (47 mm) nucleopore filter. Aliquots of the filtrate were retained for carbohydrate (reducing sugar) assays.

The residue on the filter paper was washed with distilled water until the filtrate pH was 5. The filter paper plus residue was dried overnight at 65 °C and weighed. The filter and residue were then transferred to a tared crucible and ashed overnight at 550 °C. Lignin was reported as the fraction of the residue (exclusive of the filter) which vaporized. The filter contained less than 0.5 mg ash.

Protein was measured by the Lowery method, with crystalline egg albumin as a standard.

III. RESULTS

A. PURE AND MIXED CULTURE FERMENTATIONS

1. Nutritional Requirements of C. thermocellum Q

Extensive studies (10) on a limited number of strains has revealed the need for vitamins and amino acids commonly found in yeast extract. However, our preliminary attempts to grow Q on a vitamin supplemented casamino acid medium or a defined medium were unsuccessful. An optimum yeast extract concentration was, therefore, determined after investigating three different levels (Table I). Increasing the yeast extract concentration above 0.5% had an adverse effect on the culture. Below 0.5% yeast extract, the growth of C. thermocellum Q was more sluggish. Growth on glucose occurred only at and above 0.5% yeast extract, which is consistent with the observed decrease in glucose accumulation as the yeast extract level is increased. Subsequent experiments, unless otherwise indicated, employed 0.5% yeast extract in the culturing medium.

Table I

EFFECT OF YEAST EXTRACT ON C. THERMOCELLUM Q GROWTH ON CELLOBIOSE*

Yeast Extract	Doubling Time (Hrs.)	Cellulase** Activity	Glucose Accumulated (mg/ml)	Ethanol (mg/ml)	Acetic Acid (mg/ml)
0.2%	5.0	1.20	1.82	0.57	0.47
0.5%	1.5	1.65	1.09	0.83	0.66
0.7%	2.0	1.80	0.72	0.76	0.56

*pH Controlled Between 6.5-7.0

**mg/ml of Glucose Equivalents, 24 Hrs. Avicel Assay

2. Growth substrates and products

C. thermocellum Q grows on cellulose, cellobiose, and glucose. Growth on cellobiose results in the production of glucose, lactic acid, ethanol, acetic acid, hydrogen,

and carbon dioxide. Glucose represents about half of the soluble sugar production arising from growth on cellulose, and the remainder was assumed to be cellobiose. C. thermosaccharolyticum is noncellulolytic but forms a very stable mixed culture with C. thermocellum and grows on cellobiose, glucose, and xylose. The fermentation products of C. thermosaccharolyticum from growth on cellobiose are similar to those of C. thermocellum except for variable amounts of butyric acid. Both cultures require relatively high levels (0.2 - 0.5%) of yeast extract for growth. A quantitative fermentation balance was deferred until a defined medium was developed.

The comparative growth of Q on glucose, cellobiose, and cellulose is shown in Figure 2. Growth on glucose is preceded by a long lag period, which was shortened but not eliminated by adaptation of the culture to glucose. Growth on cellobiose is rapid and proceeds to completion in about 60 hr. Growth on cellulose is slower than that observed on cellobiose, as expected if saccharification of cellulose is rate limiting. The similarity in the initial rates of cellobiose and cellulose depletion rates probably results from carry over of cellulase-saturated cellulose. These particular experiments employed 5% cellulose-grown inoculums.

Product formation by C. thermocellum Q was found to depend on substrate (Figure 3). The higher ethanol concentration arising from growth on cellobiose resulted from greater substrate utilization. Glucose accumulation during growth on cellobiose was about four times higher than that observed during growth on cellulose. During the growth on cellulose, the supply of soluble sugars is limited, and this condition may lead to an increase in the rate of glucose utilization or decrease in its rate of excretion into the medium.

3. Effect of Exogenous Glucose

The observed dependence of glucose accumulation on substrate prompted us to examine the effect of exogenous glucose on the growth of Q of cellobiose (Figure 4). Optical density measurements did not reveal a dramatic difference in growth rate or cell mass. The modest increase in cell mass may reflect the partial utilization of exogenous glucose. The kinetic profiles of soluble sugar utilization are shown in Figure 5.

Figure 2. Growth of *C. thermocellum* Q on cellulose, cellobiose and glucose

The presence of glucose had no effect on the initial rate of cellobiose uptake. Cellobiose appeared to be the preferred substrate. After approximately 24 hr, which corresponds to the maximum cell mass production, glucose levels declined. In the case of 0.1% exogenous glucose, the decreased cellobiose and glucose levels indicated that both substrates were utilized. With 0.5% exogenous glucose, glucose utilization appeared to overtake and retard cellobiose utilization. Glucose is a product of cellobiose utilization; therefore, one would expect to see an increase in the levels of glucose similar to that observed in the absence of exogenous glucose. Further study is required to understand substrate preference and glucose accumulation, and their dependence on the concentration of added glucose.

4. Cellulase from *C. thermocellum* Q

Cellulase production on cellulose, cellobiose, and glucose varied somewhat, but the crude activity was generally about 1.6 mg/ml of glucose equivalents on a 24 hr Avicel assay. These findings suggest that *C. thermocellum* cellulase is constitutive.

Figure 3. Product Formation by *C. thermocellum* Q grown on Cellulose, Cellobiose and Glucose

During *C. thermocellum* Q growth on cellulose, the cellulase activity was primarily located in the supernatant and on cellulose. When cells were separated from the cellulose particles by settling, very little cell-associated avicelase activity was detected even after an eight-fold concentration of the cells. Glucose production during incubation of cell-free filtrate or the cell mass pellet with Avicel was not detected. Neither the whole culture nor the cell-free filtrate showed detectable β -glucosidase activity as measured by the liberation of p-nitrophenol from p-nitrophenylglucoside.

Figure 4. Effect of Exogenous Glucose on *C. thermocellum* Q Growth on Cellobiose (0.5%)

Figure 5. Effect of Exogenous Glucose on Cellobiose (0.5%) Utilization by *C. thermocellum* Q

The thermal stability of C. thermocellum Q cellulase (pH optimum 6.0) was examined between 40 °C and 60 °C (Table II). At 60 °C, some loss of activity was observed; however, after 24 hr, further inactivation was not apparent.

Table II
THERMAL STABILITY OF CELL-FREE CELLULASE

Temperature	Cellulase Activity* at Different Incubation Times			
	0 Hours	24 Hours	48 Hours	60 Hours
40 °C	100	98	100	100
50 °C	100	89	94	100
60 °C	100	87	83	83

* Expressed as Percent of Original

Glucose or cellobiose, in concentrations from 0.1 to 0.5% was not found to affect the cellulase activity of C. thermocellum.

5. Growth of C. thermosaccharolyticum ZC

C. thermosaccharolyticum ZC grew equally well on cellobiose, glucose, and xylose. A lag period of about 20 hr was observed during growth on cellobiose (Figure 6). Ethanol and acetic acid were produced in comparable amounts, and the ratio was generally between 1 and 1.5. The yield of ethanol was higher on glucose than on cellobiose. Product formation was growth-associated but continued to increase after cell growth leveled off. As with C. thermocellum Q, C. thermosaccharolyticum ZC also excreted glucose during growth on cellobiose. The growth of ZC on mixed substrates that are found in the pre-treated wood was also investigated.

When a ZC inoculum was incubated for 72 hr with equal concentrations of cellobiose, glucose, and xylose (Table III), only cellobiose was utilized. However, in separate experimental runs with the individual sugars alone, ZC utilized each substrate during the same time period, indicating a substrate preference similar to C. thermocellum.

Figure 6. Growth of *C. thermosaccharolyticum* ZC on 1% Cellobiose

Table III

GROWTH OF *C. THERMOSACCHAROLYTICUM* ZC ON XYLOSE, CELLOBIOSE, AND GLUCOSE

		Cellobiose mg/ml	Glucose mg/ml	Xylose mg/ml
A	T ₀	2.9	3.1	2.9
	T _{72 hr}	0.2	2.8	2.8
B	T ₀	2.9	---	---
	T _{72 hr}	0.02	---	---
C	T ₀	---	3.1	---
	T _{72 hr}	---	0	---
D	T ₀	---	---	2.9
	T _{72 hr}	---	---	0

6. Mixed-Culture Cellulose Degradation

Coculture of *C. thermosaccharolyticum* ZC with *C. thermocellum* Q on cellulose resulted in an enhanced rate of cellulose degradation and negligible soluble sugar accumulation (Figure 7). The yield of ethanol and acetic acid, generally about 25% of the cellulose consumed, was comparable with that observed in the pure culture fermentation. Soluble sugars did not accumulate in the mixed culture fermentation because of rapid utilization by ZC. However, since exogenous sugar was not observed to stimulate enzyme production or to inhibit enzyme activity, the enhanced cellulose degradation rate could not be attributed to the reduced levels of soluble sugars.

Figure 7. Cellulose Degradation by Mixed Culture and Monoculture

7. Bench Top Fermentation

Several pure and mixed culture one-liter bench top fermentations were examined to confirm earlier results from serum bottle fermentations and to gain additional insight into the course of the fermentation under environmentally controlled conditions.

The utilization of microcrystalline cellulose by *C. thermocellum* Q proceeded, after a brief lag, via first-order kinetics with a rate constant of 0.008 hr^{-1} (Figure 8). Ethanol and acetic acid production correlated with cellulose depletion, and the product yield based on cellulose utilized was 0.34 gm/gm . The ratio of ethanol to acetic acid was comparable in the early part of the fermentation and increased somewhat at the end of 160 hr. Soluble sugar production remained below 1.5 mg/ml , and the glucose level during the fermentation averaged around 0.03 mg/ml .

Figure 8. Growth of *C. thermocellum* Q on 1% Microcrystalline Cellulose

On cellobiose with media containing 0.5% yeast extract (Figure 9), *C. thermocellum* Q grew up more rapidly than in media which contained 0.2% yeast extract (Figure 10). The addition of fresh cellobiose after cell growth peaked was accompanied by a similar decline in substrate, little or no new cell growth, and continued production of ethanol

Figure 9. Growth of *C. thermocellum* Q Cellobiose Containing 0.5% Yeast Extract

and acetic acid. Upon the addition of more fresh cellobiose, the production of ethanol and acetic acid declined and was overshadowed by glucose production.

In media containing 0.2% yeast extract, the growth of *C. thermosaccharolyticum* ZC on cellobiose resulted in glucose production exceeding ethanol and acetic acid production (Figure 11). The rate of glucose accumulation paralleled the growth rate and indicated that little glucose was being utilized, probably because of nutrient limitation.

During mixed culture Q & ZC growth on microcrystalline cellulose (Figure 12), the observed rate constant for substrate depletion was 0.015 hr^{-1} . The latter was

Figure 10. Growth of *C. thermocellum* Q on 1% Cellobiose at pH 7.5

markedly faster than *C. thermocellum* Q alone under similar conditions. Glucose and soluble sugar remained relatively low during the fermentation, and comparable amounts of ethanol and acetic acid were produced. The yield of ethanol and acetic acid was calculated to be 0.25 gm/gm.

Cellobiose utilization by the mixed culture in media containing 0.2% yeast extract resulted in glucose, ethanol, and acetic acid production which paralleled cell growth. The pure and mixed culture growth on cellobiose was qualitatively similar except for

Figure 11. Growth of ZC on 1% Cellobiose

glucose accumulation. A satisfactory explanation for incomplete substrate utilization is not available, but the fermentation may have been nutrient-limited.

B. Culture Development

The necessity of improving the ethanol yield and tolerance of both *C. thermocellum* and *C. thermosaccharolyticum* was recognized at the beginning of this research program. To enhance the ethanol tolerance, cultures were either sequentially transferred into media containing incrementally (0.5%) higher amounts of ethanol or separated (via centrifugation)

Figure 12. Growth of Mixed Culture Q & ZC on Microcrystalline Cellulose (1%)

and incubated in fresh medium containing exogenous ethanol. A strain of *C. thermocellum* (Q-5) which was able to grow in media containing 5% ethanol was isolated by the latter procedure. In media containing no added ethanol, the ratio to acetic acid produced by Q-5 was significantly enhanced over that observed with the parent strain Q (Figure 14).

Similar efforts with *C. thermosaccharolyticum* were unsuccessful. More extensive subculturing and smaller incremental increases in exogenous ethanol concentrations are apparently required to select for strains with higher ethanol tolerance.

Figure 13. Growth of Mixed-Culture Q & ZC on 1% Cellobiose

B. WOOD PRETREATMENT AND EVALUATION

1. Background

The susceptibility of wood and other lignocellulosic materials to microbial, enzymatic, or acid attack can be increased by a variety of mechanical and chemical treatments. Increased sensitivity to acid hydrolysis is best correlated with the cellulose crystallinity and exemplified by amorphous cellulose, which is quantitatively hydrolyzable to glucose under mild conditions. However, the chemical and/or energy expenditure required to decrystallize cellulose is usually prohibitive, unless a high-value end-product is anticipated. Acid hydrolysis of crystalline cellulosic substrates results in unacceptably

Figure 14. Comparison of Ethanol/Acetic Acid Ratio of Q-5 and Q Grown on Cellulose, Cellobiose, and Glucose

low yields of fermentable sugars because the latter are degraded under the conditions required for hydrolysis.

Enzymatic hydrolysis of cellulosic substrates with varying degrees of crystallinity proceeds at measurable rates, and the crystallinity of cellulose is not a major impediment to saccharification. The resistance of lignocellulosic materials to enzymatic or microbial attack has been attributed to lignin, hemicellulose, lignin carbohydrate association, particle size, and pore size. Evidence to support any of these hypotheses is not well defined and not formulated in terms of mechanism of substrate alteration that might permit the rapid and complete microbial utilization of wood cellulose. Such insight is a prerequisite for the development of an efficient pretreatment process.

Our approach to wood pretreatment was based on reports of enhanced microbial utilization of lignocellulosic substrates as a result of high-temperature steaming (11,12) and treatment with gaseous sulfur dioxide (13). Steaming rapidly softens wood and allows operation in the absence of a liquid phase. However, mechanical shearing through dies and sodium hydroxide delignification was deemed necessary to observe the enhanced susceptibility. The required operating temperatures also resulted in considerable destruc-

tion of the hemicellulose fraction, and therefore, the attainable yield of fermentable sugars from wood carbohydrate. Sulfur dioxide treatment was noteworthy because the resulting delignified fiber was reported to be quantitatively converted to sugar by cellulase. The overall yield of fermentable sugar was not disclosed, and the enhanced susceptibility was ascribed to the loss of most of the original Klason lignin. In neither approach was it possible to deduce the mechanism, and we began constructing a laboratory pretreatment apparatus which would, first, allow an independent evaluation of the chemical, physical, and structural factors which limit microbial utilization of wood, and second, contribute to the development of an efficient pretreatment process.

2. Pretreatment Reactor

The principal components of the experimental apparatus for pretreating wood are schematically presented in Figure 15. The steam generator is a high-pressure bomb that can withstand 1800 psi. The capacity of the bomb is one gallon, which is large enough to supply steam to the pretreatment chamber. Both sides of the steam pretreatment chamber are connected to hydraulically operated ball valves capable of withstanding

Figure 15. Pretreatment Reactor Schematic

500 psi at 250 °C. The ball valves have a rapid response time to ensure uniform fiber discharge conditions. The use of ball valves allows for rapid front-end loading and discharge of wood chips and fiber, respectively. Under these experimental conditions, the minimum residence time is limited primarily by the time required to load the pretreatment chamber. The addition of chemicals before or after steaming is accomplished with a high-pressure gas port on the input side of the pretreatment chamber. The pretreatment chamber is a 1 in.-ID stainless steel threaded pipe that is insulated and heated to preset temperatures. The discharge port is connected via flexible tubing to a vortex sample collector.

The effect of steam pressure and reaction time on fiber composition fermentable sugar recovery, and susceptibility of poplar to cell-free enzymic hydrolysis were initially examined to determine a suitable pretreatment operating range. The yield of soluble sugars from enzymatic hydrolysis of poplar fibers steamed at 240 psi (205 °C) was about four times greater than that which could be obtained from fibers treated at 200 psi (196 °C). At these pressures, reaction time beyond 15 minutes generally resulted in decreased yields of soluble sugars; however, reaction time was less influential than temperature. Subsequently, the effect of separate additions of sulfur dioxide and ammonia were also examined and compared with the effects of steam alone. Chemically augmented steaming was performed by initially contacting the poplar in the preheated (200 °C) reactor with gaseous sulfur dioxide (30 psi) for about 2 min. The wood was decompressively discharged from the reactor and analyzed.

The composition of treated and untreated poplar (Populus tristis) fiber is given in Table IV. The unaccounted fraction (~18%) is thought to contain low-molecular-weight lignin, acid insoluble organics, and carbohydrates other than xylose and glucose. Steam and SO₂/steam treatment caused a decrease in the xylan and ash content of the fibers. However, operating on the basic side with ammonia significantly retarded xylose degradation. About 30% of the original Klason lignin was altered during each pretreatment to

the point where it became soluble in 72% H_2SO_4 (Table V). Steam or sulfur dioxide pretreatment at 240 psi resulted in reduced yields of fermentable sugars unless the pH in the reactor was controlled.

Table IV
FIBER COMPOSITION BEFORE AND AFTER PRETREATMENT^a

	Xylose (%)	Glucose (%)	Lignin (%)	Ash (%)	Unaccounted (%)
Untreated	11.6	40.6	26.4	4.5	18.9
Steam	4.9	46.5	29.3	0.2	19.1
Steam + SO_2	4.4	51.7	32.7	0.2	11.0
Steam + NH_3	12.5	49.9	27.4	0	10.2

^aBased on total dry weight used in analysis

240 psi, 15 minutes; SO_2 at 30 psi; NH_3 at 70 psi.

Table V
RECOVERY OF FERMENTABLE SUGAR AND LIGNIN AFTER PRETREATMENT^a

	Xylose ^b (%)	Glucose ^b (%)	Lignin (%)
Steam ^a	65	77	72
Steam + SO_2 ^a	47	77	72
Steam + NH_3 ^a	90	88	68

^a240 psi; SO_2 at 30 psi; NH_3 at 70 psi

^bincludes xylose and glucose in fiber and mother liquor

The susceptibilities of treated and untreated poplar fibers to acid hydrolysis and enzymatic saccharification are summarized in Table VI. Hydrolysis in 6N HCl provides a rough measure of the amorphous carbohydrate content of the fiber, and the results indicate that little or no decrystallization of cellulose occurred as a result of the pretreatment. The relatively higher soluble sugar production from untreated fibers results from their higher xylan content.

Enzymic hydrolysis of cellulose with T. reesei cellulase leads to the formation of both glucose and cellobiose. Since the soluble sugar yields reported in Table VI were

Table VI
SACCHARIFICATION OF PRETREATED POPLAR

	Acid ^a	Soluble Sugar Production (mg)	
		Enzyme ^b	
		24 hr	48 hr
Untreated	6.3	1.4 (6.1%)	2.1 (9.2%)
Steam	3.0	7.1 (30.6%)	10.3 (44.5%)
Steam + SO ₂	2.0	12.9 (50.0%)	18.3 (70.0%)
Steam + NH ₃	1.5	--- (3.8%)	---

^a50 mg of fiber in 5 ml 6N HCl, incubated at 96 °C for 1 hr; soluble sugars were determined by dinitrosalicylic acid method.

^b50 mg of fiber in 2 ml enzyme solution which had Avicelase activity of 15 mg/ml (10% Avicel incubated for 24 hrs); sugars were determined by glucostat method.

determined by glucostat assay, the yields of soluble sugars will be higher once the cellobiose is accounted for. Since extending the incubation time from 24 to 48 hr results in additional soluble sugar production, it is likely that for SO₂-treated fibers, all of the carbohydrate can be saccharified in a reasonable period of time. Steam pretreatment alone resulted in a less dramatic improvement in fiber accessibility, and ammonia-treated fibers were as resistant to saccharification as untreated fibers.

The ability of *C. thermocellum* Q to utilize sulfur dioxide pretreated poplar fibers for ethanol production was evaluated at 60 °C in a pH-controlled (7.2) fermentor (Figure 16). Approximately 70% of the fiber was hydrolyzed and converted into acid and alcohol.

Treated and untreated fibers were examined by transmission electron microscopy to ascertain the nature of the structural alterations in fiber morphology which occurred as a result of pretreatment. Unstained samples cut from Noble agar and fixed in 2% KMnO₄ were observed in a Sieman Elmiskop 101 at 60 kV after dehydration, spurr infiltration, and thin sectioning.

Figure 16. Growth of *C. Thermocellum* Q on SO₂-Treated Fiber

The principal cell wall components—intercellular substance (I), primary wall (P), outerlayer of secondary wall (S₁), middle layer of secondary wall (S₂), and inner layer of secondary wall (S₃)—were readily discernible in the electron micrograph of a cross section of untreated poplar shown in Figure 17. A representative electron micrograph of steam-treated poplar is shown in Figure 18. Steam treatment resulted in extensive swelling in the S₃ layer and partial separation of the S₂/S₃ layers. Lignin diffusion (altered or native) and coagulation at the S₂/S₃ and, in some cases, at the P/S₁ interface were also observed. Individual fibers did not constitute a significant fraction of the samples examined, and cell wall structure remained generally intact. The loss of some intercellular substance (lignin) was apparent in some electron micrographs, but more extensive analysis is required to quantify the amount of cell wall material removed. Swelling of the primary wall and the S₁ and S₂ layers was quite limited relative to that observed in the S₃ layer. Several "checks" or slits were observed across the entire swollen S₃ layer. It has not yet been determined if these were artifacts arising from sample preparation.

Figure 17. Electron Micrograph of Untreated Poplar (20,000X)

The SO₂/steam treatment resulted in more extensive alterations in fiber morphology, and frequently complete loss of cell wall structure was observed (Figure 19).

A representative electron micrograph showing the extent of alteration prior to loss of structure is shown in Figure 20. The S₃ layer was absent, and a considerable portion of the intercellular substance, the primary wall and the S₁ layer, was removed. The S₂ layer is still discernible, but the loss of structure is apparent.

Ammonia-treated poplar fibers (Figure 21) retained considerable structural integrity and displayed extensive swelling of the S₃ layer. However, separation at the S₂/S₃ interface was infrequent, and completely detached lumen walls were not observed. Loss of middle lamella lignin appeared to be comparable or somewhat less than with samples treated with steam alone. Lignin coagulation and/or diffusion into

Figure 18. Electron Micrograph of Steamed Poplar (20,000X)

the S₂/S₃ interface was not readily observed. The pH of ammonia-treated fibers was about eight, which indicated that pH or a specific chemical interaction with sulfur dioxide, or both, was required to make most of the wood carbohydrate susceptible to microbial utilization.

Figure 19. Electron Micrograph of SO₂/Steam-Treated Poplar Showing Complete Loss of Cell Wall Structure (20,000X)

Figure 20. Electron Micrograph of SO₂/Steam-Treated Poplar Showing the Extent of Alteration Prior to Loss of Structure (20,000X)

Figure 21. Electron Micrograph of Steam-Treated Poplar (20,000X)

IV. DISCUSSION

A. MICROBIOLOGY AND BIOCHEMISTRY OF *C. THERMOCELLUM* CELLULOSE FERMENTATION

1. Experimental Results in Microbiology

The experimental results permit the advancement of a tentative picture of the microbiology of pure and mixed culture utilization of cellulose by *C. thermocellum* Q and *C. thermosaccharolyticum* ZC. During growth of *C. thermocellum* Q on cellulose, the culture elaborates extracellular cellulase, which produces primarily cellobiose that is metabolized by *C. thermocellum* Q to produce glucose, lactic acid, ethanol, acetic acid, hydrogen, and carbon dioxide. Similar end-products are produced by *C. thermosaccharolyticum* ZC grown on cellobiose. Since preliminary attempts to detect glucose as an end-product of *C. thermocellum* cell-free cellulase attack on Avicel were negative, most, if not all, of the glucose in the broth arose from the metabolism of cellobiose. However, additional studies are required to substantiate this conclusion. Glucose production arising from the action of cell-free or cell-wall-associated β -glucosidase on cellobiose was rejected because of the failure of either preparation to liberate p-nitrophenol from p-nitrophenyl- β -D-glucoside. Since *C. thermocellum* endoglucanase activity would also be expected to produce glucose as one end-product of extracellular hydrolysis, endoglucanase activity may be quite low. The latter is particularly important because the absence of sufficient endoglucanase activity (required for synergistic hydrolysis of crystalline cellulose) in *C. thermocellum* Q cell-free extracts may explain the relatively low cellulose depletion rates.

Glucose accumulation during *C. thermocellum* Q growth on cellulose may also be influenced by the physiological state of the culture. If cellulose saccharification is rate limiting, then, during growth on cellulose, the concentration of the actual growth substrate (cellobiose) remains low, limits cell growth, and influences the amount of glucose that is utilized. However, during growth on cellobiose, the carbon substrate is plentiful, and glucose accumulation is controlled primarily by substrate preference.

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C. thermocellum Q and C. thermosaccharolyticum ZC display a definite preference for cellobiose over glucose. In the case of Q at least, the preference was reversed when the concentration of glucose was approximately double that of cellobiose, a result that suggests partially separate metabolic pathways and pathway regulation. McBee and Sih⁽¹⁴⁾ and Alexander⁽¹⁵⁾ have reported on the mechanism of cellobiose uptake in C. thermocellum. Hulcher and King⁽¹⁶⁾ advanced the hypothesis that the disaccharide preference in a cell-vibrio resulted from a greater energy yield per mole of hexose consumed. Later, King et al⁽¹⁷⁾ demonstrated the metabolic nonequivalence of the two glucose units in cellobiose. Glucose released into the medium by C. thermocellum or C. thermosaccharolyticum during growth on cellobiose is much less than half of the cellobiose. This suggests partial utilization of the free glucose or simultaneous uptake of both glucose and cellobiose. The effect of exogenous glucose accumulation remains unclear, and interpretation of the results is not straightforward because the measured glucose levels represent the net change arising from glucose production and consumption during growth on cellobiose. Additional studies with radiolabeled substrates should resolve these questions and suggest a way to minimize or eliminate glucose excretion.

The enhanced rate of cellulose depletion during growth of C. thermocellum Q in the presence of C. thermosaccharolyticum ZC appears not to be the result of soluble growth factor provided by the latter. No demonstrable cellulose depletion rate enhancements were observed when C. thermocellum Q was grown on cellulose in the presence of added C. thermosaccharolyticum ZC cell-free supernatant. Weimer and Zeikus⁽³⁾ reported only a slight growth rate enhancement for coculture with C. thermoautotrophicum. The potential for achieving further increases in substrate depletion rates by coculturing is apparent from a comparison of the mixed culture cellulose depletion rates of C. thermocellum Q and C. thermosaccharolyticum ZC with that observed for the contaminated C. thermocellum LQ8 strain. The latter exceeded the former by almost a factor of three, even when the basal medium yeast extract level is only 0.2%.

B. WOOD PRETREATMENT

1. Experimental Results in Chemical Augmented Steaming

The experimental results on wood pretreatment clearly confirm our original hypothesis that low-pressure sulfur dioxide-augmented hardwood steaming should produce a readily accessible substrate. Since the treated wood retains 75% or more of the original Klason lignin, the latter does not appear to be the primary barrier to enzymatic hydrolysis.

While the preliminary experimental findings do not permit identification of the primary physiochemical barrier to enzyme accessibility, they do provide considerable new insight and a basis for formulating a working hypothesis to guide future studies and the design of an optimum pretreatment process. The electron micrographs are particularly helpful in this regard. However, the results of the electron microscopy analysis are merely preliminary, and several representative samples must be examined before a general picture of the effects of steam and SO_2 /steam treatment can be established. The observed changes in fiber morphology arise primarily from the combined effects of steam temperature, pH, and rapid decompression; and these factors can be independently evaluated in the reactor.

8. Total capital investment includes contingency (15% of fixed investment) and working capital (20% of total capital investment).

9. Fixed charges for manufacturing costs include 8% depreciation, 8% interest, 1% property tax, 1% insurance, and 3% maintenance costs.

Nolan⁽¹⁷⁾ has estimated the capital investment cost for a 30×10^6 gallon/year ethanol plant using combined saccharification and vacuum fermentation. Since similar equipment was used, the fermentation equipment costs were based on the estimates of Nolan and adjusted to 1978 costs with the use of Chemical Engineering Cost Indexes of 245.5 and 226.2 for 1978 and 1979, respectively.

Based on these assumptions and the capital investment cost presented in Table VII for a 30×10^6 gallon/year 95% ethanol plant, the manufacturing cost for a gallon of ethanol is calculated to be about \$1.06 (Table VIII). Ethanol costs of about \$1.25/gallon (pure ethanol) are considered achievable⁽¹⁷⁾ even with vacuum fermentation and ethanol yields which are only 50% of theoretical. The proposed modified vacuum fermentation process would use a much smaller system coupled to a conventional fermentor, which should further reduce the capital investment.

The favorable ethanol cost estimate is a reflection of engineering simplicity, low-cost pretreatment, high carbohydrate recovery, and the integration of the fermentation and ethanol recovery operations. Biomass costs represent about 58% of the ethanol manufacturing costs. With small-scale decentralized plants, the biomass costs may be lower than the \$30/ton assumed in the foregoing calculations. Since mixed biomass feedstock could be employed, the yearly plant loading could be smoothed out with crop residues and hardwood. If the grower is also the ethanol manufacturer, the need and pressure to sell biomass for profit would be reduced because of the opportunity to recover a satisfactory profit on the sale of ethanol.

V. PRELIMINARY ECONOMIC CONSIDERATIONS

A preliminary cost analysis of the GE/CRD process was performed to estimate the costs of ethanol production and to determine areas where technical improvement would lead to significant cost reduction.

The cost of producing ethanol via the GE/CRD process was evaluated for a 30×10^6 gallon/year (300 ton/day) plant producing 95 volume percent ethanol from poplar. The size of the plant was dictated by available data and does not indicate an optimum or even desirable size. The material balance used to develop the cost estimate is shown in Figure 1, page 4.

The following assumptions were made to arrive at a calculated manufacturing cost of ethanol.

1. Poplar wood available at \$30/ton on a dry weight basis is fed into the reactor as chips containing 40 weight percent water as received.
2. Moist wood chips are contacted with gaseous sulfur dioxide (wood/SO₂ weight ratio $\cong 100/1$) under steam pressure of about 300 psi for 10 to 15 minutes.
3. Fiber recovery after pretreatment is 90% of the charge.
4. One-half of the pretreated fiber is fermentable carbohydrate, and 90% of this carbohydrate is utilized during fermentation. The ethanol yield is 40%, based on the amount of sugar utilized.
5. The ethanol concentration in the fermentor broth is 2.5%.
6. Material costs for SO₂, NH₃, and steam are \$143/ton, \$120/ton, and \$2.50/1000 pounds, respectively.
7. Lang⁽¹⁵⁾ multiplication factor of 4.6 may be used to estimate the practical investment on delivered equipment costs. When 1978 equipment was not available, costs were updated with the use of the December 1978 Chemical Engineering Plant Construction Cost Index of 245.2 (16).

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Table VII
CAPITAL INVESTMENT

<u>Pretreatment</u>	<u>Fermentation</u>
Two 120 ton/hr 10" x 120" screw conveyors	Twenty-one 500 x 10 ³ gallon fermentors
Two 72 ton/hr 10" x 120" screw conveyors	Twenty-one agitators (100 hp)
Two 3 x 12" cylindrical autoclaves	Distillation System
Two 500 x 10 ³ gallon storage tanks	Vacuum System and Compressor
Two 4000 gallon collector tanks	Fermentation Condenser
	Process Piping
	Transfer Pump
	Package Bioler Plant
	Cooling Tower (6000 gal/min)
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Fixed Capital: 1.72 x 10 ⁶	Fixed Capital: 24.38 x 10 ⁶
Total Capital: 2.24 x 10 ⁶	Total Capital: 35.06 x 10 ⁶

Table VIII
PRELIMINARY COST-ESTIMATES*

	<u>Raw Material</u>	<u>Pretreatment</u>	<u>Fermentation</u>
Total Capital Investment		\$2.24 x 10 ⁶	\$35.06 x 10 ⁶
Fixed Charge (\$/gal)		1.73	24.5
Direct Cost (\$/gal)			
Fiber	57.9		
Chemical		2.79	7.56
Steam + Lab + Utility		3.37	7.20
Manufacturing Cost (\$/gal)	57.9	7.70	39.30

* Cost does not include general expense, plant overhead cost, laboratory charge, land purchasing, building, service facility, yard improvement and startup.

12. Esdale, W., *Nutritional Release No. 2, Bulletin 05E71, Stake Technology, Ltd., 20 Enterprise, Ottawa, Canada, 1971.*
13. Millett, M.A., Baker, A.J., and Satter, L.D., "Pretreatment of Enhance Chemical, Enzymatic, and Microbiological Attack of Cellulosic Materials," *Biotechnol. and Bioeng. Symp. No. 5, 1975, pp. 193-219.*
14. Sih, C.J., and McBee, R.H., "A Cellobiose-Phosphorylase in *Clostridium thermocellum*," *Proc. of the Montana Acad. Sci. 15, 1955, pp. 21-22.*
15. Alexander, J.K., "Characteristics of Cellobiose Phosphorylase," *J. Bacteriol. 81, 1961, pp. 903-910.*
16. Hulcher, F.H., and King, K.W., "Metabolic Basis for Disaccharide Preference in a *Cellvibrio*," *J. Bacteriol. 76, 1958, pp. 571-577.*
17. Swisher, E.J., Storvick, W.O., and King, K.W., "Metabolic Nonequivalence of the Two Glucose Moieties of Cellobiose in *Cellvibrio gilvus*," *J. Bacteriol. 88, 1964, pp. 817-820.*
18. Peters, M., and Timmerhause, K., *Plant Design and Economics for Chemical Engineers*, McGraw-Hill, New York, 1968, p. 120.
19. "Economic Indicators," *Chem. Eng. 86, 3, 1979, p. 7.*
20. Nolan, E.J., General Electric Company, Philadelphia, Pa., personal communication, Jan., 1979.

VI. SUMMARY

Based on these studies, the direct mixed culture thermophilic fermentation of SO_2 /steam pretreated hardwood to ethanol is technically feasible and offers the potential of becoming economically attractive. The yield of ethanol and the ethanol tolerance of the microorganisms must be increased to justify the assumptions used in the cost estimate. Adaptive mutation in the presence of ethanol has proven to be an effective means of achieving improvements in culture performance. Additionally, several avenues for improving the pretreatment operation and the overall productivity of the process have been suggested for future study.

REFERENCES

1. Hungate, R.E., "A Roll Tube Method for Cultivation of Strict Anaerobes," *Methods in Microbiology*, Vol. 3B, J.R. Norris and D.W. Ribbons, eds., Academic Press, New York, 1969, pp. 117.
2. Miller, T., and Wolan, M.J., "A Serum Bottle Modification of the Hungate Technique for Cultivating Obligate Anaerobes," *Appl. Microbiol.*, 27, 1974, pp. 285-287.
3. Weimer, P.J., and Zeikus, J.G., "Fermentation of Cellulose and Cellobiose by *Clostridium thermocellum* in the Absence and Presence of *Methanobacterium Thermoautotrophicum*," *Appl. Environ. Microbiol.* 33, 1977, pp. 289-297.
4. Pinnegar, M.A., in *Automation in Analytical Chemistry*, L.T. Skeggs, ed., Mediad Incorporated, New York, 1966, p. 80.
5. "Dextranase" in *Worthington Enzyme Manual*, L. Decker, ed., Worthington Biochemical Corp., 1977, p. 183.
6. Freitas, R., Wilke, C.R., Long, B., and Sciamanna, A., *Procedures for Analysis of Solids and Liquids from Cellulosic Sources*, Report No. LB6-5967, Lawrence Berkeley Laboratory, University of California, Berkeley, CA., 1978.
7. Brittain, G.D., and Sullivan, J.E., "Silylation in the Presence of Water," *Recent Advances in Gas Chromatography*, I. Domszy, and J. Perry, ed., 1971, pp. 223-229.
8. Su, T.M., and Paulavicius, I., "Enzymatic Saccharification of Cellulose by Thermophilic Actinomyces," *Appl. Polym. Symp.* 28, 1975, pp. 221-236.
9. Okada, G.J., "Enzymatic Studies on a Cellulase System of *Trichoderma viride*," *J. Biochem.* 77, 1974, p. 33.
10. Fleming, R.W., Characterization of Organic Nutritional Requirements for *Clostridium Thermocellum*, PhD Dissertation, Iowa State University, Ames, Iowa, 1970.
11. Bender, F., Heaney, D.P., and Bowden, A., "Potential of Steamed Wood as a Feed for Ruminants," *For. Prod. J.* 20, 4, 1970, pp. 36-41.

12. Esdale, W., Nutritional Release No. 2, Bulletin 05E71, Stake Technology, Ltd., 20 Enterprise, Ottawa, Canada, 1971.
13. Millett, M.A., Baker, A.J., and Satter, L.D., "Pretreatment of Enhance Chemical, Enzymatic, and Microbiological Attack of Cellulosic Materials," *Biotechnol. and Bioeng. Symp. No. 5*, 1975, pp. 193-219.
14. Sih, C.J., and McBee, R.H., "A Cellobiose-Phosphorylase in *Clostridium thermocellum*," *Proc. of the Montana Acad. Sci.* 15, 1955, pp. 21-22.
15. Alexander, J.K., "Characteristics of Cellobiose Phosphorylase," *J. Bacteriol.* 81, 1961, pp. 903-910.
16. Hulcher, F.H., and King, K.W., "Metabolic Basis for Disaccharide Preference in a *Cellvibrio*," *J. Bacteriol.* 76, 1958, pp. 571-577.
17. Swisher, E.J., Storvick, W.O., and King, K.W., "Metabolic Nonequivalence of the Two Glucose Moieties of Cellobiose in *Cellvibrio gilvus*," *J. Bacteriol.* 88, 1964, pp. 817-820.
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19. "Economic Indicators," *Chem. Eng.* 86, 3, 1979, p. 7.
20. Nolan, E.J., General Electric Company, Philadelphia, Pa., personal communication, Jan., 1979.